GENETICS OF ACQUIRED ANTIMICROBIAL RESISTANCE IN ANIMAL AND ZOONOTIC PATHOGENS

EDITED BY: Axel Cloeckaert, Michel Stanislas Zygmunt and Benoît Doublet

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GENETICS OF ACQUIRED ANTIMICROBIAL RESISTANCE IN ANIMAL AND ZOONOTIC PATHOGENS

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Antimicrobial susceptibility testing by the disk diffusion method of a β-lactam resistant *E. coli* isolate.

Image: Axel Cloeckaert, INRA.

Development and spread of antimicrobial resistance is the result of an evolutionary process by which microorganisms adapt to antibiotics through several mechanisms including alteration of drug target by mutation and horizontal transfer of resistance genes. The concomitant occurrence of independent antimicrobial resistance mechanisms is a serious threat to human health and

has appeared in several emerging epidemic clones over the past decade in humans and also in animals. The increasing prevalence of antimicrobial drug resistance among animal and zoonotic foodborne pathogens is of particular concern for public health. In this Ebook, we gathered a collection of articles which deal with the most important aspects of the genetics of acquired antimicrobial resistance extending from medically-important resistance, emerging epidemic resistant clones, main mobile genetic elements spreading resistance, resistomes, dissemination between animals and humans, to the "One Health" concept.

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EDITORIAL
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Editorial: Genetics of Acquired Antimicrobial Resistance in Animal and Zoonotic Pathogens

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Keywords: one health, integron, mobile genetic element, antibiotic resistance, dissemination

Editorial on the Research Topic

Genetics of Acquired Antimicrobial Resistance in Animal and Zoonotic Pathogens

Antimicrobial resistance has become a global public health concern due to multidrug-resistant (MDR) bacteria and to the lack of novel antibiotics. Resistant bacteria, including zoonotic pathogens, can be exchanged between animals and humans through direct contact, the food chain, or contamination of the shared environment. Resistance to medically-important antibiotics such as extended spectrum beta-lactams, carbapenems, fluoroquinolones, or aminoglycosides, is of increasing magnitude among zoonotic pathogens. Acquired antimicrobial resistance is the result of an evolutionary process by which microorganisms adapt to antibiotics through several mechanisms including alteration of drug target by mutations and horizontal transfer of novel/foreign genes, referred to as resistance genes. Acquired resistance genes coding for any of the three major resistance mechanisms, i.e., enzymatic inactivation, reduced intracellular accumulation, or modification of the cellular target sites are associated with mobile genetic elements including plasmids, transposons, gene cassettes, integrative, and conjugative elements or other mobile elements (Schwarz et al., 2017).

This Research Topic is focused on acquired antimicrobial resistance mechanisms in animal and zoonotic pathogens isolated from food-producing animals, food products, companion animals, humans, and the environment. Different sets of articles document the most important aspects of the genetics of acquired antimicrobial resistance extending from medically-important resistance genes such as those conferring resistance to extended spectrum cephalosporins and more recently those conferring resistance to colistin, emerging epidemic clones, primary mobile genetic elements such as class 1 integrons, culture-independent approach of resistomes, dissemination between animals and humans, to the "One Health" concept.

First, in an elegant review article, Imperial and Ibana addressed the global problem of the spread of emerging antibiotic-resistant bacteria in the "One Health" perspective. They summarized the processes that govern the spread of antibiotic resistance in relation to resistance genes, their horizontal transfer through mobile genetic elements, the microbial ecology of resistant bacteria in the human gut microbiota, and their dissemination by international travel of humans, animals, or food. Finally, they discussed the probiotic use in both human and veterinary applications to tackle the antibiotic resistance threat and concluded on the "double-edged sword" with potential risk in propagating antibiotic resistance by probiotics.

Salmonella enterica spp. are important zoonotic pathogens related to foodborne diseases worldwide. In the present Research Topic, Zhao et al. and Zhao et al. contributed with two research articles dealing with antimicrobial resistance of *S. enterica* serotypes isolated from food-producing animals (chickens, ducks, and pigs) in farms and slaughterhouses in the Shandong province, China.

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They reported a higher prevalence of *Salmonella* in chickens (∼24%) compared to pigs (∼9%). The serotype distribution and multidrug resistance phenotypes were dependent on the animal species, serotypes Indiana, and Enteritidis being prevalent in chickens and strongly associated with MDR phenotypes. High antimicrobial resistance rates were observed for old class antibiotics such as nalidixic acid, ampicillin, and tetracyclines (>80%). The plasmid-mediated quinolone resistance genes *qnr* were also frequently detected in isolates from farm animals and in slaughterhouses contributing probably with target gene mutations to a rate of around 40% of ciprofloxacin resistance. A relatively high resistance rate to cefotaxime (∼30%) was also described, mostly *Salmonella* isolates harboring the ESBL gene $bla_{\text{CTX-M-55}}$, largely predominant in Asia. In another study from China, Huang et al. investigated the prevalence of colistin resistance and associated *mcr-1* gene on a large collection of commensal *E. coli* isolates (>4,000) from food-producing animals during the period 2013–2014. They reported an overall resistance rate of 18.7% with MIC colistin $\geq$ 4 mg/L, with a higher frequency of colistin-resistant *E. coli* isolated from pigs (24%) compared to chickens (14%). Among 200 randomly selected colistin-resistant *E. coli* isolates, they found 182 positive *mcr-1* positive isolates. Colistin being in some cases the last therapeutic option to treat infections due to carbapenemase-producing Gram-negative bacteria, the high prevalence of MCR-1-mediated colistin resistance among commensal *E. coli* recovered from food-producing animals is worrisome in China. These MCR-1-producing *Enterobacteriaceae* might transfer to humans through the food chain or to farmers via direct animal contact, and furthermore transfer the *mcr-1* resistance gene to human pathogens. Since the recent ban of colistin as animal feed additive in China, future surveillance programmes will be necessary to follow the evolution of colistin resistance in food-producing animals (Walsh and Wu, 2016).

This Research Topic includes also a panel of articles focused on resistance genes in different companion animal pathogens. Resistance to extended spectrum cephalosporins is a major concern for companion animals due to the close contact with their owners. Liu et al. investigated the occurrence of Extended Spectrum Beta Lactamase (ESBL)-producing clinical *E. coli* recovered from dogs and cats in the United States, from 2009 to 2013. They reported a prevalence of 3.8% ESBL-producing *E. coli*, the $bla_{\text{CTX-M}}$ resistance genes being the most prevalent ones followed by the cephalosporinase and carbapenemase genes, $bla_{\text{CMY-2}}$ and $bla_{\text{OXA-48}}$, respectively. CTX-M-producing MDR *E. coli* isolates were primarily of sequence types ST131, ST648, and ST405 suggesting the possible transfer of predominant human clones with companion animals in the U.S. In a similar large epidemiological study dealing with *Morganellaceae* of animal origin in France, Schultz et al. investigated the prevalence of ESBL and carbapenemase genes and their genetic supports. They found a similar prevalence of ESBL producers (∼4%) among *Proteus mirabilis* isolates from dogs, cats, and horses. Interestingly, molecular characterization showed the spread of a clonal population harboring the MDR integrative mobilizable element SGI1-V (*Salmonella* genomic island 1—variant V) carrying the ESBL gene $bla_{\text{VEB-6}}$, previously identified in human isolates in France. Xia et al. reported a study focused on 16S rRNA methylases conferring high-level resistance to aminoglycosides in *Klebsiella pneumoniae* isolates from diseased dogs and cats in a veterinary hospital in China. They highlighted the spread of genetically-related *K. pneumoniae* ST37 isolates as well as the horizontal transfer of a conjugative IncF33:A-:B- plasmid carrying the 16S rRNA methylase gene *rmtB* and the ESBL gene $bla_{\text{CTX-M-55}}$. Finally, Deng et al. described the multiresistance genes *vga*(A), $vga(A)_{\text{LC}}$, *sal*(A), and *lsa*(E) carried by plasmids and chromosomal genomic islands and conferring resistance to pleuromutilins, lincosamides, and streptogramin A in different *Staphylococci* species isolated from pet animals and sometimes their owners in China. In regard of all these articles, it is important to consider the current role of companion animals as a reservoir of resistant bacteria and their mobile resistance determinants that may be exchanged in either direction between animals and humans, warranting the prudent use of all antibiotics in veterinary medicine of companion animals.

Acquired resistance mechanisms are based on resistance mutations of a chromosomal gene or on the acquisition of mobile resistance genes. Several articles exemplified both phenomena of molecular resistance mechanisms, whose relative importance depends on the antibiotic family and the implicated bacterial species. With the recent description of plasmid-mediated resistance to colistin (*mcr* genes), a renewed interest of research took place for molecular resistance mechanisms to polymixins. Prasgasam et al. characterized carbapenem- and colistin-resistant *K. pneumoniae* isolates from bacteremia cases in India by whole genome sequencing. They described multiple mutations in the chromosomal genes coding for lipopolysaccharide (LPS) lipid A modifications including silent mutations, point mutations, insertions and/or deletions. The most significant were mutations in the *mgrB* gene. The resistance genes *mcr* were not detected in this study. The significance of other mutations observed in this study needs to be confirmed for conferring polymixin resistance. Another example of resistance-conferring mutations in chromosomal genes is given in the study by Xu et al. dealing with fosfomycin resistance in *Staphylococcus aureus*. The key resistance mechanisms to fosfomycin include the production of fosfomycin-modifying enzymes (FosA, FosB, FosC, and FosX), modifications of the target enzyme MurA or the membrane transporters GlpT and UhpT. The GlpT and UhpT transporters are responsible for fosfomycin uptake. They previously showed that mutations in *glpT* and *uhpT* genes were common in fosfomycin- and methicillin-resistant *S. aureus* (Fu et al., 2016). Using *uhpT* and/or *glpT* deletion mutants in *S. aureus*, they confirmed the role of mutations or insertional inactivation in these transporter or their regulatory genes in high-level fosfomycin resistance (MIC > 1,024 μg/ml). Cha et al. investigated the phylogenetic lineages of fluoroquinolone- and macrolide-resistant *Campylobacter jejuni* isolates recovered from Michigan patients. Resistance mechnisms to fluoroquinolones and macrolides involve acquisition of mutations of the target sites of the antibiotics, i.e., the DNA gyrase/topoisomerase IV and 23S rRNA subunit, respectively. They identified clonal spread of specific fluoroquinolone-resistant *C. jejuni* lineages, like ST464, associated with history of foreign travel and also a significant

association between tetracycline-resistant *C. jejuni* ST982 and contact with cattle, chickens and drinking well water at home. Linking genetic diversity and antimicrobial resistance profiles of *C. jejuni* from various sources is needed to better understand transmission dynamics to humans.

Acquired resistance genes are not by themselves mobile but are carried by various genetic structures allowing their horizontal mobility at the molecular or cellular level. The spread of successful multidrug resistance (MDR) mobile genetic elements (plasmids and genomic islands) between bacteria is the main driving force in the dissemination of acquired antibiotic resistance genes. The involvement of plasmids in the zoonotic spread of ESBL- and carbapenemase-resistance genes between human and animal reservoirs are documented by several articles in the present Research Topic. Seiffert et al. described the occurrence of a new plasmid variant IncK2 carrying the cephalosporinase gene $bla_{\text{CMY-2}}$ in *E. coli* isolated from poultry, poultry meat and humans. The identification of very close CMY-2-encoding IncK2 plasmids in genetically diverse *E. coli* from poultry and humans suggested an important ability of horizontal transfer by conjugation in different reservoirs. In another article also dealing with ESBL-producing *Enterobacteriaceae*, Moremi et al. analysed ESBL producers in wild fish obtained from Lake Victoria as well as in environmental samples obtained from the Mwanza city in Tanzania. Lake Victoria is the major source of fish consumed by Mwanza residents and also receives treated wastewater effluents from Mwanza city. They previously described high rates of CTX-M15-producing *Enterobacteriaceae* in the city hospital as well as in animals and humans from the community (Mshana et al., 2016). In the present study, they reported a significant proportion of ESBL-producing *Enterobacteriaceae* in fish gut and environmental samples involving both clonal spread of resistant strains and dissemination of CTX-M15 encoding IncY plasmids. This study suggests that transmission of both ESBL-producing clones and plasmids may occur between humans and wild fish, and reciprocally, through environmental contamination by anthropogenic activity and the food chain, respectively. An additional article by Bai et al. highlights the diversity of ESBL-carrying plasmids, $bla_{\text{CTX-M}}$ genes and ESBL-producing *E. coli* isolated from diarrheic patients in China. Thanks to whole genome sequencing, additional articles emphasize the important role and diversity of integrative elements implicated in multidrug resistance in animal and human pathogens, two articles from Bossé et al. and Bossé et al. and one from Simões et al., respectively.

Finally, two research articles investigated the resistomes of humans, cattle and fish in different environmental settings, by using a culture-independent approach on targeted resistance genes and/or associated mobile elements like integrons. Chainier et al. reported a high frequency of integron carriage in the gut of cattle and humans living in the same geographic area, in France and conversely Muziasari et al. described a low occurrence of resistance genes in the gut of farmed fish and in associated sediment samples in fish farms in the Northern Baltic Sea, in Finland. Using high throughput culture independent methods to study resistomes in different microbiota will provide useful information in the future to manage the spread of antimicrobial resistance.

In summary, this Research Topic addresses various issues related to the genetics of antimicrobial resistance in frame of the "One Health" concept. They emphasize the huge diversity of resistance genes, mobile genetic elements, and multidrug-resistant clones in different microbial environments. In the era of increasing antimicrobial resistance, judicious use of antibiotics is an absolute necessity in veterinary and human medicine to prolong antibiotic usefulness. On the other hand, bacteria have developed a large set of genetic weapons specialized in the acquisition and spread of acquired resistance genes. Successful MDR mobile genetic elements are one of the main driving forces in the antibiotic resistance burden. A research effort is needed to decipher the molecular basis of resistance dissemination within and between microbial niches to manage the evolution toward resistance.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Addressing the Antibiotic Resistance Problem with Probiotics: Reducing the Risk of Its Double-Edged Sword Effect

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Antibiotic resistance is a global public health problem that requires our attention. Indiscriminate antibiotic use is a major contributor in the introduction of selective pressures in our natural environments that have significantly contributed in the rapid emergence of antibiotic-resistant microbial strains. The use of probiotics in lieu of antibiotic therapy to address certain health conditions in both animals and humans may alleviate these antibiotic-mediated selective pressures. Probiotic use is defined as the actual application of live beneficial microbes to obtain a desired outcome by preventing diseased state or improving general health. Multiple studies have confirmed the beneficial effects of probiotic use in the health of both livestock and humans. As such, probiotics consumption is gaining popularity worldwide. However, concerns have been raised in the use of some probiotics strains that carry antibiotic resistance genes themselves, as they have the potential to pass the antibiotic resistance genes to pathogenic bacteria through horizontal gene transfer. Therefore, with the current public health concern on antibiotic resistance globally, in this review, we underscore the need to screen probiotic strains that are used in both livestock and human applications to assure their safety and mitigate their potential in significantly contributing to the spread of antibiotic resistance genes in our natural environments.

Keywords: antibiotic resistance, probiotics, mobile genetic elements, veterinary medicine, livestock production

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INTRODUCTION

Since its advent, antibiotics remain as the major therapeutic strategy that is used to address numerous diseases of infectious etiologies both in human and veterinary medicine (Drago et al., 2011; Schjørring and Krogfelt, 2011; Verraes et al., 2013; Allen and Stanton, 2014; Card et al., 2015). However, the indiscriminate and improper use of antibiotics has led to the decreased susceptibility and increased resistance rates observed not only in disease-causing microbes but in commensal microbes as well (Rosander et al., 2008; Drago et al., 2011; Allen and Stanton, 2014; von Wintersdorff et al., 2014; Card et al., 2015). Rampant antibiotic use has pushed microbes to adapt and survive by acquiring antibiotic resistance genes that led to antibiotic-resistant strains (Schjørring and Krogfelt, 2011; Forslund et al., 2013; Fouhy et al., 2013; Ghosh et al., 2013; Verraes et al., 2013; Allen and Stanton, 2014; Card et al., 2014). Antibiotic resistance genes are then vertically passed on to the next generation of microbes; in some cases, they are acquired through

horizontal transfer from one microbe to another, when thriving in the same microbial environment (Schjørring and Krogfelt, 2011; Broaders et al., 2013; Forslund et al., 2013; Fouhy et al., 2013; Ghosh et al., 2013; Verraes et al., 2013; Allen and Stanton, 2014; Card et al., 2014, 2015).

In the human clinical setting, these antibiotic-resistant pathogens have caused numerous treatment failures that eventually led to both hospital morbidities and mortalities (Vankerckhoven et al., 2008; Schjørring and Krogfelt, 2011; Lu et al., 2014). Overall, the prevalence of antibiotic resistance has now become a global health problem that needs urgent attention from the world health authorities (Egervärn et al., 2010; Schjørring and Krogfelt, 2011; Ghosh et al., 2013; Penders et al., 2013; Hu et al., 2014; Lu et al., 2014; von Wintersdorff et al., 2014; Card et al., 2015; van Schaik, 2015). In addressing the problem on antibiotic resistance, the use of probiotics in lieu of antibiotics for treating certain diseases of host organisms has been investigated (Rosander et al., 2008; Muñoz-Atienza et al., 2013). Numerous studies have shown that instead of killing pathogenic microbes through antibiotics, the establishment of commensal and sometimes mutualistic microbes may hinder the growth of disease-causing microbes found in the same host microbial environment (Saarela et al., 2007; Hammad and Shimamoto, 2010; Klein, 2011; Nueno-Palop and Narbad, 2011; Wei et al., 2012; Varankovich et al., 2015). In addition, it has also been demonstrated that maintaining what is considered "normal" microbiota for certain host microbial environments may prevent diseased conditions that are not necessarily of infectious etiology and may improve general health outcome (Franz et al., 2011; Nueno-Palop and Narbad, 2011; Wei et al., 2012; Téllez et al., 2015; Varankovich et al., 2015). As a result, probiotic use, defined as the application of actual live beneficial microbes, has been increasingly practiced for both human and veterinary applications (Tompkins et al., 2008; Vankerckhoven et al., 2008; Sanders et al., 2010; Xiao et al., 2010; Nueno-Palop and Narbad, 2011; Songisepp et al., 2012; Devi et al., 2015; D'Orazio et al., 2015; Fuochi et al., 2015; Senan et al., 2015; Varankovich et al., 2015). Among the modes of probiotic use, the consumption of probiotics through the gastrointestinal route may be considered the most common application in both human and veterinary uses.

However, microbes used as probiotics are not exempted from acquiring antibiotic resistance genes. Given their shared microbial environment in the gastrointestinal tract, a risk of pathogenic microbes acquiring antibiotic resistance genes from probiotic microbes exists, and vice versa (Mater et al., 2007; Rosander et al., 2008; Liu et al., 2009; Egervärn et al., 2010; Drago et al., 2011; Nueno-Palop and Narbad, 2011; Gueimonde et al., 2013; Varankovich et al., 2015). If improperly cooked, livestock treated with probiotics that are consumed by humans as food may also pose as a possible source of antibiotic resistance genes for the human gut microbiota (Devirgiliis et al., 2011; Schjørring and Krogfelt, 2011; Forslund et al., 2013; Verraes et al., 2013; Allen and Stanton, 2014; Hu et al., 2014; Woolhouse et al., 2015). To complicate the aforementioned risks, some probiotic microbes are even screened specifically for antibiotic resistance to be used concomitantly with antibiotics in treating certain medical conditions (Galopin et al., 2009; Hammad and Shimamoto, 2010). As such, there is a need to review existing studies to clarify the safety of increasing probiotic use in relation to the existence of antibiotic resistance genes.

This review aims to describe the processes that govern the spread of antibiotic resistance in relation to antibiotic resistance genes. Antibiotic resistance gene transfer in the absence of probiotics is discussed first to elucidate the ongoing problem of the prevalence of antibiotic-resistant bacterial strains. Probiotic uses in both human and veterinary applications are then described and reviewed to reaffirm their beneficial use. Screening of probiotic bacterial strains for antibiotic resistance genes is then discussed to evaluate the safety of probiotic use. Finally, probiotic use in relation to the spread of antibiotic resistance genes is tackled to clarify the potential role of probiotics in propagating antibiotic resistance.

ANTIBIOTIC RESISTANCE

Although the remarkable increase in the incidence and prevalence of antibiotic resistance were observed after the introduction and widespread use of antibiotics (Datta and Hughes, 1983; Hughes and Datta, 1983), antibiotic resistance is believed to have existed long before human antibiotic use (Hughes and Datta, 1983; Broaders et al., 2013). It is evident in multiple ecological interactions, wherein many organisms, may they be microbes or macro-organisms, have the ability to produce natural antibiotics that ultimately increase their chances of survival (Samuels et al., 2013; Cawoy et al., 2014; Timbermont et al., 2014; Pinchas et al., 2015; Sherpa et al., 2015; Téllez et al., 2015). Organisms use antibiotics to kill or inhibit growth of pathogenic microbes, while some microbes use antibiotics to compete for the same resources that other microbes consume as well (Samuels et al., 2013; Timbermont et al., 2014; Pinchas et al., 2015; Sherpa et al., 2015; Téllez et al., 2015). As a natural evolutionary response, microbes that are able to adapt to and survive these natural antibiotics gain advantage in producing the next generation of microbes. Hence, antibiotic resistance is a natural phenomenon (Sherpa et al., 2015).

Taking advantage of these ecological interactions, we have learned to harness the use of antibiotics for a variety of applications (Scanlon et al., 2014; Sherpa et al., 2015). We have used antibiotics to treat infectious diseases that were once considered very fatal, until the advent of readily available antibiotic medications (Sherpa et al., 2015). In agriculture, we have learned that the use of antibiotics greatly increases yield in rearing animals primarily as food source (Allen and Stanton, 2014; Woolhouse et al., 2015; Xiao et al., 2015). Although farmers were not entirely clear as to how antibiotic use increases growth rates of livestock animals, they have continually used antibiotics in sub-therapeutic doses as feeding supplements with observable results (Allen and Stanton, 2014). In addition, infectious diseases that once plagued farm animals are now treated successfully by antibiotics and even prevented by prophylactic use (Allen and Stanton, 2014; Woolhouse et al., 2015). As such, humans have multiple reasons to use antibiotics.

However, with rampant antibiotic use, the natural way of developing antibiotic resistance has also changed in both manner and rate. Due to different human applications, multiple avenues for microbes to encounter antibiotics have put immense selective pressure on microbes to develop antibiotic resistance (Rosander et al., 2008; Devirgiliis et al., 2011; Drago et al., 2011; Schjørring and Krogfelt, 2011; Forslund et al., 2013; Fouhy et al., 2013; Ghosh et al., 2013; Verraes et al., 2013; Allen and Stanton, 2014; Hu et al., 2014; von Wintersdorff et al., 2014; Card et al., 2015). In turn, these have hastened the development of antibiotic-resistant microbial strains. The rate of developing antibiotic-resistant strains is now occurring much faster than the rate of discovering new antibiotics (Sherpa et al., 2015).

Antibiotic Resistance Genes and the Influence of Ecology

Just like any evolutionary response, adaptive changes of an organism are passed on to the next generation via the organism's genetic material. Some microbes possess antibiotic resistance genes that confer their ability to survive exposure to antibiotics (Rosander et al., 2008; Drago et al., 2011; Schjørring and Krogfelt, 2011; Forslund et al., 2013; Fouhy et al., 2013; Ghosh et al., 2013; Verraes et al., 2013; Allen and Stanton, 2014; Hu et al., 2014; von Wintersdorff et al., 2014; Card et al., 2015). According to the Antibiotic Resistance Gene Database, there are at least 23,317 antibiotic genes established so far, which are effective against at least 249 known antibiotics (Hu et al., 2013). In several recent studies, investigators have shown that the presence of antibiotic resistance genes in microbial organisms is greatly affected by their microbial ecology (Forslund et al., 2013; Hu et al., 2013, 2014; Gibson et al., 2015). This observation reiterates the natural selection pressure toward antibiotic resistance brought about by ecological interactions found in the natural microbial environment (Sherpa et al., 2015).

In the study of Hu et al. (2013), investigators studied the presence of antibiotic resistance genes in the human gut microbiota. A total of 162 individuals from China, Denmark, and Spain were screened for antibiotic resistance genes. It was found that a total of 1,093 antibiotic resistance genes were present in the sample population. Antibiotic resistance genes against three antibiotic classes – tetracycline, macrolides, and beta-lactams – accounted for more than 75% of the total antibiotic resistance genes. Interestingly, antibiotic resistance genes found in Chinese individuals were statistically clustered in terms of similarity, when compared to the two European populations that had statistically more similar antibiotic resistance profiles. This suggests that the actions of human hosts as a population also affect the microbial ecology and, ultimately, the antibiotic resistance gene profiles of the gut microbiota. In support of the previous statement, a study of Lu et al. (2014) demonstrated that human populations of different age groups also produced different antibiotic resistance genes profiles that were statistically clustered by age. Hence, different activities of different human age groups also dictate antibiotic resistance profiles of human gut microbiota.

In addition, Forslund et al. (2013) showed that country-specific antibiotic use influences the antibiotic resistance genes found in the human gut microbiota. In addition to actual medical application of antibiotics, the investigators also considered the antibiotics used in livestock agriculture for food production. Investigators found that veterinary use of antibiotics also influenced antibiotic resistance genes profile of the human gut microbiota. This reaffirms the role of ecological interactions among humans, animals, and the microbial environment in influencing antibiotic resistance genes, ultimately found in the human gut microbiota (Schjørring and Krogfelt, 2011; Hu et al., 2014). As such, many studies recommend that in addressing the problem on antibiotic resistance, an ecological approach and perspective are needed as evidence suggests that antibiotic resistance is not entirely confined to human medical antibiotic use alone (Devirgiliis et al., 2011; Schjørring and Krogfelt, 2011; Forslund et al., 2013; Verraes et al., 2013; Allen and Stanton, 2014; Hu et al., 2014; Woolhouse et al., 2015). Agricultural use of antibiotics should be properly regulated as well as clinical prescription of antibiotics in humans (Verraes et al., 2013; Allen and Stanton, 2014; Woolhouse et al., 2015).

Antibiotic Resistance Genes and Their Spread through Mobile Genetic Elements

As mentioned earlier, antibiotic resistance genes are vertically transferred from one generation to another and favor the survival of resistant microbes (Schjørring and Krogfelt, 2011; Penders et al., 2013; Verraes et al., 2013; Allen and Stanton, 2014; Fouhy et al., 2014; Hu et al., 2014; Card et al., 2015; van Schaik, 2015). However, it is the ability of microbes to conduct horizontal gene transfer that somehow complements the rampant human antibiotic applications in hastening the development of antibiotic-resistant strains. Horizontal gene transfer in microbes is made possible through mobile genetic elements (Karami et al., 2007; Mater et al., 2007; De Vries et al., 2011; Drago et al., 2011; Haug et al., 2011; Broaders et al., 2013; Machado and Sommer, 2014; von Wintersdorff et al., 2014). The gastrointestinal tract is a prime candidate for conducting studies on horizontal gene transfer (Broaders et al., 2013).

In the human gastrointestinal tract, four mobile genetic elements are considered: plasmids, conjugative transposons, integrons, and bacteriophages (Broaders et al., 2013). Among mobile genetic elements, only bacterial plasmids, conjugative transposons, and integrons are considered well-established and well-documented to cause antibiotic genes transfer in both the natural environment and the clinical setting. It has been reported that antibiotic resistance genes were successfully transferred from a commensal bacterial strain to a pathogenic bacterial strain, and vice versa, using plasmids as vectors of horizontal gene transfer (Broaders et al., 2013). Using conjugative transposons, antibiotic resistance to tetracycline, chloramphenicol, kanamycin, and erythromycin are reported to be transferred in certain species of bacteria. The loss of efficacy of tetracycline against opportunistic *Bacteroides* spp. infection is specifically attributed to conjugative transposons (Broaders et al., 2013). Integrons, which function very similarly, like transposons, have been identified as the

cause of spread of antibiotic resistance in *Vibrio cholerae* (Broaders et al., 2013). On the other hand, the role of bacteriophages in promoting bacterial horizontal gene transfer is only suggested with the small but significant presence of phage-related proteins in the human gut microbial community (Broaders et al., 2013). Given the genetic-hacking machinery of viruses, bacterial horizontal gene transfer brought about by bacteriophage infection from one bacterium to another cannot be simply ruled out.

Besides mobile genetic elements, it was suggested that perhaps the most mobile that antibiotic resistance genes can be is through actual travel of human hosts carrying antibiotic-resistant strains. In the study of von Wintersdorff et al. (2014), the investigators demonstrated that international travel of 122 healthy Dutch travelers with documented fecal antibiotic resistance genes profiles caused significant increases in extended spectrum beta-lactamase-encoding genes and quinolone resistance-encoding genes found in their gut microbiota, immediately after their return to the Netherlands. Travel to Southeast Asia and travel to the Indian subcontinent were the most associated with increase in quinolone resistance-encoding genes alone, and increase in both beta-lactamase-encoding genes and quinolone resistance-encoding genes, respectively. In sum, these alarming findings suggest that human travel can contribute to the global spread of antibiotic resistance genes (von Wintersdorff et al., 2014).

Current Efforts to Control Antibiotic Resistance

Given the ecological nature of the problem on antibiotic resistance, several nations have adopted policies to address the issue. Perhaps the most notable is that of the member states of the European Union (EU). Since 2006, all EU countries have prohibited the use of antibiotics for the sole purpose of growth promotion in agricultural livestock industry (Cogliani et al., 2011). In addition, the European Food Safety Authority (EFSA) has instituted guidelines on the use of food additives in animal products that may potentially spread antibiotic resistance genes (Panel, 2012). However, other developed nations, such as the United States (US), has not imposed strict regulatory policies on antibiotic use for livestock growth promotion. Animal food and pharmaceutical industries in the US have strongly opposed restrictions in antibiotic use and have argued that such policies have been detrimental to food production in places where they were implemented. Furthermore, some countries do not have any known or established policy regarding the use of antibiotics as growth promoters, nor even require veterinary prescription for animal antibiotic use (Maron et al., 2013).

With the current paucity of clear regulatory policies in many countries, a global effort is needed in order to consolidate an effective strategy in controlling antibiotic resistance. Without a unifying regulatory guideline to follow, the problem on antibiotic resistance will most likely persist given the interweaving ecological interactions that govern the spread of antibiotic resistance. Furthermore, the spread of antibiotic resistance genes through human travel or trading of animal products between nations may make instituted regulatory policies of some countries less effective.

USE OF PROBIOTICS

As the development of antibiotic resistance strains continues, the use of probiotics as a substitute for antibiotics is becoming more popular in both the medical field and livestock agriculture (Collins and Gibson, 1999; Collado et al., 2012; Muñoz-Atienza et al., 2013; D'Orazio et al., 2015; Téllez et al., 2015; Varankovich et al., 2015). Probiotic use is defined as the actual application of live beneficial microbes to obtain a desired outcome, may it be prevention of a diseased state or improvement in general health outcome observed in the host organism (Collins and Gibson, 1999). The basis of its efficacy relies on the symbiosis of an established microbial ecology that resists the intrusion or overproduction of pathogens that lead to a diseased condition of the host organism (Catanzaro and Green, 1997). By limiting the use of antibiotics, probiotic use may help decrease the rate of development of antibiotic-resistant strains secondary to widespread and rampant antibiotic use (Collins and Gibson, 1999; Collado et al., 2012; Muñoz-Atienza et al., 2013; Varankovich et al., 2015).

Probiotics and Livestock Benefits

Many farm animals are considered to be adapted in a symbiotic relationship with microbes as shown by specialized gastrointestinal organs that enable microbial fermentation (Téllez et al., 2015). The capacity of farm animals to ferment complex polysaccharides with the help of their intestinal microbiota gives as much as 70% of energy acquired by ruminants and up to 30% of energy acquired by monogastric animals (Téllez et al., 2015). However, it is only fairly recent that this symbiotic relationship between farm animals and their microbiota is taken advantage of by farmers with the use of probiotics. With the increasing problem on antibiotic resistance, studies have shown that probiotic use can replace antibiotics in preventing diseased conditions and promoting growth in livestock animals (Muñoz-Atienza et al., 2013; Téllez et al., 2015).

Probiotic use in livestock agriculture of chickens and turkeys has been demonstrated to confer increased resistance in *Salmonella* spp. infections through accelerated establishment of what is considered normal and healthy microbiota for the aforementioned birds (Téllez et al., 2015). In addition, the incidence of idiopathic diarrhea in commercial turkey brooding houses was reported to be decreased by probiotic use (Téllez et al., 2015). As a whole, large-scale commercial trials of appropriate probiotic administration in chickens and turkeys demonstrated increased performance and reduced overall costs of production (Téllez et al., 2015).

In cattle raising, it was reported that probiotic use provided no significant effects in reducing cattle pathogens (Téllez et al., 2015). However, *Escherichia coli* O157:H7, a food-borne pathogen capable of causing severe hemorrhagic illness in humans, was known to be shed by livestock animals in their feces. With the use of different combinations of bacteria used

as probiotics, it was shown that there was decreased shedding of *E. coli* O157:H7 in both cattle and sheep livestock that may translate to decreased risk of *E. coli* O157:H7 human infections (Téllez et al., 2015).

Even in a different habitat such as in aquaculture, probiotic use has also been gaining ground. In the study of Muñoz-Atienza et al. (2013), it was demonstrated that lactic acid-producing bacteria of aquatic origin can exhibit antimicrobial activity against established gram-positive and gram-negative fish pathogens. However, no comprehensive *in vivo* assessment has yet to determine the beneficial and possible harmful effects of probiotic use in aquaculture (Muñoz-Atienza et al., 2013).

Probiotics and Human Health

For humans, probiotic use has become a popular practice for promoting good health. Many commercial food products are now being supplemented by probiotic bacteria with claims of promoting good health (Songisepp et al., 2012). It is no surprise that there is an increasing number of probiotic users as we begin to understand better the role and importance of human gut microbiota, as well as what is considered to be "normal" and symbiotic human gut microbiota (Catanzaro and Green, 1997; Collins and Gibson, 1999; Collado et al., 2012).

The complex microbiota of the human gut is considered to play important roles in several gastrointestinal functions. These functions include host nutrition, regulation of gut epithelial development, regulation of fat storage, stimulation of intestinal angiogenesis, inflammatory immune response, and pathogen resistance (Collins and Gibson, 1999; Ventura et al., 2009). In parallel comparisons with the macro-ecosystems of the natural environment, we can deduce that the more stable the composition of the human gut microbiota is, the more beneficial it is for the human host habitat. Indeed, there are expected human gut microbiota profiles that are considered to be symbiotic and are indicators of good health (Varankovich et al., 2015). This is supported by associations demonstrated between deviations in the composition of symbiotic adult pattern of human gut microbiota and a variety of human medical conditions such as inflammatory bowel disease, allergy, obesity, and atopic disease (Ventura et al., 2009).

Looking at current human medical applications, probiotic use is clinically proven to modulate infant gut microflora disturbance after antibiotic use (Collado et al., 2012). The use of antibiotics early in the infant's life is considered detrimental to infant gut microbial diversity, as antibiotics do not discriminate in killing microorganisms that are affected by the antibiotics' mechanism of action. As such, normal gut microbiota composed of commensal and beneficial microbes is decreased in the infant's gut, allowing the potential increase in the population of harmful microbes that are not affected by the antibiotics' mechanism of action. This process leads to diseased conditions associated with antibiotic use such as antibiotic-induced diarrhea (Collins and Gibson, 1999; Collado et al., 2012; Varankovich et al., 2015). In addition, perinatal and postnatal probiotic use are reported to have potential benefits in preventing future developments of allergies (Kukkonen et al., 2007), asthma (Luoto et al., 2010), gastrointestinal infections (Johnston et al., 2007), and obesity (Vliagoftis et al., 2008), as the aforementioned diseased conditions are all associated with the interplay of gut microbiota and the development of the host immune system (Collado et al., 2012). By disturbing the infant's gut microbiota, antibiotics pose a potential risk in pre-disposing infants to the aforementioned diseased conditions.

Unlike in infants, human adults with consistent oral intake of established probiotics have not yet shown clinically significant changes in adult gut microbiota composition, structure, and gene content (Ursell et al., 2013). However, for a specific medical condition, such as *Clostridium difficile*-associated diarrhea, studies have shown that probiotic use, sometimes in the extreme form of fecal transplantation, effectively addresses recurrent *C. difficile*-associated diarrhea (Ursell et al., 2013; Van den Abbeele et al., 2013; Varankovich et al., 2015). *C. difficile*-associated diarrhea is a disease associated with prolonged antibiotic use which kills normal human gut microbiota that supposedly hinders the growth of the *C. difficile* population (Van den Abbeele et al., 2013; Varankovich et al., 2015).

There are other human medical conditions now being connected to the human gut microbiota. Interestingly, these medical conditions associated with human gut microbiota are not confined to diseases with infectious etiologies (Ventura et al., 2009). Common medical conditions with established non-microbial pathophysiologies, such as obesity and diabetes mellitus, and much rarer gastrointestinal disorders, such as irritable bowel syndrome, Crohn's disease, and necrotizing enterocolitis, are presently being associated with dysbiosis in the human gut microbiota (Peterson et al., 2008; Emami et al., 2009; Serino et al., 2009; Salonen et al., 2010; Angelakis et al., 2012; Snedeker and Hay, 2012; Jeffery and O'Toole, 2013). Deviation from what is accepted to be "normal" human gut microbial ecology appears to be part of the aforementioned diseases' pathophysiologies. Therefore, more medical applications of probiotic use to maintain normal human gut microbial ecology are anticipated to appear in the near future.

SAFETY OF PROBIOTIC USE

Microbes used as probiotics are not exempted from the natural processes governing antibiotic resistance (Mater et al., 2007; Rosander et al., 2008; Chang et al., 2009; Egervärn et al., 2010; Drago et al., 2011; Nueno-Palop and Narbad, 2011; Gueimonde et al., 2013; Varankovich et al., 2015).

As such, it is imperative to screen microbes effectively for antibiotic resistance genes before using them as probiotics. A crucial aspect in studying antibiotic resistance in probiotic bacteria is to separate intrinsic resistance from acquired resistance (Sanders et al., 2010). Mechanisms of intrinsic resistance, such as active efflux of antibiotics by a bacterial outer membrane, is not governed by acquired antibiotic resistance genes (Chang et al., 2009; Hammad and Shimamoto, 2010). Focusing on acquired antibiotic resistance, random genetic changes on chromosomal genes should be further distinguished from the more likely transmissible type of antibiotic resistance.

However, to our knowledge, no unified world-wide health authority has taken full responsibility in screening for antibiotic resistance genes in probiotic microorganisms. Fortunately, research projects, such as the Biosafety Assessment of Probiotics used for Human Consumption (PROSAFE), the Assessment and Critical Evaluation of Antibiotic Resistance Transferability in Food Chain (ACE-ART), and the Joint International Organization for Standardization-International Dairy Federation Action Team on Probiotics (ISO-IDF) have individually contributed to address the issue (Sanders et al., 2010). In evaluating the safety of potential probiotic strains, two statuses are currently acceptable – Qualified Presumption of Safety (QPS) by the EFSA and Generally Recognized as Safe (GRAS) by the US-FDA. It is noteworthy that GRAS status is applied to microorganisms and microbial-derived ingredients used in food products while QPS is applied to any biological agent in the form of bacteria, fungi, or virus, that is intentionally added at different stages into the food chain. However, the QPS is considered by many as the more applicable and flexible criteria, given the emerging risk of spreading antibiotic resistance genes through probiotic strains (Sanders et al., 2010).

In the paper of Sanders et al. (2010), a case was discussed wherein the outcomes of aforementioned standards for evaluating potential probiotics were not consistent. The case involved the use of a probiotic bacteria intended for infant-formula that claims improved growth in developing infants. Both standards were aware that the probiotic strain involved carries a chromosomal gene for tetracycline resistance. Using the GRAS criteria, the US FDA evaluated the "safety of probiotic use" with "reasonable certainty," while the European counterpart cited "safety of probiotic use in light of the strength or weakness of the evidence for benefit" and "the lack of knowledge necessitates application of precautionary principles." As such, the US FDA granted GRAS status to the involved probiotic strain, while the EFSA did not grant QPS status.

Given the separate approaches in screening probiotic bacterial strains, multiple independent studies have demonstrated several methods in screening different probiotic strains for antibiotic resistance genes (Tompkins et al., 2008; Vankerckhoven et al., 2008; Chang et al., 2009; Galopin et al., 2009; Xiao et al., 2010; Klein, 2011; Nueno-Palop and Narbad, 2011; Cebrián et al., 2012; Songisepp et al., 2012; Wei et al., 2012; Tan et al., 2013; Devi et al., 2015; Senan et al., 2015). In places where it is not bound to either US FDA or EFSA, screening for antibiotic resistance genes in probiotic strains becomes even more important as rigid guidelines and regulations for probiotic use are lacking (Chang et al., 2009).

Lactic Acid Bacteria and Their Safety Profile

The most common group of bacteria used as probiotics belongs to the group of lactic acid bacteria (Gueimonde et al., 2013; Varankovich et al., 2015). Included in this group are *Lactobacillus* and *Enterococcus*. Both genera are currently extensively screened for species that can be used as probiotic bacteria (Tompkins et al., 2008; Vankerckhoven et al., 2008; Chang et al., 2009; Klein, 2011; Nueno-Palop and Narbad, 2011; Cebrián et al., 2012; Songisepp et al., 2012; Tan et al., 2013; Devi et al., 2015; Senan et al., 2015).

Lactobacillus probiotic strains are capable of improving digestion, absorption, and availability of nutrients in both livestock animals and humans (Téllez et al., 2015; Varankovich et al., 2015). They are also known to inhibit and kill *Helicobacter pylori*, a pathogen regarded as the major cause of gastritis and peptic ulcers, and is a risk factor for gastric malignancy in humans (Varankovich et al., 2015). In addition, the risk of human infectious disease due to *Lactobacillus* is considered negligible at less than one case per million individuals (Sanders et al., 2010).

Enterococcus probiotic strains are also known to be effective in reducing recovery periods of acute diarrhea in both animals and humans (Vankerckhoven et al., 2008; Varankovich et al., 2015). However, unlike *Lactobacillus*, the genus *Enterococcus* has member strains that are considered opportunistic pathogens and are sometimes the etiologic agents of some human nosocomial infections, such as bacteremia and infective endocarditis (Vankerckhoven et al., 2008; Sanders et al., 2010; Franz et al., 2011; Varankovich et al., 2015).

It has also been reported in several studies that some species of lactic acid bacteria have intrinsic resistance to bacitracin, kanamycin, teicoplanin, vancomycin, and beta-lactams (Varankovich et al., 2015). Given the beneficial effects of lactic acid bacteria, intrinsic resistance to certain antibiotics may be considered advantageous if an antibiotic-probiotic combination therapy is desired (Hammad and Shimamoto, 2010; Varankovich et al., 2015). In the study of Hammad and Shimamoto (2010), investigators deliberately screened for antibiotic-resistant probiotic strains in 40 commercially available Japanese probiotic supplements, which are to be used with a possible probiotic-antibiotic combination therapy. However, results showed no antibiotic resistance genes found in their isolates. As such, no isolated probiotic strain was reported feasible for a probiotic-antibiotic combination therapy.

Due to horizontal gene transfer, concerns are still raised particularly in lactic acid bacteria strains that carry mobile genetic elements such as plasmids (Varankovich et al., 2015). In the study of Mater et al. (2007), *in vivo* transfer of the vancomycin resistance gene, a plasmid encoded gene, was successfully demonstrated between lactic acid bacteria *Enterococcus faecium* strains and *Lactobacillus acidophilus* probiotic strains during digestive transit in mice. The results highlighted the risk of probiotics being a conduit for the spread of antibiotic resistance (Mater et al., 2007).

In the study of Chang et al. (2009), it was found that commercially available food and drugs with probiotic additives contained lactic acid bacteria strains positive for antibiotic resistance genes. Although the incidence of the antibiotic resistance genes was relatively low among the sample population, the antibiotic resistance genes found in the probiotic strains were located in mobile genetic elements such as plasmids and transposons. Despite the GRAS status of the specified lactic acid bacteria strains, the study's findings confirm the threat of spreading antibiotic resistance genes through the use of probiotics. This is especially the case in countries without established guidelines and regulations in biosafety testing and

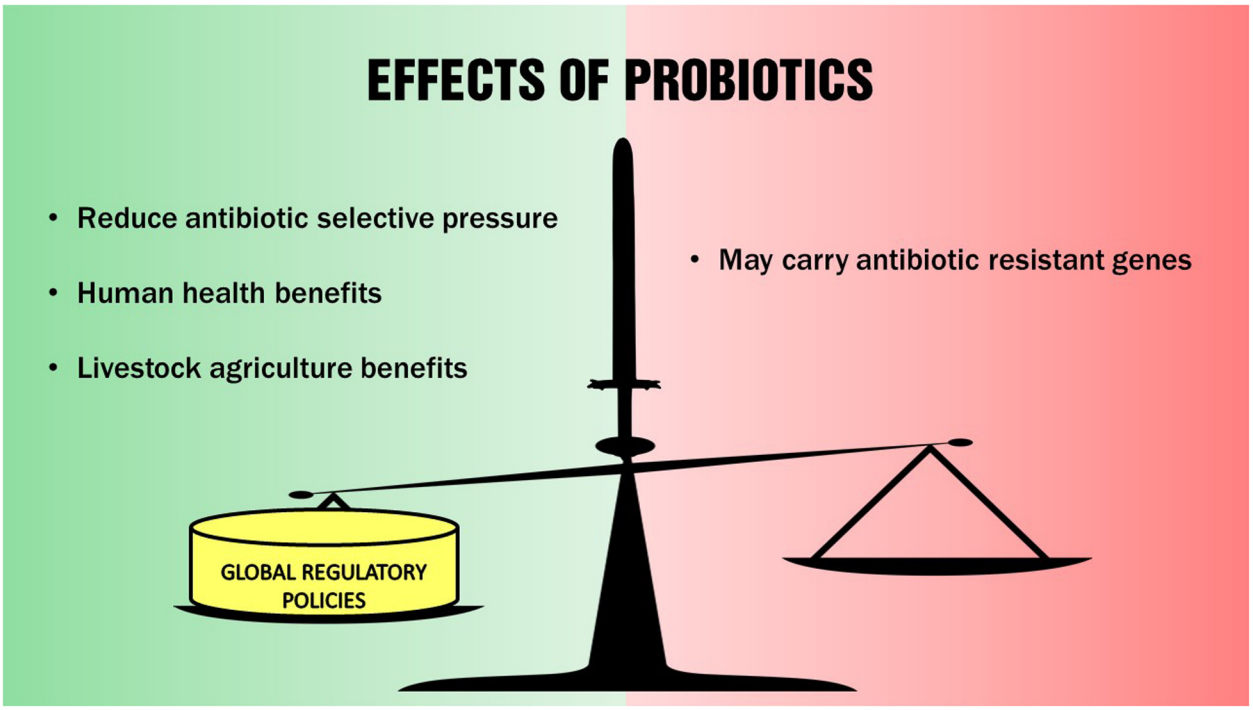

FIGURE 1 | Skewing the double-edged effects of probiotics toward positive outcomes.

rigid post-marketing surveillance. As such, a regulatory body, if not a united world-wide health authority, should always be in place to rigidly monitor probiotic use in every country (Chang et al., 2009).

Bifidobacteria and Their Safety Profile

Another group of bacteria commonly used as probiotics belong to the genus *Bifidobacterium* (Gueimonde et al., 2013; Varankovich et al., 2015). *Bifidobacterium* is a known major constituent of the gut microbiota of both animals and humans. Bifidobacteria have the capacity to metabolize non-digestable host dietary carbohydrates such as plant-derived dietary fiber.

As a probiotic, strains of *Bifidobacterium* are known to inhibit adherence of enterotoxigenic *E. coli*, enteropathogenic *E. coli*, and *C. difficile* to intestinal epithelial cells (Varankovich et al., 2015). In combination with *Lactobacillus, Bifidobacterium* was shown to alleviate side effects of *H. pylori* eradication therapy as some *Bifidobacterium* strains also suppress *H. pylori*-induced genes in human epithelial cells (Varankovich et al., 2015). In addition, *Bifidobacterium* strains are also known to alleviate infectious diarrhea as well as inflammatory bowel disease (Varankovich et al., 2015). Like *Lactobacillus*, infectious diseases due to *Bifidobacterium* are extremely rare (Gueimonde et al., 2013).

Bifidobacterium strains are known to have intrinsic resistance against ciprofloxacin, nalidixic acid, mupirocin, streptomycin, and aminoglycosides (Wei et al., 2012; Gueimonde et al., 2013; Varankovich et al., 2015). However, resistance genes for lincosamides, macrolides, streptogramin B, and tetracycline are reported to be located in transposons (Gueimonde et al., 2013). In the study of Xiao et al. (2010), investigators tested the antibiotic susceptibility of *Bifidobacterium* strains distributed in the Japanese market. A total of 23 *Bifidobacterium* strains were isolated and tested for 15 antibiotics. Results confirmed the intrinsic resistance of *Bifidobacterium* against aminoglycosides. However, *Bifidobacterium animalis* subsp. *lactis* was found to be resistant to tetracycline and was in fact harboring a tetracycline resistance gene in its chromosome. The study concluded that although there is no risk factor for safety found in *Bifidobacterium* strains distributed in the Japanese market, the presence of the tetracycline resistance gene stresses the need for future evaluation (Xiao et al., 2010).

Miscellaneous Concerns

The problem with the absence of a unified world-wide health authority that assumes responsibility for regulating probiotic use is exemplified by the case mentioned in the paper of Sanders et al. (2010). In countries with no established regulating body for probiotic use, the result is even worse as exemplified by the study of Chang et al. (2009). Keeping in mind that the likelihood of the spread of antibiotic resistance genes through human international travel was clearly indicated in the study of von Wintersdorff et al. (2014), we believe that a united global effort to screen probiotics that are marketed for human consumption is imperative.

In addition, given the established ecological nature of the emergence of antibiotic resistance problem, it is deemed logical to deduce that regulating probiotic use should also involve an

ecological approach. It should be noted that studies involving the regulation of probiotic use in animals are lacking when compared to those of human applications. We should be reminded that numerous studies have already shown the connection between antibiotic resistance genes in animals and those in humans (Devirgiliis et al., 2011; Schjørring and Krogfelt, 2011; Forslund et al., 2013; Verraes et al., 2013; Allen and Stanton, 2014; Hu et al., 2014; Woolhouse et al., 2015). Without addressing regulations in animal probiotic applications, efforts in regulating human probiotic use might be considered inadequate in the end.

CONCLUSION

The emergence of antibiotic resistant pathogens through the spread of antibiotic resistance genes is an ecological problem that is exacerbated by the widespread indiscriminate use of antibiotics in livestock agriculture, and in veterinary and human medicine. The use of probiotics in lieu of antibiotics to control some diseases in animals and humans may reduce the antibiotic selective pressures on microorganisms in our natural environments and contribute in reducing the problem of the rapid emergence of antibiotic resistant pathogens. However, probiotic bacterial strains used in both animal and human applications also have risks in becoming conduits themselves in spreading antibiotic resistance genes. We conclude that the use of probiotics to address the global problem of emerging antibiotic resistant microorganisms is a "double-edged" sword – with both beneficial effects and associated risks as depicted in **Figure 1**. Therefore, although probiotics are currently generally regarded as safe, we think that it is imperative to implement proper regulation on their use in both livestock and human applications globally to effectively mitigate their potential contribution in the spread of antibiotic resistance genes in our natural environments.

AUTHOR CONTRIBUTIONS

II and JI conceptualized and wrote the manuscript.

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frontiers in Microbiology

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Characterization of Integrons and Resistance Genes in *Salmonella* Isolates from Farm Animals in Shandong Province, China

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A total of 154 non-duplicate *Salmonella* isolates were recovered from 1,105 rectal swabs collected from three large-scale chicken farms (78/325, 24.0%), three large-scale duck farms (56/600, 9.3%) and three large-scale pig farms (20/180, 11.1%) between April and July 2016. Seven serotypes were identified among the 154 isolates, with the most common serotype in chickens and ducks being *Salmonella* enteritidis and in pigs *Salmonella* typhimurium. Antimicrobial susceptibility testing revealed that high antimicrobial resistance rates were observed for tetracycline (72.0%) and ampicillin (69.4%) in all sources. Class 1 integrons were detected in 16.9% (26/154) of these isolates and contained gene cassettes *aadA2*, *aadA1*, *drfA1-aadA1*, *drfA12-aadA2*, and *drfA17-aadA5*. Three β-lactamase genes were detected among the 154 isolates, and most of the isolates carried bla_{TEM-1}(55/154), followed by bla_{PSE-1}(14/154) and $bla_{CTX-M-55}$ (11/154). Three plasmid-mediated quinolone resistance genes were detected among the 154 isolates, and most of the isolates carried *qnrA* (113/154), followed by *qnrB* (99/154) and *qnrS* (10/154). *Fifty-four* isolates carried *floR* among the 154 isolates. Multilocus sequence typing (MLST) analysis showed that nine sequence types (STs) were identified; ST11 was the most frequent genotype in chickens and ducks, and ST19 was identified in pigs. Our findings indicated that *Salmonella* was widespread, and the overuse of antibiotics in animals should be reduced considerably in developing countries.

Keywords: *Salmonella*, antimicrobial susceptibility, class 1 integron, antimicrobial resistance gene, MLST

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INTRODUCTION

Salmonella is an important source of foodborne diseases that cause morbidity and mortality worldwide. Among 94 million cases of non-typhoid *Salmonella* infections, it was presumed that approximately 85% of the cases were induced by food origin *Salmonella* (Chiu et al., 2010). In China, *Salmonella* causes an estimated 22.2% of foodborne diseases (Wang et al., 2007). Many *Salmonella* serovars exist. More than 2,600 serovars are classified based on the reactivity of antisera to O and H antigens (Stevens et al., 2009), and the serovars from farms have a significant overlap with those causing illnesses in humans (Alcaine et al., 2006). Animals have been recognized as an important reservoir for *Salmonella*, and this pathogen can be transferred to humans via the food chain, posing a serious threat to human health (Vo et al., 2006).

The use of antimicrobials is important for the control and treatment of *Salmonella*. However, antimicrobial- and multidrug-resistant *Salmonella* strains have emerged, leading to treatment failure (Gong et al., 2013). The increasing prevalence of multidrug-resistance among *Salmonella*, not only against the front-line antimicrobials, chloramphenicol and trimethoprim/sulfamethoxazole but also against clinically important antimicrobial agents, such as β-lactams and fluoroquinolones, is also an emerging problem (Lunguya et al., 2013).

The spread of the antibiotic resistant potential in *Salmonella* is mainly attributed to integrons. Integrons are DNA elements, capable of capturing antimicrobial resistant genes and disseminating them using a mobile genetic element (MGE) such as a plasmid among bacteria. The class I integron is the most common integron type identified in multidrug-resistant (MDR) *Salmonella* and plays an important role in the dissemination of resistance genes among pathogens (Wright, 2010).

In developed countries, many surveys have been conducted at the molecular level to monitor the incidence of antibiotic-resistant *Salmonella* in animal farms (Melendez et al., 2010; Graciela et al., 2016). However, the extent of antibiotic-resistant *Salmonella* in many developing countries and the molecular mechanisms underlying this resistance remain unclear. Therefore, we selected large-scale animal farms as sample sites, collected swab samples, isolated *Salmonella* and characterized the molecular mechanisms of antimicrobial resistance.

MATERIALS AND METHODS

Samples and *Salmonella* Isolation

From April to July 2016, rectal swabs were collected from healthy animals on farms in Qingdao, Jinan and Zibo regions in Shandong Province, China. All of the sampling sites were visited only once. In total, 1,105 samples were collected in a random manner from chickens ($n = 325$), ducks ($n = 600$), and pigs ($n = 180$). The samples were independently collected from individual animals, and the sample collection conformed to the cluster random sampling principle. Farms were chosen based on their scale with the following requirements: for chickens, the breeding stock was >150,000 heads; for ducks, the breeding stock was >100,000 heads, and for pigs, the breeding stock was >1,000 heads. The owners of each farm gave permission for rectal swab samples to be collected. The animals from which samples were extracted remained alive and did not undergo any surgery. Therefore, ethical approval was not required for the study because the sampling process did not harm the animals. All of the collected samples were transported in an ice box to our laboratory within 6 h for further bacteriological analysis.

Isolation and identification of *Salmonella* were performed as described previously (Yan et al., 2010), with some modifications. Briefly, swabbing samples were placed into a sterile plastic bag containing 100 ml of buffered peptone water (BPW) and mixed vigorously for 3 min. The BPW mixture was then incubated for 24 h at 37°C for pre-enrichment. Approximately 1 ml of pre-enrichment cultures were incubated in 10 ml of selenite cysteine (SC) broth and 10 ml of rappaport-vassiliadis (RV) broth at 42°C for 24 h, respectively. After selective enrichment, a loop-full of SC and RV broth cultures were streaked onto xylose lysine tergitol 4 (XLT4) agar and incubated at 37°C overnight. A minimum of two presumptive *Salmonella* colonies was confirmed by PCR using a previously described method (Malorny et al., 2003).

Salmonella Serotyping

According to the manufacturer's instructions, the serogroup and serovars of *Salmonella* isolates were determined according to the Kauffmann-White scheme by slide agglutination with O and H antigens (Tianrun Bio-Pharmaceutical, Ningbo, China).

Antimicrobial Susceptibility Testing

A minimal inhibition concentration (MIC) assay, as described by the Clinical and Laboratory Standard Institute (Clinical Laboratory Standards Institute, 2013), was used in this study to test the susceptibility of 12 commonly used antibiotics (**Table 1**), including ampicillin (AMP), amikacin (AMK), enrofloxacin (ENO), ciprofloxacin (CIP), nalidixic acid (NA), florfenicol (FFN), tetracycline (TET), ceftiofur (CEF), gentamicin (GEN), neomycin (NEO), levofloxacin (LVX), and fosfomycin (FOS). *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 700603) were used as the quality control strains in this study. *Salmonella* isolates resistant to more than three classes of antimicrobials were defined as MDR isolates.

Detection of Class I Integrons and Antimicrobial Resistance Genes

Bacterial DNA was extracted using a TIANamp Bacteria DNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Conserved primers were used for the detection and identification of class I integrons using previously described primers and procedures (Kerrnet et al., 2002). PCR screening for β-lactamase-encoding genes bla_{TEM}, $bla_{\mathrm{PSE-1}}$, bla_{SHV}, and $bla_{\mathrm{CTX-M}}$ was performed as previously described (Li et al., 2013).

TABLE 1 | Antimicrobials and the range of concentrations tested.

Antimicrobials	Abbreviation	Concentration range (μg/mL)
Ampicillin	AMP	0.06∼256
Amikacin	AMK	0.5∼512
Enrofloxacin	ENO	0.06∼512
Ciprofloxacin	CIP	0.015∼512
Nalidixic acid	NA	0.06∼512
Florfenicol	FFN	0.5∼512
Tetracycline	TET	0.5∼512
Ceftiofur	CEF	0.06∼512
Gentamicin	GEN	0.5∼512
Neomycin	NEO	0.5∼512
Levofloxacin	LVX	0.06∼512
Fosfomycin	FOS	1∼2.048

Furthermore, PCR amplification was used to screen for plasmid-mediated quinolone resistance genes, *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*, which were the most frequently observed in China, using previously described primers (Ahmed et al., 2013). Finally, the florfenicol resistance gene, *floR*, was detected using previously described primers (Ahmed et al., 2013). The PCR products were purified and subsequently sequenced (Invitrogen, Beijing, China). The obtained DNA sequences were compared with those in GenBank using Basic Local Alignment Search Tool (BLAST).

MLST

Seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) were used to characterize *Salmonella* by MLST. MLST was performed as described online (http://mlst.warwick.ac.uk/mlst/dbs/Senterica/documents/primersEnterica_html). All polymerase chain reaction products were purified and sequenced (Invitrogen, Beijing, China), and the alleles and STs were assigned according to the MLST scheme at http://mlst.warwick.ac.uk/mlst/dbs/Senterica.

Data Analysis

The statistical package SPSS (version 15.0, SPSS, Chicago, IL, USA) was used to compare the prevalence and MDR resistance rate of *Salmonella* isolated from chickens, ducks and pigs, and a *P*-value less than 0.05 was considered significant.

RESULTS

Prevalence and Serotypes of *Salmonella*

In this study, a total of 154 non-duplicate *Salmonella* isolates (154/1105, 13.9%) were recovered. From chickens, 78 *Salmonella* isolates were recovered (78/325, 24.0%) (**Table 2**), which was significantly higher than the *Salmonella* isolated from ducks and pigs ($P < 0.05$). Seventy-eight *Salmonella* isolates were divided into six serovars. The most common serovar was *Salmonella* enteritidis (69/78, 88.5%) (**Table 3**).

From ducks, 56 *Salmonella* isolates were recovered (56/600, 9.3%) (**Table 2**), and they were divided into two serovars. The most common serovar was *Salmonella* enteritidis (38/56, 67.9%) (**Table 3**).

From pigs, 20 *Salmonella* isolates were recovered (20/180, 11.1%) (**Table 2**), and they were divided into three serovars. The most common serovar was *Salmonella* typhimurium (13/20, 65.0%) (**Table 3**).

Antimicrobial Susceptibility Testing

Among 78 isolates from chickens, they were susceptible to amikacin, levofloxacin and fosfomycin. Most isolates were resistant to ampicillin (69/78, 88.5%) and tetracycline (61/78, 78.2%). In addition, 63 isolates (63/78, 80.8%) exhibited MDR (**Table 3**).

Among 56 isolates from ducks, they were susceptible to amikacin, levofloxacin and fosfomycin. Most isolates were resistant to tetracycline (52/56, 92.9%) and ciprofloxacin (45/56, 80.4%). In addition, 50 isolates (50/56, 89.3%) exhibited MDR (**Table 3**), which was significantly higher than the *Salmonella* isolated from chickens and pigs ($P < 0.05$).

Among 20 isolates from pigs, they were susceptible to amikacin and levofloxacin. Most isolates were resistant to ampicillin (15/20, 75.0%) and tetracycline (9/20, 45.0%). In addition, 9 isolates (9/20, 45.0%) exhibited MDR (**Table 3**).

Characteristics of Class I Integrons and Antimicrobial Resistance Genes

Among the 78 isolates recovered from chickens, 17 isolates (17/78, 21.8%) contained four groups of resistance gene cassettes, consisting of *drfA1-aadA1* (1.7 kb, $n = 7$), *aadA2* (1.2 kb, $n = 5$), *drfA17-aadA5* (2 kb, $n = 3$), and *aadA1* (1.2 kb, $n = 2$). Three β-lactamase genes were detected among the isolates, and $bla_{\mathrm{TEM-1}}$ ($n = 25$) was the most commonly isolated β-lactamase gene, followed by $bla_{\mathrm{PSE-1}}$ ($n = 7$) and $bla_{\mathrm{CTX-M-55}}$ ($n = 4$). Three plasmid-mediated quinolone resistance genes were detected among the isolates. *qnrA* ($n = 53$) was the most commonly isolated plasmid-mediated quinolone resistance gene, followed by *qnrB* ($n = 44$) and *qnrS* ($n = 7$). In addition, 23 isolates carried *floR* (**Table 3**).

Among the 56 isolates recovered from ducks, eight isolates (8/56, 14.3%) contained three groups of resistance gene cassettes, consisting of *aadA2* (1.2 kb, $n = 4$), *drfA1-aadA1* (1.7 kb, $n = 3$), and *drfA12-aadA2* (2 kb, $n = 1$). Three β-lactamase genes were detected among the isolates. $bla_{\mathrm{TEM-1}}$ was the most commonly isolated β-lactamase gene ($n = 20$), followed by $bla_{\mathrm{PSE-1}}$($n = 2$) and $bla_{\mathrm{CTX-M-55}}$ ($n = 1$). Three plasmid-mediated quinolone resistance genes were detected among the isolates. *qnrA* was the most commonly isolated plasmid-mediated quinolone resistance gene ($n = 44$), followed by *qnrB* ($n = 40$) and *qnrS* ($n = 2$). In addition, 13 isolates carried *floR* (**Table 3**).

Among the 20 isolates recovered from pigs, one isolate (1/20, 5.0%) contained one group of a resistance gene cassette, consisting of *aadA2* (1.2 kb, $n = 1$). Three β-lactamase genes were

TABLE 2 | Prevalence of *Salmonella* isolates from farm animals.

	Chicken		Duck		Pig	
Locations	**No. of samples**	**No. of posotive samples (%)**	**No. of samples**	**No. of posotive samples (%)**	**No. of samples**	**No. of posotive samples (%)**
Qingdao	100	17 (17%)	200	22 (11.0%)	60	7 (11.7%)
Jinan	115	34 (29.6%)	200	19 (9.5%)	60	8 (13.3%)
Zibo	110	27 (24.5%)	200	15 (7.5%)	60	5 (8.3%)
Total	325	78 (24.0%)	600	56 (9.3%)	180	20 (11.1%)

TABLE 3 | Resistance phenotype, ST, incidence of class I integron, and resistance gens in *Salmonella* isolated from animals in farms.

No.	Location	Farms	Serovar	ST	Resistance phenotype	Integrons/resistance genes
1	Qingdao	Chicken	*S.* Enteritidis	11	AMP, CEF, ENO, TET	bla_{TEM-1},*qnrA*
2	Qingdao	Chicken	*S.* Enteritidis	11	AMP, TET	*qnrA*
3	Qingdao	Chicken	*S.* Enteritidis	11	AMP, CEF, NA, NEO, TET	Class I (*drfA1-aadA1*), bla_{TEM-1},*qnrA*, *qnrS*
4	Qingdao	Chicken	*S.* Enteritidis	11	AMP, ENO, NA, TET	bla_{TEM-1}, *qnrA*, *qnrB*
5	Qingdao	Chicken	*S.* Enteritidis	11	AMP, CEF, CIP, ENO, GEN, NA, NEO	Class I (*aadA2*), bla_{PSE-1}, *qnrB*, *qnrS*
6	Qingdao	Chicken	*S.* Enteritidis	11	AMP, CIP, ENO, FFN, GEN, NA, NEO	bla_{TEM-1}, $bla_{CTX-M-55}$, *qnrB*, *floR*
7	Qingdao	Chicken	*S.* Enteritidis	11	AMP, TET	bla_{TEM-1}
8	Qingdao	Chicken	*S.* Enteritidis	11	AMP, ENO, NA, TET	$bla_{CTX-M-55}$, *qnrB*
9	Qingdao	Chicken	*S.* Enteritidis	11	AMP, CEF, CIP, ENO, NA, TET	*qnrA*, *qnrB*
10	Qingdao	Chicken	*S.* Enteritidis	11	AMP, CIP, TET	bla_{TEM-1},*qnrA*, *qnrB*
11	Qingdao	Chicken	*S.* Indiana	17	AMP, CEF, CIP, ENO, FFN, NA, TET	Class I (*aadA2*), bla_{TEM-1}, *qnrA*, *qnrB*, *qnrS*, *floR*
12	Qingdao	Chicken	*S.* Enteritidis	11	AMP, CEF, ENO, GEN, NA, TET	bla_{PSE-1}, *qnrA*, *qnrS*
13	Qingdao	Chicken	*S.* Enteritidis	11		
14	Qingdao	Chicken	*S.* Enteritidis	11	AMP, CIP, TET	*qnrA*
15	Qingdao	Chicken	*S.* Enteritidis	11	AMP, NA	*qnrA*
16	Qingdao	Chicken	*S.* Enteritidis	11	AMP, ENO, NA, TET	bla_{TEM-1}, *qnrA*, *qnrB*
17	Qingdao	Chicken	*S.* Enteritidis	11	NA	
18	Jinan	Chicken	*S.* Enteritidis	11	AMP, CIP, TET	*qnrA*
19	Jinan	Chicken	*S.* Thompson	26	AMP, CEF, GEN, NA, TET	*qnrA*, *qnrB*
20	Jinan	Chicken	*S.* Enteritidis	11	AMP, CIP, ENO, FFN, GEN, NA, NEO	Class I (*aadA2*), bla_{TEM-1}, *qnrA*, *qnrB*, *floR*
21	Jinan	Chicken	*S.* Enteritidis	11	AMP, CEF, CIP, NA, TET	bla_{TEM-1}, *qnrA*, *qnrB*
22	Jinan	Chicken	*S.* Enteritidis	11	AMP, ENO, NA, TET	*qnrA*
23	Jinan	Chicken	*S.* Enteritidis	11	AMP, TET	
24	Jinan	Chicken	*S.* Enteritidis	11	AMP, CIP, ENO, FFN, GEN, NA, NEO	Class I (*aadA2*), bla_{TEM-1}, *qnrA*, *qnrB*, *floR*
25	Jinan	Chicken	*S.* Enteritidis	11	AMP, CEF, FFN, ENO, NA, TET	Class I (*aadA2*), *qnrA*, *qnrB*, *floR*
26	Jinan	Chicken	*S.* Thompson	26	AMP, ENO, NA, NEO, TET	bla_{TEM-1}, *qnrA*, *qnrB*
27	Jinan	Chicken	*S.* Enteritidis	11	AMP, CEF, FFN, NEO, NA, TET	Class I (*drfA1-aadA1*), bla_{PSE-1}, *qnrA*, *floR*
28	Jinan	Chicken	*S.* Enteritidis	11	AMP, CEF, NA, NEO, TET	*qnrA*, *qnrB*
29	Jinan	Chicken	*S.* Enteritidis	11		
30	Jinan	Chicken	*S.* Enteritidis	11	AMP, CEF, FFN, NEO, NA, TET	Class I (*aadA1*), bla_{TEM-1}, *qnrA*, *floR*
31	Jinan	Chicken	*S.* Enteritidis	11	AMP, CEF, CIP, ENO, NA, TET	*qnrA*, *qnrB*
32	Jinan	Chicken	*S.* Enteritidis	11		
33	Jinan	Chicken	*S.* Enteritidis	11	AMP, GEM, NA, TET	*qnrA*
34	Jinan	Chicken	*S.* Typhimurium	19	AMP, CEF, CIP, FFN, NA, TET	*qnrB*, *floR*
35	Jinan	Chicken	*S.* Enteritidis	11	AMP, CEF, ENO, TET	*qnrA*
36	Jinan	Chicken	*S.* Enteritidis	11	AMP, CEF, CIP, FFN, NA, TET	Class I (*drfA17-aadA5*), bla_{PSE-1}, *qnrA*, *qnrB*, *floR*
37	Jinan	Chicken	*S.* Enteritidis	11	AMP, CIP, TET	*qnrA*, *qnrB*
38	Jinan	Chicken	*S.* Enteritidis	11	AMP, CEF, CIP, ENO, FFN, GEN, NA	Class I (*drfA17-aadA5*), bla_{TEM-1}, bla_{PSE-1}, *qnrA*, *qnrB*, *floR*
39	Jinan	Chicken	*S.* Enteritidis	11	TET	
40	Jinan	Chicken	*S.* Enteritidis	11	AMP, ENO, NA, TET	*qnrA*, *qnrB*
41	Jinan	Chicken	*S.* Enteritidis	11	AMP, CIP, TET	bla_{TEM-1}, *qnrA*, *qnrB*, *floR*
42	Jinan	Chicken	*S.* Enteritidis	11	AMP, CIP, TET	*qnrA*, *qnrB*
43	Jinan	Chicken	*S.* Enteritidis	11	AMP, CEF, FFN, NA, TET	*qnrA*, *qnrB*, *floR*
44	Jinan	Chicken	*S.* Enteritidis	11	AMP, ENO, NA, TET	*qnrB*
45	Jinan	Chicken	*S.* Enteritidis	11	NA	*qnrB*
46	Jinan	Chicken	*S.* Enteritidis	11	AMP, ENO, NA, TET	bla_{PSE-1}, *qnrA*, *qnrB*
47	Jinan	Chicken	*S.* Enteritidis	11	AMP, CIP, TET	*qnrA*
48	Jinan	Chicken	*S.* Enteritidis	11		
49	Jinan	Chicken	*S.* Enteritidis	11	AMP, CIP, TET	*qnrA*, *qnrB*
50	Jinan	Chicken	*S.* Enteritidis	11	AMP, ENO, NA, TET	*qnrA*, *qnrB*

(Continued)

TABLE 3 | Continued

No.	Location	Farms	Serovar	ST	Resistance phenotype	Integrons/resistance genes
51	Jinan	Chicken	*S.* Indiana	17	AMP, CEF, CIP, ENO, FFN, NA, TET	Class I (*drfA17-aadA5*), *qnrA*, *qnrB*, *floR*
52	Zibo	Chicken	*S.* Enteritidis	11	AMP, CEF, ENO, GEN, NA, TET	Class I (*aadA1*), *qnrA*, *qnrB*, *qnrS*
53	Zibo	Chicken	*S.* Agona	28	AMP, CEF, ENO, NA, TET	bla_{TEM-1}, *qnrA*, *qnrB*
54	Zibo	Chicken	*S.* Enteritidis	11	AMP, CEF, ENO, FFN, GEN, NA, TET	Class I (*drfA1-aadA1*), bla_{TEM-1}, $bla_{CTX-M-55}$, *qnrA*, *qnrS*, *floR*
55	Zibo	Chicken	*S.* Senftenberg	14	AMP, ENO, NA, TET	*qnrA*, *qnrB*
56	Zibo	Chicken	*S.* Enteritidis	11	AMP, CEF, FFN, NEO, NA, TET	Class I (*drfA1-aadA1*), *qnrB*, *floR*
57	Zibo	Chicken	*S.* Enteritidis	11	AMP, CIP, TET	$bla_{CTX-M-55}$, *qnrA*
58	Zibo	Chicken	*S.* Enteritidis	11	AMP, CEF, ENO, NEO, NA, TET	bla_{TEM-1}, *qnrA*, *floR*
59	Zibo	Chicken	*S.* Enteritidis	11	AMP, CEF, FFN, GEN, NA, TET	bla_{TEM-1}, bla_{PSE-1}, *qnrA*, *floR*
60	Zibo	Chicken	*S.* Enteritidis	11	AMP, CIP, TET	*qnrA*, *qnrB*
61	Zibo	Chicken	*S.* Enteritidis	11	AMP, ENO, NA, TET	*qnrB*
62	Zibo	Chicken	*S.* Enteritidis	11		
63	Zibo	Chicken	*S.* Enteritidis	11	AMP, ENO, NA, TET	*qnrA*
64	Zibo	Chicken	*S.* Enteritidis	11	AMP, TET	
65	Zibo	Chicken	*S.* Enteritidis	11	AMP, ENO, NA, TET	bla_{TEM-1}
66	Zibo	Chicken	*S.* Enteritidis	11	AMP, ENO, NA, TET	*qnrA*
67	Zibo	Chicken	*S.* Enteritidis	11	AMP, CEF,GEN, NA, TET	bla_{TEM-1}, *qnrB*, *floR*
68	Zibo	Chicken	*S.* Enteritidis	11	AMP, CIP, TET	*qnrB*
69	Zibo	Chicken	*S.* Enteritidis	11	AMP, CEF, NA, NEO, TET	*qnrA*, *qnrB*, *floR*
70	Zibo	Chicken	*S.* Enteritidis	11		
71	Zibo	Chicken	*S.* Enteritidis	11	AMP, CIP, TET	
72	Zibo	Chicken	*S.* Enteritidis	11	AMP, NA	
73	Zibo	Chicken	*S.* Indiana	17	AMP, CEF, CIP, FFN, GEN, NA, TET	Class I (*drfA1-aadA1*), bla_{TEM-1}, *qnrA*, *floR*
74	Zibo	Chicken	*S.* Enteritidis	11	AMP, CEF, CIP, ENO, GEN, NA, NEO	bla_{TEM-1}, *qnrA*, *qnrB*
75	Zibo	Chicken	*S.* Indiana	17	AMP, CEF, CIP, ENO, FFN, GEN, NA	Class I (*drfA1-aadA1*), bla_{TEM-1}, *qnrA*, *qnrS*, *floR*
76	Zibo	Chicken	*S.* Enteritidis	11	AMP, CEF, CIP, FFN, GEN, TET	*qnrA*, *qnrB*, *floR*
77	Zibo	Chicken	*S.* Enteritidis	11	AMP, CEF, CIP, FFN, GEN, TET	Class I (*drfA1-aadA1*), bla_{TEM-1}, *qnrB*, *floR*
78	Zibo	Chicken	*S.* Enteritidis	11	AMP, GEN, NA, TET	*qnrA*, *qnrB*
79	Qingdao	Duck	*S.* Typhimurium	34	CIP, ENO, GEN, NA, NEO, TET	*qnrA*, *qnrB*
80	Qingdao	Duck	*S.* Typhimurium	34	CIP, NA, NEO, TET	*qnrA*
81	Qingdao	Duck	*S.* Typhimurium	19	AMP	bla_{TEM-1}
82	Qingdao	Duck	*S.* Enteritidis	11	AMP, CEF, CIP, ENO, FFN, NEO, GEN, NA, TET	Class I (*drfA1-aadA1*), bla_{TEM-1}, *qnrB*, *floR*
83	Qingdao	Duck	*S.* Enteritidis	11	AMP	bla_{TEM-1}
84	Qingdao	Duck	*S.* Typhimurium	19	AMP, NA	bla_{TEM-1}, *qnrA*, *qnrB*
85	Qingdao	Duck	*S.* Enteritidis	11	AMP, CEF, ENO, GEN, NA, NEO, TET	Class I (*drfA1-aadA1*), bla_{TEM-1}, $bla_{CTX-M-55}$, *qnrB*
86	Qingdao	Duck	*S.* Enteritidis	11	CEF, CIP, ENO, FFN, GEN, NA, TET	Class I (*aadA2*), *qnrA*, *qnrB*
87	Qingdao	Duck	*S.* Enteritidis	11	AMP, TET	bla_{TEM-1}
88	Qingdao	Duck	*S.* Enteritidis	11		bla_{TEM-1}
89	Qingdao	Duck	*S.* Typhimurium	34	CIP, ENO, GEN, NA, NEO, TET	*qnrA*, *qnrB*
90	Qingdao	Duck	*S.* Enteritidis	11	CEF, CIP, ENO, FFN, GEN, NA, TET	*qnrA*, *floR*
91	Qingdao	Duck	*S.* Enteritidis	11	AMP, CEF, CIP, ENO, FFN, NEO, GEN, NA, TET	*qnrA*, *qnrB*, *floR*
92	Qingdao	Duck	*S.* Typhimurium	34	CEF, CIP, ENO, GEN, NA, NEO, TET	Class I (*aadA2*), *qnrB*
93	Qingdao	Duck	*S.* Enteritidis	11	CEF, CIP, ENO, FFN, GEN, NA, NEO, TET	*qnrA*
94	Qingdao	Duck	*S.* Enteritidis	11	CEF, CIP, ENO, FFN, GEN, NA, TET	*qnrA*, *qnrB*
95	Qingdao	Duck	*S.* Enteritidis	11	AMP, CEF, CIP, GEN, NA, NEO, TET	bla_{TEM-1}, *qnrA*
96	Qingdao	Duck	*S.* Enteritidis	11	CEF, CIP, ENO, NA, TET	*qnrA*, *qnrB*
97	Qingdao	Duck	*S.* Enteritidis	11	CEF, CIP, ENO, NA, NEO, TET	*qnrA*
98	Qingdao	Duck	*S.* Enteritidis	11	CIP, ENO, GEN, NA, TET	*qnrA*, *qnrB*

(Continued)

TABLE 3 | Continued

No.	Location	Farms	Serovar	ST	Resistance phenotype	Integrons/resistance genes
99	Qingdao	Duck	*S.* Enteritidis	11	CEF, CIP, ENO, FFN, GEN, NA, NEO, TET	bla_{TEM-1}, *qnrA*, *floR*
100	Qingdao	Duck	*S.* Enteritidis	11	CEF, CIP, NA, TET	*qnrB*
101	Jinan	Duck	*S.* Typhimurium	19	CIP, ENO, FFN, GEN, NA, NEO, TET	*qnrA*, *floR*
102	Jinan	Duck	*S.* Enteritidis	11	AMP, CIP, ENO, FFN, NA, NEO, TET	bla_{TEM-1}, *qnrB*
103	Jinan	Duck	*S.* Typhimurium	19	AMP, CEF, CIP, NA, NEO, TET	*qnrA*
104	Jinan	Duck	*S.* Typhimurium	19	CIP, NA, NEO, TET	*qnrA*, *qnrB*
105	Jinan	Duck	*S.* Typhimurium	19	AMP, CEF, ENO, GEN, NA, NEO, TET	Class I (*aadA2*), bla_{TEM-1}, *qnrB*
106	Jinan	Duck	*S.* Typhimurium	19	CIP, ENO, GEN, NA, TET	*qnrA*, *qnrB*
107	Jinan	Duck	*S.* Enteritidis	11	CEF, CIP, ENO, FFN, GEN, NA, NEO, TET	*qnrA*, *qnrB*
108	Jinan	Duck	*S.* Enteritidis	11	AMP, CEF, CIP, ENO, FFN, GEN, NA, TET	bla_{TEM-1}, *qnrA*, *floR*
109	Jinan	Duck	*S.* Enteritidis	11	CIP, ENO, FFN, GEN, NA, TET	*qnrA*, *qnrB*
110	Jinan	Duck	*S.* Enteritidis	11	CEF, CIP, ENO, FFN, GEN, NA, TET	*qnrA*, *qnrB*, *floR*
111	Jinan	Duck	*S.* Enteritidis	11	AMP, CEF, CIP, ENO, NA, TET	bla_{TEM-1}, *qnrB*
112	Jinan	Duck	*S.* Enteritidis	11	AMP, CEF, CIP, GEN, NA, NEO, TET	bla_{TEM-1}, bla_{PSE-1}, *qnrA*
113	Jinan	Duck	*S.* Enteritidis	11	CEF, CIP, ENO, FFN, GEN, NA, TET	*qnrA*, *qnrB*, *floR*
114	Jinan	Duck	*S.* Enteritidis	11	AMP, CEF, NA, NEO, TET	bla_{TEM-1}, *qnrA*, *qnrB*
115	Jinan	Duck	*S.* Enteritidis	11	CEF, CIP, ENO, FFN, GEN, NA, NEO, TET	*qnrA*, *qnrB*, *floR*
116	Jinan	Duck	*S.* Enteritidis	11	CIP, ENO, GEN, NA, TET	*qnrA*, *qnrB*
117	Jinan	Duck	*S.* Enteritidis	11	CIP, GEN, NA, TET	*qnrA*, *qnrB*
118	Jinan	Duck	*S.* Enteritidis	11	AMP, CEF, CIP, NA, NEO, TET	Class I (*aadA2*), bla_{TEM-1}, *qnrA*, *qnrB*
119	Jinan	Duck	*S.* Enteritidis	11	CIP, ENO, GEN, NA, TET	*qnrA*, *qnrB*
120	Zibo	Duck	*S.* Enteritidis	11	CEF, CIP, ENO, FFN, GEN, NA, TET	*qnrA*, *qnrB*, *floR*
121	Zibo	Duck	*S.* Enteritidis	11	AMP, CEF, CIP, ENO, FFN, NEO, GEN, NA, TET	Class I (*drfA1-aadA1*), bla_{TEM-1}, *qnrA*, *qnrB*, *floR*
122	Zibo	Duck	*S.* Enteritidis	11	AMP, CEF, FFN, GEN, NA, NEO, TET	*qnrA*, *qnrB*
123	Zibo	Duck	*S.* Typhimurium	19	AMP, CEF, CIP, FFN, NA, NEO, TET	*qnrA*, *qnrB*
124	Zibo	Duck	*S.* Typhimurium	19	CEF, CIP, ENO, GEN, NA, TET	*qnrA*, *qnrB*
125	Zibo	Duck	*S.* Typhimurium	34	CIP, ENO, GEN, NA, NEO, TET	*qnrA*, *qnrB*
126	Zibo	Duck	*S.* Enteritidis	11	AMP, CEF, ENO, GEN, NA, NEO, TET	bla_{TEM-1}, bla_{PSE-1}, *qnrA*, *qnrB*
127	Zibo	Duck	*S.* Enteritidis	11	AMP, CEF, CIP, GEN, NA, NEO, TET	*qnrA*, *qnrB*
128	Zibo	Duck	*S.* Enteritidis	11	AMP, CEF, ENO, FFN, GEN, NA, NEO, TET	Class I (*drfA12-aadA2*), bla_{TEM-1}, *qnrB*, *qnrS*
129	Zibo	Duck	*S.* Typhimurium	34	CEF, CIP, ENO, GEN, NA, TET	*qnrA*
130	Zibo	Duck	*S.* Typhimurium	34	AMP, CEF, CIP, NA, TET	*qnrA*, *qnrB*
131	Zibo	Duck	*S.* Enteritidis	11	AMP, CIP, ENO, FFN, GEN, NA, NEO, TET	bla_{TEM-1}, *qnrA*, *qnrS*, *floR*
132	Zibo	Duck	*S.* Typhimurium	19	CIP, ENO, GEN, NA, NEO, TET	*qnrA*, *qnrB*
133	Zibo	Duck	*S.* Typhimurium	19	AMP, CEF, CIP, NA, TET	*qnrA*, *qnrB*
134	Zibo	Duck	*S.* Enteritidis	11	CEF, CIP, ENO, FFN, GEN, NA, TET	*qnrA*, *qnrB*, *floR*
135	Qingdao	Pig	*S.* Typhimurium	19	AMP, NA	*qnrA*, *qnrB*, *floR*
136	Qingdao	Pig	*S.* Typhimurium	19	AMP, TET	bla_{TEM-1}, *qnrA*, *qnrB*, *floR*
137	Qingdao	Pig	*S.* Typhimurium	19		*qnrB*, *floR*
138	Qingdao	Pig	*S.* Typhimurium	34	AMP, CIP, ENO, FFN, LVX, NA, NEO, TET	bla_{TEM-1}, $bla_{CTX-M-55}$,*qnrA*, *qnrB*, *floR*
139	Qingdao	Pig	*S.* Derby	40	TET	*floR*
140	Qingdao	Pig	*S.* Derby	40	AMP	bla_{TEM-1},*qnrA*, *floR*
141	Qingdao	Pig	*S.* Typhimurium	19	AMP, CIP, FFN	$bla_{CTX-M-55}$, bla_{PSE-1}, *qnrA*, *qnrB*, *floR*
142	Jinan	Pig	*S.* Typhimurium	19	FFN, FOS, TET	*qnrA*,*qnrB*, *floR*
143	Jinan	Pig	*S.* Derby	40	AMP	*qnrA*, *floR*
144	Jinan	Pig	*S.* Derby	40		
145	Jinan	Pig	*S.* Typhimurium	19	AMP, CIP, FFN, LVX, NA, NEO, TET	Class I (*aadA2*), bla_{TEM-1},bla_{PSE-1}, *qnrA*, *qnrB*, *floR*
146	Jinan	Pig	*S.* Typhimurium	34	AMP, CIP, FFN	bla_{TEM-1}, bla_{PSE-1}, *qnrA*, *qnrB*, *floR*
147	Jinan	Pig	*S.* Enteritidis	11	AMP, CEF, CIP, FFN, FOS, NA	bla_{TEM-1}, $bla_{CTX-M-55}$, *qnrA*, *qnrB*, *floR*
148	Jinan	Pig	*S.* Typhimurium	34	AMP, TET	$bla_{CTX-M-55}$, *qnrA*, *qnrB*, *floR*

(Continued)

TABLE 3 | Continued

No.	Location	Farms	Serovar	ST	Resistance phenotype	Integrons/resistance genes
149	Jinan	Pig	*S.* Typhimurium	34		
150	Zibo	Pig	*S.* Enteritidis	11	AMP, CIP, FFN, NA	bla_{TEM-1}, bla_{PSE-1}, *qnrA*, *qnrB*, *floR*
151	Zibo	Pig	*S.* Typhimurium	34	AMP, CIP, ENO, FFN	bla_{TEM-1}, $bla_{CTX-M-55}$, *qnrA*, *qnrB*, *floR*
152	Zibo	Pig	*S.* Typhimurium	19	AMP, CIP, TET	bla_{TEM-1}, bla_{PSE-1}, *qnrA*, *qnrB*, *floR*
153	Zibo	Pig	*S.* Typhimurium	19	AMP, TET	bla_{TEM-1}, *qnrA*, *qnrB*, *qnrS*, *floR*
154	Zibo	Pig	*S.* Enteritidis	3,007	AMP, TET	$bla_{CTX-M-55}$, *qnrA*, *qnrB*, *floR*

detected among the isolates. bla_{TEM-1} was the most commonly isolated β-lactamase gene ($n = 10$), followed by $bla_{CTX-M-55}$ ($n = 6$) and bla_{PSE-1} ($n = 5$). Three plasmid-mediated quinolone resistance genes were detected among the isolates. *qnrA* was the most commonly isolated plasmid-mediated quinolone resistance gene ($n = 16$), followed by *qnrB* ($n = 15$) and *qnrS* ($n = 1$). In addition, 18 isolates carried *floR* (**Table 3**).

MLST

A total of nine STs among the 154 isolates were found. ST11 was the most common ST in both chickens and ducks, and it was represented by 69 and 38 *Salmonella* isolates, respectively. ST19 was the most common ST in pigs, and it was represented by eight *Salmonella* isolates (**Table 3**). The STs in this study were correlated with specific serovars, such as ST11 with *Salmonella* enteritidis, ST19 and ST34 with *Salmonella* typhimurium, and ST40 with *Salmonella* derby.

DISCUSSION

In this study, *Salmonella* spp. were recovered from chickens, ducks and pigs in Qingdao, Jinan and Zibo regions. For the chickens, the prevalence (24.0%) was significantly higher than that reported in Shanghai, China (4.5%) (Liu et al., 2010) but was lower than that reported from chicken farms in Egypt (41.0%) (Hanem et al., 2017). The prevalence (9.3%) in ducks was similar to that obtained from duck farms in Sichuan province (12.0%) (Li et al., 2013) but was lower than those reported in Penang, Malaysia (39.0%) (Adzitey et al., 2012), and in South Korea (65.2%) (Cha et al., 2013). For pigs, the occurrence ratio (11.1%) was similar to those reported in previous studies of *Salmonella* spp. in food products of animal origin in China (Jiang et al., 2006; Li et al., 2013) but was higher than that reported from conventional farms (3.5%) in Korea (Migma et al., 2015). Data on the prevalence of *Salmonella* in different studies were difficult to compare based on differences in regions, collection seasons, sample types, isolation methodologies, culture methods, culture media, and environmental conditions.

For serotyping, a total of seven serovars were found among the 154 isolates, including six from chickens, two from ducks, and three from pigs. The most common serotype in chickens and ducks was *Salmonella* enteritidis. This result was consistent with those from Shanxi province (Yang et al., 2010), but it was different from other reports that the dominant serotype in chicken farms was *Salmonella* Colindale in Chad (Tabo et al., 2013). The most common serotype in duck farms was *Salmonella* typhimurium (Martelli et al., 2016). The dominant serotype in pigs was *Salmonella* typhimurium, which was the most common serovar isolated from humans and it can lead to severe human and animal diseases (Deng et al., 2012), but it was different from other studies, where the dominant serotype in pig farms was *Salmonella* IIIb in Henan province (Kuang et al., 2015), and *Salmonella* derby in England and Wales (Miller et al., 2011). The difference in dominant serotype among animals may be due to differences in the pathogenicity of two serovars, geographical regions and diversities (Volf et al., 2010; European Centre for Disease Prevention Control, 2013).

Antimicrobial resistance in *Salmonella* is a threat to human public health. As shown in **Table 3**, the high rates of antimicrobial resistance were against tetracycline (72.0%) and ampicillin (69.4%) in all sources, which was similar to reports of *Salmonella* isolates from Africa, in which chickens exhibited resistance to tetracycline (93.0%) and ampicillin (47.0%) (Zishiri et al., 2016). These high resistance rates are due to its wide use in animal feed and were consistent with other reports (Piras et al., 2011; Shao, 2011; Bai et al., 2015). In addition, resistance to ciprofloxacin in 35.9% of chickens, 80.4% of ducks, and 30.0% of pigs deserves our attention because resistance to this antimicrobial agent may lead to the delay or failure of fluoroquinolone therapies (Van et al., 2007). In this study, all of the isolates were susceptible to amikacin, which may be because this antimicrobial is not used for therapeutic purposes in veterinary medicine or as a growth promoter in conventional animal fattening, and the result was consistent with other reports (Eva et al., 2015). In this study, MDR *Salmonella* isolates were frequently observed among chickens, ducks and pigs. In addition, MDR *Salmonella* is serotype-dependent (Clemente et al., 2014): the data provided evidence that *Salmonella* indiana, *Salmonella* typhimurium and *Salmonella* enteritidis were strongly associated with MDR phenotypes. Of particular concern, MDR strains could transfer to humans via animal or animal-derived products and pose a great risk to public health (Rosangela et al., 2016).

In this study, our results related to the incidence of class I integrons (26/154, 16.9%) were similar to the report in Sichuan (Li et al., 2013) but were higher than those reported in the USA, as class I integrons were identified in only 2.8% of the *Salmonella* isolates from bulk milk and milk filters (Van et al., 2013). In the present study, the incidence of class I integrons was significantly higher in *Salmonella* from chickens (21.8%) than *Salmonella* from pigs (5.0%). In addition, in this study, the *Salmonella* isolates carrying class I integrons included *Salmonella* enteritidis, typhimurium and indiana.

Production of β-lactamases is considered to be the main mechanism of resistance in Gram-negative bacteria to overcome penicillin-derived antibiotics, and the bla_{TEM} and bla_{CTX-M} ESBLs can hydrolyse third and fourth generation cephalosporins. In this study, a total of three β-lactamase genes were detected among the *Salmonella* isolates recovered from chickens, ducks and pigs: bla_{TEM-1}, bla_{PSE-1}, and $bla_{CTX-M-55}$. Most of the isolates carried $bla_{TEM-1,}$ which was similar to the report in South Africa that bla_{TEM-1} was the most commonly identified β-lactamase gene in *Salmonella* isolates from food-producing animals (Igbinosa, 2015). In addition, in this study, most isolates carried bla_{TEM-1} and $bla_{CTX-M-55}$, which confer resistance to ampicillin.

