RESISTANCE AND TOLERANCE IN FOOD-BORNE PATHOGENS: MECHANISMS, PUBLIC HEALTH IMPACT, AND CONTROL MEASURES

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RESISTANCE AND TOLERANCE IN FOOD-BORNE PATHOGENS: MECHANISMS, PUBLIC HEALTH IMPACT, AND CONTROL MEASURES

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Editorial: Resistance and Tolerance in Food-Borne Pathogens: Mechanisms, Public Health Impact, and Control Measures

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Editorial on the Research Topic

Resistance and Tolerance in Food-Borne Pathogens: Mechanisms, Public Health Impact, and Control Measures

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Foodborne pathogens are exposed to many stress conditions present in animal hosts and the environment surrounding farms at the stage of primary production and encounter artificial stressors, including disinfectants and non-optimal growth conditions, during food processing, storage, and cooking. Foodborne pathogens can cause foodborne illnesses only when they successfully overcome stress conditions along the farm to fork continuum (Begley and Hill, 2015; Oh et al., 2018; Zhang F. et al.). Most foodborne infections are self-limiting; however, serious cases of illness require antimicrobial chemotherapy. With the increasing prevalence of antibiotic-resistant foodborne pathogens being detected, the efficacy of antimicrobial chemotherapy is significantly compromised, leading to serious patient outcomes. Bacterial tolerance to environmental stress and resistance to antibiotics significantly affect food safety and public health. Although stress tolerance and antibiotic resistance in bacteria may be considered totally different topics, there are some overarching features for both. For instance, oxidative stress is a general tolerance mechanism that affects the lethality of antibiotic treatment (Poole, 2012). Thus, understanding stress tolerance and antibiotic resistance in foodborne pathogens can provide a holistic perspective for food safety from farm to fork and beyond, including clinical treatment of foodborne illnesses. The Research Topic "*Resistance and Tolerance in Food-borne Pathogens: Mechanisms, Public Health Impact, and Control Measures*" aims to extend our knowledge of how these mechanisms influence food safety and public health.

Two articles about stress tolerance in *Listeria monocytogenes* were published. Zhang H. et al. reported that *L. monocytogenes* 1/2b isolates were the predominant strain types isolated from processing facilities of ready-to-eat meat in China irrespective of the observed hygiene levels based on aerobic plate counts and coliform detection. Whole genome sequence analysis suggested that the isolates clonally expanded possibly by forming biofilms, confirming the importance of sanitation procedures for the control of *L. monocytogenes*. Interestingly, the findings of Li et al. showed that the use of organic acids increases the transcriptional levels of genes associated with acid and bile

stress response and virulence. Exposure to acetic acid and lactic acid, both common organic acids used in the food industry, increase the virulence of *L. monocytogenes* based on testing using the *Galleria mellonella* infection model. Organic acids are frequently used for the control of *Listeria* contamination by the food industry. However, organic acids can potentially influence *Listeria's* stress tolerance and virulence.

With respect to various *in vivo* stresses encountered by microbes, foodborne pathogens also rely on effective and complex tolerance strategies to survive and establish successful infection in the host. One such study is focused on the resistance of *Vibrio vulnificus* to nitric oxide (NO), an important antimicrobial effector produced by the host innate immune system to counteract invading pathogens (Choi et al.). The investigators identified a NO-responsive transcriptional regulator NsrR, a strong repressor of *hmpA* that encodes an NO dioxygenase required for resistance of *V. vulnificus* to nitrosative stress. Further molecular studies found that NsrR could delicately cooperate with other two regulatory proteins, Lrp and CRP, to tightly control the transcription of *hmpA*, consequently contributing to the survival of *V. vulnificus* under host-derived nitrosative stress (Choi et al.).

Several articles in the eCollection ascertained that antibiotic resistance is highly common in the food supply chain worldwide. The article by Lopez-Chavarrias et al. provides a good demonstration of the high prevalence of antibiotic-resistant *Campylobacter* in healthy livestock in Spain (Lopez-Chavarrias et al.). Approximately 94.5% of *Campylobacter coli* isolates and 91.1% of *Campylobacter jejuni* isolates from broilers were resistant to ciprofloxacin, a fluoroquinolone drug of clinical importance in human health, and 66.6% of *C. coli* from pigs were resistant to erythromycin. Consistently, tetracycline and fluoroquinolone resistance are prevalent in *C. jejuni* isolates from patients in the United States. Notably, campylobacteriosis associated with fluoroquinolone resistance was significantly associated with international travel (Rodrigues et al.). Barbieri et al. reported that *mcr-1* is highly prevalent in *Escherichia coli* isolates from healthy and sick poultry with colibacillosis in Brazil due to the agricultural application of colistin, a last-resort antibiotic to treat Gram-negative infections (Liu et al., 2016). In addition, multi-drug resistance in *Yersinia enterocolitica* 4/O:3 derived from fresh pre-washed spinach was found to be the cause of the consecutive foodborne yersiniosis outbreaks in Sweden in 2019. Molecular characterization of the multidrug-resistant *Y. enterocolitica* outbreak strain revealed that this foodborne pathogen harbored the Tn*2670* transposon with resistance determinants against quaternary ammonium compounds, the heavy metal mercury, phenicols, streptomycin, and sulfonamides and an additional plasmid carrying tetracycline resistance gene. Interestingly, neither the Tn*2670* transposon nor the *tet*B resistance plasmid has previously been reported in foodborne *Yersinia* nor in isolates derived from ready-to-eat products, suggesting that horizontal gene transfer events occurring in the environment, agriculture, or animal husbandry have promoted the selection of *Y. enterocolitica* carrying multi-antibiotic and metal resistance determinants (Karlsson et al.).

An article included in this collection discussed potential intervention measures to control antibiotic resistance in the food supply chain using bacteriophages (phages). Kim et al. discovered that some phages preferentially infect *E. coli* based on the phylogenetic group and constructed a highly effective phage cocktail targeting poultry isolates of *E. coli* producing extended-spectrum β-lactamases (ESBL) that frequently contaminate retail poultry. Although antibiotic-resistant, non-pathogenic *E. coli* does not develop an infection in animals and humans, antibiotic resistance can be transferred to pathogenic bacteria. The phages strongly inhibited ESBL-producing *E. coli* on chicken skin at refrigeration temperatures, suggesting that phages have potential application for use in retail raw chicken to reduce antibiotic resistance (Kim et al.).

Humans are continuously exposed to antibiotic-resistant microorganisms through the consumption of food, and the chances of exposure to antibiotic-resistant microorganisms will continue to pose a challenge and potentially increase in prevalence if foodborne pathogenic bacteria can survive stress conditions in the pathway from farm to fork. These articles highlight the concern about food chain contamination as a potential reservoir for transmission and dissemination of antimicrobial resistance, raising concerns for food safety and public health.