Quinolones are the first choice for the treatment of invasive and systemic salmonellosis that occurs in humans and animals (Dimitrov et al., 2007). A total of three quinolone resistance genes were detected among the *Salmonella* isolates recovered from chickens, ducks and pigs: *qnrA*, *qnrB* and *qnrS*. *qnrA* was the most commonly isolated plasmid-mediated quinolone resistance gene consistent with a report in Henan, where *qnrA*, *qnrB* and *qnrS* were identified in *Salmonella* strains isolated from retail food with an incidence of 46.6, 12.7, and 19.5%, respectively (Yang et al., 2013). It is well known that *qnr* genes confer only low-level resistance to fluoroquinolones, and accumulation of quinolone resistance-determining region (QRDR) mutations is necessary for *S*. enterica to be resistant to fluoroquinolone, especially ciprofloxacin (Eaves et al., 2004). In this study, most *Salmonella* isolates containing a plasmid-mediated quinolone resistance gene were resistant to ciprofloxacin, nalidixic acid and gentamicin.

Florfenicol, a new chemosynthesis broad spectrum antibiotic of chloramphenicol analogs, is a fluorinated derivative of thiamphenicol. It is not approved for human use. In this study, *floR* was identified in 35.1% of *Salmonella* strains isolated from chickens, ducks and pigs, which was significantly higher than that reported in Egypt (1.0%) (Ahmed and Shimamoto, 2012). In addition, *floR* was identified in 90.0% of *Salmonella* strains isolated from pigs in this study. In this study, most *Salmonella* isolates containing the *floR* gene were resistant to florfenicol.

MLST results reveal that a total of nine STs were identified in this study. ST11 was the most frequent genotype that was recovered in chickens and ducks, and ST19 was the most frequent genotype that was recovered in pigs. ST11 belongs to *Salmonella* enteritidis, and ST19 belongs to *Salmonella* typhimurium; they all have continually been reported to cause human salmonellosis in recent years (Cai et al., 2016; Kang et al., 2017). In addition, our results revealed that the MLST patterns were generally associated with serotypes and provided a reliable prediction of the *Salmonella* serovars, which was consistent with previous research (Achtman et al., 2012).

CONCLUSION

The prevalence of *Salmonella* was higher in the animal farms. Moreover, many serovars reported in humans and MDR *Salmonella* were recovered in this study. The high rates of MDR *Salmonella*, class I integrons and antibiotic resistance gene positive isolates detected suggest that measures must be taken to facilitate the reasonable use of antimicrobials in animal husbandry. Therefore, continuous surveillance of *Salmonella* and associated antimicrobial resistance in *Salmonella* of animals is essential to detect emerging *Salmonella* serovars and associated resistance genes.

AUTHOR CONTRIBUTIONS

SS, XZ, contributed to the conception of the study; WC, XZ; contributed significantly to analysis and manuscript preparation; XZ, performed the data analyses and wrote the manuscript; XZ, JY, BZ: helped perform the analysis with constructive discussions.

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Serotype Distribution, Antimicrobial Resistance, and Class 1 Integrons Profiles of *Salmonella* from Animals in Slaughterhouses in Shandong Province, China

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The current study aimed to analyze the prevalence and characterization of *Salmonella* enterica isolated from animals in slaughterhouses before slaughter. A total of 143 non-duplicate *Salmonella* were recovered from 1,000 fresh fecal swabs collected from four major pig slaughterhouses (49/600, 8.2%) and four major chicken slaughterhouses (94/400, 23.5%) between March and July 2016. Among *Salmonella* isolates from pigs, the predominant serovars were *Salmonella* Rissen (28/49, 57.1%) and Typhimurium (14/49, 28.6%), and high antimicrobial resistance rates were observed for tetracycline (44/49, 89.8%) and ampicillin (16/49, 32.7%). Class 1 integrons were detected in 10.2% (5/49) of these isolates and all contained gene cassettes *aadA2* (0.65 kb). Two β-lactamase genes were detected among these isolates, and most of these isolates carried $bla_{\text{TEM-1}}$ (46/49), followed by $bla_{\text{OXA-1}}$(4/49). Seven STs (MLST/ST, multilocus sequence typing) were detected in these isolates, and the predominant type was ST469 (19.6%). Among *Salmonella* isolates from chickens, the predominant serovars were *Salmonella* Indiana (67/94, 71.3%) and Enteritidis (23/94, 24.5%), and high antimicrobial resistance rates were observed for nalidixic acid (89/94, 94.7%), ampicillin (88/94, 93.6%) and tetracycline (81/94, 86.2%). Class 1 integrons were detected in 23 isolates (23/94, 24.5%), which contained empty integrons (0.15 kb, $n = 6$) or gene cassettes *drfA17-aadA5* (1.7 kb, $n = 6$), *aadA2* (1.2 kb, $n = 5$), *drfA16-*$bla_{\text{PSE-1}}$*-aadA2-ereA2* (1.6 kb, $n = 5$) or *drfA1-aadA1* (1.4 kb, $n = 1$). Three β-lactamase genes were detected, and all 94 isolates carried $bla_{\text{TEM-1}}$, followed by $bla_{\text{CTX-M-55}}$ ($n = 19$) and $bla_{\text{SPE}-1}$ ($n = 3$). Five STs were found in these isolates, and the predominant type was ST17 (71.3%). Our findings indicated that *Salmonella* was widespread in animals at slaughter and may be transmitted from animal to fork.

Keywords: *Salmonella*, animal slaughterhouses, antimicrobial resistance, class 1 integrons, MLST/ST

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INTRODUCTION

Salmonella enterica is a major global foodborne pathogen (Chiu et al., 2010; Scallan et al., 2011). More than 2,600 different serovars have been identified worldwide (Guibourdenche et al., 2010). In China, *Salmonella* causes an estimated 22.2% of foodborne diseases, and the majority of diseases are associated with the ingestion of contaminated meat products (Wang et al., 2007). Both pigs and

chickens have been recognized as an important reservoir for antibiotic resistant *Salmonella*, and the resistance genes can be transferred to other bacteria via mobile genetic elements, such as plasmids and gene cassettes (Vo et al., 2006).

Agents of different antimicrobial classes, such as β-lactams or fluoroquinolones, are frequently used in clinical practice for *Salmonella enterica* infections. Unfortunately, *Salmonella* has gradually developed a high resistance rate to these antimicrobials, leading to the increase of healthcare costs and even clinical treatment failure (Cui et al., 2009; Gonzalez-Sanz et al., 2009). To date, numerous studies have been conducted to monitor antimicrobial resistance and molecular epidemiology of *Salmonella* isolated from pigs and chickens in slaughterhouses (Van et al., 2013; Mohamed et al., 2014).

However, little information concerning prevalence and characterization of *Salmonella* from animals in slaughterhouses in China is available. Shandong province, as a major breeding region, is the main producer of meat products in China. Therefore, major animal slaughterhouses in Shandong province, China were singled out as sampling sites to analyze the prevalence and characterization of *Salmonella* from animals in slaughterhouses.

MATERIALS AND METHODS

Description of Sampling Sites

From March to July 2016, 1,000 fresh fecal swabs were collected from four major pig slaughterhouses with process capacity of 1,500–2,500 pigs per day (150 samples per pig slaughterhouse) and four major chicken slaughterhouses with process capacity of 10,000–40,000 chickens per day (100 samples per chicken slaughterhouse). The animal slaughterhouses are respectively located in Weihai, Ciyao, Zhucheng, and Yantai regions in Shandong province, China. Sampling was carried out before slaughter, and at the time animals from different farms has been mixed.

Identification and Sreotyping of *Salmonella*

From each animal slaughterhouse, fresh fecal swabs were randomly collected from different individual animals, and transported in an ice box to our laboratory within 6 h for further bacteriological analysis. Each swab sample was added into 50 mL buffered peptone water (BPW) and was incubated at 37°C for 16 to 18 h. After that, 0.1 mL of the BPW suspensions was sub-cultured in 10 mL subpackaged Rappaport-Vassiliadis (RV) broth at 42°C for 24 h. One loopful of each RV broth culture was then plated onto xylose lysine tergitol 4 agar plates, and was incubated at 37°C for 24 to 48 h (Yan et al., 2010). Presumptive *Salmonella* colonies were identified using both the VITEK system (BioMerieux, Marcy 1'Etoile, France) and polymerase chain reaction (PCR) amplification of the inherent gene *invA* (Malorny et al., 2003).

All *Salmonella* isolates were serotyped according to the Kauffmann-White scheme by slide agglutination with O and H antigen-specific sera (Tianrun Bio-Pharmaceutical, Ningbo, China) (Grimont and Weill, 2007).

Antimicrobial Susceptibility Testing

The Kirby-Bauer disk diffusion method was used in this study to examine resistance of *Salmonella* to 10 commonly used antibiotics, including amoxicillin/clavulanic acid (AMC, 20/10 μg), ampicillin (AMP, 10 μg), cefotaxime (CTX, 30 μg), ciprofloxacin (CIP, 5 μg), florfenicol (FFC, 30 μg), gentamicin (GEN, 10 μg), nalidixic acid (NAL, 10 μg), spectinomycin (SPT, 10 μg), tetracycline (TET, 30 μg), and sulfamethoxazole/trimethoprim (SXT, 1.25/23.75 μg). *Escherichia coli* (ATCC25922) was used as a quality control. The results were interpreted based on the Clinical and Laboratory Standards Institute (CLSI) standards guidelines (CLSI, 2013). *Salmonella* isolates resistant to more than three classes of antimicrobials were defined as multidrug resistance (MDR) isolates.

Detection of Class I Integrons and β-Lactamase-Encoding Genes

Bacterial DNA was extracted using a TIANamp bacteria DNA kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The gene cassettes within the variable region of class I integrons were detected via polymerase chain reaction (PCR), using previously described primers and procedures (Kerrn et al., 2002). The PCR products were cloned into the pMD18-T vector using the pMD18-T cloning kit (Takara, Dalian, China) and submitted for sequencing (Invitrogen, Beijing, China).

PCR screening for β-lactamase-encoding genes bla_{TEM}, $bla_{\text{PSE-1}}$, $bla_{\text{CMY-2}}$, bla_{SHV}, $bla_{\text{DHA-1}}$, bla_{OXA}, and $bla_{\text{CTX-M}}$ was performed as previously described (Guerra et al., 2001; Chen et al., 2004; Batchelor et al., 2005; Hasman et al., 2005; Li et al., 2013). The PCR products were purified and subsequently sequenced.

MLST

The MLST analysis was performed by sequencing the fragments of seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*), and the alleles and STs were assigned according to the MLST scheme at http://mlst.warwick.ac.uk/mlst/dbs/Senterica. A minimum spanning tree was created using Bionumerics software 6.5 (Applied Maths, Kortrijk, Belgium), according to the instructions (the unweighted pair group method of arithmetic averages method).

Statistical Analyses

All statistical analyses were performed using package SPSS 15.0 (SPSS Inc., Chicago, IL, USA). The *chi-square* test was used to compare the prevalence, multidrug resistance rate and carriage of class 1 integron of *Salmonella* isolated from pigs and chickens, and $P < 0.05$ was considered difference significant.

RESULTS

Prevalence and Serotypes of *Salmonella*

From pig slaughterhouses, 49 *Salmonella* isolates were recovered (49/600, 8.2%), including 13 from Weihai (13/150, 8.7%), 9 from Ciyao (9/150, 6.0%), 11 from Yantai (11/150, 10.7%),

and 16 from Zhucheng (16/150, 10.7%) (**Table 1**). In terms of isolation rate of *Salmonella*, no significant difference was found between the pig slaughterhouses ($P > 0.05$). The 49 *Salmonella* belonged to 6 serovars, including *Salmonella* Rissen ($n = 28$), Typhimurium ($n = 14$), Grampian ($n = 3$), Derby ($n = 2$), Indiana ($n = 1$), and Enteritidis ($n = 1$). The most common serovars were *Salmonella* Rissen (28/49, 57.1%) and Typhimurium (14/49, 28.6%) (**Table 2**).

From chicken slaughterhouses, 94 *Salmonella* isolates were recovered (94/400, 23.5%), including 23 from Weihai (23/100, 23.0%), 33 from Ciyao (33/100, 33.0%), 17 from Yantai (17/100, 17.0%), and 21 from Zhucheng (21/150, 21.0%) (**Table 1**). In terms of isolation rate of *Salmonella*, no significant difference was found between the chicken slaughterhouses ($P > 0.05$). These 94 *Salmonella* isolates belonged to 4 serovars, including *Salmonella* Indiana ($n = 67$), Enteritidis ($n = 23$), Typhimurium ($n = 3$), and Hadar ($n = 1$). The dominant serovars were *Salmonella* Indiana (67/94, 71.3%) and Enteritidis (23/94, 24.5%) (**Table 2**).

TABLE 1 | Prevalence of *Salmonella* isolates from pigs and chickens in slaughterhouses.

Locations	Pigs		Chickens	
	No. of samples	No. of positive samples	No. of samples	No. of positive samples
Weihai	150	13 (8.7%)	100	23 (23.0%)
Ciyao	150	9 (6.0%)	100	33 (33.0%)
Yantai	150	11 (7.3%)	100	17 (17.0%)
Zhucheng	150	16 (10.7%)	100	21 (21.0%)
Total	600	49 (8.2%)	400	94 (23.5%)

Antimicrobial Susceptibility Testing

All 49 isolates from pig slaughterhouses were susceptible to amoxicillin/clavulanic acid and cefotaxime. But most isolates were resistance to tetracycline (44/49, 89.8%) and ampicillin (16/49, 32.7%). In addition, 7 isolates (7/49, 14.3%) exhibited MDR (**Table 2**). In addition, 4 isolates were susceptible to all antibiotics used in this study.

All 94 isolates from chicken slaughterhouses were susceptible to amoxicillin/clavulanic acid and sulfamethoxazole/trimethoprim. But most isolates were resistant to nalidixic acid (89/94, 94.7%), ampicillin (87/94, 92.6%), and tetracycline (81/94, 86.2%). Eighty-six isolates (86/94, 91.5%) exhibited MDR (**Table 2**). Of note, MDR rate of *Salmonella* from chickens was higher than that from pigs ($P < 0.05$). In addition, 2 isolates were susceptible to all antibiotics used in this study.

Characteristics of Class 1 Integrons and β-Lactamase-Encoding Genes

Among the 49 isolates recovered from pigs, class 1 integrons were found in 5 isolates (5/49, 10.2%), including 4 *Salmonella* Typhimurium and 1 Enteritidis. The 5 isolates only contained the single resistance gene cassette *aadA2* (0.65 kb). Two β-lactamase genes were detected among the isolates, most of the isolates carried $bla_{\text{SPE-1}}$ ($n = 46$) and $bla_{\text{OXA-1}}$($n = 4$) (**Table 2**).

Among the 94 isolates recovered from chicken, class 1 integrons were found in 23 isolates (23/94, 24.5%), including 16 *Salmonella* Indiana, 5 Enteritidis and 2 Typhimurium. Of these isolates, 5 groups of resistance gene cassettes were detected: empty integrons (0.15 kb, $n = 6$), *drfA17-aadA5* (1.6 kb, $n = 6$), *aadA2* (1.2 kb, $n = 5$), *drfA16-*$bla_{\text{PSE-1}}$*-aadA2-ereA2* (1.7 kb, $n = 5$), and *drfA1-aadA1* (1.4 kb, $n = 1$). Three β-lactamase genes were detected among these isolates. Most of the isolates carried $bla_{\text{TEM-1}}$ ($n = 94$), followed by $bla_{\text{CTX-M-55}}$ ($n = 19$) and $bla_{\text{SPE-1}}$($n = 3$) (**Table 2**).

MLST

One hundred and forty-three *Salmonella* isolates were divided into 9 STs, including 7 STs from pigs (ST11, ST17, ST19, ST34, ST40, ST358, and ST469), and 5 STs from chickens (ST11, ST17, ST19, ST33, and ST3172). The STs identified in the present study showed the following correlations with *Salmonella* serovars: ST11 with *Salmonella* Enteritidis, ST17 with Indiana, and ST469 with Rissen.

BioNumerics software version 6.5 was used to generate a minimum-spanning tree based on all the sources of STs (**Figure 1**). The dominant ST was ST17 (68/143, 47.6%), with most of isolates from chickens and only one from pigs, followed by ST469 (28/143, 19.6%), with all isolates from pigs. ST34 and ST19 belonged to one clone complex and had the same serovars of *Salmonella* Typhimurium. ST11 and ST3172 belonged to one clone complex, and had the same serovars of *Salmonella* Enteritidis.

DISCUSSION

In this study, *Salmonella* isolation rate from pigs (8.2%) was much lower than that (71.8%) in Jiangsu province, China (Cai et al., 2016), and the most common serotype in pigs was *Salmonella* Rissen, which in consistent with the result from the retail pork products in Thailand (Prapas et al., 2016). However, this finding was different from that reported in EU in which *Salmonella* Typhimurium was the most common serotype (European Food Safety Authority, 2014). Of note, *Salmonella* Rissen isolates from pigs only showed resistant to tetracycline (85.7%), which may be associated with the fact that the antimicrobial is frequently used in pig farms in China (Bai et al., 2015).

The *Salmonella* isolation rate from chickens (23.5%) was similar to the result reported for frozen chicken meat in Shandong province (26.3%), China (Cui et al., 2016). However, the result in this study was much lower than that (45.2%) from chickens in Henan province, China (Bai et al., 2015) and was higher than that (4.5%) from large-scale chicken farms in Shanghai, China (Liu et al., 2010). The difference of these isolation rates may be related with collection seasons, culture methods, and local environments. In the present study, the most common serotypes identified in chickens were

TABLE 2 | Resistance phenotype, incidence of class 1 integron, and resistance gens in Salmonella isolated from animals in slaughterhouses.

No.	Location	Slaughterhouse	Serovar	Resistance phenotype	Integrons/resistance genes
1	Weihai	Pig	*S.* Typhimurium	AMP, TET	bla_{TEM-1}
2	Weihai	Pig	*S.* Typhimurium	AMP, TET	bla_{TEM-1}
3	Weihai	Pig	*S.* Typhimurium	AMP, TET	bla_{TEM-1}
4	Weihai	Pig	*S.* Typhimurium	AMP, TET	bla_{TEM-1}
5	Weihai	Pig	*S.* Enteritidis	AMP, GEN, NAL	Class 1 (*aadA2*), bla_{TEM-1},
6	Weihai	Pig	*S.* Typhimurium	AMP, TET	bla_{TEM-1}
7	Weihai	Pig	*S.* Derby	AMP, TET	bla_{TEM-1}
8	Weihai	Pig	*S.* Rissen	TET	bla_{TEM-1}
9	Weihai	Pig	*S.* Rissen	TET	bla_{TEM-1}
10	Weihai	Pig	*S.* Derby	TET	bla_{TEM-1}
11	Weihai	Pig	*S.* Rissen	TET	bla_{TEM-1}
12	Weihai	Pig	*S.* Typhimurium	AMP, TET	bla_{TEM-1}, bla_{OXA-1}
13	Weihai	Pig	*S.* Typhimurium	TET	bla_{TEM-1}
14	Ciyao	Pig	*S.* Typhimurium	AMP, TET	bla_{TEM-1} , bla_{OXA-1}
15	Ciyao	Pig	*S.* Typhimurium	AMP, TET	bla_{TEM-1}
16	Ciyao	Pig	*S.* Typhimurium	AMP, TET	bla_{TEM-1}
17	Ciyao	Pig	*S.* Grampian	TET	bla_{TEM-1}
18	Ciyao	Pig	*S.* Indiana	CIP, FFC, NAL, SXT, TET	bla_{TEM-1}, bla_{OXA-1}
19	Ciyao	Pig	*S.* Grampian	AMP, FFC, SPT, SXT, TET	Class 1 (*aadA2*), bla_{TEM-1},
20	Ciyao	Pig	*S.* Rissen	TET	bla_{TEM-1}
21	Ciyao	Pig	*S.* Grampian	TET	bla_{TEM-1}
22	Ciyao	Pig	*S.* Typhimurium	AMP, GEN, FFC, NAL, SPT, SXT, TET	Class 1 (*aadA2*), bla_{TEM-1}
23	Yantai	Pig	*S.* Typhimurium	AMP, GEN, FFC, SPT, SXT, TET	bla_{TEM-1}, bla_{OXA-1}
24	Yantai	Pig	*S.* Typhimurium	AMP, GEN, FFC, NAL, SPT, SXT, TET	Class 1 (*aadA2*), bla_{TEM-1}
25	Yantai	Pig	*S.* Typhimurium	AMP, GEN, FFC, NAL, SPT, SXT, TET	Class 1 (*aadA2*), bla_{TEM-1},
26	Yantai	Pig	*S.* Rissen	GEN, TET	bla_{TEM-1}
27	Yantai	Pig	*S.* Rissen	TET	bla_{TEM-1}
28	Yantai	Pig	*S.* Rissen		
29	Yantai	Pig	*S.* Rissen	TET	bla_{TEM-1}
30	Yantai	Pig	*S.* Rissen	TET	bla_{TEM-1}
31	Yantai	Pig	*S.* Rissen		
32	Yantai	Pig	*S.* Rissen	TET	bla_{TEM-1}
33	Yantai	Pig	*S.* Rissen	GEN, TET	bla_{TEM-1}
34	Zhucheng	Pig	*S.* Rissen	TET	bla_{TEM-1}
35	Zhucheng	Pig	*S.* Rissen	TET	bla_{TEM-1}
36	Zhucheng	Pig	*S.* Rissen	TET	bla_{TEM-1}
37	Zhucheng	Pig	*S.* Rissen	TET	bla_{TEM-1}
38	Zhucheng	Pig	*S.* Rissen	TET	bla_{TEM-1}
39	Zhucheng	Pig	*S.* Rissen	TET	bla_{TEM-1}
40	Zhucheng	Pig	*S.* Rissen		
41	Zhucheng	Pig	*S.* Rissen	TET	bla_{TEM-1}
42	Zhucheng	Pig	*S.* Rissen	TET	bla_{TEM-1}
43	Zhucheng	Pig	*S.* Rissen	TET	bla_{TEM-1}
44	Zhucheng	Pig	*S.* Rissen	TET	bla_{TEM-1}
45	Zhucheng	Pig	*S.* Rissen	TET	bla_{TEM-1}
46	Zhucheng	Pig	*S.* Rissen		bla_{TEM-1}
47	Zhucheng	Pig	*S.* Rissen	TET	bla_{TEM-1}
48	Zhucheng	Pig	*S.* Rissen	TET	bla_{TEM-1}
49	Zhucheng	Pig	*S.* Rissen	TET	bla_{TEM-1}
50	Weihai	Chicken	*S.* Enteritidis	AMP, CTX, NAL	bla_{TEM-1}
51	Weihai	Chicken	*S.* Indiana	AMP, CIP, CTX, NAL, TET	bla_{TEM-1}

(Continued)

TABLE 2 | Continued

No.	Location	Slaughterhouse	Serovar	Resistance phenotype	Integrons/Resistance genes
52	Weihai	Chicken	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	Class 1 (*aadA2*), $bla_{\text{TEM-1}}$
53	Weihai	Chicken	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	$bla_{\text{TEM-1}}$, $bla_{\text{CTX-M -55}}$
54	Weihai	Chicken	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	$bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$
55	Weihai	Chicken	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	$bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$
56	Weihai	Chicken	*S.* Indiana	AMP, CTX, NAL	Class 1 (*drfA1-aadA1*), $bla_{\text{TEM-1}}$
57	Weihai	Chicken	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	$bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$
58	Weihai	Chicken	*S.* Typhimurium	AMP, GEN, SPT	Class 1 (*aadA2*), $bla_{\text{TEM-1}}$
59	Weihai	Chicken	*S.* Typhimurium	AMP, SPT	$bla_{\text{TEM-1}}$
60	Weihai	Chicken	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	bla_{TEM-1}, $bla_{\text{CTX-M-55}}$
61	Weihai	Chicken	*S.* Enteritidis	AMP, CTX, NAL	$bla_{\text{TEM-1}}$
62	Weihai	Chicken	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	$bla_{\text{TEM-1}}$
63	Weihai	Chicken	*S.* Enteritidis	AMP, CTX, FFC, NAL, TET	$bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$
64	Weihai	Chicken	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	Class 1 (*drfA17-aadA5*), $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$
65	Weihai	Chicken	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	$bla_{\text{TEM-1}}$
66	Weihai	Chicken	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	Class 1 (*drfA17-aadA5*), $bla_{\text{TEM-1}}$
67	Weihai	Chicken	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	$bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$
68	Weihai	Chicken	*S.* Typhimurium	AMP, SPT	Class 1 (*aadA2*), $bla_{\text{TEM-1}}$
69	Weihai	Chicken	*S.* Enteritidis	NAL	$bla_{\text{TEM-1}}$
70	Weihai	Chicken	*S.* Enteritidis	AMP, GEN, CTX, FFC, NAL, TET	$bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$
71	Weihai	Chicken	*S.* Enteritidis	AMP, CIP, CTX, NAL, TET	$bla_{\text{TEM-1}}$
72	Weihai	Chicken	*S.* Enteritidis	AMP, CTX, NAL	empty integron, $bla_{\text{TEM-1}}$
73	Ciyao	Chicken	*S.* Indiana	AMP, CTX, NAL	$bla_{\text{TEM-1}}$
74	Ciyao	Chicken	*S.* Enteritidis	NAL	$bla_{\text{TEM-1}}$
75	Ciyao	Chicken	*S.* Indiana	AMP, NAL, TET	$bla_{\text{TEM-1}}$
76	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	$bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$
77	Ciyao	Chicken	*S.* Enteritidis		$bla_{\text{TEM-1}}$
78	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	$bla_{\text{TEM-1}}$
79	Ciyao	Chicken	*S.* Indiana	AMP, NAL, TET	$bla_{\text{TEM-1}}$
80	Ciyao	Chicken	*S.* Indiana	AMP, NAL, TET	$bla_{\text{TEM-1}}$
81	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	$bla_{\text{TEM-1}}$
82	Ciyao	Chicken	*S.* Enteritidis	AMP, CIP, NAL, TET	Class 1 (*drfA17-aadA5*), $bla_{\text{TEM-1}}$
83	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	$bla_{\text{TEM-1}}$
84	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	$bla_{\text{TEM-1}}$
85	Ciyao	Chicken	*S.* Indiana	AMP, NAL, TET	$bla_{\text{TEM-1}}$
86	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	$bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$
87	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	$bla_{\text{TEM-1}}$
88	Ciyao	Chicken	*S.* Indiana	AMP, NAL, TET	$bla_{\text{TEM-1}}$
89	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	$bla_{\text{TEM-1}}$
90	Ciyao	Chicken	*S.* Indiana	AMP, CIP, GEN, NAL, TET	Class 1 (*drfA16-*$bla_{\text{PSE-1}}$*-aadA2-ereA2*), $bla_{\text{TEM-1}}$, $bla_{\text{SPE-1}}$
91	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	$bla_{\text{TEM-1}}$
92	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	$bla_{\text{TEM-1}}$
93	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	$bla_{\text{TEM-1}}$
94	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	$bla_{\text{TEM-1}}$
95	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	$bla_{\text{TEM-1}}$
96	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	$bla_{\text{TEM-1}}$
97	Ciyao	Chicken	*S.* Indiana	AMP, NAL, TET	$bla_{\text{TEM-1}}$
98	Ciyao	Chicken	*S.* Indiana	AMP, CIP, CTX, NAL, TET	Class 1 (*aadA2*), $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$

(Continued)

TABLE 2 | Continued

No.	Location	Slaughterhouse	Serovar	Resistance phenotype	Integrons/Resistance genes
99	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
100	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	empty integron, bla_{TEM-1}
101	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
102	Ciyao	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
103	Ciyao	Chicken	*S.* Indiana	AMP, CIP, CTX, NAL, TET	Class 1 (*drfA16-bla*$_{PSE-1}$*-aadA2-ereA2*), bla_{TEM-1}
104	Ciyao	Chicken	*S.* Indiana	AMP, CIP, CTX, NAL, TET	bla_{TEM-1}, $bla_{CTX-M-55}$
105	Ciyao	Chicken	*S.* Indiana	AMP, CIP, CTX, NAL, TET	bla_{TEM-1}, $bla_{CTX-M-55}$
106	Yantai	Chicken	*S.* Indiana	AMP, CIP, CTX, NAL, TET	empty integron, bla_{TEM-1}
107	Yantai	Chicken	*S.* Hadar	NAL, TET	bla_{TEM-1}
108	Yantai	Chicken	*S.* Indiana	AMP, CIP, CTX, NAL, TET	bla_{TEM-1}, $bla_{CTX-M-55}$
109	Yantai	Chicken	*S.* Indiana	AMP, CIP, CTX, NAL, TET	Class 1 (*drfA17-aadA5*), bla_{TEM-1}
110	Yantai	Chicken	*S.* Indiana	AMP, CIP, CTX, NAL, TET	bla_{TEM-1}, $bla_{CTX-M-55}$
111	Yantai	Chicken	*S.* Indiana	AMP, CIP, CTX, NAL, TET	bla_{TEM-1}, $bla_{CTX-M-55}$
112	Yantai	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
113	Yantai	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
114	Yantai	Chicken	*S.* Indiana	AMP, CIP, CTX, NAL, TET	bla_{TEM-1}, $bla_{CTX-M-55}$
115	Yantai	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
116	Yantai	Chicken	*S.* Indiana	AMP, CIP, GEN, CTX, NAL, TET	Class 1 (*drfA17-aadA5*), bla_{TEM-1}
117	Yantai	Chicken	*S.* Indiana	NAL	bla_{TEM-1}
118	Yantai	Chicken	*S.* Indiana	AMP, CIP, CTX, NAL, TET	Class 1 (*drfA16-bla*$_{PSE-1}$*-aadA2-ereA2*), bla_{TEM-1}, $bla_{CTX-M-55}$
119	Yantai	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
120	Yantai	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
121	Yantai	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	empty integron, bla_{TEM-1}
122	Yantai	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
123	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	Class 1 (*drfA16-bla*$_{PSE-1}$*-aadA2-ereA2*), bla_{TEM-1}, bla_{SPE-1}
124	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
125	Zhucheng	Chicken	*S.* Enteritidis		bla_{TEM-1}
126	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
127	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
128	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
129	Zhucheng	Chicken	*S.* Enteritidis	AMP, CIP, NAL, TET	Class 1 (*aadA2*), bla_{TEM-1}
130	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
131	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
132	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
133	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
134	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
135	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, CTX, NAL, TET	Class 1 (*drfA16-bla*$_{PSE-1}$*-aadA2-ereA2*), bla_{TEM-1}, bla_{SPE-1}
136	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
137	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	empty integron, bla_{TEM-1}
138	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	empty integrons, bla_{TEM-1}
139	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
140	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, GEN, NAL, TET	Class 1 (*drfA17-aadA5*), bla_{TEM-1}
141	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, NAL, TET	bla_{TEM-1}
142	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, GEN, NAL, TET	bla_{TEM-1}
143	Zhucheng	Chicken	*S.* Indiana	AMP, CIP, GEN, NAL, TET	bla_{TEM-1}

amoxicillin/clavulanic acid (AMC), ampicillin (AMP), cefotaxime (CTX), ciprofloxacin (CIP), florfenicol (FFC), gentamicin (GEN), nalidixic acid (NAL), spectinomycin (SPT), tetracycline (TET,) and sulfamethoxazole/trimethoprim (SXT).

Salmonella Indiana and Enteritidis, consistent with findings reported in Henan, China (Bai et al., 2015). However, this finding differed from the result reported in Sichuan province, China, in which *Salmonella* Derby and Typhimurium were the most common serotypes (Li et al., 2013). Additionally, *Salmonella* Kentucky and Enteritidis were the most common serotypes in the USA (National Antimicrobial Resistance Monitoring System, 2011), and *Salmonella* Typhimurium in the EU (European Food Safety Authority, 2014). This difference may be associated with geographical regions. In the present study, *Salmonella* Indiana showed a high MDR rate (61/68, 89.7%), similar with the result conducted in China (Lu et al., 2011), which demonstrated that most of *Salmonella* Indiana showed MDR, and these bacteria were not only resistant to streptomycin and tetracycline but also were resistant to chloramphenicol, fluoroquinolones and cephalosporin antibiotics.

In the current study, most *Salmonella* isolates showed high resistance to tetracycline, ampicillin, and nalidixic acid, similar to the report on slaughterhouses in Italy (Piras et al., 2011), suggesting that these drugs may have been widely used on animals during disease control and prevention. A high resistance rate (63.4%) of nalidixic acid was observed in *Salmonella* isolates, consistent with other reports (Piras et al., 2011; Siriken et al., 2015). The resistance rate to ciprofloxacin was up to 42.7%. The results may be related with the fact that fluoroquinolone antibiotics are the most common treatment for *Salmonella* infections. A relatively high resistance rate to cefotaxime (29.1%) was observed in this study, which may be associated with the fact that third-generation cephalosporins have become the primary drugs for the treatment of salmonellosis because of the increase in fluoroquinolone resistance. In addition, the results of the present study showed the high prevalence of multidrug resistant *Salmonella* isolates in chickens (91.5%), much higher than those reported in Henan province (46.0%), China (Bai et al., 2015) and in central China (34.7%) (Kuang et al., 2015). In this study, MDR isolate rate of *Salmonella* (91.5%) from chickens were higher than that (14.3%) from pigs, and the higher occurrence of MDR *Salmonella* isolates from chickens likely reflects the extensive use of antibiotics during intensive rearing. In addition, MDR *Salmonella* is serotype-dependent (Clemente et al., 2014): the data provided evidence that *Salmonella* Indiana, Typhimurium and Enteritidis were strongly associated with MDR phenotypes. However, these findings were different from a previous study showing that *Salmonella* Derby is commonly associated with MDR (Newell et al., 2010).

In the present study, PCR identified class 1 integrons in 19.6% of *Salmonella* isolates, which was similar to the 15.0% reported from retail meat products in the USA (Zhao et al., 2009) but higher than that of (2.8%) reported from milk products (Van et al., 2013). In the present study, the incidence of class 1 integrons was higher in *Salmonella* from chickens (24.5%) than *Salmonella* from pigs (10.2%) ($P < 0.05$). Class 1 integrons are often associated with MDR *Salmonella* isolates, consistent with the result of the present study. In addition, the *Salmonella* isolates carrying class 1 integrons included *Salmonella* Typhimurium, Enteritidis, and Indiana.

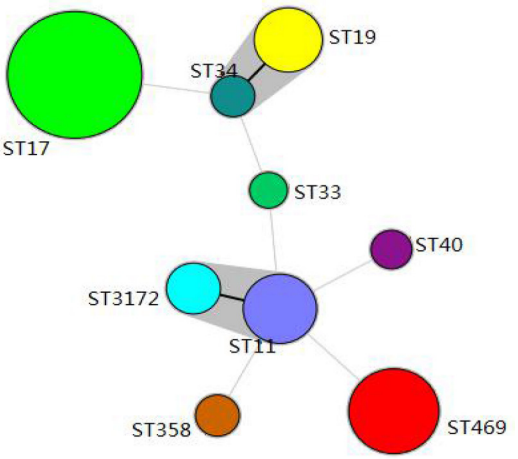

FIGURE 1 | Minimum-spanning tree analysis of the *Salmonella* isolated from pigs and chickens in slaughterhouses. Each circle represents one ST, and the area of the circle corresponds to the number of isolates, the gray region indicates that ST19 and ST34 belong to a clonal complex, ST11, and ST3172 belong to a clonal complex.

Four β-lactamase genes were detected among *Salmonella* isolates recovered from pigs and chickens: $bla_{\text{TEM-1}}$, $bla_{\text{PSE-1}}$, $bla_{\text{OXA-1}}$, and $bla_{\text{CTX-M-55}}$. Most isolates carried $bla_{\text{TEM-1}}$, consistent with the report from meat and milk products in Egypt (Ashraf et al., 2014), but different from the report from animal slaughterhouses and retail meat products in Sichuan, China, which showed the dominant β-lactamase gene was $bla_{\text{OXA-1}}$, followed by $bla_{\text{TEM-1}}$, $bla_{\text{PSE-1}}$, and $bla_{\text{CMY-2}}$ (Li et al., 2013). The fact that 46 *Salmonella* from pigs carried $bla_{\text{TEM-1}}$ whereas only 16 were resistant to ampicillin, and only 88 out of 94 *Salmonella* carrying $bla_{\text{TEM-1}}$ from chickens showed resistant to ampicillin may be associated with the expression status of $bla_{\text{TEM-1}}$ genes and is needed to be further studied.

In addition, $bla_{\text{CMY-2}}$ encodes resistance to third-generation cephalosporins, an important class of antibiotics used to treat complicated cases of salmonellosis (Gonzalez-Sanz et al., 2009). The incidence of $bla_{\text{CMY-2}}$-positive *Salmonella* in China was low and was only reported in Shanxi and Sichuan (Yang et al., 2010; Li et al., 2013).

The MLST results revealed 9 STs identified in *Salmonella* from pigs and chickens. ST19 and ST34 have continually been reported to cause human salmonellosis in recent years, and these bacteria belong to the same serotype, *Salmonella* Typhimurium (Cai et al., 2016), and this circumstance was also true for *Salmonella* Enteritidis, represented by ST11 and ST3172. These findings suggested that serovars and STs were tightly coupled (Sukhnanand et al., 2005). ST358 is rare in China and corresponds to *Salmonella* Grampian, which causes an unusual increase in human cases of *Salmonella* Grampian infections (Horvath et al., 2013). This observation indicates that *Salmonella* could spread from animals to

humans via pork and chicken products (Osman et al., 2014).

CONCLUSIONS

Collectively, our findings exhibit the prevalence and characteristics of *Salmonella* isolated from animals in slaughterhouses in Shandong province, China. In addition, this study highlights the necessity to carry out the long-term surveillance for *Salmonella* recovered from food-producing animals.

AUTHOR CONTRIBUTIONS

WC and SS: conceived and designed the study. XZ and CY: performed the experiments and analyzed the data. XZ, WC, and SS: wrote and revised the manuscript.

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High Prevalence of Colistin Resistance and *mcr-1* Gene in *Escherichia coli* Isolated from Food Animals in China

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Huang X, Yu L, Chen X, Zhi C, Yao X, Liu Y, Wu S, Guo Z, Yi L, Zeng Z and Liu J-H (2017) High Prevalence of Colistin Resistance and mcr-1 Gene in Escherichia coli Isolated from Food Animals in China. Front. Microbiol. 8:562. doi: 10.3389/fmicb.2017.00562

The objective of this study was to determine the minimal inhibitory concentration of colistin for *Escherichia coli* from food animals and the possible underlying colistin resistance mechanisms. During 2007–2014, 4,438 *E. coli* isolates of food animal origins were collected. The susceptibility of colistin was tested by the agar dilution method. Mutations in *pmrA*, *pmrB*, and *mgrB* and the presence of *mcr-1* gene were determined by PCR and DNA sequencing. Complementation experiments were carried out to evaluate the contribution of the mutations to colistin resistance. There was a high frequency of colistin resistance in *E. coli* from pigs on farm (24.1%) and at slaughter (24.3%) in 2013–2014, followed by chickens on farm (14.0%) and at slaughter (9.5%). The resistance frequency of *E. coli* in cow isolates was the lowest (0.9%). MIC distribution for colistin showed that most isolates (75.2%) were distributed at 0.25 mg/L–0.5 mg/L, followed by 4 mg/L–8 mg/L (16.8%). Compared with the isolates from pigs and chickens recovered during 2013–2014, *E. coli* isolates collected during 2007–2008 (5.5%) and 2010–2011 (12.4%) showed significantly lower frequency of colistin resistance ($P < 0.05$). DNA sequencing and complementation experiments failed to detect any insertion inactivation or mutation in *pmrA*, *pmrB*, and *mgrB* associated with colistin resistance. However, 91.0% colistin-resistant isolates were positive for *mcr-1*. The high frequency of colistin resistance and *mcr-1* gene among *E. coli* isolates from food animals in China urged the need to minimize potential risks of colistin resistance development and the spread of *mcr-1* gene.

Keywords: *Escherichia coli*, colistin, food animals, resistance, *mcr-1*

INTRODUCTION

The rising prevalence of multidrug-resistant (MDR) gram-negative Enterobacteriaceae (GNB), especially carbapenem- resistant, has resulted in a renewed interest in polymyxins, especially polymyxin E (colistin), for the management of gram-negative infections in many countries (Falagas and Michalopoulos, 2006; Cassir et al., 2014). Despite their relatively recent reintroduction in clinical practice, reports on colistin resistant isolates are on the rise (Falagas et al., 2010; Olaitan et al., 2014a). Resistance to polymyxins has been traditionally regarded as occurring via mutations

in genes regulating the synthesis of LAra4N (Falagas et al., 2010; Olaitan et al., 2014b). However, we recently described for the first time the emergence of plasmid-mediated colistin resistance gene, *mcr-1*, which now has been identified in several Enterobacteriaceae species from various sources (environment, food, animal and humans) (Liu et al., 2016).

Colistin has been used in veterinary medicine through prophylactic or metaphylactic practices, but the prevalence of colistin resistance in bacteria isolated from animals in many countries was still low (Kempf et al., 2013; Wasyl et al., 2013; Quesada et al., 2014). In China, colistin has been widely used in veterinary medicine, especially in swine and poultry for many years. We previously detected high prevalence of *mcr-1* among *E. coli* isolates from pigs at slaughter in Guangzhou (Liu et al., 2016). Soon after, *mcr-1* gene has been reported to be present in Enterobacteriaceae from animals, food and humans worldwide (Schwarz et al., 2001; Quan et al., 2017; Wang et al., 2017a). However, little is known about the prevalence of colistin resistance and *mcr-1* gene among commensal *E. coli* isolates from other food animals in China. The aim of this study was to investigate the frequency of colistin resistance among commensal *E. coli* isolates from farm animals (chicken, cattle, and pig) and food animals at slaughter recovered from 12 provinces of China and to determine the possible underlying mechanisms among part of colistin-resistant isolates.

MATERIALS AND METHODS

Origin of *E. coli* Isolates

Cloacal samples from chickens (laying hens, chickens, and broilers) and rectal swabs from pigs (piglets, weaned pigs, fattening pigs, and sows) and cattle were collected from 107 food animal farms located in different geographic areas of China (Guangdong, Henan, Jiangxi, Ningxia, Jilin, Qinghai, Sichuan, Shanghai, Jiangsu, Shandong, Beijing, and Neimeng) from May 2013 to August 2014 (**Table 1**). Animals were randomly selected for sampling on each farm based on their age and stage of production. Ten to thirty samples per stage of production per farm were collected. In addition, cecal contents of chickens from seven farmers markets and two live-bird markets and rectal swabs of pigs from two live pig markets and eight abattoirs located in Guangdong, Henan, Shandong, Liaoning, and Sichuan province were collected at slaughter between April 2013 and August 2014. No more than five animal samples per farm were analyzed. All samples were seeded on MacConkey agar plates and were incubated at 37°C for 24 h. One presumptive colony with typical *E. coli* morphology and size was selected and then inoculated on eosin-methylene blue agar. After incubation, suspected *E. coli* colony was identified using classical biochemical methods. In addition, 349 *E. coli* isolates (91 were from chicken during 2007–2008, 86 from chicken during 2010–2011, and 172 from pigs during 2010–2011) from healthy food animals mentioned in our previous study were also included in this study for comparison (Yang et al., 2014).

Antimicrobial Susceptibility Testing

The minimal inhibitory concentration (MIC) of colistin was determined by the agar dilution method according to the protocols recommended in M100-S25 of the (Clinical and Laboratory Standards Institute, 2013). For isolates from pigs at slaughter, MICs of ampicillin, cefotaxime, imipenem, gentamicin, amikacin, neomycin, apramycin, florfenicol, tetracycline, ciprofloxacin, and fosfomycin were also determined. The results were interpreted according to epidemiological cut-off (ECOFF) values recommended by EUCAST[1] (colistin, florfenicol, and neomycin) and the interpretative criteria recommended by CLSI (M100-S25) (ampicillin, cefotaxime, gentamicin, amikacin, fosfomycin, and ciprofloxacin) (Clinical and Laboratory Standards Institute, 2013).

Statistical significance for the comparison of resistance prevalence data was determined by the χ^2 test. *P* values less than 0.05 were considered statistically significant.

PCR Amplification and Sequencing

A total of 200 colistin-resistant *E. coli* isolates of different origins (127 from pigs, 70 from chickens, and 3 from cows) were randomly selected for PCR amplification of *mcr-1* (Liu et al., 2016). In addition, 50 of them were randomly selected for sequencing for genes encoding PmrA, PmrB, and MgrB. *pmrA* were amplified using primers described previously (Quesada et al., 2014). The primers used for amplification of entire *mgrB* and *pmrB* genes were as follows: EmgrB-F (5′- CCGCTGAGTAATAATCCTAT -3′) and EmgrB-R (5′- TACAACCAAAGACGCAAT -3′), EpmrB-F (5′-

[1]http://www.eucast.org

TABLE 1 | Prevalence of colistin resistance among *Escherichia coli* isolates of different origins.

Animals	Farm number	Samples	Number of isolates	Number of colistin-resistant isolates (%)
2013–2014				
Laying hens	21	357	295	25 (8.5)
Broilers	43	886	611	102 (16.7)
Chickens	6	90	67	9 (13.4)
All farm chickens	47	1333	973	13 (14.0)
Chickens at slaughter		456	325	31 (9.5)
Piglets	15	275	246	57 (23.2)
Weaned pigs	12	180	150	97 (64.7)
Fattening pigs	32	713	664	141 (21.2)
Sows	24	361	332	26 (7.8)
All farm pigs	46	1529	1392	335 (24.1)
Pigs at slaughter		1200	1063	258 (24.3)
Cows	13	370	336	3 (0.9)
Total		4888	4089	763 (18.7)
2007–2008				
Farm chickens			91	5 (5.5)
2010–2011				
Farm chickens			86	10 (11.6)
Farm pigs			172	22 (12.8)

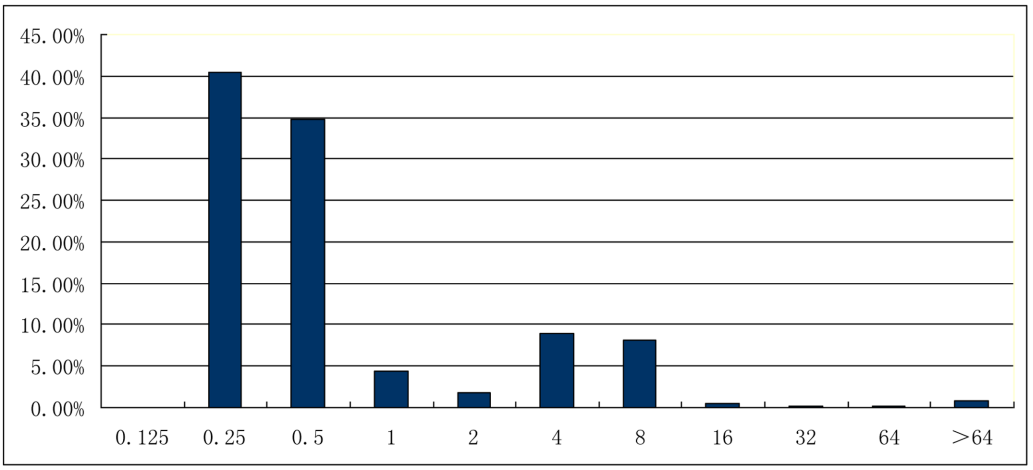

FIGURE 1 | Distribution of minimal inhibitory concentrations (mg/L) of *Escherichia coli* isolates toward colistin.

ATAAGCTGAAACGGATGGC -3′) and EpmrB-R (5′- CATA ATAATCAGGGCGAAAGT -3′). PCR products of *pmrA*, *pmrB*, and *mgrB* were sequenced and the nucleotides and deduced protein sequences were analyzed at the National Center for Biotechnology Information web site[2]. In addition, the *pmrA*, *pmrB*, and *mgrB* sequences of five colistin-susceptible *E. coli* isolates were determined as control.

Complementation Experiments

The wild-type *mgrB* and *pmrB* genes from an *E. coli* reference strain ATCC 25922 (colistin MIC of 0.25 μg/ml) were amplified by PCR using primers EmgrB-F/EmgrB-R and EpmrB-F/EpmrB-R, respectively. The non-coding mdh sequence was amplified by PCR using primers described previously (Jayol et al., 2014). The PCR products were cloned into the plasmid pCR-BluntII-TOPO (Invitrogen) encoding resistance to kanamycin and zeocin. The resulting plasmids pTOPO-mgrB, pTOPO-pmrB, and pTOPO-mdh were separately transformed into *E. coli* TOP10 strains by electroporation. Transformants were selected by on Mueller–Hinton agar supplemented with 50 mg/L of kanamycin. The recombinant plasmids were isolated and transformed into electrocompetent colistin-resistant *E. coli* with *mgrB* mutation (pTOPO-mgrB) or *E. coli* with *pmrB* mutation (pTOPO-pmrB) via electroporation. The transformants were selected on Mueller–Hinton agar supplemented with zeocin (25 mg/L) and the presence of the cloned gene was confirmed by PCR. The colistin MICs of the transformants were determined by the agar dilution method.

RESULTS

Antimicrobial Susceptibility

Overall, 4089 commensal *E. coli* collected from 973 chickens on farm, 1392 pigs on farm, 325 chickens at slaughter, 1063 pigs at slaughter, and 336 cows on farm during 2013–2014 were recovered from 4888 samples. Among them, 763 (18.7%) isolates showed resistance to colistin (MIC $\geq$ 4 mg/L) (**Table 1**). The MIC values were shown in **Figure 1**. MICs of most isolates (75.2%) were distributed at 0.25 mg/L–0.5 mg/L, followed by 4 mg/L–8 mg/L (16.8%). Only MICs of 6.1% isolates and 1.9% isolates were distributed at 1 mg/L–2 mg/L and $\geq$16 mg/L, respectively.

There was a high frequency of colistin resistance in *E. coli* from pigs on farm (24.1%) and at slaughter (24.3%), followed by chickens on farm (14.0%) and at slaughter (9.5%). The resistance frequency of cow isolates was the lowest (0.9%). Compared with the isolates recovered during 2013-2014, *E. coli* isolates collected during 2007–2008 and 2010–2011 showed significantly lower frequency of colistin resistance ($P < 0.05$, **Table 1**).

Of the 258 colistin resistant isolates from pig at slaughter, 76.7% showed resistance to 3–9 other antimicrobial agents, including tetracycline (93.8%), ampicillin (79.5%), florfenicol (64.3%), cefotaxime (13.2%), neomycin (56.6%), gentamicin (29.1%), ciprofloxacin (28.3%), apramycin (12.4%), fosfomycin (7.8%), and amikacin (0.4%) (**Table 2**). The frequencies of antimicrobial resistance to other antimicrobial agents among colistin resistant isolates were significantly higher than those of colistin susceptible isolates ($P < 0.01$), except to amikacin and gentamicin. All of the isolates were susceptible to imipenem.

mcr-1 Detection and Sequence of *pmrA*, *pmrB*, and *mgrB* Genes

Of the 200 randomly seiected colistin-resistant isolates, 182 (91.0%) were positive for *mcr-1*. The sequences of the *pmrA*, *pmrB*, and *mgrB* genes known to be involved in polymyxin resistance were determined in 50 isolates. For PmrA, no amino acid substitution was observed among the 50 isolates except one isolate that had the G144S substitution. However, G144S substitution was found to be present in colistin-susceptible

[2]http://www.ncbi.nlm.nih.gov

TABLE 2 | Comparison of antimicrobial susceptibility of colistin-susceptible isolates and colistin-resistant isolates from pigs at slaughter.

Antimicrobial agents	Colistin-susceptible isolates ($n = 805$) (%)	Colistin-resistant isolates ($n = 258$) (%)	chi-square value	*P*-value
Ampicillin	65.30	79.50	18.126	< 0.0001
Cefotaxime	6.60	13.20	11.307	0.0008
Amikacin	0.90	0.90	0	1.000
Gentamicin	23.90	29.10	2.8293	0.093
Apramycin	4.5	12.40	20.5248	< 0.0001
Neomycin	32.60	56.60	47.52	< 0.0001
Tetracycline	84.80	93.80	293.998	< 0.0001
Florfenicol	47.50	64.30	22.31	< 0.0001
Fosfomycin	2.10	7.80	18.5003	< 0.0001
Ciprofloxacin	17.60	28.30	13.7470	0.0002

isolates (Quesada et al., 2014). For MgrB, 4 isolates from different regions possessed D31G substitution. For PmrB, one isolate had two amino acid substitutions (T246I and D282N). However, the MICs of colistin remained unchanged upon transformation with plasmid pTOPO-mgrB or pTOPO-pmrB.

DISCUSSION

Despite the frequent use of colistin in animal farming for over 50 years, the occurrence of colistin resistance among *E. coli* strains isolated from food animals remains low (<1%) (Kempf et al., 2013; Kieffer et al., 2015). However, in this study, we found a very high prevalence of colistin resistance (18.7%) among commensal *E. coli* isolates from food animals, especially pigs. The frequency of resistance in commensal intestinal *E. coli* is considered to be a good marker for the selection pressure exerted by antibiotic use in the host animals and the resistance problems to be predicted in pathogenic bacteria (van den Bogaard and Stobberingh, 2000). This high prevalence of colistin resistance may be due to the increasing use of colistin in food animals in recent years. Our previous studies showed that most *E. coli* strains from chicken and pigs in China showed resistance to fluoroquinolones and florfenicol, and over 20% isolates exhibited resistance to third-generation cephalosporins, amikacin and fosfomycin (Chen et al., 2014; Rao et al., 2014). Thus, in recent years, the lack of effective drugs against *E. coli* might be attributed by the increased consumption of colistin in veterinary medicine, especially in piglets which are frequently treated with colistin sulphate for colibacillosis. This high selective pressure might result in the highest prevalence (64.7%) of colistin resistance among *E. coli* isolates from weaned piglets found in this study. Compared with pig and chicken isolates, the prevalence of colistin resistance among *E. coli* from cows was very low (0.9%) which might be associated with the infrequent use of this drug on dairy-farm.

To determine whether there was an increase of colistin resistance from 2007 to 2014, *E. coli* isolates collected in our previous study were reviewed for colistin resistance. By comparison, colistin resistance among *E. coli* isolated from chicken raised nearly three times from 2007/2008 to 2013/2014 and that among *E. coli* isolated from pigs raised nearly two times from 2010/2011 to 2013/2014. Though we could not obtain the amount of colistin consumption on each farm sampled in this study, data from China Veterinary Drug Association showed that the volume of colistin sales increased significantly from 2011 to 2013 (China Veterinary Drug Association, 2014). Taken together, our results revealed that colistin resistance in food animals was correlated with the consumption of colistin.

Interestingly, MICs of most colistin- resistant isolates were 4 or 8 mg/L. The emergence and spread of *E. coli* with low level of colstin resistance (MIC = 4 or 8) might lead to the treatment failure of diarrhea with standard colistin dosage (2–20 mg/kg, ppm). Thus, farmers have to illegally use medicated feed added with increased dosage of colistin (80–100 ppm) to prevent diarrhea in piglets (personal communication).

Colistin resistance among commensal *E. coli* isolates recovered from pigs at slaughter was also worrisomely high. These resistant bacteria might contaminate meat during slaughtering procedures and transfer to humans by food chain or to workers via direct animal contact as indicated in some previous studies (Angulo et al., 2004; Liebana et al., 2013). Recently, several studies suggested the possibility of the transference MCR-1-producing Enterobacteriaceae to humans via food chain (Campos et al., 2016; Carnevali et al., 2016; Figueiredo et al., 2016; Wang et al., 2017b). Hence it is urgent to limit the usage of polymyxins (colistin) in veterinary medicine especially as feed additives in China. Fortunately, following our discovery of *mcr-1*, the Chinese Government has banned the use of colistin in animal feed since Nov 1, 2016 (Walsh and Wu, 2016).

Similar to our previous results, colistin resistance is mainly caused by *mcr-1* gene. Quesada et al. have recently found mutations in PmrAB that confer resistance to polymyxins in *E. coli* (Quesada et al., 2014). However, in this study, we failed to detect any meaningful mutation in *pmrAB* and *mgrB* conferring resistance to colistin. Further studies are needed to understand the possible mechanism mediating colistin resistance among *mcr-1*-negative isolates.

CONCLUSION

We have detected a high prevalence of colistin resistance and *mcr-1* gene in *E. coli* from food animals. Though colistin exhibited high antimicrobial activities against GNB, including *E. coli*, *A. baumannii*, *Pseudomonas aeruginosa* isolates, and *K. pneumonia* in human (Chen et al., 2015), the frequent presence of *mcr-1*-positive *E. coli* and in food animals might be a threat to human. As colistin is the last therapeutic option against infections caused by MDR GNB, careful monitoring of the evolution of colistin resistance and the spread of *mcr-1* gene in isolates from humans in China is urgently needed.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: J-HL, XH, and ZZ. Performed the experiments: XH, LY, XC, CZ, XY, YL, SW, ZG, and LY. Analyzed the data: J-HL, XH, LY, YL, and ZZ. Wrote the paper: J-HL and XH.

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Occurrence of OXA-48 Carbapenemase and Other β-Lactamase Genes in ESBL-Producing Multidrug Resistant *Escherichia coli* from Dogs and Cats in the United States, 2009–2013

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Objective: The aim of this study was to explore the occurrence and molecular characterization of extended-spectrum β-lactamases (ESBL), plasmid-mediated AmpC β-lactamase (pAmpC) and carbapenemases among ESBL-producing multidrug resistant (MDR) *Escherichia coli* from dogs and cats in the United States.

Methods: Of 2443 *E.*coli isolated from dogs and cats collected between August 2009 and January 2013, 68 isolates were confirmed as ESBL-producing MDR ones. PCR and sequencing were performed to identify β-lactamases and plasmid-mediated quinolone resistance (PMQR) genes, and shed light on the virulence gene profiles, phylogenetic groups and ST types.

Results: Phylogenic group D and B2 accounted for 69.1% of the isolates. 50 (73.5%) isolates carried CTX-M ESBL gene, and the most predominant specific CTX-M subtype identified was $bla_{\text{CTX-M-15}}$ (n = 33), followed by $bla_{\text{CTX-M-1}}$ (n = 32), $bla_{\text{CTX-M-123}}$ (n = 27), $bla_{\text{CTX-M-9}}$ (n = 19) and $bla_{\text{CTX-M-14}}$ (n = 19), and $bla_{\text{CTX-M-123}}$ was firstly reported in *E. coli* isolates in the United States alone or in association. Other β-lactamase genes bla_{TEM}, bla_{SHV}, $bla_{\text{OXA-48}}$, and $bla_{\text{CMY-2}}$ were detected in 41.2, 29.4, 19.1, and 17.6% of 68 ESBL-producing MDR isolates, respectively. The bla_{TEM} and bla_{SHV} genes were classfied as ESBLs with the exception of the $bla_{\text{TEM-1}}$ gene. Additionally, 42.6% (29/68) of isolates co-expressed $bla_{\text{CTX-M-15}}$ and PMQR gene *aac(6′)-Ib-c*. The overall occurrence of virulence genes ranged from 11.8 (*ireA*) to 88.2% (*malX*), and most of virulence genes were less frequent among CTX-M-producing isolates than non-CTX-M isolates with the exception of *malX* and *iutA*. The 68 isolates analyzed were assigned to 31 STs with six being novel. Three pandemic clonal lineages ST131 (n = 10), ST648 (n = 9), and ST405 (n = 9) accounted for more than 41% of the investigated isolates, and ST648 and ST405 of phylogenetic D were firstly reported in *E. coli* from dogs and cats in the United States.

Conclusion: $bla_{CTX-M-123}$ of ESBLs and carbapenemase bla_{OXA-48} were firstly reported in ESBL-producing MDR *E.*coli from dogs and cats in the United States, and ST131, ST648, and ST405 were the predominant clonal groups.

Keywords: *Escherichia coli*, ESBL, OXA-48 carbapenemase, multidrug resistance, companion animals

INTRODUCTION

Extraintestinal pathogenic strains of *Escherichia coli* (ExPEC) are the most important dogs and cats bacterial pathogens associated with extraintestinal infections (Beutin, 1999). However, extended-spectrum β-lactamase (ESBL)-producing ExPEC are isolated worldwide with increasing frequency from human and animal clinical isolates (Pitout, 2012). The occurrence of β-lactamases, including ESBLs, plasmid-mediated AmpC β-lactamases (pAmpC) and carbapenemases among *E. coli* pose serious challenges to the use of penicillins, extended-spectrum cephalosporins (3rd and 4th generation cephalosporins), monobactams, and carbapenems (Karisik et al., 2008; Geser et al., 2012). Furthermore, ESBL-producing isolates are often cross-resistant to fluoroquinolones and other antimicrobial agents, thus expressed multidrug resistance (MDR). This combination of properties can significantly affect the course and outcomes of infections. β-lactamase genes commonly located on mobile genetic elements, such as plasmids, transposons, or integrons, and the resistance plasmids can easily be transferred between bacterial isolates by conjugation mechanism. Accordingly, transmission of β-lactamase genes between companion animals and owner has become a subject of active discussion as companion animals could be potential sources of ESBL-producing *E. coli* isolates causing community-acquired infections (Schmiedel et al., 2014).

Although the ESBLs, pAmpCs and carbapenemases in *E. coli* isolates from humans and animals have been characterized in various studies around the world, knowledge about the β-lactamases and population structure in MDR *E. coli* isolates from companion animals in the United States is limited. Prior to the current study only two studies have described the occurrence and the diversity of ESBLs in *E. coli* from dogs and cats in the United States (O'Keefe et al., 2010; Shaheen et al., 2011), and the isolates were collected from September 2004 to December 2007, and May 2008 to May 2009, respectively. However, the β-lactamases, particularly CTX-M-type ESBLs, are characterized by ongoing and complex evolution. Currently, greater than 150 variants have been identified, and several chimeras, e.g., $bla_{CTX-M-64}$ and $bla_{CTX-M-123}$ have been reported since 2009 (He et al., 2013). Moreover, several novel β-lactamases, e.g., bla_{KPC}, bla_{NDM-1}, and bla_{OXA-48} are emerging worldwide in *E. coli* isolated from humans or animals.

The aim of the present study was to (i) investigate the occurrence and molecular characterization of ESBL-producing MDR *E. coli* recovered from clinical cases of infection in dogs and cats in the United States, over a period of time ranging from August 2009 to January 2013, and (ii) characterize the association between β-lactamases, phylogenetic groups, virulence genes and the ST types.

MATERIALS AND METHODS

Bacterial Isolates

Between August 2009 and January 2013, a total of 2443 *E. coli* isolates from urine, wound, ear, genital tract, anal sac, nasal structure, and soft tissue samples of dogs and cats with presumed naturally-occurring infection in six geographical regions of the United States: West (California), South (North Carolina), Central (Missouri), Midwest (Ohio and Illinois), and Southeast (Alabama), Northeast (Massachusetts) were received from a nationally recognized veterinary diagnostic laboratory. Isolates were reconfirmed to be *E. coli* upon receipt by the Clinical Pharmacology Laboratory (CPL) at Auburn University based on reculture overnight on CHROMagar Orientation (BD Diagnostics, Franklin Lakes, NJ) at 37°C, and then the isolates were harvested and stored in tryptic soy broth containing 30% glycerol at −80°C until studied.

Susceptibility Testing and Initial ESBL Identification

Antimicrobial susceptibility testing was performed for all 2443 isolates using 96 well custom microdilution susceptibility plates according to the manufacturer's protocol (Trek Diagnostic Systems, Inc., Cleveland, OH). Susceptibility testing was performed using 16 antimicrobials representing six antimicrobial classes and classified into 12 antimicrobial categories: penicillins: ampicillin; penicillins + β-lactam inhibitor: amoxicillin-clavulanic acid; anti-pseudomonal + β-lactam inhibitor: ticarcillin-clavulanic acid; non-extended spectrum cephalosporins (1st generation cephalosporins): cephalothin; extended-spectrum cephalosporins (3rd and 4th generation cephalosporins): cefotaxime, ceftazidime, and cefpodoxime; cephamycins: cefoxitin; carbapenems: meropenem; tetracyclines: doxycycline; phenicols: chloramphenicol; fluoroquinolones: enrofloxacin and ciprofloxacin; aminoglycosides: gentamicin and amikacin; and folate pathway inhibitor: sulfamethoxazole-trimethoprim (Magiorakos et al., 2012; Thungrat et al., 2015). All MIC determinations were performed in triplicates and *E. coli* ATCC 25922 was used for quality control. The results were interpreted according to the guidelines of Clinical Laboratories Standards Institute (CLSI; CLSI, 2013). The MICs were recorded using the Sensititre Vizion system (Trek Diagnostic Systems), and each isolate was categorized in terms of its resistant phenotype as to: susceptible (S), non-multidrug resistance (DR) or MDR. DR was defined as resistance to 1 or 2 antimicrobial classes, and MDR was defined as resistance to three or more antimicrobial classes.

Additionally, all the 2443 *E. coli* isolates were screened for ESBL production using microdilution-based Sensititre (TREK diagnostic systems, Cleveland, Ohio) with ESBL Confirmatory

MIC plates (ESB1F) as described previouly (Aly et al., 2012). Finally, the ESBL-producing isolates expressed MDR phenotype were used in the current study.

Phylogenetic Grouping and Virulence Genotyping

The distribution of phylogenetic groups amongest the ESBL-producing MDR isolates was determined by the new quadruplex PCR as recently described by Clermont et al. (Clermont et al., 2013). Genomic DNA were extracted from bacterial preparations using the PreMan® Ultra Preparation Reagent according to the manufacturer's protocol. The presence of 17 virulence genes [*fimH*, *sfa/focDE*, *afa/draBC*, *papA*, *papC*, *papG alleles* (I, II, III), *hlyA*, *cnf1*, *kpsM* II, *fyuA*, *iutA*, *ireA*, *iroN*, *traT*, and *malX*] known for their association with pathogenicity ExPEC isolates was ascertained in each isolate by use of established PCR assay as reported previously (Johnson and Stell, 2000; Liu et al., 2015).

Identification of β-Lactamase Genes and Other Resistance Genes

The occurrence of β-lactamase genes $bla_{\text{CTX-Ms}}$, bla_{TEM}, bla_{SHV}, $bla_{\text{CMY-2}}$, bla_{KPC}, $bla_{\text{NDM-1}}$, and $bla_{\text{OXA-48}}$ were identified by PCR and subsequent sequencing using specific primers and conditions previously described (Yan et al., 2004; Poirel et al., 2011; Shaheen et al., 2011). Furthermore, the identification of plasmid-mediated quinolone resistance (PMQR) genes [*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6′)-Ib-cr* and *qepA*] was carried out as described previously (Liu et al., 2012).

Transfer of Resistance Genes by Conjugation

We tested whether the ESBL-producing *E. coli* isolates harboring $bla_{\text{CTX-Ms}}$, bla_{TEM}, bla_{SHV}, or $bla_{\text{OXA-48}}$ enzymes were transferable. Conjugation was performed by broth mating at 37°C on 10 ESBL-producing MDR isolates using plasmid-free sodium azide resistant *E. coli* J53 (J53 AZ^r) as recipient as described previously (Shaheen et al., 2011). Transconjugants were selected on tryptic soy agar plates supplemented with sodium azide (150 μg/ml) and cefotaxime (2 μg/ml). Antimicrobial susceptibility, confirmatory tests for ESBL production, and PCR detection of ESBL genes were performed on all transconjugants as described to confirm transfer of ESBL genes.

Multilocus Sequence Typing (MLST)

MLST was performed using seven conserved housekeeping genes of *E. coli* (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA,* and *recA*). A detailed scheme of the MLST procedure, including the primers, PCR conditions, allelic type and sequence type assignment methods, is available at MLST databases at the at the Warwick University website (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

Statistical Analysis

Significance was determined by Pearson's Chi-squared test with Yates continuity correction using "R" software (version 3.0.1), and the level of significance was set at $P < 0.05$.

RESULTS

Antimicrobial Susceptibility

Among the 2443 investigated *E. coli* isolates, 92 isolates (3.8%) were ESBL producers, including 68 (73.9%) MDR isolates, 20 (21.5%) DR isolates, and 4 (4.4%) S isolates. Among the 68 ESBL-producing MDR isolates (including 52 dog and 16 cat isolates), 97.1% (66/68) isolates were resistance to cephalothin, followed by cefotaxime (94.1%), ampicillin (92.6%), cefpodoxime (91.2%), amoxicillin-clavulanic acid (86.8%), ticarcillin-clavulanic acid (85.3%), ceftazidime (69.1%), cefoxitin (44.1%), and meropenem (17.6%). Moreover, some of the investigated ESBL-producing MDR isolates were also resistant to non-β-lactam agents, including ciprofloxacin (91.2%), doxycycline (88.2%), enrofloxacin (82.4%), sulfamethoxazole-trimethoprim (50%), chloramphenicol (44.1%), gentamicin (39.7 %), and amikacin (30.9%).

Phylogenetic Groups and the Virulence Genes Distribution

Phylogenetic analysis showed that the predominant phylogenetic groups were D (35.3%) and B2 (33.8%), followed by C (11.8%), A (10.3%), B1 (5.9%), E (1.5%), and F (1.5%). Fourteen of seventeen investigated virulence genes were detected, with the overall occurrence ranging from 11.8% (*ireA*) to 88.2% (*malX*) with the exception of *papG* I and *papG* II, which were not detected in any isolate. The isolates of phylogenic group B2 harbored more virulence genes (mean 7.3), and followed by group B1 (mean 5.8), group D (mean 5.6), group A (mean 4.7), and group C (mean 3.8). Furthermore, CTX-M-producing isolates possessed more virulence genes (mean 8.4) than did non-CTX-M isolates (mean 4.0; $P < 0.0001$). Several virulence genes, including *sfa/focDE*, *afa/draBC*, *papA*, *papC*, *papG* III, *hlyA*, *cnf1,* and *iroN*, were significantly more common or even exclusively present in non-CTX-M-producing isolates, whereas *traT* was significantly more common in CTX-M-producing isolates than in non-CTX-M isolates (72 vs. 22.2%, $P = 0.0008$). Additionally, the occurrence of virulence genes among ESBL-producing MDR *E. coli* was significantly lower than among non-ESBL isolates with the exception of *malX* ($P < 0.01$; **Table 1**).

Distribution of β-Lactamases and PMQR Genes

The distribution of β-lactamase and PMQR genes among the 68 ESBL-positive MDR *E. coli* isolates was shown in **Table 2**. The results showed that bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, $bla_{\text{CMY-2}}$, and $bla_{\text{OXA-48}}$ were detected in 28 (41.2%), 20 (29.4%), 50 (73.5%), 12 (17.6%), and 13 (19.1%) isolates, respectively. 94.1% (64/68) of the isolates harbored two or more β-lactamase genes, and one isolate from dog with severe urinary tract infection co-harbored eight tested genes [bla_{TEM-5}, $bla_{\text{SHV-12}}$, $bla_{\text{CMY-2}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-123}}$,and *aac(6′)-Ib-cr*; **Table 2**]. For the $bla_{\text{CTX-M}}$ positive isolates, CTX-M enzymes were clustered in CTX-M-1 ($n = 35$), CTX-M-9 ($n = 22$), and hybrid β-lactamases ($n = 27$) clusters. CTX-M-1 and CTX-M-9 double-positive group accounted for 10.3%

TABLE 1 | The occurrence of virulence genes in the ESBL-producing and non-ESBL-producing MDR *E. coli* isolates.

	Occurrence (no. %) of the virulence genes														
	malX	***fimH***	***fyuA***	***traT***	***afa/draBC***	***iutA***	***iroN***	***PapG* III**	***sfa/focDE***	***papC***	***cnf1***	***hlyA***	***papA***	***ireA***	***kpsM* II**
Non-ESBL producers (n = 36)	19 (52.8)	30 (83.3)	26 (72.2)	23 (63.9)	32 (88.9)	26 (72.2)	20 (55.6)	11 (30.6)	17 (47.2)	17 (47.2)	18 (50.0)	17 (47.2)	20 (55.6)	7 (19.4)	18 (50.0)
ESBL producers (n = 68)	60 (88.2)	47 (69.1)	45 (66.2)	41 (60.3)	39 (57.4)	26 (38.2)	23 (33.8)	16 (23.5)	14 (20.6)	12 (17.6)	11 (16.2)	11 (16.2)	10 (14.7)	7 (10.3)	4 (5.9)
CTX-M producers (n = 50)	49 (98)	29 (58.0)	29 (58.0)	36 (72.0)	21 (42.0)	22 (44.0)	9 (18.0)	5 (10)	1 (2.0)	3 (6.0)	2 (4.0)	2 (4.0)	1 (2.0)	1 (2.0)	4 (8)
non-CTX-M producers (n = 18)	11 (61.1)	18 (100)	16 (88.9)	5 (27.8)	18 (100)	4 (22.2)	14 (77.8)	11 (61.1)	13 (72.2)	9 (50.0)	9 (50.0)	9 (50.0)	9 (50.0)	6 (33.3)	0 (0)
P-value															
ESBL producers vs. nonproducers	0.0012	0.016	0.0166	0.101	0.0005	0.0013	0.0037	0.0096	0.0028	<0.0001	0.0011	0.0017	<0.0001	0.0162	<0.0001
CTX-M producers vs. nonproducers	0.0064	0.0887	0.1056	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

of isolates, and three isolates co-harbored CTX-M-1, CTX-M-9 as well as hybrid β-lactamase. $bla_{\text{CTX-M-15}}$ (n = 33) was the predominant genotype in $bla_{\text{CTX-M}}$ positive isolates, and followed by $bla_{\text{CTX-M-1}}$ (n = 32), $bla_{\text{CTX-M-123}}$ (n = 27), $bla_{\text{CTX-M-9}}$ (n = 19), and $bla_{\text{CTX-M-14}}$ (n = 19). Sequencing of bla_{TEM} gene revealed 24 $bla_{\text{TEM-1}}$, three $bla_{\text{TEM-5}}$, and one $bla_{\text{TEM-30}}$, whereas sequencing of bla_{SHV} gene revealed 17 $bla_{\text{SHV-12}}$ and two $bla_{\text{SHV-3}}$. All bla_{TEM} and bla_{SHV} genes were classfied as ESBLs with the exception of the $bla_{\text{TEM-1}}$ gene based on the sequencing. Moreover, 48.5% (33/68) of investigated isolates harbored *aac(6′)-Ib-cr*, while none of the isolates carried *qnr* and *qepA* genes. The vast majority of *aac(6′)-Ib-cr*-producing isolates were positive for $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-1}}$, and $bla_{\text{CTX-M-123}}$, but negative for CTX-M 9 group enzymes despite $bla_{\text{CTX-M-14}}$ and *aac(6′)-Ib-cr* coexisted in three isolates.

Conjugation Experiments

We tested whether bla_{CTX-M} genes or other β-lactamase genes in 10 selected isolates were transferable by conjugation experiments, and seven out of the 10 ESBL-producing isolates successfully transferred the β-lactamase genes to the recipient *E. coli*. PCR analysis showed the presence of respective bla_{CTX-M} genes and other β-lactamase genes, including two bla_{OXA-48}-carrying plasmids from all the transconjugants (**Table 3**). Meanwhile, PMQR gene *aac-(6')-Ib-cr* was co-transferred with β-lactamase genes. Generally, all donors and their transconjugants were resistant to amoxicillin-clavulanic acid, ampicillin, cefotaxime, cefoxitin, cefpodoxime, cephalothin, and ticarcillin-clavulanic acid, and all transconjugants exhibited an increase of at least eight-fold in MICs compared to the recipient, *E. coli* J53 AZ^r. The ciprofloxacin MICs for four transconjugants harboring *aac-(6′)-Ib-cr* ranged from 0.06 to 0.125 mg/L, representing an increase of two-fold to four-fold compared with the recipient (**Table 3**). Additionally, the transconjugants remained susceptible to meropenem, ciprofloxacin, gentamicin, chloramphenicol, and doxycycline, whereas one transconjugant harboring bla_{OXA-48} was resistant to sulfamethoxazole-trimethoprim and reduced the susceptibility to meropenem.

MLST

The MLST investigation revealed that the 68 isolates were assigned to 31 STs, including six new STs (**Table 2**). Twelve STs were represented by more than two isolates, and other 19 STs contained a single isolate each. ST131 (n = 10), ST648 (n = 9), and ST405 (n = 9) accounted for more than 41% (28/68) of investigated isolates and 54% (27/50) of CTX-M-producing isolates, respectively. 74.2% (23/31) of STs, especially ST131, ST648, and ST405 were positively associated with CTX-M-producing isolates, while other STs, including ST10, ST5232, ST1722, ST2175, ST1800, ST73, ST372, and ST127 seem to have no relationship with CTX-Ms. Vast majority of ST131 and ST648 isolates were positively associated with $bla_{CTX-M-15}$ and/or $bla_{CTX-M-1}$ as well as $bla_{CTX-M-123}$, whereas 77.8% of ST405 isolates were negatively associated with $bla_{CTX-M-1}$, $bla_{CTX-M-15}$, and $bla_{CTX-M-123}$ genes. Moreover, 55.6% of ST648 isolates were positively associated with bla_{OXA-48}, and 12

TABLE 2 | Occurrence, diversity, and molecular diversity of ESBL-producing MDR isolates.

Sequence types	Phylogenetic group	Total No. of isolates	β-lactamase genes (No. of isolates)				PMQR gene	Resistance profiles
			Non-ESBL	ESBL	pAmpC	Carbapenemase		
ST5174	F	1		CTX-M-1 + CTX-M-15 + CTX-M-123 (1)	CMY-2 (1)		*aac(6′)-Ib-cr* (1)	AMC, AMP, CIP, CTX, FOX, CPD, CAZ, CEP, CHL, DOX, ENR, GEN, AMK, TIM, SXT
ST1011	E	1		CTX-M-1 + CTX-M-15 (1)			*aac(6′)-Ib-cr* (1)	AMC, AMP, CIP, CTX, FOX, CPD, CAZ, CEP, CHL, DOX, ENR, GEN, AMK, TIM, SXT
ST10	A	1	TEM-1 (1)		CMY-2 (1)			AMC, AMP, CIP, CTX, FOX, CPD, CAZ, CEP, CHL, DOX, ENR, TIM, SXT
ST167	A	2	TEM-1 (1)	CTX-M-1 + CTX-M-15 + CTX-M-123 (2)	CMY-2 (1)		*aac(6′)-Ib-cr* (2)	AMC, AMP, CIP, CTX, FOX, CPD, CAZCHL, DOX, ENR, GEN, TIM, (CEP, AMK)[a]
ST5220	A	1		CTX-M-1 + CTX-M-15 + CTX-M-123 (1)			*aac(6′)-Ib-cr* (1)	AMC, AMP, CIP, CTX, CPD, CEP, DOX, TIM, SXT
ST617	A	1		TEM-5 + CTX-M-15 + CTX-M-123 (1)			*aac(6′)-Ib-cr* (1)	AMC, AMP, CIP, CTX, CPD, CAZ, CEP, DOX, TIM, SXT
ST44	A	1		CTX-M-1 + CTX-M-15 + CTX-M-9 + CTX-M-123 (1)				AMC, AMP, CIP, CTX, CPD, CAZ, CEP, DOX, ENR, TIM, SXT
ST2936	A	1		CTX-M-9 + CTX-M-14 + CTX-M-123 (1)				AMC, CIP, CTX, CPD, CEP, CHL, TIM, SXT
ST2175	B1	1		SHV-12 (1)				FOX, CEP, CHL, DOX, GEN, AMK, SXT
ST443	B1	1		CTX-M-1 + CTX-M-15 (1)			*aac(6′)-Ib-cr* (1)	AMC, AMP, CIP, CTX, CPD, CAZ, CEP, DOX, ENR, TIM
ST162	B1	1		TEM-30 + CTX-M-9 + CTX-M-14 + CTX-M-123 (1)				AMC, CIP, CTX, CPD, CHL, DOX, ENR, TIM, SXT
ST1800	B1	1			CMY-2 (1)	OXA-48 (1)		AMC, AMP, CIP, CTX, FOX, CPD, CAZ, MEM, DOX, ENR, TIM
ST5231	C	1		CTX-M-9+ CTX-M-14 (1)				AMC, AMP, CIP, CTX, CPD, CEP, DOX, ENR, TIM
ST5206	C	1		CTX-M-1+ CTX-M-15+ CTX-M-123 (1)			*aac(6′)-Ib-cr* (1)	AMC, AMP, CIP, CTX, CPD, CAZ, CEP, DOX, ENR, TIM
ST23	C	2	TEM-1 (1)	CTX-M-1 + CTX-M-15 + CTX-M-123 (1)	CMY-2 (1)		*aac(6′)-Ib-cr* (2)	AMC, AMP, CIP, CTX, CPD, CAZ, CEP, DOX, ENR, TIM, (FOX)
ST5232	C	1	TEM-1 (1)				*aac(6′)-Ib-cr* (1)	AMC, AMP, CIP, DOX, ENR, TIM
ST410	C	2	TEM-1 (2)	CTX-M-1 + CTX-M-15 (2), CTX-M-14 (1), CTX-M-123 (1)			*aac(6′)-Ib-cr* (2)	AMC, AMP, CIP, CTX, CPD, CAZ, CEP, DOX, ENR, TIM, SXT
ST1088	C	1	TEM-1 (1)	SHV-3 + CTX-M-1 + CTX-M-15 + CTX-M-123 (1)		OXA-48 (1)	*aac(6′)-Ib-cr* (1)	AMC, AMP, CIP, CTX, CPD, CAZ, CEP, MEM, GEN, AMK, TIM, SXT
ST1722	D	1	TEM-1 (1)		CMY-2 (1)			AMC, AMP, CTX, FOX, CPD, CAZ, CEP, CHL, DOX, TIM, SXT

(Continued)

TABLE 2 | Continued

Sequence types	Phylogenetic group	Total No. of isolates	β-lactamase genes (No. of isolates)				PMQR gene	Resistance profiles
			Non-ESBL	ESBL	pAmpC	Carbapenemase		
ST68	D	1		CTX-M-1 + CTX-M-15 + CTX-M-123 (1)			*aac(6′)-Ib-cr* (1)	AMC, AMP, CIP, CTX, FOX, CPD, CAZ, CEP, CHL, DOX, ENR, TIM, SXT
ST69	D	1		CTX-M-1+ CTX-M-15 (1)				AMC, AMP, CPD, CAZ, CEP, DOX, TIM, SXT
ST38	D	3	TEM-1 (1)	TEM-5 + CTX-M-9 + CTX-M-14 + CTX-M-123 (2), SHV-12 (1), CTX-M-1 (1), CTX-M-15 (1),	CMY-2 (2)		*aac(6′)-Ib-cr* (1)	AMC, AMP, CIP, CTX, FOX, CPD, CEP, ENR, DOX, TIM, (GEN, AMK, SXT)
ST405	D	9	TEM-1 (5)	CTX-M-9 + CTX-M-14 (7), CTX-M-1 + CTX-M-15 (2)	CMY-2 (3)	OXA-48 (3)	*aac(6′)-Ib-cr* (2)	AMC, AMP, CIP, CTX, FOX, CPD, CAZ, CEP, CHL, DOX, GEN, TIM, (AMK, MEM, SXT)
ST648	D	9	TEM-1 (2)	SHV-12 (2), CTX-M-1 + CTX-M-15 + CTX-M-123 (3), CTX-M-1 + CTX-M-15 + CTX-M-9 (2), CTX-M-1 + CTX-M-15 (3), CTX-M-9 + CTX-M-14 (1),	CMY-2 (1)	OXA-48 (5)	*aac(6′)-Ib-cr* (6)	AMC, AMP, CIP, CTX, FOX, CPD, CAZ, CEP, DOX, ENR, AMK, TIM, SXT, (CHL, MEM, GEN)
ST131	B2	10	TEM-1 (4)	SHV-3 (1), CTX-M-1 + CTX-M-15 + CTX-M-123 (7), SHV-12 (5), CTX-M-1+ CTX-M-9 + CTX-M-14 (2)		OXA-48 (2)	*aac(6′)-Ib-cr* (7)	AMC, AMP, CTX, CPD, CAZ, CEP, CHL, DOX, ENR, GEN, TIM, (CIP, AMK, SXT)
ST12	B2	2		CTX-M-9 (1), CTX-M-14 (1)		OXA-48 (1)		AMC, AMP, CIP, CTX, CPD, CAZ, CEP, (CHL MEM, DOX, ENR, TIM)
ST5219	B2	1		SHV-12 + CTX-M-14 (1)			*aac(6′)-Ib-cr* (1)	AMP, CTX, CEP, CHL, DOX, ENR
ST961	B2	2		SHV-12 (1), CTX-M-14 (1)			*aac(6′)-Ib-cr* (1)	AMC, CTX, FOX, CEP, CHL, GEN, (CIP, SXT)
ST127	B2	2	TEM-1 (1)	SHV-12 (2)				AMC, AMP, CIP, CTX, FOX, CPD, CEP, CHL, DOX, ENR, (GEN, AMK, TIM, SXT)
ST73	B2	3	TEM-1 (2)	SHV-12 (1)				AMC, AMP, CIP, CTX, CPD, CAZ, CEP, DOX, ENR, TIM
ST372	B2	3		SHV-12 (3)				AMC, AMP, CIP, CTX, FOX, CPD, CEP, CHL, DOX, ENR, (TIM)

AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; CPD, cefpodoxime; CEP, cephalothin; MEM, meropenem; DOX, doxycycline; CHL, chloramphenicol; CIP, ciprofloxacin; ENR, enrofloxacin; GEN, gentamicin; AMK, amikacin; TIM, ticarcillin-clavulanic acid; SXT, sulfamethoxazole-trimethoprim.

[a]*The antibiotics in parentheses indicated that the antibiotics were variability among the isolates.*

pAmpC genes bla_{CMY-2} were distributed in nine STs. Notably, all ST131, ST405, and ST648 isolates expressed resistance to ciprofloxacin and 3rd generation cephalosporins, whereas all ST131 isolates remained susceptible to cefoxitin. A strong correlation was revealed between the virulence gene profiles and STs, and the same STs showed the similar virulence gene profiles. Among the three most common STs, ST405 isolates harbored more virulence genes (mean 4.6), followed by ST131 (mean 4.4), and virulence genes were less abundant in ST648 isolates (mean 3.4). Almost all of the ST131 and ST405 isolates were positive for *afa/draBC*, *traT,* and *malX* genes, ST648 isolates were significantly associated with *fimH*, *malX* and *traT*, but negative for *afa/draBC*.

DISCUSSION

ESBLs, pAmpC and carbapenemases are mostly responsible for the emerging resistance to the β-lactam antibiotics, especially the 3rd generation cephalosporins and carbapenems in *E. coli* (Pitout, 2012). In the present study, we conducted a molecular detection and characterization of the β-lactamase genes in ESBL-producing MDR *E. coli* isolates from dogs and cats in the United States over a period of time ranging from August 2009 to January 2013, and also revealed the association between the phylogenetic groups, virulence gene profiles, genetic backbones and β-lactamase types.

The prevalence of 3.8% ESBL-producing *E. coli* found in this study is similar to that recorded in a recent study (3%; Shaheen et al., 2011) but higher than the first survey (1%; O'Keefe et al., 2010) among *E. coli* from dogs and cats in the United States. Surprisingly, 73.9% (68/92) of the ESBL-producing *E. coli* exhibited MDR phenotype, and 75% of MDR isolates were resistant to more than 10 antimicrobial agents tested. Phylogenetic groups D and B2 were the main phylogenetic groups in this study, and it was similar to the phylogenetic subtype distribution of the ESBL-producing isolates from human patients (Hu et al., 2013), which further demonstrated that isolates in phylogenetic groups D and B2 were associated with extraintestinal infections. Among the 68 ESBL-producing MDR isolates, bla_{CTX-M} was prominent and detected in 73.5% (50/68) of isolates, whereas two previous similar surveys carried out in different states in the United States showed that the corresponding prevalence of bla_{CTX-M} were 16.7% and 89.7%, respectively (O'Keefe et al., 2010; Shaheen et al., 2011). It is indicated that the geographical regions, time, resistant phenotype and the history of antimicrobial treatment of the animals can affect the prevalence of bla_{CTX-M} gene. The high prevalence of bla_{CTX-M} strongly suggests a significant role for *E. coli* isolates from companion animals as ESBL gene reservoirs, which poses an additional risk to humans. Therefore, monitoring of the spread of bla_{CTX-M} genes in *E. coli* isolates in dogs and cats is urgently needed. Althouth $bla_{CTX-M-15}$ was still the most frequently encountered gene, the specific genotype of bla_{CTX-M} is undergoing changes, which was supported by available evidence from the occurrence of CTX-M-9 group as well as the occurrence of a novel hybrid β-lactamase gene $bla_{CTX-M-123}$. $bla_{CTX-M-123}$ was firstly discovered in *E. coli* from pig feces in China in 2013 (He et al., 2013), and afterward in human specimen (Hu et al., 2013). It is interesting to note that $bla_{CTX-M-15}$ is also the most widely distributed ESBL gene among human-associated *Enterobacteriaceae* (Cantón and Coque, 2006). These finding revealed the possibility of cross-transmission between animals and humans. Moreover, several isolates appear only with bla_{TEM-1}, bla_{CMY-2}, or bla_{OXA-48}, suggesting that these isolates perhaps carry other ESBL genes, which will require further studies.

bla_{CMY-2} was the most prevalent pAmpC, and it not only confer resistance to a wide range of extended-spectrum cephalosporins but also are not affected by β-lactamase inhibitors. bla_{CMY-2} was dected in 17.6% of the isolates in our study, and it was significantly lower than the occurance of bla_{CMY-2} (89%) in *E. coli* from companion animals in a previous study in the United States (Shaheen et al., 2011). We supposed that the occurance of bla_{CMY-2} might be underestimated since only the ESBL-producing MDR isolates were characterized in this study. Meanwhile, our results showed that majority (58.3%) of CMY-2-producing isolates belonged to phylogenetic group D, consistent with a previous study in *E. coli* from human in Australia (Sidjabat et al., 2014). This similar distribution of phylogenetic group further certified that bla_{CMY-2} can also be transferred between different bacterial species and between animals and humans (Li et al., 2007; Shaheen et al., 2011). bla_{OXA-48} was initially reported in *Klebsiella pneumoniae* isolates in Turkey in 2001(Poirel et al., 2004) and afterward in other Mediterranean countries (Spain, France, Italy, Egypt, and Lebanon Turkey) (Girlich et al., 2014). In 2013, it was firstly discovered in *E. coli* from dogs in Germany (Stolle et al., 2013). bla_{OXA-48} can hydrolyze carbapenems and β-lactamase inhibitors but has no activity toward broad-spectrum cephalosporins (Mathers et al., 2013). Our data showed that about 19% of the isolates carried the bla_{OXA-48}, and they were mostly associated with meropenem resistance, sequence types ST648, ST405, and ST131 as well as different combinations of β-lactamase genes. To our knowledge, bla_{OXA-48} was firstly reported in the United States in 2012 (Poirel et al., 2012), and the present study is the first report of bla_{OXA-48} in *E. coli* from dogs and cats in the United States. Moreover, bla_{OXA-48} can transfer with other β-lactamases and *aac(6′)-Ib-cr*. This finding also revealed possibility of the transfer between humans and companion animals appears highly probable through multiple potential pathways although bla_{OXA-48} is still sporadic occurrence in animals.

aac(6′)-Ib-cr was the exclusive PMQR gene in this study, and CTX-M-producing isolates (particularly $bla_{CTX-M-15}$ positive isolates) showed significantly higher occurrence of *aac(6′)-Ib-cr* compared to non-CTX-M or non-ESBL isolates (62 vs. 11.1 vs. 10%, $P < 0.001$). The frequent combination of $bla_{CTX-M-15}$ and *aac(6′)-Ib-cr* in this study further supported the previous studies that coproduction of β-lactamases and PMQR genes could conduce to the dissemination of MDR isolates, and also reflect the fact that genes encoding resistance to β-lactams and quinolones are located on the same plasmid. Although it was not the primary focus of this study, our results coincided

TABLE 3 | Antibimicrobial susceptibility testing profiles of *E. coli* isolates used in the conjugation experiments.

Strain ID		MIC (μg/ml) of antimicrobial agents													Presence or absence of					
		AMC	AMP	CTX	FOX	CPD	CAZ	CEP	TIM	MEM	CIP	GEN	CHL	DOX	SXT	TEM	SHV	CTX-M-15	CTX-M-14	CTX-M-123	OXA-48	*aac(6′)-Ib-cr*
J53 AZ^r	Recipient	1	4	0.125	0.5	2	0.06	8	2	0.015	0.03	0.25	0.125	0.06	0.06	–	–	–	–	–	–	–
S0544451	Donor	16	512	128	4	256	32	1024	256	0.03	32	32	0.25	1	32	+	+	+	–	+	+	+
Trans-S0544451	Transconjugant	**8**	**512**	**64**	**4**	**64**	**32**	**1024**	**128**	0.015	0.125	0.25	0.125	0.5	0.25	+	–	+	–	+	+	+
H0477803	Donor	32	512	64	32	256	8	1024	64	0.03	16	1	0.25	0.5	1	+	+	+	+	+	–	+
Trans-H0477803	Transconjugant	**32**	**512**	**64**	**16**	**128**	**4**	**1024**	**64**	0.015	0.06	0.125	0.125	0.25	0.25	+	–	+	–	+	–	+
L0812551	Donor	32	512	64	4	256	0.5	1024	256	0.03	64	16	16	32	32	+	–	–	+	+	–	–
Trans-L0812551	Transconjugant	**32**	**512**	**64**	**4**	**256**	**0.5**	**1024**	**256**	0.03	0.015	0.25	0.125	1	**0.5**	–	–	–	+	–	–	–
I0447331	Donor	16	512	128	128	512	16	>1024	128	0.5	64	32	16	32	32	–	+	+	–	–	–	–
Trans- I0447331	Transconjugant	**16**	**512**	**128**	**64**	**512**	**8**	**1024**	**64**	0.015	0.03	0.125	0.125	0.5	**1**	–	+	+	–	–	–	–
I4097858	Donor	16	512	>1024	16	512	32	>1024	512	0.5	32	1	16	32	16	+	–	+	–	–	+	–
Trans-I4097858	Transconjugant	**8**	**256**	**512**	**16**	**256**	**32**	**>1024**	**512**	0.06	0.015	0.25	0.125	0.25	**32**	+	–	+	–	–	+	–
B6276651	Donor	16	512	>1024	8	256	128	>1024	>1024	0.25	128	128	32	16	2	+	+	+	–	+	–	+
Trans-B6276651	Transconjugant	**16**	**512**	**>1024**	**8**	**256**	**64**	**1024**	**>1024**	0.03	0.125	0.25	0.125	0.5	0.06	+	–	–	–	+	–	+
Y0769764	Donor	16	512	128	16	256	64	>1024	256	0.03	32	32	16	16	1	+	–	+	–	+	–	+
Trans-Y0769764	Transconjugant	**16**	**512**	**64**	**8**	**128**	**64**	**>1024**	**256**	0.015	0.125	0.125	0.125	0.5	0.25	–	–	+	–	–	–	+

Bold values mean that MIC of transconjugant increased at least eight-fold relative to those for the recipient.

with a previous study (Qin et al., 2013) that ESBL-producing isolates presented a lower occurrence of studied virulence genes compared with non-ESBL isolates (the data from another study in our laboratory) with the exception of *malX* gene, and CTX-M-producing *E. coli* harbored fewer virulence genes than non-CTX-M isolates ($P<0.0001$). A possible reason why individual virulence gene increased among ESBL-producing is that might be a fitness trade-off for the ESBL to survive antibiotics exposure (Qin et al., 2013) and the difference source of *E. coli*. The exact explanation needs additional study in the future.