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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Presence and Characterization of a Novel *cfr*-Carrying Tn*558* Transposon Derivative in *Staphylococcus delphini* Isolated From Retail Food

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Antimicrobial resistance has become a major public health threat. Food-related *Staphylococcus* species have received much attention due to their multidrug resistance. The *cfr* gene associated with multidrug resistance has been consistently detected in food-derived *Staphylococcus* species. In this retrospective study, we examined the prevalence of *cfr*-positive *Staphylococcus* strains isolated from poultry meat in different geographical areas of China from 2011 to 2016. Two *cfr*-positive *Staphylococcus delphini* strains were identified from poultry meat in China. Comparative and whole-genome analyses were performed to characterize the genetic features and overall antimicrobial resistance genes in the two *S. delphini* isolates 245-1 and 2794-1. Whole-genome sequencing showed that they both harbored a novel 20,258-bp *cfr*-carrying Tn*558* transposon derivative on their chromosomes. The Tn*558* derivative harbors multiple antimicrobial resistance genes, including the transferable multiresistance gene *cfr*, chloramphenicol resistance gene *fexA*, aminoglycoside resistance genes *aacA-aphD* and *aadD*, and bleomycin resistance gene *ble*. Surprisingly, within the Tn*558* derivative, an active unconventional circularizable structure containing various resistance genes and a copy of a direct repeat sequence was identified by two-step PCR. Furthermore, core genome phylogenetic analysis revealed that the *cfr*-positive *S. delphini* strains were most closely related to *S. delphini* 14S03313-1 isolated from Japan in 2017 and 14S03319-1 isolated from Switzerland in 2017. This study is the first report of *S. delphini* harboring a novel *cfr*-carrying Tn558 derivative isolated from retail food. This finding raises further concerns regarding the potential threat to food safety and public health safety. The occurrence and dissemination of similar *cfr*-carrying transposons from diverse *Staphylococcus* species need further surveillance.

Keywords: Tn*558*, *cfr*, *Staphylococcus delphini*, unconventional circularizable structure, multidrug resistance

INTRODUCTION

In recent years, resistance in bacteria has spread worldwide and presents a serious threat to human health. Linezolid is an oxazolidinone antibiotic and is considered as the last-resort antibiotic for the treatment of infections caused by multidrug-resistant (MDR) Gram-positive pathogens, including *Staphylococcus* species (Wilson et al., 2008). The antibiotic targets the P site in the peptidyl transferase center of the 23S ribosomal RNA of the 50S ribosomal subunit, acting on this target and blocking protein synthesis (Aoki et al., 2002). In fact, due to the synthetic nature of the drug, resistance to this antibiotic is rare. However, the *cfr* gene could mediate resistance to linezolid (Long et al., 2006). This gene encodes a methyltransferase that catalyzes the posttranscriptional methylation of adenosine at nucleotide position 2503 (*Escherichia coli* numbering) in 23S rRNA, which replaced the target of binding for linezolid (Corinna et al., 2005; Giessing et al., 2009; Anna et al., 2016). However, due to overlapping binding sites, *cfr* methylation also confers resistance to four other classes of antimicrobial agents and results in the PhLOPSA multiresistance phenotype, including resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A compounds (Long et al., 2006; Anna et al., 2016). Interestingly, *cfr* is often associated with *erm*, *fexA*, *lsa*(B), and *tet*(L), which can assist in co-selecting the *cfr* gene and in its spread (Shen et al., 2013; Mendes et al., 2014).

Generally, the *cfr* gene is often associated with mobile genetic elements (MGEs) (plasmids, integrative, and conjugative elements or transposons), which have great potential for dissemination (Shen et al., 2013). Tn*558* is one of these bacterial transposons and was first identified on the plasmid pSCFS2 harboring the antimicrobial resistance gene (ARG) *fexA* from *Staphylococcus lentus* (Kehrenberg and Schwarz, 2005). Currently, this transposon is often harbored with *cfr,* and derivatives of Tn*558* usually carry other acquired ARGs (Kehrenberg et al., 2007; Li et al., 2018). Therefore, this transposon plays an important role as vectors in the spread of transposon-borne ARGs.

Members of the genus *Staphylococcus* are widespread in nature and play vital roles in disease causation in humans and animals (McGavin and Heinrichs, 2012; Vrbovská et al., 2020). Among these species, *Staphylococcus delphini* is a pathogen that causes animal and human infections (Magleby et al., 2019; Ruiz-Ripa et al., 2019). It belongs to the *Staphylococcus intermedius* group and was first described in purulent skin lesions of dolphins (Varaldo et al., 1988). *S. delphini* is further separated into two subgroups, groups A and B, based on the phylogenetic analysis of the *sodA*, *hsp60*, and *nuc* genes and DNA–DNA hybridization (Sasaki et al., 2007). Although this staphylococcal species is poorly documented due to misidentification with *S. intermedius*, it has been isolated from humans and a wide range of diseased animals, including domestic pigeons, camels, horses, magpies, cinereous vultures, and mustelids, which serve as the natural hosts of *S. delphini* group A (Devriese et al., 2005; Sasaki et al., 2007; Sledge et al., 2010; Guardabassi et al., 2012; Sudagidan and Aydin, 2012; Stull et al., 2014; Magleby et al., 2019; Ruiz-Ripa et al., 2019).

In this retrospective study, we examined the prevalence of *cfr*-positive *Staphylococcus* isolates in poultry meat from 2011 to 2016. We determined the complete genome sequence of *cfr*-positive *S. delphini* and described their phenotypic and genotypic profiles. This is the first report of a Tn*558* derivative-embedded *cfr* in *S. delphini* isolated from retail food.

MATERIALS AND METHODS

Bacterial Isolation

From July 2011 to June 2016, we collected 4,300 retail food samples from supermarkets, fairs, and farmer markets, covering most of the provincial capitals of China (**Supplementary Figure 1**), and isolated 1,581 *Staphylococcus* strains, including *Staphylococcus aureus*, *Staphylococcus argenteus*, *S. delphini*, *Staphylococcus epidermidis*, and other staphylococci from 1,063 positive samples from all the sampling sites (Wu et al., 2018a,b). During the retrospective study of *cfr*-positive *Staphylococcus* species among these isolates, the *cfr*-positive strains 245-1 and 2794-1 were isolated from frozen duck wings in Guangzhou 2013 and frozen duck legs in Kunming 2014, respectively. The isolates were further identified as *S. delphini* by the MALDI-TOF/MS system (Bruker, Bremen, Germany) (Decristophoris et al., 2011).

PCR Detection

The presence of the resistance gene *cfr* was identified by PCR and Sanger sequencing (Kehrenberg et al., 2009). The presence of the two direct repeats (DRs) and circular intermediate translocatable units (TUs) was detected by PCR and inverse PCR (the primers and conditions are shown in **Table 2**). To minimize the detection of artificial products, a high-fidelity polymerase (PrimeSTAR GXL DNA Polymerase, Takara, Dalian, China) and an 8-min elongation step were used (Tansirichaiya et al., 2016). The amplicons obtained by PCR and inverse PCR experiments were subjected to Sanger sequencing.

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MICs) were determined using a standard broth dilution method according to the CLSI guidelines with *S. aureus* ATCC 29213 as a quality control strain (Weinstein and Clinical and Laboratory Standards Institute, 2018). The MICs for all of the following antimicrobials were determined: FFC, florfenicol; CHL, chloramphenicol; CLI, clindamycin; TIA, tiamulin; LZD, linezolid; K, kanamycin; ERY, erythromycin; FOX, cefoxitin; VAN, vancomycin; RIP, rifampicin; and DAP, daptomycin. The MIC breakpoints of each antibiotic, except florfenicol, were used as recommended by the current CLSI guidance (Weinstein and Clinical and Laboratory Standards Institute, 2018). For florfenicol, the results were interpreted according to the Veterinary CLSI (VET01-A5).