A previous review suggested that attention should be paid to the rising of *E. coli* ST131, ST648, ST405, and ST38 isolates as they can play an important role in the worldwide distribution of CTX-M-producing *E. coli* (Pitout, 2012). It was further confirmed by our results since ST131, ST648, and ST405 accounted for 54% of the CTX-M-producing MDR isolates. ST131 was the predominant clone in this study, and all ST131 isolates remained susceptible to cefoxitin, which has been recently suggested as an alternative carbapenems for the treatment of infections by ESBL-producing *E. coli* (Guet-Revillet et al., 2014). It is noteworthy that nine ST648 isolates were strongly associated with $bla_{\text{CTX-M-15}}$ (88.9%, 8/9), $bla_{\text{OXA-48}}$ (55.6%, 5/9), and severe clinical signs. The zoonotic potential of ST648 ESBL-producing isolates has been indicated in the isolates from humans, domestic and wild animals in previous studies (Nicolas-Chanoine et al., 2008; Cortes et al., 2010), and two recent studies in Europe further suggested that ST648 clone may represent a novel genotype that combines MDR phenotype, extraintestinal virulence and zoonotic potential in companion animals (Huber et al., 2013; Ewers et al., 2014). Furthermore, ST131, ST648, and ST405 isolates have the similar β-lactamase gene combinations and resistance profiles, respectively. While it is alarming that other STs have various β-lactamase gene combinations, especially one ST38 isolate, which was associated with the highest frequency of β-lactamases and *aac(6′)-Ib-cr*, high level cephalosporins resistance (MICs $\geq$ 32 μg/ml), lowest frequency of virulence genes and severe clinical signs. Nevertheless, constant attention and further investigations for ST648 and ST38 isolates in companion animals are necessary as they are now rapidly and globally disseminated as well as the companion animals are more and more considered an important source of human infections as the physical closeness.

CONCLUSION

CTX-M-producing *E. coli* tend to have less virulent properties compared with the non-CTX-M isolates. CTX-Ms represented by $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-15}}$, and $bla_{\text{CTX-M-123}}$ have spread rapidly. The occurrence of $bla_{\text{CTX-M-123}}$ of ESBLs and $bla_{\text{OXA-48}}$ carbapenemase were particularly striking, being reported here for the first time in *E. coli* from dogs and cats in the United States. ST131, ST648, and ST405 were the predominant clonal groups among the ESBL-producing *E. coli*, and all ST131 isolates remained susceptible to the cefoxitin. This information will be useful for assessing

the epidemiological risk factors and appropriate use of antimicrobials for ESBL-producing *E. coli* infections of dogs and cats.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Detection of SGI1/PGI1 Elements and Resistance to Extended-Spectrum Cephalosporins in *Proteae* of Animal Origin in France

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Proteae, and especially *Proteus mirabilis*, are often the cause of urinary tract infections (UTIs) in humans. They were reported as carriers of extended-spectrum β-lactamase (ESBL) genes, and recently of carbapenemases, mostly carried by the *Salmonella* genomic island 1 (SGI1) and *Proteus* genomic island 1 (PGI1). *Proteae* have also lately become an increasing cause of UTIs in companion animals, but antimicrobial susceptibility data in animals are still scarce. Here, we report the characterization of 468 clinical epidemiologically unrelated *Proteae* strains from animals collected between 2013 and 2015 in France. Seventeen *P. mirabilis* strains (3.6%) were positive for SGI1/PGI1 and 18 *Proteae* (3.8%) were resistant to extended-spectrum cephalosporins (ESC). The 28 isolates carrying SGI1/PGI1 and/or ESC-resistance genes were isolated from cats, dogs, and horses. ESBL genes were detected in six genetically related *P. mirabilis* harboring bla_{VEB-6} on the SGI1-V variant, but also independently of the SGI1-V, in 3 *P. mirabilis* strains (bla_{VEB-6} and $bla_{CTX-M-15}$) and 1 *Providencia rettgeri* strain ($bla_{CTX-M-1}$). The AmpC resistance genes bla_{CMY-2} and/or bla_{DHA-16} were detected in 9 *P. mirabilis* strains. One strain presented both an ESBL and AmpC gene. Interestingly, the majority of the ESBL/AmpC resistance genes were located on the chromosome. In conclusion, multiple ESC-resistance genetic determinants are circulating in French animals, even though SGI1-V-carrying *P. mirabilis* seems to be mainly responsible for the spread of the ESBL gene bla_{VEB-6} in dogs and horses. These results are of public health relevance and show that companion animals in close contact with humans should be regarded as a potential reservoir of ESC-resistant bacteria as well as a reservoir of ESC-resistance genes that could further disseminate to human pathogens.

Keywords: SGI1, PGI1, *Proteus*, ESBL, AmpC, animal, dog

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INTRODUCTION

Proteae are specific *Enterobacteriaceae* comprising bacterial species present in water, soil or in the intestinal tract of humans and animals. In humans, *Proteae*, and especially *Proteus mirabilis*, are often the cause of urinary tract infections (UTIs; Schaffer and Pearson, 2015). They are otherwise opportunistic pathogens responsible for various infections ranging from minor to life-threatening issues. In veterinary medicine, *P. mirabilis* and other *Proteae* are rarely found

as pathogens except as a cause of UTIs in companion animals (Bubenik et al., 2007). This pathology is only rarely treated with extended-spectrum cephalosporins (ESC), and the recommended antibiotics are sulphonamides, aminoglycosides, or fluoroquinolones.

Proteus mirabilis is naturally susceptible to β-lactams and β-lactamases inhibitors (Stock, 2003). In the late 1990s, the emergence of *P. mirabilis* isolates expressing acquired β-lactamases was first reported in France (Chanal et al., 2000). Extended-spectrum (ESBL) and AmpC β-lactamases are of a critical importance because they both confer resistance to nearly all β-lactams, including ESC. Interestingly, even though the corresponding genes (mostly $bla_{\text{CTX-M-types}}$ and $bla_{\text{VEB-6}}$ for ESBL and $bla_{\text{CMY-2}}$ and $bla_{\text{DHA-16}}$ for AmpC) are generally located on plasmids which allow an easy intra- or inter-species dissemination, several studies revealed chromosome-encoded ESBL/AmpC genes in *P. mirabilis* (Song et al., 2011; Harada et al., 2012). These chromosome-encoded genes are often carried by genomic islands (such as the *Salmonella* Genomic Island 1, SGI1) or integrating conjugative elements (ICEs) that may also be transmitted (Harada et al., 2010; Mata et al., 2011). While ESBL-producing *P. mirabilis* are nowadays commonly isolated from humans, the first CTX-M-55-producing *P. mirabilis* in animals was only reported in 2011 from a macaque imported from Vietnam to France (Dahmen et al., 2013).

Besides, *P. mirabilis* can also carry various genomic islands conferring multidrug resistance. For example SGI1, the genomic island widely disseminated in *Salmonella*, was first identified in a clinical *P. mirabilis* from a diabetic patient from Palestine in 2006 (Ahmed et al., 2007). SGI1 is a site-specific integrative mobilizable element conferring multidrug resistance initially described in *Salmonella enterica* serovar Typhimurium DT104 (Boyd et al., 2001).

Salmonella genomic island 1 is the first MDR genomic island identified in *S. enterica*, and contains a complex class 1 integron, named In104 (Hall, 2010). Since the identification of SGI1 in *S.* Typhimurium DT104, more than 30 different SGI1 variants carrying different combinations of antimicrobial resistance genes were described so far (Hall, 2010). The complex In104 integron variants classically possess one or two cassette attachment sites (*attI1*) carrying various resistance gene cassette arrays, contain an IS*6100* element, may contain additional resistance genes, and are bound by 25-bp inverted repeats IRi and IRt (Boyd et al., 2001) (**Figure 1**). In the great majority of these variants, the complex In104 integron or its variants are found always at the same position in the SGI1 scaffold, i.e., between the resolvase gene *res* (also named *tnpR*) and the open reading frame (ORF) S044 at the 3′ end of SGI1 (Hall, 2010). A few additional variations occurred in the SGI1 backbone of some variants, especially the insertion/deletion created by IS*Vch4* between ORF S005 and S009 that was found in several SGI1 variants (SGI1-H, -Ls, -Ks, -Ps, Qs, -*Pm*ABB, and -*Pm*MAT) in *S. enterica* and *P. mirabilis* (Doublet et al., 2008; Siebor and Neuwirth, 2013).

Salmonella genomic island 1 is found integrated most of the time within the last 18 bp of the well-conserved chromosomal *trmE* gene (also named *thdF*). SGI1 is specifically mobilized *in trans* by conjugative plasmids of the IncA/C family (Doublet et al., 2005). Only this plasmid family has been shown to be able to mobilize SGI1 (Douard et al., 2010). The main reason of this specificity is that the SGI1 excision from the chromosome is triggered by the master activator AcaDC encoded by IncA/C conjugative plasmids (Carraro et al., 2014; Kiss et al., 2015). Then, as an extrachromosomal form, SGI1 is able to hijack the conjugative apparatus encoded by IncA/C plasmids to be conjugally transferred to a recipient cell (Carraro et al., 2014).

Since 2006, *P. mirabilis* strains carrying different SGI1 variants have only been reported in China and France (Boyd et al., 2008; Siebor and Neuwirth, 2013; Qin et al., 2015). Importantly, the SGI1-V variant, which is specifically found in *P. mirabilis*, harbors the $bla_{\text{VEB-6}}$ gene and was first reported in a lethal human case in France (Siebor and Neuwirth, 2011).

Recently, a new multidrug resistant genomic island named *Proteus* Genomic Island 1 (PGI1) was described in human *P. mirabilis* isolates in France (Siebor and Neuwirth, 2014). A specific PGI1 variant, PGI1-*Pm*PEL, was shown to harbor both the $bla_{\text{VEB-6}}$ gene and the carbapenemase-encoding gene bla_{NDM-1} (Girlich et al., 2015). *P. mirabilis* isolates of animal origin were also shown to carry SGI1 or PGI1. Indeed, SGI1-positive *P. mirabilis* isolates were reported in poultry and swine farms in China (Lei et al., 2014, 2015). In France, we recently described the very first cases of SGI1 (including the VEB-6-producing SGI1-V variant) or PGI1-positive *P. mirabilis* in dogs (Schultz et al., 2015).

The SGI1/PGI1-positive *P. mirabilis* isolates reported in animals and humans so far were sporadic cases. Considering the apparent emergence of these genetic determinants in *P. mirabilis*, our aim was to investigate the prevalence of SGI1 and PGI1 in *Proteae* of animal origin in France, and to characterize molecularly the collected strains. In line with recent observations that a SGI1-V-carrying *P. mirabilis* clonal population was shared between humans and animals, we also investigated the genetic relationship of those isolates in order to draw hypotheses on a possible transfer between the two populations. Finally, as these islands in *P. mirabilis* isolates were also shown to occasionally capture ESBL or carbapenemase genes, we investigated the global prevalence of those genes in the same collection.

MATERIALS AND METHODS

Bacterial Strains and Antibiotic Susceptibility Testing

Between April, 2013 and February, 2015, a total of 468 clinical non-duplicate *Proteae* isolates (*P. mirabilis*, $n = 459$; *P. vulgaris*, $n = 1$; *P. penneri*, $n = 1$; *Morganella morganii*, $n = 2$; *Providencia rettgeri*, $n = 4$; *P. stuartii*, $n = 1$) were collected from various animal hosts corresponding to distinct and epidemiological unrelated individuals (dogs, $n = 411$; cats, $n = 25$; horses, $n = 13$; bovine, $n = 7$; rabbit, $n = 3$; ovine, $n = 3$; ferret, $n = 2$; snake, $n = 2$; chicken; $n = 1$;

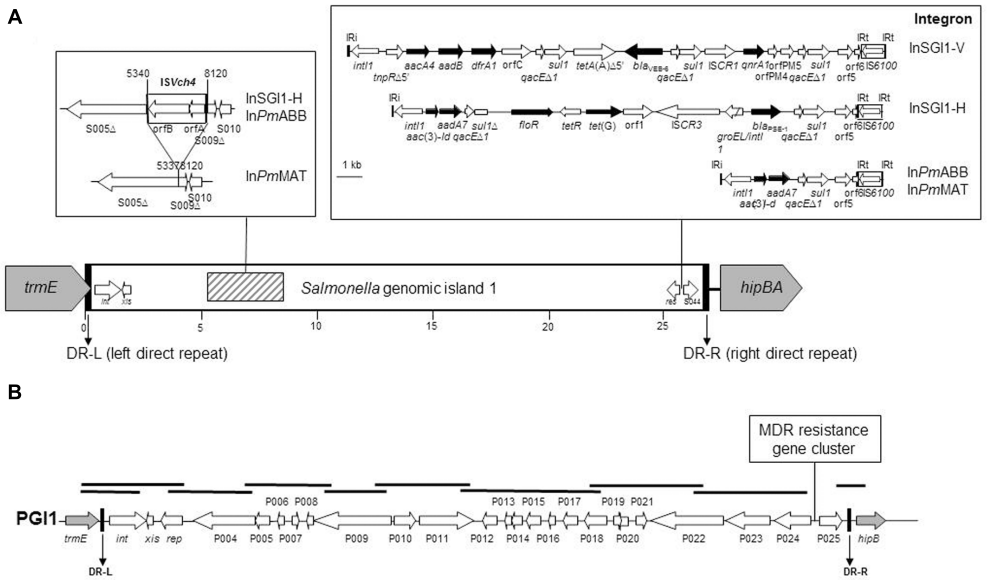


FIGURE 1 | Schematic view of SGI1 in the *Proteus mirabilis* chromosome. (A) Specific genetic traits of the SGI1 structure, the genetic rearrangement due to IS*Vch4*, and the complex class 1 integrons are represented in frames. Genes and ORFs are shown as arrows, with their orientations of transcription indicated by the arrowheads. Black arrows indicate antibiotic resistance genes. White arrows within boxes represent insertion sequence elements. IRi and IRt are 25-bp imperfect inverted repeats defining the integrase and *tni* ends of complex class 1 integrons. The complex class 1 integrons were drawn from PCR mapping results and GenBank accession numbers AF261825 (SGI1), HQ888851 (SGI1-V), AY458224 (SGI1-H), and JX121638 (SGI1-*Pm*ABB). **(B)** Schematic view of PGI1 in the *P. mirabilis* chromosome. PCRs carried out to map the PGI1 backbone and junctions with the chromosome are indicated by thick black bars. Gray and white arrows represent the chromosomal genes and the backbone of the island, respectively.

bird, $n = 1$) and originating from distant geographical areas throughout France. These isolates were collected through the Resapath, the French antimicrobial resistance surveillance network in animal pathogens[1]. Identification was performed by peripheral veterinary laboratories and then confirmed at the Anses laboratory in Lyon, France, using Api20E strips (bioMérieux, Marcy l'Etoile, France). Isolates were screened for antibiotic resistance by the disk diffusion method according to the guidelines of the CA-SFM with 30 antibiotics of veterinary or human interest[2].

SGI1/PGI1 Detection and PCR Mapping

Salmonella genomic island 1 detection was performed by PCR amplification with degenerated primers designed in order to amplify all known SGI1 and PGI1 integrase genes (FwintSGI1HR, 5′ATGTTGCGTCAGGCYGAGGC; RvintSGI1HR, 5′GAGTGYCCAAGAAGSCGAGAG). The chromosomal location was confirmed by PCR amplification of the left and right junctions in the chromosome, as previously described (Schultz et al., 2015). The genetic diversity of SGI1 was assessed by PCR mapping covering the entire island using primers previously described (Siebor and Neuwirth, 2011, 2013). PGI1 detection, chromosomal location, backbone mapping and resistance gene detection were performed by PCR, as previously described (**Figure 1**) (Siebor and Neuwirth, 2014).

[1]www.resapath.anses.fr

[2]www.sfm-microbiologie.org

Identification and Genetic Location of ESBL and AmpC Genes

β-lactamase genes detection was performed by PCR, as previously described (Dallenne et al., 2010). For the CTX-M-1 group, an additional PCR was performed using external primers (ISEcp1L1, 5′ CAGCTTTTATGACTCG; P2D, 5′ CAGCGCTTTTGCCGTCTAAG) and the amplicons were sequenced.

The chromosomal location of these genes was assessed with the *I-Ceu*I (New England Biolabs, Hertfordshire, UK) technique (Liu et al., 1993). After digestion of the complete DNA, DNA fragments were separated by Pulsed-Field Gel Electrophoresis (PFGE) in TBE 0.5 X at 14°C using a CHEF Mapper (Bio-Rad Laboratories, Richmond, CA, USA). Running conditions were 6 V/cm with a switch time of 5.3–49.9 s for 19.7 h. Southern blots were performed by transferring the DNA on a Hybond-N^+ membrane and hybridizing the membrane with DIG-labeled probes specific for the 23S rDNA, the ESBL (bla_{VEB-6}, $bla_{CTX-M-1}$, or $bla_{CTX-M-15}$) and AmpC (bla_{CMY-2} or bla_{DHA-16}) genes of interest (see detailed primers in Supplementary Table S1). Probes were prepared by PCR using labeled DIG-dUTP (PCR DIG probe synthesis kit; Roche Diagnostics, Indianapolis, IN, USA). Detection was performed using the DIG DNA Labeling and Detection Kit (Roche Diagnostics) according to the manufacturer's instructions.

The plasmidic location of these genes was assessed by Southern blots on S1-PFGE gels (New England Biolabs, Hertfordshire, UK). Running conditions were 6 V/cm with a

switch time of 1–30 s for 20 h. Hybridization was performed as described above for Southern blots on *I-Ceu*I gels, using the same ESBL/AmpC probes and the DIG DNA Labeling and Detection Kit.

Finally, all SGI1/PGI1-positive, ESBL and AmpC strains were analyzed by PCR-based replicon typing (PBRT kit; Diatheva, Fano, Italy) in order to type the plasmids carrying the ESBL/AmpC genes.

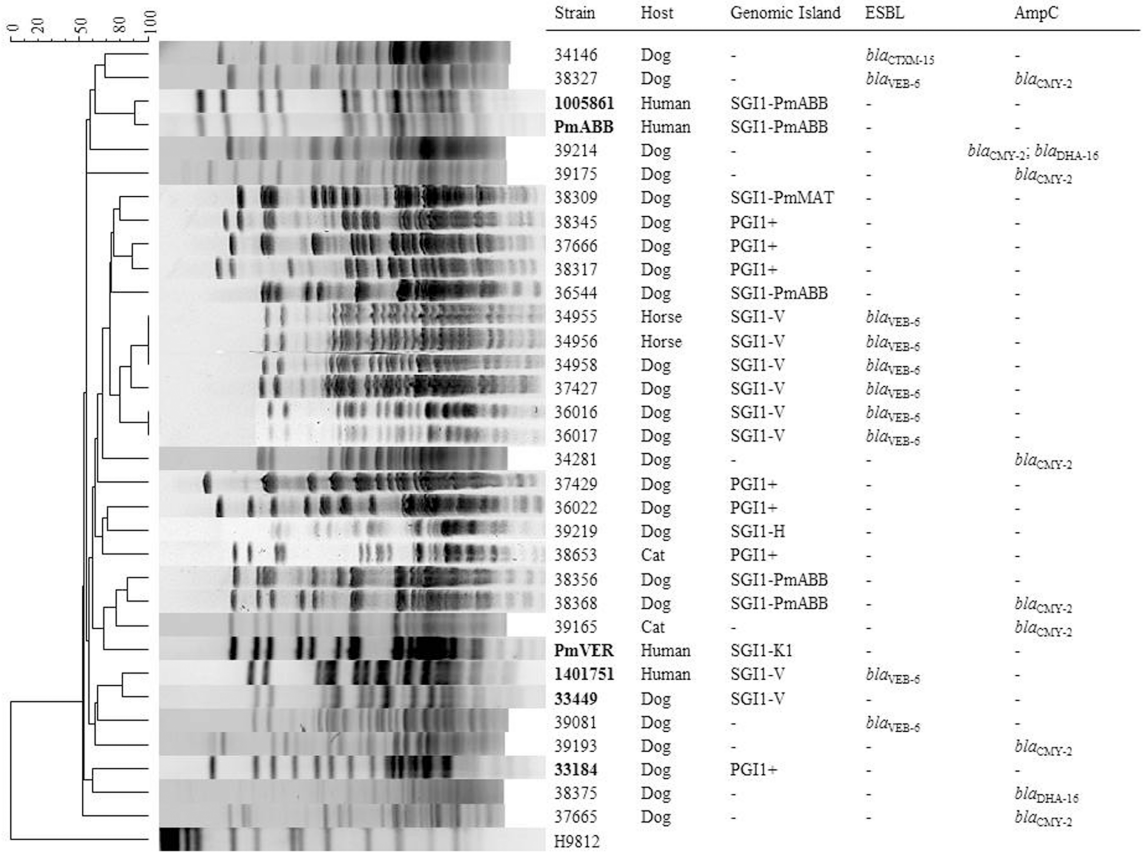
Genetic Relationship of the Isolates

The genetic diversity was investigated by PFGE. DNA plugs were digested with *Sma*I (Promega, Madison, WI, USA) and genomic DNA was separated by CHEF Mapper gel electrophoresis in TBE 0.5 X at 14°C. The running conditions were 6 V/cm with a switch time of 5–20 s for 22 h. *Xba*I-digested *S. enterica* serovar Braenderup strain H9812 was used as size ladder. The DNA patterns were analyzed using BioNumerics software version 4.5 (Applied Maths, Sint-Martens-Latem, Belgium) to construct a phylogenetic tree. Analysis was performed using the Dice coefficient with optimization set at 0.5% and tolerance at 1%.

RESULTS AND DISCUSSION

Prevalence and Molecular Characterization of the SGI1/PGI1 Genomic Islands

Salmonella genomic island 1/*Proteus* genomic island 1-carrying *P. mirabilis* are emerging pathogens in humans and animals. In a recent study, SGI1/PGI1-carrying *P. mirabilis* were reported in animals in France but no large-scale non-biased data on their prevalence were available (Schultz et al., 2015). Here, we investigated a large collection of 468 *Proteae* isolates of animal origin in France to estimate the prevalence of the SGI1/PGI1 elements. We also assessed the prevalence of resistance to broad-spectrum cephalosporins in those *Proteae* isolates considering that the bla_{VEB-6} gene was recurrently reported on the SGI1-V variant in *P. mirabilis* (Siebor and Neuwirth, 2011; Schultz et al., 2015). Among the 468 isolates studied, 17 *P. mirabilis* (17/468, 3.6%) were positive for SGI1 (11 isolates) or PGI1 (6 isolates) by PCR (**Figure 2** and **Table 1**). They were mostly recovered from dogs ($n = 13$; 13/411, 3.2%), but also from cats ($n = 2$; 2/25, 8%), and horses ($n = 2$; 2/13, 15.4%). While *Proteae* are more



Strain	Host	Genomic Island	ESBL	AmpC
34146	Dog	-	$bla_{CTXM-15}$	-
38327	Dog	-	bla_{VEB-6}	bla_{CMY-2}
1005861	Human	SGI1-PmABB	-	-
PmABB	Human	SGI1-PmABB	-	-
39214	Dog	-	-	bla_{CMY-2}; bla_{DHA-16}
39175	Dog	-	-	bla_{CMY-2}
38309	Dog	SGI1-PmMAT	-	-
38345	Dog	PGI1+	-	-
37666	Dog	PGI1+	-	-
38317	Dog	PGI1+	-	-
36544	Dog	SGI1-PmABB	-	-
34955	Horse	SGI1-V	bla_{VEB-6}	-
34956	Horse	SGI1-V	bla_{VEB-6}	-
34958	Dog	SGI1-V	bla_{VEB-6}	-
37427	Dog	SGI1-V	bla_{VEB-6}	-
36016	Dog	SGI1-V	bla_{VEB-6}	-
36017	Dog	SGI1-V	bla_{VEB-6}	-
34281	Dog	-	-	bla_{CMY-2}
37429	Dog	PGI1+	-	-
36022	Dog	PGI1+	-	-
39219	Dog	SGI1-H	-	-
38653	Cat	PGI1+	-	-
38356	Dog	SGI1-PmABB	-	-
38368	Dog	SGI1-PmABB	-	bla_{CMY-2}
39165	Cat	-	-	bla_{CMY-2}
PmVER	Human	SGI1-K1	-	-
1401751	Human	SGI1-V	bla_{VEB-6}	-
33449	Dog	SGI1-V	-	-
39081	Dog	-	bla_{VEB-6}	-
39193	Dog	-	-	bla_{CMY-2}
33184	Dog	PGI1+	-	-
38375	Dog	-	-	bla_{DHA-16}
37665	Dog	-	-	bla_{CMY-2}
H9812				

FIGURE 2 | Analysis of *Sma*I-Pulsed-Field Gel Electrophoresis (PFGE) patterns obtained from the relevant *P. mirabilis* isolates. PFGE profiles were compared using BioNumerics software version 4.5 (Applied Maths) with settings of 0.5% optimization and 1.0% tolerance. DNA of *S. enterica* serovar Braenderup strain H9812 was used as standard size marker in PFGE experiments and as outgroup in analysis (Hunter et al., 2005). The isolates in bold correspond to previously published profiles included for comparison purposes (Schultz et al., 2015).

TABLE 1 | Antibiotic resistance profiles of *Proteus mirabilis* strains of interest in this study.

Strain	Isolation date (yyyy/mm/dd)	Host	Geographic area[b]	Pathology	Antibiotic resistance profile[a]
34146	2013/04/17	dog	Haute-Savoie	Urinary tract infection	ESBL; StrKanAprTobNetSss
34381	2013/10/09	dog	Alpes-Maritimes	Urinary tract infection	AmpC; Chl
34955	2013/11/22	horse	Oise	Skin infection	ESBL; KanTobNetChlSssTmpNalEnr
34956	2013/10/25	horse	Calvados	Skin infection	ESBL; StrKanTobNetChlSssTmpNalEnr
34958	2013/07/30	dog	Paris	Urinary tract infection	ESBL; StrKanAmkAprGenTobNetChlSssTmpNalEnr
36016	2013/11/05	dog	Val-de-Marne	Abdominal infection	ESBL; StrKanAmkGenTobNetChlSssTmpNalEnr
36017	2013/22/04	dog	Val-de-Marne	Urinary tract infection	ESBL; StrKanGenTobNetChlSssTmpNalEnr
36022	2013/11/10	dog	Alpes-Maritimes	Urinary tract infection	StrSss
36544	2014/03/05	dog	Paris	Urinary tract infection	StrChlSssTmpNalEnr
37427	2014/04/04	dog	Val-de-Marne	Urinary tract infection	ESBL; KanGenTobNetChlSssTmpNalEnr
37429	2014/03/14	dog	Loire	Otitis	StrKanGenTobChlSss
37665	2014/04/22	dog	Paris	Urinary tract infection	AmpC
37666	2014/03/21	dog	Val-de-Marne	Wound	AmxStrKanGenTobSssTmpNalEnr
38309	2014/09/09	dog	Haute-Savoie	Unknown	StrSssTmpNalEnr
38317	2014/09/09	dog	Tarn	Otitis	AmxStrKanGenTobChlSssTmpNalEnr
38327	2014/09/29	dog	Oise	Skin infection	ESBL + AmpC; StrKanChlSssTmp
38346	2014/07/09	dog	Hauts-de-Seine	Urinary tract infection	AmxStrKanGenTobChlSss
38356	2014/07/15	dog	Aisne	Otitis	StrChlSssTmpNalEnr
38368	2014/08/21	dog	Loire-Atlantique	Urinary tract infection	AmpC; ChlSssTmpNalEnr
38375	2014/08/21	dog	Côtes-d'Armor	Otitis	AmpC; Chl
38653	2014/11/03	cat	Seine-St-Denis	Urinary tract infection	StrKanGenTobChlSss
39081	2014/12/01	dog	Val-de-Marne	Urinary tract infection	ESBL; StrKanAmkGenTobNetChlSssTmpNalEnr
39165	2014/10/02	cat	Loire-Atlantique	Urinary tract infection	AmpC; SssTmpNalEnr
39175	2014/10/31	dog	Val-de-Marne	Otitis	AmpC; AprChlNal
39193	2014/10/09	dog	Paris	Otitis	AmpC; StrChlTmpNal
39214	2014/11/18	dog	Val-de-Marne	Skin infection	AmpC; AprChlNal
39219	2015/01/26	dog	Somme	Unknown	StrChlSssTmpNalEnr
39465[c]	2014/09/19	cat	Gironde	Unknown	ESBL; SssTmpNalEnr

[a]*ESBL, extended-spectrum beta-lactamase; Amx, amoxicillin; Chl, chloramphenicol; Ffc, florfenicol; Amk, amikacin; Apr, apramycin; Gen, gentamicin; Tob, tobramycin; Net, netilmicin; Kan, kanamycin; Str, streptomycin; Sss, sulfonamides; Tmp, trimethoprim; Nal, nalidixic acid; Enr, enrofloxacin.* [b]*French department: The department is the administrative subdivision of France.* [c]P. rettgeri *strain.*

frequently isolated from dogs, SGI1/PGI1 elements were more prevalent in cats and horses. This would deserve confirmation due to the low number of isolates collected from these two animal categories.

Among the 11 SGI1-positive *P. mirabilis*, six showed an ESBL phenotype using the double disk synergy test by antibiogram. PCR mapping of the SGI1 resistance gene cluster and backbone revealed that all six ESBL-positive isolates harbored the SGI1-V variant carrying the bla_{VEB-6} gene (**Figure 1**). In the five other SGI1-positive *P. mirabilis* isolates, PCR mapping of the antimicrobial resistance gene cluster gave positive results for different complex class 1 integrons, i.e., InSGI1-H, In*Pm*ABB and In*Pm*MAT (**Figures 1**, **2** and **Table 1**). PCR mapping of the SGI1 backbone confirmed that SGI1 elements with InSGI1-H, In*Pm*ABB, and In*Pm*MAT harbored the same 2789 bp deletion at position 5340-8120 (GenBank accession number AF261825) in the region spanning from ORF S005 to ORF S009, as previously described. Except for SGI1-In*Pm*MAT, this deletion was replaced by the insertion of IS*Vch4* (also called IS*1359*) (**Figure 1**). Interestingly, this region contains two ORFs S007 and S006 coding for homologs of the master activator AcaDC of IncA/C conjugative plasmids and named SgaDC (SGI1 activator, subunits D and C; Poulin-Laprade et al., 2015; Muranyi et al., 2016). The SgaDC activator (also named $FlhDC_{SGI1}$) was shown to be active on the same AcaDC-dependent promoter regions, i.e., P_{xis}, and thus should be also implicated in transfer and/or maintenance of SGI1 (Muranyi et al., 2016). The absence of $flhDC_{SGI1}$ in these variants and the partial deletion of ORF S005 (*traN*) may have implications in their spread that needs to be further studied. In addition to the classical penta-resistance of SGI1 to amoxicillin, chloramphenicol, streptomycin and sulphonamides, other SGI1/PGI1-conferred resistances were amikacin, apramycin, gentamicin, tobramycin, netilmicin, kanamycin, nalidixic acid, trimethoprim, and enrofloxacin.

The six PGI1-positive *P. mirabilis* isolates were characterized by PCR mapping of the whole backbone. PCR products of the expected sizes were obtained for the complete mapping indicating a conserved PGI1 genetic structure in these isolates (**Figure 1**). All six isolates showed similar antibiotic resistance profiles as the ones previously described (Siebor and Neuwirth, 2014; Schultz et al., 2015). Interestingly PGI1, which was only recently reported in *P. mirabilis*, was represented in more than one-third of the genomic islands characterized here, thus confirming the proportion observed in a recent study on a much

smaller number of isolates (Schultz et al., 2015). This may suggest either a previously undetected situation in the *Proteae* population or a recent and rapid spread of PGI1 elements, which were first described in 2014 (Siebor and Neuwirth, 2014). Our data also indicate that the dissemination of SGI1/PGI1 in multidrug-resistant *P. mirabilis* in animals is not a sporadic phenomenon and should be considered with great attention.

Extended-Spectrum and AmpC β-Lactamases

Eighteen strains (18/468, 3.8%) harbored an ESBL profile ($n = 9$), an AmpC profile ($n = 8$), or a combination of both ($n = 1$) after antibiotic susceptibility analysis. Only one AmpC-producing strain did not present any additional resistance (**Table 1**). Otherwise, proportions of strains resistant to non-β-lactam antibiotics were particularly high for chloramphenicol (14/18, 77.8%), streptomycin (10/18, 55.6%), trimethoprim/sulphonamides (13/18, 72.2%), nalidixic acid (13/18, 72.2%), and enrofloxacin (10/18, 55.6%). Of note, none of the strains were resistant to carbapenems.

In addition to the six SGI1-V positive *P. mirabilis* described above, the ESBL phenotype was detected in four other strains. Two *P. mirabilis* isolates (38327 and 39081) harbored the bla_{VEB-6} gene independently of the SGI1 element (**Figure 2**), whereas the two last ESBL-producing isolates harbored the $bla_{CTX-M-15}$ gene (*P. mirabilis* 34146) and the $bla_{CTX-M-1}$ gene (*P. rettgeri* 39465). The prevalence of ESBL producers was lower than the one recently reported in France. However, the previous sampling was based on isolates conserved by the veterinary laboratories and a bias toward non-susceptible isolates cannot be excluded (Schultz et al., 2015). On the contrary, no ESBL-producing isolate was detected in Japan (Harada et al., 2014). In any case, *Proteae* from animal origin present much less ESC-resistant isolates than those from human origin, since the nowadays ESBL rate in animals is very close to the French situation in human medicine in the late 1990s (Chanal et al., 2000).

Concerning AmpC resistance phenotypes, the bla_{CMY-2} gene was detected alone in 7 *P. mirabilis* isolates whereas another *P. mirabilis* isolate (38375) presented a bla_{DHA-16} gene (**Figure 2**). Finally, one *P. mirabilis* isolate (39214) possessed both bla_{CMY-2} and bla_{DHA-16} genes (**Figure 2**). The presence of multiple ESC-resistance genes in two strains (bla_{VEB-6}/bla_{CMY-2} in 38327 and bla_{CMY-2}/bla_{DHA-16} in 39214) shows the capacity of *P. mirabilis* to accumulate redundant resistance genes and thus acting as a potential reservoir.

The chromosomal location of genes was assessed by Southern-blots on *I-Ceu*I-PFGE using the probes corresponding to the ESBL/AmpC genes carried by the studied isolates, as well as probes specific for the 23S rDNA. This method revealed that all bla_{CMY-2} (**Figure 3**) and bla_{DHA-16} genes (Supplementary Figure S1) were located on the bacterial chromosome. All bla_{VEB-6} genes carried by the SGI1-V variant as well as the bla_{VEB-6} gene identified in isolate 39081 were also proved to be encoded by the chromosome (Supplementary Figure S2). On the contrary, the plasmidic localization of genes was proved by Southern-blots on S1-nuclease-PFGE using adequate probes. The last non-chromosomal bla_{VEB-6} (38327; Supplementary Figure S3) and $bla_{CTX-M-1}$ (*P. rettgeri* 39465; **Figure 4**) genes were thus shown to be carried on plasmids, which were considered as non-typable because of the total absence of amplification using the PBRT kit. Finally, the *P. mirabilis* isolate 34146 carried two copies of the ESBL gene $bla_{CTX-M-15}$, one

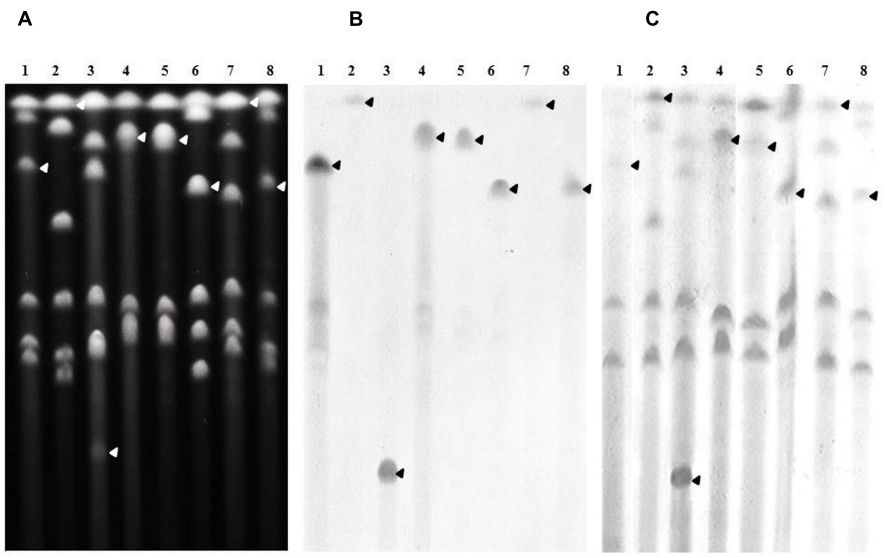


FIGURE 3 | Chromosomal localization of bla_{CMY-2} in the relevant *P. mirabilis* isolates. (A) Whole genomic DNAs of isolates 34381 (lane1), 37665 (lane 2), 38327 (lane 3), 38368 (lane 4), 39165 (lane 5), 39175 (lane 6), 39193 (lane 7), and 39214 (lane 8) were digested with *I-Ceu*I, and the restricted fragments subjected to PFGE. DNA fragments were transferred to a nylon membrane and hybridized with probes specific to bla_{CMY-2} **(B)**, and the 23S rRNA gene **(C)**. The arrows indicate the bands of interest.

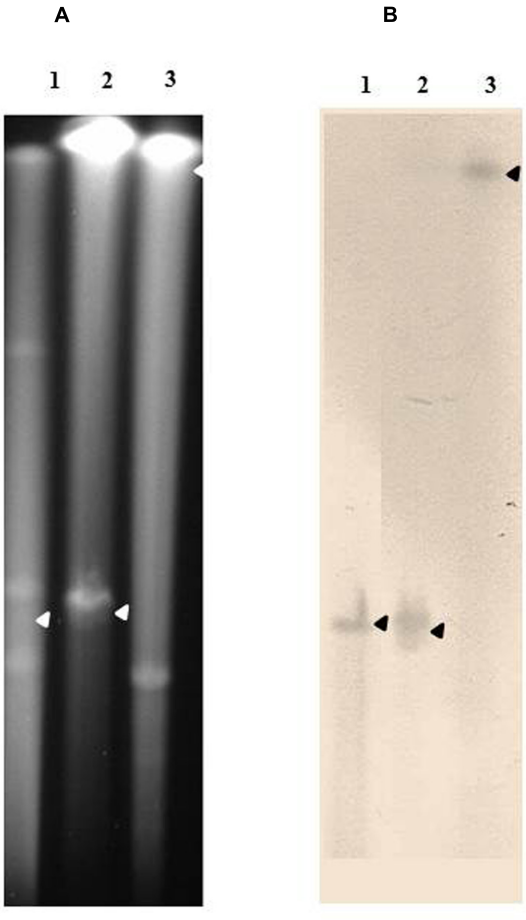


FIGURE 4 | Plasmidic localization of $bla_{CTX-M-1\ group}$ genes in the relevant isolates. (A) Whole genomic DNAs of a CTX-M-15-carrying *E. coli* isolate used as a positive control (lane 1), *P. mirabilis* isolate 34146 (lane 2), and *P. rettgeri* isolate 39465 (lane 3), were digested with S1-nuclease, and the restricted fragments subjected to PFGE. DNA fragments were transferred to a nylon membrane and hybridized with a probe specific to bla_{CTX-M} **(B)**. The arrows indicate the bands of interest.

located on the chromosome and the other on a non-typable plasmid (**Figure 4**). Whereas ESBL genes were mostly reported on plasmids in *Enterobacteriaceae*, it is noteworthy to observe that it is seemingly not the case in *Proteae*. These data strongly suggest that *Proteae* may be more prone than other *Enterobacteriaceae* to integrate resistance determinants into the chromosome, mostly on SGI1/PGI1 or other genomic islands.

Genetic Diversity of the Isolates

*Sma*I PFGE revealed that all SGI1/PGI1-positive isolates were genetically unrelated as were the ESBL/AmpC producing isolates (**Figure 2**), except the six SGI1-V positive isolates that belonged to the same cluster but interestingly differed from the ones previously reported (Schultz et al., 2015). This clustering suggests the spread of a clonal population among different and unrelated individuals. Such a dominance of a clonal population of SGI1-V/bla_{VEB-6} *P. mirabilis* strains in humans and companion animals may result from close contacts between these two populations, as also demonstrated for the transfer of other multidrug resistant bacteria or plasmids, such as those carrying ESBL genes. Indeed, the transmission of bacterial clones from animals to humans (or vice-versa) through physical contacts or contact with contaminated saliva or feces has been described (Espinosa-Gongora et al., 2015; Ljungquist et al., 2016).

CONCLUSION

In this study, we showed a significant prevalence rate (∼4%) of ESBLs/AmpC in *P. mirabilis* of animal sources. A long-term survey is now needed to decipher whether these are emerging phenotypes or only sporadic cases. The multidrug resistance genomic islands SGI1 and PGI1 play a major role in the dissemination of ESBLs/AmpC genes as well as other non-β-lactam resistance genes. Moreover, we report the spread of the SGI1-V/bla_{VEB-6}-carrying *P. mirabilis* clonal population to horses. This peculiar ESBL-producing *P. mirabilis* population was previously recognized in humans and dogs and highly suspected in poultry isolates (Seiffert et al., 2013). Altogether, these data suggest an inter-transmission pathway of public health relevance that needs further investigations to be clarified. Therefore, *P. mirabilis* should be regarded as a potential reservoir of resistance traits in companion animals making us believe that veterinarians should pay more attention to *P. mirabilis* as an opportunistic multidrug-resistant pathogen.

AUTHOR CONTRIBUTIONS

ES, MH, J-YM, BD, and AC designed the experiments. ES did the experiments. ES, MH, and BD analyzed the data. ES and MH drafted the manuscript. BD, J-YM, and AC actively contributed to the manuscript's writing. All authors approved the final version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00032/full#supplementary-material

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Clonal Spread of 16S rRNA Methyltransferase-Producing *Klebsiella pneumoniae* ST37 with High Prevalence of ESBLs from Companion Animals in China

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We screened 30 *Klebsiella pneumoniae* isolates from dogs and cats at a single animal hospital in Guangdong Province, China. Among them, 12 *K. pneumoniae* strains possessed high-level resistance to amikacin and gentamicin and these were screened for 16S rRNA methyltransferase (16S-RMTase) genes. And then the genes positive isolates were detected for ESBLs (extended spectrum β-lactamases) and analyzed by pulsed-field gel electrophoresis, multilocus sequence typing, PCR-based replicon typing and plasmid analysis. The genetic profiles of *rmtB* were also determined by PCR mapping. The twelve 16S-RMTase gene-positive isolates were *rmtB* (11/30) and *armA* (2/30) with one isolate carrying both genes. Extended spectrum β-lactamases genes were represented by $bla_{\text{CTX-M-55}}$ (9/12), $bla_{\text{CTX-M-27}}$ (2/12) and $bla_{\text{CTX-M-14}}$ (1/12). The twelve 16S-RMTase containing strains were grouped into five clonal patterns and ST37 was the most prevalent sequence type. Ten *rmtB*-bearing plasmids conjugated successfully and all belonged to IncN and IncF (F33:A-:B-) incompatibility groups. Nine of the transconjugants carried a 97 kb plasmid and the other harbored both ∼60 and ∼200 kb plasmids. *rmtB* and $bla_{\text{CTX-M-55}}$ were present on the same plasmid and indicated the co-transfer of these two genes, with the *rmtB* gene showing highly relevant relationships with IS*26* and Tn*3*. Our findings suggested a high prevalence of 16S-RMTase genes in *K. pneumonia* ST37 from dogs and cats. Additional studies are needed to trace the evolutionary path of this type of resistance among the *K. pneumonia* isolates, and to determine whether they have been transferred to humans.

Keywords: *Klebsiella pneumoniae*, 16S rRNA methyltransferases, companion animals, MLST

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INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen associated with a wide spectrum of community- and hospital-acquired infections (Lee et al., 2011). Increasing resistance to multiple antimicrobial agents has compromised the effectiveness of *K. pneumoniae* treatment options (Lee et al., 2011). Coexistence of resistance genes on the same plasmid appears to be the primary cause

of the spread of resistance determinants (Luo et al., 2011). This has resulted in the appearance of multidrug resistant (MDR) or even pan drug-resistant (PDR) strains (Luo et al., 2011). Acquisition of MDR by Enterobacteriaceae members has become a global concern especially for *K. pneumonia* PDR strains (Zacharczuk et al., 2011a; Wasyl et al., 2015; Grevskott et al., 2017).

Aminoglycoside resistance is due primarily to aminoglycoside-modifying enzymes. Acetyltransferases, nucleotidyltransferases, and phosphotransferases inactivate commonly used aminoglycosides such as gentamicin (GEN) and tobramycin (Yang et al., 2011). Recently, plasmid-encoded 16S rRNA methyltransferases (16S-RMTase) have emerged in the Enterobacteriaceae family and in a group of glucose-non-fermentative microbes (Zacharczuk et al., 2011b). This is a new resistance mechanism to 4,6-disubstituted 2-deoxystreptamines and 4,5-disubstituted 2-deoxystreptamines. These structures encompass the majority of clinically important aminoglycosides (Zacharczuk et al., 2011b). Since the first report in 2003, ten 16S-RMTase-encoding genes, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF*, *rmtG*, *rmtH*, *armA*, and *npmA*, have been identified (O'Hara et al., 2013).

Reports on the prevalence of the 16S-RMTases have increased in the past years with the majority focused on the human clinical isolates including *K. pneumoniae* (Mezzatesta et al., 2013; Belbel et al., 2014; Bartoloni et al., 2016). Moreover, the majority of reports on dissemination of *armA* and *rmtB* to various Enterobacteriaceae species were focused on *E. coli* isolated from chickens and pigs (Yao et al., 2011; Yang Y. et al., 2015). Previous research has reported the occurrence of the ArmA methyltransferase in an ST11 clone of *K. pneumoniae* isolated from cats and dogs in Spain (Hidalgo et al., 2013a). Another research focused on the dissemination of *rmtB-bla*$_{\text{CTX-M-9}}$ group genes and *rmtB-qepA* in Enterobacteriaceae isolates of dogs and cats in China mainly related to *E. coli* (Deng et al., 2011a). However, researches on prevalence and mechanisms of 16S-RMTase spread in pets via *K. pneumonia* strains in China are lacking.

Because of the common use of antimicrobial agents and the close contact with humans, companion animals may become potential sources for dissemination of antimicrobial resistance (Lloyd, 2007). Thus, we decided to identify the prevalence of 16S-RMTase genes among *K. pneumonia* isolates from dogs and cats in a veterinary hospital in Guangdong Province, China.

MATERIALS AND METHODS

Bacterial Isolates

Thirty clinical *K. pneumoniae* strains were recovered from diseased dogs and cats during July, August, and September in 2010 and from July to October in 2012 at one animal hospital in Guangdong Province, China. Further information about the antimicrobial treatments of these pets were unfortunately not available. Samples were collected from the exudates of the infection areas (urinary tract infections, skin infections, or intra-abdominal infections) or feces, then seeded on MacConkey agar at 37°C. Each isolate was from a single animal. All bacterial species were identified with classical biochemical methods and confirmed using a matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) method (Shimadzu, Japan). As 16S-RMTase can confer high-level resistance to both GEN and amikacin (AMK), all isolates were subcultured in MacConkey agar containing 64 mg/L GEN and 64 mg/L AMK to screen for GEN/AMK-resistant isolates.

Detection of Resistance Genes and Antimicrobial Susceptibility Testing

All the strains exhibiting aminoglycoside resistance were screened for 16S-RMTase genes including *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmt*F, *rmtG*, *rmtH*, *armA*, and *npmA* (Hidalgo et al., 2013b; Xia et al., 2016). Aminoglycoside-resistant isolates were further analyzed for the presence of extended spectrum β-lactamase (ESBL) genes (*bla*$_{\text{TEM}}$, *bla*$_{\text{CTX-M}}$, and *bla*$_{\text{SHV}}$) using previously described primers (Liao et al., 2015).

The minimal inhibitory concentration (MIC) of ampicillin (AMP), cefotaxime (CTX), meropenem, ciprofloxacin (CIP), norfloxacin (NOR), GEN, kanamycin (KAN), AMK, streptomycin (STR), neomycin (NEO), apramycin (APR), chloramphenicol (CHL), florfenicol (FFC), tetracycline (TET), sulfamethoxazole/trimethoprim (SXT) were determined by the agar dilution method according to Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute, 2015). *E. coli* ATCC 25922 was used as a quality control strain.

Clonal Relatedness

Chromosomal DNA digested with *Xba*I restriction enzyme was used for pulsed-field gel electrophoresis (PFGE) to analyze the genetic relatedness of all isolates containing 16S-RMTase genes (CHEF Mapper1, Bio-Rad Laboratories, Hercules, CA, USA) as previously described (Fang et al., 2015). PFGE patterns were analyzed with the Dice coefficient and the unweighted pair group method with average linkages (UPGMA) clustering method using BioNumerics software (Applied Maths, Ghent, Belgium). PFGE types were defined with >90% similarity between clusters. Multilocus sequence typing (MLST) of 16S-RMTase genes containing *K. pneumoniae* was performed according to the protocols described on the *K. pneumoniae* database website[1]. Seven chromosomal genes were PCR amplified and sequenced. Then the sequences were compared with those reference sequences, submitted to the MLST database to determine allele numbers and STs (Belbel et al., 2014).

Transfer of the 16S-RMTase Genes and Plasmids Replicon Typing

Isolates positive for 16S-RMTase genes were selected for conjugation experiments by the broth-mating method using STR-resistant *E. coli* C600 as the recipient to determine the

[1]http://bigsdb.pasteur.fr/klebsiella/

transferability of 16S-RMTase genes. Transconjugants were selected on MacConkey agar plates supplemented with STR (1000 μg/mL) and AMK (64 μg/mL). Transconjugants harboring 16S-RMTase genes were confirmed by PCR and antimicrobial susceptibility testing as described above (Fang et al., 2015). Antimicrobial susceptibility testing of the transconjugants and co-transfer of other resistance genes as mentioned above were also determined.

Plasmids were preliminarily classified according to their incompatibility group by using the PCR-based replicon typing (PBRT) scheme described previously (Yao et al., 2011). To better characterize IncF plasmids, replicon sequence typing of IncF plasmids was performed according to the protocol previously described (Yang Q.E. et al., 2015). The A- and B-symbols indicate the absence of the FIA and FIB replicons, respectively.

Plasmid Analysis

PFGE with S1 nuclease (TakaRa Biotechnology, Dalian, China) digestion of genomic DNA was performed for all the transconjugants as described previously (Xia et al., 2015). After Southern blotting, fix the DNA to a Hybond-N^+ membrane (GE Healthcare, Little Chalfont, UK), the plasmids were probed with the 16S-RMTase genes and $bla_{\text{CTX-M-1G/9G}}$ gene (DIG High Prime DNA Labeling and Detection Starter Kit I, Roche Applied Science, Mannheim, Germany). All the transconjugants were subjected to restriction enzyme digestion (*Eco*RI) analysis to clarify whether a specific plasmid had been disseminated among the isolates (Deng et al., 2011b). *Eco*RI-RFLP types were defined with >80% similarity between clusters using the method described as PFGE analysis.

Genetic Profiles of 16S-RMTase Genes

Reports on the genetic contexts of the 16S-RMTase genes indicated that IS*26*, Tn*3*, IS*CR1*, and IS*CR3* played an important role in the dissemination of these genes. These transfer events enabled horizontal spread among different bacterial lineages (Deng et al., 2011b). Here we focused on the link between 16S-RMTase genes and mobile elements by PCR mapping. The primers using in PCR mapping were listed in Supplementary Table S1.

Ethics Statement

In this study, the owners of the companion animals from which fecal swabs and the exudates of the infection areas were taken gave permission for their animals to be used in this study. Written informed consent was obtained, and the study protocol in our research was approved by the South China Agriculture University

TABLE 1 | 16S rRNA methyltransferases gene-positive *K. pneumoniae* isolates from this study.

Strains[a]	Origin	Year	Resistance profiles[b]	Resistant genes[c]	MLST	Replicon types[d]	Plasmids size (kb)
KP01	Dog	2010	GEN, CTX, SXT, AMP, CIP, TET, STR, KAN, AMK, CHL, NEO, NOR	*rmtB, armA*, $bla_{\text{TEM-1}}$, $bla_{\text{SHV-1}}$	ST2018	NT	60 (*rmtB*), 200 (*armA*)
KP04	Cat	2010	GEN, SXT, AMP, FFC, CIP, TET, KAN, AMK, CHL, NEO, APR, NOR	*armA*	ST395	ND	ND
KP07	Dog	2012	GEN, CTX, SXT, AMP, FFC, CIP, TET, STR, KAN, AMK, CHL, NEO, NOR	*rmtB*, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$, $bla_{\text{SHV-1}}$	ST37	F33:A-:B-, N	97
KP16	Cat	2012	GEN, CTX, SXT, AMP, FFC, CIP, TET, STR, KAN, AMK, CHL, NEO, APR, NOR	*rmtB*, $bla_{\text{SHV-1}}$	ST147	ND	ND
KP21	Dog	2012	GEN, CTX, SXT, AMP, FFC, CIP, TET, STR, KAN, AMK, CHL, NEO, NOR	*rmtB*, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$, $bla_{\text{SHV-1}}$	ST37	F33:A-:B-, N	97
KP22	Cat	2012	GEN, CTX, SXT, AMP, FFC, CIP, TET, STR, KAN, AMK, CHL, NEO, NOR	*rmtB*, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$, $bla_{\text{SHV-1}}$	ST37	F33:A-:B-, N	97
KP23	Dog	2012	GEN, CTX, SXT, AMP, FFC, CIP, TET, STR, KAN, AMK, CHL, NEO, NOR	*rmtB*, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$, $bla_{\text{CTX-M-27}}$, $bla_{\text{SHV-1}}$	ST37	F33:A-:B-, N	97
KP24	Dog	2012	GEN, CTX, SXT, AMP, FFC, CIP, TET, STR, KAN, AMK, CHL, NEO, NOR	*rmtB*, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$, $bla_{\text{CTX-M-27}}$, $bla_{\text{SHV-1}}$	ST37	F33:A-:B-, N	97
KP25	Dog	2012	GEN, CTX, SXT, AMP, FFC, CIP, TET, STR, KAN, AMK, CHL, NEO, NOR	*rmtB*, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$, $bla_{\text{SHV-1}}$	ST37	F33:A-:B-, N	97
KP26	Dog	2012	GEN, CTX, SXT, AMP, FFC, CIP, TET, STR, KAN, AMK, CHL, NEO, NOR	*rmtB*, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{SHV-1}}$	ST37	F33:A-:B-, N	97
KP29	Dog	2012	GEN, CTX, SXT, AMP, FFC, CIP, TET, STR, KAN, AMK, CHL, NEO, NOR	*rmtB*, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$	ST37	F33:A-:B-, N	97
KP30	Dog	2012	GEN, CTX, SXT, AMP, FFC, CIP, TET, STR, KAN, AMK, CHL, NEO, NOR	*rmtB*, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$, $bla_{\text{SHV-1}}$	ST37	F33:A-:B-, N	97

[a] *Isolates with transconjugants are underlined.*

[b] *Resistance phenotypes transferred to the recipient by conjugation are underlined. GEN, gentamicin; CTX, cefotaxime; SXT, sulfamethoxazole/trimethoprim; AMP, ampicillin; FFC, florfenicol; AMK, amikacin; CIP, ciprofloxacin; TET, tetracycline; STR, streptomycin; KAN, kanamycin; CHL, chloramphenicol; NEO, neomycin; APR, apramycin; NOR, norfloxacin.*

[c] *Genes that were co-transferred by conjugation are underlined.*

[d] *ND, not determined; NT, non-typeable.*

Animal ethics committee and carried out in accordance with relevant guidelines.

RESULTS

Prevalence of 16S-RMTase Genes and Antimicrobial Susceptibility Testing

Among 30 *K. pneumoniae* isolates from the hospital, 12 were resistant to AMK and GEN. However, all *K. pneumoniae* strains contained the 16S-RMTase genes *rmtB* (11/30) and/or *armA* (2/30) and a single isolate carried both genes (1/30). The AMK/GEN-resistant *K. pneumoniae* isolates showed an MDR phenotype (resistant to three or more classes of antimicrobials). Apart from aminoglycoside antibiotics, there was a very high frequency (>90.0%) of resistance to AMP, CTX, SXT, TET, CIP, NOR, CHL, and FFC. The most frequently observed pattern of MDR was GEN-CTX-SXT-AMP-CIP-TET-STR-KAN-AMK-CHL-NEO-NOR (**Table 1**).

Characterization of Resistance Genes

We examined the prevalence of ESBL and AME genes in the 16S-RMTase gene-positive isolates. Among these, nine isolates contained $bla_{\text{CTX-M}}$ that were distributed as $bla_{\text{CTX-M-55}}$ (6), $bla_{\text{CTX-M-55}}$ and $bla_{\text{CTX-M-27}}$ (2), and $bla_{\text{CTX-M-55}}$ and $bla_{\text{CTX-M-14}}$ (1). Two other ESBLs were also present in these isolates as $bla_{\text{TEM-1}}$ (10/12) and $bla_{\text{SHV-1}}$ (10/12) (**Table 1**).

PFGE Typing and MLST

PFGE was successfully performed in all twelve 16S-RMTase genes positive *K. pneumoniae* isolates and were grouped into five clonal patterns designated PFGE types A to E (**Figure 1**). The predominant PFGE type was type C that accounted for 67% (8/12) of the isolates, suggesting that one small clonal outbreak had occurred. The MLST results revealed that one ST was the most prevalent in this collection: ST37 (9/12). ST2018, ST395, and ST147 each had one isolate (**Table 1**). The results obtained by MLST were consistent with those obtained by PFGE; eight *rmtB*-positive *K. pneumoniae* strains showing similar PFGE patterns were belonged to ST37.

Transfer of the 16S-RMTase Genes and Plasmid Analysis

Plasmids carrying *rmtB* from 10 isolates were successfully transferred to recipients by conjugation. All the transconjugants were highly resistant to AMK, GEN, and KAN (MICs $\geq$ 512 μg/mL). PBRT of the plasmid incompatibility groups showed that nine *rmtB*-bearing plasmids from *K. pneumoniae* isolates carried two replicons. The two replicons were confirmed to be IncFII in combination with IncN, and the other *rmtB*-bearing plasmid was non-typeable. All the IncFII plasmids were further determined to be F33:A-:B-.

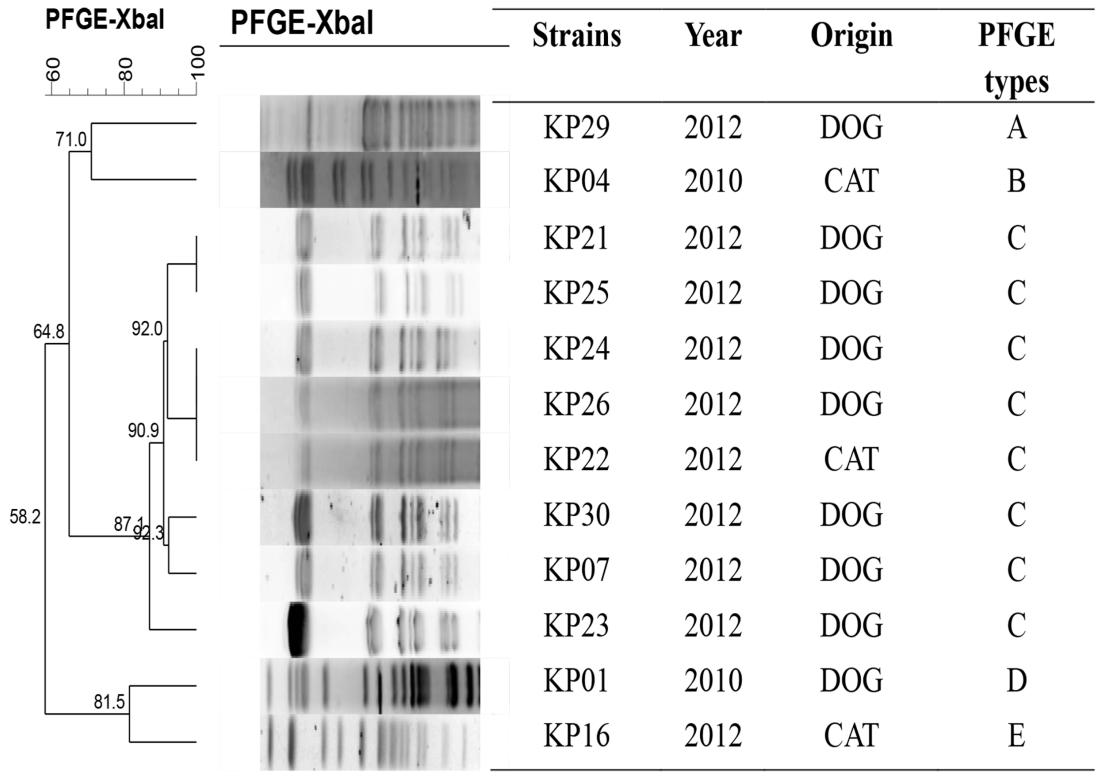
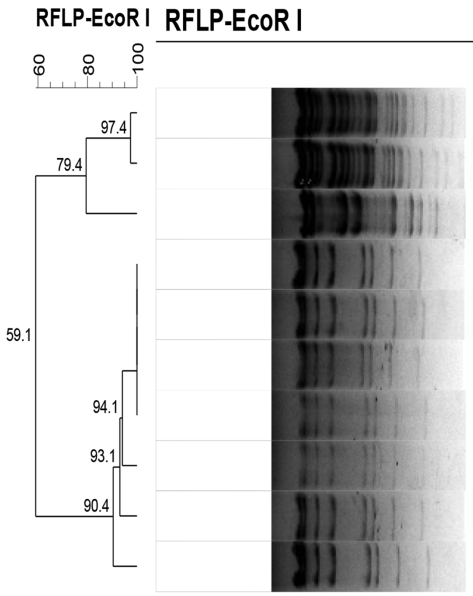


FIGURE 1 | PFGE analysis of the twelve 16S-RMTase-positive *K. pneumoniae* isolates.



Strains	Year	Origin	RFLP types
KP07	2012	DOG	A
KP25	2012	DOG	A
KP01	2010	DOG	B
KP21	2012	DOG	C
KP26	2012	DOG	C
KP29	2012	DOG	C
KP30	2012	DOG	C
KP22	2012	CAT	C
KP24	2012	DOG	C
KP23	2012	DOG	C

FIGURE 2 | *Eco*RI-RFLP analysis of the 10 transconjugants in the 16S-RMTase-positive *K. pneumoniae* isolates.

Nine of the 10 transconjugants carried one approximately 97 kb plasmid. The other one harbored both ∼60 kb (*rmtB* positive) and ∼200 kb (*armA* positive) plasmids at the same time. Moreover, *rmtB* and $bla_{\text{CTX-M-55}}$ genes were located on the same 97 kb plasmid. The RFLP analysis indicated that 7 out of the 10 transconjugants had identical plasmid restriction patterns. The two remaining plasmids shared the same RFLP type (**Figure 2**).

Genetic Profile Analysis

The genetic profiles were successfully obtained in the 10 *rmtB*-positive transconjugants. We found four new genetic profiles in the 10 *rmtB*-positive transconjugants and all possessed Tn*3* and $bla_{\text{TEM-1}}$ directly upstream of *rmtB*. Tn*3* was truncated by IS*26* in 9 of the 10 transconjugants, which were inserted at the same site (3573 of Tn*3*). The other isolate KP01 harbored the intact *tnpA–tnpR* genetic structure of Tn*3*, with a Na^+/H^+-exchanging protein gene and IS*CR1* downstream *rmtB*. IS*26* was also found directly downstream of *rmtB* in seven plasmids, which was located in the same orientation to the 5′ IS*26* elements. Moreover, the Na^+/H^+-exchanging protein gene located between *rmtB* and IS*26* (in the opposite orientation) occurred in strain KP30 plasmid (**Figure 3**). The four unique sequences were deposited in GenBank under accession numbers KY402260, KY402261, KY402262, and KY402263.

DISCUSSION

The 16S-RMTase genes confer broad and high-level resistance to most clinically available aminoglycosides (Habeeb et al., 2013). This has become a worldwide concern especially when these genes are combined with other classes of antimicrobial resistance determinants. This has led to the emergence of MDR strains (Habeeb et al., 2013).

In the present study, our results highlighted a high prevalence of 16S-RMTase genes in *K. pneumoniae* isolates from pets compared with other reports of human infections (Yan et al., 2004; Yu et al., 2009). Similarly, these 16S-RMTase-positive *K. pneumoniae* isolates were all MDR strains and mostly resistant to β-lactams, aminoglycosides, TETs, amphenicols, sulfonamides, and fluoroquinolones. Fortunately, the isolates we investigated in this study were susceptible to carbapenem antibiotics, which was different from other studies (Zacharczuk et al., 2011b; Li et al., 2012).

The twelve 16S-RMTase-positive *K. pneumoniae* isolates were successfully typed into five different groups by PFGE. Group C predominated and contained eight isolates indicating that the *rmtB*-positive isolates were spread by clonal dissemination. MLST analysis indicated four STs: ST37, ST2018, ST395, and ST147. The most prevalent ST in this collection was ST37 containing eight isolates belonging to the same PFGE type C group.

Previous reports had documented that ST11 was the most prevalent 16S-RMTase-positive *K. pneumoniae* in human isolates. ST101 was the second frequently reported ST type, which often co-harbored KPC-2 (*K. pneumoniae* carbapenemase) in 16S-RMTase-positive *K. pneumoniae* (Li et al., 2012; Mezzatesta et al., 2013; Hu et al., 2014; Seiffert et al., 2014). However, the sharing of ST37 between isolates in our study is important because KPC-producing

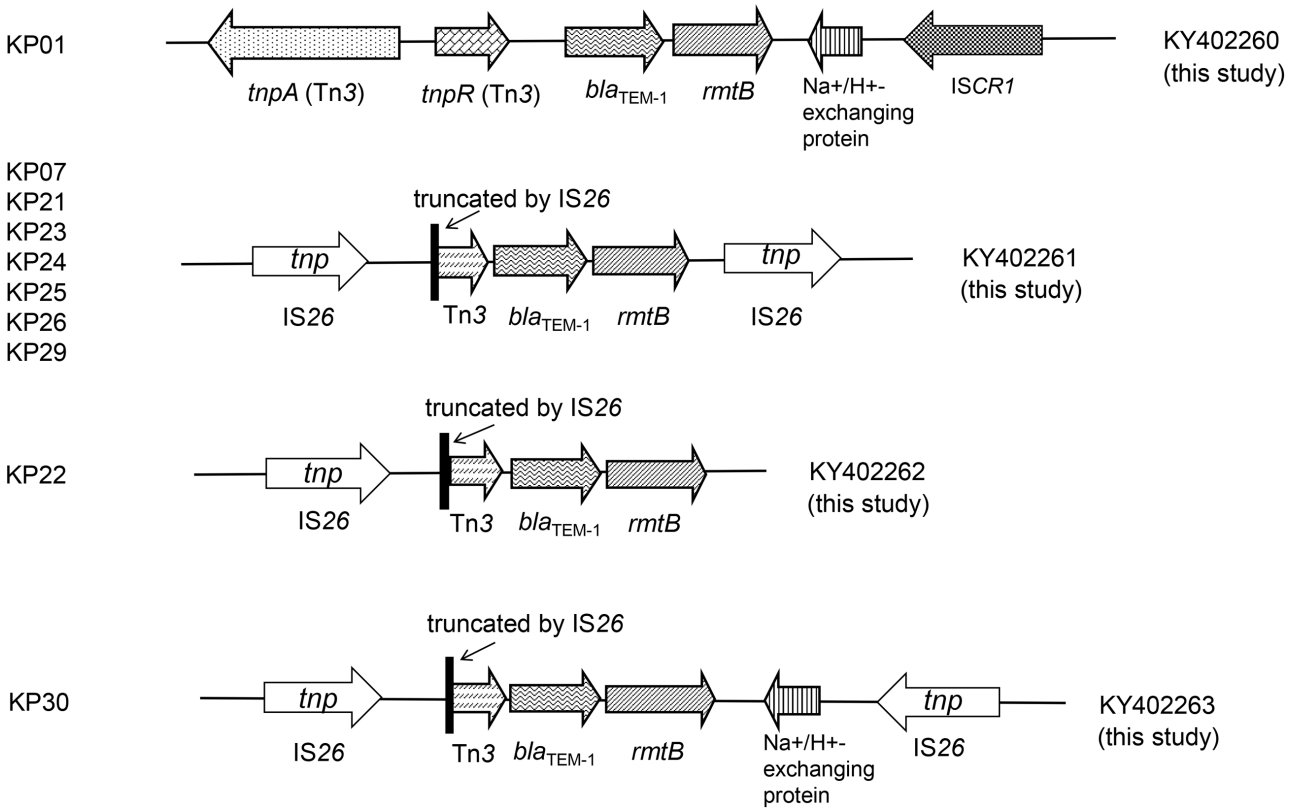


FIGURE 3 | Genetic profiles of the *rmtB* gene.

K. pneumoniae ST37 of human origin has been detected only sporadically in China (Yang et al., 2013; Liu et al., 2015). This observation might provide further support in concerns about transmission of resistance between companion animals and humans.

ESBL genes, especially CTX-M genes, frequently coexist and co-transfer with 16S-RMTase genes in Enterobacteriaceae members including *K. pneumoniae* (Yan et al., 2004; Bogaerts et al., 2007; Zacharczuk et al., 2011a). In this study, PCR and sequence analyses revealed that $bla_{\text{CTX-M-55}}$ and $bla_{\text{TEM-1}}$ were co-transferred with *rmtB* in 9 and 10 transconjugants of *K. pneumoniae* strains, respectively. This confirmed an intimate association between ESBLs and 16S-RMTase genes (Deng et al., 2011a; Xia et al., 2016). In addition, PCR-based plasmid replicon typing indicated that the nine transconjugants were in the FII and N incompatibility groups. All IncFII plasmids belonged to the same F33:A-:B- subgroup. This finding is similar with reports of *rmtB*-positive *E. coli* from food animals, pets and humans in China (Yu et al., 2010; Deng et al., 2011a,b; Yao et al., 2011; Xia et al., 2016).

This data indicated the horizontal transmission of the prevailing plasmids from variety bacterial species between different hosts. Moreover, our plasmid digestion profiles and S1-PFGE analysis confirmed that the prevalence of the *rmtB*-positive *K. pneumoniae* isolates was due to the dissemination of a ∼97 kb plasmid containing $bla_{\text{CTX-M-55}}$.

Previous studies indicated that Tn*3*, IS*26* and IS*CR3* were associated with the transmission of *rmtB* (Doi and Arakawa, 2007; Deng et al., 2011b). In this study, the *rmtB* gene showed highly relevant relationships with IS*26* and Tn*3*. The presence of a truncated Tn*3* inserted *via* IS*26* upstream of *rmtB*, and IS*26* inserting in the same direction located downstream of *rmtB*, was the most prevalent structure in seven transconjugants. Within this group, five plasmids of the corresponding transconjugants shared similar *Eco*RI-RFLP patterns (**Figures 2**, **3**). This indicated a plasmid with this particular genetic profile was the origin of *rmtB* in the *K. pneumoniae* isolates from this group of pets.

In conclusion, *rmtB* was the most prevalent 16S-RMTase gene of *K. pneumoniae* that originated in pets. The dissemination of *rmtB*-producing *K. pneumoniae* isolates in this study appeared to be clonal spread and horizontal transfer. ST37 *K. pneumoniae* and F33:A-:B- plasmids sized ∼97 kb with the same genetic profile mediated by IS*26* transmission both contributed to the dissemination of *rmtB*. In this study, we investigated only a single veterinary hospital. An organized surveillance effort is required to better understand the scope of this problem and identify control measures among hospitals.

AUTHOR CONTRIBUTIONS

Y-HL and X-PL designed and organized the study. JX and L-XF did the research. KC, G-HX, and X-RW did the assisted help. JS analyzed the data and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00529/full#supplementary-material

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Detection and Genetic Environment of Pleuromutilin-Lincosamide-Streptogramin A Resistance Genes in Staphylococci Isolated from Pets

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Increasing emergence of staphylococci resistant to pleuromutilins, lincosamides, and streptogramin A (PLS_A) and isolated from humans and pets is a growing public health concern worldwide. Currently, there was only one published study regarding one of the PLS_A genes, *vga*(A) detected in staphylococci isolated from cat. In this study, eleven pleuromutilin-resistant staphylococci from pets and two from their owners were isolated and further characterized for their antimicrobial susceptibilities, plasmid profiles, genotypes, and genetic context of the PLS_A resistance genes. The gene *sal*(A) identified in 11 staphylococcal isolates was found for the first time in *Staphylococcus haemolyticus*, *Staphylococcus epidermidis*, and *Staphylococcus xylosus*. Moreover, these 11 isolates shared the identical regions flanking the *sal*(A) gene located in the chromosomal DNA. Two *S. haemolyticus* isolates from a cat and its owner carried similar *vga*$(A)_{LC}$ plasmids and displayed indistinguishable PFGE patterns. A novel chromosomal multidrug resistance genomic island (MDRGI) containing 13 resistance genes, including *lsa*(E), was firstly identified in *S. epidermidis*. In addition, *vga*$(A)_{LC}$, *sal*(A), and *lsa*(E) were for the first time identified in staphylococcal isolates originating from pet animals. The plasmids, chromosomal DNA region, and MDRGI associated with the PLS_A resistance genes *vga*(A), *vga*$(A)_{LC}$, *sal*(A), and *lsa*(E) are present in staphylococci isolated from pets and humans and present significant challenges for the clinical management of infections by limiting therapeutic options.

Keywords: PLS_A genes, staphylococci, pet, human, multidrug resistance

INTRODUCTION

Transferable resistance to three chemically distinct classes of antimicrobial agents (pleuromutilins, lincosamides, and streptogramin A; PLS_A) in staphylococci has been attributed to ABC transporters of the Vga, Lsa, or Sal families. All of the corresponding resistance genes, including *vga*(A), *vga*$(A)_V$, *vga*$(A)_{LC}$, *vga*(B), *vga*(C), *vga*(E), *vga*$(E)_V$, *lsa*(E), and *sal*(A), were mainly identified in staphylococci from food-producing isolates (Allignet and El Solh, 1997;

Haroche et al., 2000; Kadlec and Schwarz, 2009; Jung et al., 2010; Lozano et al., 2012; Hauschild et al., 2012; Wendlandt et al., 2013; Li et al., 2014; Hot et al., 2014). However, the *vga*(A) gene was also identified in *Staphylococcus epidermidis* isolates originating from one cat, clinic environment and an employee (Weiß et al., 2013).

To date, the antimicrobial resistance mechanisms of staphylococci in pets have received less attention than those in food-producing animals. Given the close relationship between pets and their owners, resistant bacteria and their mobile resistance determinants may be exchanged in either direction between pets and humans (Schwarz et al., 2016). The increasingly frequent isolation of methicillin- and multidrug-resistant staphylococci from pets, particularly from dogs with pyoderma and otitis (Cain, 2013), and the occurrence of the same resistance genes in pets and in humans (Butaye et al., 2001; Simjee et al., 2002), may underline the potential role of pets in the transmission of antimicrobial-resistant staphylococci to humans. Currently, there is one published study regarding one of the PLS$_A$ genes detected in staphylococci isolated from a cat (Weiß et al., 2013). In the present study, we investigated the distribution of PLS$_A$ resistance genes in staphylococci of pet origin and analyzed the locations and genetic environments of these genes.

MATERIALS AND METHODS

Ethics Statement

This research was carried out according to the principles of the Declaration of Helsinki. The involved pet animals were treated with the best practice veterinary care and the informed consent was obtained from pets' owners. The study protocol was approved by the Ethics Committee of China Agricultural University.

Bacterial Strains and Antimicrobial Susceptibility Testing

A total of 300 anal swabs or nasal samples were used in this study which were collected from dogs ($n = 269$), cats ($n = 10$), and some of their owners ($n = 21$) in the Veterinary Teaching Hospital of China Agricultural University, Beijing in 2013. The pleuromutilin-resistant isolates were selected by incubation on mannitol salt agar (Luqiao, Beijing, China) supplemented with 2 μg/ml of valnemulin at 37°C for 16–24 h.

The MIC (Minimal Inhibitory Concentration) determinations were done by a standard broth microdilution test following the recommendations of the Clinical and Laboratory Standards Institute [CLSI] (2015). *Staphylococcus aureus* ATCC 29213 was served as the quality control strain.

Molecular Methods

The genomic DNAs of the valnemulin-resistant isolates were obtained using a Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), and were screened for the *vga*, *lsa*, and *sal* gene types by PCR assays (Novotna and Janata, 2006; Hot et al., 2014; Li et al., 2014; Wendlandt et al., 2015). Furthermore, the species assignment of the resistant isolates was done by 16S rDNA sequencing and confirmed by MALDI-TOF MS (Bruker Daltonik, Bremen, Germany).

The clonality of the valnemulin-resistant isolates was analyzed by pulsed-field gel electrophoresis (PFGE) as described previously (Wang et al., 2011), and the PFGE results were analyzed using InfoQuestFP software (version 4.5).

The S1 nuclease-PFGE (S1-PFGE) and subsequent Southern blot hybridization were performed to locate the valnemulin resistance genes as described before (Barton et al., 1995). The Low Range PFGE Marker (New England BioLabs, Beverly, MA, USA) served as the size marker.

Characterization and Sequence Analysis of the PLS$_A$ Genes

Genomic DNAs of *S. sciuri* isolate 100N carrying *sal*(A) and *S. epidermidis* isolate 138N carrying *sal*(A) and *lsa*(E) were submitted to high-throughput whole-genome sequencing (WGS), and preceded by library construction on a HiSeq 2500, which produced 150 bp paired-end reads (Berry Genomics Company, Beijing, China).

Draft assembly of the genomic DNA sequences was analyzed by CLC Genomics Workbench 5 (CLC Bio, Aarhus, Denmark). All contigs with the average coverage of >100-fold were searched for the PLS$_A$ genes using BLAST analysis. The regions flanking the PLS$_A$ genes were identified using *de novo* assembly as earlier described (Zerbino and Birney, 2008) and the random primer walking strategy (Zhang et al., 2009). Sequence analysis was conducted using the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and BLAST functions (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To obtain more information about the genetic environments of the PLS$_A$ genes in valnemulin-resistant isolates, the random primer walking strategy and inverse PCR were performed.

Nucleotide Sequence Accession Numbers

The PLS$_A$-carrying segments of various isolates in this study have been deposited in GenBank, and their accession numbers are KX712120 (*Staphylococcus haemolyticus* plasmid p131A carrying *vga*(A)$_{LC}$), KX712121 (*S. epidermidis* plasmid p132R carrying *vga*(A)), KX712119 (*S. epidermidis* 138N carrying *sal*(A)), and KX712118 (*S. epidermidis* 138N carrying *lsa*(E)).

RESULTS

Characterization of Valnemulin-Resistant Staphylococcal Isolates and Antimicrobial Resistance Profiles

Amongst the 300 anal swabs and nasal samples, 13 (4.3%) valnemulin-resistant isolates with valnemulin MICs of ≥8 μg/ml were detected, which were identified to species level as *Staphylococcus sciuri* ($n = 8$), *Staphylococcus haemolyticus* ($n = 2$), *S. epidermidis* ($n = 2$), and *Staphylococcus xylosus* ($n = 1$) (**Table 1**).

TABLE 1 | Characteristics of the 13 valnemulin-resistant staphylococci isolates identified in this study.

Isolate	Species	Host	Origin of samples	Gene	PFGE subtype	Location of PLS$_A$[a]	MIC (μg/ml)[b]							
							VAL	TIA	RET	LIN	VIR	SPE	GEN	ERY
139N	*S. sciuri*	Dog	Nasal	*sal*(A)	A	C	**32**	**128**	**16**	**8**	**4**	**≥8192**	1	0.125
140N	*S. sciuri*	Dog	Nasal	*sal*(A)	A	C	**32**	**128**	**32**	**4**	**4**	**≥8192**	1	0.5
145N	*S. sciuri*	Dog	Nasal	*sal*(A)	A	C	**32**	**128**	**32**	**4**	**4**	**1024**	0.5	0.25
90A	*S. sciuri*	Dog	Anal	*sal*(A)	B	C	**64**	**128**	**64**	**8**	**4**	**≥8192**	**64**	0.125
96A	*S. sciuri*	Dog	Anal	*sal*(A)	C1	C	**32**	**128**	**128**	**8**	**4**	**≥8192**	4	0.125
96N	*S. sciuri*	Dog	Nasal	*sal*(A)	C2	C	**64**	**128**	**32**	**8**	**4**	**≥8192**	0.5	**4**
100N	*S. sciuri*	Cat	Nasal	*sal*(A)	D	C	**32**	**128**	**8**	**8**	**4**	**≥8192**	2	0.125
123N	*S. sciuri*	Cat	Nasal	*sal*(A)	E	C	**32**	**64**	**8**	**128**	**4**	**≥8192**	**32**	**128**
131R	*S. haemolyticus*	Human	Nasal	*vga*(A)$_{LC}$	H	P	**16**	**32**	**16**	**16**	**4**	**≥8192**	2	**32**
131A	*S. haemolyticus*	Cat	Anal	*vga*(A)$_{LC}$, *sal*(A)	H	P, C	**16**	**64**	**8**	**16**	**4**	**≥8192**	1	**32**
132R	*S. epidermidis*	Human	Nasal	*vga*(A)	O	P	**16**	**64**	**16**	**64**	**2**	**≥8192**	4	**32**
138N	*S. epidermidis*	Dog	Nasal	*lsa*(E), *sal*(A)	P	C	**>128**	**128**	**128**	**256**	**16**	**≥8192**	**>128**	**>128**
95N	*S. xylosus*	Dog	Nasal	*sal*(A)	n.t.[c]	C	**8**	**32**	**16**	**8**	**8**	**2048**	2	0.125

[a]*The PLS$_A$ resistance gene is located on the chromosome (C), on a plasmid (P), or both (P, C).* [b]*VAL, valnemulin; TIA, tiamulin; RET, retapamulin; LIN, lincomycin; VIR, virginiamycin M1; SPE, spectinomycin; GEN, gentamicin; ERY, erythromycin. The MICs that classify an isolate as resistant or high MICs (in cases where no applicable breakpoints are available to classify an isolate as resistant) are displayed in bold.* [c]*PFGE was performed only for ≥ two isolates of the same species.*

Nine (eight *S. sciuri* and one *S. xylosus*) of the 13 isolates carried only *sal*(A), one *S. haemolyticus* and one *S. epidermidis* isolate carried *vga*(A)$_{LC}$ and *vga*(A), respectively, one *S. haemolyticus* isolate carried both *vga*(A)$_{LC}$ and *sal*(A), and one *S. epidermidis* isolate carried both *lsa*(E) and *sal*(A) (**Table 1**). Notably, the *lsa*(E) gene was only detected in one *S. epidermidis* isolate of dog origin, and the *vga* genes were detected in staphylococcal isolates originating from both humans and cats. In contrast, *sal*(A) was widespread among the valnemulin-resistant staphylococcal isolates from pets. As the *sal*(A) gene has been previously found exclusively in *S. sciuri* isolates (Hot et al., 2014; Wendlandt et al., 2015), this finding describes for the first time the occurrence of *sal*(A) in *S. haemolyticus*, *S. epidermidis*, and *S. xylosus*.

All 13 valnemulin-resistant isolates displayed resistance or high MICs to pleuromutilins (valnemulin, tiamulin, and retapamulin), lincomycin, virginiamycin M1 and spectinomycin (**Table 1**).

Genotyping by PFGE

Using a cut-off of 90% similarity, the eight *S. sciuri* isolates, which carried highly conserved (>99% nucleotide identity) *sal*(A) genes, clustered into five PFGE types (A–E), with type A accounting for the majority (3/8) of the isolates (**Table 1**; **Figure 1**). Remarkably, the feline *S. haemolyticus* isolate 131A and the human *S. haemolyticus* isolate 131R from the corresponding pet owner, which carried identical copies of *vga*(A)$_{LC}$, showed the same PFGE type H, indicating that they were closely related. This finding supports the idea that these isolates might have been exchanged between the cat and its owner.

Genetic Environment of PLS$_A$ Genes

S1-PFGE and Southern blot hybridization indicated that all *vga*-type genes in the three isolates were located on plasmids, while all *lsa*(E) and *sal*(A) genes in the respective staphylococci were located in chromosomal DNA (**Table 1**; **Figure 2**).

To determine the regions flanking the *vga*-type genes in isolates 131R, 131A, and 132R, inverse PCRs were performed using the primers vga-F (5′-CAAGCTGAAAAGCCAACAAGG-3′) and vga-R (5′-CCTCGTCAATTTCCCATATAGT-3′), which are located inside the conserved regions of *vga*(A) and *vga*(A)$_{LC}$ genes. Three amplicons of 6056-bp (plasmids p131A and p131R) and 7209-bp (p132R) were obtained (**Figure 3A**). Plasmids p131A and p131R from the feline and human *S. haemolyticus* isolates differed in only nine base pairs, and showed 99% nucleotide sequence identity to plasmid pUR2355 from human *S. aureus* (accession no. JQ312422). Plasmid p132R shared 99% nucleotide identity with the *S. epidermidis* plasmid pUR3036 of cat origin (accession no. JQ312423). The most notable difference between p132R and p131A/p131R was the presence of 13 amino acid substitutions in the Vga protein. Surprisingly, the lincomycin MICs of isolates 131A, 131R, and 132R were 16, 16, and 64 μg/ml, respectively, which is in contrast to the finding that *vga*(A)$_{LC}$ confers higher MICs to lincosamides than does *vga*(A) (Novotna and Janata, 2006).

Genomic DNA of *S. sciuri* isolate 100N carrying *sal*(A) and *S. epidermidis* isolate 138N carrying *sal*(A) and *lsa*(E) were sequenced by WGS. Comparative analysis of the draft genomes of isolates 100N and 138N with the corresponding sequenced genomes of staphylococci from NCBI revealed the presence of a very similar 12.5-kb DNA segment (97% nucleotide identity) containing the *sal*(A) gene inserted between the housekeeping genes *aspS* and *alaS* of isolates 100N and 138N (**Figure 3B**). This DNA segment contains 10 ORFs, of which only *sal*(A) is an antimicrobial resistance gene. Additionally, two pairs of primers, aspS-F (5′-CGTTGTTGAAGATGGTGCGTCT-3′), salA-R (5′-GGACCGAACCTTGAAATGATTG-3′), and salA-F (5′-GATGGATACCTTATAGAAGGTG-3′), alaS-R (5′-GTCTGTATCATAGTTCGTTGG-3′), which are located

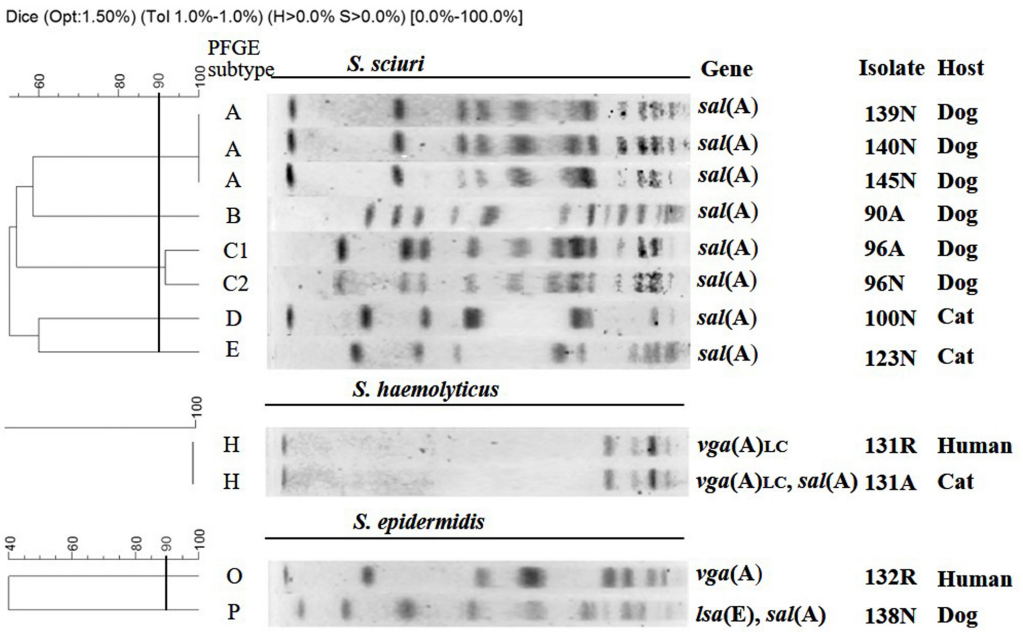

FIGURE 1 | SmaI-PFGE typing of 12 valnemulin-resistant staphylococcal isolates for which more than a single isolate per species was available.

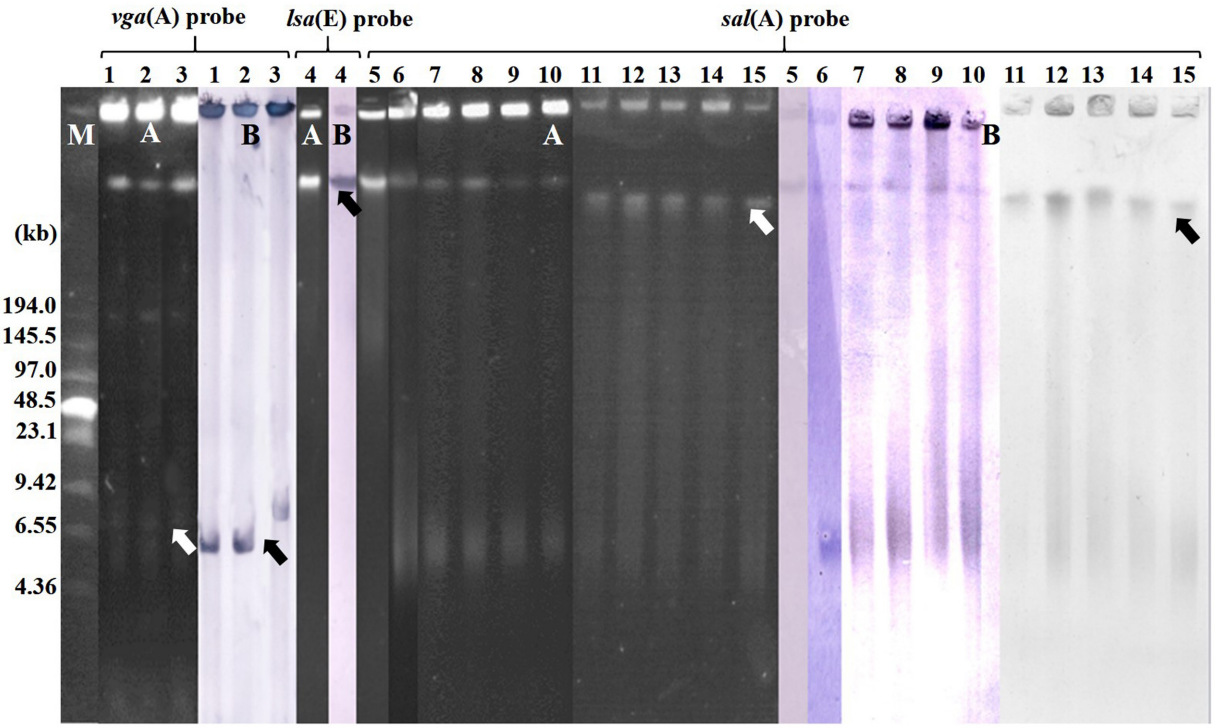

FIGURE 2 | Localization of pleuromutilins, lincosamides, and streptogramin A (PLS$_A$) in the valnemulin-resistant staphylococcal isolates by S1 nuclease-PFGE (A) and Southern blot hybridization **(B)**. M indicates the Low Range PFGE Marker. The black arrows indicate the location of PLS$_A$ gene in Southern blot hybridization and white arrows indicate the position of PLS$_A$ gene in genome according to the S1-PFGE and marker. 131A and 131R (lanes 1–2, respectively) were *vga*(A)$_{LC}$-positive *S. haemolyticus* isolates. 132R (lane 3) was a *vga*(A)-positive *S. epidermidis* isolate. 138N (lane 4) was a *lsa*(E)-positive *S. epidermidis* isolate. 90A, 95N, 96A, 96N, 100N, 123N, 131A, 138N, 139N, 140N, and 145N (lanes 5–15, respectively) were *sal*(A)-positive staphylococcal isolates.

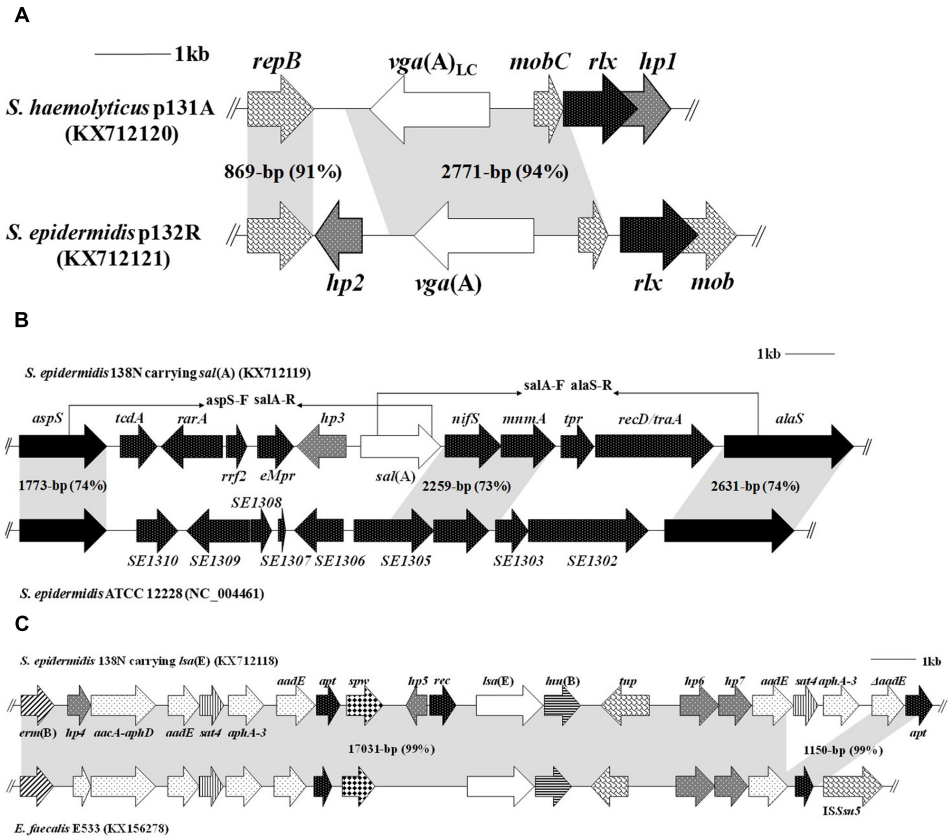

FIGURE 3 | Comparison of genetic variations of the PLS$_A$ genes (*vga*(A)$_{LC}$, *vga*(A), *sal*(A), and *lsa*(E)) of staphylococcal isolates. Four accession numbers (KX712120, KX712121, KX71211, and KX712118) were newly submitted in this study. The arrows indicate the positions and orientations of the genes. The similarities between the different structures are marked by gray shading. **(A)** Genetic environments of *vga*(A)$_{LC}$ in plasmid p131A from a *S. haemolyticus* isolate of cat origin and of *vga*(A) in p132R from a *S. epidermidis* isolate of human origin. **(B)** Structure of the novel genetic environment of *sal*(A) in 11 staphylococcal isolates (138N was the representative). The locations of primers used to detect and verify the genetic environment of *sal*(A) in other isolates are indicated by black arrowheads. **(C)** Genetic environment of *lsa*(E) in *S. epidermidis* 138N and structural comparison with the corresponding regions in *E. faecalis* E533.

in the conserved regions of the *aspS*, *sal*(A), and *alaS* genes (**Figure 3B**), respectively, were designed, and long-range PCRs were preformed to investigate the genetic environments of *sal*(A) in the other nine *sal*(A)-positive staphylococcal isolates (**Table 1**). Two amplicons of 7.4 and 7.7 kb were obtained, and identical sequences were found in all nine isolates.

A 20,154-bp *lsa*(E)-carrying fragment containing 21 ORFs was obtained in the canine *S. epidermidis* isolate 138N (**Figure 3C**) after the conjoint analysis of the results of WGS and modified random primer walking strategy. Similar to the corresponding regions in porcine *E. faecalis* E533 (accession no. KX156278), this *lsa*(E)-carrying fragment also contained the macrolide-lincosamide-streptogramin B resistance gene *erm*(B), the aminoglycoside resistance genes *aacA-aphD*, *aadE*, and *aphA-3*, the spectinomycin resistance gene *spw*, the streptothricin resistance gene *sat4*, and the lincosamide resistance gene *lnu*(B). Further downstream of the *lnu*(B) gene, a second copy of the genes *aadE-sat4-aphA-3* was detected (**Figure 3C**). This multidrug resistance gene region confers resistance to seven classes of antimicrobial agents and explained why isolate 138N exhibited resistance not only to PLS$_A$ antibiotics, but also to spectinomycin, gentamicin and erythromycin (**Table 1**). In addition, the *hp4* gene encoded a predicted protein related to an acetyltransferase enzyme GNAT (GCN5-related N-acetyltransferase, with no confirmed function) from *Enterococcus*.

DISCUSSION

Increasing emergence of staphylococci that are resistant to PLS$_A$ and are isolated from humans and pets is a growing public health concern worldwide. Each of these three classes of antimicrobial agents contains important drugs for human and veterinary medicine. The pleuromutilin antibiotics valnemulin and tiamulin are frequently used in veterinary medicine. Retapamulin was the first pleuromutilin approved for topical use in skin infections of humans caused by staphylococci (Jacobs, 2007). Lincosamides, such as clindamycin and lincomycin, have generally been indicated for the treatment of osteomyelitis but also skin and soft tissue infections caused by staphylococci in humans and pets (Xue et al., 1996; Zetner et al., 2003). Virginiamycin

is a two-component streptogramin with a broad spectrum of activity against Gram-positive bacteria. Some antimicrobial agents approved for use in human medicine are also applied to nonfood-producing animals under specific regulations, such as the Animal Medicinal Drug Use Clarification Act (AMDUCA) in the USA. In this study, all 13 valnemulin-resistant isolates displayed resistance or high MICs to pleuromutilins (valnemulin, tiamulin, and retapamulin), lincomycin, virginiamycin M1, indicating that all PLS$_A$ genes including *vga*(A), *vga*(A)$_{LC}$, *sal*(A), and *lsa*(E) of these isolates were functional. Interestingly, genes *sal*(A) and *vga*(A)$_{LC}$ coexisted in one feline *S. haemolyticus* isolate 131A, and genes *sal*(A) and *lsa*(E) were co-located in the chromosomal DNA of the canine *S. epidermidis* isolate 138N. Isolate 138N exhibited significant differences in the MICs of valnemulin, lincomycin, and virginiamycin M1 compared with other isolates that carried only one type of PLS$_A$ genes (**Table 1**). There have been no studies regarding to *sal*, *vga*, and *lsa* coexisting in the same isolate, while both *vga*(A) and *lsa*(E) were identified in four bovine staphylococcal isolates (Wendlandt et al., 2015). Notably, *vga*(A)$_{LC}$, *sal*(A), and *lsa*(E) were for the first time identified in staphylococcal isolates originating from pet animals.

The presence of PLS$_A$ resistance genes in pet- and human-associated staphylococci indicates the adaptation of these bacteria to antibiotic pressure, given that PLS$_A$ resistance genes confer resistance to antimicrobial agents used in both pets and humans. Additionally, *S. epidermidis* 138N carried at least 13 resistance genes conferring resistance to seven classes of antimicrobial compounds (pleuromutilins, lincosamides, streptogramins, macrolides, aminoglycosides, aminocyclitols, and streptothricins), indicating that the use of any of the abovementioned antimicrobial agents may lead to co-selection of the MDR genomic island (MDRGI) in isolate 138N. The plasmids, chromosomal DNA region, and MDRGI associated with the multiresistance genes *vga*(A), *vga*(A)$_{LC}$, *sal*(A), and *lsa*(E) exist in staphylococci isolated from pets and humans, which enhances the dissemination of PLS$_A$-resistant staphylococci among pets and humans and presents significant challenges for the clinical management of infections by limited therapeutic options.

The three canine *sal*(A)-positive *S. sciuri* isolates 139N, 140N, and 145N not only shared the identical regions flanking the *sal*(A) gene, but also had the uniform PFGE pattern (subtype A), suggesting that these isolates have originated from a single clone. Remarkably, plasmids p131A and p131R, the two almost identical *vga*(A)$_{LC}$-carrying plasmids present in isolates with the same PFGE subtype (subtype H), were derived from a cat and its owner. This fact suggests that these two isolates might have been exchanged between the cat and its owner due to their extensive contact. In this regard, it is important to consider the current role of dogs and cats as actual family members in many households and, consequently, the close contact to their owners and other family members (Schwarz et al., 2016).

CONCLUSION

This is the first description of the PLS$_A$ genes in staphylococci of pet origin and also the description of a novel *lsa*(E)-carrying MDRGI in *S. epidermidis*. Moreover, we identified the *sal*(A) gene for the first time in *S. haemolyticus*, *S. epidermidis*, and *S. xylosus*. Pets are likely reservoirs of antimicrobial-resistant bacteria, warranting the prudent use of all antimicrobials in pet animal medicine.

AUTHOR CONTRIBUTIONS

YW and SS designed research; FD, HW, and YL performed research; FD, JL, AF, and GM analyzed data; FD, SS, and YW wrote the paper. All authors listed have approved research for publication.

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Molecular Mechanisms of Colistin Resistance in *Klebsiella pneumoniae* Causing Bacteremia from India—A First Report

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Colistin has long been a reserve drug used for the treatment of carbapenem resistant *Klebsiella pneumoniae*. Carbapenem resistance in *K. pneumoniae* has been increasing and is as high as 44% in India. Although a reserve agent, with rise in rates of resistance to carbapenems, the usage of colistin has increased over the years leading to slow emergence of resistance. Colistin resistance is mainly mediated by the alteration in the LPS of bacterial outer membrane with the addition of L-Ara4-N and PEtN molecules. These alterations are mediated by mutations in several genes involved in lipidA modifications and most commonly mutations in *mgrB* gene has been reported. Recently there is emergence of plasmid mediated resistance due to *mcr-1* and *mcr-2* genes which poses a threat for the rapid global spread. This study aims at characterizing eight colistin resistant *K. pneumoniae* from bacteremia by whole genome sequencing. Eight *K. pneumoniae* were isolated from blood culture during 2013 and 2014 at the Department of Clinical Microbiology, Christian Medical College, India. Antimicrobial susceptibility testing was performed and minimum inhibitory concentration (MIC) was determined for colistin and polymyxin B by broth-micro dilution method. Whole genome sequencing was performed using Ion Torrent and the genome of all eight isolates was analyzed. The eight isolates were resistant to all the antimicrobials expect tigecycline. MIC of colistin and polymyxin B were ranged from 4 to 1024 μg/ml and 0.5 to 2048 μg/ml respectively. Multiple mutations were observed in the chromosomal genes involved in lipid A modifications. *mcr-1* and *mcr-2* gene was absent in all the isolates. The most significant were mutations in *mgrB* gene. Among the eight isolates, four, three and one were belonged to sequence types ST 231, ST14 and ST147 respectively. Seven isolates had $bla_{\text{OXA-48 like}}$, one co-expressed $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48 like}}$ genes leading to carbapenem resistance. Overall, multiple numbers of alterations have been observed.

This includes silent mutations, point mutations, insertions and/or deletions. Mutations in *mgrB* gene is responsible for resistance to colistin in this study. Due to emergence of resistance to reserve drugs, there is a need for combination therapies for carbapenem resistant *K. pneumoniae* and colistin must be judiciously used.

Keywords: colistin, polymyxin B, resistance, *Klebsiella pneumoniae*, whole genome sequencing

INTRODUCTION

Klebsiella pneumoniae is a gram negative bacteria and is a common cause of severe community and hospital acquired infections. It readily colonizes human mucosal surfaces especially the gastrointestinal (GI) tract and oropharynx (Bagley, 1985; Dao et al., 2014; Rock et al., 2014). In immunocompromised hosts, it can invade other sites leading to severe infections (Paczosa and Mecsas, 2016). In India, carbapenems are commonly used for treatment of *K. pneumoniae* due to high rates of ESBL producers (Ah et al., 2014). Increasing carbapenem resistance has resulted in colistin (polymyxin E) and tigecycline being used as last resort drugs for treatment of multidrug resistant (MDR) isolates (Carmeli et al., 2010).

Polymyxins are cationic polypeptides with five different compounds, known as polymyxin A–E (Storm et al., 1977). However, only polymyxin B and polymyxin E (colistin) are used in clinical practice to treat bacterial infections. When initially introduced, polymyxins were not widely used due to their adverse effects of nephrotoxicity and neurotoxicity (Falagas and Kasiakou, 2006). However, with the rise of carbapenem resistant organisms, polymyxins have gained attention as one of the few agents available for treatment (Falagas et al., 2005). Polymyxins are positively charged antimicrobial peptide molecules, which act by biding to negatively charged phosphate groups in the lipidA moiety of lipopolysaccharides (LPS). This causes disruption and loss of cell membrane integrity, leading to cell death (Yahav et al., 2012).

Due to its rising and sometimes inappropriate use, colistin resistance is increasing. Resistance to colistin is mainly mediated by modification of the LPS moiety with the addition of positively charged L-Ara4-N and PEtN molecules (Falagas et al., 2010). This modification decreases the negative charge of the outer membrane, reducing its interaction with colistin (Velkov et al., 2013). It is mainly due to mutations in the two component regulatory systems. The most common mutations are in *mgrB, phoP/phoQ, pmrA, pmrB, pmrC,* and *crrABC* (Cheng et al., 2010; Poirel et al., 2015; Wright et al., 2015).

Recently, plasmid mediated colistin resistance named *mcr-1* (plasmid Mediated Colistin Resistance) encoding for phosphoethanolamine transferase has been reported in *Escherichia coli* and *K. pneumoniae* from China (Liu et al., 2016). Following this, *mcr-1.2*, a variant of *mcr-1*, was identified in KPC producing *K. pneumoniae* in Italy (Di Pilato et al., 2016) followed by the *mcr-2* gene reported from Belgium, This had 76% identity with the *mcr-1* gene previously reported (Xavier et al., 2016). The increasing number of resistance mechanisms reported in such a short time period is alarming.

The emergence of colistin resistance is a serious cause for concern for both clinicians and patients, particularly in countries with high rates of carbapenem resistant *Enterobacteriaceae* such as China, India, and Greece. Hence, it is necessary to perform surveillance studies for colistin resistance organisms in Indian hospitals. In this study, we report eight colistin resistant *K. pneumoniae* isolated from bloodstream infections from Christian Medical College, Vellore, India. Whole genome sequencing was employed to study the mechanisms of colistin resistance such as chromosomal genes and plasmid mediated *mcr-1* and *mcr-2* genes.

MATERIALS AND METHODS

Bacterial Strains

A total of eight clinical isolates of colistin resistant (CR) *K. pneumoniae* (CRKP1–CRKP8) were included in this study. These CR-*K. pneumoniae* were isolated in blood culture from patients admitted at Christian Medical College, Vellore, South India between 2013 and 2015. All the eight isolates were identified up-to species level as per standard biochemical tests (Versalovic et al., 2011) and by Vitek2 (bioMerieux)

Demographic and Clinical Details of Patients

These were obtained from electronic medical records available in the hospital intranet.

Antimicrobial Susceptibility Testing

The Kirby Bauer disk diffusion method was performed to ascertain antimicrobial susceptibility to commonly used agents. This included ceftazidime (30 μg), cefotaxime (30 μg), cefoxitin (30 μg), cefepime (30 μg), piperacillin/tazobactam (100/10 μg), imipenem (10 μg), meropenem (10 μg), ciprofloxacin (5 μg), gentamicin (10 μg), netilmicin (30 μg), amikacin (30 μg), polymyxin B (300 units), and tigecycline (15 μg). The susceptibility results were interpreted as per CLSI (2013, 2014, 2015) guidelines, respectively (CLSI M100S-23; CLSI M100S-24; CLSI M100S-25). Tigecycline results were interpreted according to FDA criteria (http://www.accessdata.fda.gov/drugsatfda_docs/label/2009/021821s016lbl.pdf).

The minimum inhibitory concentration (MICs) were determined by broth micro dilution method (BMD) for both polymyxin agents, colistin and polymyxin B. The results were interpreted as per EUCAST guidelines (EUCAST, 2013, 2014, 2015, respectively), (EUCAST susceptibility breakpoint v 3.0, v 4.0 and v 5.0). *E. coli* ATCC 25922 was used as a quality control.

Carbapenemase Detection by CarbaNP and Multiplex PCR

The CarbaNP test was performed for all isolates by the previously described method (Nordmann et al., 2012). *K. pneumoniae* ATCC BAA 1705 and *K. pneumoniae* ATCC BAA 1706 were used as positive and negative controls, respectively.

For molecular testing, all the isolates were grown overnight on blood agar and DNA was extracted using Qiagen kit as per manufacturer's protocol (Qiagen Bacterial DNA mini kit). The presence of carbapenemase genes such as bla_{SPM}, bla_{IMP}, bla_{VIM}, bla_{NDM}, $bla_{KPC,}$ and $bla_{OXA-48\ like}$ genes were determined by conventional multiplex PCR as described previously (Anandan et al., 2015). The products were analyzed on 2% agarose gel stained with ethidium bromide. Respective positive controls were used for each run (Courtesy: IHMA, Inc., USA).

Molecular Characterization of Colistin Resistance

PCR and Sequencing for LPS Modifications Genes

Genes involved in LPS modification including *mgrB*, *phoP*/*phoQ*, and *pmrA*/*pmrB*/*pmrD* were amplified and sequenced as previously described (Jayol et al., 2015; Wright et al., 2015).

Whole Genome Sequencing (WGS) for CR- *K. pneumoniae*

WGS was performed using the Ion Torrent PGM platform using 400 bp read chemistry. Sequencing was performed as per the protocol recommended by Life Technologies. Raw reads were assembled *de novo* using Assembler SPAdes software v4.4.0.1 in Torrent suite server version 4.4.3. The genome was annotated using the PATRIC (Pathosystems Resource Integration Center -https://www.patricbrc.org) and RAST (Rapid Annotations using Subsystems Technology) databases. Upon annotation, antimicrobial resistance genes were found as per ARDB and CARD, respectively. These genes were further taken for mutational analysis. The whole genome sequences were deposited at GenBank under the following accession numbers: CRKP1 (*MOXM00000000*); CRKP2 (*MIEJ00000000*); CRKP3 (*MPCT00000000*); CRKP4 (*MDZG00000000*); CRKP5 (*MOXL00000000*); CRKP6 (*MEBR00000000*); CRKP7 (*MOXN00000000*); CRKP8 (*LZYN00000000*).

TABLE 1 | Minimum inhibitory concentration (MIC) for colistin and polymyxin B by broth micro dilution (BMD) for *K. pneumoniae.*

MICRO No.	MIC (BMD)	
	Colistin (μg/ml)	Polymyxin B (μg/ml)
CRKP1	4	0.5
CRKP2	8	32
CRKP3	8	64
CRKP4	16	64
CRKP5	16	64
CRKP6	16	64
CRKP7	16	64
CRKP8	1024	2048

Molecular Typing of CR *K. pneumoniae* by MLST

The MLST database at the Center of Genomic Epidemiology was used to identify the sequence types (ST) of the study isolates using WGS data (https://cge.cbs.dtu.dk/services/MLST/).

eBURST

The program eBURST v 3.0 was used to identify clonal complexes.

RESULTS

Demographic and Clinical Details of Patients

The demographic and clinical details of each patient were obtained from electronic medical records. The patients' ranged from 25 to 59 years in age. Five were male. Six patients were under the care of the hematology team, one was under the care of neurology, and one under nephrology. All but one patient was immunocompromised. *K. pneumoniae* sepsis was hospital acquired in all cases and the sources of infection were neutropenic sepsis *(n = 4),* pneumonia *(n = 1),* urosepsis *(n = 1),* intra-abdominal infection *(n = 1),* and line infection *(n = 1).* Meropenem was administered to all patients before *K. pneumoniae* was isolated and colistin was administered to five patients. Four patients expired within 30 days of infection and seven within 90 days of infection.

Antimicrobial Susceptibility Testing

All isolates were extremely drug resistant (XDR) as per the definition of Magiorakos et al. (2012) All isolates were resistant to cephalosporins, carbapenems, fluoroquinolones, aminoglycosides, and polymyxin B by disk diffusion but were susceptible to tigecycline. The MIC-values determined by BMD for colistin and polymyxin B ranged from 4 to 1024 and 0.5 to 2048 μg/ml, respectively. The MIC results of the eight isolates are summarized in **Table 1**.

Carbapenemase Detection

CarbaNP test was positive for all the eight isolates tested indicating the production of carbapenemases. Multiplex PCR for carbapenamase genes revealed the presence of $bla_{OXA-48\ like}$ gene in seven isolates. One isolate co-expressed $bla_{NDM-1\ +\ OXA-232}$ genes.

Whole Genome Sequence Analysis

For whole genome sequencing analysis, PATRIC, and RAST databases have been used to analyse the annotated sequences (http://patricbrc.org/) (http://rast.nmpdr.org/). As these eight isolates were XDR strains, multiple resistance genes against various classes of antimicrobial agents have been identified. These are summarized in **Table 2**. For cephalosporin resistance, the most common resistance genes were bla_{TEM}, $bla_{SHV,}$ and $bla_{CTX-M-15}$. For carbapenem resistance, $bla_{OXA-232}$, a variant of $bla_{OXA-48\ like}$ gene was found in six of the study isolates, one

TABLE 2 | Antimicrobial resistance genes identified against various antimicrobial agents in CR *K. pneumoniae*.

Isolate	β-Lactamase	Aminoglycoside	Fluoroquinolones	Fosfomycin	Macrolides	Phenicol	Rifampicin	Sulphonamide	Tetracycline	Trimethoprim
CRKP1	*TEM-1A, OXA-232*, OXA-1, SHV-28, CTX-M-15*	*aadA2, aacA4, armA*	*aac(6′)Ib-cr, oqxB, oqxA,*	*fosA*	*msr(E), mph(E)*	*catB3*	–	*sul1*	*tet(D)*	*dfrA14, dfrA12, dfrA1*
CRKP2	*OXA-1, CTX-M-15, OXA-232*, TEM-1B, SHV-28*	*aadA2, aacA4, armA*	*aac(6′)Ib-cr, oqxB, oqxA*	*fosA*	*msr(E), mph(A), erm(B), mph(E)*	*catB3*	–	*sul1*	*tet(D)*	*dfrA12, dfrA1, dfrA14*
CRKP3	*OXA-232**	–	–	–	–	–	–	*sul1*	–	–
CRKP4	*TEM-1B, CTX-M-15, SHV-12, OXA-232**	*aadA2, aacA4*	*aac(6′)Ib-cr, QnrS1*	*fosA*	*mph(A), erm(B)*	*catA1*	*ARR-2*	*sul1*	–	*drfA12*
CRKP5	*TEM-124, OXA-232*, SHV-12, CTX-M-15*	*aadA2, aacA4, rmtF*	*aac(6′)Ib-cr, oqxA*	*fosA*	*mph(A), erm(B)*	*catA1*	*ARR-2*	*sul1*	–	*drfA12*
CRKP6	*OXA-1, CTX-M-15, OXA-232*, NDM-1, SHV-28, TEM-1A*	*aadA2, aacA4, armA, aph(3′)VIa*	*aac(6′)Ib-cr, oqxA, oqxB*	*fosA*	*msr(E), mph(A),*	*catB3*	*fosA*	*sul1*	*tet(D)*	*dfrA14, dfrA12, dfrA1*
CRKP7	*TEM-1B, CTX-M-15, SHV-12, OXA-232**	*aadA2, aacA4*	*aac(6′)Ib-cr, oqxA, QnrS1*	*fosA*	*mph(A), erm(B)*	*catA1*	*ARR-2*	*sul1*	–	*drfA12*
CRKP8	*TEM-1B, CTX-M-15, OXA-181*, SHV-11*	*strA, rmtF, aacA4*	*QnrB66, oqxB, oqxA, aac(6′)Ib-cr*	*fosA*	*mph(A)*	–	*ARR-2*	*sul2*	–	*dfrA12, dfrA14*

**Variant of $bla_{OXA-48\ like}$ gene.*

TABLE 3 | Cumulative results of amino acid substitutions in various genes contributing to colistin resistance found upon WGS analysis of CR—*K. pneumoniae*.

Strain ID	*mgrB*	*phoP*	*phoQ*	*pmrA*	*pmrB*	*pmrC/ eptA*	*eptB*	*phoB*	*phoR*	*arnB*	*arnC*	*arnT*	*arnA_FT*	*pagP*
CRKP1	None	R 114 A	D 150 G	None	A 246 T L 344 P	V 39 L S 257 L A 279 G	None	None	None	G 47 D A 112 D I 126V	S 19 T	A 55 G S 56 L A 57 R T 58 Y Y 59 F	I 260 L N 442 K	F 170 I
CRKP2	None	R 114 A	D 146 G	None	None	V 42 L S 260 L	None	None	None	G 47 D A 112 D I 126V	S 30 T	A 55 G S 56 L A 57 R T 58 Y Y 59 F	I 260 L N 442 K	F 170 T
CRKP3	Premature stop codon (truncated protein 27 amino acid)	R 114 A	D 150 G	None	A 246 T L 344 P	V 39 L S 257 L A 279 G	None	None	None	G 47 D A 112 D I 126V	S 30 T	A 55 G S 56 L A 57 R T 58 Y Y 59 F	I 260 L N 442 K	F 170 I
CRKP4	Deletion of A at position 10	R 114 A	D 146 G	None	L 344 P	V 39 L R 152 H A 279 G D 477 N	None	None	R 69 C	A 112 D D 285 E	S 30 T	A 55 G S 56 L A 57 R T 58 Y Y 59 F K 372 R I 474 N	L 161 C I 260 L N 442 K	None
CRKP 5	None	R 114 A	D 146 G	None	L 344 P	V 39 L R 152 H A 279 G D 477 N	None	None	R 69 C	A 112 D D 285 E	S 30 T	A 55 G S 56 L A 57 R T 58 Y Y 59 F K 372 R I 474 N	I 260 L N 442 K	None
CRKP6	Premature stop codon (truncated protein 27 amino acid)	R 114 A	D 146 G	None	A 246 T L 344 P	V 42 L S 260 L	None	None	None	G 47 D A 112 D I 126V	S 19 T	A 55 G S 56 L A 57 R T 58 Y Y 59 F	I 260 L N 442 K	F 170 I

(Continued)

TABLE 3 | Continued

Strain ID	*mgrB*	*phoP*	*phoQ*	*pmrA*	*pmrB*	*pmrC/ eptA*	*eptB*	*phoB*	*phoR*	*arnB*	*arnC*	*arnT*	*arnA_FT*	*pagP*
CRKP7	Deletion of A at position 10	R 114 A	D 146 G	None	T 157 P L 344 P	V 42 L R 155 H D 480 N	None	None	R 69 C	A 112 D D 285 E	S 19 T	A 55 G S 56 L A 57 R T 58 Y Y 59 F K 372 R I 474 N	I 260 L N 442 K	None
CRKP8	None	R 128 A	D 150 G	None	R 256 G L 344 P	C 27 F V 39 L V 50 L A 135 P A 279 G	None	None	None	None	S 30 T	A 55 G S 56 L A 57 R T 58 Y Y 59 F L 114 M I 117 V H 156 Q	S 18 A T 185 A I 260 L N 442 K	None

isolate co-producing $bla_{OXA-232}$ and bla_{NDM-1}, while one isolate had $bla_{OXA-181}$, also a variant of the $bla_{OXA-48\ like}$ gene.

For colistin resistance, RAST classified 11 genes involved in lipid A modification with L-Ara4N pathway. These included *ugd, arnA_DH, arnA_FT, arnB, arnC, arnT, pmrJ, pmrL, pmrD, pmrM,* and *pmrG*. In addition, 10 genes were classified under genes involved in Lipid A modifications. These include *pagP,pagL, lpxO, phoQ, phoP, pmrA, pmrB, eptA, eptB,* and *yijP*. Of these 21 genes, a few gene sequences could not be retrieved from the annotated genomes of the study isolates from RAST/PATRIC databases and hence analysis was not done. However, for the available genes, sequences were retrieved and analyzed for mutations. Upon analysis, multiple mutations were observed in various genes. In seven isolates, there was a change from arginine to alanine at position 114 of *phoP*. In one isolate, there was a change from arginine to alanine in position 128. In *phoQ*, five isolates had a substitution of glycine for aspartic acid at position 146 and three isolates had the same substitution at position 150. The mutations in other genes such as *pmrB, pmrC, phoB*, *arnB, arnC, arnT, arnA_FT,* and *pagP* are listed in **Table 3** and have not been reported in other studies to date. No mutations were found in *pmrA*, e*ptB,* and *phoR* genes in any of the isolates analyzed. *mcr-1* and *mcr-2* genes were absent in all the study isolates.

Targeted Gene Sequencing Analysis of Mutations in Genes Involved in LPS Modifications

Of the eight isolates screened for mutations, CRKP3 and CRKP6 had a premature stop codon in *mgrB* gene resulting in a truncated protein of 27 amino acids. Whereas, a frame shift mutation in CRKP4 and deletion of nucleotide A at 10th position of *mgrB* gene in CRKP7 was noted, this could be responsible for the resistant phenotype. Three isolates, CRKP1, CRKP2, and CRKP5 did not have any mutations in the *mgrB* gene. Sequencing of two component systems *phoP/phoQ* and *pmrA/pmrB* and *pmrD* found multiple silent mutations. Since targeted sequencing results did not reveal much information, WGS data analysis was performed to further understand the additional resistance mechanisms.

Molecular Typing of CR *K. pneumoniae* by MLST

MLST database available at https://cge.cbs.dtu.dk/services/MLST/ by the Center of Genomic Epidemiology was used to identify the sequence type (ST) of the study isolates using WGS data. Of the eight CR-*K. pneumoniae*, three different sequence types were identified (ST231, ST14, and ST147). Four isolates belonged to ST231 (CRKP3, CRKP4, CRKP6, and CRKP7), three to ST14 (CRKP1, CRKP2, and CRKP5) and one to ST147 (CRKP8).

eBURST

eBURST analysis found no clustering of colistin resistant isolates into a clonal complex. The result is depicted in **Figure 1**.

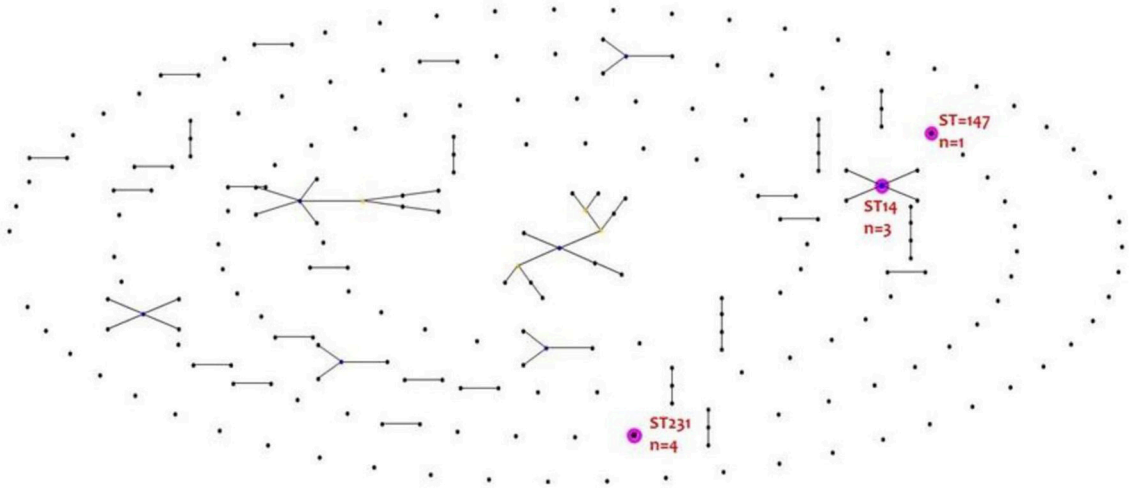

FIGURE 1 | eBURST analysis of colistin resistant *K. pneumoniae*.

DISCUSSION

Antimicrobial resistance is on the rise globally. The limited treatment options available for carbapenem resistant *K. pneumoniae* have resulted in increasing use of colistin, despite its adverse effects. Colistin resistance is now emerging, leaving clinicians with very few options to treat these extensively drug resistant bacteria.

The main challenge in screening colistin resistance lies in determining polymyxin susceptibility (Balaji et al., 2011). Polymyxins are large cationic peptide molecules. They do not diffuse through agar well, hence disk diffusion is neither reliable nor recommended. A reliable screening test is awaited. Other susceptibility methods produce variable and discrepant results. Further, polymyxin B and colistin produce discrepant results (Hindler and Humphries, 2013; Humphries, 2015). Colistin susceptibility testing by disk diffusion has resulted in very major errors of up to 32% when compared with broth or agar dilution methods for colistin (Tan and Ng, 2006; van der Heijden et al., 2007). *E*-test may also produce discrepant results, hence broth microdilution is considered the reference method (Tan et al., 2007).

From this study results, the MIC was found to be two to eight fold higher for polymyxin B when compared to colistin by BMD method. Such discordant results could be due to the non-reliability of MIC testing for polymyxin B. These findings concur with previous studies where the MIC was found to be higher for polymyxin B than colistin (van der Heijden et al., 2007).

A number of studies have reported discrepant results when testing polymyxin B and colistin susceptibility by disk diffusion and MIC methods (Behera et al., 2010). Similar observations were noted in this study; all eight CR-*K. pneumoniae* were found to be resistant on BMD testing with variable MIC values for polymyxin B and colistin as shown in **Table 1**. Detection of molecular resistance mechanisms are based on preliminary screening with polymyxins. This emphasizes the need for a reliable screening test.

mgrB is the commonest chromosomal gene mutation involving modification of LPS which includes insertional inactivation causing colistin resistance. The second commonest cause is multiple mutations in the *pmrAB* and *phoPQ*, the two component systems involved in the LPS modifications.

Of the genes analyzed, mutations in *mgrB* with truncation to 27 amino acids concurs with previous studies (Poirel et al., 2015). The T157P mutation in *pmrB* has also been previously identified (Jayol et al., 2014, 2015; Olaitan et al., 2014). Multiple new mutations were identified in various genes in this study. These include mutations in *phoP* (R114A), *phoQ* (D146G), *pmrB* (L334P, A246T), *pmrC* (V39L, R152H, A279G, D477N, D480N, S260L, C27F, V50L, A235P), *phoR* (R69C), *arnB* (A112D, D285E, G47D, I126V), *arnC* (S30T), *arnA_FT* (I260L, N442K, L161C, S18A, T185A), and *pagP* (F170I). However, the significance of these mutations and their contribution to colistin resistance needs to be studied further.

One isolate (CRKP8) had very high MICs of 1024 μg/ml and 2048 μg/ml by BMD for colistin and polymyxin B respectively. This is a huge variation in MIC between two different methodologies.

The clonality distribution of colistin resistant *K. pneumoniae* is of great interest. Worldwide, sequence types (ST) associated with colistin resistant *K. pneumoniae* are ST258, ST512, and ST147 (Mammina et al., 2012; Cannatelli et al., 2014). However, in our study it was ST231, ST14 and ST147. Our study agrees with Jayol et al who reported ST14 *K. pneumoniae* producing $bla_{\text{OXA-48 like}}$ (Jayol et al., 2014, 2015). However, in this study, the resistance mechanisms were *mgrB* mediated rather than due to mutation in *pmrB*. One of the study isolates belonging to ST14 (CRKP6) was a $bla_{\text{NDM-1}}$ producer. Isolates with mutated and/or truncated *mgrB* were of different sequence types. This further indicates that resistance determinants are irrespective of the sequence types.

Interestingly, isolates CRKP3 and CRKP6, both of which had a mutation in *mgrB* gene resulting in a truncated product, were not of the same clone. However, isolate CRKP4 and CRKP7 which had a frame shift mutation in *mgrB* gene, both belonged to ST231.

CONCLUSION

Inappropriate usage of colistin in the clinical setting should be avoided, as selection pressure may contribute to colistin resistance due to mutations. Further, use of colistin in agricultural practices should be forbidden to prevent the further spread of resistance. The global demand for colistin in agriculture is estimated to reach 16,500 tons by 2021 (QYResearch Medical Research Centre, 2015). India is also one of the leading producers of colistin for veterinary use (Liu et al., 2016). In this study, multiple mutations in the genes coding for LPS modification have been observed. These include silent mutations, point mutations, insertions, and/or deletions. Mutation profiles were observed to be divergent. This signifies the fact that clinical isolates do develop mutations for survival. Altogether, mutations in the *mgrB* gene were found to be the most common, followed by mutations in the other genes such as *pmrB*, *pmrC*, *arnB,* and *arnAFT*. This observation emphasizes that colistin resistance is multifactorial. However, the significant effect of these mutations observed in this study needs to be validated. Therefore, further studies should be focused on the role of these genes in pathways involving LPS modifications conferring resistance to colistin.

ETHICS STATEMENT

Ethical approval was obtained for the study from the Ethical Committee of Christian Medical College, Vellore, India.

AUTHOR CONTRIBUTIONS

AP, CS, FI: lab methods, data analysis, manuscript writing. BV, IB: study design and manuscript writing. LN, BG, SV: study design and data collection.

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Mutations of the Transporter Proteins GlpT and UhpT Confer Fosfomycin Resistance in *Staphylococcus aureus*

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With the increasing spread of methicillin-resistant *Staphylococcus aureus* worldwide, fosfomycin has begun to be used more often, either alone or in combination with other antibiotics, for treating methicillin-resistant *S. aureus* infections, resulting in the emergence of fosfomycin-resistant strains. Fosfomycin resistance is reported to be mediated by fosfomycin-modifying enzymes (FosA, FosB, FosC, and FosX) and mutations of the target enzyme MurA or the membrane transporter proteins UhpT and GlpT. Our previous studies indicated that the *fos* genes might not the major fosfomycin resistance mechanism in *S. aureus*, whereas mutations of *glpT* and *uhpT* seemed to be more related to fosfomycin resistance. However, the precise role of these two genes in *S. aureus* fosfomycin resistance remains unclear. The aim of the present study was to investigate the role of *glpT* and *uhpT* in *S. aureus* fosfomycin resistance. Homologous recombination was used to knockout the *uhpT* and *glpT* genes in *S. aureus* Newman. Gene complementation was generated by the plasmid pRB473 carrying these two genes. The fosfomycin minimal inhibitory concentration (MIC) of the strains was measured by the *E*-test to observe the influence of gene deletion on antibiotic susceptibility. In addition, growth curves were constructed to determine whether the mutations have a significant influence on bacterial growth. Deletion of *uhpT*, *glpT*, and both of them led to increased fosfomycin MIC 0.5 μg/ml to 32 μg/ml, 4 μg/ml, and >1024 μg/ml, respectively. By complementing *uhpT* and *glpT* into the deletion mutants, the fosfomycin MIC decreased from 32 to 0.5 μg/ml and from 4 to 0.25 μg/ml, respectively. Moreover, the transporter gene-deleted strains showed no obvious difference in growth curves compared to the parental strain. In summary, our study strongly suggests that mutations of *uhpT* and *glpT* lead to fosfomycin resistance in *S. aureus*, and that *uhpT* mutation may play a more important role. The high resistance and low biological fitness cost resulting from *uhpT* and *glpT* deletion suggest that these strains might have an evolutionary advantage in a fosfomycin-rich clinical situation, which should be closely monitored.

Keywords: *Staphylococcus aureus*, fosfomycin, resistance, membrane transporter, *glpT*, *uhpT*

INTRODUCTION

Staphylococcus aureus is one of the most common bacterial pathogens worldwide in both community and hospital settings. Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important multi-resistant pathogen. To date, vancomycin has remained the cornerstone drug in the management of invasive MRSA infections. However, the continuous rise in the vancomycin minimum inhibitory concentration (MIC), known as the "vancomycin MIC creep" phenomenon, poses a significant challenge to MRSA therapy; therefore, fosfomycin has recently been used alone or in combination with other antibiotics in treating MRSA infections (del Rio et al., 2016; VanEperen and Segreti, 2016). Nevertheless, this situation has inevitably led to the emergence of fosfomycin-resistant MRSA strains. In a recent review, the susceptibility of *S. aureus* to fosfomycin ranged between 33.2 and 100% in the nine available studies (frequency = 91.7%, 95% confidence interval 88.7–94.9%); in seven of the studies susceptibility rate was >90% (Vardakas et al., 2016). According to the CHINET surveillance program in China in 2010, 29.5% of the MRSA clinical isolates were resistant to fosfomycin (Guo et al., 2013). And Yu et al. (2010) reported a fosfomycin susceptible rate of 33.2%.

The mechanisms of action and resistance of fosfomycin have been studied for decades. Fosfomycin was first discovered in 1969 as an effective bactericidal agent against Gram-positive and Gram-negative organisms. The mechanism of action of fosfomycin differs from that of most commonly used antimicrobials. In general, fosfomycin is transported across the bacterial wall primarily with the help of the glycerol-3-phosphate (G-3-P) transport (GlpT) system. In the presence of glucose-6-phosphate (G-6-P), the hexose phosphate uptake transport (UhpT) system is induced, and provides an alternative route to the GlpT system. UhpT are important membrane transporter proteins for small molecules, including fosfomycin (Castaneda-Garcia et al., 2009). When transported into the cytosol of a bacterium, fosfomycin deactivates the target protein UDP-*N*-acetylglucosamine-3-enolpyruvyltransferase (MurA, encoded by the *murA* gene), thereby preventing the formation of *N*-acetylmuramic acid from *N*-acetylglucosamine and phosphoenolpyruvate, which is the initial step in peptidoglycan chain formation of the bacterial wall (Kahan et al., 1974). The key resistance mechanisms to fosfomycin include the loss or reduced production of transporters, reduced affinity to MurA, and production of fosfomycin-modifying enzymes (Sastry and Doi, 2016).

However, to date, the mechanisms contributing to fosfomycin resistance have been mostly studied in Gram-negative bacteria, with few related studies on Gram-positive bacteria. We have conducted several studies to investigate the fosfomycin resistance mechanisms in Gram-positive cocci, including *Enterococcus faecium* and *S. aureus* (Xu et al., 2013; Chen et al., 2014; Fu et al., 2016a,b). These previous studies indicated that the *fos* gene was not the major mechanism of fosfomycin resistance in MRSA isolates from our hospital, whereas mutations of *glpT* and *uhpT* seemed to be more closely related to fosfomycin resistance. However, the exact roles of these two genes in *S. aureus* fosfomycin resistance remain unclear. Thus, we designed the present study to investigate the roles of *glpT* and *uhpT* in *S. aureus* fosfomycin resistance.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The strains and plasmids used in this study are presented in **Table 1**. The clinical fosfomycin-resistant MRSA strains SA2, SA94, and SA30 were collected from the blood or cerebral spinal fluid of patients at Huashan Hospital and were characterized previously (Fu et al., 2016b). And the strain names are in accordance with that in the **Supplementary Table S1** of the previous article (Fu et al., 2016b). Each of the clinical MRSA strains was with a different type of transporter gene mutation (**Table 1**). The *S. aureus* strains Newman and RN4220, and the plasmid pKOR1 were used in the homologous recombination assay (Bae and Schneewind, 2005; Wang et al., 2015). In addition, *S. aureus* ATCC29213 (American Type Tissue Culture Collection, Manassas, VA, United States) was used for the quality control of susceptibility testing. These strains and plasmids were laboratory collection.

Allelic Gene Deletion by Homologous Recombination

Knockout of the transporter genes *glpT* and *uhpT* was conducted as previously described (Bae and Schneewind, 2005; Wang et al., 2015). The plasmids and primers used are listed in **Tables 1**, **2**, respectively. Proper gene deletion was verified by analytical polymerase chain reaction (PCR) and sequencing of the genomic DNA at the borders of the PCR-derived regions. Sequencing was then performed to confirm the nucleotides. The amplified fragments were used to construct the homologous recombinant pKOR1-Δ*uhpT/glpT* with Gateway® BP Clonase™ II Enzyme mix (Thermo Fisher Scientific, Waltham, MA, United States).

pKOR1-Δ*uhpT* and pKOR1-Δ*glpT* were introduced into *S. aureus* RN4220 by electroporation for modification. The plasmid extracted from strain RN4220 was then introduced into *S. aureus* Newman. The desired *uhpT* and *glpT* deletion mutants were selected as described previously (Bae and Schneewind, 2005).

The successful generation of the Newman-Δ*uhpT* and Newman-Δ*glpT* strains was further confirmed by PCR and sequencing. PCRs were performed using the primers attB1-*uhpT*-up-F/attB2-*uhpT*-CF and attB1-*glpT*-up-F/attB2-*glpT*-CF in the strains *S. aureus* Newman, Newman-Δ*uhpT*, and Newman-Δ*glpT*, respectively.

Construction of the Complemented Strain

Fragments were PCR-amplified from *S. aureus* Newman using the primers C-*uhpT*-F/R and C-*glpT*-F/R. The PCR products and vector pRB473 were double-digested with the designed restriction enzymes BamHI and EcoRI (for *uhpT*), or BamHI and KpnI (for *glpT*), and ligation was performed with T4

ligase. The resulting plasmids were transferred into *S. aureus* RN4220, and then introduced into the deletion and clinical strains with defects on *uhpT* and/or *glpT*, SA2, SA94, and SA30.

Antimicrobial Susceptibility Testing

Fosfomycin susceptibility of the knockout and clinical strains with defects on *uhpT* and/or *glpT*, and their complemented strains were tested with the *E*-test (BioMerieux SA, La Balme Les Grotts, France), according to the manufacturer's guidance. Results were interpreted according to European committee on antimicrobial susceptibility testing criteria (European Committee on Antimicrobial Susceptibility Testing [EUCAST], 2017) (susceptible, ≤32 mg/L; resistant, ≥64 mg/L).

Measurement of Growth Curves

To evaluate the influence of deletion of the transporter genes on bacterial growth, we measured the *in vitro* growth curves of *S. aureus* Newman, Newman-Δ*uhpT*, Newman-Δ*glpT*, Newman-Δ*uhpT*&*glpT,* and the clinical strains. The strains were cultivated in tryptic soy broth overnight at 37°C. The bacterial solution was diluted to an optical density at 600 nm (OD_{600}) of 0.1 and cultivated again. The OD_{600} was then measured at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h for each strain, by

TABLE 1 | Strains and plasmids used to make the deletion mutations.

Strain, plasmid, or primer	Description	Source
***S. aureus* strains**		
SA2	MRSA carrying mutation on *glpT* and *uhpT*, fosfomycin MIC > 1024 μg/ml	Clinical strain 2 (Fu et al., 2016b)
SA94	MRSA carrying mutation on *uhpT*, fosfomycin MIC = 256 μg/ml	Clinical strain 94 (Fu et al., 2016b)
SA30	MRSA carrying mutation on *glpT*, fosfomycin MIC = 128 μg/ml	Clinical strain 30 (Fu et al., 2016b)
Newman	A fosfomycin-sensitive *S. aureus* strain, fosfomycin MIC = 0.5 μg/ml	Bae and Schneewind, 2005
RN4220	A non-a-hemolytic, non-restricting strain of *S. aureus*	Bae and Schneewind, 2005
Newman-Δ*uhpT*	*S. aureus* Newman with deletion of *uhpT*	This study
Newman-Δ*glpT*	*S. aureus* Newman with deletion of *glpT*	This study
Newman-Δ*uhpT*&*glpT*	*S. aureus* Newman with deletion of both *uhpT* and *glpT*	This study
Newman-Δ*uhpT*+pRB473-*uhpT*	Newman-Δ*uhpT* complemented with *uhpT* by plasmid pRB473	This study
Newman-Δ*glpT*+pRB473-*glpT*	Newman-Δ*glpT* complemented with *glpT* by plasmid pRB473	This study
SA2+pRB473-*uhpT*	SA2 complemented with *uhpT* by plasmid pRB473	This study
SA2+pRB473-*glpT*	SA2 complemented with *glpT* by plasmid pRB473	This study
SA94+pRB473-*uhpT*	SA94 complemented with *uhpT* by plasmid pRB473	This study
SA94+pRB473-*glpT*	SA94 complemented with *glpT* by plasmid pRB473	
SA30+pRB473-*uhpT*	SA30 complemented with *uhpT* by plasmid pRB473	
SA30+pRB473-*glpT*	SA30 complemented with *glpT* by plasmid pRB473	This study
Plasmids		
pKOR1	*E. coli* – *S. aureus* shuttle vector; Amp^r in *E. coli*; Cm^r in *S. aureus*	Bae and Schneewind, 2005
pKOR1-Δ*uhpT*	pKOR1 with deletion mutation of *uhpT*	This study
pKOR1-Δ*glpT*	pKOR1 with deletion mutation of *glpT*	This study
pRB473	*E. coli* – *S. aureus* shuttle vector; Cm^r in *S. aureus*	Wang et al., 2015
pRB473-*uhpT*	pRB473 ligated with *uhpT*	This study
pRB473-*glpT*	pRB473 ligated with *glpT*	This study

TABLE 2 | Primers for PCR and sequencing.

Primers	Sequence (5′–3′)	Application
attB1-uhpT-up-F	ggggacaagtttgtacaaaaaagcaggctAAATGCCTCTACACCAG	Allelic replacement
uhpT-NR-EcoRI	CCGgaattcTTGTTCGGAATCTTATGG	
attB2-uhpT-CF	ggggaccactttgtacaagaaagctgggtAATTGCAGACAAAGTAGG	
uhpT-CR-EcoRI	CCGgaattcTCTATGTTGCATTATTCCTA	
attB1-glpT-up-F	ggggacaagtttgtacaaaaaagcaggctATCGGCGTTATCTTTGTTG	
glpT-NR-EcoRI	CCGgaattcGGATGGGATGTCGGTTT	
attB2-glpT-CF	ggggaccactttgtacaagaaagctgggtAACCTTGTGGTGCTAATGTC	
glpT-CR-EcoRI	CCGgaattcCAGCGTAACCGATGAAAAT	
C-uhpT-F	CGCggatccGATTATTGTAAGCAAGCAA	Construction of complemented strain
C-uhpT-R	CCGgaattcTAACGCCATATTCAACTG	
C-glpT-F	CGCggatccTTAATGATGAACAGTTTCTT	
C-glpT-R	CGGggtaccTATTCATACTATCCCTCCT	

spectrophotometer (UNICO, Shanghai, China). The procedure was repeated three times for each strain, and the mean OD_{600} values were used to draw the growth curves.

Phenotype Microarray (PM) Analysis

Phenotype Microarray analysis was performed using BIOLOG Phenotype Microarray™ (BIOLOG, Hayward, CA, United States) according to the manufacturer's recommendations. The deletion mutants, namely Newman-Δ*uhpT*, Newman-Δ*glpT*, Newman-Δ*uhpT*&*glpT*, and the parental strain Newman, were tested with the 96-wells plates PM1 and PM2, containing 190 carbon substrates, including G-6-P (PM1 plate, well C1). To assess the altered phenotypes in carbon metabolism of the deletion mutants, the growth was compared to the parent *S. aureus* Newman.

RESULTS

The deletion mutants showed considerably increased MIC values to fosfomycin compared to that of the parental strain. The Newman-Δ*uhpT*&*glpT* strain, in which both transporter genes were knocked out, showed high-level resistance (MIC > 1024 μg/ml) to fosfomycin, as determined by the *E*-test (**Table 3**). When *uhpT* or *glpT* was knocked out from *S. aureus* Newman, the fosfomycin MICs increased from 0.5 to 32 μg/ml or 4 μg/ml, respectively.

Complementing *uhpT* and *glpT* led to a reduced fosfomycin MIC in the deletion mutants and the clinical fosfomycin-resistant *S. aureus* strains with defects at both sites. By complementing plasmid pRB473-*uhpT* into Newman-Δ*uhpT*, the strain's fosfomycin MIC decreased from 32 to 0.5 μg/ml. Similarly, by complementing *glpT* into Newman-Δ*glpT*, strain's fosfomycin MIC decreased from 4 to 0.25 μg/ml. *S. aureus* SA2, SA94, and SA30 were clinical fosfomycin-resistant strains, with mutations of both *uhpT* and *glpT, uhpT* only, and *glpT* only, respectively. When complemented with the functional transporter genes, the fosfomycin MICs decreased considerably, as shown in **Table 3**.

TABLE 3 | Fosfomycin MIC (μg/ml) of *S. aureus* mutant strains and complemented strains.

S. aureus strains	Fosfomycin MIC
Newman-Δ*glpT*	4
Newman-Δ*glpT*+pRB473-*glpT*	0.25
Newman-Δ*uhpT*	32
Newman-Δ*uhpT*+pRB473-*uhpT*	0.5
Newman-Δ*uhpT*&*glpT*	>1024
SA2	>1024
SA2+pRB473-*glpT*	>1024
SA2+pRB473-*uhpT*	16
SA94	256
SA94+pRB473-*glpT*	256
SA94+pRB473-*uhpT*	16
SA30	128
SA30+pRB473-*glpT*	32
SA30+pRB473-uhpT	64

In vitro bacterial growth curves of the wild-type strain *S. aureus* Newman and the deletion mutants were compared to evaluate the potential fitness cost of these resistant-conferring mutations. As shown in **Figure 1**, no significant depression in growth was observed in Newman-Δ*uhpT* and Newman-Δ*glpT* compared to the wild-type strain. However, the strain Newman-Δ*uhpT*&*glpT* presented slight growth inhibition compared to the wild-type.

Phenotype Microarray analysis was performed using carbon utilization panels, PM1 and PM2, in 190 carbon substrates. The changes in carbon metabolism were listed in **Figure 2**. *S. aureus* Newman showed metabolic advantage over Newman-Δ*uhpT* and Newman-Δ*uhpT*&*glpT* in wells containing G-6-P (**Figure 2**, PM1, well C1). G-3-P was not included in the substrate list, and there was no obvious change found in Newman-Δ*glpT*.

DISCUSSION

Intravenous fosfomycin is broadly used in the treatment of multidrug-resistant pathogens in Europe and Asia owing to its unique antibiotic mechanism, high permeability, and high susceptibility rate (Falagas et al., 2009). Falagas et al. (2010) performed fosfomycin susceptibility testing in non-urinary MRSA isolates, among which 99.2% (129/130) were found to be susceptible. The same group reviewed the susceptibility data of Gram-positive cocci, and reported a cumulative susceptibility rate of 87.9% (4240/4892) in *S. aureus* (Falagas et al., 2009).

Fosfomycin-resistance mechanism has been well described for Gram-negative bacteria such as *E. coli* (Kim et al., 1996; Horii et al., 1999; Huang et al., 2003; Takahata et al., 2010). In *E. coli*, GlpT and UhpT are responsible for fosfomycin uptake. Mutations or insertional inactivation in the *glpT* and/or *uhpT* genes or their regulatory genes lead to the loss of function of the transporters and fosfomycin resistance. The inactivation of either *uhpT* or *glpT* conferred a moderate fosfomycin resistance, (MICs increased from 2 to 8 μg/ml and 32 μg/ml, respectively, compared to the wild type) (Takahata et al., 2010). In *P. aeruginosa*, the inactivation of *glpT* produced significant decrease in fosfomycin MIC, from 8 to 1024 μg/ml (Castaneda-Garcia et al., 2009; Takahata et al., 2010). Modification or overexpression of *murA*, production of fosfomycin-modifying enzymes, are also associated with fosfomycin resistance (Garcia et al., 1995; Kim et al., 1996; Bernat et al., 1997; Horii et al., 1999; Fillgrove et al., 2003; Roberts et al., 2013).

There is less known of fosfomycin resistance mechanism in Gram-positive cocci. In previous works, we collected MRSA clinical strains, and found that only the minority of the fosfomycin-resistant MRSA strains carried the fos gene or murA mutation, while glpT and uhpT mutations were common (82.1%, 55/67, vs. 77.6%, 52/67, respectively) (Fu et al., 2016a,b). This fact indicated that, fosB or murA mutation is not the major contributor to fosfomycin resistance in MRSA, while mutations within the glpT and/or uhpT genes might play an important

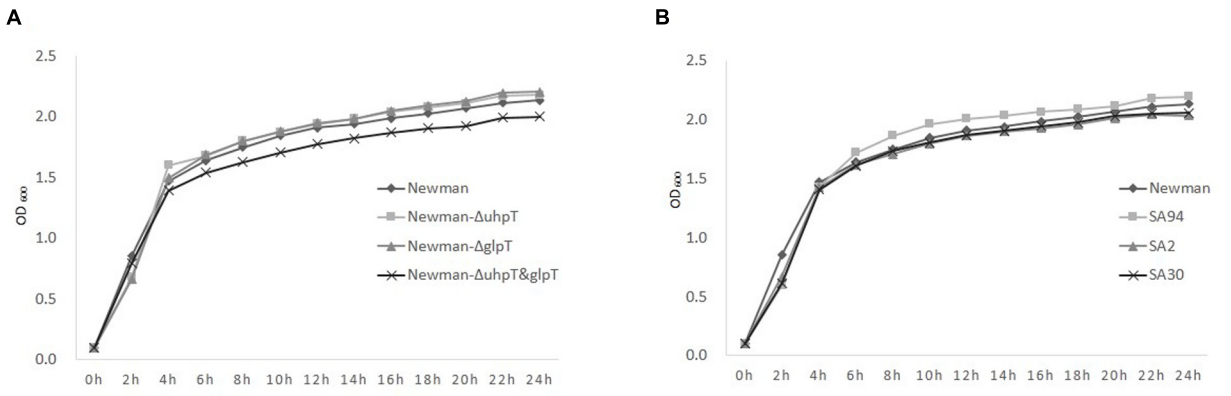

FIGURE 1 | The *in vitro* growth curves of *S. aureus* strains. The strains were cultivated in tryptic soy broth overnight at 37°C. The bacterial solution was diluted to an optical density at 600 nm (OD_{600}) of 0.1 and cultivated again. The OD_{600} was then measured at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h to draw the curves. **(A)** Newman (◆), Newman-Δ*uhpT* (■), Newman-Δ*glpT* (▲), and Newman-Δ*uhpT&glpT* (×). **(B)** Newman (◆) and the clinical *uhpT/glpT* mutants: SA2 (▲), with mutations of both *glpT* and *uhpT*; SA94 (■), with mutation of *uhpT*; SA30 (×), with mutation of *glpT*.

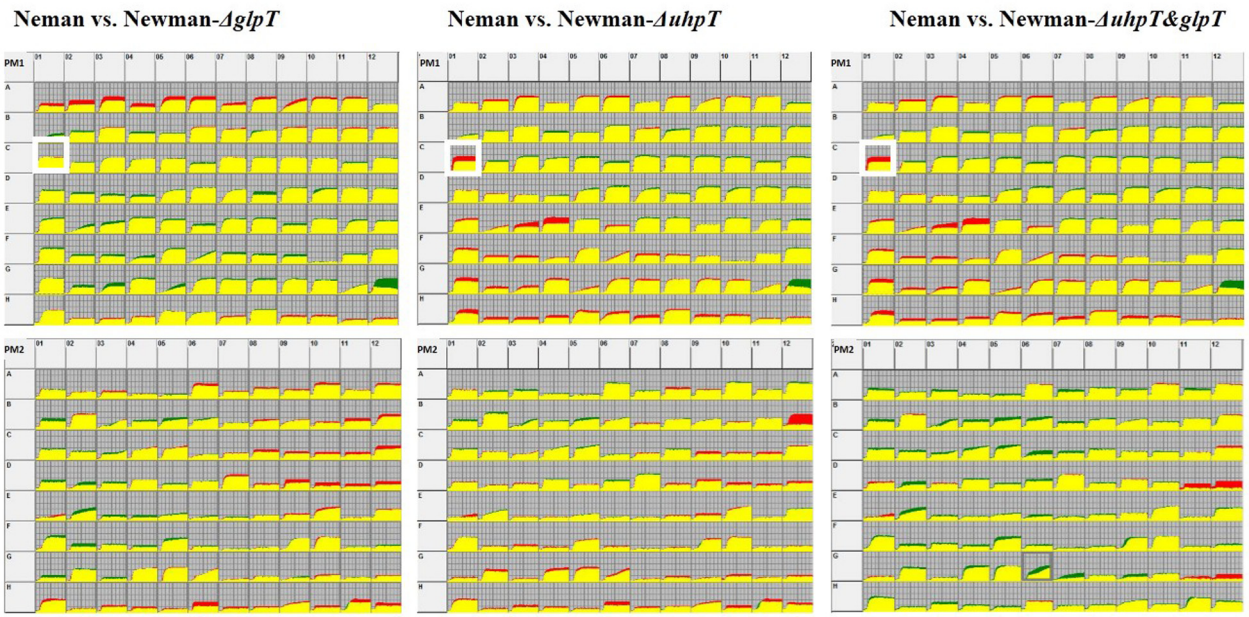

FIGURE 2 | PM analysis for carbon metabolism. The strains were grown in a 96-well plate under 37°C over two nights. Carbon utilization kinetics was measured with OmniLog instrument. Data were superimposed using OmniLog software. The kinetic results show consensus data comparing *S. aureus* Newman and its transporter gene deletion mutants. A metabolic advantage by *S. aureus* Newman is indicated by red, while a metabolic advantage by the deletion mutants is shown by green. The yellow part indicates that both strains have equal metabolism in the well. The white box around PM1 plate, well C1, highlights the G-6-P metabolism. Detailed substrate information of PM1 and PM2 were shown in the **Supplementary Table S1**.

role in *S. aureus* fosfomycin resistance. In the present study, we established uhpT and/or glpT deletion mutants. Knocking out both genes resulted in high-level fosfomycin resistance (MIC > 1024 μg/ml). Complementing either of the two genes into the deletion mutants and clinical mutated strains resulted in a decreased fosfomycin MIC. Direct comparison of uhpT and glpT according to the level of increase of the MIC suggested that uhpT has a greater effect on the strain's MIC to fosfomycin than glpT.

To evaluate the possible, the *in vitro* fitness cost of the transporter gene mutation, we compared growth curves between the fosfomycin-sensitive wild-type strain, laboratory deletion mutant strain, and clinical strains with defects on *uhpT* and/or *glpT*. Previous reports have shown that mutations of *uhpT* and *glpT* can compromise the growth of strains of *E. coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* (Li Pira et al., 1987; Marchese et al., 2003). A probable mechanism might be that mutations of the *glpT* and/or *uhpT* transporting systems prevent carbon

source getting into the cytoplasm, and therefore disturb cell metabolism (Venkateswaran and Wu, 1972; Nilsson et al., 2003). But in *P. aeruginosa*, the *glpT* mutation was found to lead to fosfomycin resistance with no obvious fitness cost (Castaneda-Garcia et al., 2009). In the present study, there was only a slight reduction of growth observed in the strain Newman-Δ*uhpT*&*glpT* compared to the wild type, and no significant growth suppression was observed either in the laboratory deletion mutant strains or in clinical strains with defects on *uhpT* and/or *glpT*, which is similar as observed in *P. aeruginosa*. So *S. aureus* might also compensate the disadvantage in energy obtainment caused by *uhpT* and/or *glpT* mutation through other transporting systems. But further study is still in need for verification.

We observed that G-6-P utilization was defected in both Newman-Δ*uhpT* and Newman-Δ*uhpT*&*glpT*. UhpT is the membrane transporter of this substrate, deletion mutants of *uhpT* showed defects in G-6-P metabolism is as expected. The G-3-P metabolism in *S. aureus* seems to be more complicated. G-3-P seems to be an intermediate product in carbon/phosphorus metabolism pathway. As another low G+C Gram-positive bacteria, *B. subtilis* shares similar carbon metabolism pattern as *S. aureus*. In *B. subtilis*, G-3-P is produced from glycerol with glycerol kinase. And G-3-P dehydrogenase can oxidize G-3-P to dihydroxyacetone phosphate, an intermediated in glycolysis (Holmberg et al., 1990). We have not observed significant change in metabolism. This may be because that G-3-P utilization defect is easily compensated by other pathways.

In summary, the results of our study strongly suggest that mutations of uhpT and glpT lead to fosfomycin resistance in *S. aureus*, and that the uhpT mutation may play a more important role. The high resistance and low fitness cost resulting from uhpT and glpT mutations suggest that these mutated strains might have an evolutionary advantage in a fosfomycin-rich clinical situation. The widely observed uhpT or glpT mutation in *S. aureus* might be a threat in hospital settings. Further studies are needed to evaluate the frequency of *S. aureus* fosfomycin mutants, and virulence of these mutants.

AUTHOR CONTRIBUTIONS

Designed and conceived the experiments: YL, XX, and MW. Performed the experiments: SX, ZF, and YZ. Analyzed the data: SX, ZF, and YZ. Wrote and reviewed the manuscript: SX, ZF, XX, and YL.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00914/full#supplementary-material

TABLE S1 | Carbon sources tested in PM1 and PM2 plates.

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Antimicrobial Susceptibility Profiles of Human *Campylobacter jejuni* Isolates and Association with Phylogenetic Lineages

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Campylobacter jejuni is a zoonotic pathogen and the most common bacterial cause of human gastroenteritis worldwide. With the increase of antibiotic resistance to fluoroquinolones and macrolides, the drugs of choice for treatment, *C. jejuni* was recently classified as a serious antimicrobial resistant threat. Here, we characterized 94 *C. jejuni* isolates collected from patients at four Michigan hospitals in 2011 and 2012 to determine the frequency of resistance and association with phylogenetic lineages. The prevalence of resistance to fluoroquinolones (19.1%) and macrolides (2.1%) in this subset of *C. jejuni* isolates from Michigan was similar to national reports. High frequencies of fluoroquinolone-resistant *C. jejuni* isolates, however, were recovered from patients with a history of foreign travel. A high proportion of these resistant isolates were classified as multilocus sequence type (ST)-464, a fluoroquinolone-resistant lineage that recently emerged in Europe. A significantly higher prevalence of tetracycline-resistant *C. jejuni* was also found in Michigan and resistant isolates were more likely to represent ST-982, which has been previously recovered from ruminants and the environment in the U.S. Notably, patients with tetracycline-resistant *C. jejuni* infections were more likely to have contact with cattle. These outcomes prompt the need to monitor the dissemination and diversification of imported fluoroquinolone-resistant *C. jejuni* strains and to investigate the molecular epidemiology of *C. jejuni* recovered from cattle and farm environments to guide mitigation strategies.

Keywords: *Campylobacter jejuni*, antimicrobial resistance, multilocus sequence typing, epidemiology

INTRODUCTION

Campylobacter spp. are Gram negative bacteria responsible for the greatest number of cases of bacterial gastroenteritis worldwide (World Health Organization [WHO], 2013). It is estimated that 1.3 million *Campylobacter* infections occur every year in the U.S., resulting in 13,000 hospitalizations and 120 deaths, with *C. jejuni* comprising almost 90% of the cases

(Centers for Disease Control and Prevention [CDC], 2015). Furthermore, previous studies have demonstrated an association between campylobacteriosis and autoimmune diseases such as Guillain Barré syndrome (Nachamkin et al., 1998), reactive arthritis (Zia et al., 2003), and irritable bowel syndrome (Connor, 2005). *Campylobacter* spp. have a broad host range and can colonize the intestinal tracts of chickens, turkeys, pigs and ruminants without causing clinical signs (Altekruse et al., 1999; Stanley and Jones, 2003). These pathogens can also survive in water and soil for extended periods of time, up to several months (Jones, 2001; Pitkänen, 2013). The consumption of contaminated poultry is the primary source of sporadic human *Campylobacter* infections (Kaakoush et al., 2015), while approximately 66% of *Campylobacter* outbreaks are attributed to dairy products, mostly raw milk or cheese (Interagency Food Safety Analytics Collaboration Project, 2015). Direct transmission from animal sources including household pets such as dogs and cats, has also been reported (Damborg et al., 2004).

The most common clinical presentation of campylobacteriosis is self-limiting gastroenteritis with vomiting, cramping, and diarrhea lasting for 7–10 days in most cases (Peterson, 1994). Many individuals develop more severe and prolonged infections. Extraintestinal spread of the bacterium, for example, can lead to bacteremia and infection of other organs in some individuals (Man, 2011). Infants, geriatric patients, and immunocompromised individuals more commonly require treatment with antibiotics to combat *C. jejuni* infections (Guerrant et al., 2001). Ciprofloxacin, a fluoroquinolone that inhibits DNA synthesis by targeting *gyrA* and macrolides such as azithromycin and erythromycin, which hinder bacterial protein biosynthesis by targeting 23S rRNA, have been recommended as the first line antimicrobials. Yet, resistance to both antimicrobials has emerged and increases in resistance frequencies have been reported (Zhou et al., 2015). Indeed, the proportion of *Campylobacter* isolates resistant to fluoroquinolones has increased from 14.2% in 1998 to 25.3% in 2012 in the U.S. (Centers for Disease Control and Prevention [CDC], 2014).

Campylobacter resistance to fluoroquinolones and macrolides is conferred by point mutations in their target sites, the *gyrA* and 23S rRNA genes, respectively (Payot et al., 2006). The C257T point mutation in *gyrA* that yields a Thr-86-Ile amino acid change is the most frequently observed mutation that leads to high-level resistance to quinolone, i.e., nalidixic acid, and fluoroquinolone (Iovine, 2013). For macrolides, point mutations of A2074C, A2074G, and A2075G in domain V of the 23S rRNA gene, have been found to confer a high-level of resistance [Minimal inhibitory concentration (MIC) >128 μg/mL], while A2074T has been shown to confer a low-level of resistance (MIC = 8 μg/mL) (Vacher et al., 2005). The presence of the 23s rRNA methyltransferase gene *ermB* and an amino acid change in L4/L22 ribosomal proteins also have been described as resistance mechanisms for macrolides (Pérez-Boto et al., 2010; Wang et al., 2014). In addition, the active efflux pump, CmeABC, works synergistically with point mutations in these gene targets to simultaneously resist the action of fluoroquinolone, macrolide, tetracycline, beta-lactam, and ketolide antimicrobials (Zhang, 2008).

Tetracycline has been suggested as an alternative treatment for patients with systemic *Campylobacter* infections (Guerrant et al., 2001), but it is rarely used in practice. On the other hand, tetracycline is widely used in food animals like chickens and cattle for preventive and growth promoting purposes and treatment, e.g., in sheep for abortion (Giguere et al., 2006). In *Campylobacter*, resistance to tetracycline is conferred by *tet*(O), which has been found widely in isolates recovered from various sources (Connell et al., 2003). *tet*(O) encodes a ribosomal protection protein that induces a conformational change upon binding to the bacterial ribosome, the target site for tetracycline. This binding results in the release of the bound tetracycline molecule. A prior study has identified the *tet*(O) gene in plasmids in the majority of isolates from campylobacteriosis cases (Gibreel et al., 2004), though it has also been identified in the bacterial chromosome (Wu et al., 2014).

In the U.S., the FoodNet surveillance system was designed to monitor the incidence of common foodborne pathogens, including *Campylobacter*, while the National Antimicrobial Resistance Monitoring System (NARMS) was implemented to examine antimicrobial resistance trends. Michigan is not one of the 10 states included in the FoodNet system, however, *Campylobacter* was the most common foodborne pathogen reported through the Michigan Disease Surveillance System (MDSS) in the past decade (2004–2013). As a result, this study was undertaken to determine the frequency of antimicrobial resistance in a subset of *C. jejuni* isolates collected in Michigan between 2011 and 2012, and to estimate the genetic diversity of both susceptible and resistant isolates using multilocus sequence typing (MLST). We hypothesized that the frequency of antimicrobial resistance in *C. jejuni* isolates recovered from Michigan patients is similar to national frequencies, but that specific risk factors, which may be unique to this patient population, are associated with resistant infections. Identifying which lineages are associated with resistance and estimating their frequency in different populations is important for disease prevention efforts aimed at controlling resistance emergence and more rapidly detecting resistant infections.

MATERIALS AND METHODS

Study Population and *Campylobacter* Isolation

From 2011 to 2012, we obtained *C. jejuni* isolates from patients with campylobacteriosis identified via the Michigan State University (MSU) Enteric Research Investigative Network (ERIN) surveillance system. This system was established in collaboration with the Michigan Department of Health and Human Services (MDHHS) and four major hospitals in Michigan. All protocols were approved by the Institutional Review Boards at Michigan State University (IRB# 10-736SM), the MDHHS (842-PHALAB), and each participating hospital.

Isolates were cultured on tryptone soy agar plates with sheep blood and cefoperazone (20 μg/mL), amphotericin B (4 μg/mL), and vancomycin (20 μg/mL) using microaerophilic conditions at 37°C for 48 h. Multiplex PCR was performed on the extracted

DNA to confirm the species following previously described protocol (Yamazaki-Matsune et al., 2007). The isolates were stored in tryptone soy broth with 10% glycerol at −80°C until further testing.

Epidemiological Data

Demographic and epidemiological data were retrieved from the MDSS[1] and managed using Microsoft Access and Excel. Three cases were not Michigan residents, but developed campylobacteriosis while traveling in Michigan and thus, epidemiological data was transferred to the respective states. The *C. jejuni* isolates from these cases were included in the overall genetic diversity and resistance prevalence estimates, but were excluded from further analyses. A patient was considered to have a history of travel only when the traveling period was within 1 week prior to the onset of symptoms. For animal contact, birds were defined as poultry, including chickens, ducks, and turkeys, while ruminants were defined as cattle, sheep and goats, and domestic animals were defined as household pets such as dogs and cats. The season was classified based on the onset date of symptoms: spring (March, April, May), summer (June, July, August), fall (September, October, November), and winter (December, January, February). Food history represents consumption of specific foods (e.g., frozen chicken, home-prepared chicken, etc.) within a week prior to onset of symptoms.

Phenotypic Antimicrobial Susceptibility Profiling

The MICs of nine antimicrobials were determined by a standard broth microdilution test following the guidelines of the Clinical and Laboratory Standards Institute [CLSI] (2015). The Sensititre system (Trek Diagnostic Systems, Thermo Fisher Scientific Inc., Cleveland, OH, USA) was used for each isolate following the manufacturer's instructions. Antimicrobials included ciprofloxacin (fluoroquinolone), nalidixic acid (quinolone), azithromycin (macrolide), erythromycin (macrolide), tetracycline, florfenicol, telithromycin, clindamycin, gentamicin, and *C. jejuni* ATCC 33560 was used as the quality control strain for every batch. The breakpoints for each antimicrobial were determined using epidemiologic cut-off values (ECOFFs), following the guidelines of European Committee on Antimicrobial Susceptibility Testing [EUCAST] (2015), per current protocol of the NARMS (Centers for Disease Control and Prevention [CDC], 2014). If bacterial growth was observed at the highest MIC tested (e.g., 64 μg/mL for tetracycline), then the MIC for the isolate was interpreted as greater than the highest MIC, i.e., > 64 μg/mL.

Whole Genome Sequencing

DNA was extracted from all 94 *C. jejuni* isolates using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA) and the concentrations were measured using a Qubit fluorometer (Life Technologies; Invitrogen, Carlsbad, CA, USA). A total of 1ng of DNA per isolate was used for library preparation with the Nextera XT kit (Illumina, San Diego, CA, USA) following manufacturer's instruction. Validation of the library size and quantity was performed using Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and KAPA library quantification kit (Kapa Biosystems, Woburn, MA, USA), respectively. The libraries were pooled together for denaturing and sequencing on a MiSeq (Illumina) platform for 2 × 250 reads at the Research Technology Support Facility at MSU. Genomic assemblies were performed *de novo* using Velvet, 1.2.07 (Zerbino and Birney, 2008) after trimming with Trimmomatic (Bolger et al., 2014), followed by quality checking with FastQC[2]. Assemblies were constructed using different kmer values (31, 33, and 35), and the assembly yielding the best N50 value for each isolate was used for downstream analyses.

Multilocus Sequence Typing

The MLST profile of each sample was initially determined using the web-based server[3] with both the assembled contigs and extracted alleles from whole genome sequences. Each gene sequence was also confirmed by PCR-based MLST, as described previously (Dingle et al., 2001). Allele, sequence type (ST), and clonal complex (CC) assignments were made using the PubMLST database[4] (Jolley and Maiden, 2010). New alleles ($n = 4$) and STs ($n = 6$) identified in this study were deposited in the PubMLST database (id 24892-28175).

In Silico Analysis of Resistance Genes

Sequences specific for *gyrA* and 23S rRNA were extracted from the draft genomes based on reference sequences available via the National Center for Biotechnology Information using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) (*gyrA*: GenBank KU693342-KU693435; 23S rRNA: GenBank KU891692-KU891785). Regions of the 23S rRNA and gyrA genes, which include the typical point mutation sites associated with resistance to macrolides and fluoroquinolones, respectively, were aligned by MegAlign (DNAstar, Madison, WI, USA). Sanger sequencing was used to confirm the point mutations identified in 47 strains relative to the ATCC 33560 *C. jejuni* strain (*gyrA*: GenBank KU693436; 23S rRNA KU891786), as described in prior studies (Vacher et al., 2003; Said et al., 2010). The presence of *tet*(O) was determined from the genome sequences and further confirmed by PCR using a published protocol (Gibreel et al., 2004).

Data Analysis

The frequency map of all campylobacteriosis cases reported in Michigan between 2011 and 2012 ($n = 1{,}449$) was generated using ArcMap GIS software (version 10.2; ESRI, Redlands, CA, USA) using the data extracted from MDSS.

To identify evolutionary relationships between *C. jejuni* isolates, a Neighbor-joining phylogeny (p-distance) with 1,000

[1] http://www.michigan.gov/mdhhs/0,5885,7-339-71550_5104_31274---,00.html

[2] http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[3] www.cbs.dtu.dk/services/MLST

[4] http://pubmlst.org/campylobacter/

bootstrap replications was constructed in MEGA6 (Tamura et al., 2013) based on seven MLST loci. Clusters or CCs were classified as STs that grouped together with >70% bootstrap support, and parsimonious informative sites were further evaluated for evidence of genetic recombination using Splitstree4 (Huson and Bryant, 2006).

Statistical analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC, USA). Differences in the frequencies of antimicrobial resistance across ST, CC, and other variables including disease presentation, were examined using χ^2 and Fisher's exact tests for dichotomous variables, and the student's *t*-test for continuous variables; $p < 0.05$ was considered significant. Multivariate analyses were performed using logistic regression with any independent variables that yielded a *p*-value of less than 0.2 or were plausibly linked to resistant *C. jejuni* infections, e.g., age, sex. The model was built using a forward stepwise method with the requirement for a significance level of ≤0.1 to remain in the model.

RESULTS

Description of *Campylobacter* Cases Identified in Michigan

Ninety four *C. jejuni* isolates were recovered from clinical cases of campylobacteriosis identified at four hospitals between January 2011 and December 2012. Among the 94 cases, more than 50% ($n = 52$) were male, while 39 cases were female patients; the sex was not known for three cases. Children 2 years and younger (22.3%; $n = 21$) and adults older than 50 years (25.5%; $n = 24$) comprised about half of the total cases. Race was available for 80 of the 94 cases, and the majority identified as Caucasian ($n = 60$; 75.0%).

A total of 1,449 laboratory confirmed *Campylobacter* cases were reported in Michigan in 2011 and 2012 and the frequency of reported cases varied across counties (**Figure 1**). The participating hospitals and most of the residences of the cases included in this study were in counties with higher population densities and a greater frequency of campylobacteriosis. Particularly, cases residing in Wayne, Washtenaw and Oakland counties, which represent the counties with the highest disease frequency, comprised 66.7% ($n = 58$) of the total cases.

Sixty-eight cases had travel history information, among which nine (13.2%) had a history of foreign travel, while 13 (19.1%) had a history of domestic travel in the week prior to the onset of symptoms (**Table 1**). Four cases had a history of domestic travel outside Michigan, while three cases from other states (Ohio, New Jersey, and Georgia) developed symptoms and were diagnosed with campylobacteriosis while traveling in Michigan. Among the 64 cases with a history of animal contact, 38 (59.4%) had contact with domestic animals including dogs and cats. Contact with ruminants, which were all identified as cattle, was reported in seven cases (10.9%), while six cases (9.4%) reported contact with birds such as chickens and ducks. Six of the cases reporting contact with cattle (85.7%) and five of the cases with bird contact (83.3%) also reported contact with dogs and cats.

Antimicrobial Resistance Profiles of *C. jejuni* Isolates and Mechanisms of Resistance

Thirty isolates (31.9%) were susceptible to all nine antimicrobial agents tested, while 64 isolates (68.1%) were resistant to one or more agents. The highest frequency of resistance was observed for tetracycline ($n = 58$ isolates; 61.7%), followed by resistance to both ciprofloxacin and nalidixic acid ($n = 18$ isolates; 19.1%) (**Table 2**). All *C. jejuni* isolates resistant to ciprofloxacin and nalidixic acid showed high MICs (4–32 μg/mL, ≥64 μg/mL, respectively). Resistance to florfenicol was detected in one isolate (1.1%), and all isolates were susceptible to gentamicin. A total of 15 isolates (16.0%) were resistant to two or more classes of antibiotics. Thirteen (13.8%) of these isolates were resistant to ciprofloxacin, nalidixic acid, and tetracycline, while two isolates (2.1%) were resistant to both azithromycin and erythromycin as well as ciprofloxacin, nalidixic acid, telithromycin, and clindamycin.

All 18 *C. jejuni* isolates that were phenotypically resistant to ciprofloxacin had a point mutation at 257 in *gyrA*; 17 isolates had the C257T mutation resulting in an amino acid change of Thr-86-Ile, while one isolate had double mutations (C257G and A258G) resulting in a Thr-86-Arg change. Two isolates that were resistant to azithromycin and erythromycin had an A2074T point mutation in 23S rRNA, and all 58 tetracycline resistant isolates harbored *tet*(O).

Epidemiological Associations with Antimicrobial Resistant *C. jejuni* Infections

To identify factors associated with antimicrobial resistant *C. jejuni* infections, we conducted univariate analyses using demographic and epidemiological data (Supplementary Table S1). Notably, cases reporting a history of foreign travel had a higher likelihood of fluoroquinolone-resistant *C. jejuni* infections (Fisher's $p < 0.0001$) with an odds ratio (OR) of 35.7 (exact 95% CI; 4.6, 395.3). In detail, among the nine cases with foreign travel history, seven were resistant to ciprofloxacin and nalidixic acid; six of these isolates were also resistant to tetracycline, yielding a significant association between foreign travel and the following resistance profile: ciprofloxacin-, nalidixic acid-, tetracycline-resistance (CipNalTet) (Fisher's $p < 0.0001$; OR = 35.3). Cases with CipNalTet infections were significantly more common during the winter months of December, January, and February (Fisher's $p < 0.05$; OR = 5.7), while eating chicken prepared at home was protective for both CipNalTet infections ($p < 0.01$; OR = 0.0) and fluroquinolone-resistant infections ($p < 0.01$; OR = 0.08). By contrast, contact with cattle was associated with tetracycline-resistant infections (Fisher's $p < 0.05$; OR = infinity) and cases reporting consumption of frozen chicken were less likely to have tetracycline-resistant infections ($p < 0.05$; OR = 0.2).

Multivariate analysis was conducted to identify predictors of fluoroquinolone-resistant infections in all 94 cases using the factors with significant associations ($p < 0.2$) identified in the

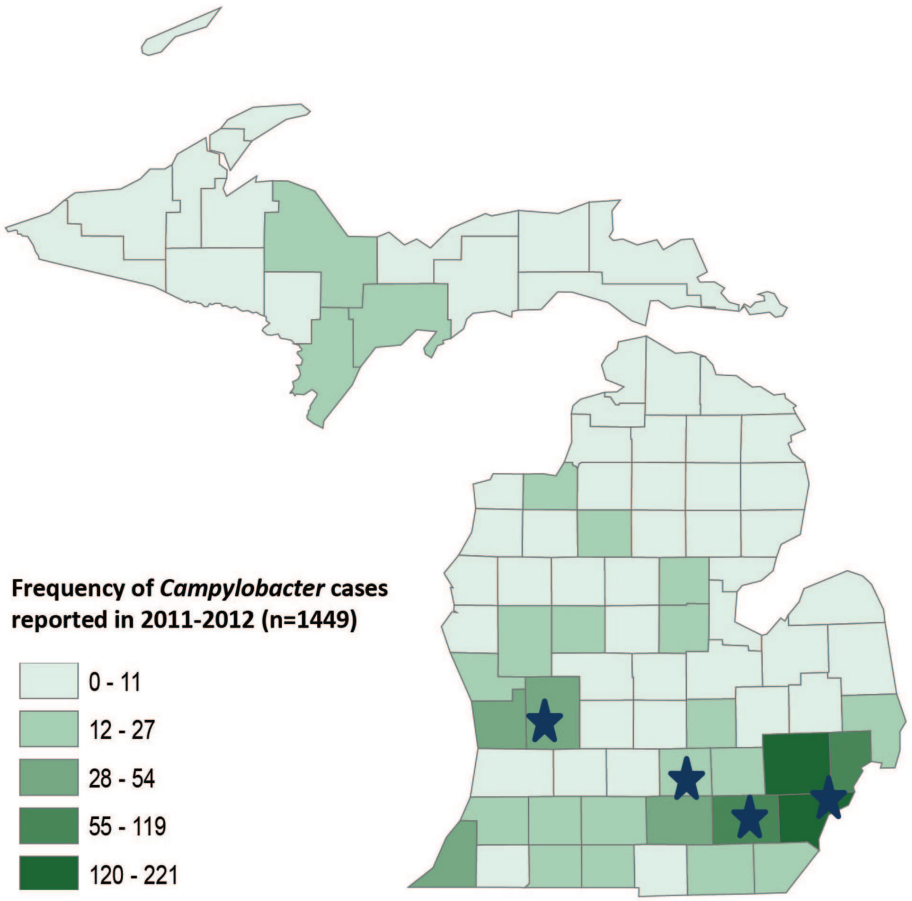

FIGURE 1 | GIS map of Michigan by county showing the frequency of *Campylobacter* cases reported in 2011–2012. The stars represent the location of four hospitals where the samples were collected.

univariate analyses as well as biologically plausible factors such as age, sex (**Table 3**). History of foreign travel was associated with fluoroquinolone-resistant infections regardless of age and sex; the OR was 33.4 (95% CI = 3.9–285.2) in the final model. When the same multivariate analysis was performed for CipNalTet infections, history of foreign travel (OR = 54.2; 95% CI = 4.1–717.1) and winter (OR = 25.3; 95% CI = 1.6–405.7) were independently associated with infection. However, there was no association between foreign travel history and season, including winter. Although having chicken prepared at home was a protective factor in the univariate analysis, this variable could not be examined in the multivariate models as it significantly reduced the sample size due to missing data in 37 of the cases.

Genetic Diversity and Phylogenetic Structure of *C. jejuni*

A total of 49 different STs, including six novel STs, were represented among the 94 *C. jejuni* isolates recovered in Michigan (**Figure 2**). These STs were assigned to 17 CCs, while 11 STs were singletons. The six new STs were assigned to ST-6749 (CC-353), ST- 6751 (CC-61), ST-6752 (CC-353), ST-6788 (CC-1332), ST-7009 (CC unassigned), and ST-7010 (CC unassigned). The most prevalent STs were ST-982 ($n = 10$; 10.6%) and ST-353 ($n = 9$; 9.6%), followed by ST-45 ($n = 7$; 7.4%), ST-50 ($n = 5$; 5.3%) and ST-48 ($n = 4$; 4.3%). Thirty four of the remaining STs had only one isolate assigned to each ST.

The MLST-based Neighbor-joining phylogeny for all 94 isolates showed that some STs were closely related, though the bootstrap values were low, which is likely due to the high diversity and frequent recombination among STs in this isolate population. Indeed, an evaluation of the 144 parsimonious informative sites provided evidence of significant recombination [pairwise homoplasy index (PHI) = 0.0] among the STs via a Neighbor-net analysis (Supplementary Figure S1). In order to elucidate the ST distribution and evolutionary relationships of isolates that were restrictively derived from Michigan, we excluded cases with foreign travel and history of travel outside of Michigan as well as cases with missing data. A total of 36 different STs, including four novel STs, represented the 52 Michigan cases without any history of travel ($n = 46$) or travel only within Michigan ($n = 6$). Although there was still evidence of recombination between these isolates, the phylogenetic tree

showed enhanced bootstrap support, and six distinct clusters with bootstrap support values exceeding 70% were identified (**Figure 3**, Supplementary Figure S2).

Association between Phylogenetic Lineage, Epidemiologic Data, and Resistance

Multiple epidemiological factors were associated with specific *C. jejuni* genotypes. For example, individuals reporting a history of foreign travel were more likely to have infections caused by ST-464 (CC-464) isolates (Fisher's $p < 0.05$), while infection with ST-982 was associated with cattle contact (Fisher's $p < 0.05$). Drinking water obtained from a well at home (Fisher's $p < 0.05$), and contact with birds, i.e., chickens (Fisher's $p < 0.01$) were also associated with ST-982 infections, while contact with birds, i.e., chickens (Fisher's $p < 0.05$) and female gender (χ^2 $p < 0.05$) were both associated with CC-21 ($n = 25$) infections. In addition, cases older than 50 years of age were more likely to have isolates

TABLE 1 | Description of cases included in the study.

Demographic data	No. of cases†	(%)‡	Epidemiologic data	No. of cases†	(%)‡
Sex			**Travel**		
Male	52	57.1%	No travel	46	67.6%
Female	39	42.9%	Domestic travel	13	19.1%
Age group (years)			Foreign travel	9	13.2%
≤2	21	22.3%	**Animal contact**		
3–23	25	26.6%	Reptile	0	0%
24–50	24	25.5%	Livestock	7	10.9%
>50	24	25.5%	Birds/poultry	6	9.4%
Race			Domestic	38	59.4%
Caucasian	60	75.0%	Others	5	7.8%
African American	9	11.3%	**Food consumption**		
Asian	1	1.2%	Ground meats	33	55.0%
Others	10	12.5%	Home prepared chickens	30	50.0%
Residence (county)			Frozen chickens	14	23.3%
Clinton	4	4.6%	Restaurant chickens	19	31.7%
Ingham	8	9.2%	Raw sprouts	4	6.7%
Livingston	6	6.9%	Raw milk	4	6.7%
Macomb	3	3.4%	**Water at home**		
Oakland	10	11.5%	Well	12	19.0%
Washtenaw	17	19.5%	Municipal	42	66.7%
Wayne	31	35.6%	Bottled	7	11.1%
Others	8	9.2%	Others	2	3.2%

† *Counts for sex, age group, race, travel history, and water source at home are mutually exclusive for each category; counts for animal contact and food consumption are repeated across categories as they are reported.* ‡*The percentages are based on the number of cases for which information was available. Total number of cases varies between variables due to the difference in missing information.*

TABLE 2 | Frequency (%) of antimicrobial resistance and minimum inhibitory concentration (MIC) of human *C. jejuni* isolates in Michigan.

CLSI Antimicrobial class	Antimicrobial agent	% Resistance ($n = 94$)	MIC (μg/mL)		
			Range	MIC_{50}	MIC_{90}
Fluoroquinolone	Ciprofloxacin	19.15% (18)	<0.015 ~ 32	0.12	8
Quinolone	Nalidixic acid	19.15% (18)	<4 ~ > 64	4	>64
Macrolide	Azithromycin	2.13% (2)	<0.015 ~ > 64	0.06	0.12
	Erythromycin	2.13% (2)	< 0.03 ~>64	0.5	1
Aminoglycoside	Gentamicin	0	< 0.12 ~ 1	0.5	1
Tetracycline	Tetracycline	61.7% (58)	< 0.06 ~ > 64	64	>64
Lincosamide	Clindamycin	2.13% (2)	< 0.03 ~ > 16	0.12	0.25
Ketolide	Telithromycin	2.13% (2)	<0.015 ~ > 8	1	2
Phenicol	Florfenicol	1.06% (1)	< 0.03 ~ 32	1	2

† *CLSI: Clinical and Laboratory Standards Institute.*

TABLE 3 | Univariate and multivariate analyses of factors associated with fluoroquinolone resistant *C. jejuni* infections among all cases ($n = 94$).

Characteristic	Univariate analysis			Multivariate analysis		
	OR	95% CI†	*p*	OR	95% CI†	*p*
Foreign travel	35.7	5.78–220.38	<0.0001	33.4	3.9–285.2	0.0013
Season (Winter)	3.27	0.92–11.58	0.056	8.1	0.9–72.7	0.0614
Age (years)*	–	–	–	1.05	0.99–1.1	0.0536
Sex (Female)	0.92	0.32–2.68	0.88	–	–	–
Domestic animal contact	0.37	0.10–1.33	0.19	0.26	0.041–1.659	0.1542
Home prepared chicken**	0.082	0.0095–0.71	0.0095	–	–	–

**Age (years) is a continuous variable; not proper for univariate analysis used (χ^2 or Fisher's exact test). **Consumption of home prepared chicken was a significant protective factor by univariate analysis; however, it was not used for multivariate analysis because of the small sample size left when the characteristic was included in the base model. †Wald Confidence interval*

belonging to CC-45 ($n = 9$) relative to all other CCs (Fisher's $p < 0.05$).

Among the 52 cases without any history of travel or travel only within Michigan, similar associations were observed. Notably, contact with birds, i.e., chickens, was associated with both ST-982 (Fisher's $p < 0.01$) and CC-21 (Fisher's $p < 0.05$) infections. Because the phylogenetic clusters were better defined in the isolate population recovered exclusively from Michigan cases (**Figure 3**), we also analyzed epidemiologic associations by clusters identified in the Neighbor joining phylogeny (**Figure 3**). Isolates belonging to Cluster V, which includes ST-982 and the 4 other STs, all representing CC-21, were significantly associated with contact with birds, i.e., chickens (Fisher's $p < 0.05$). Additionally, when compared to other clusters, the two cases reporting contact with cattle were included in Cluster V; however, the association was not significant (Fisher's $p = 0.0571$), which may be due to the small sample size.

It is also notable that the three isolates assigned to ST-464 had the same profile with resistance to ciprofloxacin, nalidixic acid and tetracycline (CipNalTet). A significant association was observed between ST-464 and resistance to ciprofloxacin and nalidixic acid (Fisher's $p < 0.01$), as well as CipNalTet (Fisher's $p < 0.01$). Similarly, the isolates belonging to ST-982 had a higher likelihood of having resistance to tetracycline when compared to all other lineages (Fisher's $p < 0.05$). No other significant associations were observed for other STs or CCs with specific resistance profiles.

DISCUSSION

Increasing frequencies of resistance to antimicrobials in *C. jejuni* is a growing public health concern worldwide. In the U.S., NARMS monitors resistance trends by testing *C. jejuni* isolates collected from 10 states across the country; however, Michigan is not included in this system. Hence, this represents the first study reporting the antimicrobial resistance profiles and genetic diversity of *C. jejuni* recovered from patients in Michigan collected via active surveillance. The high frequency of resistance to several important antimicrobials including the fluoroquinolones and macrolides, which are commonly used to treat human infections, is of concern as are the risk factors for resistant infections. Indeed, we found that a history of foreign travel is the most important risk factor for fluoroquinolone-resistant *C. jejuni* infections in Michigan, which is consistent with prior reports in other geographic locations in the U.S. (Smith et al., 1999; Kassenborg et al., 2004) and Europe (Engberg et al., 2001; Rautelin et al., 2003; Norström et al., 2006). A case-control study conducted using FoodNet surveillance sites in the U.S. during 1998–1999, for instance, demonstrated that history of foreign travel resulted in a higher likelihood of acquiring fluoroquinolone-resistant *Campylobacter* infections (OR = 7.6; 95% CI = 4.3-13.4) (Kassenborg et al., 2004). This same study also found that consumption of poultry prepared outside of the home was a risk factor for domestically acquired fluoroquinolone-resistant infections (OR = 10.0, 95% CI = 1.3–78). The latter result is similar to our finding that chicken prepared at home was protective and resulted in a decreased likelihood of having a fluoroquinolone-resistant infection. These data are important as they demonstrate that the major sources of fluoroquinolone-resistant *C. jejuni* infections are similar in different parts of the U.S. even though disease frequencies may vary. They also emphasize the importance of monitoring patients with foreign travel history and considering individual treatment schemes in order to more readily combat resistant infections.