Whole-Genome Sequence and Analysis

Genomic DNA for whole-genome sequencing was extracted from the *cfr*-positive strains using a genomic extraction

kit (Magen Biotech, Guangzhou, China) according to the manufacturer's instructions. Whole-genome sequencing of the *cfr*-positive strains was performed using the Illumina HiSeq Xten platform (800-bp paired-end reads with 100-fold average coverage) and a PacBio Sequel II sequencing instrument (100-fold average read depth). The chromosome sequences were assembled into one scaffold using the software SMRT Portal, version 3.2.0. The genomic DNA annotation was performed in Prokka NCBI-BLASTP/BLASTX (Torsten, 2014). The single-nucleotide polymorphisms (SNPs) between strains 245-1 and 2794-1 were identified with Snippy software[1].

The acquired antibiotic resistance genes were identified by ResFinder 3.0[2] and were further verified through a BLAST search against the Comprehensive Antibiotic Resistance Database (Ea et al., 2012). The genetic environment of the *cfr* gene was analyzed using BLAST[3], followed by visualization of the comparative *cfr* multiresistance region (MRR) with Easyfig, v2.2.2 (Sullivan et al., 2011).

Phylogenetic Analysis

All publicly available draft genome sequences of *S. delphini* strains were acquired (22 strains with at least 50 × read coverage), and core SNP alignments were produced *via* Snippy using the *S. delphini* 8086 complete genome sequence (ASM30811v1) as a reference (see text footnote 1). The maximum-likelihood (ML) phylogenetic tree was constructed with RAxML-NG based on the ML optimality criterion (Kozlov et al., 2019). The locations of recombined regions on each branch were detected, and this tree was reconstructed by ClonalFrameML (Didelot and Wilson, 2015). FigTree, v1.4.3, was used to finalize the tree visualization (Morariu et al., 2008).

Nucleotide Sequence Accession Numbers

The complete genomic sequences of 245-1 and 2794-1 have been deposited in GenBank: 245-1 (GenBank ID: CP063368) and 2794-1 (GenBank ID: CP063367).

RESULTS

Phenotypic Characteristics of *cfr*-Positive *S. delphini*

In this study, 245-1 and 2794-1 displayed the same MDR profiles. Antimicrobial susceptibility testing showed that these strains were resistant to chloramphenicol, florfenicol, tiamulin, clindamycin, and linezolid, exhibiting a high level of resistance to florfenicol (MIC = 256 μg/ml), chloramphenicol (MIC > 128 μg/ml), and tiamulin (MIC > 128 μg/ml). Moreover, the isolates were susceptible to vancomycin, daptomycin, and rifampicin (**Table 1**).

[1]https://github.com/tseemann/snippy

[2]https://cge.cbs.dtu.dk/services/ResFinder/

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

Basic Genomic Information for *cfr*-Positive *S. delphini*

To understand the molecular characteristics and resistomes of the two strains of *S. delphini*, they were submitted for whole-genome sequencing. Basic information related to the complete genome sequence of *cfr*-positive *S. delphini* is shown in **Figure 1**. The chromosomes of 245-1 and 2794-1 consisted of 2,708,646 bp with 2,486 predicted ORFs along with 102 RNAs and 2,707,963 bp with 2,486 predicted ORFs along with 102 RNAs, respectively. The genome analysis of the complete chromosomal DNA revealed that there were 166 variants between the chromosomes of 245-1 and 2794-1, and there were multiple ARGs located on their chromosomes, including *fexA* (conferring resistance to chloramphenicol), *aacA-aphD* and *aadD* (resistance to aminoglycosides), *ble* (resistance to bleomycin), and the multiresistance gene *cfr* (resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A).

Core Genome Phylogenetic Analysis of *cfr*-Positive *S. delphini*

To further investigate the potential sources of *cfr*-positive *S. delphini* 245-1 and 2794-1, we performed a core genome phylogenetic analysis of all publicly available draft genome sequences of *S. delphini* strains. The phylogenetic analysis shows that 245-1 and 2794-1 are most closely related to *S. delphini* 14S03313-1 (GCA_002374125.1) isolated from Japan in 2017 and 14S03319-1 (GCA_002369675.1) isolated from Switzerland in 2017 (**Figure 2**). This phylogenetic analysis did not reveal the origin of 245-1 and 2794-1, indicating that the scarcity of genomic sequences may be the constraint, and further genomic sequencing is needed to identify the source of the *cfr*-positive strains.

Genetic Environment of *cfr* Located on a Novel Tn*558* Transposon Derivative

Genomic mining revealed that the *cfr* gene, along with four other ARGs, namely, *fexA*, *aacA-aphD*, *aadD*, and *ble*, was located on a 20,258-bp (62,847–83,104 nt on the chromosomes of 245-1 and 2794-1 in **Figure 1**) MRR on the chromosomes. Further BLAST analysis showed that the ARGs *aacA-aphD*, *aadD*, *ble*, and *cfr* were flanked by two DRs oriented in the same direction within the MRR and that the two DRs both belonged to Tn*558* (**Figure 3**). The presence of the two DRs was further identified by PCR assays followed by sequencing of the amplicons (primers shown in **Table 2**). Both DRs were 1,326 bp in size, except for 18-bp exchanges in DR_B compared to DR_A. DR_A contained partial *fexA* (430 bp) and *orf138* sequences, while DR_B comprised partial *orf1* (430 bp) and *orf2* sequences. Further analysis revealed that the single-nucleotide exchange TAG (*orf138*) → TAC (*orf2*) caused the termination codon to mutate to a Tyr codon, resulting in an extension of the open reading frame that transformed *orf138* to *orf2*.

To further determine whether these unknown DRs in 245-1 and 2794-1 could mediate the formation of circular intermediate TUs, inverse PCR (P3, P4) was performed, followed by sequencing of the amplicons. Two identical PCR products

TABLE 1 | Phenotypic and genotypic characteristics of *Staphylococcus delphini*.

Bacterial isolate	MIC (μg/mL)											Resistance genes
	FFC	CHL	CLI	TIA	LZD	K	ERY	FOX	VAN	RFP	DAP	
245-1	256	128	4	128	8	16	0.25	0.5	1	<0.015	0.25	*cfr*, *fexA*, *ble*, *aacA-aphD*, *aadD*
2794-1	256	128	4	128	8	16	0.25	0.5	1	<0.015	0.5	*cfr*, *fexA*, *ble*, *aacA-aphD*, *aadD*
29213	8	8	0.0625	0.25	4	1	0.125	4	0.5	0.0078	0.5	NONE

FFC, florfenicol; CHL, chloramphenicol; CLI, clindamycin; TIA, tiamulin; LZD, linezolid; K, kanamycin; ERY, erythromycin; FOX, cefoxitin; VAN, vancomycin; RIP, rifampicin; DAP, daptomycin.

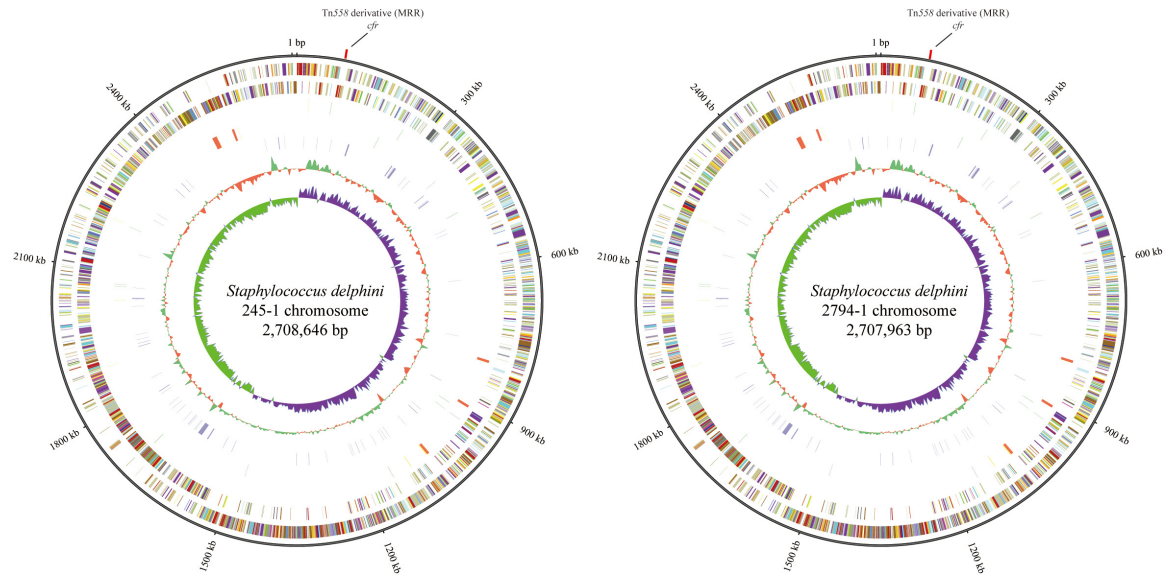

FIGURE 1 | Circular representation of the *cfr*-positive *Staphylococcus delphini* 245-1 and 2794-1 genomes. From the outer to the inner circles in the chromosome circular map: slot 1 (ARGs) and slots 2–9 (slot 2, genome size; slot 3, forward strand gene, colored according to the cluster of orthologous groups classification; slot 4, reverse strand gene, colored according to the cluster of orthologous groups classification; slot 5, forward strand ncRNA; slot 6, reverse strand ncRNA; slot 7, repeat; slot 8, GC content; and slot 9, GC skew).

(1,824 bp) were acquired from 245-1 and 2794-1, including a copy of DR_B, *orf138*, and part of *fexA*, as determined by sequencing (**Figure 3**). The TUs (13,613 bp) resulted from the recombination between DR_A and DR_B, including multi-ARGs and one copy of DR_A. The PCRs (P1, P2) containing one copy of DR_B detected the remaining structures after the excision of unconventional circularizable structures (UCSs) on chromosomes, and the results were consistent with the inverse PCR results (**Figure 3**). Importantly, the remaining structures were Tn*558*. These results confirmed the excision and cyclization of the structure (**Figure 3**). Further BLAST analysis revealed that the left Δ*fexA*-UCS exhibited 99.88% nucleotide identity to the corresponding region of the plasmids pWo28-1 (KX982171.1) and pWo28-3 (KY601170.1) from *Staphylococcus sciuri* and the plasmid pJP2 (KC989517.1) from *Staphylococcus rostri* lacking DR_B (**Figure 3**).

The sequence alignment analysis showed that the *cfr* MRR consisted of a Tn*558* homologous region (6,644 bp) and a 13,613-bp region (**Figure 3**). This arrangement is a novel derivative of the Tn*558* transposon. Compared to the *fexA*, *orf138*, *tnpC*, *tnpB*, and *tnpA* genes in Tn*558* (Kehrenberg and Schwarz, 2005), a closer inspection of the Tn*558* derivative showed that several nucleotide exchanges were identified in *fexA* (14 bp), *tnpB* (20 bp), and *tnpA* (14 bp), except for *orf138*. To further explain the genetic environment of the *cfr* MRR in this study, the plasmids pWo28-1 (KX982171.1) and pWo28-3 (KY601170.1) from *S. sciuri* and plasmid pJP2 (KC989517.1) from *S. rostri* are also shown in **Figure 3**. Analysis of the regions flanking the Tn*558* derivative insertion in the chromosome identified a reading frame encoding a putative protein of 114 aa (62,844–62,846 and 83,105-83,443 nt on chromosomes 245-1 and 2794-1) that shared 98.54% nucleotide identity with a 148-aa DNA repair protein from *Macrococcus canis* (CP021059.1) (Gobeli et al., 2017). Additionally, a minicircle of Tn*558*, an indication of Tn*558* having transposition activity, was identified *via* PCR (primers shown in **Table 2**) and sequencing of the derivative.

DISCUSSION

Naturally, *S. delphini* is widely susceptible to clinically relevant classes of antibiotics. In a previous study from Denmark,

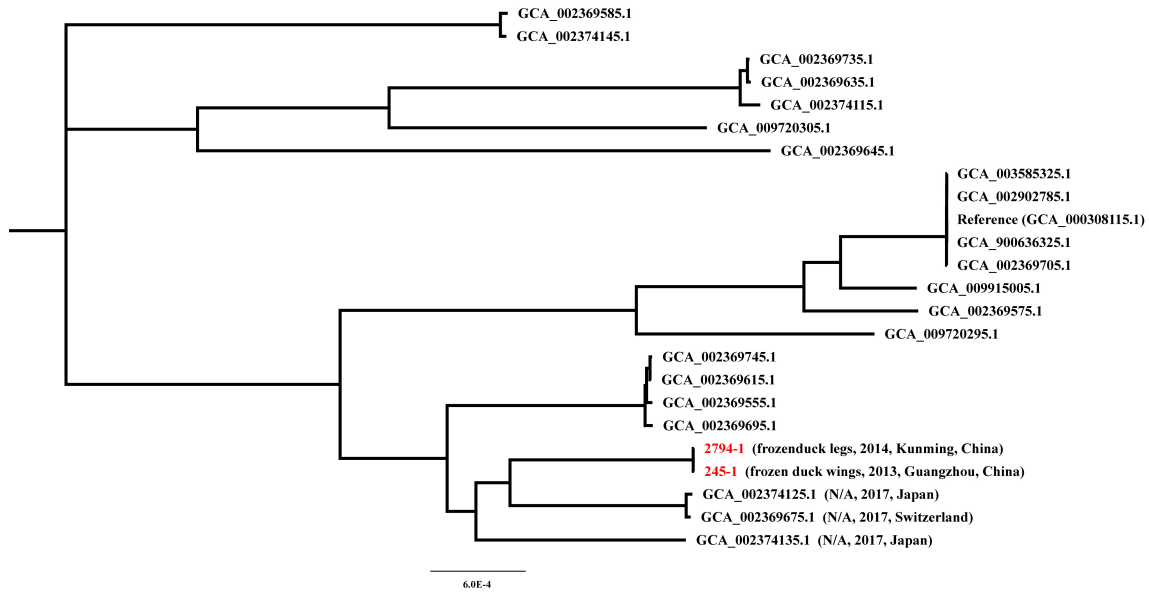

FIGURE 2 | Maximum-likelihood (ML) core genome phylogeny of *cfr*-positive *Staphylococcus delphini* 245-1 and 2794-1 based on the ML method.