Prior studies have suggested that a high frequency of travel-associated fluoroquinolone-resistant *Campylobacter* infections may be due to a high prevalence of fluoroquinolone resistance in the destination areas (Piddock, 1995; Smith et al., 1999). Recently, clonal spread of specific fluoroquinolone-resistant lineages, e.g., ST-464, have been reported in Europe (Cody et al., 2012; Kittl et al., 2013). According to the PubMLST database (Jolley and Maiden, 2010), ST-464 has been frequently reported in Europe and Asia, however, no prior reports have been noted in the U.S. All three isolates assigned to ST-464 had the same resistance profile (CipNalTet) and two of these isolates were from cases that had traveled to Cambodia and to Italy, Turkey, Greece, and France; travel status was missing for the remaining case. These data suggest the dissemination of a fluoroquinolone-resistant ST-464 lineage outside of Europe, warranting the need to enhance surveillance efforts and identify factors associated with global spread.

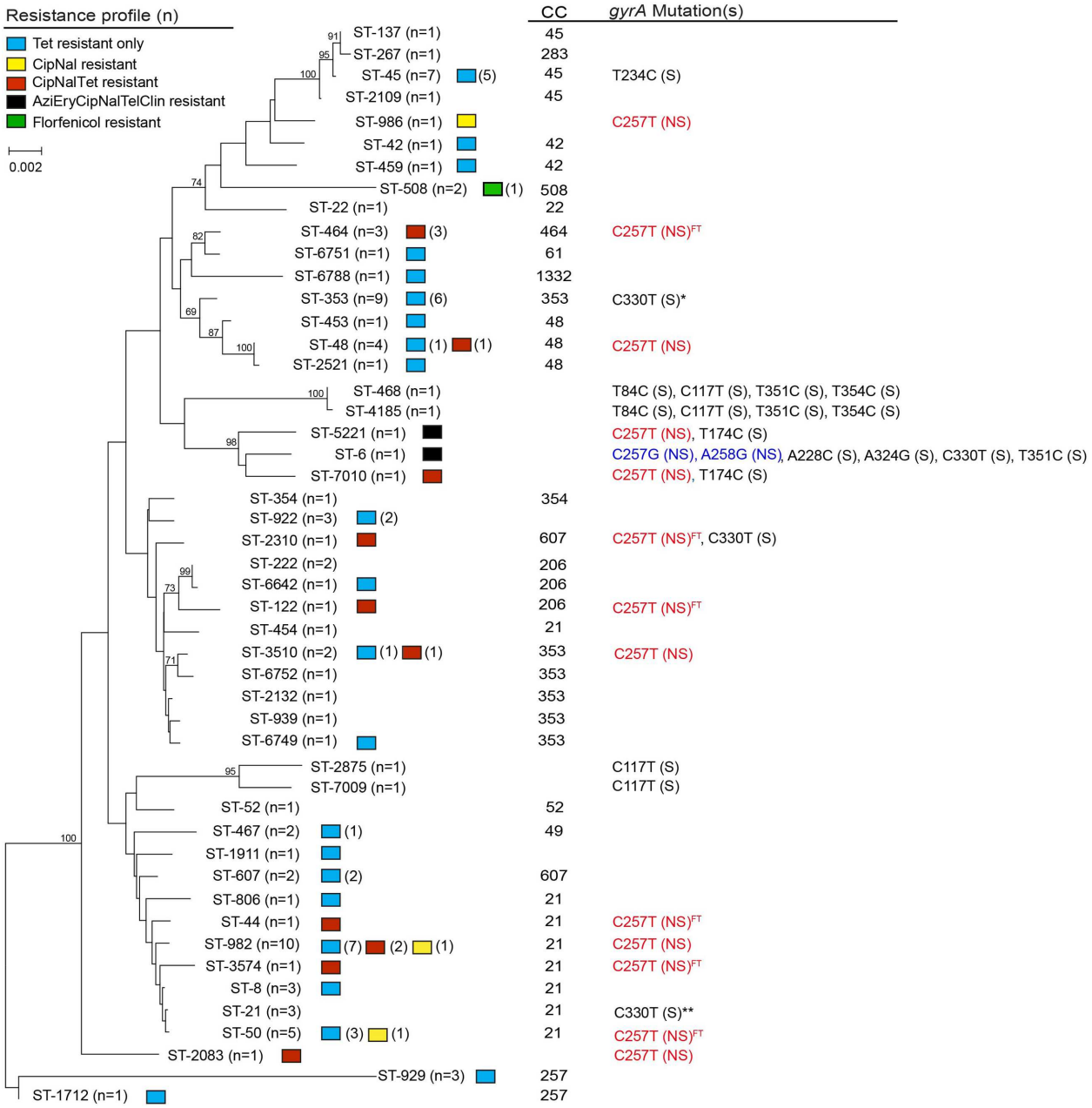

FIGURE 2 | Neighbor-joining phylogeny of sequence types (STs) by antimicrobial resistance patterns, clonal complexes (CCs), and type of *gyrA* mutation. The numbers at the end of each branch represent the STs found in this study, while the numbers at the nodes represent the bootstrap value from 1,000 replications. Non-synonymous mutation is noted by NS in parentheses; S in parentheses indicates synonymous mutation. Superscript FT indicates isolates recovered from a case with foreign travel history. *gyrA* mutations were determined using *C. jejuni* ATCC 33560 as the reference; location 243, 357, 360 were defined as CT variable regions. *6 of 9 isolates had a synonymous point mutation at C330T. **1 of 3 isolates had a synonymous point mutation at C330T.

From a total of 94 isolates, thirty six (38.3%) had at least one point mutation in *gyrA* compared to the ATCC 33560 reference strain that is susceptible to all antimicrobials tested (McDermott et al., 2004). Eighteen isolates had non-synonymous point mutations conferring resistance to fluoroquinolones; the amino acid changes, Thr-86-Ile and Thr-86-Arg, were observed in 17 isolates and 1 isolate, respectively. Although the Thr-86-Arg mutation was described as one of the mutations inducing high MIC values for fluoroquinolones in *Campylobacter* spp. (Rossi et al., 2015), this mutation is rarely reported. It is also notable that only this isolate with Thr-86-Arg had transversion mutations, C257G and A228C in *gyrA* (**Figure 2**). Interestingly, among the 17 isolates with a Thr-86-Ile mutation, 13 different STs were represented and five of these were recovered from patients with a

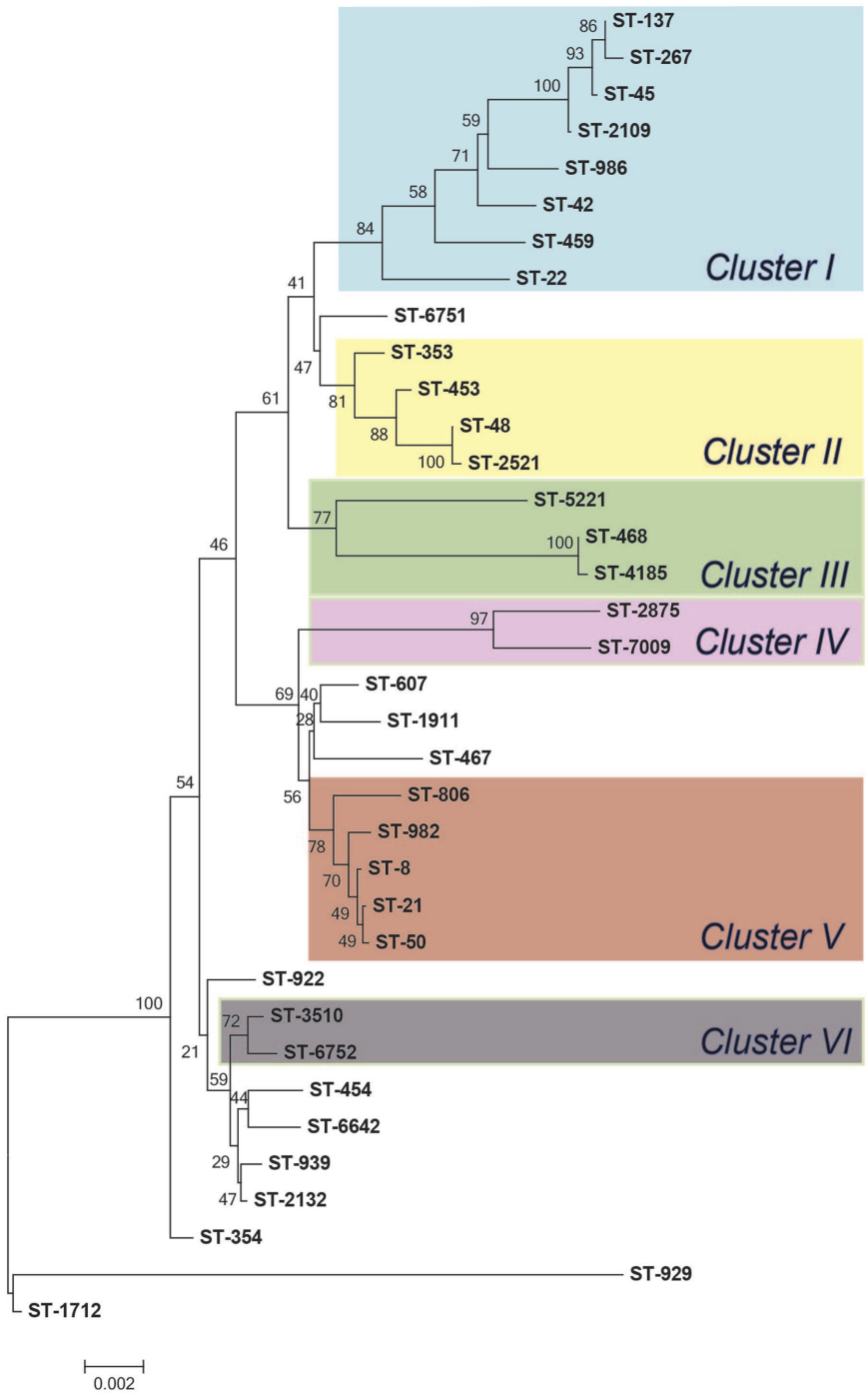

FIGURE 3 | Neighbor-joining phylogeny of sequence types (STs) from Michigan (*n* = 52). The numbers at the end of each branch represent the STs and numbers at the nodes are bootstrap values following 1,000 replications.

history of foreign travel to multiple geographic locations. These data demonstrate that fluoroquinolone resistance can develop via similar point mutations in *gyrA* among phylogenetically distinct *Campylobacter* lineages. No other pattern or association was observed in the type or frequency of synonymous point mutations in *gyrA* and specific resistance profiles or increased MICs. However, notable associations were observed between STs and the types of point mutations. For example, all seven isolates assigned to ST-45 had a T234C point mutation, while the two closely related lineages, ST-2875 and ST-7009, ST-468 and ST-4185, respectively, contained identical synonymous *gyrA* point mutations (**Figure 2**). Indeed, prior studies have suggested the

use of *gyrA* as a genetic marker to investigate the relatedness of *C. jejuni* strains (Ragimbeau et al., 2014). It is also important to note that not all mutations were restricted to closely related lineages. The C330T synonymous mutation in *gyrA*, for instance, was found in ST-353, ST-6, ST-2310, and ST-21, distinct lineages located in different branches of the phylogenetic tree.

Prior studies have detected variation in the level of resistance to macrolides based on the type of mutation in 23S rRNA as well. The A2074T mutation in 23S rRNA, for example, has been linked to low macrolide resistance levels previously (Vacher et al., 2005), while a recent study detected a high resistance level (MIC $>$ 512 μg/ml) in a *C. jejuni* strain from a human clinical case (Ohno et al., 2016). Similar to the latter finding, the two macrolide resistant isolates recovered from Michigan had the same A2074T 23S rRNA mutation with high resistance levels to both erythromycin and azithromycin (MIC $>$ 64 μg/ml). Notably, these isolates were multiple drug resistant (MDR) with resistance to four classes of antimicrobials including macrolides, a fluoroquinolone, a lincosamide (clindamycin) and a ketolide (telithromycin), suggesting that other resistance mechanisms could impact the overall level of resistance. One example is the *cmeABC* efflux pump, which has been linked to resistance to multiple antimicrobials, including fluoroquinolones, macrolides, and tetracycline. Both of the Michigan strains, however, were susceptible to tetracycline. It is important to note that these isolates, representing ST-5221 and ST-6, respectively, clustered together in the Neighbor-joining phylogeny with ST-7010 (**Figure 2**), all of which contained a high frequency of similar synonymous point mutations in 23S rRNA (Supplementary Table S2). This finding suggests that some *C. jejuni* lineages may be more susceptible to point mutations, which may lead to antimicrobial resistance. Further studies are required to determine whether specific synonymous mutations impact the mutability of some isolates resulting in greater mutation rates or enhanced fitness *in vivo*.

A significantly higher tetracycline resistance rate (61.7%) was observed in the study samples when compared to the 2012 NARMS report (47.8%) (χ^2 $p < 0.01$). All of the tetracycline-resistant *C. jejuni* isolates ($n = 58$) harbored *tet*(O), which was confirmed by both PCR and *in silico* analysis. Although we could not determine whether *tet*(O) was plasmid-derived in all of the isolates examined, the gene was found in multiple lineages throughout the phylogeny, suggesting that plasmid-mediated resistance and horizontal gene transfer across lineages is likely. In addition, a significant association was observed between ST-982 and tetracycline resistance as well as cattle contact. According to the PubMLST database, ST-982 has previously been reported from cattle ($n = 14$), cow's milk ($n = 4$), the farm environment ($n = 3$) and a lamb ($n = 1$) in the U.S., while it was linked to human clinical cases in Canada and the U.K. Another study conducted in the state of Washington (Davis et al., 2013), however, recovered ST-982 isolates from both humans and cattle. Taken together, these data suggest that this resistant genotype has the ability to cross species and may be more likely to acquire resistance to tetracycline. A significant association was also observed between ST-982 and contact with chickens and drinking well water at home, suggesting that this lineage may be readily adaptable to different hosts and environments. Further investigation of the genetic diversity and antimicrobial resistance profiles of *C. jejuni* recovered from reservoir hosts and the environment, however, is needed to better understand transmission dynamics and host specificity.

Recent studies in Europe show mounting evidence that clonal spread of specific fluoroquinolone-resistant *C. jejuni* lineages may be contributing to the increasing trend of fluoroquinolone resistance observed worldwide (Cody et al., 2012; Wimalarathna et al., 2013; Kovač et al., 2014). Our study affirms foreign travel as a major risk factor for fluoroquinolone-resistant infections in the U.S., and further suggests that specific resistant lineages, like ST-464, may be emerging in the country. With continuing globalization through imported food and foreign travel, unified diagnostic guidelines on resistance detection and global surveillance is warranted to track the emergence and spread of these resistant lineages so effective mitigation strategies can be implemented.

AUTHOR CONTRIBUTIONS

WC, JR, SM designed the study; WC, RM, SW, PS, SM generated data; WC, DN, HS, PL, WK, LM, JR, and SM analyzed the data; and WC, SM drafted the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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frontiers in Microbiology

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Characteristics of Quinolone Resistance in *Escherichia coli* Isolates from Humans, Animals, and the Environment in the Czech Republic

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Escherichia coli is a common commensal bacterial species of humans and animals that may become a troublesome pathogen causing serious diseases. The aim of this study was to characterize the quinolone resistance phenotypes and genotypes in *E. coli* isolates of different origin from one area of the Czech Republic. *E. coli* isolates were obtained from hospitalized patients and outpatients, chicken farms, retailed turkeys, rooks wintering in the area, and wastewaters. Susceptibility of the isolates grown on the MacConkey agar with ciprofloxacin (0.05 mg/L) to 23 antimicrobial agents was determined. The presence of plasmid-mediated quinolone resistance (PMQR) and ESBL genes was tested by PCR and sequencing. Specific mutations in *gyrA*, *gyrB*, *parC*, and *parE* were also examined. Multilocus sequence typing and pulsed-field gel electrophoresis were performed to assess the clonal relationship. In total, 1050 *E. coli* isolates were obtained, including 303 isolates from humans, 156 from chickens, 105 from turkeys, 114 from the rooks, and 372 from wastewater samples. PMQR genes were detected in 262 (25%) isolates. The highest occurrence was observed in isolates from retailed turkey (49% of the isolates were positive) and inpatients (32%). The *qnrS1* gene was the most common PMQR determinant identified in 146 (56%) followed by *aac(6′)-Ib-cr* in 77 (29%), *qnrB19* in 41 (16%), and *qnrB1* in 9 (3%) isolates. All isolates with high level of ciprofloxacin resistance (>32 mg/L) carried double or triple mutations in *gyrA* combined with single or double mutations in *parC*. The most frequently identified substitutions were Ser(83)Leu; Asp(87)Asn in GyrA, together with Ser(80)Ile, or Glu(84)Val in ParC. Majority of these isolates showed resistance to beta-lactams and multiresistance phenotype was found in 95% isolates. Forty-eight different sequence types among 144 isolates analyzed were found, including five major clones ST131 (26), ST355 (19), ST48 (13), ST95 (10), and

ST10 (5). No isolates sharing 100% relatedness and originating from different areas were identified. In conclusion, our study identified PMQR genes in *E. coli* isolates in all areas studied, including highly virulent multiresistant clones such as ST131 producing CTX-M-15 beta-lactamases.

Keywords: *Escherichia coli*, quinolone resistance, human, animals, wastewater, MLST, PFGE

INTRODUCTION

Escherichia coli is a common part of the gastrointestinal flora of humans and animals but some strains can cause serious diseases. Quinolones are the antimicrobial agents of choice for treatment of various infections caused by *E. coli* or other Gram-negative bacteria. Because of extensive use for multiple clinical indications in human or veterinary medicine, bacterial resistance to quinolones has developed over the time (Andriole, 2005). The World Health Organization indicated fluoroquinolones as critically important drugs for human medicine due to a strong correlation observed between the consumption and an increase in the resistance, and they recommended prudent use of fluoroquinolones both in human and veterinary medicine (World Health Organization, 2012).

According to the European Antimicrobial Resistance Surveillance Network (EARS-Net), an interactive database, an alarming emergence of fluoroquinolone-resistant invasive *E. coli* isolates is evident in European countries (European Centre for Disease Prevention and Control, 2015). Quinolone antibiotics are one of the most widely used antimicrobial agents in the treatment of urinary tract infections. Unfortunately, the extensive use has led to the increase of the rate of *E. coli* isolates resistant to fluoroquinolones all over the world (Lautenbach et al., 2004; Dalhoff, 2012). Resistance to nalidixic acid and ciprofloxacin was reported as high or extremely high in isolates from broilers in European countries (ECDC, EFSA, and EMA, 2015). *E. coli* isolates resistant to fluoroquinolones has been described in food-producing and companion animals (Platell et al., 2011; Gosling et al., 2012; Literak et al., 2013; Agabou et al., 2016) as well as in wild animals and the environment (Jiménez Gómez et al., 2004; Colomer-Lluch et al., 2013).

Target sites for quinolones in *E. coli* strains are the bacterial topoisomerases, namely DNA gyrase (topoisomerase II) as the primary site and topoisomerase IV as a secondary target. Both enzymes are essential for bacterial DNA replication. Mutations in specific domains of *gyrA*, *gyrB*, *parC*, and *parE* cause single aminoacid changes in either gyrase or topoisomerase IV that contribute to quinolone resistance. Multiple mutations in the quinolone-determining resistant regions (QRDR) of topoisomerase enzymes are usually associated with high-level of fluoroquinolone resistance in *E. coli* strains (Hopkins et al., 2005). Target-mediated resistance represents the most common and clinically most significant form (Ruiz, 2003; Aldred et al., 2014).

The first identified plasmid-mediated quinolone resistance (PMQR) gene was discovered in 1998 and termed *qnrA1* (Martínez-Martínez et al., 1998). So far, three families of plasmid-mediated mechanisms associated with quinolone resistance have been identified: i/Qnr proteins protecting target enzymes DNA gyrase and topoisomerase IV from quinolone inhibition ii/ aminoglycoside acetyltransferase Aac(6′)-Ib-cr acetylating several fluoroquinolones such as ciprofloxacin and norfloxacin and iii/ efflux pumps QepA and OqxAB removing antibiotics from bacterial cells. PMQR provide only a low level of quinolone resistance, not reaching the clinical breakpoints defined by the Clinical and Laboratory Standard Institute criteria (CLSI, 2015). However, PMQR genes may facilitate the selection of higher-level resistance in the presence of quinolones and lead to treatment failure (Strahilevitz et al., 2009; Jacoby et al., 2014).

The aims of this study were to determine the occurrence of PMQR in *E. coli* isolates from humans, food-producing animals, wild animals and wastewater samples from one defined area in the Czech Republic and to compare genetic characteristics of PMQR-positive *E. coli* isolates from various sources as well as to outline possible ways of their transmission between humans and animals.

MATERIAL AND METHODS

Sample Collection

Between May 2013 and December 2014, *E. coli* isolates were obtained from human clinical materials (urine, stool, sputum, blood, bile, endotracheal aspirate, samples from cannulas, and bronchoalveolar lavages) of patients hospitalized at the University Hospital Olomouc (UHO) in the Czech Republic and from urine samples from outpatients in the community of Olomouc Region. The community subjects had neither been hospitalized in the previous 3 months nor had been living in nursing homes.

In the same period (May 2013 and December 2014), *E. coli* isolates were obtained from environmental samples from chicken farms ($n = 2628$) and cloacal swabs from market-weight turkeys at slaughterhouses ($n = 120$) in the eastern part of the Czech Republic (Olomouc and South Moravian Region). Environmental samples were taken from bedding using gauze shoe covers worn by a worker who walked through a poultry house. Turkey cloacal swabs were collected at slaughterhouses and placed into Amies transport medium.

Wastewaters were taken during 2013–2015 in six sampling sites that included wastewater treatment plants (WWTP) from four hospitals located in three towns of the Olomouc Region (Olomouc, Prostejov and Sternberk) (WWTP1-4), the abattoir in Prerov (WWTP5) and WWTP near Henčlov (WWTP6). In total, 124 wastewater samples were examined including 21 samples from WWTP1 (in 2013-6 samples; 2014-13; 2015-2), 25 from WWTP2 (2014-20, 2015-5), 19 from WWTP3 (in 2013-6;

2014-11, 2015-2), 19 from WWTP4 (in 2013-6; 2014-11, 2015-2), 21 from WWTP5 (collected weekly for a period of 4 months in 2013/2014, plus 3 control samplings were made in May and August 2014) and 19 from WWTP6 (collected weekly for a period of 4 months in 2013/2014). Cellulose swabs were immersed into wastewater at the inflow and/or outflow of sampling points for 48 h according to the procedure described (Moore et al., 1952).

Faecal rook (*Corvus frugilegus*) samples ($n = 595$) were collected from roosting places used by rook flocks in the Olomouc Region. The samples were collected in Prerov in October, November and December 2012, in Tovacov from January till March 2013, and in Troubky in November 2013. The sampling method has been described previously (Literak et al., 2012). Smears from fresh feces were taken by cotton swabs tampons and inserted into Amies transport medium.

Selective Cultivation of *E. coli* and Detection of PMQR Genes

After delivery to the laboratory, collected samples were handled according to the following procedures in order to isolate *E. coli*.

Samples from the environment of chicken farms were placed into peptone water and incubated aerobically for 24 h at 37°C. Subsequently, the peptone water was inoculated onto MacConkey agar with ciprofloxacin (0.05 mg/L; MCA_{CIP}) and cultivated overnight. Swabs from retailed turkeys were subcultivated directly on MCA_{CIP} overnight. One isolate per sample was selected for further phenotypic and genetic analysis.

Swabs taken from wastewaters were inserted into a sterile bottle with peptone water and incubated at 37°C for 24 h. The enriched samples of peptone water were subcultivated on MCA_{CIP} overnight. One to ten colonies of lactose-positive colonies showing different morphology were taken from each MCA_{CIP} and subjected to further examination.

Faecal swabs from rooks were incubated in buffered peptone water at 37°C overnight and subsequently cultivated on MCA_{CIP} overnight. In this type of sample, also only one isolate per sample was selected.

Human clinical material was handled according to the types of material and cultivated aerobically for 24 h at 37°C. A total number of 3521 *E. coli* isolates were collected and from these, three hundred and three isolates were randomly selected for detection of PMQR genes. The collection analyzed included 270 resistant (MIC of ciprofloxacin >0.5 mg/L) and 33 sensitive (MIC of ciprofloxacin ≤0.5 mg/L) isolates according to the European Committee in Antimicrobial Susceptibility Testing (EUCAST) criteria.

The species identification of all isolates was confirmed using matrix-assisted laser desorption/ionization-time of flight mass spectrometer (MALDI-TOF MS) (Biotyper Microflex, Bruker Daltonik GmbH, Bremen, Germany).

Genomic DNA of all *E. coli* isolates obtained using heat lysis was used as a template for PCR detection of PMQR genes (*aac(6′)-Ib-cr*, *qepA*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *oqxAB*), followed by sequencing of the amplicons. As positive controls, well-known characterized strains were included in each reaction (Table S1).

Antimicrobial Susceptibility Testing of PMQR-Positive Isolates

For each PMQR-positive *E. coli* isolate, susceptibilities to ampicillin (the ranges of tested concentrations for the antimicrobial substance were 0.5–64 mg/L, ampicillin/sulbactam (0.5–64), cefazolin (0.5–64), cefuroxime (0.5–64), gentamicin (0.25–32 or 0.5–64), trimethoprim/sulfamethoxazole (1–128), colistin (0.25–32), oxolinic acid (0.25–32 or 0.5–64), ofloxacin (0.125–16 or 0.25–32), tetracycline (0.25–32), aztreonam (1–64), piperacillin (2–256), piperacillin/tazobactam (2–256), cefoperazone (0.25–32), cefotaxime (0.125–16), ceftazidime (0.125–16), cefepime (0.125–16), cefoperazone/sulbactam (0.5–64), meropenem (0.125–16), ciprofloxacin (0.125–16 or 0.25–32), tigecycline (0.06–8), tobramycin (0.25–32), and amikacin (0.5–32) were tested using the standard microdilution method according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing) breakpoint criteria (European Centre for Disease Prevention and Control, 2015). Microdilution antibiotic panels were prepared using dispensing instrument DYNAMIC 3000 automated system (DYNEX, Czech Republic). *E. coli* ATCC 25922 and *E. coli* ATCC 35218 were used as reference strains for quality control.

Detection of ESBL Genes in PMQR-Positive Isolates

All PMQR-positive isolates of *E. coli* with the minimum inhibitory concentration (MIC) of the tested 3rd and 4th generation cephalosporins ≥1 mg/L were screened for extended-spectrum beta-lactamase production (ESBL) using Jarlier's double-disk synergy test (DDST) which was modified by including a disk with cefepime and another disk with ceftazidime and ceftazidime/clavulanic acid (Jarlier et al., 1988; Htoutou Sedlakova et al., 2011). Each ESBL-producing isolate was screened for $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV} genes by PCR followed by sequencing of amplicons of genes responsible for ESBL phenotype (Table S1).

Detection of Mutations in the Topoisomerase Genes and MLST Analysis of PMQR-Positive Isolates

From a group of 262 PMQR-positive *E. coli* isolates, 144 isolates from all of the studied areas were selected for further characterization that included the detection of specific mutations in *gyrA*, *gyrB*, *parC* and *parE* genes and multilocus sequence typing (MLST). Isolates were selected randomly in order to cover all the studied areas.

Total genomic DNA from *E. coli* strains was prepared from an overnight culture (16 h, 37°C) grown on meat-peptone agar using DNeasy Blood & Tissue kit (QIAGEN, Germany) according to the manufacturer's recommendations.

PCR amplification of the part of *gyrA*, *gyrB*, *parC* and *parE* genes that included the sequence of QRDRs was performed using previously described primers (Oram and Fisher, 1991; Vila et al., 1994, 1996; Ruiz et al., 1997). The reaction mixture contained complete reaction buffer with $MgCl_2$ (containing 100 mmol/L Tris-HCl (pH 8.8), 500 mmol/L KCl, 1% Triton X-100,

15 mmol/L $MgCl_2$ (Top-Bio, Czech Republic), 0.5 U of *Taq* DNA polymerase (Top-Bio), 0.4 μmol/L primer concentration for each primer, 40 μmol/L concentration of deoxynucleoside triphosphates and 1 μL of template DNA. The PCR was run in the Light Cycler96 instrument (Roche, USA) under the following conditions: initial denaturation at 95°C for min, 30 cycles of denaturation (95°C for 30 s), annealing (58°C for 30 s), extension (72°C for 60 s), and final extension at 72°C for 7 min.

MLST of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, *recA*) was performed according to the MLST protocol standardized for *E. coli* (http://mlst.warwick.ac.uk/mlst/).

For each of the 144 samples, 11 amplicons (4 topoisomerase and 7 MLST amplicons) were pooled in an equimolar ratio to generate one single sample and sent to the laboratories of the Institute of Applied Biotechnologies (Prague, Czech Republic) for sequencing. DNA libraries were constructed using the Nextera XT DNA Sample Preparation Kit (Illumina, Inc., San Diego, CA) and two multiplex sequencing assays (1 × 96 samples, 1 × 48 samples) were performed on the Illumina MiSeq platform (Illumina, Inc. San Diego, CA).

NGS Data Analysis

A quality control check of pair-end FASTQ files was performed as a first step. Bases with Phred Quality Score lower than defined threshold (threshold = 30) were filtered out. Quality control included check for possible adapter contamination. Then all variants of reference sequences of single housekeeping genes of *E. coli* (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, *recA*) in FASTA file were downloaded (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) and indexed by Burrows-Wheeler Aligner (BWA version 0.7.13; Li and Durbin, 2010). Paired-end FASTQ files were aligned against all indexed reference FASTA files using BWA-mem algorithm with minimum seed length = 19, matching score = 1, mismatch penalty = 4, gap open penalty = 6, and gap extension penalty = 1. Generated Sequence Alignment Map (SAM format) was converted into its binary form (BAM format) and sorted by coordinates. Unmapped sequences reads were filtered out from BAM files. Algorithms Samtools (samtools version 0.1.18) and VarScan (Koboldt et al., 2009) were applied for variant calling using the following parameters: minimum coverage = 10, minimum mapping quality = 10, minimum reads = 2, minimum variant frequency = 0.05, and $p = 0.05$ were used for variant calling. Generated Variant Calling Format (VCF format) was statistically analyzed and a minimum number of variants (required 0, this number means that our FASTQ files have 100% match with a variant of the reference gene) was evaluated like a gene form of selected housekeeping gene of *E. coli*. All bioinformatics analyses were performed on Operating System Linux (Ubuntu 14.04 LTS) and using programming language Python.

Single point mutations in selected part of *gyrA*, *gyrB*, *parC* and *parE* including QRDRs were evaluated using the Integrative Genomics Viewer IGV version 2.3. (Broad Institute, Cambrige, UK) (https://www.broadinstitute.org/software/igv). The genome sequence *E. coli* MG1655 (NC_000913.2) was selected as a reference sequence for alignment.

Sanger sequencing was performed with 11 samples to confirm the results of MLST analysis carried out by NGS. This was done because some problems occurred in the assignment of the appropriate allele in MLST analysis. Gene sequences were subsequently submitted to the *E. coli* MLST Database, University of Warwick (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/). Obtained MLST allele sequences were compared with the results generated by NGS.

Pulsed-Field Gel Electrophoresis

E. coli isolates belonging to the same sequence type (ST) according to MLST analysis and originating from different sources (human, animal or wastewater) were subjected to examination of their epidemiological relationship using pulsed-field gel electrophoresis (PFGE). The total genomic DNA was isolated from overnight bacterial culture according to the procedure published (Husickova et al., 2012). All samples were digested with *Xba*I (30 U for 3 h) (Takara, Bio, Otsu, Shiga, Japan) and subjected to PFGE. The resulting restriction profiles were analyzed with the GelCompar II software (Applied Maths, Kortrijk, Belgium) using the Dice coefficient (1,5%) for comparing similarity and unweighted pair group method using arithmetic averages for cluster analysis. The results were interpreted according to criteria described by Tenover et al. (1995).

RESULTS

PMQR Genes in *E. coli* Isolates with Reduced Susceptibility or Resistance to Ciprofloxacin

A collection of 1050 isolates of *E. coli* with reduced susceptibility or resistance to ciprofloxacin was obtained from humans, food producing, or wild animals and wastewater samples from the area of the Olomouc and South Moravian Regions, Czech Republic (**Table 1**). The highest occurrence of *E. coli* strains isolated from MCA_{CIP} of animal originwas detected from turkey samples (105 isolates per 120 samples), the lowest from chicken samples (156 isolates per 2628 samples). In human population, 303 *E. coli* isolates were selected according to the procedure described in material and methods section. From the total amount of 124 wastewater samples, 372 *E. coli* isolates were analyzed.

PMQR genes were identified in 262 (25%) *E. coli* isolates (**Table 1**). The gene *qnrS1* was the most prevalent, found in 56% of PMQR-positive isolates from all studied areas. It was identified in 76% PMQR-positive isolates from poultry farms, turkey, rooks and wastewater. The second most common gene was *aac(6′)-Ib-cr* found in 82% of human PMQR-positive clinical isolates followed by *qnrB19* that dominated in isolates from turkey and wastewater samples. The *qnrA, qnrC, and qepA* genes were not found.

Twenty-three isolates carried two different PMQR genes of the following combination: *qnrB19*+*qnrS1* (12 isolates), *qnrB1*+*aac(6′)-Ib-cr* (7), *qnrB4*+*aac(6′)-Ib-cr* (2), *qnrS1*+*oqxAB* (1) *qnrB10*+*qnrS1* (1).

TABLE 1 | Distribution and characterization of PMQR-positive isolates of *E. coli* from different areas of collection.

Origin	No. of collected samples	No. of collected isolates	No. of PMQR-positive isolates	*qnrB1*	*qnrB4*	*qnrB8*	*qnrB10*	*qnrB19*	*qnrD1*	*qnrS1*	*qnrS2*	*aac(6′)-Ib-cr*	*oqxAB*	No. of isolates subjected to NGS
Hospital	18954	220	70 (31.8%)	7	1	–	–	2	–	6	–	60	1	33
Community	nd	83	14 (16.9%)	1	1	–	–	–	–	4	–	9	–	9
Chicken	2628	156	17 (10.9%)	–	–	1	–	4	–	12	–	–	1	10
Turkey	120	105	51 (48.6%)	–	–	–	–	16	–	46	–	–	–	50
Rook	595	114	20 (17.5%)	–	–	–	–	5	2	9	–	4	–	11
Wastewater	124	372	90 (24.2%)	1	–	–	1	14	–	69	4	4	–	31
Total		1050	262 (25.0%)	9	2	1	1	41	2	146	4	77	2	144

nd, data for community samples not available.

From a total number of 124 wastewater samples 26 contained at least one PMQR-positive isolate of E. coli.

In case of wastewaters where more than one *E. coli* colony per sample was examined, the occurrence of several *E. coli* isolates carrying different PMQR genes and originating the same water sample was detected. Six wastewater samples contained *E. coli* isolates carrying *qnrS1* together with *E. coli* carrying *qnrB19* were detected. In one sample *E. coli* harboring *qnrB1* and *E. coli* with *qnrS1* were found. The last sample contained two different types of *E. coli*, one carrying *aac(6′)-Ib-cr* and the other with *qnrS1*.

Antimicrobial Susceptibility of PMQR-Positive Isolates

For each PMQR-positive *E. coli* isolate, susceptibility to 23 antimicrobial agents was tested (Table S2). Isolates from hospitalized patients showed the highest level of resistance including ampicillin (97%), piperacillin (96%), third generation cephalosporins (63% for cefotaxime and 43% for ceftazidime), tetracycline (83%), and tobramycin (86%). Isolates from the community displayed high level of resistance to ampicillin (93%), tetracycline (93%), piperacillin (93%), tobramycin (64%), and resistance to third generation cephalosporins (50.0% for cefotaxime and 43% for ceftazidime). On the other hand, wastewater isolates were less resistant to tested antibiotics. High level of resistance was detected only for ampicillin (79%), tetracycline (83%) and piperacillin (78%).

Resistance to ampicillin was common also among isolates from retailed turkeys (96%), chicken farms (71%) and rook feces (75%). Other frequent resistance phenotypes included tetracycline and piperacillin found in 77 and 59% of chicken, in 86 and 90% from turkey and in 75 and 70% from rook isolates, respectively. The majority of *E. coli* isolates from food-producing animals were susceptible to third generation cephalosporins while 20% of rook isolates showed resistance. Most isolates from the inpatients and the community showed resistance to ciprofloxacin. A total of 270 isolates were resistant (MIC of ciprofloxacin >0.5 mg/L) and 33 sensitive (MIC of ciprofloxacin ≤0.5 mg/L) according to the EUCAST criteria. However, this results is influenced by different selection criteria for human isolates since isolates with MIC of ciprofloxacin >0.5 mg/L were preferably included in the study. In contrast, only 50 of all PMQR-positive isolates from other studied areas showed resistance to ciprofloxacin with the highest level observed in isolates from chicken farms (41%). A total of 36% of animal *E. coli* isolates (chicken, turkey, and rook) were resistant to oxolinic acid, 32% to ofloxacin and 28% to ciprofloxacin. In the group of wastewater samples, 28% of isolates displayed resistance to oxolinic acid as well as to ciprofloxacin, and 34% to ofloxacin. The distribution of MIC values of tested quinolones in selected isolates ($n = 144$) and the comparison with the results of genetic detection is summarized in the **Table 2**.

A total number of 66 isolates was screened for ESBLs using phenotypic and genetic methods. Production of ESBL enzymes was found in 61 *E. coli* isolates. Subsequent genetic analysis revealed the presence of $bla_{\text{CTX-M-15}}$ in 51 isolates (hospital, community, wastewater). Two isolates from chicken and wastewater carried the gene $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$ was found in two isolates from hospital and wastewater and $bla_{\text{SHV-12}}$

TABLE 2 | Distribution of substitutions in topoisomerase subunits, PMQR determinants and MIC of tested quinolones in *E. coli* isolates.

Substitutionsin topoisomerase subunits				PMQR	No. of isolates	MIC range (mg/L)			Origin of isolates
GyrA	GyrB	ParC	ParE			OXO	OFL	CIP	
Ser(83)Leu	–	–	–	*qnrB1*	2	16–>32	0.5–2	0.5–2	Hospital, community
Ser(83)Leu	–	–	–	*qnrB19*	3	64	2	0.5	Wastewater
Ser(83)Leu	–	–	–	*qnrS1*	4	16–64	0.25–8	<0.125–4	Wastewater, hospital, chicken
Ser(83)Leu	–	Ser(80)Arg	Ser(458)Pro	*qnrS1*	1	>32	16	16	Chicken
Ser(83)Leu; Asp(87)Asn	–	Ser(80)Arg	Ser(458)Pro	*qnrS1*	1	>64	16	>16	Turkey
Ser(83)Leu; Asp(87)Asn	–	Ser(80)Ile	Ser(458)Ala	*aac(6′)-Ib-cr*	3	>64	>16	>16	Rook
Ser(83)Leu; Asp(87)Asn	–	Ser(80)Ile	–	*qnrS1*	1	>128	2	8	Chicken
Ser(83)Leu; Asp(87)Asn	–	Ser(80)Ile*	Leu(445)His	*qnrS1*	1	>32	>16	>32	Hospital
Ser(83)Leu; Asp(87)Asn	–	Ser(80)Ile*	Ser(458)Ala	*aac(6′)-Ib-cr*	6	>32–>64	16–32	>16–>32	Hospital, community
Ser(83)Leu; Asp(87)Asn	–	Ser(80)Ile*	Ser(458)Ala	*qnrB1*	2	>64	>16	>32	Hospital
Ser(83)Leu; Asp(87)Asn	–	Ser(80)Ile*	Ser(458)Ala	*qnrS1*	1	>16	>32	>32	Hospital
Ser(83)Leu; Asp(87)Asn	–	Ser(80)Ile*	–	*qnrB19, qnrS1*	3	>64	8–32	8–>16	Chicken
Ser(83)Leu; Asp(87)Asn	–	Ser(80)Ile*	–	*qnrS1*	4	>32–>64	4–16	8–32	Community, chicken, turkey
Ser(83)Leu; Asp(87)Asn	–	Ser(80)Ile*; Glu(84)Val	Ser(458)Ala	*aac(6′)-Ib-cr*	1	>32	>32	>32	Hospital
Ser(83)Leu; Asp(87)Asn	–	Ser(80)Ile*; Ser(57)Thr	Leu(57)Phe	*qnrB1*	1	>32	>16	32	Hospital
Ser(83)Leu; Asp(87)Asn	–	Ser(80)Ile; Glu(84)Val	–	*aac(6′)-Ib-cr*	21	>32–>64	8–16	16–>32	Hospital, community, wastewater
Ser(83)Leu; Asp(87)Asn	–	Ser(80)Ile; Glu(84)Val	–	*qnrB1*	1	>32	16	>32	Hospital
Ser(83)Leu; Asp(87)Asn	–	–	–	*qnrS1*	1	>32	8	16	rook
Ser(83)Leu; Asp(87)Gly	–	Ser(80)Arg	–	*qnrS1*	1	>32	4	8	Chicken
Asp(87)Asn	–	–	–	*qnrS1*	1	16	2	2	Chicken
Ser(83)Leu; Ala(84)Val; Asp(87)Asn	–	Ser(80)Ile; Glu(84)Val	–	*aac(6′)-Ib-cr*	1	>32	8	32	Hospital
–	–	–	Arg(378)His	*qnrS1*	1	4	0,5	0.5	Chicken
–	–	–	Asp(475)Glu	*qnrB19, qnrS1*	1	8	0,5	0.5	Turkey
–	–	–	Asp(475)Glu	*qnrS1*	17	8–>32	<0.25–4	0.25–8	Turkey
–	–	–	–	*qnrB1*	2	<0.5–4	<0.125–0,25	<0.125–0.25	Hospital
–	–	–	–	*qnrB1, aac(6′)-Ib-cr*	1	2	0.5	0.5	Hospital
–	–	–	–	*qnrB19*	14	4–32	0.25–1	<0.125–0.5	Chicken, hospital, wastewater, rook
–	–	–	–	*qnrB19, qnrS1*	7	4–8	0.25–0.5	0.25–0.5	Turkey
–	–	–	–	*qnrD1*	1	8	0.5	0.125	Rook
–	–	–	–	*qnrS1*	39	1–32	0.25–4	0.125–4	wastewater, hospital, community, rook, chicken, Turkey
–	–	–	–	*qnrS1, qnrB10*	1	16	4	2	Wastewater

PMQR, plasmid-mediated quinolone resistance; OXO, oxolinic acid; OFL, ofloxacin; CIP, ciprofloxacin; MIC, minimum inhibitory concentration; nucleotide change ATT (marked as Ile) and ATC (marked as Ile).*

was detected in three isolates from wastewater. Three isolates showing ESBL phenotype were negative for all tested *bla* genes.

Mutations in the Topoisomerase Genes in PMQR-Positive Isolates

Various mutations in *gyrA*, *parC* or *parE* genes were found in 60 out of 144 PMQR-positive isolates while no isolate with mutation in *gyrB* was observed (**Table 2**).

The most common GyrA substitution was Ser(83)Leu found in 59 isolates (41%), followed by Asp(87)Asn, detected in 49 isolates (34%). A total of 49 isolates carried double or triple substitution in GyrA.

Two aminoacid substitutions in ParC were found in 25 isolates (17%) with Ser(80)Ile and Glu(84)Val being the most common combination ($n = 23$). The most common substitution in ParE was Asp(475)Glu found in 18 isolates (13%).

All the *E. coli* isolates with a high level of ciprofloxacin resistance (>32 mg/L) were shown to carry double mutations in *gyrA* in combination with single or double mutations in *parC*. Substitutions at codon 83 and 87 in GyrA along with substitutions at codon 80 and 84 in *parC* gene were the most common. One isolate carried three mutations in *gyrA* with aminoacid changes at positions 83, 84, and 87. The majority of high-level ciprofloxacin resistant isolates were obtained from hospitalized patients and possessed the gene *aac(6′)-Ib-cr*. Other isolates with high level ciprofloxacin resistance originated from community and WWTP3 also carried *aac(6′)-Ib-cr*.

In most isolates susceptible to ciprofloxacin according to the EUCAST criteria (≤0.5 mg/L), no mutations in *gyrA*, *gyrB*, or *parC* were detected. However, five isolates possessed single aminoacid substitution at the codon 83 in GyrA (MIC of ciprofloxacin was 0.5 mg/L). All isolates were susceptible to almost all tested antimicrobial agents except ofloxacin (the MIC was 16–64 mg/L) and three of them were resistant to ofloxacin (MIC = 2 mg/L). Substitutions at position 378 [Arg(378)His)] or 475 [Asp(475) Glu] in ParE were found in 18 isolates susceptible to ciprofloxacin (MIC of ciprofloxacin was 0.2 or 0.5 mg/L). These strains displayed high level of resistance to ampicillin and piperacillin.

Clonal Relationship of PMQR-Positive Isolates

In the group of 144 isolates, analyzed by MLST, 48 different STs were identified (Table S3) including five major clones ST131 ($n = 26$), ST355 (19), ST48 (13), ST95 (10), and ST10 (5). Isolates of ST131 were obtained from various types of human clinical materials and from one wastewater sample, while the second most common clone ST355 carrying the *qnrS1* or the combination of *qnrS1*+*qnrB19* genes was found predominantly from retailed turkey. MLST analysis also showed the presence of identical STs in samples of different origin. Isolates of ST88 or ST226 were identified from chicken farms as well as from retailed turkeys. ST10, ST48, and ST95 were found in the poultry and wildlife (chicken farms, retailed turkey, and rooks) and in wastewater samples. Other sequence types identified in samples of different origin included ST428, ST533, and ST617. The determination of relevant ST failed in four samples. In these samples bioinformatics analysis did not assign appropriate allele variant and Sanger sequencing did not provide relevant sequences, suggesting the isolates belonged to atypical *E. coli* isolates untypable by MLST.

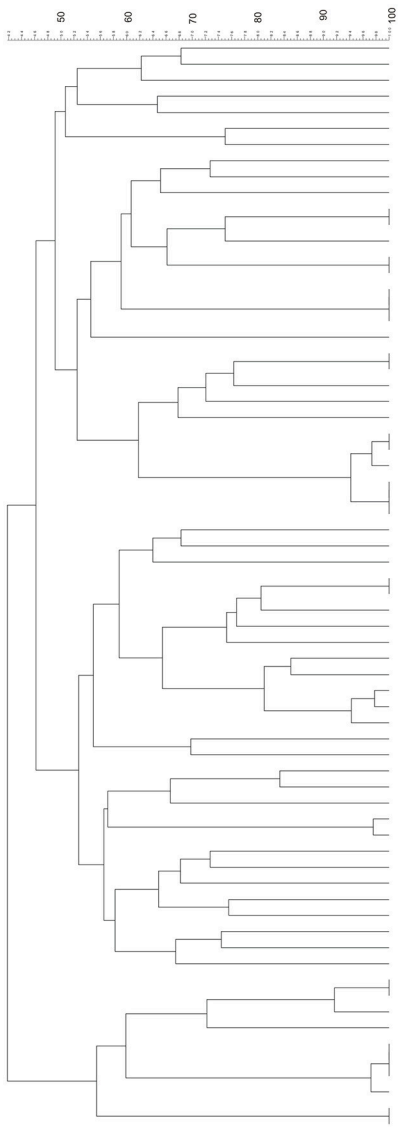
ST131 was most often associated with $bla_{\text{CTX-M-15}}$ and *aac(6′)-Ib-cr*, however one strain harbored the combination of $bla_{\text{CTX-M-15}}$ a *qnrB1*. The production of CTX-M-15 enzyme was also detected together with *qnrB1* in one ST393 and two ST410 strains. In three ST405 and one ST617 a combination of $bla_{\text{CTX-M-15}}$ and *aac(6′)-Ib-cr* was identified. One ST48 *E. coli* isolate harbored $bla_{\text{CTX-M-15}}$, together with *qnrB1* and *aac(6′)-Ib-cr*. In one *qnrS1*-postitive ST43 strain, $bla_{\text{CTX-M-1}}$ gene was detected. Two *qnrS1*-positive and SHV-12 producing strains, one belonging to ST540 and the other to ST58 were identified. The other CTX-M-14 or CTX-M-15 producing strains were not analyzed using MLST.

E. coli isolates belonging to the same ST and originating from different sources (human, animal or environmental) were subjected to PFGE to determine the level of their genetic relatedness (**Figure 1**). Based on these criteria a total of 68 isolates of the following STs were analyzed: ST10 ($n = 5$), ST48 ($n = 13$), ST58 ($n = 2$), ST88 ($n = 3$), ST95 ($n = 10$), ST131 ($n = 26$), ST226 ($n = 2$), ST428 ($n = 2$), ST533 ($n = 2$), and ST617 ($n = 3$). Overall, a high diversity of restriction patterns was observed in isolates of the same ST. No isolates with 100% identity of PFGE profiles originating from different areas (human, animal, or environment) were identified.

Globally disseminated ST131 was predominant in samples from hospitalized patients as well as from community subjects while no isolates of this clonal group were found in food-producing animals or rooks. The majority (69%) of *E. coli* ST131 isolates collected from these samples displayed high-level of ciprofloxacin resistance (>32 mg/L), harbored double or triple mutations in *gyrA* along with double mutations in *parC* gene and the PMQR gene aac*(6′)-Ib-cr*. The overall similarity of PFGE patterns of ST131 isolates was <50% confirming high genetic diversity of this clonal group. Only two *E. coli* ST131 isolates originating from the same patient from which the samples were taken 17 days apart shared 100% identity.

Significant clonal similarity was detected only in isolates originating from single collection areas, including ST10, ST48, ST88, and ST95 from retailed turkeys. In the ST10 group two isolates shared 100% similarity of their PFGE profiles. Both isolates were susceptible to ciprofloxacin, carried *qnrS1,* and did not contain gyrase or topoisomerase mutations. A group of six closely related strains was detected in ST48 group. All of them were collected from turkeys, displayed susceptibility or a low level of resistance to ciprofloxacin (MIC 1 - 2 mg/L), harbored *qnrS1* and were originated from the same turkey farm. Another three ST48 isolates from WWTP5 displayed 100% identity of their PFGE patterns, originated from wastewater samples obtained from the same collection date and shared only 52% similarity of the PFGE patterns with ST48 isolates from turkey.

In the ST95 group, two and three strains from turkeys showed identical PFGE profiles. All isolates of the ST95 group were susceptible to ciprofloxacin, did not possess any mutations in gyrase or topoisomerase genes and most of them carried both *qnrB19* and *qnrS1* genes.



ST	Origin	Type of sample	PMQR gene	Gyrase and topoisomerase substitutions	MIC of ciprofloxacin(mg/L) }	ESBL
ST95	turkey	cloacal swab	*qnrB19, qnrS1*	-	0.2	
ST95	WWTP5	wastewater sample	*qnrS1*	-	0.2	
ST88	chicken	environmental sample	*qnrS1*	*gyr*A[D(87)N]	1	
ST10	WWTP6	wastewater sample	*qnrS1*	-	0.5	
ST131	hospital	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I;E(84)V]	>16	
ST58	WWTP5	wastewater sample	*qnrS1*	-	0.5	SHV-12
ST58	WWTP6	wastewater sample	*qnrS1*	-	4	
ST10	rook	faecal sample	*qnrS1*	-	0.2	
ST533	chicken	environmental sample	*qnrS1*	*gyr*A[S(83)L;D(87)N]*par*C[S(8o)I*]	8	
ST48	chicken	environmental sample	*qnrS1*	-	0.2	
ST10	turkey	cloacal swab	*qnrS1*	-	0.2	
ST10	turkey	cloacal swab	*qnrS1*	-	0.2	
ST48	WWTP1	wastewater sample	*qnrB1, aac(6´)-Ib-cr*	-	0.5	CTX-M-15
ST88	turkey	cloacal swab	*qnrS1*	-	0.2	
ST88	turkey	cloacal swab	*qnrS1*	-	0.2	
ST48	WWTP5	wastewater sample	*qnrB19*	-	0.2	
ST48	WWTP5	wastewater sample	*qnrB19*	-	0.2	
ST48	WWTP5	wastewater sample	*qnrB19*	-	0.2	
ST10	chicken	environmental sample	*qnrS1*	*gyr*A[S(83)L]	<0.1	
ST48	turkey	cloacal swab	*qnrB19*	-	<0.1	
ST48	turkey	cloacal swab	*qnrB19*	-	<0.1	
ST617	WWTP6	wastewater sample	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I*] *parE*[S(458)A]	>8	
ST617	hospital	wound swab	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I*;E(84)V] *parE*[S(458)A]	>32	
ST617	hospital	wound swab	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I*] *parE*[S(458)A]	>16	CTX-M-15
ST48	turkey	cloacal swab	*qnrS1*	-	0.5	
ST48	turkey	cloacal swab	*qnrS1*	-	0.5	
ST48	turkey	cloacal swab	*qnrS1*	-	2	
ST48	turkey	cloacal swab	*qnrS1*	-	0.2	
ST48	turkey	cloacal swab	*qnrS1*	-	1	
ST48	turkey	cloacal swab	*qnrS1*	-	0.5	
ST131	community	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	CTX-M-15
ST533	rook	faecal sample	*qnrS1*	*gyr*A[S(83)L;D(87)N]	16	
ST131	hospital	throat swab	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32 C	CTX-M-15
ST131	community	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	
ST131	community	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	
ST131	hospital	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>16	CTX-M-15
ST131	hospital	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>16	CTX-M-15
ST131	hospital	bile	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	CTX-M-15
ST131	hospital	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	CTX-M-15
ST131	WWTP3	wastewater sample	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	CTX-M-15
ST131	hospital	wound swab	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	CTX-M-15
ST131	hospital	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	CTX-M-15
ST131	hospital	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	CTX-M-15
ST131	hospital	wound swab	*qnrS1*	-	0.1	
ST131	hospital	sputum	*aac(6´)-Ib-cr, qnrB1*	-	<0.1	
ST131	hospital	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	CTX-M-15
ST131	hospital	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	CTX-M-15
ST131	hospital	urine	*qnrB1*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	
ST428	hospital	wound swab	*qnrS1*	*gyr*A[S(83)L]	4	
ST428	WWTP5	wastewater	*qnrS1*	*gyr*A[S(83)L]	4	
ST131	hospital	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	CTX-M-15
ST131	community	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	CTX-M-15
ST131	hospital	bronchoalveolar lavage	*aac(6´)-Ib-cr, qnrB1*	*gyr*A[S(83)L]	16	CTX-M-15
ST131	hospital	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	CTX-M-15
ST131	hospital	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	8	CTX-M-15
ST131	hospital	bronchoalveolar lavage	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	CTX-M-15
ST131	hospital	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)I; E(84)V]	>32	CTX-M-15
ST131	hospital	urine	*aac(6´)-Ib-cr*	*gyr*A[S(83)L;A(84)V;D(87)N] *par*C[S(80)I; E(84)V]	>32	CTX-M-15
ST95	turkey	cloacal swab	*qnrB19, qnrS1*	-	0.2	
ST95	turkey	cloacal swab	*qnrB19, qnrS1*	-	0.2	
ST95	turkey	cloacal swab	*qnrB19, qnrS1*	-	0.5	
ST95	turkey	cloacal swab	*qnrS1*	-	0.5	
ST95	turkey	cloacal swab	*qnrB19, qnrS1*	-	0.2	
ST95	turkey	cloacal swab	*qnrB19, qnrS1*	-	0.2	
ST95	turkey	cloacal swab	*qnrB19, qnrS1*	-	0.5	
ST95	turkey	cloacal swab	*qnrB19*	-	0.2	
ST226	turkey	cloacal swab	*qnrS1*	*gyr*A[S(83)L;D(87)N] *par*C[S(80)R] *parE*[S(458)P]	>16	
ST226	chicken	environmental sample	*qnrS1*	*gyr*A[S(83)L] *par*C[S(80)R] *parE*[S(458)P]	16	

FIGURE 1 | Dendrogram of *E. coli* isolates including information about origin, PMQR genes, gyrase and topoisomerase substitutions, MIC of ciprofloxacin and beta-lactamases.

DISCUSSION

Quinolones are antimicrobial agents widely used in hospitals, community and veterinary practice. According to the combined data of ECDC, EFSA, EMA (ECDC, EFSA, and EMA, 2015), the consumption rate of fluoroquinolones reached 3.2 tons in primary care sector in the Czech Republic in 2012. The same data reported that 1.2 tons of fluoroquinolones were used in food producing animals in the country. However, the consumption of this class of antibiotics is still relatively low compared to other countries such as Germany, France, or Italy (ECDC, EFSA, and EMA, 2015).

The first aim of this study was to investigate the occurrence of *E. coli* isolates with PMQR genes in humans, food-producing animals, wild animals and wastewater samples from a defined area of the Czech Republic. The results pointed at 25% occurrence of *E. coli* with PMQR genes in all areas studied. The most common PMQR gene in *E. coli* from humans was *aac(6′)-Ib-cr* found mainly in ST131 clone with a high level of fluoroquinolone resistance. It has been demonstrated that *aac(6′)-Ib-cr* gene is more prevalent in *E. coli* ST131 than other PMQR genes (Cerquetti et al., 2009; Peirano et al., 2010; Hussain et al., 2012) and it is carried mainly by plasmids of incompatibility group F (IncF) along with genes encoding extended-spectrum beta-lactamase CTX-M or resistance to other antimicrobial classes (Woodford et al., 2009; Nicolas-Chanoine et al., 2014).

Regarding other types of PMQR determinants, *qnrS1* gene was identified in all areas examined. It was found (alone or

in combination with *qnrB19*) in 73% of isolates that showed reduced susceptibility to ciprofloxacin and originated from wastewater samples or food-producing animals. Currently, this PMQR variant seems widespread in *E. coli* isolates from various types of food-producing animals in Europe (Forcella et al., 2010; Veldman et al., 2011). The second most common *qnr* variant, *qnrB19*, was found predominantly in the isolates from turkeys and wastewater samples. Both *qnrB19* and *qnrS1* were previously detected in chicken broilers in the Czech Republic (Literak et al., 2013). In *E. coli* isolates from rooks, *qnrB19, qnrD1, qnrS1* and *aac(6′)-Ib-cr* were identified. These PMQR variants have been previously detected in *E. coli* from rooks sampled in several European countries including the Czech Republic (Literak et al., 2012; Jamborova et al., 2015).

All *E. coli* isolates with high levels of ciprofloxacin resistance (MIC >32 mg/L), including ST131 clone, carried double or triple mutations in *gyrA* in combination with single or double mutations in *parC*. *E. coli* ST131 with specific substitutions in GyrA or ParC as found in our study have been previously described (Cerquetti et al., 2010; Platell et al., 2011; Paltansing et al., 2013). Isolates from poultry and rooks that displayed clinical levels of ciprofloxacin resistance linked to single or double mutations in *gyrA* and *parC* were found in our study. Strains of various animal origin with these types of mutations have been recently found worldwide (Sáenz et al., 2003; Liu et al., 2012; Castillo et al., 2013; Johnning et al., 2015; Balakrishnan et al., 2016).

ST131 was the predominant clone in our study found in the hospitalized patients as well as in the community subjects and also in one sample from the hospital WWTP. *E. coli* ST131 is a worldwide multidrug resistant clone responsible mainly for urinary but also for other types of infections and widely disseminated in hospital and community patients worldwide (Johnson et al., 2003; Cerquetti et al., 2010; Lee et al., 2010; Nicolas-Chanoine et al., 2014). In the Czech Republic, isolates of this ST producing CTX-M beta-lactamases were found in patients suffering from community or hospital acquired infections (Dolejska et al., 2012; Micenková et al., 2014; Papagiannitsis et al., 2015). Our results also pointed at the occurrence of CTX-M-15-producing ST131 clone in UHO. The ST131 clone has also been isolated from wildlife, companion animals, and retail chicken meat (Ewers et al., 2010; Ghodousi et al, 2016; Oh et al., 2016) as well as from wastewater or water samples (Dolejska et al., 2011a; Colomer-Lluch et al., 2013; Varela et al., 2015).

ST10 clonal complex (including ST10, ST34, ST43, ST48, ST167, and ST617) was the second most widespread in our study. It represents one of the largest clonal complexes within the *E. coli* MLST database, generally being antimicrobial-susceptible, with low virulence (Manges and Johnson, 2012) but it has also been associated with strains producing ESBL or AmpC beta-lactamases (Alcalá et al., 2016; Ben Said et al., 2016; Wang et al., 2016). Based on our results, one ST48 *E. coli* producing CTX-M-15 from wastewater, one ST617 strain with CTX-M-15 from wound swab and one ST43 *E. coli* with production of CTX-M-1 beta-lactamase from chicken were identified. In contrast to other studies (Huijbers et al., 2014; Agabou et al., 2016), no epidemiological link between human and animal isolates of this clonal group was observed in our study.

ST95 was the third largest clonal group in our study including isolates from cloacal swabs from market-weight turkeys and one isolate from a wastewater sample. This clone has been associated with infections in domestic (Mora et al., 2013) and wild birds (Jamborova et al., 2015) as well as urinary tract infections in humans (Riley, 2014). In contrast to ST131, ST95 lineage includes less multidrug resistant strains (Gibreel et al., 2012; Adams-Sapper et al., 2013). ST355 prevalent in our samples from market-weight turkey, seems to be not as widespread in the world as previous ones. It has been documented in *E. coli* isolates from urine and rectal samples in a multinational report by Adler et al. (2014) and in a study from the U.S. (Tartof et al., 2007).

Several authors suggested potential transmission of *E. coli* clones from animals to humans (Platell et al., 2011; Agabou et al., 2016; Nüesch-Inderbinen and Stephan, 2016) and the role of contaminated food in the local spread of resistant *E. coli* strains (Vincent et al., 2010). In a study from Platell et al. (2011), a high level of relatedness of *E. coli* ST131 strains from companion animals and humans was observed. Moreover, Agabou et al. (2016) demonstrated a clonal relationship between human and chicken ciprofloxacin-resistant *E. coli* isolates in North-Eastern Algeria. In contrast to these studies, we did not find any correlation between samples of human, animal, or environmental origin. No strains sharing 100% relatedness and originating from different areas of collection were identified using PFGE. However, some limitations of our study might affect the final results. These include the preferable selection of *E. coli* isolates resistant to ciprofloxacin from clinical materials of hospitalized patients and community subjects, the preferable selection of urine samples and the absence of rectal samples from a healthy population.

AUTHOR CONTRIBUTIONS

Conception and design of the study: JB, AC, MK, IL. Sample collection: MR, DH, IP, MM, VH, VP, MH, PS, JB, and AC. Data analysis and interpretation, Final approval of the version to be published and drafting the article: MR, DH, IP, MD, MM, VH, VP, PB, MH, PS, JB, AC, MK, and IL. Critical revision of the article: MR, DH, IP, MD, MK, and IL. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: MR, DH, IP, MD, MM, VH, VP, PB, MH, PS, JB, AC, MK, and IL.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.02147/full#supplementary-material

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frontiers in Microbiology

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Characteristics of Quinolone Resistance in *Salmonella* spp. Isolates from the Food Chain in Brazil

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Salmonella spp. is an important zoonotic pathogen related to foodborne diseases. Despite that quinolones/fluoroquinolones are considered a relevant therapeutic strategy against resistant isolates, the increase in antimicrobial resistance is an additional difficulty in controlling bacterial infections caused by *Salmonella* spp. Thus, the acquisition of resistance to quinolones in *Salmonella* spp. is worrisome to the scientific community along with the possibility of transmission of resistance through plasmids. This study investigated the prevalence of plasmid-mediated quinolone resistance (PMQR) in *Salmonella* spp. and its association with fluoroquinolone susceptibility in Brazil. We evaluated 129 isolates, 39 originated from food of animal sources, and 14 from environmental samples and including 9 from animals and 67 from humans, which were referred to the National Reference Laboratory of Enteric Diseases (NRLEB/IOC/RJ) between 2009 and 2013. These samples showed a profile of resistance for the tested quinolones/fluoroquinolones. A total of 33 serotypes were identified; *S.* Typhimurium (63) was the most prevalent followed by *S.* Enteritidis (25). The disk diffusion test showed 48.8% resistance to enrofloxacin, 42.6% to ciprofloxacin, 39.53% to ofloxacin, and 30.2% to levofloxacin. According to the broth microdilution test, the resistance percentages were: 96.1% to nalidixic acid, 64.3% to enrofloxacin, 56.6% to ciprofloxacin, 34.1% to ofloxacin, and 30.2% to levofloxacin. *Qnr* genes were found in 15 isolates (8 *qnr*S, 6 *qnr*B, and 1 *qnr*D), and the aac(6′)-Ib gene in 23. The integron gene was detected in 67 isolates with the variable region between ±600 and 1000 bp. The increased detection of PMQR in *Salmonella* spp. is a serious problem in Public Health and must constantly be monitored. Pulsed-field gel electrophoresis was performed to evaluated clonal profile among the most prevalent serovars resistant to different classes of quinolones. A total of 33 pulsotypes of *S.* Typhimurium were identified with a low percentage of genetic similarity (≤65%). This result demonstrates the presence of high diversity in the resistant clones evaluated in this study.

Keywords: foodborne diseases, *Salmonella* spp., quinolone resistance, plasmid mediated quinolone resistance, clonal profile

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INTRODUCTION

Foodborne diseases caused by *Salmonella* spp. are a serious public health problem in many parts of the world. The variety of food sources, particularly foods of animal origin, and routes of transmission can lead to human infection (Scallan et al., 2011).

In addition, the progressive increase of antimicrobial resistance in foodborne Salmonella isolates is observed as due to the uncontrolled use of these drugs for therapeutic and prophylactic purposes in foods of animal origin such as poultry, pigs, and cattle. These events have reinforced the need for epidemiological studies describing the prevalence and patterns of resistance in these bacteria (Yang et al., 2010; Tamang et al., 2011). Antimicrobial-resistant bacteria emerge from the use of antimicrobial drugs to treat and prevent diseases and promote growth in large-scale animal production.

Quinolones, particularly fluoroquinolones, are commonly used for the treatment of multi-drug resistant salmonellosis "in human and veterinary medicine" because of their broad spectrum antimicrobial activity (Dalhoff, 2012).

Point mutations in DNA gyrase and topoisomerase IV genes are directly related to quinolone resistance in *Enterobacteriaceae* "by changes in the action target site called quinolone resistance-determining regions (QRDR)." "In *Salmonella* spp., these mutations are related to resistance to nalidixic acid (NAL) and reduced susceptibility to FQs such as that of ciprofloxacin (Cip) (Cavaco and Aarestrup, 2009)." It is believed that the resistance to quinolones is mediated only by this mechanism. However, the situation changed with the discovery of a variety of determinants of plasmid-mediated quinolone resistance (PMQR).

Currently three mechanisms are recognized as PMQRs. The *qnr* genes with five different qnr families, each with different numbers of alleles "(qnrA1–7, qnrS1–4, qnrB1–31, qnrC, and qnrD) (Jacoby et al., 2009)"; "a modified aminoglycoside acetyl-transferase gene [aac(6′)-1b-cr] (Robicsek et al., 2005)"; and a specific quinolone efflux pump (qepA) (Yamane et al., 2007) and multidrug resistance pumps such as oqxAB (Zhao et al., 2010). PMQR-positive isolates present a low-level of resistance to quinolones (only a small reduction in susceptibility to nalidixic acid). However, the ability to highlight pre-existing resistance mechanisms, such as chromosomal mutations in target regions of quinolones that still allow the selection of resistant mutants to quinolone concentrations (therapeutic doses), emphasize the importance of studying these genes (Cui et al., 2014).

The present study identified the occurrence of some PMQR in *Salmonella* spp. isolated between 2009 and 2013 from the food chain in Brazil, and characterized the genetic similarity profile of serovars of greatest importance in the dispersion of resistance to quinolones in Brazil.

MATERIALS AND METHODS

Bacterial Isolates

129 *Salmonella* spp. strains with resistance to quinolone and/or fluoroquinolone were evaluated. Of these, 51.9% (67/129) were from human clinical isolates, 30.2% (39/129) from food products for human consumption (beef, eggs, milk), 7.1% (9/129) from food of animal origin for human consumption (poultry, swine, cattle), and 10.8% (14/129) from environmental samples (water and drag swabs); all samples were selected from a database. The studied strains were sent to the National Reference Laboratory of Enteric Diseases (NRLEB/IOC/RJ) between 2009 and 2013 and stored in phosphate-buffered agar at room temperature and/or in BHI/glycerol broth −70°C. The isolates were inoculated in Nutrient Broth (DIFCO) and incubated at 37°C for 12–18 h for subsequent tests, as confirmation of the biochemical, serological and antimicrobial resistance profile.

Antigenic Characterization

The serological determination of *Salmonella* serotypes was determined according to the Kauffmann-White scheme using slide agglutination with O and H antisera prepared in the LRNEB/IOC/RJ.

Antimicrobial Susceptibility

The obtained resistance profiles were confirmed by the disk diffusion test according to Clinical and Laboratory Standards Institute (2013, 2014). According to Pribul et al. (2016), this test was performed using representatives of the quinolone class (OXOID) for human and veterinary therapeutic use, such as Nalidixic Acid (NAL), Ciprofloxacin (CIP), Enrofloxacin (ENO), Ofloxacin (OFL), and Levofloxacin (LVX).

MIC determinations for Nalidixic Acid (SIGMA), Ciprofloxacin (SIGMA), Enrofloxacin (SIGMA), Levofloxacin (SIGMA), and Ofloxacin (SIGMA) were performed in 96-well-microplates and according to the Clinical and Laboratory Standards Institute (2013) broth microdilution assay.

Detection of PMQR

Total DNA was extracted using the DNEASY Tissue Qiagen® kit. The studied genes were detected by PCR amplification using primer sequences reported in Pribul et al. (2016). The *qnrA, qnrB*, and *qnrS* genes were amplified through multiplex PCR reactions; the *rrs* gene was used as the reaction control. The *qnrC, qnrD, aac(6′)-Ib, integrase*, and variable *integron* region genes were amplified by simplex PCR.

PFGE

The isolates from serovars *S.* Typhimurium, *S.* Muenchen, *S.* Infantis, and *S.* Heidelberg were subjected to molecular typing by pulsed-field gel electrophoresis, which clonally evaluates isolates. The PulseNet protocol was used in this study including DNA preparation according to Heir et al. (2000) and digestion with XbaI restriction enzyme, according to Pfaller et al. (1992), Tenover et al. (1997), and Cooper et al. (2006). The definition of clones was based on the recommendations of Tenover et al. (1997) and Barrett et al. (2006). *S.* Braenderup H9812, which is considered the universal strain for PulseNet (Hunter et al., 2005), was used as the standard. The restriction patterns were analyzed in the BioNumerics software IV (Applied Maths).

RESULTS

Serovar Identified

Altogether, 26 different *Salmonella* serovars were identified. *Salmonella* Typhimurium (48.8%, 63/129) was the predominant serovar followed by *Salmonella* Enteritidis (19.4%, 25/129). The prevalent serovars associated with resistance to quinolones are presented in **Table 1**.

Most of the studied samples were isolated in 2012 (88 of 129).

Among these 129 isolates that were previously resistant to Nalidixic Acid, five were sensitive to all tested quinolones (including Nalidixic Acid), 55 (42.6%) were resistant to Ciprofloxacin, 63 (48.8%) to Enrofloxacin, 51 (39.53%) to Ofloxacin, and 48 (37.2%) to Levofloxacin in the disc diffusion test.

The broth microdilution test identified 36.4% (47/129) isolates with decreased susceptibility to Ciprofloxacin (MICs between 0.125 and 0.5 mg/ml), 20.1% (26/129) to Enrofloxacin, 9.3% (12/129) to Ofloxacin, and 6.2% (8/129) to Levofloxacin (MICs between 0.5 and 1 mg/ml). The decreased susceptibility breakpoint to Nalidixic Acid is not reported by Clinical and Laboratory Standards Institute (2015). Seventy-three (56.6%) isolates were resistant to Ciprofloxacin, 83 (64.3%) to Enrofloxacin, 44 (34.1%) to Ofloxacin, and 39 (30.2%) to Levofloxacin. A total of 124 isolates (96.1%) were resistant to Nalidixic Acid.

The resistance profile obtained with the microdilution test showed that 37 (28.7%) isolates were resistant to all tested quinolones, 30 (23.2%) to Ciprofloxacin, Enrofloxacin, and Nalidixic Acid, 16 (12.4%) to Enrofloxacin and Nalidixic Acid, 2 (1.5%) to Ciprofloxacin and Nalidixic Acid, and 39 (30.2%) to Nalidixic Acid only.

The detection of resistance genes showed six isolates carrying the *qnr*B gene, eight the *qnr*S gene, and one the *qnr*D gene. Among these 15 positive isolates, 10 strains were recovered from human samples, 3 from food of animal origin, 1 from environmental samples, and 1 from animal samples. The most *qnr*-positive prevalent serovar was *S.* Typhimurium followed by *S.* Saintpaul and *S.* Livingstone. None of the isolates presented the *qnr*A or *qnr*C genes.

A total of 23 isolates showed the *aac*(6′)-Ib gene, which is prevalent in *S.* Typhimurium (14/23). The most prevalent source of isolation was human (10/23), followed by foodborne (7/23), animal (3/23), and amibental (3/23). Thirteen isolates *aac*(6′)-Ib positive were resistant to all tested quinolones.

Three *qnr*-positive isolates presented the *aac*(6′)-Ib gene in association: two *S.* Typhimurium and one *S.* Saintpaul. These two *Salmonella* ser. Typhimurium were resistant to all tested quinolones/fluoroquinolones in the broth microdilution assay at the highest concentration.

Sixty-seven isolates showed the presence of integrase gene within 600 to 1000 bp variable region range and were mainly identified in human samples (38/67) followed by food samples (15/67), ambiental (8/67), and animal (6/67). The *S.* Typhimurium serovar was the most frequent (39/67) isolate with the conserved region of class 1 integron and variable regions between ±900 and >1000 bp. Nine isolates of serovar *S.* Typhimurium carrying the *aac(6′)-Ib* gene were positive for the integron with ±900 bp; two of these were also positive for *qnr*.

TABLE 1 | Distribution of quinolone-resistant *Salmonella* spp. serovars isolated from food chain diseases.

Salmonella spp. Serotype	Number of NTS[a] from				
	Human	Food	Environment	animal	Total
S. Typhimurium	35	22	4	2	63
S. Enteritidis	24	1	–	–	25
S. Muenchen	2	2	–	–	4
S. Infantis	1	1	–	–	3
S. Heidelberg	2	–	–	1	3
Others	3	11	8	5	26
Total	67	37	12	8	

[a] *Non-typhoidal Salmonella.*

Figure 1 shows the clonal profile comparison between the quinolones/fluoroquinolones resistant strains and other sensitive strains.

The genetic similarity among isolates with resistance to quinolones was approximated 84% in *S.* Infantis, 92% in *S.* Heidelberg, 88% in *S.* Muenchen, and 63% in *S.* Typhimurium despite their isolation in different periods, regions, and sources.

Thirty-three distinct pulsotypes were identified among strains with low percentage genetic similarity in serovar *S.* Typhimurium (≤65%), representing the highest diversity among resistant clones.

The **Table 2** presents resistance profiles obtained with the microdilution test, detection of PMQR, size of variable integron region, and the pulsotype identified by the pfge technique.

DISCUSSION

The variation in resistance to the different tested quinolones can be explained by the mechanism of resistance when the resistance level depends on the affected target enzyme, the number of accumulated mutations, and presence of PMQRs. Furthermore, there is a relationship between the level of specific resistance and potency of each drug, especially in the newest quinolones (Sanders, 2001; Ruiz et al., 2012).

Chong et al. (2010) reported that an increased resistance to fluoroquinolones based on the acquisition of *qnr* genes could be related with reduction in the clinical efficacy "of this class" of antimicrobial. "However, Jacoby et al. (2009)" argue that the genes involved in plasmid-level resistance to fluoroquinolones are still poorly understood when compared to other resistance mechanisms.

A high prevalence of isolates carrying PRQM genes is reported in the present study (27%, 35/129). The most prevalent serovar associated with the presence of PMQR genes was *Salmonella* ser. Typhimurium (18/35). A high level of detection of *S.* Typhimurium was expected because this serovar is directly related to detected genotypic and phenotypic profiles of antimicrobial resistance (Herrero et al., 2008; Kingsley et al., 2009). The presence of PMQR genes is related "to decreased susceptibility to fluoroquinolones," accelerating the selection of

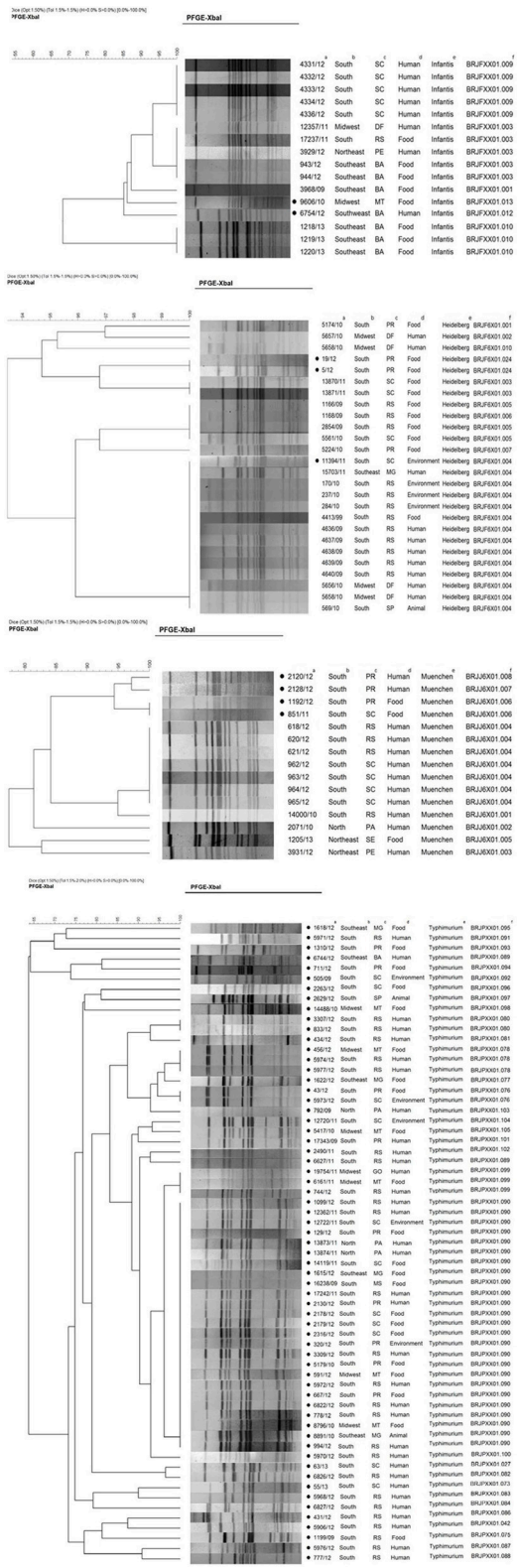

FIGURE 1 | Distribution of phylogenetic groups of *Salmonella* ser. Infantis, Heidelberg, Muenchen, and Typhimurium according to PFGE. Isolates with resistance to quinolones; [a]ID of IOC/year of isolation; [b]region; [c]state; [d]source of isolation; [e]serovar; [f]pulsotype.

fluoroquinolone-resistant mutants (Rodríguez-Martínez et al., 2011).

Three isolates presented association between the *qnr* and *aac*(6′)-Ib genes. A similar association has been reported by Park et al. (2006) in the United States, Xiong et al. (2011) while investigating the *aac*(6′)-Ib and *qnr* genes in *Enterobacter cloacae* "in China, and" Kim et al. (2013) in enterobacteria isolated from clinical samples in Korea. Not sequencing the *aac*(6′)-Ib gene to determine the cr variant was one limitation in the present study.

Regardless that some authors recognize the location of the *aac*(6′)-Ib gene mostly in class 1 integrons, our results show the absence of this gene in all analyzed strains [12 *aac*(6′)-Ib positive isolates without the integron region] (Rodríguez-Martínez et al., 2011; Kim et al., 2013).

The Enteritidis serovar was not assessed by PFGE because, according to the literature, these isolates have low clonal diversity (Spiliopoulou et al., 2007).

Six distinct pulsotypes were detected in *S.* Infantis serovar isolates. Those with resistance to quinolones are placed in two separate pulsotypes (BRJFXX01.13 and BRJFXX01.12) with a genetic similarity of ∼85%. The quinolone resistance isolates were obtained from different sources, regions, and periods, and the resistance to quinolones showed variations. The 6754/12 isolate showed resistance to ciprofloxacin, nalidixic acid, and enrofloxacin and carried the *qnr*D gene; the 9606/10 isolate showed resistance to nalidixic acid only and did not carry resistant genes.

The *S.* Heidelberg serovar presented eight distinct pulsotypes. The resistant isolates showed a clonal ratio of 100% similarity between the isolates 5/12 and 19/12, and ∼94% between them and isolate 11394/11. The 11394/11 isolate (environmental source from the Southern region) was detected in the BRJF6X01.004 pulsotype (pulsotype with 13 susceptible isolates). Isolates 5/12 and 19/12, within the same pulsotype, were foodborne and originated in the Southern region.

Three pulsotypes were identified in serovar Muenchen isolates with quinolone resistance profiles (pulsotypes BRJJ6X01008, BRJJ6X01007, and BRJJ6X01006), showing 88% of genetic similarity. Among the isolates resistant, the isolates of human origin presenting a profile similarity of ∼97%. The isolates of foodborne origin presenting the same origin clonal being from different periods and states. The 2128/12, 2120/12, and 1192/12 isolates show similar quinolone resistance profiles. However, isolate 851/11 show a resistance profile to ciprofloxacin and ofloxacin. The 2120/12 and 2128/12 isolates show similar resistance profile and are carriers of the *qnr*S gene.

The detection of 33 different pulsotypes of *S.* Typhimurium indicates that different clones with resistance to quinolones are circulating in Brazil. The most prevalent pulsotype (BRJPXX01.090) is mainly represented in samples from the Southern region and are related to food and human sources. Most isolates of this pulsotype show the same resistance profile to quinolones/fluoroquinolones (except isolates 3309/11, 5179/10, 591/12, and 667/12) demonstrating a resistance profile to all tested quinolones/fluoroquinolones. Out of the 25 isolates showing resistance to quinolones, 4 did not carry resistance genes. Twelve isolates show an integron with a variable region of

TABLE 2 | Resistance profile and resistance genes in isolates evaluated by PFGE.

Serovar	Source	IOC ID/Year[d]	Pulsotype	PMQR[c]	Integron (bp)	Resistance profile (MIC[b])
Infantis	F	6754/12	BRJFXX01.13	*qnr*D	–	CIP NAL ENO[a]
Infantis	H	9606/10	BRJFXX01.012	–	–	NAL
Heidelberg	F	19/12	BRJF0X01.024	–	700	CIP NAL ENO
Heidelberg	F	5/12	BRJF0X01.024	–	–	CIP NAL ENO
Heidelberg	H	11394/11	BRJF0X01.004	–	900	CIP NAL ENO
Muenchen	H	2120/12	BRJJ6X01.008	*qnr*S	–	NAL ENO
Muenchen	H	2128/12	BRJJ6X01.007	*qnr*S	–	NAL ENO
Muenchen	F	1192/12	BRJJ6X01.006	*aac*(6′)-Ib	600	NAL ENO
Muenchen	F	851/11	BRJJ6X01.006	-	700	CIP NAL ENO OFL
Typhimurium	F	1618/12	BRJPXX01.095	*aac*(6′)-Ib	–	CIP NAL ENO LVX OFL
Typhimurium	H	5971/12	BRJPXX01.091	*aac*(6′)-Ib	1000	NAL
Typhimurium	F	1310/12	BRJPXX01.093	*qnr*S	–	NAL ENO
Typhimurium	H	6744/12	BRJPXX01.089	*qnr*B	1000	NAL ENO
Typhimurium	F	711/12	BRJPXX01.094	*qnr*D	–	NAL
Typhimurium	E	505/09	BRJPXX01.092	–	900	CIP NAL ENO
Typhimurium	F	2263/12	BRJPXX01.096	–	–	CIP NAL ENO LVX OFL
Typhimurium	A	2629/12	BRJPXX01.097	*aac*(6′)-Ib	900	CIP NAL ENO LVX OFL
Typhimurium	F	14488/10	BRJPXX01.098	–	900	CIP NAL ENO
Typhimurium	H	3307/12	BRJPXX01.080	–	1000	CIP NAL ENO
Typhimurium	H	833/12	BRJPXX01.080	–	–	CIP NAL ENO
Typhimurium	H	434/12	BRJPXX01.081	–	–	CIP NAL ENO
Typhimurium	F	456/12	BRJPXX01.078	–	1000	NAL
Typhimurium	H	5974/12	BRJPXX01.078	–	1000	NAL OFL
Typhimurium	H	5977/12	BRJPXX01.078	–	900	CIP NAL ENO OFL
Typhimurium	F	1622/12	BRJPXX01.077	–	–	NAL ENO
Typhimurium	F	43/12	BRJPXX01.076	–	–	NAL
Typhimurium	E	5973/12	BRJPXX01.076	–	1000	CIP NAL ENO
Typhimurium	H	792/09	BRJPXX01.103	–	900	CIP NAL
Typhimurium	E	12720/11	BRJPXX01.104	–	900	CIP NAL ENO LVX OFL
Typhimurium	F	5417/10	BRJPXX01.105	–	900	CIP NAL ENO LVX OFL
Typhimurium	H	17343/09	BRJPXX01.101	–	1000	CIP NAL ENO
Typhimurium	H	2490/11	BRJPXX01.102	–	900	CIP NAL ENO LVX OFL
Typhimurium	H	6627/11	BRJPXX01.089	–	900	CIP NAL ENO LVX OFL
Typhimurium	H	19754/11	BRJPXX01.099	–	–	CIP NAL ENO LVX OFL
Typhimurium	F	6161/11	BRJPXX01.099	–	–	CIP NAL ENO LVX OFL
Typhimurium	H	744/12	BRJPXX01.099	–	900	CIP NAL ENO LVX OFL
Typhimurium	H	1099/12	BRJPXX01.090	–	1000	CIP NAL ENO LVX OFL
Typhimurium	H	12362/11	BRJPXX01.090	–	900	CIP NAL ENO LVX OFL
Typhimurium	E	12722/11	BRJPXX01.090	–	–	CIP NAL ENO LVX OFL
Typhimurium	F	129/12	BRJPXX01.090	*aac*(6′)-Ib	–	CIP NAL ENO LVX OFL
Typhimurium	H	13873/11	BRJPXX01.090	–	900	CIP NAL ENO LVX OFL
Typhimurium	H	13874/11	BRJPXX01.090	–	900	CIP NAL ENO LVX OFL
Typhimurium	F	14119/11	BRJPXX01.090	–	900	CIP NAL ENO LVX OFL
Typhimurium	F	1615/12	BRJPXX01.090	–	–	CIP NAL ENO LVX OFL
Typhimurium	F	16238/09	BRJPXX01.090	–	900	CIP NAL ENO LVX OFL
Typhimurium	H	17242/11	BRJPXX01.090	–	900	CIP NAL ENO LVX OFL
Typhimurium	H	2130/12	BRJPXX01.090	*aac*(6′)-Ib	–	CIP NAL ENO LVX OFL
Typhimurium	F	2178/12	BRJPXX01.090	*aac*(6′)-Ib	–	CIP NAL ENO LVX OFL
Typhimurium	F	2179/12	BRJPXX01.090	*aac*(6′)-Ib	–	CIP NAL ENO LVX OFL

(Continued)

TABLE 2 | Continued

Serovar	Source	IOC ID/Year[d]	Pulsotype	PMQR[c]	Integron (bp)	Resistance profile (MIC[b])
Typhimurium	F	2316/12	BRJPXX01.090	–	900	CIP NAL ENO LVX OFL
Typhimurium	E	320/12	BRJPXX01.090	*aac*(6′)-Ib	900	CIP NAL ENO LVX OFL
Typhimurium	H	3309/12	BRJPXX01.090	*qnr*B/*aac*(6′)-Ib	900	CIP NAL ENO
Typhimurium	F	5179/10	BRJPXX01.090	–	–	CIP NAL ENO
Typhimurium	F	591/12	BRJPXX01.090	–	900	NAL
Typhimurium	H	5972/12	BRJPXX01.090	*aac*(6′)-Ib	900	CIP NAL ENO LVX OFL
Typhimurium	F	667/12	BRJPXX01.090	–	–	CIP NAL ENO
Typhimurium	H	6822/12	BRJPXX01.090	–	900	CIP NAL ENO LVX OFL
Typhimurium	H	778/12	BRJPXX01.090	*qnr*B	–	CIP NAL ENO LVX OFL
Typhimurium	F	8796/10	BRJPXX01.090	–	900	CIP NAL ENO LVX OFL
Typhimurium	A	8891/10	BRJPXX01.090	–	900	CIP NAL ENO LVX OFL
Typhimurium	H	994/12	BRJPXX01.090	–	900	CIP NAL ENO OFL
Typhimurium	H	5970/12	BRJPXX01.100	*aac*(6′)-Ib	–	CIP NAL ENO LVX OFL
Typhimurium	H	63/13	BRJPXX01.027	–	1000	CIP NAL ENO
Typhimurium	H	6826/12	BRJPXX01.082	–	1000	NAL
Typhimurium	H	55/13	BRJPXX01.073	*qnr*D	–	CIP NAL ENO LVX OFL
Typhimurium	H	5968/12	BRJPXX01.083	–	1000	NAL
Typhimurium	H	6827/12	BRJPXX01.084	–	1000	NAL
Typhimurium	H	431/12	BRJPXX01.086	–	–	–
Typhimurium	H	5906/12	BRJPXX01.042	–	–	NAL
Typhimurium	F	1199/09	BRJPXX01.075	–	600	CIP NAL ENO
Typhimurium	H	5976/12	BRJPXX01.087	*aac*(6′)-Ib	700	CIP NAL ENO LVX OFL
Typhimurium	H	777/12	BRJPXX01.088	*qnr*B/*aac*(6′)-Ib	–	CIP NAL ENO LVX OFL

[a]*CIP, Ciprofloxacin; ENO, Enrofloxacin; NAL, Nalidixic Ácid; LVX, Levofloxacina; OFL, Ofloxacin;* [b]*MIC, Minimum Inhibitory Concentration;* [c]*PMQR, Plasmid-Mediated Quinolone Resistance;* [d]*IOC ID/Year, Institut Oswaldo Cruz Identification by Year;* [e]*F, Food; A, Animal; E, Environment; H, Human.*

900 bp and one with >1000 bp. One isolate shows the *qnr*B gene and, four show the *aac*(6′)-Ib gene. Two isolates show the 900 bp integron and the *aac*(6′)-Ib gene; one isolate shows the 900 bp integron and the *qnr*B, *acc*(6′)-Ib gene.

The profiles identified in the PFGE analysis show relatively high diversity in all serovars and, indicate that cases of resistance to quinolones are probably sporadic. This interpretation is in accordance with other results reported in the literature (Feasey et al., 2012).

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Plasmids Carrying $bla_{CMY-2/4}$ in *Escherichia coli* from Poultry, Poultry Meat, and Humans Belong to a Novel IncK Subgroup Designated IncK2

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The $bla_{CMY-2/4}$-carrying IncB/O/K-like plasmids of seven *Escherichia coli* strains from poultry, poultry meat and human urine samples were examined using comparative analysis of whole plasmid sequences. The incompatibility group was determined by analysis of the *inc*RNAI region and conjugation assays with strains containing the IncK and IncB/O reference plasmids. Strains were additionally characterized using MLST and MIC determination. The complete DNA sequences of all plasmids showed an average nucleotide identity of 91.3%. Plasmids were detected in *E. coli* sequence type (ST) 131, ST38, ST420, ST1431, ST1564 and belonged to a new plasmid variant (IncK2) within the IncK and IncB/O groups. Notably, one *E. coli* from poultry meat and one from human contained the same plasmid. The presence of a common recently recognized IncK2 plasmid in diverse *E. coli* from human urine isolates and poultry meat production suggests that the IncK2 plasmids originated from a common progenitor and have the capability to spread to genetically diverse *E. coli* in different reservoirs. This discovery is alarming and stresses the need of rapidly introducing strict hygiene measures throughout the food chain, limiting the spread of such plasmids in the human settings.

Keywords: ESBL, pAmpC, food, antibiotic resistance, animals

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INTRODUCTION

The worldwide dissemination of *Enterobacteriaceae* along with resistance plasmids carrying β-lactamase-encoding genes (*bla*) is posing an increasing threat to the public health system (Mathers et al., 2015). Poultry and poultry meat have been revealed as a large reservoir of extended-spectrum β-lactamases (ESBL) and plasmid-mediated AmpC (pAmpC) enzymes in several countries (Endimiani et al., 2012; Dierikx et al., 2013; Nilsson et al., 2014; Hansen et al., 2016; Mo et al., 2016). These resistance genes are carried by various mobile genetic elements; primarily located on plasmids which are self-replicating extrachromosomal elements (Couturier et al., 1988; Carattoli, 2009). Such plasmids not only vary in their size and compatibility to other plasmid groups, but also in their capability of transferring to various bacterial species (Carattoli, 2009, 2013).

The involvement of plasmids in the zoonotic spread of β-lactamase- and carbapenemase-encoding genes between human and animal reservoirs has already been emphasized

based on the presence of similar plasmids in *Escherichia coli* from humans, poultry, and poultry meat (Leverstein-van Hall et al., 2011; Börjesson et al., 2013; Seiffert et al., 2013b; Voets et al., 2013; Guerra et al., 2014; Wang et al., 2014). However, there is a lack of firm evidence since there are only few completely sequenced and assembled plasmids to demonstrate the spread and exchange of the same plasmids in *E. coli* of human and poultry origin.