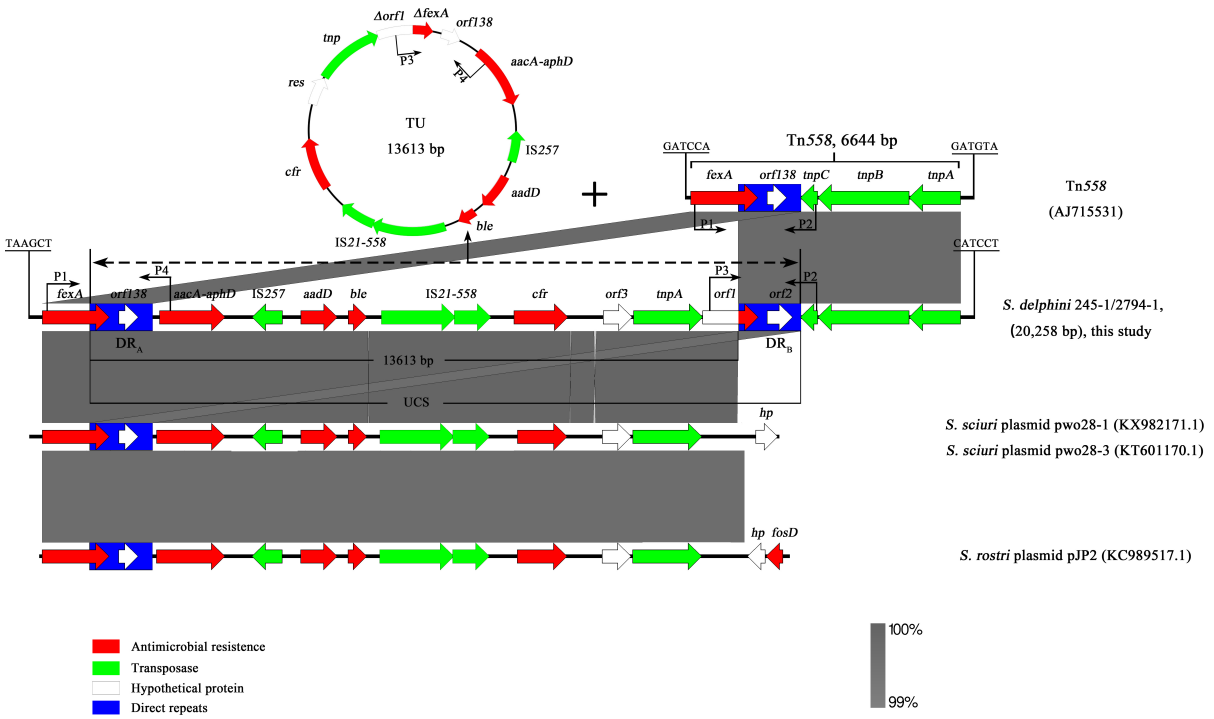

FIGURE 3 | Schematic presentation of the environment of the *cfr* gene on chromosomes 245-1 and 2794-1 and the formation of translocatable units (TUs) mediated by direct repeats (DRs). The TUs derived from the region between DR_A and DR_B and the remaining structures after the excision of unconventional circularizable structures on chromosomes. Areas shaded in gray indicate homologous regions of ≥99% nucleotide sequence identity. Arrows indicate the orientations of the open reading frames, and the colors are based on their predicted gene functions. Frames with blue represent DRs. "*Delta*" represents a truncated gene. The figure is drawn to scale.

among 55 *S. delphini* isolates recovered from mink, only some isolates were resistant to tetracycline (51%), penicillin (47%), and erythromycin (20%), whereas all the isolates tested susceptible to a vast majority of the antimicrobials assayed, including cefoxitin (Nikolaisen et al., 2017). In 2019, Magleby et al. also reported the first human case of *S. delphini* infection and found that

TABLE 2 | Primers used for detecting antibiotics resistance genes, the circular forms and the structures not included in the corresponding region of the unconventional circularizable structures.

Primer	Sequence (5′ to 3′)	Product size (bp)	Annealing temperature (°C)	Purpose
Tn-F	CGGTGCCTAATCATTCGTATGC	872	55	Detection of minicircle form of Tn*558*
Tn-R	CGCTTAACCGGTTCTATGACTTCA			
P1	GAAAAACGGTTGGCACGGTA	1824	65	Detection of the formation of translocatable units (TUs) between DR_A and DR_B
P2	CTTCATCTTCCCAAGGCTCTGT			
P3	GGCAGAATCCGTAGGAAGCA	1817	65	Detection of remaining structures after the excision of UCSs on chromosomes
P4	CCCTCGTTCAGAGGACGTAT			
A-F	TCGTCCCATTGCTAGTCGTT	1683	55	Detection of DR_A
A-R	AAAACTTCATCTTCCCAAGGCT			
B-F	TGCCTGGAATCGAAAAACGG	1680	55	Detection of DR_B
B-R	CCCTCGTTCAGAGGACGTATT			
cfr-F	TGAAGTATAAAGCAGGTTGGGAGTCA	746	58	Detection of *cfr*
cfr-R	ACCATATAATTGACCACAAGCAGC			

Primers Tn-F/Tn-R were used for detecting the formation of minicircle of Tn558 derivative; primers P1/P2 were used for detecting the formation of translocatable units (TUs) between DR_A and DR_B; primers P3/P4 were used for detecting the remaining structures after the excision of UCSs on chromosomes; primers A-F/A-R, B-F/B-R were used for detecting DR_A and DR_B; primers cfr-F/cfr-R were used for detecting resistance gene cfr.

the isolate exhibited low MIC values for all the antimicrobials assayed, including oxacillin (Magleby et al., 2019). Remarkably, the multiresistance gene *cfr* was shown to encode Cfr, an RNA methyltransferase that affects the binding of at least five chemically unrelated antimicrobial classes, namely, phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics, ultimately leading to a multidrug resistance phenotype (Long et al., 2006). Thus, the emergence and the global spread of the multiresistance gene *cfr* reduce the efficacy of a number of antibiotics in the control of Gram-positive bacteria. In this study, we identified the *cfr* gene in two food-related *S. delphini* strains. To the best of our knowledge, this study is the first report of the *cfr* gene existing in *S. delphini*. Furthermore, the *cfr* gene was located in an MRR with a number of antibiotic genes (*fexA*, *aacA-aphD*, *aadD*, and *ble*). The coexistence of *cfr* and other ARGs limits the choice of antibiotic therapy and may lead to the co-selection of these genes even without direct selection pressure, thereby increasing the retention and dissemination of these ARGs in *Staphylococcus*.

In this study, MRRs, including *cfr* and other ARGs, were confirmed as novel derivatives of the Tn*558* transposon. Tn*558* is a 6.6-kb bacterial transposon. It was first identified on the plasmid pSCFS2 harboring ARG *fexA* from *S. lentus*, and then numerous derivatives harboring numerous ARGs were found (Kehrenberg and Schwarz, 2005; Kehrenberg et al., 2007; Li et al., 2018). With a few exceptions, *cfr* is often harbored in the Tn558 transposon as coexisting with other ARGs, such as *fexA*, *mecA*, *erm*(A/B/C), *tet*(K/L/M), and *drf*(K/G) in the plasmids pSCFS3, pSCFS6, and pSCFS7 in previous studies (Witte and Cuny, 2011), but in this study, the derivative of the Tn*558* transposon harbored *cfr*, *fexA*, *aacA-aphD*, *aadD*, and *ble* on the chromosomes. In addition, the additional DRs within the Tn*558* derivative further confirm the particularity of this transposon. As previously reported for Tn*558* derivatives, there are no inverted repeats at the ends and no duplication of the target sequence at the integration site of the Tn*558* derivative. The typical 6-bp core sequences 5′-GATGTA-3′ at the left-end junction and 5′-GATCCA-3′ at the right-end junction were replaced by 5′-CATCCT-3′ and 5′-TAAGCT-3′ in the novel derivative. The disappearance of target duplication and the alteration of the typical core sequences may have occurred during the transposition process (Diaz-Aroca et al., 1987; Murphy, 1990). Moreover, the reading frame, including the insertion site of the Tn*558* derivative, is similar to the protein containing the Tn*558* site, and the excision of TUs in this Tn*558* derivative could lead to the formation of Tn*558*, indicating that the DR_A and DR_B in this study may be involved in the evolution of Tn*558* and that this derivative may be the ancestor of Tn*558* (Kehrenberg and Schwarz, 2005). Although multiple conjugation assays failed, the presence of a circular Tn*558* structure is indicative of the functional activity, suggesting that this novel Tn*558* derivative is a transposable element and may mediate the transfer of the *cfr* gene in the process of transposition (Kehrenberg and Schwarz, 2005).

Generally, the *cfr* gene often coexists with other ARGs on transposons or plasmids and is often in close proximity to insertion sequences (ISs), such as IS*21-558*, IS*256*, or IS*Enfa4*, which play a crucial role in the mobility of *cfr* (Witte and Cuny, 2011; Wendlandt et al., 2015). These mobile structures have been detected among several Gram-positive bacteria, such as staphylococci, *Enterococcus faecalis*, *Macrococcus caseolyticus*, *Jeotgalicoccus pinnipedialis*, *Bacillus* spp., and *Streptococcus suis*, as well as in Gram-negative bacteria, such as *E. coli* and *Proteus vulgaris* (Shen et al., 2013). However, mobile structures can form UCSs (Palmieri et al., 2013). UCSs lack recombinase genes

and can be excised in circular form due to the extensive DRs flanking the DNA segment undergoing excision (Locke et al., 2012; Palmieri et al., 2012, 2013). Thus, they are very important for the horizontal transmission of ARGs. In this study, the ARGs *aacA-aphD*, *aadD*, *ble*, and *cfr*, bracketed by DRs, formed a novel genetically mobile structure. The particular genetic structures identified by the analysis were referred to as UCSs. Two-step PCR results indicated that this structure can be looped out and excised from the chromosome, leading to the formation of Tn*558* (**Figure 3**), which suggests that the DR is active and involved in the mobility of the Tn*558*-carried *cfr* gene in this study. Further BLAST analysis revealed that the left Δ*fexA*-UCS exhibited 99.88% nucleotide identity to the corresponding region of the plasmids pWo28-1 (KX982171.1) and pWo28-3 (KY601170.1) from *S. sciuri* and plasmid pJP2 (KC989517.1) from *S. rostri* lacking DR_B (**Figure 3**). Therefore, the DR_A and DR_B in this study, similar to ISs, might facilitate the dissemination and accumulation of ARGs in Tn*558* (Palmieri et al., 2013; Harmer et al., 2014). Of course, the functions of these two unknown DRs still need to be further studied and explored in the future.

Unconventional circularizable structures are widely distributed in Gram-negative and Gram-positive bacteria and play an important role in the dissemination of ARGs (Palmieri et al., 2013; Chanchaithong et al., 2019). The DRs in UCSs are usually long and are more than 100 times longer than the *att* sites functioning in traditional MGEs (Frost et al., 2005). The DRs may contain genes, such as *erm(B)*, *mef*, (macrolide efflux), and *ofr138* in this study, but they are not involved in transposition (Locke et al., 2012; Hao et al., 2019). The exact mechanism of mobilization has not been determined, although hypotheses have been proposed (Azpiroz et al., 2011). This transfer mechanism may be similar to that of IS*26 via* site-specific recombination, including a multistep process that requires the formation of a TU, precise excision of the TU, and integration targeting the preexisting DR (Harmer et al., 2014; Harmer and Hall, 2015). The endogenous instability of UCSs endows the encompassed niche adaptation determinants with the ability to be transferred. Moreover, they are often carried by MGEs, which prompts the updating of MGEs (such as the derivative of Tn*558*) and further accelerates the spread of UCSs. Furthermore, the presence of DRs on this novel *cfr*-carrying Tn*558* derivative may accelerate the spread and persistence of ARGs among staphylococci and exacerbate the threat of superbugs, such as methicillin-resistant *S. aureus*. The proliferation of the transferable ARG *cfr* kidnapped by transposons or other MGEs has impaired the efficiency of oxazolidiones in clinical settings and threatens public health (Li et al., 2018).

CONCLUSION

To the best of our knowledge, this study is the first report of *S. delphini* harboring a novel *cfr*-carrying Tn*558* derivative. The constant occurrence of the *cfr* gene in new staphylococcal host species underlines its strong transmissibility and wide distribution. This finding raises further concerns regarding the potential threat to food safety and public health safety. The occurrence and the dissemination of similar *cfr*-carrying transposons from diverse *Staphylococcus* species need further surveillance.

DATA AVAILABILITY STATEMENT

The complete genomic sequences of 245-1 and 2794-1 have been deposited in GenBank: 245-1 (GenBank ID: CP063368) and 2794-1 (GenBank ID: CP063367).

AUTHOR CONTRIBUTIONS

QW, JZ, SW, and TL conceived and designed the experiments. FZ, JH, and JD performed the experiments. FZ, SW, and RY analyzed the data. YD, LX, MC, and JW contributed reagents, materials, and analysis tools. FZ, SW, and JW contributed to the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.598990/full#supplementary-material

Supplementary Figure 1 | Sample collection locations for this study in China.