By comparing datasets from poultry, poultry meat, and clinical human isolates from Switzerland, we noticed that the pAmpC bla_{CMY-2} and bla_{CMY-4} genes were associated with plasmids belonging to the IncB/O/K groups (Endimiani et al., 2012; Seiffert et al., 2013a; Vogt et al., 2014). Other studies investigating pAmpC-producing *E. coli* have detected the nearly identical kind of plasmid in healthy humans (Baudry et al., 2009; Porres-Osante et al., 2015; Hansen et al., 2016), poultry (Dierikx et al., 2010; Hansen et al., 2016; Mo et al., 2016), poultry meat (Egervärn et al., 2014), and dogs (Hansen et al., 2016). This prompted us to perform an in-depth analysis of $bla_{CMY-2/4}$-carrying IncB/O/K plasmids from *E. coli* isolated in three settings in Switzerland to determine their genetic relatedness, revealing a novel IncK-group variant, the IncK2.

MATERIALS AND METHODS

Bacterial Strains

All strains were obtained from previous studies characterizing 3rd-generation cephalosporin-resistant *E. coli* (3GC-R-*Ec*) from poultry, poultry meat, and humans isolated at the Institute of Veterinary Bacteriology and at the Laboratory of Microbiology of the Institute of Infectious Diseases, University of Bern, Switzerland (Endimiani et al., 2012; Seiffert et al., 2013a; Vogt et al., 2014). Strains were collected between 2011 and 2014 (**Table 1**) and were selected based on the presence of $bla_{CMY-2/4}$ by PCR/DNA sequencing (Seiffert et al., 2013a; Vogt et al., 2014) and on ambiguous results with the PCR-based replicon typing (PBRT) of plasmids, giving cross-reaction with either IncK and/or IncB/O PCRs (Carattoli et al., 2005).

Plasmids were transformed by electroporation into either *E. coli* ElectroMax DH10B competent cells or into *E. coli* One Shot® TOP10 Electrocomp cells (Invitrogen, Life Technologies) as previously described (Seiffert et al., 2013a; Vogt et al., 2014). The MIC of antibiotics were obtained from donors and transformants using the microdilution Sensititre panels ESB1F and EUMVS2 (Trek Diagnostics) and interpreted following the CLSI guidelines (CLSI, 2016). Sequence types (ST) were assigned following the multi-locus sequence typing (MLST) Achtman scheme[1].

Whole Plasmid Sequencing (WPS)

Plasmids were purified using the Genopure Plasmid Midi Kit (Roche Diagnostic) according to the manufacturer's procedures and used for Whole-Plasmid Sequencing (WPS). WPS was performed using the 454-Junior Genome Sequencer procedure (Roche Diagnostic). The libraries of plasmid DNA were constructed using the GS FLX Titanium Rapid Library Preparation Kit.

De novo Assembly of DNA Reads, Gap-Closure and Annotation

Contigs with at least a 70-fold coverage were obtained using the GS *de novo* Assembler software (Roche Diagnostics). Scaffolding was first done *in silico* by using the 454 ReadStatus output file to identify reads with overlapping adjacent contigs. The assembly and gap-closure, as well as insertion/deletion events, single nucleotide polymorphisms and shufflons were confirmed and verified by PCR followed by Sanger DNA sequencing. The annotation was done using the Artemis Version 8 (Sanger Institute) in combination with a pairwise alignment using a BLASTN and BLASTP homology search[2].

Phylogenetic Analysis of IncB/O/K Plasmids

For the phylogenetic analysis of the IncB/O/K-like plasmids, two fully sequenced plasmids, namely pCT belonging to IncK (GenBank acc. No. FN868832.1; Cottell et al., 2011) and p3521 belonging to IncB (GU256641; Papagiannitsis et al., 2011) were included as reference. These plasmids were chosen as they represent IncK and IncB plasmids whose complete nucleotide sequence is available in the GenBank. The phylogenetic trees and the homology matrices were constructed by UPGMA method and Jukes-Cantor correction [Multiple alignment (open gap penalty (OG): 100%, unit gap penalty (UG): 0%, gap penalty: 100%), 2000 Bootstrap trials] using Bionumerics 7.6 (Applied Maths, Kortrijk, Belgium).

The analyses were performed using the full length sequences of the IncK2 plasmids with and without shufflon region. The deleted shufflon region started at the shufflon-specific DNA recombinase (*rci*) and ended with the last shufflon gene which varies from plasmid to plasmid (position 62686–65450 in pTMSA970). In the case of the reference IncK and IncB plasmids, in addition to the shufflon region, the 3,144-bp $bla_{CTX-M-14}$-carrying element was removed from the IncK reference plasmid (position 68837–71981 in FN868832) and the 26,548-bp large bla_{ACC-4} containing element was removed from the IncB reference plasmid (position 59569–86117 in GU256641).

Conjugation for Incompatibility Testing

Mating experiments were performed crossing the transformant strain containing plasmid p5312.29 (strain SNS5312.29) or pTMSA970 (strain TMSA970) with reference plasmids Rhh72 (IncB/O), and R387 (IncK; provided by the Istituto Superiore di Sanità, Rome, Italy) which are used for incompatibility testing (Couturier et al., 1988). Conjugations were performed on Luria-Bertani (LB) agar solid media streaking two crossing lines, one for each mating strain, and incubating the mating plates overnight at 37°C.

[1] http://mlst.warwick.ac.uk/mlst/dbs/Ecoli

[2] http://blast.ncbi.nlm.nih.gov/Blast.cgi

TABLE 1 | Origin, characteristics and resistance profile of the original *E. coli* strains and of the *E. coli* strains transformed with the $bla_{CMY-2/4}$ containing IncK2 plasmids.

Strain	Origin (date of isolation)	Sequence type	Antibiotic resistance profile	Reference
4809.66	Human UTI, hospital (2011)	ST1431	FOX CPD CRO CTX CAZ CFZ CEF CIP NAL TMP SMZ	Seiffert et al., 2013a
5312.29	Human UTI, hospital (2014)	ST131	FOX CPD CRO CTX CAZ CFZ CEF PIP/TAZ NAL	This study
DV10	Poultry retail meat, MPP from slaughterhouse A (Apr 2013)	ST38	FOX CPD CRO CTX CAZ CFZ CEF NAL	Vogt et al., 2014
DV45	Poultry retail meat, MPP from slaughterhouse A (Apr 2013)	ST1564	FOX CPD CRO CTX CAZ CFZ CEF TMP SMZ NAL TET KAN	Vogt et al., 2014
MSA1088	Broiler cloaca, slaughterhouse A (Dec 2012)	ST38	FOX CPD CRO CTX CAZ CFZ CEF TMP TET	Endimiani et al., 2012; this study
MSA992	Broiler cloaca, slaughterhouse A (Nov 2012)	ST420	FOX CPD CRO CTX CAZ CFZ CEF PIP/TAZ NAL	Endimiani et al., 2012; this study
MSA970	Broiler cloaca, slaughterhouse B (Nov 2012)	ST420	FOX CPD CRO CTX CAZ CFZ CEF PIP/TAZ NAL	Endimiani et al., 2012; this study
SNS4809.66	DH10B transformed with bla_{CMY-2} plasmid p4809.66	–	FOX CPD CRO CTX CAZ CFZ CEF	This study
SNS5312.29	DH10B transformed with bla_{CMY-2} plasmid p5312.29	–	FOX CPD CRO CTX CAZ CFZ CEF	This study
ALC74	TOP10 transformed with bla_{CMY-4} plasmid pDV10	–	FOX CPD CRO CTX CAZ CFZ CEF	This study
ALC76	TOP10 transformed with bla_{CMY-2} plasmid pDV45	–	FOX CPD CRO CTX CAZ CFZ CEF	This study
TMSA1088	TOP10 transformed with bla_{CMY-2} plasmid pTMSA1088	–	FOX CPD CRO CTX CAZ CFZ CEF	This study
TMSA992	TOP10 transformed with bla_{CMY-2} plasmid pTMSA992	–	FOX CPD CRO CTX CAZ CFZ CEF PIP/TAZ NAL	This study
TMSA970	TOP10 transformed with bla_{CMY-2} plasmid pTMSA970	–	FOX CPD CRO CTX CAZ CFZ CEF PIP/TAZ	This study
TOP10	One Shot® TOP10 Electrocomp™ cells	–		Invitrogen, Life Technologies
DH10B	ElectroMax DH10B competent cells	–		Invitrogen, Life Technologies

The three isolates from broiler cloacae were from three different herds. The three isolates from poultry meat were from three different lots originating from the meat packing plant (MPP) of slaughterhouse A; The three isolates from human infections were from patients hospitalized at the same hospital. CEF, cephalotin; CFZ, cefazolin; FEP, cefepime; CTX, cefotaxime; CTX-CLA, cefotaxime/clavulanic acid; FOX, cefoxitin; CPD, cefpodoxime; CAZ, ceftazidime; CRO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; CST, colistin; GEN, gentamicin; IPM, imipenem; KAN, kanamycin; MEM, meropenem; NAL, nalidixic acid; TZP, piperacillin/tazobactam; STR, streptomycin; SMZ, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim.

Donor and transconjugant colonies were counted after plating 10-fold serial dilutions on LB plates containing 50 μg/ml of ampicillin (as resistance marker for p5312.29 and pTMSA970), 50 μg/ml kanamycin (as resistance marker for Rhh72), 25 μg/ml chloramphenicol (as resistance marker for R387), 50 μg/ml of ampicillin plus 50 μg/ml kanamycin, and 50 μg/ml of ampicillin plus 25 μg/ml chloramphenicol.

Stability of the plasmids within the conjugant bacterial cells was determined as follows: three independent colonies resulting from each mating and exhibiting the double resistance (ampicillin/kanamycin, or ampicillin/chloramphenicol) were grown 24 h at 37°C in 50 ml of LB without antibiotics. Ten-fold dilutions of the conjugant cultures were plated on LB plates containing either ampicillin, kanamycin or chloramphenicol alone, and on plates containing both ampicillin and kanamycin, or both ampicillin and chloramphenicol. Colonies were counted after an overnight incubation at 37°C. Stability was determined as the ratio between the number of ampicillin-resistant colonies and the number of ampicillin/kanamycin-resistant colonies for the p5312.29 × Rhh72 and pTMSA970 × Rhh72 transconjugants, and between the number of ampicillin-resistant colonies and the number of ampicillin/chloramphenicol-resistant colonies for the p5312.29 × R387 and pTMSA970 × R387 transconjugants, respectively.

Nucleotide Sequence Accession Numbers

The complete nucleotide sequence of the seven IncK2 plasmids have been deposited into the GenBank database under accession numbers KR905385 (p5312.29), KR905389 (p4809.66), KR905387 (pTMSA992), KR905388 (pTMSA970), KR905386 (pTMSA1088), KR905384 (pDV45), and KR905390 (pDV10).

RESULTS AND DISCUSSION

Plasmid Sequencing

Whole plasmid sequencing of seven $bla_{CMY-2/4}$-containing plasmids [poultry cloacae: pTMSA970, pTMSA992, pTMSA1088;

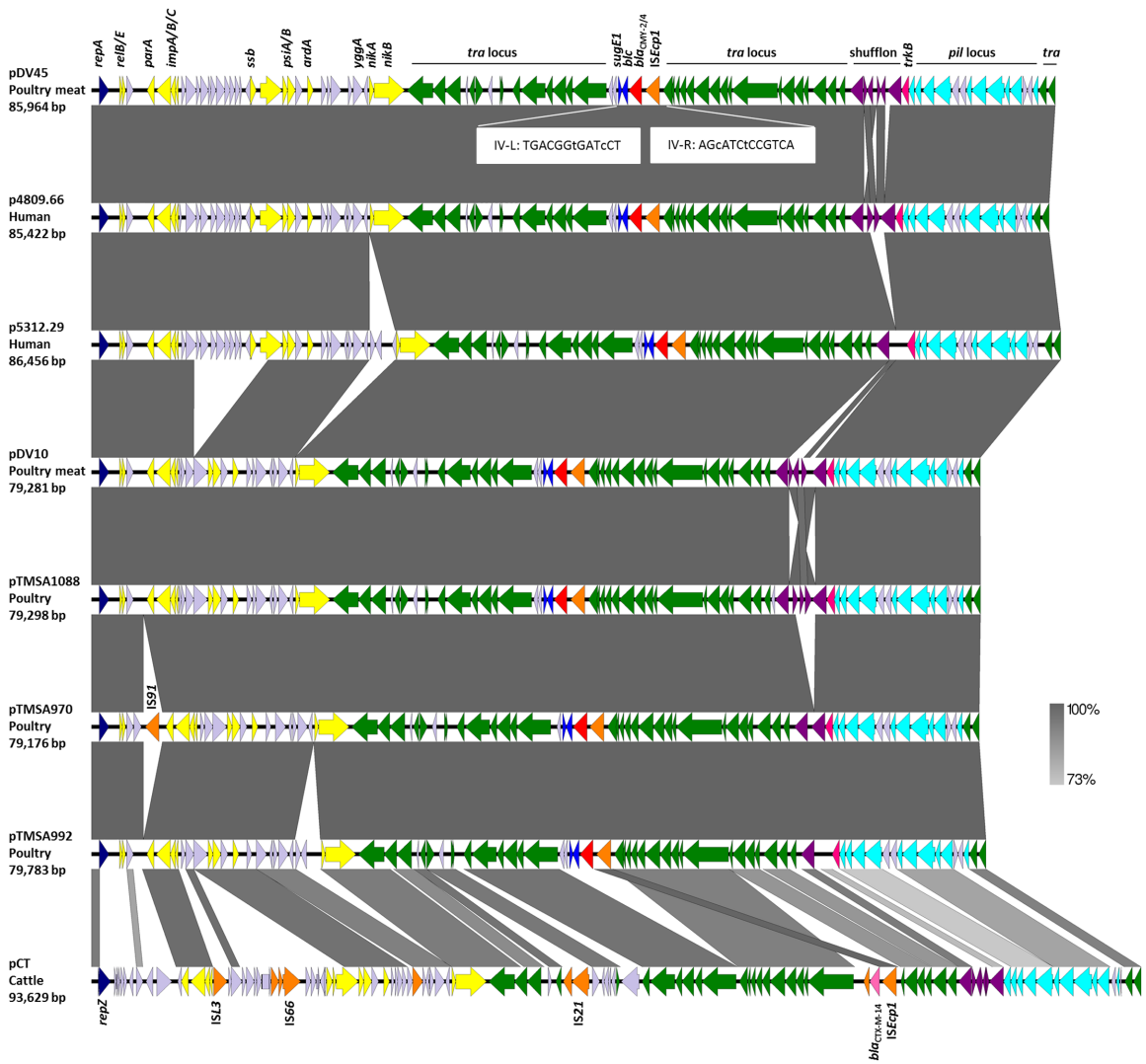

FIGURE 1 | Structural features of seven fully sequenced IncK2 plasmids encoding CMY-2/4 β-lactamases and of the IncK plasmid pCT. The open reading frames are illustrated by arrows pointing in direction of their respective orientation. The figure was generated with EasyFig 2.1 (Sullivan et al., 2011). Imperfect inverted repeats (IV-R and IV-L) are shown in white boxes. The color code is as follows: The *tra* and *trb* transfer loci are in green, antibiotic resistance genes in red ($bla_{CMY-2/4}$) and pink ($bla_{CMY-2/4}$), insertion sequence element (IS) family in orange, replicase genes in dark blue, partitioning genes and toxin/anti-toxin and other stabilization systems in yellow, shufflon-associated genes in violet, pilus genes in pale blue, *sugE1* and *blc* genes in blue, and genes with unknown function are in lavender. Important genes and loci are indicated above the first plasmid sequence. Plasmid sequences of pTMSA970 and pCT carry additional IS elements which are indicated in the figure. The black lines above the figure indicate the different loci and the shufflon-region.

poultry meat: pDV10, pDV45; human urine isolates: p4809.66, p5312.29] revealed the presence of highly related plasmids in both genetically related and diverse *E. coli* from poultry (ST38, ST420), poultry meat (ST38, ST1564) and human clinical samples (ST131, ST1431).

Plasmids mainly differed from each other within the highly variable shufflon region, and a region situated between *impC* and *nikA* (**Figure 1**). A major deletion of approximately 6 kb, that starts after *yfbA* and ends before *psiB*, distinguished plasmids pDV10, pTMSA1088, pTMSA970, pTMSA992 from the larger plasmids pDV45, p4809.66, and p5312.29 (**Figure 1**).

Another fragment of 2.3 kb situated between *yggA* and *nikA* distinguished plasmids p5312.29 and pTMSA992 from the other plasmids (**Figure 1**). Plasmid pTMSA970 also had an extra insertion of a previously unreported IS-element which is closely related to IS*Sbo1* (GenBank acc. no. CP001062) and belongs to the IS*91* family. Integrations or losses of these regions might have occurred as single events during the transfer of the plasmids from one bacterial host to the other rather than during a longer evolutionary process. Outside of these regions, the plasmid sequences were virtually identical, only differing by a few SNPs, which have all been confirmed by Sanger sequencing.

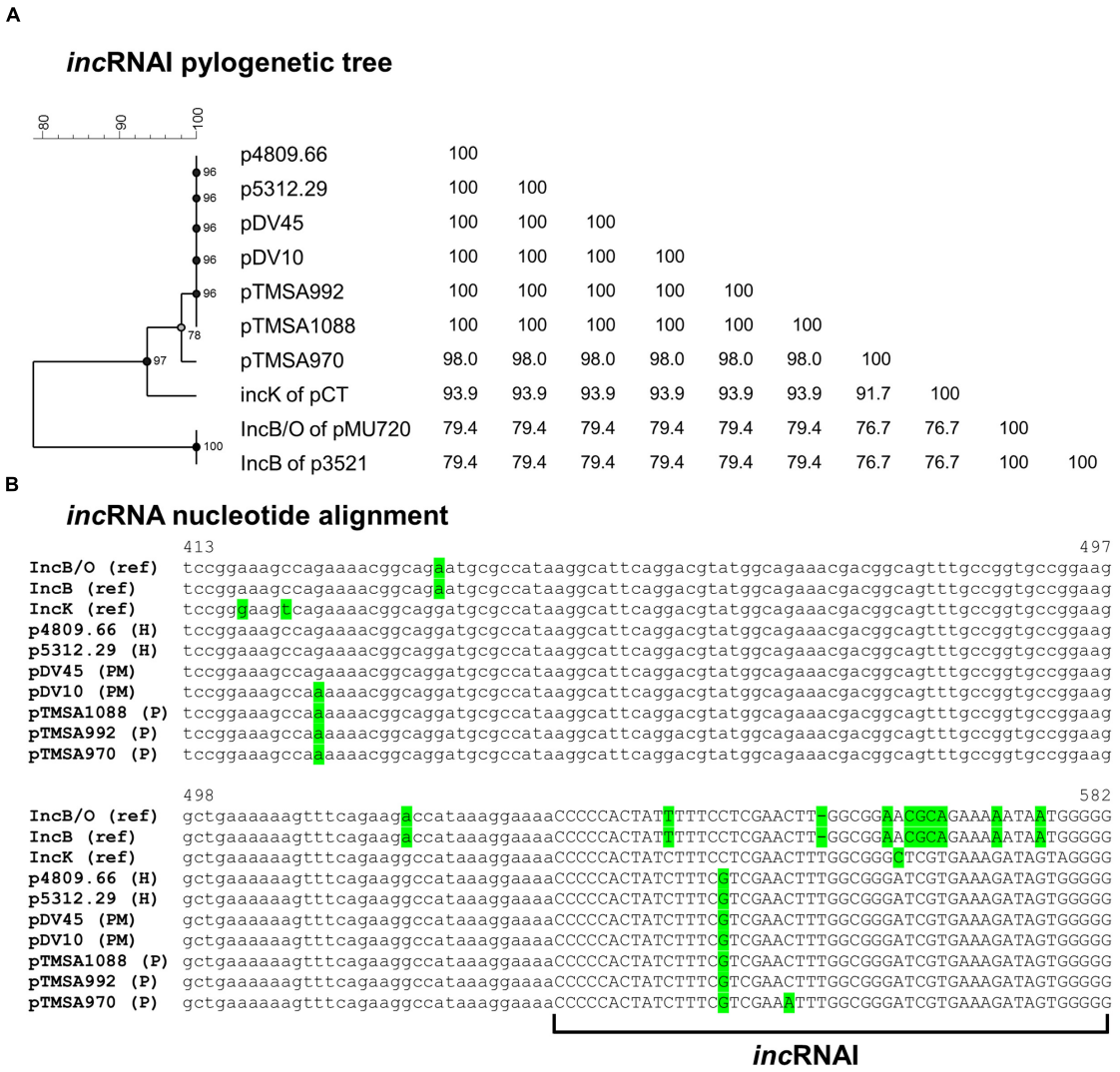

FIGURE 2 | (A) Phylogenetic tree and **(B)** sequence alignment of the *inc*RNAI-II of the IncB/O/K-group plasmids including references. The sequences were extracted from the IncK2 plasmids as well as from the reference plasmids of IncB and IncK. The IncB/O *inc*RNAI-II reference sequence was derived from the paper of Couturier et al. (1988). Due to the absence of the complete nucleotide sequence of the IncB/O plasmid, pMU720 was not included into **Figure 3**. The phylogenetic tree was constructed by UPGMA method and Jukes-Cantor correction [Multiple alignment (open gap penalty (OG): 100%, unit gap penalty (UG): 0%, gap penalty: 100%), 2000 Bootstraps] using Bionumerics 7.6 (Applied Maths, Kortrijk, Belgium). H, human urine sample; P, poultry; PM, poultry meat.

Of note, except for the variable shufflon regions, plasmid pDV45 from *E. coli* from poultry meat was identical to plasmid p4809.66 from human *E. coli*, and plasmid pDV10 *E. coli* from poultry meat was identical to plasmid pTMSA1088 from poultry (**Figure 1**), which strongly suggests transfer of plasmids between genetically diverse *E. coli* along the food chain.

The variable region between *impC* and *nikA* also harbors, along with many genes encoding for proteins with unknown function the single-stranded DNA-binding (Ssb) protein. One of the main functions of the Ssb protein is binding of ssDNA and subsequent protection from degradation by nucleases (Meyer and Laine, 1990). The direct consequence of the loss of Ssb for the IncB/O/K-group plasmids is difficult to determine and needs further investigations.

The shufflon is a biological switch that controls the C-terminal end of the PilV proteins via site-specific recombination. This mechanism has been described in R64 as well as in pCT where the shufflon determines the recipient specificity in liquid mating despite being part of a minor component of the thin pilus (Komano, 1999; Cottell et al., 2014). The shufflon of R64 consists of four invertible DNA fragments embedded with seven 19-bp repeat sequences. These 19-bp repeats can be subdivided into a 7-bp core-site region where the DNA crossover occurs and the highly conserved 12-bp right arm region (Komano, 1999; Brouwer et al., 2015).

The shufflon of the seven plasmids is highly diverse and contains between two and eight recombination sites. Four of the 19-bp repeat sequences found in R64 were also

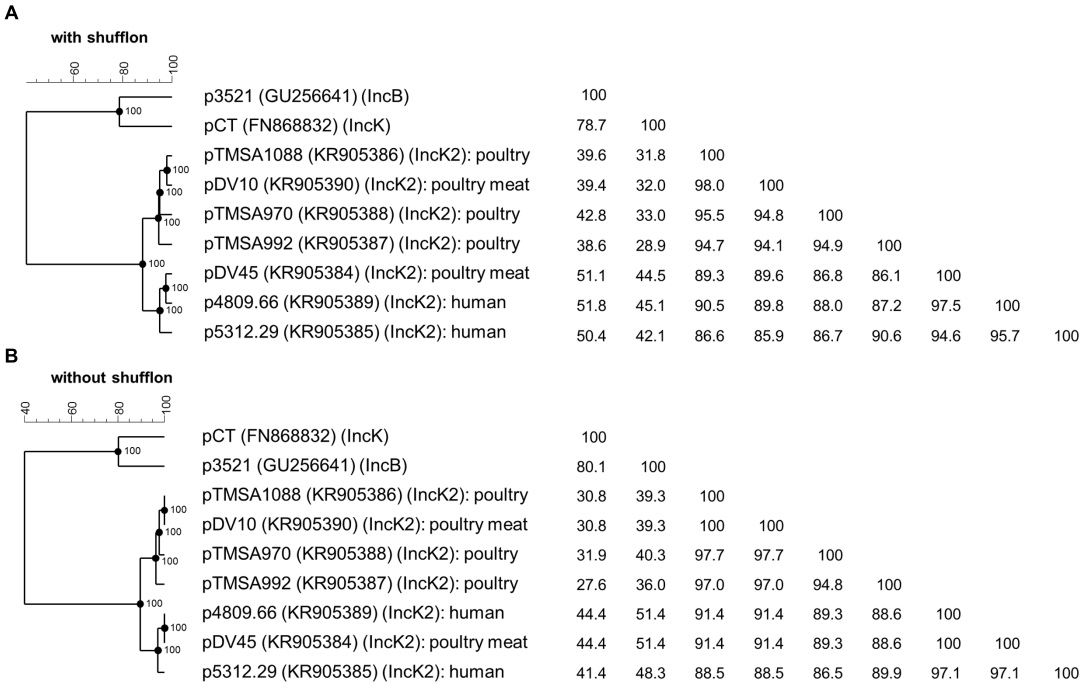

FIGURE 3 | Phylogenetic analysis and identity matrix of the IncK2 group of plasmids of *E. coli* from poultry, poultry meat, and human. The analysis includes the plasmids of the novel IncK2 group with **(A)** and without shufflon **(B)** as well as core sequences of the reference plasmids pCT of the IncK group and p3521 of the IncB group; the reference plasmid pMU720 of the IncB/O group was not included in the tree due the absence of complete plasmid sequence. GenBank accession numbers are indicated in parentheses. The phylogenetic trees and the homology matrices were obtained by UPGMA method and Jukes-Cantor correction [Multiple alignment (open gap penalty (OG): 100%, unit gap penalty (UG): 0%, gap penalty: 100%), 2000 Bootstraps] using Bionumerics 7.6 (Applied Maths, Kortrijk, Belgium).

identified in the plasmids. Interestingly, the 7-bp core-site region is identical to the ones identified in the IncI1/I2 plasmids. The terminology of 19-bp repeat sequences follows the designation of the repeats in R64 (Komano, 1999) (i.e., repeat-e: *GTGCCAA*TCCGGTtcgTGG; repeat-f: *GTGCCAA*TCCGGTatcTGG; repeat-g: *GTGCCAA*TCCGGTGtgtGG; repeat-h: *GTGCCAA*TCCGGTttgTGG; letters in *italic* represent the core-site, small letters for the variable bases). The three plasmids pTMSA970, pTMSA992, and p5312.29 have only two repeat-e sequences, followed by p4809.66 with four repeat sequences in the following order: e-e-e-g. The most complex shufflons with eight repeat sequences each were detected in pDV10 (g-g-e-e-e-e-h-f), pDV45 (e-e-e-e-g-g-h-f), and pTMSA1088 (e-e-h-f-e-e-g-g). Although, no apparent correlation between the numbers of recombination sites could be detected (neither with the size of the plasmid nor with their origin), the observation of such great diversity may have an impact on the mating preferences of the various plasmids.

Incompatibility Assays

Sequence analysis of the region upstream of the *repA* including the *inc*RNAI revealed that the DNA sequences of the seven plasmids diverged from the previously reported *inc*RNAI of the IncK, IncB, and IncB/O plasmids (**Figure 2**).

This region before the *repA* is highly conserved among the specific plasmid Inc groups and is crucial for the incompatibility behavior among plasmids of the same group (Couturier et al., 1988; Praszkier et al., 1991). The key element for this behavior is the *inc*RNAI that forms a stem and loop structure and negatively regulates the transcription of *repA*. Consequently, if two plasmids of the same or similar Inc group(s) reside in the same bacterial cell the *inc*RNAIs hybridize with each other and prohibit translation (Couturier et al., 1988). Additionally, a phylogenetic tree constructed using only the *inc*RNAI sequences containing the stem-and loop structure strengthened the previous observations that the seven plasmids are highly similar to each other, and are more closely related to the IncK group than the IncB/O groups (**Figure 2**).

The divergent *inc*RNAI sequences suggested that these plasmids could be compatible with the IncK and IncB/O reference plasmids. Therefore, incompatibility assays were performed crossing SNS5312.29 and TMSA970 strains with both IncB/O Rhh72 and IncK R387 reference strains, respectively. Transconjugants were obtained from all mating experiments, but at different frequency. Both SNS5312.29 and TMSA970 showed higher frequencies of conjugation when crossed with IncB/O (1×10^{-5} transconjugant/donor cell) than when crossed with the IncK (1×10^{-7} transconjugant/donor cell) reference plasmid. This suggests that the plasmids under study are more closely related to IncK than to IncB/O. Furthermore, IncB transconjugants demonstrated higher stability (>90%) when grown in liquid medium without antibiotics then the IncK

transconjugants (>50%). Based on both conjugation results and *inc*RNAI sequence analysis, the plasmids were assigned to a novel variant of the IncK group designated as IncK2.

Phylogenetic Analysis of the IncK2 Plasmids

The 4,198-bp long $bla_{CMY-2/4}$-like-carrying mobile element (IS*Ecp1*-$bla_{CMY-2/4}$-*blc*-*sugE1*) which is flanked by two 13-bp imperfect inverted repeats (IV-R and IV-L) was integrated between the *traT* and *traU* in all seven plasmids. Integration of IS*Ecp1*-$bla_{CMY-2/4}$-*blc*-*sugE1* at the same site inside the *tra* locus is a solid indication that all the IncK2 plasmids analyzed have a common ancestor (**Figure 1**).

The same structure (IS*Ecp1*-$bla_{CMY-2/4}$-*blc*) has already been reported in IncK plasmid from *E. coli* of human origin in Spain and from *E. coli* from chicken meat in Norway (Porres-Osante et al., 2015; Mo et al., 2016), as well as in IncA/C plasmids from *Salmonella enterica* subsp. *enterica* serotype Newport in the USA (Cao et al., 2015). Notably, the only bla_{CMY-4}-carrying plasmid pDV10 found has also developed from the same progenitor as the other plasmids and was subjected to subsequent evolution via a Trp221Arg substitution in CMY-2.

The phylogenetic analysis of the IncK2 plasmids supports the hypothesis of a common ancestor. All the IncK2 plasmids share a nucleotide identity ranging from 86.1 to 98.0% which increases to 86.5 to 100% once the shufflon region has been removed. This similarity is remarkable given that the percentage of identity for the two reference plasmids IncK and IncB is maximally 78.7% and without the shufflon region 80.1% (**Figure 3**). The IncK2 plasmids differed from the IncK plasmid pCT not only by their incompatibility and replication gene, but also in their structure and base composition (**Figure 1**).

Taken together, these results lead to the conclusion that the IncK2 plasmids are an overall highly homogenous cluster although they are distributed in genetically diverse *E. coli* isolated from different sources and timeframes. The phylogenetic analysis points toward a common ancestor that has subsequently spread between the various reservoirs. Indeed, the most striking characteristic of the IncK2 group is its occurrence in different reservoirs (including poultry, poultry-meat, and humans isolates) from which they were isolated. There is strong evidence that all IncK2 plasmids originated from a common source due to: (i) the identical integration site of the bla_{CMY}-carrying mobile element; (ii) the overall 91.3% identity among all the plasmids.

Publications from Denmark, Japan, the Netherlands, Norway, Spain, and Sweden investigating pAmpC-producing *E. coli* isolates from healthy and hospitalized humans as well as strains from poultry and poultry-meat have reported a high prevalence of bla_{CMY-2} in both reservoirs (Dierikx et al., 2010; Börjesson et al., 2013; Voets et al., 2013; de Been et al., 2014; Egervärn et al., 2014; Huijbers et al., 2015; Porres-Osante et al., 2015; Hansen et al., 2016; Mo et al., 2016). These publications were all using the PBRT kit for characterization of their bla_{CMY-2}-carrying plasmids, whereby a substantial part of them was either identified as IncK plasmids or as IncB/O plasmids.

Based on the present study, it has to be noted that the IncK2 plasmid-carrying strains may have been falsely identified as positive for the IncK or IncB/O plasmid groups using the PBRT. Indeed, sequence alignment of our IncK2 plasmids with DNA sequences from the GenBank revealed that such plasmids have already been identified in *E. coli* from poultry origin in different countries. One plasmid (GenBank acc. no. JXMX01000007.1) isolated from *E. coli* strain 53C from retail chicken meat in the Netherlands (de Been et al., 2014) was almost identical to pDV45 (also from chicken meat) with 98.2% nucleotide identity and 100% without considering the shufflon. Another plasmid, pNVI1292, from *E. coli* strain 2012-01-1292 (GenBank acc. no. KU312044) from retail chicken meat in Norway (Mo et al., 2016) was nearly identical to pDV10 (also from poultry meat) and to pTMSA1088 (poultry) with 98.7 and 97.8% nucleotide identity, respectively, and 100% without considering the shufflon. Of note, strain 2012-01-1292 also belonged to ST38 like both strains harboring pDV10 and to pTMSA1088 indicating that specific clones may also contribute to the spread of the IncK2 plasmids.

The novel IncK2 group seems to be an emerging epidemic plasmid with a potential for dissemination in *E. coli* from different reservoirs including human and poultry. To our knowledge, this is the first time that a highly conserved plasmid family is described in isolates from human clinical samples, poultry, and poultry meat all coming from the same geographic region. However, further epidemiological studies are necessary to assess the scope of the spread of this plasmid in both human and animals. Nevertheless, the presence of a common plasmid in three different settings is alarming and should stimulate rapid introduction of adequate measures in the meat production chain to limit the spread of drug-resistant plasmids.

AUTHOR CONTRIBUTIONS

Conception and design (VP, AE, SNS, AC); acquisition of data (VP, AE, SNS, AC, ACo, SS); analysis and interpretation of data (VP, AE, SNS, AC); drafting the work (VP, AE, SNS, AC); critical revison for important intellectual content (all authors); final approval of the version to be published (all authors); agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved (all authors).

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Predominance of CTX-M-15 among ESBL Producers from Environment and Fish Gut from the Shores of Lake Victoria in Mwanza, Tanzania

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Extended-Spectrum Beta-Lactamase (ESBL)-producing bacteria are a common cause of healthcare and community-associated infections worldwide. The distribution of such isolates in the environment and their presence in fish as a result of sewage contamination is not well-studied. Here we examined fish and environmental samples from Mwanza city for the presence of ESBL-producing bacteria. From 196 fish sampled from local markets, 26 (13.3%) contained lactose-fermenting ESBL-producing bacteria, while 39/73 (53.4%) environmental samples from the same area were ESBL producers. Antibiotic resistance genes, multi locus sequence types (MLST) and plasmid replicon types in 24 selected isolates from both populations were identified with whole genome sequencing using Illumina MiSeq. Nine of eleven sequenced fish isolates had the $bla_{\text{CTX-M-15}}$ gene whereas 12/13 from environment carried $bla_{\text{CTX-M-15}}$. Antibiotic resistance genes encoding resistance to sulfonamides (*sul1/sul2*), tetracyclines [*tet(A)/tet(B)*] fluoroquinolones [e.g., *aac(6′)-Ib-cr*, *qnr*S1], aminoglycosides [e.g., *aac(3)-lld, str*B, *str*A,] and trimethoprim (e.g., *dfr*A14) were detected. *E. coli* sequence type ST-38 (2) and ST-5173 (2) were detected in isolates both from the environment and fish. IncY plasmids carrying $bla_{\text{CTX-M-15}}$, *qnr*S1, *str*A, and *str*B were detected in five environmental *E. coli* isolates and in one *E. coli* isolate from fish. Our data indicate spillage of resistant environmental isolates into Lake Victoria through the sewage system. Persistence of $bla_{\text{CTX-M-15}}$ in the Mwanza city environment is complex, and involves both clonal spread of resistant strains as well as dissemination by commonly occurring IncY plasmids circulating in isolates present in humans, the environment as well as in the food chain.

Keywords: $bla_{\text{CTX-M-15}}$, **fish, environment**

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INTRODUCTION

The extended-spectrum beta-lactamases (ESBL)-producing Gram-negative bacteria *Escherichia coli* and *Klebsiella* spp., particularly those producing CTX-M enzymes, have emerged as important causative agents of healthcare-associated infections across the world (Hawkey and Jones, 2009). Apart from being present in hospitals and clinics, ESBL-producing *E. coli* strains are prevalent in

the community and are reported to be responsible for community-acquired bacterial infections (Arpin et al., 2005; Pitout et al., 2005). Studies from different areas of the world show an association between the presence of ESBL-producing *E. coli* and exposure to either food or contaminated water (Ho et al., 2011; Laube et al., 2013; Xi et al., 2015). The presence of ESBL-producing *E. coli* in the community has led to the hypothesis that there could be a transmission of these strains from human waste to the environment (Martinez, 2009a). Evidence in the literature has also documented the probable horizontal transfer of resistance genes from either human sewage or clinical isolates to fish in rivers or lakes in which drainage of wastewater from treatment plants occurs (Kümmerer, 2009; Martinez, 2009b; Jiang et al., 2012; Blaak et al., 2014). Multiple genotypes of ESBL-producing *E. coli* have been found in animals and humans in Mwanza (Mshana et al., 2011, 2016; Seni et al., 2016). There are few studies from developed countries on the presence of ESBL-producing isolates in fish and environment samples and the role played by the food chain in transmission of resistance genes through contamination by human and animals sewage and anthropogenic activities in relation to water bodies (Zurfluh et al., 2013; Abgottspon et al., 2014). In Mwanza, a port city with a population of 750,000 located on the southern shores of Lake Victoria (Fitzpatrick et al., 2015), effluents from wastewater treatment plants and pit-latrines used by most of households located in the hills drain into the lake, which is also a source of fish consumed by residents as staple food. We previously used conventional phenotypic characterization and whole genome sequencing to examine samples obtained from hospitals, from rural farming communities including animals, farmers and households, in Mwanza for the presence of ESBL-producing *Enterobacteriaceae*. Our data indicate clonal spread of bacteria belonging to a small number of STs present in all populations investigated, but also suggest that commonly occurring promiscuous plasmids are involved in resistance dissemination (Fortini et al., 2015; Mshana et al., 2016). Here we examined for the presence of ESBL-producing bacteria in fish obtained from Lake Victoria as well as environmental samples obtained from the city. Our results indicate that *E. coli* genotypes that were observed in humans and animals before are also present in environment and Fish.

MATERIALS AND METHODS

Isolation and Identification of Bacterial Isolates

Ten fish markets located in different urban and rural sections of the Mwanza region were randomly selected for the study. These markets receive fishes obtained from different fishing sites within Lake Victoria. A total of 196 Nile tilapia (*Oreochromis niloticus*) fish from randomly picked vendors were sampled with an average of 15 to 20 fish at each market between July and September 2015. Fish were first washed with saline, following which a sterile surgical blade was used to open the carcass and to make a longitudinal incision along the gut. The incision was opened and the gut contents were swabbed using a sterile swab.

In addition, at ten sites including the Ngerengere River that crosses the city, drain waste and possible sewage from households located in the surrounding hills were sampled (environmental samples). Environmental samples included dirty muddy water samples from different location in the city (**Figure 1**). For each site between six to seven samples were taken from different locations (**Figure 1**). About 3 ml of each sample was collected using sterile 25 ml falcon tube (BD, Nairobi, Kenya). A sample was mixed with sterile 0.9% saline at ratio of 1:1 and vortexed to produce a homogenous solution.

Using swabs, MacConkey agar (Oxoid, Basingstoke, UK) plates supplemented with 2 mg/L cefotaxime (Medochemie Ltd, Limassol, Cyprus) were inoculated to screen for ESBL-producing *Enterobacteriaceae*. Enteric bacilli were identified using colony morphology and differentiated based on lactose fermentation on MacConkey agar. Single colonies from predominant lactose fermenting bacteria were picked for further identification using several biochemical tests (Triple Sugar Iron Agar, Simmons' citrate Agar, Sulfur-Indole-Motility test and Urease test) (Murray et al., 1995). In case of ambiguous results, the VITEK® 2 system (BioMérieux, Marcy l'Etoile, France) was used to confirm identification. A confirmed ESBL-producing *E. coli* isolate was used as a positive and *E. coli* ATCC 25922 as a negative control. ESBL isolates were stored as glycerol cultures at −80°C and used for further characterization.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was done by disk diffusion method as recommended by the Clinical Laboratory Standards Institute (CLSI) guidelines (Wayne, 2012). A bacterial suspension of 0.50 McFarland standard turbidity was prepared from pure culture. An inoculum was then plated on Mueller-Hinton Agar plates (HiMedia, Mumbai, India) and the following antibiotic disks were set: Tetracycline (2 μg), ciprofloxacin (5 μg), gentamicin (10 μg), or trimethoprim/sulphamethoxazole (1.25/23.75 μg) (Oxoid, Hampshire, UK). The plates were incubated aerobically at 37°C for 18–24h. The diameters of the respective zone of inhibitions were measured and interpreted following CLSI 2012 guidelines (Wayne, 2012). Disk approximation method based on CLSI guidelines was used to confirm ESBL production and for selected isolates ESBL production was further identified using VITEK® 2 system (BioMérieux, Marcy l'Etoile, France) and in addition the MIC for cefepime, carbapenems and colistin were determined in all selected isolates.

Using STATA version 11 the two-sample test of proportion was done to compare the rates of resistance between ESBL isolates from fish and those from environment. A p-value < 0.05 was used to indicate a significant difference at 95% confidence interval.

Whole Genome Sequencing

Eleven ESBL-producing isolates obtained from fish and 13 *E. coli* from the environmental samples were chosen for whole genome sequencing (WGS). DNA was isolated using the Purelink Genome DNA Mini kit (Invitrogen, Darmstadt, Germany) according to the manufacturer's instruction. WGS was carried

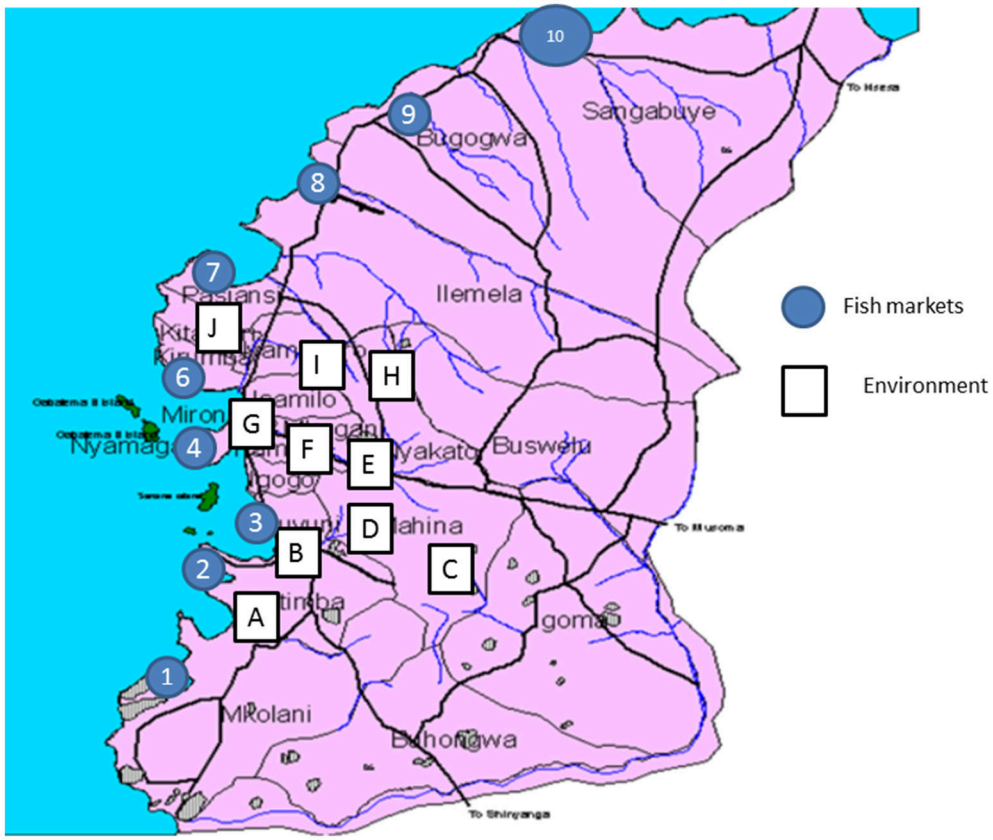

FIGURE 1 | Map of Mwanza City showing fish markets and sites from which environmental samples were obtained.

out using an Illumina Nextera XT library with 2x300bp paired-end reads on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA). The raw data was assembled using SPAdes (version 3.0) (Bankevich et al., 2012). Contigs from *E. coli* isolates were ordered by using MAUVE (Rissman et al., 2009) and *E. coli* MG1655 (accession number U00096.3) were chosen as a reference for all *E. coli* isolates, *Citrobacter freundii* strain P10159 for *Citrobacter braakii* isolates (accession number CP012554; no complete genome available for *C. braakii*), *Klebsiella pneumoniae* strain ATCC BAA-2146 (accession number CP006659) for *K. pneumoniae* and *Enterobacter cloacae* strain 34977 (accession number CP010376) for *E. cloacae* isolates. Pseudogenomes were created and whole genome phylogenetic analysis was subsequently performed by using ParSNP package of Harvest Suite (Treangen et al., 2014). The raw sequencing data of the sequenced isolates are available at the European Nucleotide Archive (ENA) under the project number PRJEB12361.

In silico Analyses of Resistance Genes, MLST, Plasmid Replicon Types, and Quinolone Resistance-Determining Regions (QRDR) Mutations

Sequences were analyzed for their multi locus sequence types, transferrable resistance genes, plasmid replicon types and pMLST using MLST 1.8, ResFinder, Plasmidfinder and pMLST software from the Center for Genomic Epidemiology (Larsen et al., 2012; Zankari et al., 2012; Carattoli et al., 2014). Search for plasmid-encoded heavy metal resistances and detergence resistance was performed using blastn with the references given in Supplementary Table 1.

The location of $bla_{CTX-M-15}$ was determined by analyzing the contigs harboring $bla_{CTX-M-15}$ using blastn. The whole genome sequences were compared with the plasmid pPGRT46 (accession number KM023153.1) using BRIG and blastn (Alikhan et al., 2011; Fortini et al., 2015). Quinolone resistance-determining regions (QRDR) mutations were identified by *silico* analysis by comparing the sequence using a reference sequence from a quinolone-susceptible *Enterobacteriaceae* strain (Weigel et al., 1998; Liu et al., 2012).

Ethical Approval

The protocol of this study was approved by CUHAS/BMC joint ethics and scientific review committee with reference CREC/019/2014.

RESULTS

Bacterial Isolates

A total of 26 (13.3%) lactose-fermenting ESBL-producing bacteria were isolated from gut samples of 196 wild Nile tilapia fish from Lake Victoria. Diverse bacterial species were

detected and included *C. braakii* (11/26, 42.3%), *E. cloacae* (5/26, 19.2%), *K. pneumoniae* (5/26, 19.2%), *E. coli* (4/26, 15.4%) and *Klebsiella oxytoca* (1/26, 3.9%). From 73 environmental samples 39 (53.4%) ESBL-producing enteric bacteria were isolated. Of these 39 isolates, 20 (51.3%) were *E. coli* and 19 (48.7%) were *K. pneumoniae*.

Antimicrobial Susceptibility Testing

All isolates had MICs for cefotaxime and cefepime of >32 and >8 μg/ml respectively. High resistance rates to co-trimoxazole ($n = 19$, 73.1%), ciprofloxacin ($n = 19$, 73.1%), and gentamicin ($n = 19$, 73.1%) and tetracycline ($n = 16$, 61.5%) were observed among the 26 ESBL-producing isolates from fish. Environmental isolates were resistant to co-trimoxazole ($n = 34$, 87.2%), ciprofloxacin ($n = 14$, 38%), and gentamicin ($n = 18$, 46.1%). ESBL-producing isolates from fish were significantly more resistant to gentamicin and ciprofloxacin ($p < 0.01$: **Table 1**). In addition, all isolates were sensitive to meropenem and imipenem (MIC <0.25 μg/ml), and all were sensitive to colistin.

Analysis of Antibiotic Resistance Genes

Each of the bacterial genome-sequenced fish and environmental isolates carried up to four different β-lactamase genes (ESBL, AmpC and other β-lactamases, **Table 2**). The most common ESBL gene was $bla_{CTX-M-15}$, present both in fish (9/11) and environmental isolates (12/13). The non-ESBL β-lactamase gene bla_{TEM-1} was present in 7/11 fish isolates and 9/13 environmental isolates, whereas bla_{OXA-1} was present in 6/11 fish isolates and 1/13 environmental isolates. *K. pneumoniae* isolates from fish were found to harbor bla_{SHV-11} and bla_{SHV-1} due to the fact that *K. pneumoniae* chromosomally possesses $bla_{SHV-1/-11}$. In addition, the fish isolates also harbored a number of AmpC type β-lactamase genes (bla_{ACT-15}, bla_{MIR-3}, bla_{CMY-37}, bla_{CMY-49}), which were not present in the environmental isolates. The latter two results might simply reflect the fact that while different types of enterobacterial species were isolated from fish, only *E. coli* were obtained from the environmental samples studied.

Commonly occurring aminoglycoside resistance genes detected in both fish and environmental isolates were *aac(6′)-Ib-cr* (6/24), *str*A/*str*B (15/24), *aac(3)-IId* (5/24), and *aad*A1 (4/24) (**Table 2**). Other aminoglycoside resistance genes were present either only in fish isolates (*aad*A2, *aac(3)-IIa*) or in environmental samples (*aad*A5). Quinolone resistance genes were detected in 8/11 (72.7%) of fish isolates and in 7/13 (53.7%) of environmental isolates that were sequenced. Quinolone resistance genes detected in both populations comprised of the *aac(6′)-Ib-cr* (6/24), *qnr*B1 (5/24), and *qnr*S1 alleles. Other quinolone resistance genes were only present in fish isolates (*qnr*B29, *qnr*B48, *oqx*A, *oqx*B).

Of the 24 sequenced isolates, 20 (83.3%) harbored sulfonamide resistance genes (*sul1* or *sul2*), and 19 of these isolates harbored in addition a resistance gene encoding trimethoprim resistance (*dfr*A14, $n = 11$; *dfr*A17, $n = 3$; *dfr*A1, *dfr*A18, *dfr*A30, *dfr*A5, *dfr*A7, each $n = 1$). Tetracycline resistance genes, [*tet(A)*, $n = 15$; *tet(D)*, $n = 3$], were present in 18/24 isolates. Altogether, there was a direct correlation between resistance phenotype and resistance genotype in all 24 isolates.

Using *in silico* analysis *par*C mutations were detected in 7 *E. coli*, only 2 isolates had S80I mutation that is associated with fluoroquinolone resistance, while *gyr*A mutations were observed in 9 isolates (2 *E. cloacae* and 7 *E. coli*). S83 (S83T, S83L, and S83A) mutations that are associated with fluoroquinolone resistance were observed in 5/9 isolates. One isolate had both S83L and D87N.

TABLE 1 | Rate of resistance of ESBL-producing isolates from fish and environment to SXT, TET, CIP, and CN.

Antibiotic	ESBL fish (26)	ESBL environment (39)	*P*-value
SXT	19 (73%, 95% CI 55.9–90.6)	34 (87.2%, 95% CI 76–97)	0.0776
TET	17 (65.4%, 95% CI 47–83)	27(69.2%, 95% CI 54.7–83.6)	0.3741
CIP	19 (73%, 95% CI 55.9–90.6)	15 (38.5%, 95% CI 23–53)	0.0032
CN	19 (73%, 95% CI 55.9–90.6)	18 (46.1%, 95% CI 37–50	0.001

SXT, sulphamethoxazole/trimethoprim; TET, tetracycline; CIP, ciprofloxacin, CN, gentamicin.

Plasmid-Encoded Heavy Metal Resistance Operons and Detergence Genes

An analysis for the presence of plasmid-located heavy metal- and detergent- resistance genes revealed, that 8/24 isolates harbored the *qacEdelta* gene, conferring resistance to tertiary ammonium compounds. Seven (29.2%) of isolates sequenced carried a mercury resistance operon originally described in plasmid R478, and 4/24 (16.6%) isolates had genes mediating resistance to silver. Only one isolate carried genes involved in resistance to copper together with nickel/cobalt efflux system (Supplementary Table 2).

Location of the bla_{CTX-M} Gene and AmpC Genes

Analysis of the whole genome sequences was used to identify and map the location of bla_{CTX-M} alleles. For 8/24 isolates, the $bla_{CTX-M-15}$ gene was located in the chromosome. In two isolates $bla_{CTX-M-15}$ was located on a phage-like plasmid, similar to the *E. coli* phage-like plasmid p1303_95 isolated from wound swab (isolate SO007, accession number of the reference: CP009168.1) and pECOH89 (isolate SO053, accession number of pECOH89: HG530657.1) (Falgenhauer et al., 2014). The isolate SO069 harbored $bla_{CTX-M-15}$ on an IncI1 plasmid similar to pESBL-EA11 (Ahmed et al., 2012). Six of 24 sequenced isolates (one fish and five environmental isolates, F044, SO005, SO008, SO025, SO037, SO063, all *E. coli*) harbored a resistance cassette similar to the one present in plasmid pPGRT46 (accession number KM023153.1) that included both *qnrS*1 as well as $bla_{CTX-M-15}$. The other isolates displayed $bla_{CTX-M-15}$ or $bla_{CTX-M-55}$ containing resistance cassettes with similarities to other ESBL-encoding plasmids (F025: pEC_L46, accession number GU371929.1; F102: pSTm-A54650, accession number LK056646.1; SO042: pSKLX3330, accession number KJ866866.1; SO060: pCA14, accession number CP009231.1). The isolates F016 and F017 did not harbor any ESBL

TABLE 2 | Antibiotic resistance phenotype and genotype of the sequenced isolates.

No	Species	Phenotypic resistance besides beta-lactams	Beta-Lactam Resistance genes	Trimethoprim resistance genes	Sulfonamide resistance genes	Aminoglycosides resistance genes	Quinolone resistance genes	Tetracycline resistance genes
F006	*Enterobacter cloacae*		*$bla_{CTX-M-15}$, bla_{TEM-1B}, bla_{ACT-15}, bla_{OXA-1}	–	–	–	–	–
F009	*Enterobacter cloacae*	CIP, SXT, TET, CN	*$bla_{CTX-M-15}$, bla_{TEM-1B}, bla_{ACT-15}, bla_{OXA-1}	*dfrA14*	*sul1*	*aadA1, aac(6′)-Ib-cr, aac(3)-IIa*	*aac(6′)-Ib-cr, qnrB1*	*tet(A)*
F016	*Enterobacter cloacae*		bla_{MIR-3}	–	–	–	–	–
F017	*Enterobacter cloacae*	CIP, TET, CN	bla_{TEM-1B}	–	*sul1*	*aadA1, aadA2*	–	*tet(A)*
F025	*Citrobacter braakii*	CIP, SXT, TET, CN	$bla_{CTX-M-15}$, bla_{OXA-1}, bla_{TEM-1B}, bla_{CMY-37}	*dfrA18*	*sul1, sul2*	*aac(3)-IIa, aac(6′)-Ib-cr, aadA2*	*aac(6′)-Ib-cr, qnrB29*	*tet(D)*
F044	*Escherichia coli*	CIP, SXT, TET, CN	$bla_{CTX-M-15}$, bla_{TEM-1B}	*dfrA14*	*sul2*	*strB, strA*	*qnrS1*	*tet(A)*
F080	*Escherichia coli*	CIP	*$bla_{CTX-M-15}$	–	–	–	*qnrB1*	–
F085	*Klebsiella pneumoniae*	CIP, SXT, TET, CN	*$bla_{CTX-M-15}$, bla_{OXA-1}, bla_{SHV-11}	*dfrA14*	*sul2*	*acc(3)-IId, aac(6′)-Ib-cr, strA, strB*	*aac(6′)-Ib-cr, qnrB1, oqxA, oqxB*	*tet(D)*
F086	*Klebsiella pneumoniae*	CIP, SXT, TET, CN	*$bla_{CTX-M-15}$, bla_{OXA-1}, bla_{SHV-11}	*dfrA14*	*sul2*	*acc(3)-IId, aac(6′)-Ib-cr, strA, strB*	*aac(6′)-Ib-cr, qnrB1, oqxA, oqxB*	*tet(D)*
F096	*Klebsiella pneumoniae*	CIP, SXT, TET, CN	bla_{TEM-1B}, $bla_{CTX-M-15}$, bla_{SHV-1}	*dfrA30*	*sul2*	*aac(3)-IId*	*oqxA, oqxB*	–
F102	*Citrobacter braakii*	CIP, SXT, TET, CN	$bla_{CTX-M-15}$, bla_{CMY-49}, bla_{TEM-1B}, bla_{OXA-1}	*dfrA14*	*sul2*	*aac(3)-IIa, aac(6′)-Ib-cr, aadA1, strA, strB*	*aac(6′)-Ib-cr, qnrB48*	–
SO005	*Escherichia coli*	SXT, TET, CN	$bla_{CTX-M-15}$, bla_{TEM-1B}	*dfrA14*	*sul2*	*strA, strB*	*qnrS1*	*tet(A)*
SO007	*Escherichia coli*	SXT, TET, CN	$bla_{CTX-M-15}$, bla_{TEM-1B}	*dfrA7*	*sul1, sul2*	*strA, strB*		*tet(A)*
SO008	*Escherichia coli*	CIP, SXT, TET, CN	$bla_{CTX-M-15}$, bla_{TEM-1B}	*dfrA14*	*sul2*	*strA, strB*	*qnrS1*	*tet(A)*
SO009	*Escherichia coli*	SXT, TET, CN	*$bla_{CTX-M-15}$	*dfrA17*	*sul1*	*aadA5*		*tet(A)*
SO025	*Escherichia coli*	SXT, TET, CN	$bla_{CTX-M-15}$	*dfrA14*	*sul2*	*strA, strB*	*qnrS1*	*tet(A)*
SO035	*Escherichia coli*	SXT, TET, CN	*$bla_{CTX-M-15}$, bla_{TEM-1B}	*dfrA1*	*sul2*	*aadA1, strA, strB,*		*tet(A)*
SO037	*Escherichia coli*	SXT, TET	$bla_{CTX-M-15}$, bla_{TEM-1B}	*dfrA14*	*sul2*	*strA, strB*	*qnrS1*	*tet(A)*
SO038	*Escherichia coli*	CIP	*$bla_{CTX-M-15}$	–	–		*qnrB1*	–
SO042	*Escherichia coli*	SXT, TET, CN	$bla_{CTX-M-15}$, bla_{TEM-1B}	*dfrA17*	*sul1, sul2*	*aac(3)-IId,aadA5,strA, strB*	–	*tet(A)*
SO053	*Escherichia coli*	CIP, SXT, TET, CN	$bla_{CTX-M-55}$, bla_{TEM-1B}	*dfrA5*	*sul2*	*strA, strB*	–	*tet(A)*
SO060	*Escherichia coli*	CIP, SXT, TET, CN	$bla_{CTX-M-15}$, bla_{OXA-1}	*dfrA17*	*sul1, sul2*	*aac(6′)-Ib-cr, aadA5, strA, strB*	*aac(6′)-Ib-cr*	*tet(A)*
SO063	*Escherichia coli*	CIP, SXT, TET, CN	$bla_{CTX-M-15}$, bla_{TEM-1B}	*dfrA14*	*sul2*	*strA, strB*	*qnrS1*	*tet(A)*
SO069	*Escherichia coli*	SXT, TET	$bla_{CTX-M-15}$, bla_{TEM-1B}	*dfrA14*	*Sul1*	*aac(3)-IId,strA, strB*		*tet(A)*

* *in these isolates $bla_{CTX-M-15}$ was located in the chromosome, CIP, ciprofloxacin; SXT, trimethoprim/sulphamethoxazole; CN, gentamicin; TET, tetracycline.*

resistance gene. It should be noted that because conjugation experiments were not done plasmid location could only be suggested.

In 13 of 22 ESBL-encoding isolates, the environment of the ESBL gene was characterized by a Tn*3* transposon deletion in the vicinity of the *bla*CTX-M gene (**Figure 2**). This included four isolates harboring a chromosomally located *bla*CTX-M-15 allele. The other isolates, including two chromosomally located *bla*CTX-M-15 and the single *bla*CTX-M-55 isolate, did not have any Tn*3*-related sequences. All ampC genes detected were located in the chromosomes (F006, F009, F016, F025, F102).

Multi Locus Sequence Types and Whole Genome Phylogeny

E. coli isolates sequence type (ST) ST-38 and ST-5173 were present in both fish and environmental isolates. Among the 13 *E. coli* environmental isolates 12 different STs were observed. The fifteen *E. coli* isolates, 13 from the environment and 2 from fish, grouped into five clusters using genome-based phylogenetic analysis. The tree was generated on the basis of a core genome accounting for 78% (3.62 Mbp) of the reference genome (*E. coli* MG1655). The two *E. coli* isolates from fish have 100% similarity with respective ST isolates derived from the environment (SO025 and F044, ST-38; F080 and SO038, ST-5137; **Figure 3**). *E. cloacae*

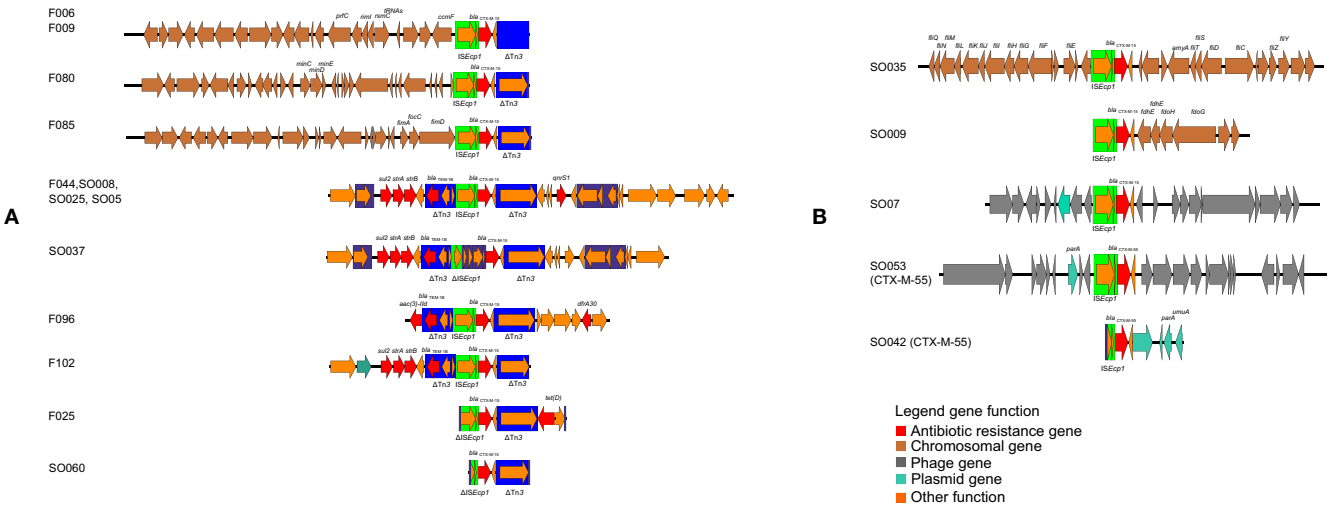

FIGURE 2 | Schematic depiction of the genetical environment of *bla*CTX-M genes in the *bla*CTX-M-encoding isolates. (A) Genetical environments associated with Tn*3* transposon deletion, **(B)** gentical environments not associated with Tn*3* transposon deletion.

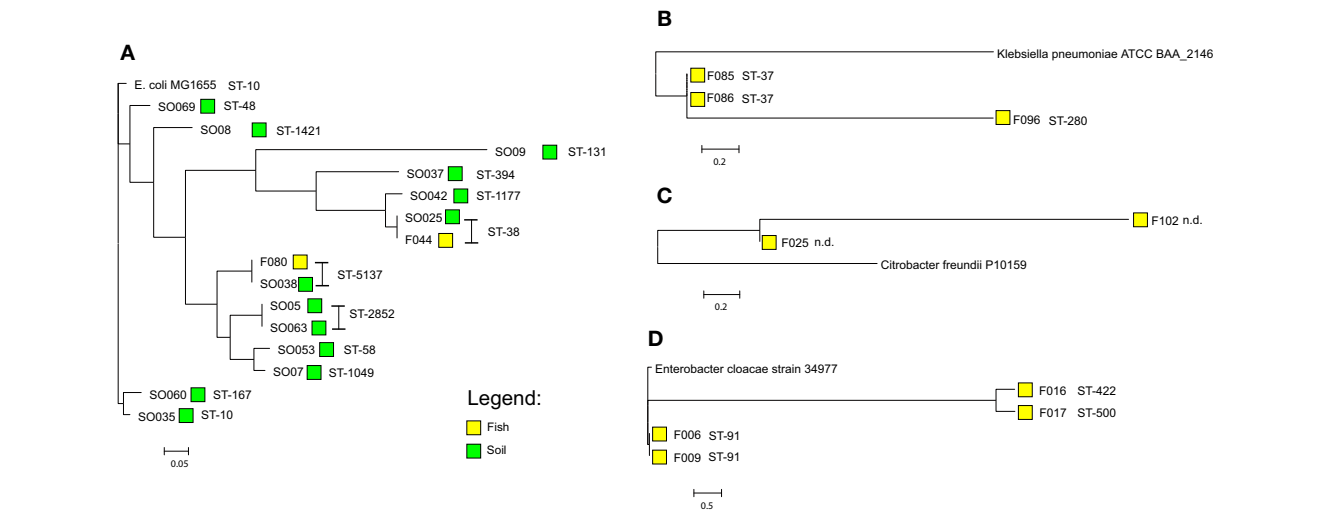

FIGURE 3 | Core-genome based phylogeny of the sequenced isolates according to their species (A) *Escherichia coli* (reference *E. coli* MG1655, accession number U00096.3), **(B)** *Klebsiella pneumoniae* (reference *Klebsiella pneumoniae* strain ATCC BAA-2146, accession number CP006659), **(C)** *Citrobacter braakii* (using *Citrobacter freundii* P10159, accession number CP012554.1 as a reference as no complete genome of *Citrobacter braakii* is presently available), **(D)** *Enterobacter cloacae (E. cloacae* strain 34977, accession number CP010376). The phylogenetic analysis was performed using Harvest Suite.

TABLE 3 | Baseline characteristics of the sequenced isolates.

No	Species	Sequence type	Phylogroups	Source
F006	*Enterobacter cloacae*	ST-91		Fish
F009	*Enterobacter cloacae*	ST-91		Fish
F016	*Enterobacter cloacae*	ST-422		Fish
F017	*Enterobacter cloacae*	ST 500		Fish
F025	*Citrobacter braakii*	*		Fish
F044	*Escherichia coli*	ST-38	E	Fish
F080	*Escherichia coli*	ST-5173	B1	Fish
F085	*Klebsiella pneumoniae*	ST-37		Fish
F086	*Klebsiella pneumoniae*	ST-37		Fish
F096	*Klebsiella pneumoniae*	ST-280		Fish
F102	*Citrobacter braakii*	*		Fish
SO005	*Escherichia coli*	ST-2852	B1	Environment
SO007	*Escherichia coli*	ST-1049	B1	Environment
SO008	*Escherichia coli*	ST-1421	A	Environment
SO009	*Escherichia coli*	ST-131	B2	Environment
SO025	*Escherichia coli*	ST-38	E	Environment
SO035	*Escherichia coli*	ST-10	E	Environment
SO037	*Escherichia coli*	ST-394	E	Environment
SO038	*Escherichia coli*	ST-5173	B1	Environment
SO042	*Escherichia coli*	ST-1177	E	Environment
SO053	*Escherichia coli*	ST-58	B1	Environment
SO060	*Escherichia coli*	ST-167	E	Environment
SO063	*Escherichia coli*	ST-2852	B1	Environment
SO069	*Escherichia coli*	ST-48	E	Environment

*There is no MLST scheme available for Citrobacter braakii.

isolates from fish displayed three different STs (ST-91 $n = 2$; ST-422 $n = 1$; ST-500 $n = 1$), based on an analysis of core genome content of around 68% (3.33 Mbp) using *E. cloacae* strain 34977 as reference. The two ST-91 isolates were more closely related to each other, than the other two isolates. *K. pneumoniae* isolates were typed as ST-37 (2/3) with a single isolate as ST-280 (1/3). The core genome with *K. pneumoniae* strain ATCC BAA-2146 (accession number CP006659) as reference accounted for 88% (4.78 Mbp) of its genome size. The ST-37 and ST-280 are quite different from one other. The *C. braakii* isolates could not be assigned to any ST, because there is no typing scheme available. Based on 82% (4.17 Mbp) core genome size using *C. freundii* strain P10159 as a reference genome, these two isolates are very distinct from each other (**Figure 3**). *E. coli* were grouped in different phylogroups based on scheme of Clermont et al. (2000). A total of 7/15(46%) of *E. coli* isolates belong to the newly described phylogroups E (**Table 3**).

Plasmid Replicon Types

Of the eleven fish isolates tested, seven (63.6%) were found to carry IncF plasmid replicon types as compared to 5/13 (38.5%) of environmental isolates (**Table 4**). The IncF pMLST types detected in fish isolates were F-: A-:B36, K4:A-:B- and K5:A-:B-. For the environmental isolates IncF pMLST types detected were F2: A-:B-, F29:-A-:B10 and F31:A4:B1. The IncI1 plasmids detected in *E. coli* from environment were classified using pMLST either as ST-31 and unknown pMLST while that in *E. coli* from fish had an unknown IncI1 pMLST. Two out of four *E. cloacae* isolates from fish harbored IncF plasmids with identical pMLST (F-:A-:B36). We extracted contig sequences of the F044, SO005, SO008, SO025, SO037, SO063, carrying the resistance cassette present in pPGRT46 (Fortini et al., 2015) (accession no. KM023153) and compared them to this plasmid to examine for overall homology. These isolates showed an overall overlap of between 76 and 90% in their nucleotide sequences with plasmid pPGRT46 (**Figure 4**).

DISCUSSION

Lake Victoria is Africa's largest lake by area, and borders three East-African countries: Tanzania (49%), Uganda (45%), and Kenya (6%). It is not only the major source of fish consumed by Mwanza residents but also receives biologically treated wastewater effluent from Mwanza city, where a tertiary hospital serving eight regions in Tanzania is located. Studies performed in this hospital have shown high rates of ESBL-producing *K. pneumoniae* and *E. coli* carrying $bla_{CTX-M-15}$ in IncF plasmids (Mshana et al., 2011, 2013). In addition recent studies involving isolates from animals and humans from community have shown the $bla_{CTX-M-15}$ allele to be predominant (Mshana et al., 2016; Seni et al., 2016).

In the present study, a significant proportion of fish gut and environmental samples were positive for ESBL-producing bacteria. As previously observed in isolates from humans in hospitals (Mshana et al., 2011, 2013), the majority of these isolates were multiply resistant to co-trimoxazole, gentamicin, tetracycline, and ciprofloxacin. In addition, it was observed that ESBL isolates from fish were significantly more resistant to ciprofloxacin and gentamicin. This could be explained by the fact majority of the environment isolates were *E. coli* while other genera such as Citrobacter and Enterobacter which tend to be resistant to ciprofloxacin and gentamicin (Dos Santos et al., 2015) formed majority of isolates from Fish. Such high AMR rates detected in the isolates from gut contents of wild fish are of concern and indicate strong anthropogenic environmental contamination of the Lake Victoria. The presence of ESBL isolates in the environmental samples suggests that the Lake Victoria is being contaminated by sewage from the hospitals and from animals (Mshana et al., 2009; Kayange et al., 2010; Moremi et al., 2014; Seni et al., 2016). Farming activities that use of animal manure together with agricultural waste from the community generate steady contaminated effluents along the borders of Lake Victoria. Climatic conditions including sustained periods of rain or man-made erosion may further contribute to increasing the presence of these bacteria in the lake.

The prevalence of ESBL-producing *E. coli* from wild fish was 2% (4/196) which is comparable to the results previously reported from China (Jiang et al., 2012) but lower than in Egypt which reported a prevalence of 4% (Ishida et al., 2010). Unlike the two studies from Egypt and China which were conducted among farmed fish with antibiotic exposure, the investigated isolates here were from uncultured free-living fish and emphasize the

TABLE 4 | Plasmid characteristics of the sequenced isolates, n.t.: not typable using IncHI1 pMLST scheme (no pMLST alleles present); # variants with homology or coverage less than 100%.

No	Species	Plasmid Replicon type	pMLST	ESBL gene	accession number of best reference	location
F006	*Enterobacter cloacae*	IncFII, IncFIB	F-:A-:B36#	$bla_{CTX-M-15}$	CP011650.1	Chromosome
F009	*Enterobacter cloacae*	IncFII, IncFIB	F-:A-:B36#	$bla_{CTX-M-15}$	CP011650.1	Chromosome
F016	*Enterobacter cloacae*	IncFIB(K)	F-:A-:B-	no ESBL gene	/	/
F017	*Enterobacter cloacae*	IncFII, IncR	F-:A-:B-	no ESBL gene	/	/
F025	*Citrobacter braakii*	No replicon		$bla_{CTX-M-15}$	GU371929.1	Plasmid
F044	*Escherichia coli*	IncI1, IncY	I1: new	$bla_{CTX-M-15}$	KM023153.1	Plasmid
F080	*Escherichia coli*	No replicon	–	$bla_{CTX-M-15}$	CP011018.1	Chromosome
F085	*Klebsiella pneumoniae*	IncFII, IncFIB(K), IncHI1B	F: K4:A-:B-, IncHI1: n.t.	$bla_{CTX-M-15}$	CP006659.2	Chromosome
F086	*Klebsiella pneumoniae*	IncFII, IncFIB(K), IncHI1B	F: K4:A-:B-, IncHI1: n.t.	$bla_{CTX-M-15}$	CP006659.2	Chromosome
F096	*Klebsiella pneumoniae*	IncFII(K), IncR	K5:A-:B-	$bla_{CTX-M-15}$	KM023153.1	Plasmid
F102	*Citrobacter braakii*	No replicon	–	$bla_{CTX-M-15}$	LK056646.1	Plasmid
SO005	*Escherichia coli*	IncY	–	$bla_{CTX-M-15}$	KM023153.1	Plasmid
SO007	*Escherichia coli*	IncI1, IncP, IncY	I1: new	$bla_{CTX-M-15}$	CP009168.1	Plasmid
SO008	*Escherichia coli*	No replicon	–	$bla_{CTX-M-15}$	KM023153.1	Plasmid
SO009	*Escherichia coli*	IncFII, IncFIA	F2:A1:B-	$bla_{CTX-M-15}$	CP013658.1	Chromosome
SO025	*Escherichia coli*	IncY	–	$bla_{CTX-M-15}$	KM023153.1	Plasmid
SO035	*Escherichia coli*	No replicon	–	$bla_{CTX-M-15}$	LM995868.1	Chromosome
SO037	*Escherichia coli*	IncFII, IncY	–	$bla_{CTX-M-15}$	KM023153.1	Plasmid
SO038	*Escherichia coli*	No replicon	–	$bla_{CTX-M-15}$	CP011018.1	Chromosome
SO042	*Escherichia coli*	IncFII, IncFIB	F29:A-:B10	$bla_{CTX-M-55}$	KJ866866.1	Plasmid
SO053	*Escherichia coli*	IncFII, IncFIB, IncQ1	F2:A-:B1	$bla_{CTX-M-15}$	HG530657.1	Plasmid
SO060	*Escherichia coli*	IncFII, IncFIA, IncFIB, IncFII	F31#:A4:B1	$bla_{CTX-M-15}$	CP009231.1	Plasmid
SO063	*Escherichia coli*	IncY	–	$bla_{CTX-M-15}$	KM023153.1	Plasmid
SO069	*Escherichia coli*	IncI1	I1: ST-31	$bla_{CTX-M-15}$	CP003290.1	Plasmid

role of environmental pollution with AMR isolates as previously published (Kümmerer, 2009; Guenther et al., 2011).

As was already noted in studies from the Asian, European and the African continents which documented the emergence and spread of $bla_{CTX-M-15}$ in humans and cattle (Madec et al., 2012; Zhang et al., 2013; Rafaï et al., 2015), CTX-M-15 was also found to be the predominant allele in this study. The gene was located on the chromosome or on different plasmids indicating extreme environmental mobility. The $bla_{CTX-M-15}$ allele has been reported in other studies (Dobiasova et al., 2015; Fortini et al., 2015; Ibrahim et al., 2015) of Gram-negative bacterial isolates from humans, fish, and animals, suggesting a transmission/circulation of this gene among different settings.

Two plasmid-mediated AmpC enzymes were detected in this study from fish isolates: bla_{MIR-1} and bla_{ACT-15}, this is in contrast with a previous report that showed the presence of plasmid-mediated bla_{CMY-2} allele in fish (Welch et al., 2009). No isolates carrying bla_{CMY-2} were detected in this study. However, a chromosomally located bla_{CMY-37} which had previously been described in *C. freundii*, was detected in a *C. braakii*, (Ahmed and Shimamoto, 2008). One resistant isolate harbored neither a plasmid-based AmpC nor a detectable ESBL enzyme (F017) suggesting the resistance observed might be due to unknown mechanisms or the presence of resistance genes in the chromosome. The chromosomally located genes cannot be detected using the software from the Center for Genomic Epidemiology because the database targets for transferable resistance genes only. As in previous studies performed in China and Egypt among farmed fish (Ishida et al., 2010; Jiang et al., 2012) plasmid-mediated quinolone resistance genes such as *aac(6′)-Ib-cr, qnr*B and *qnr*S were also detected in this study in both sampled populations.

E. coli ST-38 which was also observed in humans in hospitals, humans in the community and in livestock/companion/domestic animals in Mwanza city (Mshana et al., 2011, 2016; Seni et al., 2016) was detected in this study in both fish and environmental samples. According to the new phylogroup scheme by Clermont et al. (2000), all these Isolates (human, livestock/companion animal, fish/environment) were grouped as the new phylogroup E emphasizing the uniformity of these strains in our setting. The ST-38 *E. coli* from fish and the environment as well as other ST types from environment harbor resistance cassettes that include CTX-M-15 and QnrS1. A similar resistance cassette was previously detected on an IncY plasmid detected in isolates from healthy pregnant women in Nigeria, and from ESBL-producing isolates from livestock and companion animal isolates in the Mwanza region (Fortini et al., 2015; Seni et al., 2016). The sequence overlap with the plasmid pPGRT46 was very high (up to 90%). These findings draw attention to the likelihood that this resistance cassette and its

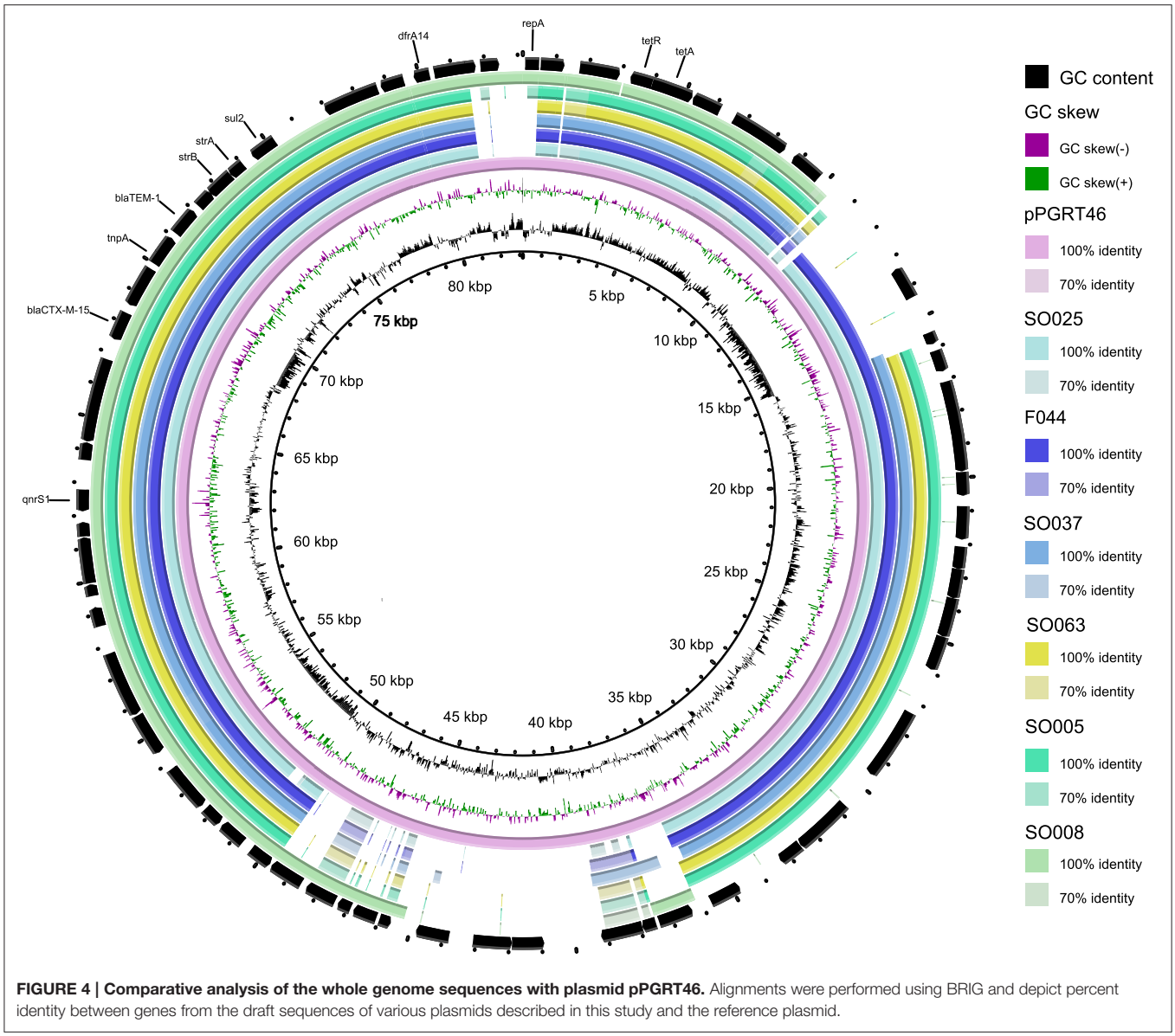

FIGURE 4 | Comparative analysis of the whole genome sequences with plasmid pPGRT46. Alignments were performed using BRIG and depict percent identity between genes from the draft sequences of various plasmids described in this study and the reference plasmid.

plasmid have spread centrally across the African continent into multiple *E. coli* genotypes and in different environments.

The *E. coli* ST-38 was observed in both fish and environment isolates as documented above was previously detected in humans and animals in the same setting. This finding is worrisome as zoonotic transmission is possible and its association with human colonization and infection might pose treatment challenges to human health. The transmission and persistence of ESBL-producing bacteria through the food chain and environment through sewage contamination has been documented previously (Beninati et al., 2015; van Hoek et al., 2015). The findings of this study suggest the possibility of the transmission of both ESBL-producing clones and plasmids between humans and wild fish through environmental contamination and indicate that anthropogenic activities and the food chain as potential factors for the persistence and dissemination of *bla*$_{CTX-M-15}$ in Mwanza City. The current study did not observed *E. coli* ST-131 from fish however it was obtained from environment; this could be due to that only 2 out 4 isolates from fish were sequenced. Further studies with large number of *E. coli* from fish are needed to confirm the role of food chain in the persistence of *bla*$_{CTX-M-15}$ in Mwanza city.

CONCLUSION

This is the first report on the epidemiology of ESBL-producing Gram-negative bacteria from fish in Lake Victoria and its surrounding environment. More than 70% of the sequenced bacterial isolates carried quinolone and aminoglycoside resistance genes. Detection of isolates/plasmids carrying *bla*$_{CTX-M-15}$ which have been also found in humans and

companion/livestock animals in the same region suggest environmental contamination with sewage effluents from humans an animal sources. Our data suggest that additional efforts to implement better sanitation and control sewage management are warranted. Many common illnesses in particular diarrhea, can be attributed to poor sanitation and unsafe water (Cheng et al., 2005) which exacerbates antibiotic use and contributes significantly to the problem of antibiotic resistance. Policy measures to improve water and sanitation quality could greatly contribute to the reduction of ESBL-resistance in this region.

AUTHOR CONTRIBUTIONS

NM, MM, CI, TC, and SM conceived, designed and executed the study; EM, NM, and SM collected the data and samples; NM, EM, and SM performed laboratory analysis; LF and TC performed WGS; LF, HG, TC, and SM analyzed the data; NM, LF, TC, and SM wrote the manuscript which was critically reviewed by all authors. All authors have read and approved the final draft of the manuscript.

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SUPPLEMENTARY MATERIAL

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frontiers in Microbiology

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Prevalence and Molecular Characteristics of Extended-Spectrum β-Lactamase Genes in *Escherichia coli* Isolated from Diarrheic Patients in China

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Background: The emergence and spread of antimicrobial resistance has become a major global public health concern. A component element of this is the spread of the plasmid-encoded extended-spectrum β-lactamase (ESBL) genes, conferring resistance to third-generation cephalosporins. The purpose of this study was to investigate the molecular characteristics of ESBL-encoding genes identified in *Escherichia coli* cultured from diarrheic patients in China from 2013 to 2014.

Materials and Methods: A total of 51 *E. coli* were confirmed as ESBL producers by double-disk synergy testing of 912 *E. coli* isolates studied. Polymerase chain reaction (PCR) and DNA sequencing were performed to identify the corresponding ESBL genes. Susceptibility testing was tested by the disk diffusion method. Plasmids were typed by PCR-based replicon typing and their sizes were determined by S1-nuclease pulsed-field gel electrophoresis. Multi-locus sequence typing (MLST) and phylogrouping were also performed. Broth mating assays were carried out for all isolates to determine whether the ESBL marker could be transferred by conjugation.

Results: Of the 51 ESBL-positive isolates identified, $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{OXA}, and bla_{SHV} were detected in 51, 26, 3, 1 of these isolates, respectively. Sequencing revealed that 7 $bla_{\text{CTX-M}}$ subtypes were detected, with $bla_{\text{CTX-M-14}}$ being the most common, followed by $bla_{\text{CTX-M-79}}$ and $bla_{\text{CTX-M-28}}$. Of the 26 TEM-positive isolates identified, all of these were $bla_{\text{TEM-1}}$ genotypes. All isolates contained one to three large plasmids and 10 replicon types were detected. Of these, IncFrep ($n = 50$), IncK/B ($n = 31$), IncFIB ($n = 26$), IncB/O ($n = 14$), and IncI1-Ir ($n = 8$) replicon types were the predominating incompatibility groups. Twenty-six isolates demonstrated the ability to transfer their cefotaxime resistance marker at high transfer rates. MLST typing identified 31 sequence

types and phylogenetic grouping showed that 12 of the 51 donor strains belonged to phylogroup B2.

Conclusion: This study highlights the diversity of the ESBL producing *E. coli* and also the diversity of ESBL genes and plasmids carrying these genes in China, which poses a threat to public health.

Keywords: *Escherichia coli*, human, extended-spectrum β-lactamase genes, horizontal gene transfer, conjugation, S1 nuclease digestion, China

INTRODUCTION

Extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* are a frequent cause of community- and hospital-acquired infections and one of the leading causative agents of infections worldwide (Hampton, 2013; Nischal, 2014). *E. coli* can become resistant to extended-spectrum cephalosporins by mutational overproduction of AmpC and/or by expression of acquired ESBLs. The latter emerged in the 1980s as derivatives of TEM (named after the patient Temoneira) and SHV (sulfhydryl reagent variable) enzyme types (Gutkind et al., 2013). The genes encoding these acquired enzymes are associated with plasmids with the potential for horizontal dissemination, and the most widespread ESBL type identified is CTX-M (Gutkind et al., 2013). These ESBL genotypes have spread in a pandemic manner and been associated with outbreaks in hospitals and communities worldwide (Gutkind et al., 2013).

Plasmid-mediated transfer of drug resistance-encoding genes among bacterial species is considered to be one of the most important mechanisms driving the dissemination of multi-drug resistance (MDR). An important element of this MDR dissemination is the transmission of the plasmid-encoded ESBL genes, which confer resistance to third-generation cephalosporins. Infection with ESBL-positive bacteria frequently results from inappropriate use of antimicrobial compounds in human and veterinary medicine a feature that also constitutes a risk factor for selection and dissemination of resistant clones (Gutkind et al., 2013; Hamprecht et al., 2016). Consequently, the treatment options for infections caused by ESBL-producing microorganisms are limited.

ESBL-producing *E. coli* has been reported from China, mostly from extra-intestinal *E. coli* cultured from patient specimens such as bloodstream infections (BSIs), along with intra-abdominal infections (IAIs) and urinary tract infections (UTIs). Some 67.8% of the isolates from BSIs were identified to be positive for the predominant CTX-M enzymes, CTX-M-14 and CTX-M-15 (Wang S. et al., 2016), 68.2% isolates were from UTIs and ICU and which carried the CTX-M-15 gene (Liu et al., 2015) and 69.6% were from IAIs and were also found to be ESBL-producing *E. coli* (Fan et al., 2016). However, there is limited epidemiological information on ESBL-producing *E. coli* from diarrheic patients. In this paper, we report on the isolation and characterization of a collection of ESBL-producing *E. coli* isolated from diarrheic patients in China. The aims of this study were (i) to identify the plasmids carrying ESBL-encoding genes, (ii) to determine the phylogenetic grouping, the multi-locus sequence typing (MLST), and the pathogenic grouping, and (iii) to determine the plasmid replicon types and S1 nuclease-based plasmid profiles.

MATERIALS AND METHODS

Ethics Statement

Fecal samples were acquired with the written consent from all patients. This study was reviewed and approved by the ethics committee of China Center for Disease Prevention and Control, according to the medical research regulations of the Ministry of Health, in China. All of this research work was conducted within China.

Bacteria and Growth Conditions

Fifty-one isolates including 27 from Beijing, 9 from Guangxi province, 9 from Henan province, and 6 from Sichuan province were confirmed as ESBL producers by the double-disk synergy test from 912 *E. coli* recovered from diarrhea cases. All human samples were collected in urban areas, and each individual was tested only once. The protocols for isolating *E. coli* were described previously (Wang et al., 2013).

Isolates from patients with typical *E. coli* phenotypes were confirmed by the API 20E biochemical gallery (bioMérieux, Beijing, China). Those *E. coli* expressing an ESBL phenotype were selectively enriched for study using Luria Bertani (LB) supplemented with 2 mg/L cefotaxime. All *E. coli* isolates that were recovered were further screened for ESBL production by determination of synergy between 0.25 and 128 mg/L ceftazidime or cefotaxime and 4 mg/L clavulanic acid (CLSI, 2012). Isolates showing a $\geq$3 twofold concentration decrease in an minimal inhibitory concentration (MIC) for either ceftazidime or cefotaxime tested in combination with clavulanic acid versus the MIC of the agent when tested alone were considered as ESBL-producing (for example, ceftazidime MIC = 8 mg/L; ceftazidime–clavulanic acid MIC = 1 mg/L; CLSI, 2012). *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as quality control microorganisms in antimicrobial susceptibility tests (CLSI, 2012). All confirmed isolates were maintained in brain heart infusion broth containing 50% [v/v] glycerol in a −70°C freezer for subsequent genotypic and phenotypic characterization.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of all *E. coli* isolates was determined using the agar dilution method and interpreted according to the Clinical and Laboratory Standards Institute guidelines

(CLSI, 2012) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST-2012)[1]. The following antimicrobial compounds were assessed: ampicillin (AMP, 1–128 mg/L), cefazolin (CZO, 0.5–64 mg/L), cefotaxime (CTX, 0.015–128 mg/L), cefotaxime–clavulanic acid, ceftazidime (CAZ, 0.03–64 mg/L), ceftazidime–clavulanic acid, chloramphenicol (CHL, 1–128 mg/L), ciprofloxacin (CIP, 0.015–512 mg/L), gentamicin (GEN, 0.125–64 mg/L), imipenem (IMP, 0.03–16 mg/L), tetracycline (TET, 0.25–64 mg/L), tigecycline (TGC, 0.015–32 mg/L), and trimethoprim–sulfamethoxazole (SXT, 0.06/1.19-16/304 mg/L). MDR was defined as resistance to three or more different classes of agent (Wang et al., 2013).

PCR Amplification and DNA Sequence Analysis of ESBLs

The following ESBLs resistance determinants were investigated by polymerase chain reaction (PCR): bla_{carB}, bla_{CMY-2}, $bla_{\text{CTX-M}}$, bla_{OXA}, bla_{SHV}, bla_{TEM} (Xu et al., 2005; Karczmarczyk et al., 2011). All PCR products amplified from bla_{CTX} and bla_{TEM} genes were commercially sequenced (Takara Biotechnology Cooperation, Dalian, China) and subsequently analyzed using the BLAST program[2].

Plasmid Characterization: S1 Nuclease Digestion and PCR-Based Plasmid Replicon Typing

S1-nuclease (Promega, Madison, WI, USA) digestion as well as pulsed-field gel electrophoresis (PFGE) analysis was performed for all 51 isolates. Briefly, the procedure included a lysis step of the bacterial cells embedded in agarose plugs followed by digestion with 8 U S1 nuclease at 37°C for 30 min. Finally, each plasmid sample was resolved by PFGE in a Chef-Mapper® XA System (Bio-Rad, USA) at 14°C, with a switch time between 1 and 12 s, at 6 V/cm on a 120° angle in 0.5× tris-boric acid-ETDA (TBE) buffer for 18 h. Each DNA band identified was considered as a unit length of linear plasmid. The approximate sizes of plasmids were determined by comparing profiles with *Xba*I-digested DNA from *Salmonella* serotype Braenderup H9812. Plasmids were assigned to incompatibility groups on the basis of the presence of specific replicon sequences identified by PCR using the primers previously designed and the corresponding amplification protocols described (Wang et al., 2013).

Conjugation-Based Mating Experiments and Verification

Conjugation experiments were performed using a broth mating protocol to determine if plasmids coding for CTX-M and TEM enzymes could be transferred (Barton et al., 1995). Fifty-one donors were used for mating with a sodium azide-resistant *E. coli* J53 as previously described (Wang et al., 2013). Transconjugants were selected on LB agar plates containing sodium azide (100 mg/L) and cefotaxime (2 mg/L). PCR amplification, antimicrobial susceptibility testing, plasmid replicon typing, and S1-PFGE were performed for all the transconjugations to determine the presence of ESBL genes, antibiotic phenotypes and incompatibility groups, respectively (Wang et al., 2013).

Phylogenetic Grouping

Phylogenetic groups were determined for each *E. coli* isolate using an established multiplex PCR targeting *chuA* (279 bp), *yjaA* (211 bp), and *tspE4* (152 bp) according to the protocol of Clermont et al. (2000). The method was previously developed to classify *E. coli* into four phylogenetic groups designated A, B1, B2, and D.