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Identification of *Listeria monocytogenes* Contamination in a Ready-to-Eat Meat Processing Plant in China

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Listeria monocytogenes is the etiologic agent of listeriosis, which remains a significant public health concern in many countries due to its high case-fatality rate. The constant risk of *L. monocytogenes* transmission to consumers remains a central challenge in the food production industry. At present, there is very little known about *L. monocytogenes* contamination in ready-to-eat (RTE) processing plants in China. In this study, *L. monocytogenes* in an RTE meat processing plant in Shanghai municipality was characterized using pulsed-field gel electrophoresis (PFGE) and whole genome sequencing (WGS). Furthermore, the biofilm formation ability of the pathogen was also tested. Results revealed that *L. monocytogenes* isolates were present in 12 samples out of the 48 samples investigated. Most of them (66.7%, 8/12) were identified from the processing facilities irrespective of observed hygiene levels of aerobic plate count (APC) and coliforms. Coliforms were present in only one processing area. ST5 (1/2b) isolates were predominant (83.3%, 10/12) and were identified in two dominant pulsotypes (PTs) (three in PT3 and seven in PT4, respectively). Results of the core-genome multi-locus sequence typing (cgMLST) showed that ST5 in three PTs (PT1, PT3, and PT4) had 0–8 alleles, which confirmed that clonal transmission occurred in the RTE meat processing facilities. In addition, the biofilm formation test confirmed that the isolates from the processing facilities could form biofilms, which helped them colonize and facilitate persistence in the environment. These results indicated that common sanitation procedures regularly applied in the processing environment were efficient but not sufficient to remove *L. monocytogenes* isolates, especially biofilm of *L. monocytogenes*. Furthermore, the ST5 isolates in this study exhibited 12 alleles with one ST5 clinical isolate, which contributes to the understanding of the potential pathogenic risk that *L. monocytogenes* in RTE meat processing equipment posed to consumers. Therefore, strong hygienic measures, especially sanitation procedures for biofilms eradication, should be implemented to ensure the safety of raw materials. Meanwhile, continuous surveillance might be vital for the prevention and control of listeriosis caused by *L. monocytogenes*.

Keywords: *Listeria monocytogenes*, RTE meat processing plant, PFGE, WGS, cgMLST

INTRODUCTION

Listeria monocytogenes is an important foodborne pathogen, which can cause severe human listeriosis, particularly in older adults, newborns, pregnant women, and immune-compromised individuals (Lomonaco et al., 2009). Listeriosis remains a significant public health concern due to the high case-fatality rate (Thomas et al., 2015). Various types of meat, especially ready-to-eat (RTE) meat products, are often vehicles of listeriosis outbreaks (Currie et al., 2015; Jensen et al., 2016; Thomas et al., 2020). A risk assessment report from the United States in 2003 attributed 90% of listeriosis cases to the consumption of contaminated RTE deli meats (Richard Whiting, 2003). In the United States, *L. monocytogenes* must not present in RTE foods at any point (USDA FSIS, 2003). A similar requirement in China is that *L. monocytogenes* is not detectable in 25 g or 25 ml RTE food according to GB29921 (GB29921-2013, 2013).

L. monocytogenes survives well in the environment and can even colonize food production facilities for extended periods (Leong et al., 2017). *L. monocytogenes* is known to colonize niche areas such as drains and hard-to-clean surfaces, which allows the bacteria to survive or even proliferate and thus make it challenging to completely eradicate it (Ferreira et al., 2014). An epidemiological investigation of listeriosis outbreaks revealed significant lack of hygiene in processing facilities (Angelo et al., 2017). Several reported outbreaks caused by *L. monocytogenes* have been linked to contaminated food-contact surfaces, packing lines, and processing environments (McCollum et al., 2013; Angelo et al., 2017). A high prevalence of *L. monocytogenes* in food processing environments is often reported. However, a smaller number of studies have evaluated the incidence and identified the potential of *L. monocytogenes* contamination in RTE meat processing plants (Nastasijevic et al., 2017). The risk of *L. monocytogenes* transmission to consumers remains a central challenge for the food industry (Almeida et al., 2013; Malley et al., 2015).

Molecular typing of *L. monocytogenes* isolates can help to establish links between isolates from different sources and assist in tracing the original source of contamination (Chen et al., 2013). It has been reported that certain serotypes and clonal complexes (CCs) are more commonly encountered in clinical cases (Chenal-Francisque et al., 2011; Maury et al., 2016). In China, ST87 and ST8 were the most prevalent types of isolates from patients (Li et al., 2019). However, ST9 was the most common type of isolates from foods (Wang et al., 2012).

Pulsed-field gel electrophoresis (PFGE), as the "gold standard" typing method, has been used to characterize clusters of *L. monocytogenes* isolates through the National Molecular Tracing Network for Foodborne Disease Surveillance (TraNet) in China (Li et al., 2019). Whole genome sequencing (WGS) is a powerful tool for obtaining genomic data, which can help to determine sequence types (STs), serogroups, virulence, and resistance gene profiles (Li et al., 2017). More importantly, several molecular typing methods have been developed using WGS data such as core-genome multiple locus sequence typing (cgMLST) and single nucleotide polymorphism (SNP). Recently, WGS has been used to determine the contamination and/or colonization routes of pathogens within food processing environments (Food and Agriculture Organization of the United Nations, 2016; Wang et al., 2016). The aim of this study was (1) to identify the transmission routes of *L. monocytogenes* using PFGE and WGS via tracking *L. monocytogenes* isolates in an RTE meat processing plant in Shanghai, as well as (2) to provide a basis for measures to prevent and control the transmission of *L. monocytogenes* in RTE meat processing plants.

MATERIALS AND METHODS

Processing of RTE Meat Products

An RTE cooked meat product processing plant in Shanghai was used to investigate *L. monocytogenes* contamination in RTE-processing environments and products as well as to track its transmission route. The plant is the one of the most important companies producing RTE meat products in Shanghai, and the RTE meat products are common food in Shanghai. The RTE meat products were processed as follows. Firstly, frozen meats were bought from trade companies as raw materials. Secondly, water thawing took place, and meats were pickled in a pickling liquid with many accessory materials including oil, salt, sauce, vinegar, herbs, and spices. Thirdly, the pickled meat products were boiled to produce intermediate products. These intermediate products were eventually processed by activities such as weighing and cutting into shapes to create end products. Finally, end products were packaged and transported to retail stores to be eventually purchased and used by consumers.

Sampling

As shown in **Table 1**, a total of 48 samples were collected during one visit in July 2019, including 21 processing environmental samples, 10 processing facility samples, 3 raw materials, 3 accessory materials, 8 intermediate products, and 3 end products. After the cleaning and sanitation procedures were complete, the processed environmental samples and facility samples were collected from associated surfaces using pre-moistened swabs (**Table 1**). Air samples were collected using Anderson six-stage sampler. The raw materials were frozen meat products bought from trade companies. Intermediate products during the processing stage included pickled products and boiled products. The end products were RTE meat products, which were destined for retail and consumption. Samples of these products were delivered to the laboratory within 2 h, in a cold chain. Here, the levels of aerobic plate count (APC) and coliforms were determined, as well as the presence of *L. monocytogenes*.

Microbiological Analysis

Enumeration of APC and coliforms were determined according to the food safety national standards GB4789.2 (2016) and GB4789.3 (2016), respectively. *L. monocytogenes* was determined according to the food safety national standard GB4789.30 (2016). Regarding the standard, environmental swabs and raw and accessory materials were placed into *L. monocytogenes* Broth 1 (LB1) for pre-enrichment at 30°C for 24 h. Afterward, 100 μl LB1 was placed into *L. monocytogenes* Broth 2 (LB2) at 30°C for

TABLE 1 | Sampling locations in an RTE processing plant and detection of *L. monocytogenes* isolates.