Multi-locus Sequence Typing of Isolates

MLST analysis was conducted by sequencing fragments of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icdF*, *mdh*, *purE*, *recA*) and sequence types (STs) were assigned using the *E. coli* MLST website (Wirth et al., 2006). eBURST was performed for MLST analysis.

Detection of Virulence Genes of Diarrheagenic *E. coli*

Five distinct classes of diarrheagenic *E. coli* (DEC) are recognized as being associated with diarrheal disease: enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and enterotoxigenic *E. coli* (ETEC). PCR was used to distinguish these *E. coli* pathotypes by amplification of the following gene targets: typical EPEC (*eae* and *bfp*), atypical EPEC (*eae* or *bfp*), STEC (*eae* and *stx1* and/or *stx2*), ETEC (*elt* and/or *estIa* or *estIb*), EIEC (*invE* and *ipaH*), and EAEC (*aggR* and/or *aatA* or *aaiC*) (Imuta et al., 2016; Tian et al., 2016).

RESULTS

Antimicrobial Susceptibility Testing of 51 ESBLs Isolates

All 51 ESBL-producing *E. coli* isolates were found to be resistant to ampicillin, cefazolin, cefotaxime, ceftazidime, and susceptible to imipenem and tigecycline. Resistance to tetracycline (27/51, 53.0%) was most common, followed by resistance to trimethoprim–sulfamethoxazole and gentamicin (49.0%, 25/51). All *E. coli* isolates expressed a MDR phenotype (being resistant to at least three different classes of antimicrobial compounds; **Figure 1**).

Identification of β-Lactamase Resistance-Encoding Genes

Of the 51 isolates, $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{OXA}, and bla_{SHV} were detected in 51, 26, 3, and 1 isolates, respectively. Sequencing revealed seven $bla_{\text{CTX-M}}$ subtypes: $bla_{\text{CTX-M-14}}$ ($n = 15$), $bla_{\text{CTX-M-79}}$ ($n = 14$), $bla_{\text{CTX-M-28}}$ ($n = 10$), $bla_{\text{CTX-M-27}}$ ($n = 5$), $bla_{\text{CTX-M-15}}$ ($n = 3$), $bla_{\text{CTX-M-24}}$ ($n = 2$), and $bla_{\text{CTX-M-3}}$ ($n = 2$). Of the 26 TEM-positive strains, all were $bla_{\text{TEM-1}}$.

[1]http://www.eucast.org

[2]http://www.ncbi.nlm.nih.gov/BLAST/

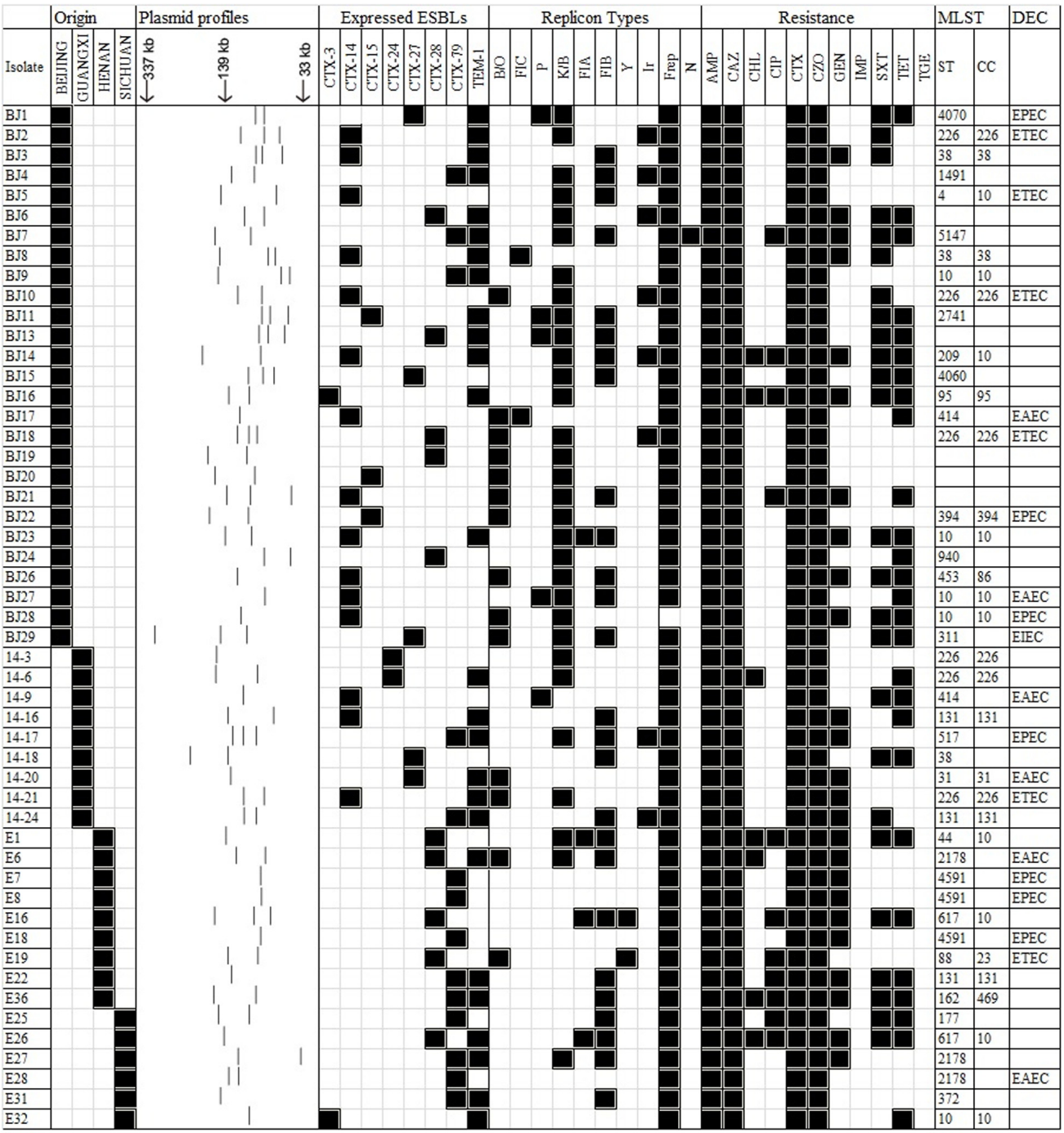

FIGURE 1 | A heat-map summary of the sources; a schematic showing the S1 nuclease plasmid profile; ESBL markers identified by PCR and sequencing, the resistance profile, the corresponding Inc plasmid type(s), MLST and DEC type for all 51 ESBL-positive *E. coli* previously reported from humans in China. Black squares shown indicate a feature present in that isolate denoting its original source the ESBL marker(s) detected, its corresponding antibiogram profile and the Inc types detected. White squares denote features that are lacking in the corresponding bacterial isolate. Antimicrobial compounds used are abbreviated as follows: AMP, ampicillin; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; CZO, cefazolin; GEN, gentamicin; IMP, imipenem; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline, TGC, tigecycline.

S1 Nuclease Digestion and PCR-Based Plasmid Replicon Typing

S1 nuclease-based plasmid analysis showed that all 51 ESBL-positive isolates contained detectable large plasmids; most possessed two plasmids ($n = 24$, 47.1%) and some ($n = 12$, 23.5%) had three plasmids. All of the remaining *E. coli* isolates harbored a single plasmid. Heterogeneity among the profiles was a common feature noted (**Figure 1**). Eighteen plasmid replicons were detected by qualitative PCR-based plasmid replicon typing (PBRT) among those 51 isolates carrying large plasmids. PBRT typing identified 10 of 18 replicons. Interestingly, IncA/C, IncT, IncW, IncFIIA, IncX IncHI1, IncHI2, or IncL/M types were not be detected by PCR analysis in our collection. IncFrep ($n = 50$), IncK/B ($n = 31$), IncFIB ($n = 26$), IncB/O ($n = 14$), and IncI1-Ir ($n = 8$) replicon types were the predominated types. Several isolates were positive for more than one replicon type ($n = 46$). A summary of these features along with the corresponding antimicrobial resistance profiles for all 51 isolates is shown as a heat-map in **Figure 1**.

Conjugational Transfer of Resistance Carried by the 26 *E. coli* Isolates

Each of the 51 *E. coli* isolates expressing an ESBL phenotype, was tested for its ability to transfer the phenotype, by conjugation under laboratory conditions. Twenty-six isolates transferred the cefotaxime resistance marker to a susceptible *E. coli* recipient with frequencies ranging from 3.8×10^{-2} to 4.2×10^{-1} transconjugants *per* donor cell (**Figure 2**). Following conjugation the antimicrobial susceptibility profiles of all 26 transconjugants was determined by the disk diffusion method. In addition to cefotaxime resistance, resistance to several non-β-lactam-based antimicrobial compounds such as gentamicin, tetracycline, and trimethoprim–sulfamethoxazole were also transferred to the recipient, suggesting that these might be located on the same plasmid. For transconjugants, the most commonly detected $bla_{\text{CTX-M}}$-encoding genes included $bla_{\text{CTX-M-79}}$ followed by $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-28}}$. It should be noted that nearly half of those genes both in the $bla_{\text{CTX-M-1}}$ group and the $bla_{\text{CTX-M-9}}$ group were transferable to the recipient bacterium, under laboratory conditions. S1-PFGE analysis showed that the donor isolates carried multiple plasmids with sizes ranging from 34- to 294-kb.

Phylogenetic Grouping and MLST

In all 51 isolates tested, 12 were assigned to B2 and 10 assigned to group D. Twenty isolates were assigned to group A and 9 were assigned to group B1 (**Figure 1**). MLST sub-typing identified 27 types along with 5 new STs not previously registered in the *E. coli* MLST database [including the isolates BJ6 (58-53-12-58-24-01-42), BJ13 (43-41-15-08-11-08-06), BJ19 (261-160-02-63-55-04), BJ20 (261-160-02-63-55-04), BJ20 (56-11-04-10-07-08-06)]. The most prevalent ST was ST266, followed by ST10, ST38, ST131, ST2178, and ST4591. Most of these strains belonged to clonal complexes (cc) of ST10cc, ST266cc, ST131cc, and ST38cc in **Figure 1**.

Occurrence of Pathogens

PCR screening of the typical virulence genes, revealed that 20 of the 51 ESBL-producing study isolates can be assigned to a pathotype with seven EPEC, six ETEC, six EAEC, and one ETEC isolate, being noted (**Figure 1**).

DISCUSSION

ESBL-producing *E. coli* are important causative agents of food-borne infections worldwide (Nischal, 2014). In China, 60.7, 88.8, and 57.1% ESBL-positive *E. coli* were detected from isolates sampled from chicken farms in Henan province (Yuan et al., 2009), healthy broilers in Shandong province (Li et al., 2016), and in piglets (Zhang et al., 2015) separately which in turn may transmit to the human population causing food-borne infections (Leverstein-van Hall et al., 2011). Therefore, tracking the transmission routes of food-borne *E. coli* in community settings is a necessary step required as efforts are made to limit its continuous spread. In China, there is limited information available to describe the nature of ESBL-positive bacteria cultured from patients with diarrhea (Liu et al., 2015; Fan et al., 2016).

In this study, the observed a prevalence of 5.6% (51/912) that was similar to data published previously (Valenza et al., 2014; Hamprecht et al., 2016). Plasmids confer positively selectable phenotypes including antimicrobial resistance genes among others (Andersson and Hughes, 2010). These mobile genetic elements represent an important pool of adaptive and transferable genetic information (with large plasmids defined as being >30-kb in size; Andersson and Hughes, 2010).

Resistance to third-generation cephalosporins was most commonly associated by ESBLs of the CTX-M-1 group in the present study (54.9%), followed by ESBLs of the CTX-M-9 group (45.1%). When comparing these data with other studies from China investigating only *E. coli*, the prevalence of the CTX-M-1 group (55.7%) and CTX-M-9 group (43.3%) among *E. coli* ESBLs was similar to those values reported in an earlier study detected from BSIs (Wang S. et al., 2016), but different compared with the prevalences in UK, the Netherlands, and Germany in which CTX-M-1 was the dominant group compared to CTX-M-9 not only from human isolates but also in those cultured from food-producing animals (Wu et al., 2013; Hamprecht et al., 2016). Genotypes corresponding to several CTX-M enzymes were identified, the majority of which were found to be of the $bla_{\text{CTX-M-14}}$ sub-type, compared with other CTX-M types (such as $bla_{\text{CTX-M-15}}$) identified in hospitals (Liu et al., 2015; Wang L.H. et al., 2016) which differed from data reported from a single survey of retail foods (which identified $bla_{\text{CTX-M-55}}$) in China (Xu et al., 2014).

As TEM-1 is the most common plasmid-mediated β-lactamase identified in enteric Gram-negative bacilli, high rates of ampicillin-resistant, corresponding with $bla_{\text{TEM}-1}$ positive isolates are to be expected. In the current study, the $bla_{\text{TEM}-1}$ gene was also identified in six transconjugants (**Figure 2**). Particular plasmid incompatibility groups are more frequently encountered among *E. coli* and these are thought to play a major role in the dissemination of specific resistance

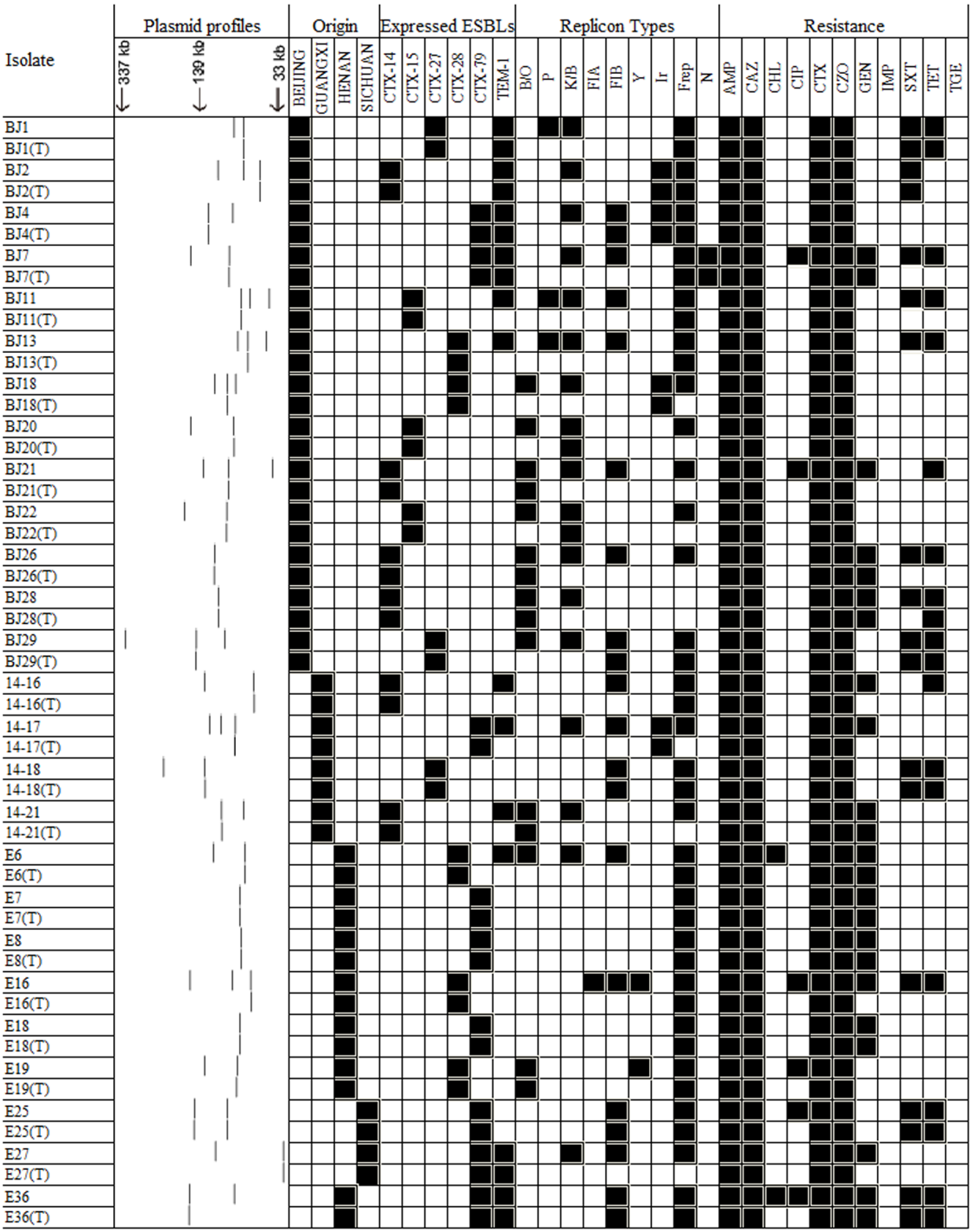

FIGURE 2 | A heat-map showing the comparison of ESBL-positive *E. coli* donors and the resultant transconjugants, characterized on the basis of their plasmid profiles; ESBL-markers identified by PCR; antimicrobial resistance profile; and plasmid replicon type(s). Antimicrobial compounds are abbreviated as described in the legend to **Figure 1**.

genes. Interestingly, $bla_{\text{CTX-M}}$ genes were found to be located predominantly on IncFrep or IncK/B types and conjugation experiments showed that $bla_{\text{CTX-M}}$ from 26 isolates were transferable, with IncFrep and IncFIB being the most prevalent replicon types, a feature that differed from data reported previously (Wang et al., 2013; Xie et al., 2016). Most of the transferable isolates belonged to the phylogenetic group A followed by groups-B2, -B1, and -D. Since virulent organisms causing infections are commonly associated with phylogroup B2, and to a lesser extent group D, most of the isolates in this study belonged to phylogroups-B1 and -A, which are regarded as commensal bacteria (Wirth et al., 2006). Based on data obtained from the *in vitro* conjugation experiments, some 51% of the isolates that were capable of transferring one or more plasmids were phylogrouped as commensal isolates. This finding is consistent with previous reports showing that commensal *E. coli* in food-producing animals were likely to be a reservoir of ESBL-encoding genes and these bacteria played a role in the dissemination of such mobile elements (Smet et al., 2010). To our surprise, nearly half of the isolates are DEC. It would appear that the CTX-M resistance mechanism has spread to several types of *E. coli*. Moreover EAEC O25:H4/CG131 is thought to have evolved to harbor CTX-M plasmids (Imuta et al., 2016).

The diversity of MLST profiles identified suggested that the $bla_{\text{CTX-M}}$ genes were acquired as a result of horizontal transfer from existing resistant organisms, rather than clonal expansion of specific resistant strains (Adler et al., 2016). ST10 clone complex was the most prevalent STcc detected in our study and which are associated with some are pathogenic *E. coli* types such as ETEC, EAEC, EPEC. Also this ST10cc was reported earlier in Europe (Day et al., 2016) and was associated with the production of different CTX-M enzymes. Further it has been observed that the latter can harbor the recently reported *mcr-1* gene (Bernasconi et al., 2016). ST266 was also detected and this is a ST type that had not previously been reported, therefore it may represent a new MLST type identified in China. Most of the ST266 types observed were identified as ETEC isolates, which is a major cause of acute secretory diarrhea and often referred to as the cause of travelers' diarrhea, being one of the major causes of diarrheal disease in children from developing countries (Joffre et al., 2016). ST131 is a globally spread clonal group of extraintestinal pathogenic *E. coli* (ExPEC), comprising different sub-lineages with the ability to acquire and spread antibiotic resistance and virulence genes *via* plasmids. It is seldom detected in companion and food-producing animals (Lanza et al., 2014; Day et al., 2016; Imuta et al., 2016; Wang L.H. et al., 2016). In this study, only three ST131-B2 isolates associated with the production of different CTX-M enzymes (including the genotypes $bla_{\text{CTX-M-14}}$ or $bla_{\text{CTX-M-79}}$ with $bla_{\text{TEM}-1}$) were identified in contrast to earlier reports (Lanza et al., 2014). This ST poses a major threat to public health because of its global distribution. Identification of ST131 isolates that produce CTX-M has important implications for the future treatment of community-associated infections. The potential widespread dissemination of ST131 with its distinctive combination of resistance and virulence requires further investigation.

CONCLUSION

The $bla_{\text{CTX-M}}$ positive *E. coli* characterized in this study pose a serious challenge for the treatment of human infections. These isolates were found to be not only resistant to cephalosporins but to other classes of antimicrobial compound. Continuous investigation and surveillance will extend our understanding of the transmission dynamics and the evolution of these isolates.

AUTHOR CONTRIBUTIONS

LB, RL, SF, and FL designed the experiments. LB, SF, and FL wrote the paper. LB, LW, and JW did the experiments and analyzed the data. XY, XG, WW, and QC collected the strains and analyzed the data. JX discussed the results.

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ICE*Apl1*, an Integrative Conjugative Element Related to ICE*Hin1056*, Identified in the Pig Pathogen *Actinobacillus pleuropneumoniae*

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ICE*Apl1* was identified in the whole genome sequence of MIDG2331, a tetracycline-resistant (MIC = 8 mg/L) serovar 8 clinical isolate of *Actinobacillus pleuropneumoniae,* the causative agent of porcine pleuropneumonia. PCR amplification of *virB4*, one of the core genes involved in conjugation, was used to identify other *A. pleuropneumoniae* isolates potentially carrying ICE*Apl1*. MICs for tetracycline were determined for *virB4* positive isolates, and shotgun whole genome sequence analysis was used to confirm presence of the complete ICE*Apl1*. The sequence of ICE*Apl1* is 56083 bp long and contains 67 genes including a Tn*10* element encoding tetracycline resistance. Comparative sequence analysis was performed with similar integrative conjugative elements (ICEs) found in other members of the *Pasteurellaceae*. ICE*Apl1* is most similar to the 59393 bp ICE*Hin1056,* from *Haemophilus influenzae* strain 1056. Although initially identified only in serovar 8 isolates of *A. pleuropneumoniae* (31 from the UK and 1 from Cyprus), conjugal transfer of ICE*Apl1* to representative isolates of other serovars was confirmed. All isolates carrying ICE*Apl1* had a MIC for tetracycline of 8 mg/L. This is, to our knowledge, the first description of an ICE in *A. pleuropneumoniae*, and the first report of a member of the ICE*Hin1056* subfamily in a non-human pathogen. ICE*Apl1* confers resistance to tetracycline, currently one of the more commonly used antibiotics for treatment and control of porcine pleuropneumonia.

Keywords: animal infections, antibiotic resistance, respiratory tract, conjugation, *Pasteurellaceae*

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INTRODUCTION

Actinobacillus pleuropneumoniae is a major contributor to swine respiratory disease worldwide, causing considerable economic losses. Isolates can be differentiated into 15 established serovars, based on capsular polysaccharides, and a recently proposed serovar 16 identified on the basis of serology alone (Sárközi et al., 2015). There are geographical differences in the distribution of serovars. Within the UK, clinical isolates are predominantly serovar 8, with serovars 2, 6, 7, and 12 also represented (O'Neill et al., 2010).

There is growing concern regarding antimicrobial resistance in bacteria from food-producing animals (Michael et al., 2015). In Europe, tetracyclines are still the most commonly used antimicrobial for treatment of swine pleuropneumonia (European Medicines Agency, 2012). The genes *tetB*, *tetH*, *tetL,* and *tetO*, reported to mediate tetracycline resistance in *A. pleuropneumoniae*, are usually carried on small plasmids (Blanco et al., 2006, 2007). We recently sequenced the genome of MIDG2331, a serovar 8 UK clinical isolate of *A. pleuropneumoniae* (Bossé et al., 2016), and identified chromosomally encoded tetracycline resistance genes within a putative integrative conjugative element (ICE). Similar to genomic islands, ICE have the ability to integrate into bacterial chromosomes at specific sites, often in tRNA loci, via the action of an integrase (predominantly tyrosine recombinases) (Boyd et al., 2009; Wozniak and Waldor, 2010). However, ICE differ from genomic islands in that they are self-mobilizing, encoding all of the genes necessary for excision from the chromosome and conjugal transfer (Boyd et al., 2009). The core genes of ICE tend to group into functional modules, with syntenic regions responsible for maintenance, dissemination and regulation, which may be interspersed with accessory genes carried on transposons or other insertion elements (Burrus and Waldor, 2004; Wozniak and Waldor, 2010). The genes encoding the type 4 secretion

TABLE 1 | Clinical isolates of *Actinobacillus pleuropneumoniae* with ICE*Apl1*.

Isolate ID	Serovar	Location of isolation[a]	Year of isolation	Length of ICE*Apl1*[b]	5′ tRNAs[c]	Accession number for ICE*Apl1* sequence
MIDG2331	8	Thirsk	1995	56,083 bp	GLGLL	LN908249 (bases 1570419–1570505)[d]
MIDG2427	8	Aberdeen	1998	56,083 bp	GLGLL	KU551309
MIDG2648	8	Bury St Edmunds	2005	**54,898 bp**	**GLGL**	KU551310
MIDG2652	8	Thirsk	2005	56,083 bp	GLGLL	KU551311
MIDG2654	8	Winchester	2005	**56,070 bp**	GLGLL	KU551312
MIDG2657	8	Winchester	2005	56,083 bp	**GLLGL**	KU551313
MIDG2663	8	Thirsk	2005	56,083 bp	**GLLGL**	KU551314
MIDG2664	8	Bury St Edmunds	2005	56,083 bp	**GLLGL**	KU551315
MIDG3200	8	Thirsk	2006	56,083 bp	GLGLL	KU551316
MIDG3201	8	Bury St Edmunds	2006	56,083 bp	GLGLL	KU551317
MIDG3221	8	Langford	2006	56,083 bp	GLGLL	KU551318
MIDG3229	8	Thirsk	2007	56,083 bp	GLGLL	KU551320
MIDG3232	8	Thirsk	2007	56,083 bp	GLGLL	KU551321
MIDG3339	8	Winchester	2008	56,083 bp	GLGLL	KU551322
MIDG3344	8	Langford	2005	56,083 bp	**GLLGL**	KU551323
MIDG3346	8	Thirsk	2005	56,083 bp	**GLLGL**	KU551324
MIDG3349	8	Thirsk	2006	56,083 bp	**GLLGL**	KU551325
MIDG3357	8	Shrewsbury	2008	56,083 bp	GLGLL	KU551326
MIDG3368	8	Thirsk	2008	56,083 bp	GLGLL	KU551327
MIDG3370	8	Thirsk	2009	56,083 bp	**GLLGL**	KU551328
MIDG3371	8	Thirsk	2009	56,083 bp	**GLLGL**	KU551329
MIDG3372	8	Thirsk	2009	56,083 bp	**GLLGL**	KU551330
MIDG3378	8	Bury St Edmunds	2009	**56,047 bp**	GLGLL	KU551331
MIDG3381	8	Thirsk	2009	56,083 bp	GLGLL	KU551332
MIDG3386	8	Bury St Edmunds	2009	56,083 bp	GLGLL	KU551333
MIDG3388	8	Thirsk	2009	56,083 bp	GLGLL	KU551334
MIDG3389	8	Thirsk	2009	56,083 bp	GLGLL	KU551335
MIDG3395	8	Thirsk	2010	**56, 011 bp**	GLGLL	KU551336
MIDG3401	8	Bury St Edmunds	2011	56,083 bp	GLGLL	KU551337
MIDG3409	8	Bury St Edmunds	2011	56,083 bp	GLGLL	KU551338
MIDG3458	8	Cyprus	2011	**56,012 bp**	GLGLL	KU551339
MIDG3469	8	Thirsk	2012	56,083 bp	GLGLL	KU551340

[a]*All isolates except MIDG2427 and MIDG3458 were cultured from pigs submitted to the then Veterinary Laboratories Agency (now Animal and Plant Health Agency) regional laboratories in the UK, as indicated.*

[b]*The length of each ICEApl1 sequence, calculated as the predicted circular form, is shown for each isolate. Size variation from that in MIDG2331 is indicated in bold. The sequence in MIDG2648 is missing 3 genes and has a truncated copy of the site-specific recombinase gene; whereas small deletions in the other sequences are all intergenic.*

[c]*Order of tRNA genes upstream of ICEApl1: G, tRNA-Gly (GCC), L, tRNA-Leu (TAA). Variation from the order seen in MIDG2331 is indicated in bold.*

[d]*ICEApl1 identified in MIDG2331 in previous study,[4] all other ICEApl1 sequences were identified in this study.*

system (T4SS), required for transport of DNA into recipient cells, include a ubiquitous ATPase encoded by *virB4* or *traU* (Guglielmini et al., 2011).

ICEs are the most abundant conjugative elements identified in prokaryotes, and there is evidence of cross-clade transfer (Guglielmini et al., 2011). Within the *Pasteurellaceae*, ICE have been identified and characterized in *Haemophilus influenzae* and *Haemophilus parainfluenzae* (Juhas et al., 2007b), *Pasteurella multocida* (Michael et al., 2012), and *Mannheimia haemolytica* (Eidam et al., 2015). Here we report characterization of ICE*Apl1*, to our knowledge the first ICE described in *A. pleuropneumoniae*.

MATERIALS AND METHODS

Comparative Sequence Analysis

The full sequence of ICE*Apl1,* identified within the genome of MIDG2331 (accession number LN908249) was analyzed using BLASTn and BLASTx[1]. A comparative alignment was generated for sequences most similar to ICE*Apl1* using Mauve version 2.3.1[2]. Default parameters were used for all programs.

Detection of Other Isolates Containing ICE*Apl1*

We screened 185 isolates of *A. pleuropneumoniae* (clinical isolates collected between 1995 and 2012 from the UK, Denmark, the Czech Republic, Cyprus, and Greece) for *virB4* by PCR using primers virB4_for (CCTTCACGGTTAAAGAATCG AC)/virB4_rev (GCATCGTTTATTGGAAATGGAT). Primers were designed based on the *virB4* gene in MIDG2331, amplifying the region from 1532511 to 1532894 in the genome sequence. Serovars 1 (1.2%), 2 (11.7%), 5 (2.4%), 6 (4.7%), 7 (10.6%), 8 (58.8%), 9/11 (2.4%), 10 (2.9%), and 12 (5.3%), were represented, and 84% of the isolates were from the UK. Genome sequence data was generated and assembled as previously described (Howell et al., 2013; Bossé et al., 2015) for 31 *virB4* positive isolates. Sequences matching ICE*Apl1* were identified by BLASTn, assembled using Geneious 9.0.4, and deposited to Genbank (see **Table 1** for accession numbers).

Minimum Inhibitory Concentrations (MICs) for tetracycline were determined for isolates containing ICE*Apl1*, according to the CLSI M37-A3 guidelines (Clinical and Laboratory Standards Institute [CLSI], 2008).

Conjugal Transfer of ICE*Apl1*

MIDG2331Δ*ureC*::*nadV* was used as the conjugal donor, with matings performed as previously described (Bossé et al., 2015). Plasmid-free, tetracycline-sensitive, nalidixic acid-resistant clinical isolates of serovars 6 (MIDG3376), 7 (MIDG2465), 8 (MIDG3217), and 12 (MIDG3347) were used as recipients. Transconjugants were selected on Brain Heart Infusion agar supplemented with 0.01% NAD, 5 mg/L tetracycline and 40 mg/L nalidixic acid. PCR was used to confirm the presence of the *virB4* gene (as above), as well as serovar of, and the absence of *nadV*, in selected transconjugants using previously described primers (Bossé et al., 2014, 2015). Chromosomal insertion sites in transconjugants were determined by PCR using primers ICE5′_out1 (TGAGGGAGTAACAAGCAACACAG)/mfd3′_out (TTTACCGCTTGCCGATAATGCG) for the 5′ junction, and ICE3′_out1 (CAATGGAGAAAGAGAGTTGTTGGAC) /hybF5′_out (GACATCTCGTGCATAACCATTCC) for the 3′ junction, respectively. Amplicons were sequenced using internal primers ICE5′_out2 (GGAAGGTTCAATATCA CGACGG) or ICE3′_out2 (AGGCATACAGCAGCAACAAATC), as appropriate. For comparison, the region between *mfd* and *hybF* in the conjugal recipients (prior to conjugation) was amplified using mfd3′_out/hyb5′_out and sequenced in both directions.

Confirmation of the Circular Extrachromosomal form of the ICE by Nested PCR

DNA was extracted from MIDG2331Δ*ureC*::*nadV* and selected transconjugants, and nested PCR was performed as previously described (Eidam et al., 2015), using primers ICE5′_out1/ICE3′_out1 followed by primers ICE5′_out2/ICE3′_out2. Amplicons were sequenced using primers ICE5′_out2 and ICE3′_out2.

RESULTS AND DISCUSSION

Sequence of ICE*Apl1* and Comparative Analysis

ICE*Apl1*, a 56083 bp element, is inserted into a tRNA-Leu (TAA) gene, a common insertion site for ICE in the family *Pasteurellaceae* (Dimopoulou et al., 2002; Michael et al., 2012; Eidam et al., 2015), in a tRNA cluster located between genes *hybF* and *mfd*. In the MIDG2331 genome (Bossé et al., 2016), this tRNA-Leu (TAA) gene is annotated as MIDG2331_01481, and is located between bases 1570419 and 1570505. Although all of the tRNA genes in the cluster, as well as *hybF* and *mfd*, are on the complement strand in the MIDG2331 genome, all further references to these genes, and the location of ICE*Apl1*, will be with respect to the forward orientation. Insertion of ICE*Apl1* generated 66 bp imperfect direct repeats (DRs) at the left and right attachment sites, *attL* and *attR* (**Figure 1A**). The three bases that differ in the DRs reflect sequence variation in the tRNA-Leu (TAA) genes in *A. pleuropneumoniae* and *H. influenzae* (**Figure 1B**). The *attI* site in the closed circular form of ICE*Apl1* (confirmed by PCR; see below) is identical to that in ICE*Hin1056* (**Figure 1C**), both having 100% identity with the last 65 bases of the *H. influenzae* tRNA-Leu (TAA) gene followed by a T. These data suggest that insertion of ICE*Apl1* in MIDG2331 has generated an altered tRNA-Leu (TAA) gene, resulting from cross-over of the circular plasmid form of the ICE into the chromosome between the G at position 25 and the A at position 54 of MIDG2331_01481.

[1]http://blast.ncbi.nlm.nih.gov/

[2]http://darlinglab.org/mauve/mauve.html

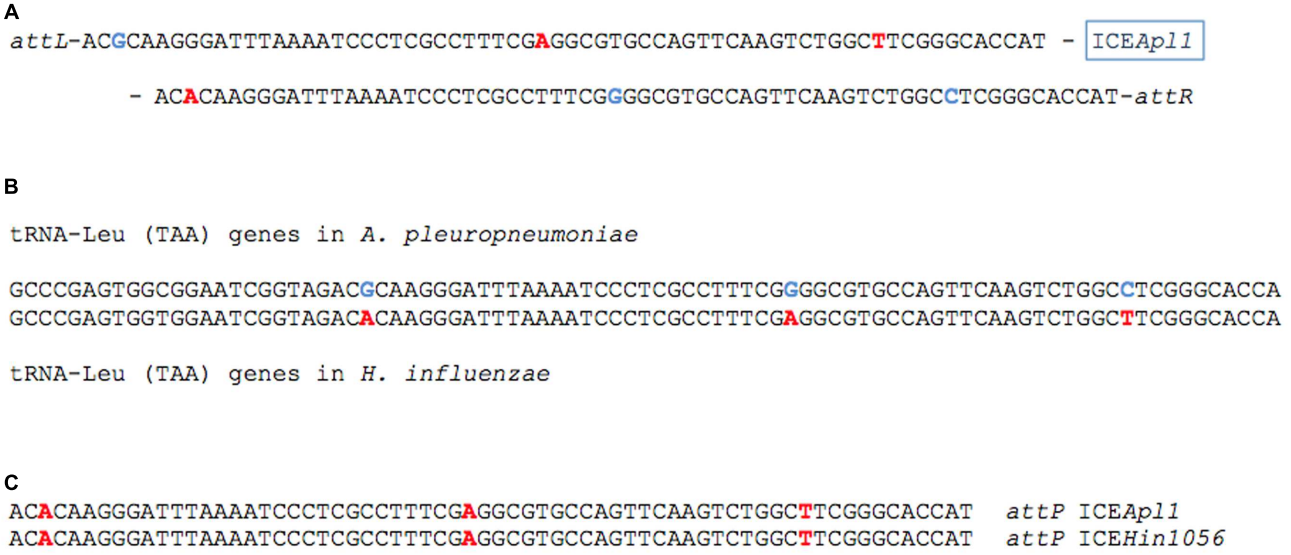

FIGURE 1 | Analysis of ICE*Apl1* insertion site. The imperfect direct repeats (DRs) **(A)** flanking ICE*Apl1* share sequence identity with the last 65 bases of tRNA-Leu genes **(B)** from *Actinobacillus pleuropneumoniae* (e.g., MIDG2331_01482 and _01484) and *Haemophilus influenzae* (e.g., accession number LK008335), which differ at 3 bases (in light blue for bases normally found in *A. pleuropneumoniae* and red for bases normally found in *H. influenzae*). The *attI* sequences **(C)** in the closed circular forms of ICE*Apl1* and ICE*Hin1056* are identical, and match the end of the *H. influenzae* tRNA-Leu (TAA) gene sequence, with an additional T which is also present in the DRs **(A)**. Insertion of ICE*Apl1* in the *A. pleuropneumoniae* tRNA-Leu (TAA) gene MIDG2331_01481 has resulted in an altered sequence, indicating cross-over of the circular ICE*Apl1* into the chromosome occurred between the G at position 25 and the A at position 54.

Comparative sequence analysis revealed that ICE*Apl1* is related to the ICE*Hin1056* subfamily of elements (**Figure 2**) found in *H. influenzae* and *H. parainfluenzae* (Mohd-Zain et al., 2004; Juhas et al., 2007b). ICE*Apl1* encodes 67 genes that share extensive sequence homology and gene order with ICE*Hin1056* and other members of this subfamily. The first 14687 bp of ICE*Apl1* shares 99% identity with the region of ICE*Hin1056* reported to contain replication and stabilization genes (Juhas et al., 2007a,b, 2013). The 8933 bp Tn*10* element in ICE*Apl1*, although in the same location and orientation as that in ICE*Hin1056*, more closely resembles that in ICE*HpaT3T1* (99% identity, but inverted), with tetracycline resistance genes *tetR*, *tetB*, *tetC*, and *tetD*; and *gltS* encoding glutamate permease (Juhas et al., 2007b). The Tn*10* element in ICE*Hin1056* has a further IS*5* insertion (encoding chloramphenicol resistance) within it that is not seen in ICE*Apl1* (Juhas et al., 2007b). The 20466 bp following the Tn*10* insertion shares 99% identity with the region containing genes encoding components of the type IV secretion system (required for conjugal transfer) in ICE*Hin1056* (Juhas et al., 2007a,b, 2013). This region is well conserved in all of the members of the ICE*Hin1056* family (Juhas et al., 2007a,b, 2013). The gene order in ICE*Apl1* remains syntenic with that of ICE*Hin1056* up to *traC*, where in ICE*Hin1056* there is a Tn*3* insertion (encoding beta-lactamase resistance) that is not found in ICE*Apl1*. From *traC* in ICE*Apl1*, the nucleotide sequence and gene order more closely resemble those in ICE*Hin2866* up to the site-specific tyrosine recombinase gene, which is the final gene in ICE*Apl1* on the *attR* side. This 8.5 kb region shares 98% identity with sequences in ICE*Hin2866*, and includes accessory genes encoding a type I restriction enzyme M subunit, and a transposon gamma-delta resolvase, as well as four hypothetical genes of unknown function (Juhas et al., 2007b).

Surprisingly, the ICE*Hin1056* subfamily of conjugative elements has previously only been reported in *H. influenzae* and *H. parainfluenzae*, two human species of *Haemophilus*, where they appear to be evolving by descent (Dimopoulou et al., 2007; Juhas et al., 2007b). To our knowledge, this is the first report of a member of the ICE*Hin1056* subfamily in a *Pasteurellaceae* species that infects livestock. There have been ICE reported for bovine isolates of *P. multocida* (ICE*Pmu1*) and *M. haemolytica* (ICE*Mh1*), as well as an uncharacterized putative ICE in *Histophilus somni* strain 2336, which are related and appear to have evolved from a common ancestor, but are part of a different subfamily than ICE*Hin1056* elements (Juhas et al., 2007b; Michael et al., 2012; Eidam et al., 2015). The identity of the *attI* sites in both ICE*Apl1* and ICE*Hin1056* with the last 65 bases of the *H. influenzae* tRNA-Leu (TAA) gene would suggest more recent acquisition of an ICE*Hin1056* element in *A. pleuropneumoniae*.

Distribution of ICE*Apl1* in *A. pleuropneumoniae* Isolates

PCR analysis revealed the presence of a *virB4* amplicon in 32/185 *A. pleuropneumoniae* isolates, including MIDG2331. All 32 (31 from the UK, 1 from Cyprus) were serovar 8 and had an MIC for tetracycline of 8 mg/L, i.e., above the CLSI breakpoint of $\geq$ 2 mg/L for *A. pleuropneumoniae* (Clinical and Laboratory Standards Institute [CLSI], 2008). ICE*Apl1* sequences were detected in the whole genomes of the 32

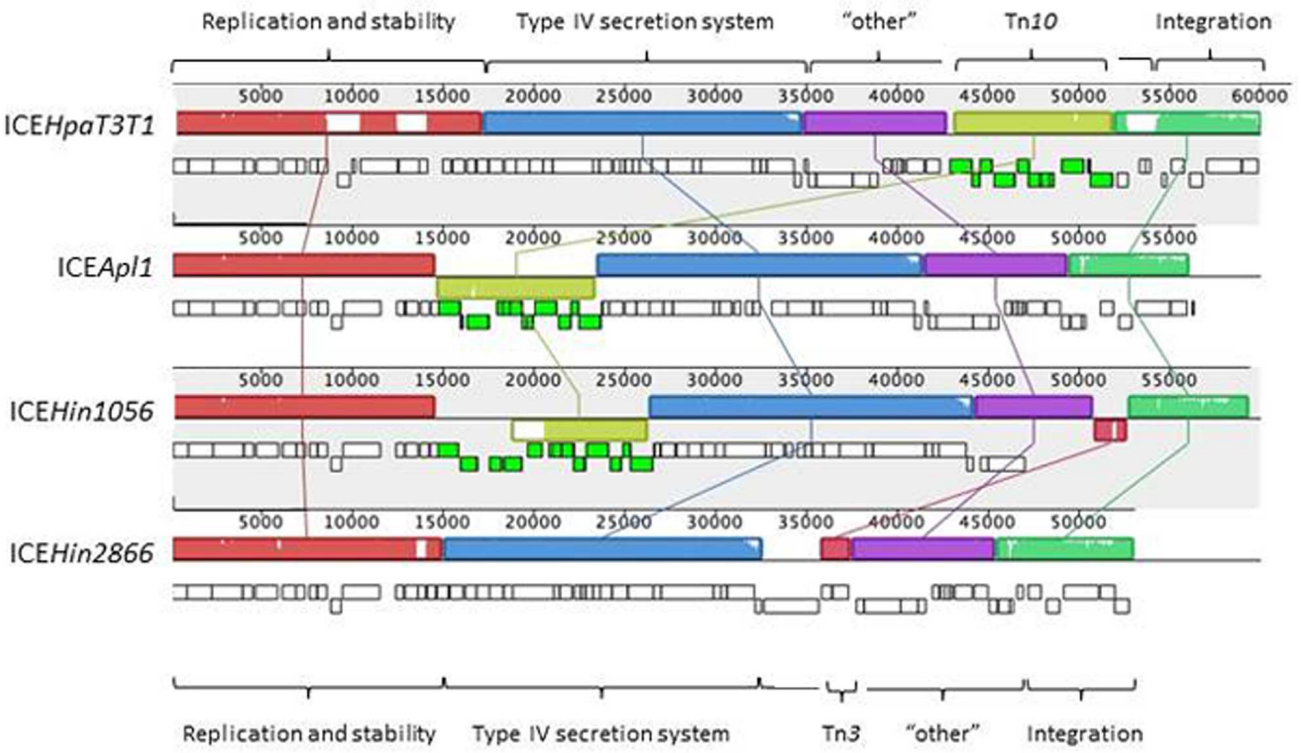

FIGURE 2 | Mauve alignments of ICE*Apl1* and closely related members of the ICE*Hin1056* subfamily of elements. The orientation and relative size of genes in each ICE are indicated by the small rectangular blocks (lower blocks are on the complementary strand), with those colored green indicating genes of Tn*10* elements present in all except ICE*Hin2866*. Note the extra genes in the Tn*10* insertion in ICE*Hin1056* are due to the presence of a further IS*5* insertion not seen in the other ICE. Regions containing contiguous genes of related function are indicated by colored blocks above the genes, and are connected by lines to matching blocks in each ICE sequence. The labels at the top and bottom of the figure indicate functional sets of genes defined by Juhas et al. (2007b).

isolates, and comparative analysis revealed that, other than minor nucleotide differences in some, all of the sequences were complete except the element from MIDG2648, which was lacking three genes (encoding a putative DNA-binding protein and two hypothetical proteins) in the accessory gene region, and has a truncated copy of the tyrosine recombinase gene (**Table 1**).

Conjugal Transfer of ICE*Apl1* and Detection of Circular Intermediate Form

As ICE*Apl1* appeared to be present only in serovar 8 isolates of *A. pleuropneumoniae*, it was possible that other serovars blocked conjugal entry of the ICE, either due to restriction modification systems or CRISPR mediated restriction (Elhai et al., 1997; Garneau et al., 2010). We therefore tested the ability to conjugally transfer ICE*Apl1* to clinical isolates of *A. pleuropneumoniae* representing serovars 6, 7, 8, and 12 (MIDG3376, MIDG2465, MIDG3217, and MIDG3347, respectively) that are commonly found in the UK (O'Neill et al., 2010). All tested isolates produced transconjugants, as initially confirmed by PCR (data not shown). The frequencies of conjugation were similar for the serovar 7, 8, and 12 recipients (between 10^{-4} and 10^{-5}), but much lower (5×10^{-8}) for the serovar 6 isolate tested. Similar frequencies have been reported for ICE*Hin1056* elements in *H. influenzae*, with strain related differences also noted (Juhas et al., 2007b). More serovar 6 isolates would need to be tested in order to determine if the difference in frequency of conjugation for ICE*Apl1* is serovar-specific. The presence of a circular intermediate form of ICE*Apl1* was confirmed in the donor strain and in transconjugants by nested PCR. Sequenced amplicons confirmed a single copy of the 66 bp *attI* (**Figure 1C**) at the closed junction of the circular intermediates.

Sequencing PCR products generated at both the *attL* and *attR* ends in the transconjugants confirmed insertion of ICE*Apl1* in the same tRNA cluster between *mfd* and *hybF* as in the donor strain (MIDG2331Δ*ureC*::*nadV*). However, in all transconjugants tested, the sequences between *mfd* and *hybF* contained only the altered tRNA-Leu (TAA) gene in which the ICE inserted, flanked by tRNA-Gly (GCC) genes at either end of the cluster (**Figure 3B**). This is in contrast to the cluster in the donor strain where there are 5 tRNA genes on the *attL* side, and a tRNA-Gly (GCC) gene on the *attR* side (**Figure 3C**). Sequencing across the tRNA cluster in the recipient strains prior to conjugation revealed that, although a different order of tRNA genes was present in MIDG3376 compared to the other strains (**Figure 3A**), all contained 3 copies each of tRNA-Gly

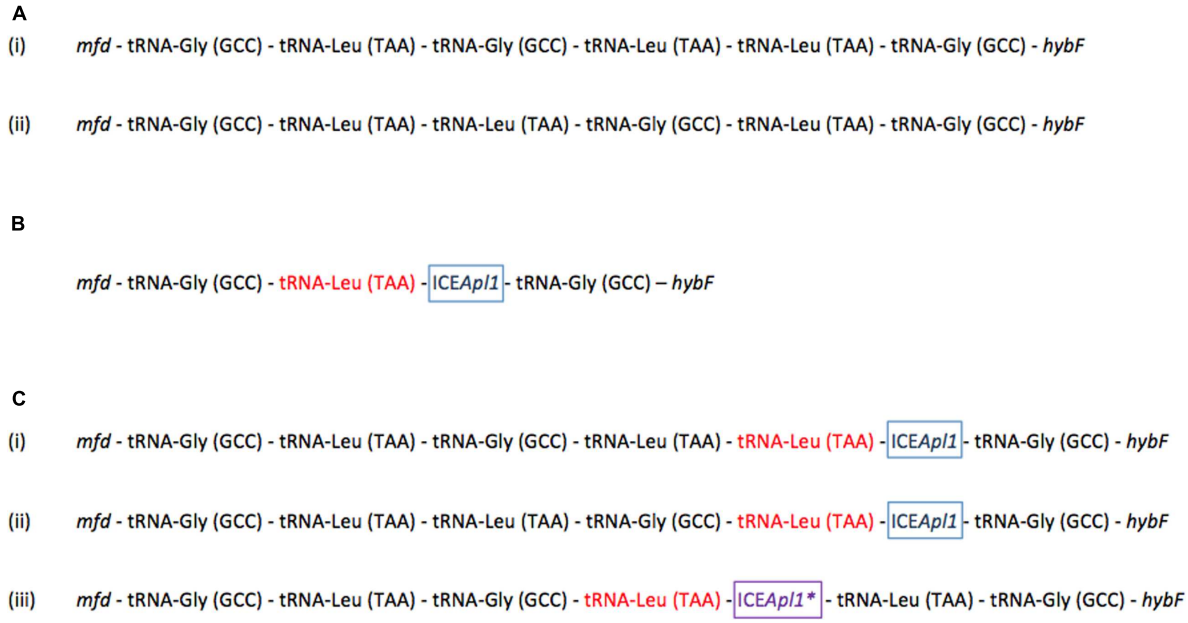

FIGURE 3 | Schematic representation of the tRNA cluster located between *mfd* and *hybF* in *A. pleuropneumoniae* isolates. Note that all sequences are shown in the forward orientation for simplicity. In the MIDG2331 genome, these sequences are on the complement strand. **(A)** The tRNA genes in isolates used as conjugal recipients are in the same order in (i) MIDG2465 (serovar 7), MIDG3217 (serovar 8), and MIDG3347 (serovar 12); and a different order in (ii) MIDG3376 (serovar 6). Note that all of the tRNA-Leu (TAA) genes have the sequence shown in **Figure 1B**. **(B)** Following conjugation, ICE*Apl1* integration resulted in loss of 3 tRNA genes, with all of the transconjugants (MIDG2465::ICE*Apl1*, MIDG3217::ICE*Apl1*, MIDG3347::ICE*Apl1*, and MIDG3376::ICE*Apl1*) showing identical sequences flanking the insertion (ICE*Apl1* shown boxed in blue). Note that the tRNA-Leu (TAA) gene shown in red has the altered bases of the *attL* DRs in **Figure 1A**. **(C)** In the 32 isolates with endogenous ICE*Apl1*, the tRNA genes are found in three different orders in (i) MIDG2331 and 21 other isolates; (ii) 9 other isolates; and (iii) MIDG2648. Note the truncated element in MIDG2648 is shown as ICE*Apl1** (in purple text, boxed in purple). See **Table 1** for details of specific isolates. Again, the tRNA-Leu (TAA) gene shown in red has the altered bases of the *attL* DRs in **Figure 1A**.

(GCC) and tRNA-Leu (TAA) genes. These results indicate that in all transconjugants tested, a deletion of 3 tRNA genes was associated with ICE*Apl1* insertion. However, examination of the insertion sites in the serovar 8 clinical isolates with endogenous ICE*Apl1* (**Table 1**) revealed conservation of the 6 tRNA genes normally found in this cluster – i.e., 3 copies each of tRNA-Gly (GCC) and tRNA-Leu (TAA) – with the order of genes showing one of 3 patterns (see **Figure 3C** and **Table 1** for details). In MIDG2648, the truncated element present in this isolate is located in a different copy of the tRNA-Leu (TAA) gene than seen in isolates with intact ICE*Apl1* (**Figure 3C**), with 4 tRNA genes on the *attL* side, and a tRNA-Leu (TAA) followed by tRNA-Gly (GCC) gene on the *attR* side. It would appear that, although the majority of clinical isolates show integration in the same tRNA-Leu (TAA) gene (i.e., the final copy in the tRNA cluster in the forward orientation), ICE*Apl1* has the ability to integrate into different copies of this gene, as has been reported for ICE*Hin1056* in *H. influenzae* (Dimopoulou et al., 2002). In *Pseudomonas knackmussii*, ICE*clc* was found to insert into different copies of the tRNA-Gly (GCC) gene, with double integration in some transconjugants (Sentchilo et al., 2009). In that study, excision and reintegration was associated with generation of a heterogenous population in which ICE*clc* was found to move from its original insertion site to alternate tRNA-Gly genes, but only those with the GCC anticodon (Sentchilo et al., 2009). Similarly, following *in vitro* conjugal transfer of ICE*Kp1* into a recipient strain of *Klebsiella pneumoniae,* integration was found at any of four tRNA-Asn genes, with insertion in multiple copies in some transconjugants (Lin et al., 2008). Furthermore, in some transconjugants, there was evidence of deletions between copies of the tRNA-Asn genes associated with integration of ICE*Kp1*, which may have been due to recombination between multiple insertion sites (Lin et al., 2008). We did not detect multiple insertions of ICE*Apl1* in our transconjugants, as only one PCR product was generated using the outward facing primers designed to amplify the closed junction of the circular ICE. These primers would also have generated a secondary product in the presence of tandem insertions, given the proximity of the copies of the tRNA-Leu (TAA) genes in *A. pleuropneumoniae*. It is also possible that recombination may occur between the DRs found in alternate copies of the target tRNA, with or without the presence of an integrated ICE, resulting in deletion of the intervening sequences. However, given the results of Lin et al. (2008), it is likely that conditions during *in vitro* conjugation favor integration of multiple copies of ICE leading to deletions, whereas this does not appear to be common amongst clinical isolates. Little is known regarding the signals that govern initiation of horizontal transfer of ICE between pathogens in a host animal environment.

CONCLUSION

Identification of ICE*Apl1* in only serovar 8 clinical isolates of *A. pleuropneumoniae* may simply be a reflection of this being the most common in the UK (O'Neill et al., 2010), and thus in our collection. It may also indicate a tendency for ICE to be inherited by vertical transmission rather than horizontal transfer. The similar *in vitro* conjugation frequencies of ICE*Apl1* into isolates of serovars 7, 8, and 12 suggests there are no restriction endonuclease or CRISPR barriers to transfer between these serovars. Futhermore, the variation in order of tRNA genes flanking ICE*Apl1* suggests that horizontal transfer may have occurred independently into different isolates, as a similar variation in tRNA gene order was also seen in clinical isolates lacking ICE*Apl1*. As reported for other ICE, ICE*Apl1* has the ability to integrate into different copies of the target tRNA gene, in this case tRNA-Leu (TAA). Although we did not detect multiple insertions following *in vitro* conjugal transfer of ICE*Apl1*, it is likely that recombination between tandem insertions was responsible for the deletions detected in transconjugants.

To our knowledge, this is the first description of an ICE identified in *A. pleuropneumoniae*, and the first report of a member of the ICE*Hin1056* subfamily found in a non-human pathogen. The presence of ICE*Apl1* in isolates of *A. pleuropneumoniae* confers resistance to tetracycline, which is commonly used for treatment and control of porcine pleuropneumonia (European Medicines Agency, 2012). Although currently only found in serovar 8 isolates, the ability to transfer to other serovars was confirmed *in vitro*, and has implications for the spread of antimicrobial resistance in this important pig pathogen.

AUTHOR CONTRIBUTIONS

JB, PL, AR, BW, DM, and AT conceived the study; JB, YL, RFC, RRC, MH, and JR produced the data; JB, YL, RFC, and RRC analyzed the data; JB and PL wrote the paper.

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Whole Genome Sequencing for Surveillance of Antimicrobial Resistance in *Actinobacillus pleuropneumoniae*

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The aim of this study was to evaluate the correlation between antimicrobial resistance (AMR) profiles of 96 clinical isolates of *Actinobacillus pleuropneumoniae*, an important porcine respiratory pathogen, and the identification of AMR genes in whole genome sequence (wgs) data. Susceptibility of the isolates to nine antimicrobial agents (ampicillin, enrofloxacin, erythromycin, florfenicol, sulfisoxazole, tetracycline, tilmicosin, trimethoprim, and tylosin) was determined by agar dilution susceptibility test. Except for the macrolides tested, elevated MICs were highly correlated to the presence of AMR genes identified in wgs data using ResFinder or BLASTn. Of the isolates tested, 57% were resistant to tetracycline [MIC $\geq$ 4 mg/L; 94.8% with either *tet*(B) or *tet*(H)]; 48% to sulfisoxazole (MIC $\geq$ 256 mg/L or DD = 6; 100% with *sul2*), 20% to ampicillin (MIC $\geq$ 4 mg/L; 100% with bla_{ROB-1}), 17% to trimethoprim (MIC $\geq$ 32 mg/L; 100% with *dfrA14*), and 6% to enrofloxacin (MIC $\geq$ 0.25 mg/L; 100% with GyrAS83F). Only 33% of the isolates did not have detectable AMR genes, and were sensitive by MICs for the antimicrobial agents tested. Although 23 isolates had MIC $\geq$ 32 mg/L for tylosin, all isolates had MIC $\leq$ 16 mg/L for both erythromycin and tilmicosin, and no macrolide resistance genes or known point mutations were detected. Other than the GyrAS83F mutation, the AMR genes detected were mapped to potential plasmids. In addition to presence on plasmid(s), the *tet*(B) gene was also found chromosomally either as part of a 56 kb integrative conjugative element (ICE*Apl1*) in 21, or as part of a Tn*7* insertion in 15 isolates. Our results indicate that, with the exception of macrolides, wgs data can be used to accurately predict resistance of *A. pleuropneumoniae* to the tested antimicrobial agents and provides added value for routine surveillance.

Keywords: animal infections, antimicrobial resistance genes, integrative conjugative elements, plasmids, genomics, respiratory tract, *Pasteurellaceae*

INTRODUCTION

Antimicrobial resistance (AMR) in bacteria from food-producing animals is a growing concern (Michael et al., 2015). Extensive use of antimicrobial agents for treatment and prevention of diseases fosters an environment in which resistance determinants are acquired and maintained by pathogens, as well as commensal bacteria. In the UK, the swine industry accounts for a large proportion of the antimicrobial agents sold for use in food-producing animals; with tetracyclines, beta-lactams, and trimethoprim/sulphonamides being the most commonly used antimicrobial agents (Burch, 2005; Borriello, 2013).

Actinobacillus pleuropneumoniae is a major contributor to swine respiratory disease, causing considerable economic losses worldwide. Strategies to reduce the incidence and severity of disease include good husbandry, vaccination, and antibiotic treatment. The latter is essential to limit the severity and spread of pleuropneumonia. Knowledge of resistance profiles for *A. pleuropneumoniae* is required to inform treatment decisions. Furthermore, this information contributes to the larger picture of AMR in bacteria of animal origin (Hendriksen et al., 2008; El Garch et al., 2016).

Typically, determination of antimicrobial susceptibility is done either by disk diffusion or minimum inhibitory concentration (MIC) assays. Identification of the genetic determinants of resistance not only corroborates phenotypic results, but is also useful for epidemiological purposes, as there are often multiple different genes that can confer resistance to a given antimicrobial agent. Both PCR and microarray hybridization have been used to detect the presence of genes encoding resistance phenotypes, but these assays are limited to detecting the sequences tested, and do not allow direct detection of mutations conferring resistances (Frye et al., 2010; Ledeboer and Hodinka, 2011). It has recently been proposed that whole genome sequencing (wgs) may be an alternative for routine surveillance of resistance profiles and for identification of emerging resistances (Zankari et al., 2013). We recently used wgs to identify (for the first time) *dfrA14* as the genetic determinant of trimethoprim resistance detected in 16 clinical isolates of *A. pleuropneumoniae* (Bossé et al., 2015b). In this study, we compare the MIC profiles for nine antimicrobial agents with detection of resistance genes in wgs data of 96 isolates.

MATERIALS AND METHODS

Bacterial Strains and Antimicrobial Susceptibility Testing

A total of 96 clinical *A. pleuropneumoniae* isolates from the UK, previously tested for trimethoprim resistance (Bossé et al., 2015b), were analyzed using the agar dilution susceptibility assay, according to the CLSI VET01-A4 guidance (Clinical and Laboratory Standards Institute [CLSI], 2013), for determination of MICs for tetracycline, ampicillin, sulfisoxazole, enrofloxacin, erythromycin, tilmicosin, tylosin, and florfenicol. Some samples were re-tested for resistance using the disk diffusion susceptibility test according to the CLSI VET01-A4 guidance (Clinical and Laboratory Standards Institute [CLSI], 2013). *A. pleuropneumoniae* ATCC 27090 and *Histophilus somni* ATCC 70025 were used as controls for all susceptibility tests. All clinical *A. pleuropneumoniae* isolates had been cultured from pneumonic lungs of pigs submitted for post-mortem to the then Animal Health and Veterinary Laboratory Agency (now Animal and Plant Health Agency) diagnostic laboratories in England (see Supplementary Table S1 for details). The serovar of each isolate was determined by PCR as previously described (Bossé et al., 2014).

Genome Sequencing and Analysis

Genomic DNA was prepared from all isolates using the FastDNA Spin kit (MP Biomedicals), and 0.5 μg were used for library preparation and paired-end sequencing (Illumina HiSeq 2000), as previously described (Howell et al., 2013; Bossé et al., 2015b; Weinert et al., 2015). Cutadapt (Martin, 2011) was used to remove Illumina adapter sequences, and Sickle[1] was used to trim low-quality sequences from the ends of reads, prior to assembly into contigs using Velvet 1.2.08 (Zerbino and Birney, 2008) and VelvetOptimiser 2.2.5 (Gladman and Seemann, 2012). Assemblies with N50 < 10000 were excluded from further analysis. The draft genome sequence for each isolate in this study has been deposited in the European Nucleotide Archive[2], and accession numbers are listed in Supplementary Table S1.

AMR genes were identified in the draft genomes using ResFinder (Zankari et al., 2012), with a threshold of 98% identity and minimum length of 60%. Alternatively, the genomes were queried by BLASTn and tBLASTn using sequences of known resistance genes from *A. pleuropneumoniae* and other members of the *Pasteurellaceae* found in GenBank (Supplementary Table S2). Clustal alignments were used to compare the *gyrA, gyrB*, *parC*, and *parE* genes in isolates with elevated MICs for enrofloxacin (≥0.25 mg/L) with those genes in all remaining isolates in order to identify any mutations that could contribute to enrofloxacin resistance. The profiles of resistance genes identified were then compared to the results of the MIC assays, and where discrepancies were found, isolates were re-tested for susceptibility by disk diffusion, and by PCR for the presence of the specific AMR genes using primers listed in **Table 1**. Correlations between resistance phenotypes and genotypes were calculated using Fisher's exact test function ('fisher.test') in R (version 3.3.2).

RESULTS

Full results of the testing and identification of AMR genes for each isolate are also shown in Supplementary Table S1. The correlations between presence of identified AMR genes and resistance phenotype are shown in **Table 2**. The majority of the 96 isolates tested in this study, representing serovars 2 (11.5%), 6 (7.3%), 7 (10.4%), 8 (68.8%), and 12 (2.1%), were collected between 2005 and 2009, with smaller numbers representing the other years between 1998 and 2011 (Supplementary Table S1).

[1]https://github.com/najoshi/sickle

[2]http://www.ebi.ac.uk/ena

TABLE 1 | Primers used in this study.

Primer name	Sequence	Amplicon size	Source
sul2_for	TCAACATAACCTCGGACAGTTTCTC	212 bp	Bossé et al., 2015a
sul2_rev	GGGAATGCCATCTGCCTTGAGC		
dfrA14_for	CATTGATAGCTGCGAAAGCGAAAAACGGC	343 bp	Bossé et al., 2015b
dfrA14_rev	ATCGTCGATAAGTGGAGCGTAGAGGC		
blaRob_for	GCTGACATTAACGGCTTGTTCGC	820 bp	This study
blaRob_rev	TTTGGCTTCTTCGGTAAATTGCG		
tetB_for	TTTGCGCTGTAGTGCTCCAAT	944 bp	This study
tetB_rev	AACAAATAAAGTTGCTCGAAAGTA		
tetH_for	TAAATACGGCAGAAAACCCATCTTGC	106 bp	This study
tetH_rev	GCGCCCAATATAGAGCATCCAAAGTG		

TABLE 2 | Correlation of phenotypic resistance to selected antimicrobial agents and presence of specific resistance genes detected in draft genomes of 96 *Actinobacillus pleuropneumoniae* isolates from the UK.

Antimicrobial agent[a]	Number of isolates resistant by genotype[b]	Number of isolates resistant by phenotype (associated MIC)	Correlation of genotype with phenotype[c]
Tetracycline	55	58 (MIC $\geq$ 4 mg/L)	94.8% ($p < 2.2e-16$)
Ampicillin	19	19 (MIC $\geq$ 4 mg/L)	100% ($p < 2.2e-16$)
Sulfisoxazole	46	46 (MIC $\geq$ 256 mg/L, or DD = 6 mm)[d]	100% ($p < 2.2e-16$)
Trimethoprim	16	16 (MIC $\geq$ 32 mg/L)	100% ($p < 2.3e-16$)
Enrofloxacin	6	6 (MIC $\geq$ 0.25 mg/L)	100% ($p < 1.1e-09$)

[a]*No isolates were resistant to florefenicol, and no florfenicol resistance genes were detected in the genomes. No specific macrolide resistance genes were identified, and phenotypic resistance to erythromycin, tilmicosin and tylosin was not clear from the MIC values obtained (see Supplementary Table S1 for further details).* [b]*Specific genes detected for each resistance phenotype: tet(B) or tet(H) for tetracycline, bla_{ROB-1} for ampicillin, sul2 for sulfisoxazole; dfrA14 for trimethoprim, and GyrAS83F for enrofloxacin resistant isolates.* [c]*Correlation between resistant phenotype and genotype was calculated using Fisher's exact test function ('fisher.test') in R (version 3.3.2).* [d]*Initially, two isolates with sul2 identified in their genomes had an MIC $\leq$ 16 mg/L, however re-test by disk diffusion showed a zone of inhibition of 6 mm, indicative of resistance. Poor solubility of sulfisoxazole may have caused the discrepancy.*

Only 33% of the isolates did not have any AMR genes, whereas 20% carried a single, and 47% two or more AMR genes. None of the isolates tested were resistant to florefenicol, and no florfenicol resistance genes were detected in the genomes.

The MICs for ampicillin showed clear separation into low MIC (MIC $\leq$ 2 mg/L) for isolates with no resistance gene detected, and high MIC (MIC $\geq$ 8 mg/L) for 18 isolates with $bla_{\mathrm{ROB-1}}$ detected by ResFinder. One isolate (MIDG3354) with an MIC = 4 mg/L also had $bla_{\mathrm{ROB-1}}$ detected by ResFinder. Thus, there was 100% correlation between the presence of $bla_{\mathrm{ROB-1}}$ and an MIC $\geq$ 4 mg/L for ampicillin. In all cases, the $bla_{\mathrm{ROB-1}}$ gene identified showed 99.9% identity with that found in the plasmid pB1000 (accession number DQ840517) from *Haemophilus parasuis* (San Millan et al., 2007). For all but one isolate, the full 918 bp $bla_{\mathrm{ROB-1}}$ sequence was identified on a single contig. In MIDG3443, where only 857/918 bp were identified by ResFinder, BLASTn confirmed that the entire $bla_{\mathrm{ROB-1}}$ sequence was present, but split over two contigs.

For tetracycline, 94.8% of isolates considered phenotypically resistant (MIC $\geq$ 4 mg/L) were found to carry either *tet*(B) or *tet*(H). A 100% correlation was found between the presence either of these genes and an MIC $\geq$ 8 mg/L. We found of four isolates with an MIC = 4 mg/L, only one (MIDG2567) carried a resistance gene, *tet*(B), whereas three other isolates (MIDG3342, MIDG3352, and MIDG3356) had no detectable tetracycline resistance gene. The *tet*(B) gene identified by ResFinder showed 99.9% to 100% identity with a 1206 bp gene found in the *Shigella flexneri* 2a SRL pathogenicity island (accession number AF326777) or the *S. flexneri* 2b plasmid R100 (accession number AP000342). In some cases, the gene was split over two contigs (with only one part identified by ResFinder), as confirmed by BLASTn. In five isolates, *tet*(H) genes were identified that had either 99.9% identity with the 1179 bp gene found in *Pasteurella aerogenes* plasmid pPAT1 (accession number AJ245947) or 100% identity with the 1203 bp sequence found in *Pasteurella multocida* plasmid pPMT1 (accession number Y15510). These two genes differ at their 3′ ends, and BLASTn analysis confirmed the presence of the 1203 bp gene in four *A. pleuropneumoniae* isolates, and the 1179 bp sequence in one.

For trimethoprim, there was a 100% correlation between an MIC $\geq$ 32 mg/L and the presence of *dfrA14* in 16 isolates, as previously described (Bossé et al., 2015b). Isolates lacking *dfrA14* had an MIC $\leq$ 4 mg/L, showing clear separation from the trimethoprim-resistant isolates. There was also a 100% correlation between an MIC $\geq$ 256 mg/L for sulfisoxazole and the presence of the *sul2* gene in 44 isolates (MIC $\geq$ 512 mg/L for 39 of these). In two isolates, MIDG2652 and MID3362, initial results (MIC $\leq$ 16 mg/L) indicated susceptibility despite the presence of the *sul2* gene. PCR confirmed the presence of *sul2* in these isolates, and resistance to sulfisoxazole was confirmed by disk diffusion (zone of inhibition = 6 mm). The *sul2* gene identified by ResFinder shared 100% with an 816 bp gene from *Acinetobacter*

bereziniae (accession number GQ421466). In cases where less than 816 bp of the gene were identified by ResFinder, BLASTn confirmed the presence of the remaining sequence on a separate contig.

The six isolates with elevated MICs for enrofloxacin (≥0.25 mg/L) all had identical sequences for their *gyrA*, *gyrB*, *parA,* and *parE* genes. The amino acid sequence for each conserved protein encoded by these genes was used to search (by tBLASTn) the remaining genomes in order to determine if any mutations could account for the increased resistance to enrofloxacin. Compared to the sequences found in the enrofloxacin-sensitive isolates, no mutations were found in GyrB, ParA or ParC, however, a substitution in the GyrA sequence (S83F) was found only in the six isolates with MICs for enrofloxacin ≥ 0.25 mg/L.

All isolates had an MIC ≤ 16 mg/L for both erythromycin and tilmicosin, whereas there was a clear separation into low (MIC ≤ 2 mg/L) and high (MIC ≥ 32) levels of resistance to tylosin. However, no macrolide resistance genes were identified by ResFinder in any of the isolates, nor were any mutations detected in the 23S rRNA gene, or in the genes encoding ribosomal proteins L4 and L22 (*rplD* and *rplV*, respectively).

DISCUSSION

Recently, several groups have investigated the use of genome sequencing as an alternative/adjunct to phenotypic testing for detection and surveillance of AMR in different bacteria (Zankari et al., 2013; Gordon et al., 2014; Köser et al., 2014; Walker et al., 2015; Zhao et al., 2016). For each bacterium, it is necessary to determine the correlation between genetic detection of a resistance gene with phenotypic determination of resistance in order to validate the usefulness of a wgs approach.

In this study, we investigated 96 clinical isolates of *A. pleuropneumoniae*, an important respiratory pathogen of pigs, and found a high correlation between the presence of specific AMR genes and elevated MICs for the corresponding antimicrobial agents tested. However, the results of macrolide testing are inconclusive. Although 20 isolates had an MIC ≥ 32 mg/L for tylosin (seven of which had an MIC ≥ 64 mg/L), all isolates had an MIC ≤ 16 mg/L for both erythromycin and tilmicosin, and no macrolide resistance genes were detected by ResFinder. Furthermore, no point mutations known to confer resistance to macrolides (e.g., in *rplV*, *rplD*, *rumA*, or the 23S rRNA genes) were detected by BLASTn in any of the isolates with elevated MICs for tylosin. In other studies, where specific macrolide resistance genes were identified in members of the *Pasteurellaceae* (Olsen et al., 2015; Dayao et al., 2016), higher MIC values (≥64 mg/L) were reported for tilmicosin and erythromycin. There is no established CLSI clinical MIC breakpoint for tylosin resistance in *A. pleuropneumoniae*, and a recent VetPath survey (El Garch et al., 2016) also showed that although only 1/158 *A. pleuropneumoniae* isolates tested had an MIC ≥ 32 mg/L for tilmicosin (considered resistant), 144/158 had an MIC ≥ 32 mg/L for tylosin (none considered resistant due to lack of established breakpoint). From these and our results, it is not clear if there is a specific resistance to tylosin associated with an MIC ≥ 64 mg/L, and if so, what is the mechanism of this resistance.

All six isolates with an MIC ≥ 0.25 mg/L for enrofloxacin had an identical amino acid exchange (S83F) in the GyrA protein, which is in agreement with a previous study in *A. pleuropneumoniae* (Wang et al., 2010). Wang et al. (2010) also reported that combinations of this and other mutations in *gyrA*, *parC,* and *parE* were associated with higher levels of resistance to enrofloxacin (MIC ≥ 1 mg/L), but these were not detected in our isolates. The six isolates with reduced susceptibility to enrofloxacin were isolated from samples submitted to the same APHA laboratory (Langford), and five were in the same year. Enrofloxacin resistance does not appear to be widespread in *A. pleuropneumoniae* isolates in the UK.

For trimethoprim resistance, there was a 100% correlation between the presence of *dfrA14* and an MIC ≥ 32 mg/L, as previously reported (Bossé et al., 2015b). Although only 16 of the tested isolates were resistant to trimethoprim, all of these also carried the *sul2* gene and had an MIC ≥ 512 mg/L for sulfisoxazole. The *sul2* gene was detected in a total of 46 isolates, however, phenotypic detection of resistance to sulfisoxazole was problematic in some of these. Although 85% of the isolates carrying *sul2* had an MIC ≥ 512 mg/L, and 96% an MIC ≥ 256 mg/L, two isolates initially showed an MIC ≤ 16 mg/L. When re-tested by disk diffusion, these two isolates showed a zone of 6 mm, consistent with a resistant phenotype (Clinical and Laboratory Standards Institute [CLSI], 2013). Issues with the solubility of sulfisoxazole may account for the discrepancies. Co-location of the *dfrA14* and *sul2* genes in two distinct plasmids (Bossé et al., 2015b) accounts for the 16 isolates with resistance to both trimethoprim and sulfisoxazole. Analysis of the sequences flanking *sul2* in the isolates lacking *dfrA14* indicate other possible plasmids that will require further investigation. Only two of the isolates carrying *sul2* had no other detectable AMR genes. The remaining 44 all had *tet*(B) or *tet*(H), with eighteen also carrying $bla_{\mathrm{ROB-1}}$, and eight of these with *dfrA14* as well. A further eight isolates carried a combination of *tet*(B), *sul2* and *dfrA14*, but lacked the $bla_{\mathrm{ROB-1}}$ gene (Supplementary Table S1).

There was a 100% correlation between the presence of $bla_{\mathrm{ROB-1}}$ in 19 isolates and an MIC ≥ 4 mg/L for ampicillin; with an MIC ≥ 8 mg/L in 18 of these. The sequences flanking $bla_{\mathrm{ROB-1}}$ indicate possible plasmids. Further investigation is required in order to determine if these represent known plasmid(s) described in other *Pasteurellaceae*, such as pB1000 (San Millan et al., 2007) or pJMA-1 (Moleres et al., 2015) or unique plasmids not yet described.

In 98% of isolates carrying either *tet*(B) or *tet*(H), an MIC ≥ 8 mg/L for tetracycline was detected. One isolate with an MIC = 4 mg/L contained the *tet*(B) gene, whereas a further three isolates with an MIC = 4 mg/L did not have any tetracycline resistance genes detected. The reason for this discrepancy is not clear. The sequences flanking *tet*(H) indicate possible plasmid(s), whereas *tet*(B) was found either on small contigs with sequences related to possible plasmid(s), or on larger contigs with sequences indicating a chromosomal insertion

site. In 21 isolates with the latter, we recently identified a 56 kb integrative conjugative element (ICE), ICE*Apl1*, inserted in a tRNA-Leu (TAA) gene, which contains *tet*(B) as part of a Tn*10* insertion (Bossé et al., 2016). In 15 other isolates with *tet*(B) on large contigs, the sequence appears to part of a Tn*7* insertion disrupting the competence related gene, *comM* (see Supplementary Table S1). The majority (55/96) of isolates tested were resistant to tetracycline, and only 11 of these carried no other detectable AMR gene.

Reproducibility of phenotypic results has been an issue with surveillance of AMR, with calls for a more standardized method to allow direct comparisons between labs (Michael et al., 2015; El Garch et al., 2016). Our results confirm that wgs is a valuable method that can be used, either in combination with phenotypic testing or on its own, for surveillance of AMR in *A. pleuropneumoniae*. In each isolate where an AMR gene was identified, phenotypic results confirmed resistance. In the case of macrolides, no genes were identified and it is not clear what MIC for tylosin should be considered as a breakpoint indicating resistance. Genome sequencing not only identifies specific AMR genes, but also gives an indication of their locations, either within the chromosome or on plasmids. ResFinder was useful for identifying and locating known AMR gene sequences within the draft genomes, though in cases where a sequence was split over multiple contigs, only the contig with the larger proportion of the gene was identified. ResFinder is also not capable of detecting point mutations in genes such as *gyrA* that lead to a resistance phenotype. In these cases, BLASTn or tBLASTn can be used to either detect mutations or to determine the location of missing sequences not identified by ResFinder.

Plasmids are the biggest contributors to the spread of AMR, with dissemination occurring not only through clonal expansion of the isolates harboring them, but also by conjugal transfer when genes expressing the required machinery are present. Tracking the spread of AMR genes/plasmids provides important epidemiological information that can best be provided by wgs data. Plasmid sequences are often distributed over multiple small contigs within draft genomes, suggesting either repeat sequences within the plasmid, or the presence of multiple plasmids sharing common backbone sequences. Plasmid isolation and sequencing can determine if a single or multiple small plasmids are present, as has been described for *P. multocida* (San Millan et al., 2009), especially in the case of isolates resistant to multiple antimicrobial agents. Most small plasmids that have been characterized in *A. pleuropneumoniae* and other *Pasteurellaceae* are mobilisable, but do not harbor genes encoding conjugal transfer machinery (Blanco et al., 2007; Matter et al., 2008; Kang et al., 2009; San Millan et al., 2009; Bossé et al., 2015a,b). These plasmids can be disseminated either via clonal expansion or conjugation. The latter mechanism requires the presence of other replicons encoding the required machinery, such as the recently described ICE (ICE*Apl1*, ICE*Mh1*, ICE*Pmu1*), discovered in the genomes of different *Pasteurellaceae* species (Michael et al., 2012; Eidam et al., 2015; Bossé et al., 2016). As more wgs become available for members of the *Pasteurellaceae*, it is likely that more ICE will be identified, and their role in dissemination of small plasmids can be further investigated.

In summary, genome sequencing is likely to be used increasingly in the surveillance of AMR, providing a standard method that can be easily compared between different laboratories in different countries. This method can be used on its own for identification of known resistance genes, or in conjunction with sensitivity testing where resistance mechanisms have yet to be identified. Furthermore, wgs data can provide more information than just AMR genotypes, it can also give insights into the mechanisms allowing spread of resistance amongst isolates.

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AUTHOR CONTRIBUTIONS

JB, PL, AR, BW, DM, AT conceived the study; JB, YaL, JR, RF, YiL, RRC, and MH produced the data; JB, YaL, RF, RRC analyzed the data; JB, PL wrote the paper.

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Single-Molecule Sequencing (PacBio) of the *Staphylococcus capitis* NRCS-A Clone Reveals the Basis of Multidrug Resistance and Adaptation to the Neonatal Intensive Care Unit Environment

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The multi-resistant *Staphylococcus capitis* clone NRCS-A has recently been described as a major pathogen causing nosocomial, late-onset sepsis (LOS) in preterm neonates worldwide. NRCS-A representatives exhibit an atypical antibiotic resistance profile. Here, the complete closed genome (chromosomal and plasmid sequences) of NRCS-A prototype strain CR01 and the draft genomes of three other clinical NRCS-A strains from Australia, Belgium and the United Kingdom are annotated and compared to available non-NRCS-A *S. capitis* genomes. Our goal was to delineate the uniqueness of the NRCS-A clone with respect to antibiotic resistance, virulence factors and mobile genetic elements. We identified 6 antimicrobial resistance genes, all carried by mobile genetic elements. Previously described virulence genes present in the NRCS-A genomes are shared with the six non-NRCS-A *S. capitis* genomes. Overall, 63 genes are specific to the NRCS-A lineage, including 28 genes located in the methicillin-resistance cassette SCC*mec*. Among the 35 remaining genes, 25 are of unknown function, and 9 correspond to an additional type I restriction modification system ($n = 3$), a cytosine methylation operon ($n = 2$), and a cluster of genes related to the biosynthesis of teichoic acids ($n = 4$). Interestingly, a tenth gene corresponds to a resistance determinant for nisin (*nsr* gene), a bacteriocin secreted by potential NRCS-A strain niche competitors in the gut microbiota. The genomic characteristics presented here emphasize the contribution of mobile genetic elements to the emergence of multidrug resistance in the *S. capitis* NRCS-A clone. No NRCS-A-specific known virulence determinant was detected, which does not support a role for virulence as a driving force of NRCS-A emergence in NICUs worldwide. However, the presence of a nisin resistance determinant on the

NRCS-A chromosome, but not in other *S. capitis* strains and most coagulase-negative representatives, might confer a competitive advantage to NRCS-A strains during the early steps of gut colonization in neonates. This suggests that the striking adaptation of NRCS-A to the NICU environment might be related to its specific antimicrobial resistance and also to a possible enhanced ability to challenge competing bacteria in its ecological niche.

Keywords: bacteremia, multiple drug resistance, late-onset sepsis, SMRT, nisin, comparative genomics, *Staphylococcus capitis*

INTRODUCTION

Coagulase-negative staphylococci (CoNS) are common commensals of the human skin and mucosa and are also opportunistic pathogens responsible for infections associated with indwelling medical devices and bloodstream infections (Otto, 2009). Among CoNSs, *Staphylococcus epidermidis* is the most prevalent species and is classically involved in nosocomial bacteremia in patients with co-morbidities including very low-birth weight preterm infants (Otto, 2009). However, the *Staphylococcus capitis* has also been incriminated in sepsis throughout the world in neonatal intensive care units (NICUs) (Van Der Zwet et al., 2002; Venkatesh et al., 2006; D'mello et al., 2008; Rasigade et al., 2012; Boghossian et al., 2013; Cui et al., 2013). We recently reported that a group of closely related multidrug-resistant (MDR) *S. capitis* strains belonging to the same clone, named NRCS-A, were present in most French NICUs but absent in the other pediatric or adult ICUs (Rasigade et al., 2012). In subsequent work, we demonstrated the dissemination of NRCS-A strains in distant NICUs throughout the world (Butin et al., 2016). This dissemination is of major concern because of the extensive drug resistance of NRCS-A as well as its ability to become highly endemic in some NICUs, representing up to 40% of all bacteremia cases (Stoll et al., 2002, 2005; Rasigade et al., 2012). Due to the presence of the type V-related staphylococcal chromosome cassette *mec* (SCC*mec*), all *S. capitis* NRCS-A strains are resistant to beta-lactams and exhibit reduced susceptibility to other antimicrobial agents in common use in NICUs, including resistance to aminoglycosides and resistance or heteroresistance to vancomycin (Venkatesh et al., 2006; Rasigade et al., 2012). Overall, the worldwide diffusion of a multidrug-resistant *S. capitis* clone that is highly adapted to NICU antibiotic selective pressure raises questions about the potential genetic support of its epidemiological success.

Few genome sequences of *S. capitis* are publicly available to date. In addition, only a single study using the genome of a *S. capitis* isolate collected from a bloodstream infection in an adult has reported the genomic comparison of virulence factors between *S. capitis* and *S. epidermidis* (Cameron et al., 2015). Considering the specific importance NRCS-A in the NICU setting and its international dissemination, we present here the first closed genome sequence of the French NRCS-A prototype strain CR01, a comparison of the whole-genome sequences (WGS) of three other NRCS-A strains collected from distant countries (Australia, Belgium, the United Kingdom), and a description of the genomic features of this NRCS-A clone.

METHODS

Genome Sequencing and Assembly

The four strains of *S. capitis*, CR01, CR03, CR04, and CR05, used in this study were part of a previously published collection, with all strains being of the international *S. capitis* NRCS-A clone (Butin et al., 2016). The strains were isolated from blood cultures of preterm infants diagnosed with neonatal sepsis in Neonatal Intensive Care Units (NICUs) from four different countries: France (isolate CR01), Belgium (isolate CR03), Australia (isolate CR04) and the United Kingdom (isolate CR05) (for further details see Supplementary Data).

Initially, whole-genome pyrosequencing (454 Life Sciences/Roche) was performed for all 4 isolates, as previously reported (Lemriss et al., 2014, 2015). Single-molecule real-time (SMRT) sequencing was then performed for strain CR01 to obtain a closed reference genome for this clone (Supplementary Data).

Sequencing for *de novo* assembly was performed using PacBio RS II (Menlo Park, CA, USA). High molecular weight DNA was sheared in a Covaris g-TUBE (Covaris, Woburn, MA, USA) to obtain 20 kb fragments. After shearing, the DNA size distribution was checked using a Fragment Analyzer (Advanced Analytical Technologies, Ames, IA, USA), and 5 μg of the sheared DNA was used to prepare a SMRTbell library with PacBio SMRTbell Template Prep Kit 1 (Pacific Biosciences, Menlo Park, CA, USA) according to the manufacturer's recommendations. The resulting library was size selected using a BluePippin system (Sage Science, Inc. Beverly, MA, USA) for molecules larger than 11 kb. The recovered library was sequenced using 1 SMRT cell with P6-C4 chemistry and MagBeads with a PacBio RSII system (Pacific Biosciences, Menlo Park, CA, USA) at 240 min movie length.

Based on the SMRT cell sequencing, we generated 50,876 post-filter polymerase reads with an average read length of 15.6 kb and an N50 read length of 21.1 kb. The average coverage was 265X; for *de novo* assembly, the PacBio module "RS_HGAP_Assembly.2" in SMRTpipe version v2.3.0 was used for continuous long reads (CLR) after polishing and error correction with Quiver, as described previously (Chin et al., 2013). DNA methylation was determined using the RS_Modification_and_Motif_Analysis protocol within SMRT Portal v2.30, with a standardized *in silico* false positive error of ∼1%. Only motifs with a mean modification quality value (QV) >50 and a mean coverage of >100X were validated as being modified (https://github.com/PacificBiosciences/Bioinformatics-Training/wiki/Methylome-Analysis-Technical-Note). Two polished contigs,

one corresponding to the chromosome and the other to the plasmid, were obtained. Circularization of both contigs was achieved by manual comparison and removal of regions of overlaps.

Computational Analysis

Automatic syntactic and functional annotation of the closed, reference genome of strain CR01 [EMBL accession number: LN866849 (chromosome) and LN866850 (plasmid)] was performed using the MicroScope platform pipeline (Vallenet et al., 2006, 2013). The annotations of the draft genomes of strains CR03 (EMBL accession number: CVUF01000001-CVUF01000031), CR04 (EMBL accession number: CTEM01000001-CTEM01000038) and CR05 (EMBL accession number: CTEO01000001-CTEO01000039) were also performed using the MicroScope platform pipeline, as published previously (Lemriss et al., 2014). Visual inspection and manual curation were carried out using the MaGe platform and BLAST searches against NCBI databases.

Virulence factors were identified using the Virulence Factors database (VFDB; http://www.mgc.ac.cn/VFs/, Chen et al., 2012) and tblastn (using the ncbi-blast-2.2.27+ suite, Camacho et al., 2009) searches against in-house databases of known virulence genes of staphylococci.

Prophage regions were identified using the PHAST server (http://phast.wishartlab.com, Zhou et al., 2011). The predicted results were confirmed using the MaGe platform.

For comparison, we obtained publicly available assemblies for six additional whole-genome sequences of *S. capitis*: strains QN1 (NCBI Ref. Seq.: NZ_AJTH00000000.1), VCU116 (NCBI Ref. Seq.: NZ_AFTX00000000.1), SK14 (NCBI Ref. Seq.: NZ_ACFR00000000.1), LNZR-1 (NCBI Ref. Seq.: NZ_JGYJ00000000.1), C87 (NCBI Ref. Seq.: NZ_ACRH00000000.1), and AYP1020 (NCBI Ref. Seq.: NZ_CP007601.1). All six genomes were aligned against the closed genome of isolate CR01 using Mauve Progressive v.2.3.1 (Darling et al., 2010) with default settings.

Resistance genes were identified by a combination of ResFinder v.2.1 (Kleinheinz et al., 2014) and tblastn searches using the complete, curated Antibiotic Resistance Proteins database (multifasta file) available at the Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al., 2013). For ResFinder, cutoffs of 80% minimum length and 90% identity were used to search for resistance genes in CR03, CR04, and CR05 assemblies previously performed against the reference CR01 using Mauve Progressive to determine their location and genomic context. For detection of resistance genes using the CARD complete, curated Antibiotic Resistance Proteins database, tblastn searches against a database composed of the four genomes of strains CR01, CR03, CR04, and CR05 were performed.

Genes specific to the NRCS-A clone were identified using the MaGe platform "Gene Phyloprofiler" tool (Vallenet et al., 2013) and the four annotated genomes of NRCS-A and public *S. capitis* genomes. Briefly, genomes were compared in terms of gene content using pre-computed homologies and synteny groups. Pairwise comparisons between predicted protein sequences of the studied genome and the proteins of another genome allowed computation of ranked hits and determination of bidirectional best hits (BBH) (for each protein, the three best hits were retained). Putative orthologous relationships between two genomes were defined as gene couples satisfying the BBH criterion and an alignment threshold for homology set as a minimum of 50% sequence identity along 80% of the length of the smallest protein. Mauve alignments were also used to complement the genomic context, followed by manual curation of the putative genes exclusively found in only the four NRCS-A genomes.

Insertion sequences (IS) in the genomes were identified using the ISfinder analysis tool (Siguier et al., 2006). Both blastn and blastp queries were performed with default settings. Matches with an *e*-value smaller than 0.5 were manually curated. To confirm whether a given IS position was conserved in the other genomes analyzed, the closed genome of isolate CR01 was aligned with the three other NRCS-A genomes and with the six public genomes using Mauve Progressive v.2.3.1 (Darling et al., 2010) with default settings. ORFs within immediate proximity of conserved IS loci in the NRCS-A genomes were compared with the *S. capitis* reference genome to check for potential alterations in coding sequences, using both Mauve Progressive and Mage platform. genomic islands (GIs) were predicted using IslandViewer 3 (Dhillon et al., 2015) and confirmed by alignment with the three NCRS-A genomes and the six public *S. capitis* genomes using Mauve Progressive. IS insertions within GIs were predicted as mentioned above. NRCS-A-specific GIs were searched in the NCBI database using megablast (Camacho et al., 2009) query, limiting to the *Staphylococcus* taxon (taxid: 1279). Published GIs from reference genomes were searched against *Staphylococcus aureus* Mu50 (NCBI Reference Sequence: NC_002758.2), COL (NCBI Reference Sequence: NC_002951.2), MW2 (NCBI Reference Sequence: NC_003923.1), N315 (NCBI Reference Sequence: NC_002745.2), FPR3757 (NCBI Reference Sequence: NC_007793.1) and S0385 (NCBI Reference Sequence: NC_017333.1), and *S. epidermidis* RP62A (NCBI Reference Sequence: NC_002976.3) using the Mage platform [15]. Syntonomes of more than 3 ORFs and with identity greater than 33% were considered to be GIs.

Investigation of *nsr* Gene Presence in Clinical Bacterial Strains

A panel of 15 strains (**Table 1**) comprising clinical strains of *S. capitis* (NRCS-A and non-NRCS-A) were used for nisin susceptibility testing. All staphylococcal species were originally isolated from blood, and species assignment was determined using Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF Vitek MS, Biomerieux, France). After isolation, the strains were cultured on Horse Blood Agar (Biomerieux) and then stored at −80°. Testing for susceptibility to nisin was performed using the disk diffusion test adapted from EUCAST[1]. Briefly, nisin solutions were

[1]European Committee on antimicrobial Susceptibility Testing, 2015, http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/Manual_v_5.0_EUCAST_Disk_Test.pdf

TABLE 1 | Bacterial strains used in the nisin susceptibility test.

Species	NRCS-A clone	Strain	Setting	Country	*nsr* gene
SC	yes	CR01	NICU	FR	+
SC	yes	CR03	NICU	BE	+
SC	yes	CR04	NICU	AUS	+
SC	yes	CR05	NICU	UK	+
SC	yes	CR07	NICU	FR	+
SC	yes	BC69	NICU	South K	+
SC	yes	AQ62	NICU	NO	+
SC	yes	AW77	NICU	NZ	+
SC	yes	BI76	NICU	USA	+
SC	no	AB51	ICU	AUS	−
SC	no	CR02	ICU	FR	−
SC	no	AR22	ICU	DK	−
SC	no	AY18	ICU	GR	−
SC	no	AZ72	ICU	Sing	−
SC	no	BA24	ICU	USA	−

SC, Staphylococcus capitis; FR, France; BE, Belgium; UK, United Kingdom; AUS, Australia; South K, South Korea; NO, Norway; NZ, New Zealand; USA, United States of America; DK, Denmark; GR, Greece; Sing, Singapore; NICU, neonatal intensive care unit; ICU, intensive care unit (adults).

prepared using 2.5% nisin powder (Sigma Aldrich, USA) according to a previously described protocol (Piper et al., 2009). Sterile paper disks (Thermofisher Diagnostics, France) were soaked with 30 μg of nisin solution. After an overnight incubation at 36°C on Horse blood Agar (Biomerieux, France), a 0.5 McF was seeded on Mueller Hinton agar (MHE) plates (Biomerieux, France). A disk containing 30 μg of nisin was placed in the middle of the plate. The diameter of the inhibition zones was measured after overnight incubation of the inoculated MHE plates at 36°C. The average diameter of inhibition and SEM were determined from four independent experiments.

PCRs for detecting the *nsr* gene were performed using consensus primers: pF—5′GGAGATATGGGACCTATGA TTGCA 3′ and pR—5′GCTGTAAkTTCwCCkGAACTkG C 3′. These primers were designed based on alignment of the *nsr* gene sequence found in strain CR01 (*S. capitis* clone NRCS-A) and sequences retrieved from NCBI after a blastn (ref) search for homologous sequences in staphylococcal WGS genomes [*S. hyicus* ATCC 11249 (acc. num. CP008747), *Staphylococcus* sp. TE8 (acc. num. NZ_JMGB00000000.1), *S. epidermidis* MC28 (acc. num. NZ_ATCZ00000000.2), *S. epidermidis* VCU128 (acc. num. NZ_AHLI00000000.1)]. PCR amplification was conducted in a final volume of 25 μL, with 2.0 μL bacterial DNA (QuickExtract kit, Qiagen), 2.5 μL 10x Reaction Buffer, 0.75 μL 50 mM MgCl2, 1 μL 10 mM forward primer, 1 μL 100 mM reverse primer, 4 μL 5 mM dNTPs, 1 μL each primer and 0.125 μL Taq polymerase (Eurobio, France). The cycling parameters were as follows: (i) initial denaturation for 3 min at 95°C; (ii) 34 cycles, with 1 cycle consisting of 30 s at 95°C, 60 s at 60°C, and 90 s at 72°C; and (iii) a final extension for 10 min at 72°C.

RESULTS

WGSs of four strains belonging to *S. capitis* clone NRCS-A and originating from NICUs in four distant countries were obtained to investigate lineage-specific virulomes, resistomes, and mobilomes as well as the presence of unshared genes using 454 pyrosequencing technology, as reported previously (Lemriss et al., 2014, 2015). Depending on the isolate, assembly of the draft genomes produced 26 to 39 contigs. Re-sequencing of strain CR01 using SMRT technology (PacBio) allowed for the generation of closed complete sequences for the chromosome and the single plasmid that was subsequently used to produce scaffolds for the three remaining WGSs.

The length of CR01's chromosome is 2,522,871 bp and exhibits a low G + C content (33.02%), as is expected for staphylococci. We identified and annotated 2419 protein-coding regions, 19 rRNAs, 62 tRNAs, 34 ncRNAs (including RNAIII), and 20 transposases associated with insertion sequence elements and transposons. Of note, gaps (identified in the draft genome assemblies of the four NRCS-A isolates (based on 454 pyrosequencing)) frequently occur within the vicinity of transposases, suggesting that some of the 454 short read assemblies were unable to bridge the gaps associated with repetitive genomic elements. These data clearly emphasize the suitability of third-generation sequencing technologies, such as PacBio SMRT, for obtaining fully defined *de novo* assemblies and, thus, for overcoming the issue of both local and global repeats (Koren and Phillippy, 2015). The genomic characteristics of the NRCS-A isolates tested in this study are summarized in **Table 2**.

Virulome

The presence of virulence-associated genes in the closed genome of strain CR01 and subsequently the draft genomes of strains CR03 (Be), CR04 (Aus), and CR05 (UK) were inferred by comparison with known virulence factors previously reported for *S. aureus*, *S. epidermidis,* and *S. haemolyticus* using the VFDB database (Chen et al., 2012). The findings were augmented with BLAST searches of well-characterized staphylococcal virulence factors and key regulators (**Table 3**). Comparison of the four NRCS-A genomes with the six published genomes of non-NRCS-A *S. capitis*, including QN1, VCU116, SK14, LNZR-1, C87, and AYP1020 strains, showed that none of the known staphylococcal virulence genes are exclusively carried by the NRCS-A clone.

Interestingly, the virulome of the NRCS-A *S. capitis* genome was quite similar to that of *S. epidermidis* RP62A, including the *ica*ABDCR and *cap*ABC biofilm-related operons, Clp proteases and multiple copies of PSM beta type 1b in tandem (75% aa identity to PSMbeta1b of strain RP62) and one PSM alpha (59% aa identity) (Otto, 2012). All PSMs were found in the same genetic environment as in the *S. epidermidis* RP62A genome, with conservation of upstream and downstream genes.

As previously observed for the fully closed *S. epidermidis* RP62a and *S. epidermidis* ATCC 12228 genomes (Zhang et al., 2003; Gill et al., 2005), no *S. aureus* toxins, except beta- and delta-hemolysins, were identified in any of the 10 *S. capitis* genomes

TABLE 2 | General genomic features of *S. capitis* strain CR01 compared with another three WGS NRCS-A genomes.

Features	*S. capitis* strain CR01 (Fr)	*S. capitis* strain CR03 (Be)	*S. capitis* strain CR04 (Aus)	*S. capitis* strain CR05 (UK)
GC% content	33.02	32.81	32.80	32.84
Nb of phages	1	1	1	3
Nb of plasmids	1	1[a]	1[a]	0
Nb and type of insertion sequence (nb on chromosome + nb on plasmid)	IS256: 10 + 0 IS1272: 3 + 1 IS431mec-like: 2 + 3	IS256: 1[b] + 0 IS1272: 1 + 0 IS431mec-like: 2 + 4	IS256: 1[b] + 0 IS1272: 1 + 0 IS431mec-like: 2 + 0	IS256: 1[b] IS1272: 0 IS431mec-like: 2

[a] *Putative plasmid, no definitive data about circularization.*

[b] *Insertion sequence isolated in a small contig, indicating probable multiple insertions in the complete genome.*

(four NRCS-A and six public non-NRCS-A). Similarly, none of the genes associated with secretion systems (*esx*A, *esx*B, *esa*A, *esa*B, *esa*C, *ess*A, *ess*B, and *ess*C) or *S. aureus* serine proteases (*spl*A, B, C, D, E, and F) are present in the *S. capitis* genomes.

Resistome

Several antibiotic resistance-associated genes were identified and correlated with the specific resistance phenotype previously reported for the NRCS-A clone (Rasigade et al., 2012; **Table 4**). All are located on mobile genetic elements (MGEs). Resistance to aminoglycosides is related to the bifunctional aminoglycoside-modifying gene *aacA-aphD*, which is carried on the transposon *Tn*4001 (GenBank accession no. AB682805.1; Lyon et al., 1984). Resistance to methicillin is due to the presence of a composite SCC*mec*-SCC*cad*/*ars*/*cop* cassette exclusively present in the NRCS-A clone, as recently published by our team (Martins Simões et al., 2013). Genomic comparison between the four NRCS-A strains showed that this mobile element is nearly identical: all ORFs are conserved (100% aa homology), and the few nucleotide sequence variations occur only in the CRISPR repeat region, as is expected for these regions. As previously reported (Martins Simões et al., 2013), no other antibiotic resistance genes were detected for this composite SCC element. Finally, the plasmidic *bla* operon, coding for *bla*Z beta-lactamase and its regulators, is present in all NRCS-A strains, except strain CR05 (UK).

With regard to other antimicrobial families, for which variability in resistance profiles have been observed among NRCS-A isolates, phenotypic antimicrobial susceptibility testing matched the specific resistance gene contents of each NRCS-A strain. Plasmidic *msr*A and *tet*K genes, respectively involved in erythromycin resistance (with a negative D-test) and tetracycline resistance, were only identified in strain CR04 (Australia). In strain CR05 (UK), the gene *far*1, which is responsible for fusidic acid resistance, is carried by a putative phage.

Mobile Elements

As mentioned previously, the shortest circular sequence obtained by SMRT for strain CR01 corresponds to a plasmid of 26.140 bp (GC content of 29.25%). It harbors 35 ORFs, including (i) the *bla* operon coding for penicillinase resistance to penicillins (see above) and (ii) copper resistance-related genes such as *cop*Z, *cop*A, and *cso*R (copper transcriptional repressor; **Figure 1**). Genome comparison of strain CR01's plasmid with the three other NRCS-A draft genomes revealed that the plasmid is not conserved in all NRCS-A strains (**Figure 1**). In strain CR03 (Belgium), one contig presents 31 of the 35 ORFs identified on the CR01 plasmid. Conversely, in strain CR04 (Australia), only 9 of the 35 ORFs (corresponding to the *blaZRI* operon and copper resistance operon) were detected in a contig corresponding to a putative plasmid. Finally, no putative plasmid was detected in strain CR05 (UK).

One intact prophage region was identified in all four NRCS-A genomes. This region shows 100% aa identity to the genomes of strains CR01, CR03, and CR04, but it is degenerate in CR05 (**Figure 2**). Moreover, this phage exhibits strong homology to a phage present in strain VCU116 [57.1, 90.2, 76.2% aa homology for regions 1, 2 and 3, respectively (**Figure 2**)] and less than 30% homology with phage Staphy_StB20_(NCBI Ref. Seq. NC_019915), the closest phage found in the NCBI database. Of note, two additional incomplete prophage regions were also detected in strain CR05.

Nineteen distinct insertion sequence (IS) elements, clustering into three distinct families (type IS256, $n = 4$; type IS1272, $n = 5$; IS431mec, $n = 10$), were identified in the CR01 genome. The presence of type IS256 was confirmed in all four NRCS-A strains, with two IS256 elements being associated with the aminoglycosides resistance gene aacA-aphD (′aac(6′)-aph(2″) on transposon *Tn*4001. Type IS1272 was also found in strains CR03 and CR04 but not in strain CR05, and their genomic locations are not conserved. Two IS431*mec* cassettes are present in all NRCS-A genomes, as they are carried within the SCC*mec*-SCC*cad*/*ars*/*cop* element that is specific to and conserved among the NRCS-A lineage (Martins Simões et al., 2013).

Finally, other GIs ($n = 5$) were predicted for CR01 (**Table 2**) and found in the CR03, CR04, and CR05 strains. However, only one was found exclusively in the NRCS-A lineage. This GI (bp position: 150,780–158,140 in the strain CR01 genome) contains six ORFs: one putative phosphoglycolate phosphatase, one conserved protein of unknown function and three pseudogenes of phage proteins (bp position: 151,470–152,238). Unexpectedly, two of these pseudogenes present 40 and 46.5% aa identity to *Listeria monocytogenes* phage B025 protein gp27 and the third pseudogene presents 60.6% aa identity to *L. monocytogenes* phage B025 protein gp28. Both gp27 and gp28 proteins have unknown functions.

TABLE 3 | Comparison of virulence factors in *S. capitis* NRCS-A, non-NRCS-A, and *S. epidermidis* after exclusion of virulence factors present only in *S. aureus*.

Function	Gene	*S. epidermidis*		*S. capitis* NRCS-A clone				*S. capitis* non-NRCS-A				
		ATCC 12228	RP62A	CR01 (Fr)	CR03 (Be)	CR04 (Aus)	CR05 (UK)	SK14	VCU116	C87	QN1	LNZR-1
Adherence	*atl*	+	+	+	+	+	+	+	+	+	+	+
	ebh	+	+	+	+	+	+	+	+	+	+	+
	ebp	+	+	+	+	+	+	+	+	+	+	+
	icaR	0	+	+	+	+	+	+	+	+	+	+
	icaA	0	+	+	+	+	+	+	+	+	+	+
	icaD	0	+	+	+	+	+	+	+	+	+	+
	icaB	0	+	+	+	+	+	+	+	+	+	+
	icaC	0	+	+	+	+	+	+	+	+	+	+
	sdrF	+	+	0	0	0	0	0	0	0	0	0
	sdrG	+	+	0	0	0	0	0	0	0	0	0
	sdrH	+	+	+	+	+	+	+	+	+	+	+
Exozymes	*sspB*	+	+	0	0	0	0	0	0	0	0	0
	sspC	0	+	0	0	0	0	0	0	0	0	0
	lip	+	+	+	+	+	+	+	+	+	+	+
	geh	+	+	+	+	+	+	+	+	+	+	+
	sspA	+	+	0	0	0	0	0	0	0	0	0
	nuc	+	+	+	+	+	+	+	+	+	+	+
Host immune invasion	*capABC*	+	+	+	+	+	+	+	+	+	+	+
Toxins	*hlb*	+	+	+	+	+	+	+	+	+	+	+
	hld	+	+	+	+	+	+	+	+	+	+	+
	hemolysin	0	+	+	+	+	+	+	+	+	+	+
	hlIII	+	+	+	+	+	+	+	+	+	+	+
Proteases	*ClpP*	+	+	+	+	+	+	+	+	+	+	+
	ClpB	+	+	+	+	+	+	+	+	+	+	+
	ClpC	+	+	+	+	+	+	+	+	+	+	+
	ClpX	+	+	+	+	+	+	+	+	+	+	+
Phenol-soluble modulins	*psm α*	+	+	+	+	+	+	+	+	+	+	+
	psm β1a	+	+	0	0	0	0	0	0	0	0	0
	psm β1B	+	+	+	+	+	+	+	+	+	+	+
	psm β2	+	+	+	+	+	+	+	+	+	+	+
	psm β3	+	+	0	0	0	0	0	0	0	0	0
	psm δ	+	+	+	+	+	+	+	+	+	+	+
	psmε	+	+	+	+	+	+	+	+	+	+	+
	psm-mec	+	+	0	0	0	0	0	0	0	0	0
Esterases	*esterase1*	+	+	+	+	+	+	+	+	+	+	+
	esterase2	+	+	+	+	+	+	+	+	+	+	+
HTH transcription factors	*SarA*	+	+	+	+	+	+	+	+	+	+	+
	SarR	+	+	+	+	+	+	+	+	+	+	+
	SarV	+	+	+	+	+	+	+	+	+	+	+
	SarX	+	+	+	+	+	+	+	+	+	+	+
	SarZ	+	+	+	+	+	+	+	+	+	+	+
	MgrA	+	+	+	+	+	+	+	+	+	+	+
	Rot	+	+	+	+	+	+	+	+	+	+	+

The presence of virulence genes is indicated by the "+" sign.

TABLE 4 | Genomic profiles of antibiotic resistance for clone NRCS-A strains from four different countries.

Antibiotic	Gene	Strains				Genomic context
		CR01 (FR)	CR03 (Be)	CR04 (Aus)	CR05 (UK)	
Penicillin	*blaZ*	+	+	+	–	Plasmid
Methicillin	*mecA*	+	+	+	+	Chromosome
Tetracycline	*tetK*	–	–	+	–	Plasmid*
Aminoglycosides	*aac(6′)-aph(2″)*	+	+	+	+	Chromosome
Macrolide, Lincosamide and Streptogramin B	*msr(A)*	–	–	+	–	Plasmid*
Fusidic acid	*far/fusB*	–	–	–	+	Phage (chromosome)*

*All antibiotic resistance genes were found in MGEs. Putative regions in the draft genomes are indicated by an *.*

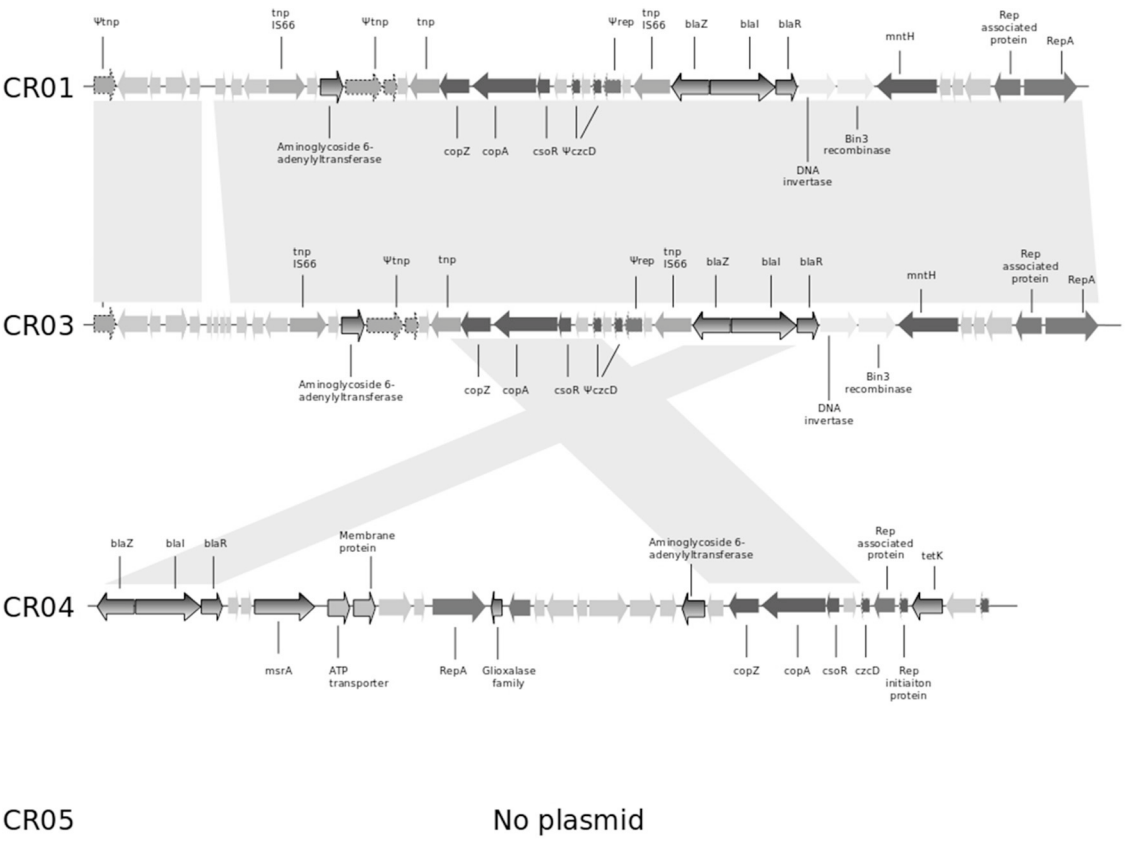

FIGURE 1 | Comparison of the genetic content of the strain CR01 plasmid with the draft genomes of the other three NRCS-A strains. Open reading frames (ORFs) are shown as arrows indicating the direction of transcription. Homologous gene clusters are indicated by a light gray shadow connecting ORFs present in distinct plasmid sequences. Light gray arrows represent unknown proteins, unless specified otherwise. Arrows with dotted lines represent partial or truncated ORFs. Antibiotic resistance genes are colored with a gray gradient; genes associated with resistance to heavy metals are colored in dark gray. Abbreviations: *tnp*, transposase; *copZ*, copper insertion chaperone and transporter component; *cop*A, copper transporter ATPase; *cso*R, copper-sensing transcriptional repressor; *czc*D, potassium/proton-divalent cation antiporter; *rep*, replication-associated family protein; *rep*A, replication-associated protein RepA; *blaZ*, beta-lactamase resistance gene; *bla*R, regulatory protein BlaR1; *bla*I, penicillinase repressor; *mnt*H, divalent metal cation transporter MntH, *tet*K, tetracycline resistance gene.

Methylome and Restriction Modification Systems

SMRT technology (PacBio) allows for genome-wide detection of modified nucleotides based on the rate at which DNA polymerase incorporates bases during sequencing (Flusberg et al., 2010, Nat. Methods; Roberts et al., 2013). Analysis of polymerase kinetic profiles in strain CR01 identified 1333 methylated positions (Table S2) including 94% ($n = 1253$) corresponding to adenine methylations (m6A) and 0.006% ($n = 8$) to cytosine methylations (m4C). The adenine modifications observed correlate with the presence of two adenine restriction-modification systems (*hsd*MSR 1 pos: 476,473–482,360 bp;

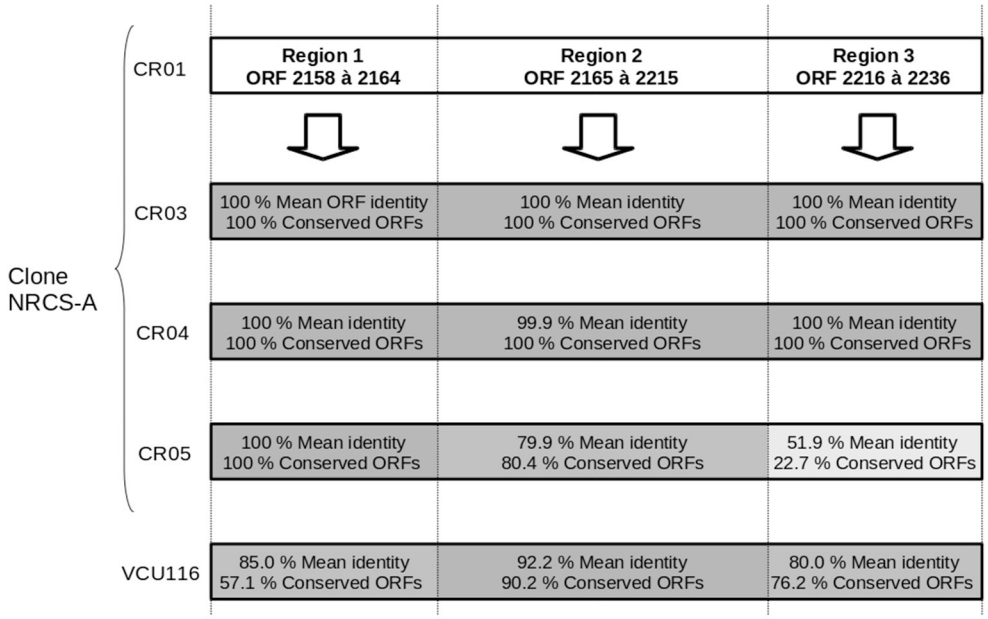

FIGURE 2 | Comparison of the genetic content of the strain CR01 intact prophage present in the other three draft genomes of NRCS-A strains. Prophage prediction was performed using PHAST software; cR01's prophage is divided into three regions according to the degree of conservation compared to the other strains. The mean percent of aa identity in each region is indicated, and a gray gradient is also used to represent the percent similarity.

*hsd*MSR 2 pos: 686,750–691,866 bp) in the strain CR01 genome, whereas the presence of an *mcr*BC 5-methylcytosine restriction system (bp position 487,030–492,276) correlates with the cytosine modifications detected. Interestingly, the latter is associated in tandem with the specific additional *hds*MSR 1 operon located immediately after the SCC*mec*-SCC*cad*/*ars*/*cop* element (see above).

NRCS-A Clone Specific Genes

Comparison of the closed genome of strain CR01 with the draft genomes of the three other NRCS-A genomes and with the six public non-NRCS-A genomes revealed a unique set of 63 genes present exclusively in the NRCS-A clone genome (Table S1). Of these, 28 ORFS are carried by the composite SCC*mec*-SCC*cad*/*ars*/*cop* mobile element, which is related to the acquisition of methicillin resistance (*mec*A gene). Interestingly, within this cassette, the CRISPR element is specific to the NRCS-A lineage (Martins Simões et al., 2013). Of note, both the clone-specific cassette (28 genes) and its genetic environment (downstream and upstream regions) are fully conserved in the four NRCS-A strains, which confirmed that the four isolates belong to the same clonal population, even though they were isolated from distant countries/continents. Indeed, it is highly unlikely that the same SCC*mec* element (100% identity) could be acquired in four independent events in four distant countries.

Among the 35 remaining genes found exclusively in the NRCS-A lineage, 25 are of unknown function, and 10 correspond to the following: (i) an additional type I restriction modification system (*hsd*MSR, $n = 3$ genes), (ii) a cytosine methylation operon (*mcr*BC, $n = 2$), (iii) a cluster of four genes known to be involved in the biosynthesis of teichoic acids [*isp*D (2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase), *tar*J (ribitol-5-phosphate dehydrogenase), *tar*K (Glycosyl glycerophosphate), and *tag*F (CDP-glycerol glycerophosphotransferase), $n = 4$]. Of note, the tenth gene encodes a 316 aa protein presenting 41% aa homology with the determinant for resistance to nisin (*nsr* gene) that is present in some *Lactococcus lactis* strains (Froseth and McKay, 1991; Liu et al., 2005) and which shows protease activity. Contrary to the plasmidic localization of the *nsr* gene in nisin-resistant *L. lactis* strains, the *nsr* gene in the NRCS-A clone is located on the chromosome (position: 521,747–522,694 bp) immediately upstream from the potassium (K+) transporter *kdp*EDABC operon conserved in all *S. capitis* isolates and downstream from the four teichoic acid biosynthesis genes (*isp*D, *tar*J, *tar*K, and *tag*F; see above). No IS or transposon-like region was identified near the *nsr* gene.

Phenotypic Assay of Clone NRCS-A Nisin Resistance

To assess functional expression of the nisin resistance gene (nsr) found exclusively in the NRCS-A clone, we performed a disk diffusion test with nisin-charged disks using a collection of *S. capitis* strains, including nine strains belonging to the NRCS-A clone and six *nsr*-negative, non-NRCS-A strains. The *S. capitis* strains belonging to the NRCS-A clone presented significantly lower nisin inhibition zones (mean of four independent measurements ± SEM: 8.81 mm ± 1.31) than the strains isolated from adults that do not carry the *nsr* gene (16.5 ± 1.5; the Student *t*-test, $p < 0.001$ vs. 8.8 mm ± 1.3 in NRCS-A). This confirms that the *nsr* gene found exclusively in *S. capitis* strains

belonging to the neonatal NRCS-A clone is functional and confers resistance to nisin. Nonetheless, it is known (i) that *S. aureus* (SA) can modulate its resistance to nisin and other small antibacterial peptides via two-component systems (TCSs) GraSR, NsaSR, BraSR in association with ABC transporters (Howden et al., 2010; Blake et al., 2011; Falord et al., 2011; Kolar et al., 2011; Kawada-Matsuo et al., 2013) and (ii) that SA strains resistant or heteroresistant to vancomycin (VISA and hVISA) usually present increased cell wall thickening (Cui et al., 2003). The same mechanisms have been observed in *L. lactis* resistant to nisin (Kramer et al., 2006; Shin et al., 2016). Thus, to assess whether the resistance to nisin observed in the NRCS-A strains is due exclusively to expression of the *nsr* gene and not to a cell wall-thickening adaptation, complementary experiments are required using *nsr+/nsr−* isogenic isolates. Such analyses are beyond the scope of the present paper.

DISCUSSION

The NRCS-A clone is of major interest due to its worldwide high dissemination in NICUs and its prevalence as an agent of neonatal sepsis in preterm newborns (Butin et al., 2016). Comparison between the complete genome of the prototype strain belonging to the *S. capitis* NRCS-A clone (strain CR01, France) with the draft genomes of clinical *S. capitis* isolates belonging or not to the NRCS-A clone revealed the presence of multiple MGEs mediating the atypical antibiotic resistance profile of this clone. These data confirm (i) the ability of the NRCS-A clone to adapt to the specific selective pressure of the antibiotics used in NICUs and (ii) emphasize the ability of WGS to provide accurate predictions of the resistance phenotypes of staphylococci and to become a promising alternative for culture methods.

Based on genomic comparisons using previously characterized staphylococcal virulence genes, the NRCS-A virulome is highly similar to that of other non-NRCS-A *S. capitis* as well as *S. epidermidis* RP62A strains, which suggests that the success of this clone in neonates is likely not due to increased virulence. Nonetheless, we identified the exclusive presence of a gene encoding a functional nisin resistance (*nsr*) gene in the NRCS-A lineage that is seldom found in staphylococci.

Nisin, a 34 aa antimicrobial peptide produced by a group of gram-positive *Lactococcus* and *Streptococcus* species (Shin et al., 2016), is active against a wide range of gram-positive bacteria, including staphylococci. Its mode of action is dual, involving (i) inhibition of cell wall biosynthesis (transglycosylation step), as it binds to and sequesters lipid II from its functional location, and (ii) pore formation (Wiedemann et al., 2001; Peschel and Sahl, 2006; Egan et al., 2016). Nisin has been described as a key player of the gut barrier, and it is widely used as food preservative due to its potent bactericidal activity. Moreover, it has been shown that (i) expression of the *nsr* gene, identified in *Streptococcus agalactiae*, in *L. lactis* not producing nisin induces resistance to nisin and (ii) the presence of human nisin-producing lactic acid bacteria in the gut reduces intestinal colonization by vancomycin-resistant enterococci (Millette et al., 2008).

Taken together, these data and our results strongly suggest that nisin resistance might be responsible for the potential increase in the ability of the NRCS-A clone to establish itself as part of the initial microflora of neonates, in which the *Lactococcus* genus is one of the dominant bacterial taxa (Park et al., 2005; Morelli, 2008). Expression of the NSR peptidase might enable NRCS-A isolates to establish themselves in the gut of neonates and also to colonize it and survive longer than nisin-susceptible bacteria. This may explain the over-representation of these isolates in sepsis processes, either through direct translocation in blood (Taft et al., 2015) or via colonization of indwelling devices. Although it remains unknown how *S. capitis* NRCS-A strains are able to translocate into the bloodstream of infants, it is hypothesized that the entry point might be the digestive tract, which is immature in very preterm infants (Taft et al., 2015). Further studies are needed to fully characterize the digestive microflora of very low-weight preterm-infants and its potential impact on the selection of and the fitness advantage to NRCS-A isolates.

AUTHOR CONTRIBUTIONS

PM: work, study design, data analysis, and manuscript preparation. HL: data analysis and manuscript preparation. YD: data analysis, work, and manuscript preparation. SL: work and manuscript preparation. JR, SA, AI, and SE: manuscript preparation. MB and FL: study design and manuscript preparation.

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Integron Digestive Carriage in Human and Cattle: A "One Health" Cultivation-Independent Approach

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Objectives: Dissemination of antimicrobial resistance (AMR) is a global issue that requires the adoption of a "One-Health" approach promoting integration of human and animal health. Besides culture-dependent techniques frequently used for AMR surveillance, cultivation-independent methods can give additional insights into the diversity and reservoir of AMR genetic determinants. Integrons are molecular markers that can provide overall and reliable estimation of AMR dissemination. In this study, considering the "One-Health" approach, we have analyzed the integron digestive carriage from stools of humans and cattle living in a same area and exposed to different antibiotic selection pressures.

Methods: Three collections of human [general population (GP) and intensive care unit patients (ICUs)] and bovine (BOV) stool samples were analyzed. The three main classes of integrons were detected using a multiplex qPCR both from total DNA extracted from stools, and from Gram-negative bacteria obtained by culture after an enrichment step.

Results: With the cultivation-independent approach, integron carriage was 43.8, 52.7, and 65.6% for GP, ICU, and BOV respectively, percentages being at least twofold higher to those obtained with the cultivation-dependent approach. Class 1 integrons were the most prevalent; class 2 integrons seemed more associated to cattle than to humans; no class 3 integron was detected. The integron carriage was not significantly different between GP and ICU populations according to the antibiotic consumption, whatever the approach.

Conclusion: The cultivation-independent approach constitutes a complementary exploratory method to investigate the integron digestive carriage of humans and bovines, notably within subjects under antibiotic treatment. The high frequency of carriage of integrons in the gut is of clinical significance, integrons being able to easily acquire and exchange resistant genes under antibiotic selective pressure and so leading to the dissemination of resistant bacteria.

Keywords: integrons, digestive carriage, antimicrobial resistance, humans, bovine, One Health

INTRODUCTION

Antimicrobial resistance (AMR) has increased to a dramatic extend and is a public health threat (Roca et al., 2015). This is particular true for Gram-negative bacteria (GNB) with the expansion of extended-spectrum beta-lactamase (ESBL) or carbapenemase-producing Enterobacteriaceae and with the emergence of new resistance mechanisms, as plasmid-mediated colistin resistance (Meletis, 2016). AMR is a global issue encompassing human, animal, and the environment. Efforts to tackle AMR dissemination thus require the adoption of a "One-Health" approach that promotes integration of public health and veterinary disease, food, and environmental surveillance (Sikkema and Koopmans, 2016). Culture-dependent techniques are frequently used for AMR surveillance in GNB. However, given the role of horizontal gene transfer in AMR dissemination, cultivation-independent methods give additional insight into the diversity and reservoir of AMR determinants.

Several studies indicated that integrons are molecular markers that can provide overall and reliable estimation of AMR dissemination (Amos et al., 2015; Gillings et al., 2015). Integrons are bacterial genetic elements capable of promoting the acquisition and expression of antibiotic resistance genes embedded within gene cassettes (Cambray et al., 2010). Three main classes of integrons are involved in the spread of AMR and several studies have underlined the link between integrons and multidrug resistance in GNB (Leverstein-van Hall et al., 2003; Barraud et al., 2014). In humans and animals, class 1 integrons are the most prevalent, followed by class 2 integrons (Barlow et al., 2004; Cocchi et al., 2007; Vinue et al., 2008; Barraud et al., 2014), whereas very few class 3 integrons are found, this class being mostly associated with environmental samples (Simo Tchuinte et al., 2016).

Nowadays, it is impossible to separate the animal and human resistance issues as genetic transfers exist between these two entities. In this study, considering a "One-Health" approach and the integron biomarker, we have taken benefit from a large collection of stools to analyze the digestive carriage of integrons in humans and bovines, in a cattle-rearing area. This study highlights the added-value of using two complementary approaches for detecting integrons, a cultivation-independent one performed directly from stools and a cultivation-dependent one from GNB and emphasizes the importance of the gut as a reservoir of genetic elements encoding antibiotic resistance.

MATERIALS AND METHODS

Stool Samples

A collection of 567 stool samples from human and bovines has been previously established by our team. These samples were consecutively collected from 2006 to 2010 and stored at −80°C (human collection number DC-2008-604). All subjects originated from the same cattle-rearing area located in the southwestern France (Haute-Vienne, 5.520 km^2). The stools were obtained from (i) subjects from the general population working in non-clinical units of Limoges hospital and attending industrial medicine clinics ('GP,' $n = 194$), (ii) patients hospitalized in the intensive care unit of the hospital ('ICU,' $n = 245$), (iii) bovines, mainly cows, from 55 different farms ('BOV,' $n = 128$). Only one stool sample per subject was collected. For the two human stool collections, on-going antibiotic therapies and previous antibiotic therapies during the last 3 months were reported; for bovines, no data regarding antibiotic consumptions were available.

Bacterial Culture

Two hundred milligrams of stools were inoculated into 5 ml of Brain Heart Infusion broth (AES Laboratory, France) at 37°C overnight without agitation. The day after, 10 µl were inoculated onto Drigalski agar plates (bioMérieux, France) incubated for 18–24 h at 37°C. GNB with different morphotypes were selected for identification (ID) and antibiotic susceptibility testing (AST). ID was performed using the Vitek 2 system (Vitek2-ID-GNB, bioMérieux, France). AST was determined using the disk diffusion method according to the recommendations of the French Society for Microbiology with the 21 following drugs: amoxicillin (AMX), amoxicillin clavulanic acid (AMC), ticarcillin (TIC), ticarcillin clavulanic acid (TCC), cefotaxim (CTX) ceftazidim (CAZ), imipenem (IPM), kanamycin (K), gentamicin (GM), tobramycin (TM), amikacin (AN), streptomycin (S), spectinomycin (SPT), nalidixic acid (NA), pefloxacin (PEF), ciprofloxacin (CIP), sulfamethoxazole (SSS), trimethoprim (TMP), trimethoprim-sulfamethoxazole (SXT), chloramphenicol (C), and tetracycline (TET). Results categorized intermediate were reported as resistant in the results section.

Integron Detection

Total DNA was extracted from 200 mg of stool samples using the QIAamp DNA stool mini kit (Qiagen, Courtaboeuf, France) following manufacturer's instructions.

Class 1, 2, and 3 integrons were detected both from stools and from GNB using a multiplex qPCR, as previously described (Barraud et al., 2010). For all qPCR runs, plasmids pBAD18::*intI1*, pGEM®-T Easy::*intI2* and pBAD18::*intI3* as positive controls. So as to check for PCR inhibitors, a qPCR targeting the 16S DNA gene was performed (Relman, 1993). All qPCR assays were performed on the MX3005P system (Agilent®).

For each stool sample, all isolates belonging to a same species but with different AST and/or integron profiles were considered as different strains.

Statistical Analysis

Mc Nemar Chi^2 test for paired data was used to evaluate the proportions of integrons (all integrons, class 1 integrons only, class 2 integrons only and class 1 and 2) detected with the cultivation-dependent approach and the cultivation-independent approach. The association between previous or on-going antibiotherapy and integron content was tested using Chi^2 test or Fisher exact test according to the theoretical effectives.

Link between integron content and AMR phenotypes was analyzed using the Chi^2 test and the Fisher's exact test for group smaller than 5, as well as to compare integron carriage results of different groups.

Ethics Statement

For this study, an ethics review process was not needed as stool samples were obtained through standard care. The stools samples from GP patients working in non-clinical units of Limoges hospital and attending industrial medicine clinics were sampled for detection of pathogens like *Salmonella* and stool samples from ICU patients were sampled for digestive screening of multidrug-resistant bacteria carriage. All subjects were informed that their stool samples could be used for a clinical research purpose and they were able to express their opposition. We declared the human stool collections to the French "Ministère de l'Enseignement et de la Recherche" under CODECOH number DC-2008-604. Moreover, this study was approved by the scientific committee of the sponsor, Limoges hospital.

RESULTS

Human and Animal Populations

Characteristics of the three populations (number of subjects, sex ratio, mean age and on-going or previous antibiotic therapy) are described in Supplementary Table S1. Antibiotic consumption was predominant in ICU patients.

Bacterial Culture

Culture was negative for 30% of ICU stools, whereas only for 2% of GP and BOV. A total of 1012 different GNB were isolated from the 488 stools with a positive culture (Supplementary Table S2). The average number of GNB per stool was 1.9, 1.4, and 2.3 for GP, ICU, and BOV, respectively. *Escherichia coli* was the most frequent species: 67.1% of all GNB (81.1, 55.5 and 63.0% for GP, ICU, and BOV respectively). Other major species were *Citrobacter freundii* ($n = 10$) and *Klebsiella pneumoniae* ($n = 9$) for GP, *K. pneumoniae* ($n = 32$) followed by *Pseudomonas aeruginosa* ($n = 23$) and *K. oxytoca* ($n = 22$) for ICU, *E. fergusonii* ($n = 25$) and *Acinetobacter lwoffi* ($n = 22$) for BOV.

GNB and Integron Content

Prevalence of integrons was 16.2, 23.8, and 14.2% among GNB isolated from GP, ICU, and BOV respectively, with mainly class 1 integrons (Supplementary Table S2). *E. coli* was the main species carrying integrons in the three populations with respectively 57, 52, and 36 integron-positive isolates in GP, ICU and BOV. In GP and BOV, respectively 3 and 6 GNB other than *E. coli* were integron-positive: *Proteus mirabilis* (3), *Proteus vulgaris* (1), *Providencia rettgeri* (1), *Enterobacter cloacae* (1), *Citrobacter braakii* (1), *Salmonella* Typhimurium (1) and *Acinetobacter lwoffi* (1). In ICU, 32 other GNB were integron-positive, mainly *K. pneumoniae* ($n = 11$), *K. oxytoca* ($n = 8$), and *P. aeruginosa* ($n = 5$).

Analysis of the link between integrons and antibiotic resistance was determined for *E. coli*. We observed a gradual increase in the percentage of integron-positive *E. coli* correlated with the number of acquired resistances (Supplementary Table S3). Globally, the percentage of *E. coli* with resistance to at least two antibiotics was higher among integron-positive isolates than among integron-negative isolates (95.3% versus 23.9%; $p < 0.001$). Moreover, a statistically significant link was observed between integron and acquired resistance to most antibiotics: penicillins, penicillins and inhibitors, almost all aminoglycosides (except amikacin), quinolones, sulfamethoxazole, trimethoprim, tetracycline, and chloramphenicol (Supplementary Table S4). In all three populations, negative predictive values (NPVs) of integron carriage for antibiotic resistance were up to 97% for third generation cephalosporins, up to 98% for aminoglycosides used in therapeutics (i.e., gentamicin, amikacin, and tobramycin), up to 93% for fluoroquinolones and up to 92% for SXT.

Digestive Carriage of Integrons

With the cultivation-dependent approach based on integron detection from GNB isolates (number of patients with at least 1 GNB carrying an integron), the integron carriage of the subjects was 23.2, 26.9% for GP and ICU patients respectively and 24.2% for bovines (**Table 1**). With the cultivation-independent approach (number of patients with integron detection by qPCR directly from stools), the integron carriage was at least twofold higher in the three populations: 43.8, 52.7, and 65.6% for GP, ICU, and BOV respectively (**Table 1**). These differences between the two approaches were statistically significant for the three populations ($p < 0.01$). In humans (GP/ICU), integron carriage was mainly due to class 1 integrons, class 2 integrons being present in only 5–6% of the subjects. In bovines, integron carriage was due to both class 1 and class 2 integrons, 57.1 and 39.1% respectively. No class 3 integron was detected. We did not find any significant difference in integron carriage along the 4 years of the study (data not shown).

Concordance between the Two Approaches

Results between the cultivation-dependent and the cultivation-independent approaches were compared for all the 567 samples. Results were concordant for 383 samples (67.5%) with 255 being integron-negative and 128 integron-positive with the two approaches. For the remaining samples, the cultivation-independent approach allowed the detection of integrons in 170 samples for whom the culture did not find integron-positive isolates. On the other hand, for 14 patients, we detected integrons with the cultivation-dependent approach and not with the cultivation-independent approach.

Integron Carriage and Antibiotic Selection Pressure

Differences of integron carriage between the two human populations (GP and ICU) were not significant whatever the approach ($p = 0.37$ with data from the cultivation-dependent approach; $p = 0.065$ with data from the cultivation-independent approach). The integron carriage was also not significantly different between GP and ICU populations according to the antibiotic consumption, whatever the approach (**Tables 2A,B**).

TABLE 1 | Digestive carriage of integrons.

		Cultivation-dependent approach	Cultivation-independent approach	*p**
GP (*n* = 194)	**All integrons**	**45 (23.2%)**	**85 (43.8%)**	<0.01
	Class 1 integrons only	41 (21.2%)	75 (38.7%)	<0.01
	Class 2 integrons only	2 (1.0%)	6 (3.1%)	0.04
	Class 1 and 2 integrons	2 (1.0%)	4 (2.1%)	0.41
ICU (*n* = 245)	**All integrons**	**66 (26.9%)**	**129 (52.7%)**	<0.01
	Class 1 integrons only	63 (25.7%)	114 (46.5%)	<0.01
	Class 2 integrons only	1 (0.4%)	7 (2.9%)	0.03
	Class 1 and 2 integrons	2 (0.8%)	8 (3.3%)	0.06
BOV (*n* = 128)	**All integrons**	**31 (24.2%)**	**84 (65.6%)**	<0.01
	Class 1 integrons only	27 (21.1%)	34 (26.6%)	0.25
	Class 2 integrons only	3 (2.3%)	11 (8.6%)	0.03
	Class 1 and 2 integrons	1 (0.8%)	39 (30.5%)	<0.01

*Numbers indicate subjects being integron carriers among each population. Class 3 integrons were not detected. *Test used was the Mc Nemar Chi^2 test for paired data.*

TABLE 2 | Integron digestive carriage and antibiotic therapy with data from the cultivation-dependent approach.

(A)			
	GP* (*n* = 188)		
	Integron-positive	Integron-negative	*p*
No antibiotic therapy	43 (24.9%)	130 (75.1%)	0.32¤
Previous or on-going antibiotic therapy	2 (13.3%)	13 (86.7%)	
	ICU (*n* = 245)		
	Integron-positive	Integron-negative	*p*
No antibiotic therapy	9 (25.0%)	27 (75.0%)	0.78#
Previous or on-going antibiotic therapy	57 (27.3%)	152 (72.7%)	
(B)			
	GP* (*n* = 188)		
	Integron-positive	Integron-negative	*p*
No antibiotic therapy	77 (55.5%)	96 (44.5%)	0.40#
Previous or on-going antibiotic therapy	5 (66.7%)	10 (33.3%)	
	ICU (*n* = 245)		
	Integron-positive	Integron-negative	*p*
No antibiotic therapy	23 (36.1%)	13 (63.9%)	0.14#
Previous or on-going antibiotic therapy	106 (50.7%)	103 (49.3%)	

*Numbers indicate subjects being integron-positive or integron-negative carriers. *Patients with no data for antibiotic therapy were excluded. Test used was the Chi^2 test# or the Fisher's exact test¤.*

DISCUSSION

In this study based on a "One Health" approach, we have retrospectively taken benefit from a stool collection from human and bovine subjects living in a cattle-rearing area in France, to check for their digestive carriage of integrons. Indeed, integrons are now well-recognized as genetic biomarkers of acquired resistance (Leverstein-van Hall et al., 2003; Barraud et al., 2014) and they can provide a reliable estimation of AMR dissemination (Amos et al., 2015; Gillings et al., 2015). The "One Health" approach is now required to tackle AMR, human and animal health being interconnected, and international declarations on AMR pay particular attention to this aspect in action plans.

In our work, the integron carriage was studied with both methods: a cultivation-dependent approach based on GNB, as classically determined (Skurnik et al., 2005, 2008; Vasilakopoulou et al., 2009; Bailey et al., 2010; Kheiri and Akhtari, 2016) and a cultivation-independent approach with a qPCR directly applied from stool DNA. This cultivation-independent approach is novel and complementary to the classical approach. To our knowledge, such an approach was previously used only once for determination of the integron carriage in cattle, not directly from stools but from an enrichment broth (Barlow et al., 2004). Even though their methodology was slightly different than ours, the authors observed integron carriage rates from 50% for class 1 integrons and 28% for class 2 integrons with the cultivation-dependent approach to 86 and 94% using a direct detection of integrons from stool DNA after an enrichment step. In our study, we also observed an increase of the integron carriage with the cultivation-independent approach, with at least a twofold increase (**Table 1**) and the cultivation-independent approach allowed detecting 170 integron carriers that were considered as non-carriers with the cultivation-dependent approach. This increase was expected. First, some bacteria may have been not collected during the culture process: (i) GNB colonies were visually selected and some strains may have been missed, notably if morphotypes were similar; (ii) culture of one integron-positive GNB could have been not observed due to competition with other more predominant GNB; (iii) not all GNB were able to grow on Drigalski agar plates, like *Campylobacter* or *Pasteurella* (Lee et al.,

2002; Kehrenberg and Schwarz, 2011). Moreover, it has been shown that Gram-positive bacteria, especially Corynebacteria, may contain integrons (Nesvera et al., 1998; Nandi et al., 2004; Barraud et al., 2011). However, our method using Drigalski agar plates only allowed isolating GNB. Using the cultivation-independent method, these Gram-positive bacteria containing integrons can be detected. Lastly, bacteria from patients under antibiotics may have been unable to grow. This is in agreement with the fact that in ICU patients exposed to a high antibiotic selective pressure, culture was negative for 30% of stools. Lastly, we know that culturing allows detection of a very limited part of all the species present in the gut microbiota (Harmsen and de Goffau, 2016) and we can expect that non-cultivable bacteria can also contain integron. All these explanations argue that the cultivation-independent approach would constitute a complementary approach for the determination of the digestive carriage of integrons than cultivation-dependent approaches, notably when an antibiotic treatment is ongoing. The benefit of cultivation-independent approaches for the diagnostic of antibiotic resistance has been previously shown (Decousser et al., 2017; Dunne et al., 2017). However, for 14 subjects, a signal was positive *via* the cultivation-dependent approach but negative *via* the cultivation-independent approach. Hypothesis of inhibitors from stools was excluded thanks to correct amplification of the 16S-DNA from all the samples. For these patients, the number of integron-positive strains was probably very low, under the threshold of detection by qPCR. The culture method with an enrichment broth allowed to isolate these bacteria. This confirms that both cultivation-dependent and cultivation-independent approach are complementary.

For humans, independently of the method used, integron carriage was mainly due to class 1 integrons (79 out of 85 integron carriers for GP, and 122 out of 129 for ICU), class 2 being present in only 11% of the integron carriers. This is in agreement with previous studies on integron detection in commensal *E. coli* (Bailey et al., 2010; Copur-Cicek et al., 2014; Kheiri and Akhtari, 2016) or in clinical isolates (Barraud et al., 2014; Malek et al., 2015; Xu et al., 2017). For bovines, results were different from humans, notably when looking at the cultivation-independent approach. Even if class 1 integrons remained the most prevalent (73 out of the 84 carriers), class 2 integrons were detected among 50 out of the 84 bovine integron carriers. These results were not obtained when looking at the culture with only 2 out of the 36 bovine carriers being positive for class 2 integrons. This discrepancy was also retrieved by Barlow et al. (2004) who obtained 94% of cattle fecal samples positive for class 2 integrons from enrichment broths *versus* 28% from GNB. Other studies indicated that class 2 integrons seemed more related to animals than to humans (Lapierre et al., 2008; Barlow et al., 2009). One can hypothesize that bacteria from the bovine microbiota constitute a yet unknown reservoir for class 2 integrons. Animal and human gut constitute a huge reservoir of resistances genes (Fitzpatrick and Walsh, 2016). If animals are reservoirs for class 2 integrons, one can expect that presence of such a class within humans may be related to contacts with animals or via the food chain. It is noteworthy that no class 3 integron was detected even using the cultivation-independent approach. It has been previously hypothesized that class 3 integrons are mainly distributed in the environment (Stalder et al., 2014; Simo Tchuinte et al., 2016), and exceptionally within humans or animals.

Rates of integron carriage in human obtained with the cultivation-dependent approach are similar to data found in previous studies, between 15 and 30% (Leverstein-Van Hall et al., 2002; Vinue et al., 2008; Vasilakopoulou et al., 2009). In previous studies, antibiotic therapy was identified as a risk factor for integron acquisition, both in humans (Nijssen et al., 2005; Skurnik et al., 2005; van der Veen et al., 2009) and in animals (Barlow et al., 2009; Wu et al., 2011). Skurnik et al. (2005, 2008) first reported different *E. coli* integron carriage in animals according to their degrees of exposure to humans. Authors observed a gradual increase in the prevalence of class 1 integrons correlated with the increase in exposure to human. In our study, antibiotic selection pressure was very different between GP and ICU with 8% of GP subjects exposed to antibiotics *versus* 83% of ICU patients. However, in our study, we did not find statistical difference in integron carriage between GP and ICU, whatever the approach.

Escherichia coli was the predominant species in all three populations. A significant link was observed in *E. coli* between presence of integrons and acquired resistance to at least two antibiotics ($p < 0.001$). This was demonstrated previously whatever the definition of the multiresistance (Martinez-Freijo et al., 1998; Leverstein-van Hall et al., 2003; Shahi and Kumar, 2015; Ochoa et al., 2016). Here, we observed that when an integron was present in an *E. coli* isolate, the most frequent phenotype was the association of a low level penicillinase conferring resistance to amoxicillin and ticarcillin with co-resistances to trimethoprim, sulfamethoxazole and tetracycline (data not shown). As we showed recently (Barraud et al., 2014), integrons alone clearly cannot explain the resistance to all of the tested antibiotics, but integrons are usually carried by plasmids or transposons, which can also host other resistance genes. It has been shown in humans and animals that *E. coli* can harbor plasmids with different genes encoding these resistances (to ampicillin, tetracycline, trimethoprim, and sulfonamides) and that some of these plasmids often carry integrons (Kirchner et al., 2014; Jackson et al., 2015). A statistically positive significant link was also observed between integrons and each antibiotic, except for carbapenems, third generation cephalosporins and amikacin. Finally, NPVs – i.e., when an integron is absent, the strain has a high probability to be susceptible – were >97% for third generation cephalosporins, up to 98% for aminoglycosides used in therapeutics, up to 93% for fluoroquinolones and up to 92% for SXT. This confirms our previous data aiming at considering integrons as relevant biomarkers of antibiotic resistance with a good NPV (Barraud et al., 2014). On the other hand, most positive predictive values (PPV) – i.e., when an integron is present, the strain has a high probability to be resistant – were below 95% or even lower for all the antibiotics (Supplementary Table S3). This may be explained by the low prevalence of multi-drug resistant enterobacteria in our county.

Our study aimed at determining the digestive carriage of integrons among human and bovine populations. We showed that the cultivation-independent approach increased significantly, by twofold, the rates of integron carriers compared to the cultivation-dependent approach. It is likely that non-cultivable bacteria of the gut harbor integrons. This high frequency of carriage of integrons in the gut is of clinical significance. Indeed, integrons are known to be very efficient genetic elements for capture, expression and dissemination of antibiotic resistance gene cassettes and they can harbor a wide diversity of gene cassettes encoding resistance to almost all antibiotic families. Antibiotics are known to alter the gut microbiota and lead to selection and emergence of resistant bacteria (Ianiro et al., 2016). Furthermore, it is known that antibiotics induce the mechanism of acquisition of antibiotic resistance gene cassettes by integrons (Guerin et al., 2009). Our findings thus highlight that the gut is a reservoir for integrons that can easily acquire and exchange resistant genes under antibiotic selective pressure leading to the dissemination of resistant bacteria.

Here, we showed that both cultivation-dependent and -independent methods are complementary for the detection of integrons. However, although the cultivation-independent approach gives new insights about the epidemiology of integrons, we advocate for the maintenance of the cultivation-dependent approach that is the sole approach able to provide, at the species level, the link between integrons and antibiotic resistance. This study also highlights that the gut, in both humans and animals, is a high potential reservoir of genetic determinants for antibiotic resistance, that can be further acquired by pathogens.

AUTHOR CONTRIBUTIONS

OB performed analysis, wrote the manuscript; DC performed analysis, wrote the manuscript; GM performed analysis; EC-D reviewed the manuscript; BF enrolled ICU patients, reviewed the manuscript; C-YC collected animal stool samples; M-CP designed the study, wrote and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01891/full#supplementary-material

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The Resistome of Farmed Fish Feces Contributes to the Enrichment of Antibiotic Resistance Genes in Sediments below Baltic Sea Fish Farms

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Our previous studies showed that particular antibiotic resistance genes (ARGs) were enriched locally in sediments below fish farms in the Northern Baltic Sea, Finland, even when the selection pressure from antibiotics was negligible. We assumed that a constant influx of farmed fish feces could be the plausible source of the ARGs enriched in the farm sediments. In the present study, we analyzed the composition of the antibiotic resistome from the intestinal contents of 20 fish from the Baltic Sea farms. We used a high-throughput method, WaferGen qPCR array with 364 primer sets to detect and quantify ARGs, mobile genetic elements (MGE), and the 16S rRNA gene. Despite a considerably wide selection of qPCR primer sets, only 28 genes were detected in the intestinal contents. The detected genes were ARGs encoding resistance to sulfonamide (*sul1*), trimethoprim (*dfrA1*), tetracycline [*tet(32), tetM, tetO, tetW*], aminoglycoside (*aadA1, aadA2*), chloramphenicol (*catA1*), and efflux-pumps resistance genes (*emrB*, *matA, mefA, msrA*). The detected genes also included class 1 integron-associated genes (*intI1*, *qacE*Δ*1*) and transposases (*tnpA*). Importantly, most of the detected genes were the same genes enriched in the farm sediments. This preliminary study suggests that feces from farmed fish contribute to the ARG enrichment in farm sediments despite the lack of contemporaneous antibiotic treatments at the farms. We observed that the intestinal contents of individual farmed fish had their own resistome compositions. Our result also showed that the total relative abundances of transposases and *tet* genes were significantly correlated ($p = 0.001$, $R^2 = 0.71$). In addition, we analyzed the mucosal skin and gill filament resistomes of the farmed fish but only one multidrug-efflux resistance gene (*emrB*) was detected. To our knowledge, this is the first study reporting the resistome of farmed fish using a culture-independent method. Determining the possible sources of ARGs, especially mobilized ARGs, is essential for controlling the occurrence and spread of ARGs at fish farming facilities and for lowering the risk of ARG spread from the farms to surrounding environments.

Keywords: rainbow trout, whitefish, fish intestinal content, class 1 integrons, transposons, mobile genetic elements, qPCR array, culture-independent method

INTRODUCTION

Fish farms have been suggested as one reservoir of antibiotic resistance genes (ARGs) in the environment due to the prophylactic and therapeutic use of antibiotics (Cabello et al., 2013; Miranda et al., 2013). Because the occurrence of ARGs at fish farms leads to inefficiencies in antibiotic treatments and there is a potential risk of ARG spread from the farms to the surrounding environments, determining the sources of ARGs at fish farms is essential.

The use of antibiotics in fish farming in Finland is controlled by the Finnish Food Safety Authority (EVIRA) and requires a guideline from veterinary professionals. Oxytetracycline, a combination of sulfonamide-trimethoprim, and florfenicol are the antibiotics authorized for use in Finnish fish farms (EVIRA, 2015a,b). From 2001 to 2014, ∼2.3 metric tons of sulfonamide, 0.6 metric ton of trimethoprim, 1.2 metric tons of oxytetracycline, and 0.04 metric ton of florfenicol were used in fish farming in Finland (EVIRA, 2015a,b). The antibiotics are used mainly against fish pathogens, such as *Aeromonas salmonicida, Flavobacterium psychrophilum*, and *Flavobacterium columnare,* and pathogens which occur only in sea farms, such as *Vibrio anguillarum, Yersinia ruckeri, Pseudomonas anguilliseptica*, and *Pseudomonas edwardsiellloosi* (Viljamaa-Dirks, 2016).

Previously, we investigated the presence of ARGs in sediments below two fish farms in the Northern Baltic Sea, Finland (Tamminen et al., 2011a; Muziasari et al., 2014, 2016). Both farms raise rainbow trout (*Oncorhynchus mykiss*, Walbaum) and European whitefish (*Coregonus lavaretus*, Linnaeus). In the studied farms, the combination of sulfonamide-trimethoprim mixed in with fish feed was used against fish pathogens such as furunculosis-causing *A. salmonicida*. Occasionally, florfenicol was also used. Oxytetracycline had not been used since year 2000. However, records of the amount of antibiotics used at the studied farms were not available. Our previous study showed that certain ARGs encoding resistance to tetracycline, sulfonamide, trimethoprim, and aminoglycoside, an antibiotic that has never been used at the farms, were enriched in the two farm sediments even though the concentration of antibiotics in the sediments was low (1–100 ng/g of sediment). The ARGs were abundant and persistent in the farm sediments for several years but were not detected in sediments at a distance of 200 m from the fish farms. Genes associated with class 1 integrons and transposons were also the genes enriched in the farm sediments. The presence of class 1 integrons and transposons of mobile genetic elements (MGEs) in the environment can contribute to the spreading of ARGs via horizontal gene transfer systems (Aminov, 2011). Sources of the gene enrichment in the farm sediments have yet to be elucidated.

We assumed that a constant influx of farmed fish feces contributes to the enrichment of ARGs in the farm sediments. Farmed fish consume antibiotic-containing feed and selection for resistant bacteria may occur in the fish intestine during antibiotic treatment (Giraud et al., 2006). Thus, resistant bacteria carrying ARGs in the fish intestine may be secreted into fish feces and released to the surrounding water and finally to the sediments (Kümmerer, 2009). Both farms use an open cage system in which fish are kept in a net that allows free transfer of fish farming waste, containing fish feces, uneaten feed, and antibiotics, from the farms to surrounding water environments and eventually to sediments. Since the Baltic Sea has no tides and water circulation is slow (Ojaveer et al., 2010), the fish farming waste directly impacts the sediments beneath the farms.

The antibiotic resistome consists of all the existing ARGs that are capable of conferring resistance to antibiotics (Wright, 2007). Previous studies on the resistome of farmed fish detected up to 10 ARGs in cultured resistant-bacteria from skin, gills, intestinal contents, and meat (Schmidt et al., 2001; Furushita et al., 2003; Akinbowale et al., 2007; Jacobs and Chenia, 2007; Ndi and Barton, 2011). To study the antibiotic resistome in environmental samples, in which the majority of bacteria are not cultivable, culture-independent methods can be used (Perry and Wright, 2013). Highly parallel qPCR arrays provide a culture-independent method that permits the combination of hundreds of assays to detect and quantify selected known genes in a single experiment. This method has been used to analyze the resistome composition in environmental samples (Looft et al., 2012; Zhu et al., 2013; Wang et al., 2014; Su et al., 2015; Karkman et al., 2016) and fish farming environments (Muziasari et al., 2016).

In the present study, we used a highly parallel qPCR array with 364 primer sets for the detection and quantification of ARGs, other genes encoding resistance to antibacterial compounds, a mercury resistance gene, MGE associated genes, and the 16S rRNA gene. The qPCR array was used to analyze the composition of intestinal content resistomes of rainbow trout and whitefish farmed at the two Baltic Sea fish farms. The parts of farmed fish that are exposed to the surrounding water environments, such as fish skin and gills, were also analyzed to detect the possible risk of ARG spread in the fish farming environments.

MATERIALS AND METHODS

Sampling

Rainbow trout, *O. mykiss* (Walbaum), and European whitefish, *C. lavaretus* (Linnaeus) were sampled from two fish farms in the Northern Baltic Sea, Finland, in September 2014. Each farm produces ∼50 metric tons of both rainbow trout and whitefish annually. The two farms used an open cage system in which each cage was 20 m in diameter and 5 m deep. The average depth was 7.5 m (±SD 1.3 m) and the average water temperature was 14.2°C (±0.85°C) during the sampling time. Detailed information of the two farms has been described previously (Pitkänen et al., 2011; Tamminen et al., 2011a,b; Muziasari et al., 2014, 2016).

Five fish from each group of small rainbow trout, big rainbow trout, small whitefish and big whitefish were sampled (**Table 1**). The 20 sampled fish were randomly picked from healthy fish in the net cages. The fish were bought and slaughtered using the normal fish farming process. The fish were put in boxes of ice, transported to the laboratory (duration of transport 6 h), and directly processed. The fish were individually measured and weighed. Mucosal skin and gill filaments were sampled from each fish using sterile cotton swabs before the fish abdominal cavity was opened using sterile surgical tools. The tissues and visceral

TABLE 1 | Farmed fish samples from the Northern Baltic Sea farms.

Farmed fish samples	Rainbow trout (rt)		Whitefish (wf)	
	Small_rt	Big_rt	Small_wf	Big_wf
Average weight (g)	540 (±160)	2800 (±480)	420 (±70)	780 (±100)
Time raised at the farm (months)	3	3*	3	15
No. of samples	5	5	5	5
History of antibiotic treatments at the Baltic Sea farms**	1.5% of sulfonamide-trimethoprim in fish feed at the farm in August 2014	No antibiotic treatment at the farms	No antibiotic treatment at the farms	No antibiotic treatment at the farms

The Baltic Sea farms also raise fish that are already mature fish.

***History of antibiotic treatments before entering the Baltic Sea farms was not available.*

fat surrounding the digestive system were removed. The portion of the digestive system consisting of the small and large intestines was removed. The content of the fish intestinal tract was gently squeezed into a zipper plastic bag and homogenized manually. The intestinal contents of each fish were analyzed individually as biological replicates. The mucosal skin and gill filaments from groups of five fish were pooled. Five fish per group were chosen to account for the minimum number of incremental samples to be taken, in line with EU commission regulation No 252/2012. The sample materials were kept at −80°C until DNA extraction.

DNA Extraction

Total DNA was extracted directly from 200 mg of each homogenized intestinal content sample using the QIAamp DNA Stool Mini Kit (Qiagen Sciences, Germantown, MD) and the pre-treatment using FastPrep method (MP Biomedicals, Irvine, CA, USA) following the manufacturer's instructions. In addition, total DNA from a swab of mucosal skin and gill filaments was extracted using the Cador Pathogen Mini Kit (Qiagen Sciences, Germantown, MD) and the pre-treatment B2 according to the manufacturer's instructions. The pre-treatment step was added in each DNA extraction method to improve the quality and yield of DNA for the qPCR array measurement. The DNA quality and concentration were analyzed with a Nanodrop 1000™ spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

The qPCR Array

The qPCR reactions were performed using 364 primer sets (**Table S1**). Validation of the primer sets was confirmed in the previous studies (Looft et al., 2012; Pitkänen et al., 2011; Tamminen et al., 2011a; Zhu et al., 2013; Muziasari et al., 2014). Of the 364 primer sets; 307 primer sets were used for ARGs encoding resistance to the nine main classes of antibiotics (aminoglycoside, beta-lactam, (flor)/(chlor)/(am)phenicol, macrolide (MSLB), multidrug-efflux, sulfonamide, tetracycline, trimethoprim, and vancomycin) and covering the three major mechanisms of antibiotic resistance (antibiotic efflux-pumps, cellular protection and antibiotic deactivation); 21 primer sets for other genes encoding resistance to antibacterial compounds, such as antiseptic and antibacterial peptides; 34 primer sets for genes associated with MGEs, such as plasmids, transposons, and insertion sequences (IS) as well as integrons; one primer set for a mercury resistance gene (*merA*) to study a possible co-selection of ARGs with mercury resistance gene; and one primer set for the 16S rRNA genes. Each primer set was designed to target sequence diversity within a gene to assess the environmental resistome and was therefore analyzed independently.

The qPCR array was performed in the SmartChip Real-time PCR system (WaferGen Biosystem, Freemont, CA, USA). In short, the SmartChip has 5184 reaction wells with a volume of 100 nL each, filled using the SmartChip Multisample Nanodispenser (WaferGen Biosystem, Freemont, CA, USA). PCR cycling conditions and initial data processing were as previously described (Wang et al., 2014). However, the detection limit was at a threshold cycle (C_T) of 27 (Zhu et al., 2013; Karkman et al., 2016; Muziasari et al., 2016). The melting curve of each PCR product was also analyzed to monitor the specificity of the primer sets. The abundance of the detected genes in proportion to the 16S rRNA gene in each DNA sample was calculated using relative quantification with the $2^{-\Delta CT}$ method, in which $\Delta C_T = (C_T \text{ detected gene} - C_T \text{ 16S rRNA gene})$ (Schmittgen and Livak, 2008).

Data Analysis

To see if the MGEs are involved in the prevalence of ARGs, the correlation between the total relative abundances of transposases and the most prevalent ARG in the intestinal contents of the farmed fish were analyzed using linear regression. A $p < 0.05$ were considered to be significant. In addition, the notched box-plot was used to see the difference between the detected genes' relative abundances. The detected genes were grouped based on the three mechanisms of ARGs (antibiotic deactivation, cellular protection, and efflux-pumps), resistance genes to antiseptics, and MGE-associated genes. The notch in the box-plot displays the 95% confidence interval of the median (McGill et al., 1978). The linear regression and notched box-plot was plotted using a graphing package for R, ggplot2 v.0.9.3.1.

The 20 farmed-fish were grouped based on fish species (rainbow trout and whitefish), size, and age (small and big fish), farming time (3 and 15 months), and the history of antibiotic treatment at the farms (treated and non-treated) as shown in **Table 1**. To compare the resistome composition of the intestinal contents between the farmed fish groups, the genes' presence in each sample and their relative abundance values were used to calculate the distance matrix of non-metric multidimensional scaling (NMDS) or matrix of dissimilarities.

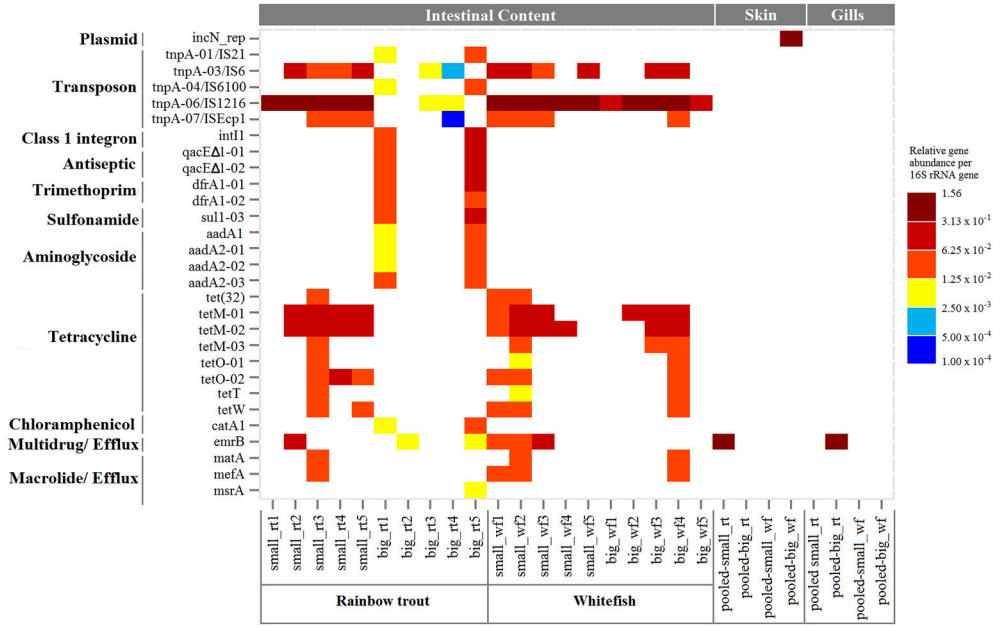

FIGURE 1 | Resistome composition of fish raised in the Northern Baltic Sea farms, Finland. The Y axis presents the gene assays of the qPCR array grouped by mobile genetic element (MGE) and by classification of the antibiotics the genes confer resistance to. The X axis presents the farmed fish organized by the type of samples: the intestinal contents, mucosal skin, and gill filaments of small rainbow trout (small_rt), big rainbow trout (big_rt), small whitefish (small_wf), and big whitefish (big_wf). The color scale indicates five-fold changes in the genes' relative abundance in proportion to the 16S rRNA gene. White indicates that the respective gene was not detected or below the detection limit of each assay (C_T cut-off was at cycle 27) in the qPCR array.

The NMDS distance matrix between samples was performed based on the Bray-Curtis method using function metaMDS from the Vegan package in R (Oksanen et al., 2014). Values missing from the data due to no detection in the qPCR array were replaced with zero for the data analysis. Function ordiplot and ordiellipse from Vegan were used, respectively, to plot the NMDS distance matrix results and to add the 95% confidence region of the farmed fish groups. All analyses were performed using RStudio v.0.98.501 (RStudio, Boston, MA, 2012).

RESULTS

Antibiotic Resistance Genes in the Intestinal Contents

We analyzed the ARG composition in the intestinal contents of 20 fish from two Northern Baltic Sea farms. The 20 farmed-fish consisted of five fish from each group of small rainbow trout, big rainbow trout, small whitefish, and big whitefish. The WaferGen qPCR array used 307 primer sets to detect and quantify ARGs, 21 primer sets for antibacterial or antiseptic resistance genes, 34 primer sets for MGE-associated genes, and one primer set for a mercury resistance gene. Altogether 28 out of the 363 genes were detected in the intestinal contents of the farmed-fish (**Figure 1**). The detected genes included transposases (*tnpA*) associated with IS21, IS6, IS6100, IS1216, and ISEcp1, an integrase gene of class 1 integron (*intI1*), a gene encoding resistance to an antiseptic which is also known as a backbone gene of class 1 integrons (*qacEΔ1*), a sulfonamide resistance gene (*sul1*), a trimethoprim resistance gene (*dfrA1*), tetracycline resistance genes [*tet(32), tetM, tetO, tetT,* and *tetW*], aminoglycoside resistance genes (*aadA1* and *aadA2*), a chloramphenicol resistance gene (*catA1*), a multidrug-efflux resistance gene (*emrB*), and macrolide (MLSB)-efflux resistance genes (*matA, mefA,* and *msrA*).

Three of the 28 detected genes were found in all four groups of the farmed-fish: small rainbow trout (small_rt), big rainbow trout (big_rt), small whitefish (small_wf), and big whitefish (big_wf) (**Figure S1**). The three genes were all transposases that associated with IS1216, IS6, and ISEcp1. The transposase associated with IS1216 was the most abundant gene detected in almost every intestinal content with a relative abundance of ca. 10^{-3}–10^{-1} in proportion to the 16S rRNA gene (**Figure 1**). The tetM-01 and tetM-02, targeting variants of the tetracycline resistance gene, *tetM*, showed these genes to be the most prevalent and abundant among the detected genes with relative abundances of ca. 10^{-2}–10^{-1} in proportion to the 16S rRNA gene. Other tetracycline resistance genes [*tet(32), tetO, tetT,* and *tetW*] were found in at least one of the five intestinal contents from small_rt, small_wf, and big_wf. The total relative abundances of the *tet* genes and transposases were significantly correlated [$F_{(1, 9)} = 22.3$, $p = 0.001$, $R^2 = 0.71$] indicating that the transposases could be connected to the prevalence of the *tet* genes in the intestinal contents (**Figure 2**). *tet* genes were not found in the big_rt samples. Two samples of the big rainbow trout, big_rt1 and big_rt5, differed from the rest of the intestinal

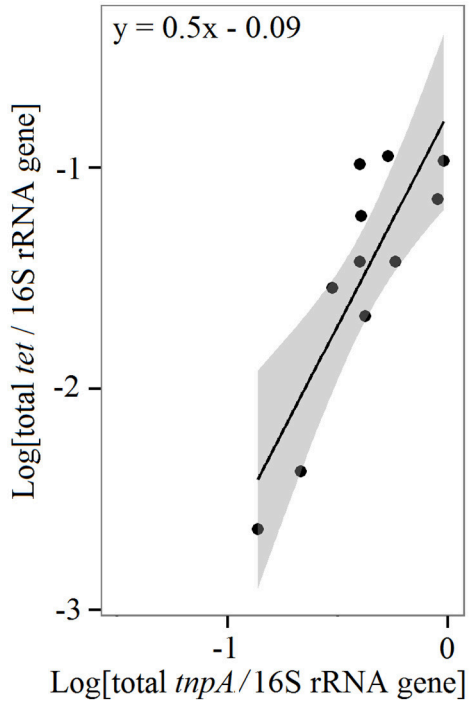

FIGURE 2 | Correlation analysis. Linear regression model with log transformed variables between the transposases (*tnpA* genes) and tetracycline resistance genes (*tet* genes) in the intestinal contents of the fish farmed at the Northern Baltic Sea farms [$F_{(1, 9)} = 20.3$; $p = 0.001$; $R^2 = 0.71$]. Each point presents the total relative abundances of the genes in proportion to the 16S rRNA gene in every intestinal content sample. The line indicates the regression model and the gray area the 95% confidence intervals.

content samples (**Figures S1**, **S2**) and carried genes that were not detected in the other intestinal content samples (**Figure 1**). These genes were transposases associated with IS21 and IS6100, *intI1*, *qacEΔ1, sul1, dfrA1, aadA1, aadA2,* and *catA1*. The efflux-pump resistance genes, *emrB, matA, mefA*, and *msrA*, were found in at least one of the intestinal contents. The mercury resistance gene (*merA*) was not detected in any of the samples.

In all, the detected ARGs in the intestinal contents of the farmed fish included the three major resistance mechanisms: antibiotic deactivation, cellular protection, and efflux pumps (**Table S4**, **Figure 3A**). The detected genes' relative abundances were ca. 10^{-4}–10^{-1} in proportion to the 16S rRNA gene (**Figure 3B**, **Tables S2**, **S3**). Relative abundances of the MGE, cellular protection and antiseptic resistance genes were similar and higher compared to the efflux-pump and antibiotic deactivation resistance genes (**Figure 3B**).

We compared the resistome composition of the intestinal contents between groups of the farmed-fish (**Table 1**). The groups based on fish species, size, farming time, and the history of antibiotic treatment at the farms did not separate in the non-metric Bray-Curtis dissimilarity (**Figures S2A–D**). However, the intestinal content resistome of each individual fish within the groups seemed to have its own composition, as shown in **Figure 1**.

Antibiotic Resistance Genes in the Mucosal Skin and Gill Filaments

The 364 assays of the WaferGen qPCR array were used to observe and quantify the resistome not only in the intestinal contents of the farmed fish but also in the fish mucosal skin and gill filaments. The skin and gill samples were pooled according to fish species and size to obtain a sufficient amount of DNA for the qPCR array analysis. In total, two genes were detected in the mucosal skin and only one gene in the gill filaments (**Figure 1**). In the mucosal skin, the two detected genes were *rep,* a specific replicon region of broad-host-range IncN plasmid, and *emrB,* a multidrug-efflux resistance gene. In the mucosal gill filaments, the *emrB* was the only detected gene. The *emrB* was the only gene found in each sample type: the intestinal contents, mucosal skin and gill filaments of the farmed fish.

DISCUSSION

Farmed Fish Feces as a Source of Gene Enrichment in Sediments

There is growing concern that antibiotic use in fish farming promotes the enrichment of ARGs in the environment (Cabello et al., 2013). The prevalence of resistant bacteria carrying ARGs (Nonaka et al., 2007; Shah et al., 2014) and enrichment of ARGs in sediments below fish farms have been observed globally (Tamminen et al., 2011a; Gao et al., 2012; Muziasari et al., 2014, 2016; Xiong et al., 2015). However, the causes of ARG enrichment in the farm sediments have received very little attention. We assumed that farmed fish feces are a plausible source of ARG enrichment in sediments below fish farms in the Northern Baltic Sea, Finland. In this study, we analyzed the resistome composition of the intestinal contents of 20 fish raised at the Baltic Sea farms. We used the WaferGen qPCR array with 364 primer sets of known target genes. We compared the composition of the genes detected in the intestinal contents to our previous data of the sediment resistome at the farms (Tamminen et al., 2011a; Muziasari et al., 2014, 2016). We found that most of the detected genes in the intestinal contents (20 of the 28 genes) reflected the composition of the genes enriched in the farm sediments. **Table 2** shows the 20 same genes that were detected in both farmed fish intestinal contents and farm sediments including transposases, class 1 integron-associated genes, and ARGs encoding resistance to tetracycline, sulfonamide, trimethoprim, and aminoglycoside. Average relative abundance of the genes in the intestinal contents was ca. 10^{-2}–10^{-1} in proportion to the 16S rRNA gene and ca. 10^{-4}–10^{-3} in proportion to the 16S rRNA gene in the farm sediments (**Table 2**). Bacterial community changes in the sediments in response to fish farming in the Baltic Sea also have been reported, with *Actinobacteria, Chloroflexi*, and *Firmicutes* prominent in the farm sediments (Tamminen et al., 2011b). *Actinobacteria and Firmicutes* are known as the core intestinal microbiota of salmonids (Pond et al., 2006; Navarrete et al., 2010; Wong et al., 2013). These findings provide indirect evidence supporting our assumption that certain ARGs are being introduced into the sediments below the fish farms in the

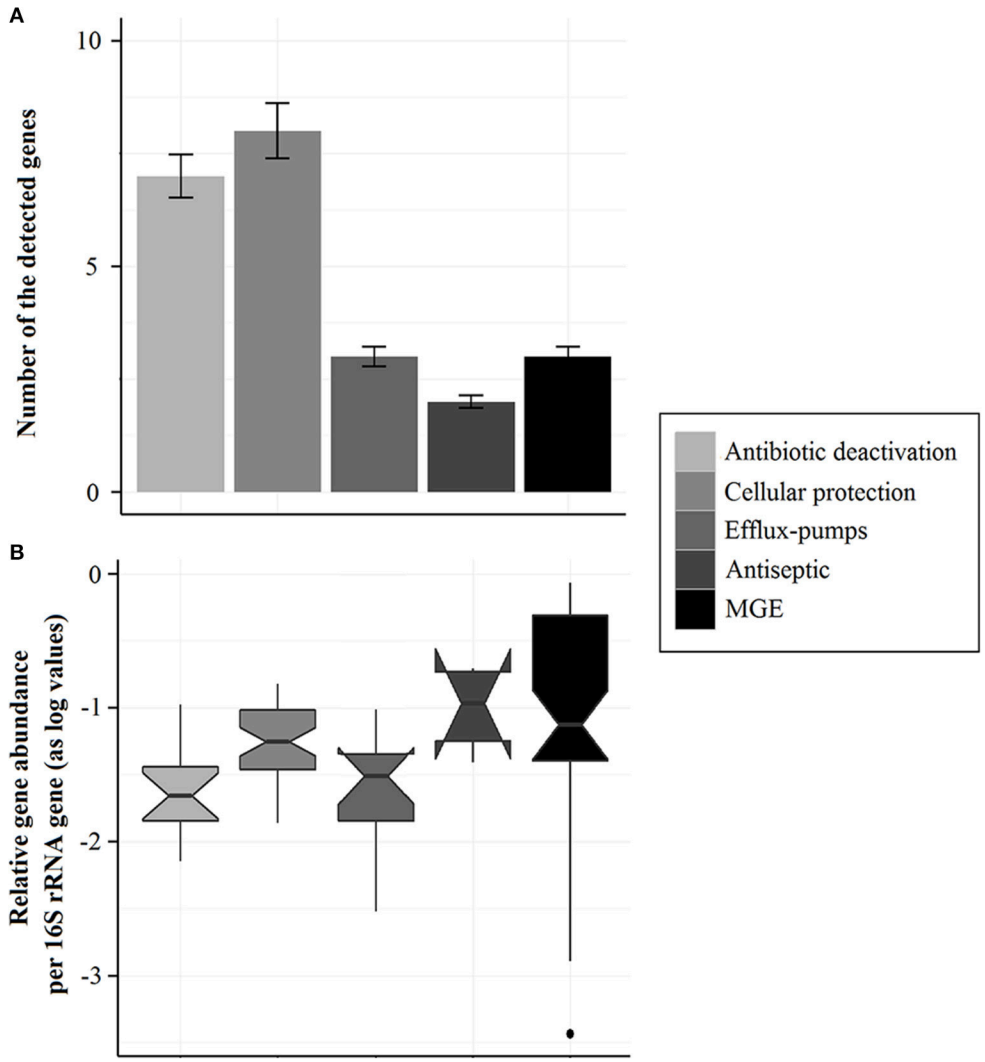

FIGURE 3 | (A) Number of the detected genes. Bars represent the numbers of genes detected in the intestinal contents of the Baltic Sea farmed fish. Error bars indicate standard error ($n = 20$). The detected genes included ARGs, antiseptic resistance genes, and genes associated with mobile genetic elements (MGEs). The ARGs are grouped by the mechanism of resistance: antibiotic deactivation, cellular protection, and efflux-pumps. **(B)** Relative abundances of the genes detected in the farmed fish in proportion to the 16S rRNA gene (as log values). The box-plot presents the biological replicates of the farmed fish ($n = 20$) and the notch the 95% confidence intervals of the median.

Northern Baltic Sea via the bacteria contained in the feces of the farmed fish.

The enrichment of ARGs also could be caused by the presence of antibiotics in the sediments. Although concentrations of tetracycline, oxytetracycline, sulfamethoxazole, sulfadiazine, and trimethoprim in the sediments were very low (ca. 1–100 ng/g of sediment), selection of resistance by antibiotics might have occurred in the past with the ARGs persisting in the sediments (Tamminen et al., 2011a; Muziasari et al., 2014). Enrichment of ARGs in the absence of selective antibiotic pressure could also be caused by co-selection with heavy metals (Baker-Austin et al., 2006). While mercury can be found in fish feed (Choi and Cech, 1998), biological and chemical analyses of the sediments revealed only very low background concentrations of this metal (Pitkänen et al., 2011). However, other heavy metals (copper, cadmium, zinc) can potentially be present in fish feed or antifouling substances and exert co-selective pressures in these sediments (Seiler and Berendonk, 2012).

On the other hand, the mercury resistance gene *merA* was not detected in the intestinal contents, but it was enriched in the farm sediments (Pitkänen et al., 2011). Similarly, not all sediment-enriched ARGs were detected in the intestinal contents. This might be due to the amount of the genes in the intestinal contents that were below the detection limit of the qPCR array, which varies between the primer sets (ca. 10^{-2}–10^{-4} copy number of target genes). Alternatively, the enriched genes in the farm sediments might be coming from other sources such as uneaten medicated feed (Kerry et al., 1995). The deposition

TABLE 2 | Twenty of the 28 genes detected in the farmed fish intestinal contents.

Classification of the antibiotics the genes confer resistance to	qPCR assay	Average relative abundance to the 16S rRNA gene	
		Fish intestinal contents (sampled in 2014)	Fish farm sediments sediments (sampled in 2006–2012)
Aminoglycoside	aadA1	2×10^2	[a] 3×10^4
Aminoglycoside	aadA2-01	2×10^2	[a] 2×10^4
Aminoglycoside	aadA2-02	2×10^2	[a] 4×10^4
Aminoglycoside	aadA2-03	4×10^2	[a] 8×10^4
Trimethoprim	dfrA1	6×10^2	[a] 3×10^3
Trimethoprim	dfrA1-02	4×10^2	[b] 1×10^3
Class 1 integron	intI1	6×10^2	[b] 3×10^3
Other (Antiseptic)	qacEΔ1-01	1×10^2	[a] 5×10^3
Other (Antiseptic)	qacEΔ1-02	1×10^1	[a] 3×10^3
Sulfonamide	sul1	7×10^2	[b] 4×10^3
Tetracycline	tet(32)	3×10^2	[a] 1×10^3
Tetracycline	tetM-01	1×10^1	[a] 3×10^3
Tetracycline	tetM-02	9×10^2	[a] 2×10^3
Tetracycline	tetM-03	4×10^2	[c] 1×10^3
Tetracycline	tetO-01	2×10^2	[a] 2×10^3
Tetracycline	tetW-01	4×10^2	[a] 4×10^4
Transposon	tnpA-01	3×10^2	[a] 6×10^4
Transposon	tnpA-04	4×10^2	[a] 2×10^4
Transposon	tnpA-06	5×10^1	[a] 1×10^3
Transposon	tnpA-07	4×10^2	[a] 4×10^3

[a] *Muziasari et al. (2016).*
[b] *Muziasari et al. (2014).*
[c] *Tamminen et al. (2011a).*
[a, c] *The quantification of the genes using standard qPCR.*
The 20 genes were the same genes found to be enriched in the sediments below fish farms in the Northern Baltic Sea, Finland. The table also shows the average relative abundance of the genes to the 16S rRNA gene in the intestinal contents and in the farm sediments. The gene assays of the qPCR array grouped by classification of the antibiotics the genes confer resistance to, class 1 integron and transposon.

of uneaten feed that increases the amount of organic matter in the farm sediments might be also resulting in the enrichment of indigenous sediment bacteria carrying the enriched ARGs (Tamminen et al., 2011b; Buschmann et al., 2012).

Because the ARGs were detected in the intestinal contents of the farmed fish with no history of antibiotic treatment at the farms, the ARGs could potentially be derived from resistant bacteria in the surrounding water environment that were able to be transferred to the intestinal microbiota of fish (Giatsis et al., 2015). The ARGs also may have already been selected in the fish intestine before entering the Baltic Sea farm cages, possibly during the processes of hatching or rearing juvenile fish in freshwater ponds. Specific record of antibiotics use in the juvenile fish prior to entering the Baltic Sea farms was not available. However, it has been reported that antibiotics were used against *F. psychrophilum* and *F. columnare* in Finnish hatchery farms (EVIRA, 2007). Antibiotic resistant bacteria can be found in fish eggs and juvenile fish due to the routine use of antibiotics during the rearing process (Hansen and Olafsen, 1999). The ARGs are still present in the fish intestine even after transferred to different water environments at the Baltic Sea farms, maybe because the resistant bacteria carrying the ARGs are the core intestinal microbiota of the farmed fish (Ghanbari et al., 2015). Although our data does not refute the prior understanding on the occurrence of antibiotic resistance in the fish farming environment due to antibiotic use at farms, it suggests that the juvenile fish may have a greater role as the source of antibiotic resistance than has been expected before. The routine use of antibiotics in fish hatchery and rearing farms should not be recommended because it may affect the natural microbiota of fish larvae and juvenile (Ringø et al., 1995). It is, therefore, important to monitor the antibiotic treatments of juvenile fish to minimize the potential risk of ARG emergence in fish farm environments.

Resistome Composition of the Intestinal Contents

Tetracycline resistance (*tet*) genes were the most prevalent ARGs in the intestinal contents of the farmed fish. The detected *tet* genes were genes which only encode ribosomal protection proteins (RPP), the *tet(32)*, *tetM*, *tetO*, *tetT*, and *tetW*. *tetM* was found to be the most prevalent of the detected ARGs. This was expected, since *tetM* has the widest host range of all known *tet* genes and is well known to be associated with a very wide range of conjugative transposons (Roberts, 2005). Beside *tetM* our results were in contrast to other studies in which the efflux-pump genes, *tetA, tetD*, and *tetE* were commonly found in resistant bacteria isolated from intestines of farmed fish (Furushita et al., 2003; Akinbowale et al., 2007). The transposase-IS1216 is predominant in the intestinal contents of the Baltic Sea farmed fish and known to be associated with conjugative transposons (Ciric et al., 2011). The RPP genes are usually associated with conjugative transposons, while the efflux-pump genes are generally associated with large plasmids (Roberts, 2012). Moreover, we observed a significant correlation between the total relative abundances of the *tet* genes and transposases ($p = 0.001$, $R^2 = 0.71$). Transposases are, therefore, likely involved in the prevalence of the *tet* genes in the Baltic Sea farmed fish intestinal contents. A highly significant correlation between the abundance of transposases and *tet* genes has been observed also in swine manure (Zhu et al., 2013).

In this study, the *tet* genes were not found in the intestinal contents of the big rainbow trout. However, some genes were only found in the intestinal contents of two big rainbow trout. Those genes were the transposases associated with IS21 or Tn21 and IS6100, genes associated with class 1 integrons, *intI1* and *qacEΔ1*, and the ARGs *sul1, dfrA1, aadA1, aadA2*, and *catA1*. Class 1 integrons are known to be able to capture and incorporate gene cassettes consisting of *sul1*, *dfrA1, aadA1, aadA2* (Partridge et al., 2009), and *catA1* (Soto et al., 2003). In the farm sediments, class 1 integrons carry the *sul1* and *aadA1* genes (Pärnänen et al., 2016). This explains the presence of *aadA1* aminoglycoside resistance genes at the farms even though aminoglycosides have never been used there. The prevalence of class 1 integrons could be cause by the association of class 1 integrons with Tn21 (Liebert et al., 1999) or with IS6100 (Partridge et al., 2001). The presence of

sul1, dfrA1, aadA1, *aadA2*, and *catA1* in the intestinal contents of two big rainbow trout may have been mediated by the class 1 integrons carried by MGEs such as Tn21 or IS6100.

The chloramphenicol and macrolide resistance genes were found in the intestinal contents and are also known to be associated with conjugative transposons (Hegstad et al., 2010). This suggests that the presence of ARGs in the farmed fish intestinal contents may be connected to the prevalence of transposases. Otherwise, the ARGs, especially efflux-pump resistance genes, could be intrinsically possessed by fish bacteria since the efflux-pump resistance genes are also known to have other functions beside antibiotic resistance, such as intracellular metabolite detoxification (Martinez et al., 2009).

We observed that the resistome composition of the intestinal contents varied between individual farmed fish. Beside the connection with the MGEs, the resistome composition in the fish intestine may be also correlated with the composition of bacterial communities (Forsberg et al., 2014). It has been reported that the bacterial communities in the intestines of individual farmed fish varies greatly and stable regardless of the variations of diet and rearing system (Wong et al., 2013; Lyons et al., 2017). Our results also show that the resistome composition of the intestinal contents might not connected to the types of fish species, size, farming time, and antibiotic treatments at the farms. This suggests that the resistome of the farmed-fish intestinal contents has its own composition and is influenced less by the fish farming processes. However, these results may vary if more farmed fish are sampled and included in the future studies. To our knowledge, this is the first study reporting the resistome composition of farmed-fish using a culture-independent method.

The intestinal content resistome of the farmed-fish covered the three mechanisms of resistance genes: antibiotic deactivation, cellular protection, and efflux-pumps. The relative abundance of cellular protection resistance genes was higher than the other two mechanisms of resistance genes. This is because the cellular protection resistance genes included all the detected RPP-tetracycline resistance genes that were the most abundant ARGs in the intestinal contents of the farmed-fish. In the farm sediment resistome, however, the efflux-pump resistance genes were the most abundant (Muziasari et al., 2016). The low frequency of detection of efflux-pump resistance genes in the resistomes of farmed-fish intestines in this study might be due to the qPCR array targeting only known ARGs since the qPCR array requires prior knowledge of ARG sequences for primer design. Insight on the ARG mechanisms within fish farming-associated resistomes is essential for predicting the emergence of ARGs at the fish farm facilities (Miranda et al., 2013).

Resistome Composition in the Mucosal Skin and Gill Filaments

In the mucosal skin and gill filaments of the farmed-fish, only one of the 307 targeted ARGs, the multidrug-efflux resistance gene, *emrB*, was detected. The *emrB* was also detected in the intestinal contents. Since the *emrB* was found in the three different sample types taken from the farmed fish, the *emrB* gene maybe intrinsically possessed by fish bacteria (Martinez et al., 2009). In other studies, ARGs encoding resistance to tetracycline, sulfonamide, and trimethoprim have been frequently found in resistant bacteria isolated from the skin and gills of farmed fish (Schmidt et al., 2001; Akinbowale et al., 2007). The different results might be due to the difference between ARG detection methods, as the qPCR array detection limit may prevent detection of a low abundance of ARGs in fish mucosal skin and gill filaments.

CONCLUSION

This study indicates that the resistomes of the intestinal contents of farmed fish contribute to the enrichment of ARGs in sediments below fish farms in the Northern Baltic Sea. Using high-throughput methods to study fish resistomes, we found a significant correlation between the relative abundances of the transposases in them and tetracycline resistance genes. This might lead to the risk of ARG mobilization from piscine bacteria to bacteria in the surrounding environments. The presence of transposases and class 1 integrons might also affect the prevalence of certain ARGs in the intestinal contents of individual fish and shape the composition of their resistomes. Determining the sources of ARGs at fish farms is thus important for managing and minimizing the emergence of ARGs at the fish farms and for lowering the risk of ARG spreading to the surrounding farm environments.

AUTHOR CONTRIBUTIONS

WM and MV: contributions to the experimental design. WM and LP: sampling. WM, LP, and RS: acquisition and analysis of data for the work. HS, JT, and MV: analysis, interpretation and adding important intellectual content. WM, LP, HS, RS, JT, and MV: drafting and revising the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.02137/full#supplementary-material

Figure S1 | Venn diagram. Number of genes shared by the groups of the Baltic Sea farmed fish: small rainbow trout (small_rt), big rainbow trout (big_rt), small

whitefish (small_whitefish), and big whitefish (big_wf). Each group consisted of five farmed fish.

Figure S2 | Non-metric multidimensional scaling (NMDS) of the intestinal content resistomes of fish farmed at the Baltic Sea farms. The distance matrix between the intestinal content resistomes was based on Bray-Curtis dissimilarity. The farmed fish ($n = 20$) were grouped based on **(A)** Fish species: rainbow trout (dark red) and whitefish (rose). **(B)** Fish size: big fish (dark red) and small fish (rose). **(C)** Farming time at the farms: 3 months (dark red) and 15 months (rose). **(D)** The history of antibiotic treatment at the farms: Non-treated (dark red) and treated with a combination of sulfonamide-trimethoprim 1 month before the fish were sampled (rose). Ellipse line indicates 95% confidence regions of the farmed fish groups.

Table S1 | The list of primer sets used in the qPCR array.

Table S2 | Gene abundance values relative to the 16S rRNA gene in Rainbow trout raised at the Northern Baltic Sea farms.

Table S3 | Gene abundance values relative to the 16S rRNA gene in European whitefish raised at the Northern Baltic Sea farms.

Table S4 | Number of positive qPCR assays and the total assays.

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