Sampling	Samples source/No. of samples	Samples source/No. of *L. monocytogenes*
Processing environments	Water[b]/2; power switches[b]/2; doorknobs[b]/1; floors[b]/3; wall[b]/1; worker's hand and shoes[b]/3; mops[b]/3; air samples from different rooms[d,e]/6	–
Processing facilities	Conveyor apparatus[c]/2; cutting boards[d]/2; knives[d]/1; weighing tools[d]/2; inside surface of facilities[d]/1; outside surface of facilities[d]/1; packing bag[d]/1	Cutting boards/2; conveyor apparatus/1; knives/1; weighing tools/2; inside surface of facilities/1; outside surface of facilities/1
Raw materials	Thawing meat products[a]/3	–
Accessory materials	Pickling liquid[a]/3	Pickling liquid/3
Intermediate products	boiled products[b]/5; cooled products[c]/3	–
End products	RTE meat products[d]/3	RTE meat product/1

Production unit:[a]thawing room.
[b]Thermal processing room.
[c]Cooling room.
[d]Packaging room.
[e]Sterilization room.

24 h. One inoculation loop of LB2 was streaked on Polymyxin Acriflavine Licl Ceftazidime Esculin Mannitol Agar Plate (PALCAM). The isolates were identified as *L. monocytogenes* by standard biochemical tests (catalase; fermentation of dextrose, xylose, rhamnose, and mannitol; β-hemolysis; motility; and gram-staining). The positive control strain used in this study was *L. monocytogenes* ATCC 19114.

PFGE

PFGE for *L. monocytogenes* was performed with the PulseNet International protocol (Centers for Disease Control and Prevention, 2013). Based on the protocol, *L. monocytogenes* isolates were embedded into agarose plugs. Afterward, slices of the agarose plugs were digested using *Asc*I (Takara, Dalian, China) for 3 h at 37°C. XbaI-digested *Salmonella* Braenderup H9812 DNA was used as a molecular size marker, and electrophoresis was conducted using the CHEF-DRII apparatus (Bio-Rad Laboratories, Hercules, CA, United States). Images were captured using the Gel Doc 2000 system (Bio-Rad) and were converted to TIFF files, which were analyzed by the BioNumerics software (version 7.7 Applied Maths, Kortrijk, Belgium). Finally, clustering was performed using the unweighted pair group method with arithmetic mean (UPGMA).

WGS

Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN, Germany) according to the manufacturer's protocol except that the cells were pre-lysed with lysozyme for 30 min at 37°C and the proteinase K treatment was extended to 30 min. A Qubit Fluorometer (Invitrogen, United States) and a NanoDrop Spectrophotometer (Thermo Fisher Scientific, United States) were used to determine the concentration, quality, and integrity of the DNA. Sequencing libraries were generated using the TruSeq DNA Sample Preparation Kit (Illumina, United States). Afterward, genome sequencing was performed using the Illumina Hiseq platform (Illumina). Finally, the reads were trimmed and assembled using the CLC Genomics Workbench v7.0 (CLC Bio, Aarhus, Denmark), and the assembled contigs were exported as FASTA files for further analysis.

Ten ST5 *L. monocytogenes* isolates from foods and three isolates from patients were analyzed using WGS to be compared to 10 isolates in this study.

Serotypes, MLST, and Pathogenic Island Determination

Serotypes of *L. monocytogenes* isolates were identified using a commercially available *L. monocytogenes* antiserum test kit (Denka Seiken, Tokyo, Japan).

Multi-locus sequence typing (MLST) was defined by the Pasteur scheme (Burall et al., 2011) and based on the sequence analysis of the seven housekeeping genes, which were extracted from WGS data using the BioNumerics software.

The virulence associated genes extracted from WGS data using BioNumerics software were put into the Virulence Factor Database (VFDB) (MOH Key Laboratory of Systems Biology of Pathogen, Institute of Pathogen Biology, Beijing, China)[1] in order to identify LIPI-1, LIPI-2, LIPI-3, and LIPI-4.

cgMLST Characterization

cgMLST typing was conducted based on the profile of 1,748 coding loci in the BigsDB Pasteur cgMLST[2]. Cluster analysis was conducted by applying a complete linkage using the BioNumerics software.

Biofilm Formation

The biofilm formation test was conducted according to Pang and Yuk (2018) with minor modifications. Stainless steel (304, Tull Metals Company, Atlanta, GA) coupons (2 cm × 1 cm × 0.2 cm) were soaked in an acetone solution for 3 h. After being wiped clean, these coupons were soaked in 70% (v/v) ethanol overnight and then rinsed with distilled water. After being air-dried and autoclaved at 121°C for 15 min, the coupons were ready for use.

Biofilm was developed on sterile coupons in tryptic soy broth with yeast extract (TSBYE). Firstly, the *L. monocytogenes* were inoculated into TSBYE and cultured overnight at 26°C. Secondly, the inoculum was washed two times with 0.9% sodium chloride and then added to 5 ml TSBYE at the final concentration of 10^5 CFU/ml for use on the coupons (one coupon in one tube). Afterward, the inoculated TSBYE samples were incubated at 26°C. After incubation for 24 and 48 h, the coupons were removed and placed into 5 ml 0.9% sodium chloride and vortexed

[1]http://www.mgc.ac.cn
[2]http://bigsdb.pasteur.fr/listeria/listeria.html

for 10 s. Next, the liquid was discarded, and the coupons were placed into a centrifuge (12,000 rpm) with 10 ml 0.9% sodium chloride and glass beads. Finally, after vortexing for 2 min, the liquid was used to enumerate the biofilm cells.

Statistical Analysis

Each set of experiments was repeated three times, and its mean values (APC, coliforms) and standard deviation were calculated using the SPSS software (17.0, IBM, United States).

RESULTS

APC and coliforms testing in various samples revealed the hygiene levels within the RTE meat processing plant. APC levels were the highest in raw and accessory materials, while coliforms levels were the highest in one processing facility (**Table 2**). The level of APC in air samples from processing rooms was 33 CFU/plate.

The *L. monocytogenes* isolate occurrence rate was 25% (12/48) (**Table 1**). Briefly, eight (8/12, 66.7%) *L. monocytogenes* isolates were detected in the processing equipment, which included nearly all production area that had direct contact with products, e.g., the cutting board, conveyor apparatus, knives, inside and outside surfaces of the equipment, and weighing tools (**Table 1**). The presence of *L. monocytogenes* was confirmed in all RTE processing facilities irrespective of the observed hygiene levels (**Table 2**).

The PFGE analysis of the comprised *Asc*I divided the 12 isolates into 4 pulsotypes (PT1–4) (**Figure 1**). PT3 and PT4 values were similarly high (97.9%). They accounted for 83.3% (10/12) of the isolates. Two PTs (PT1, PT2) were presented by only one single isolate. Seven isolates in PT3 and PT4 were processing facility samples, two were from accessory material, and one was from an end product.

The molecular typing using WGS data showed that 11 of 12 *L. monocytogenes* isolates belonged to ST5 (1/2b) with LIPI-1 (*Listeria* pathogenicity island-1). These ST5 isolates were compared with 10 other ST5 (1/2b) isolates from food products and patients using cgMLST. Nine clusters (CL1-CL9) were obtained (**Figure 2**). The *L. monocytogenes* isolates of three PTs (PT1, PT3 and PT4) and one clinical isolate belonged to CL8 and had 0–12 alleles based on cgMLST. However, >70 alleles were found between the isolates in CL8 and other CLs.

After incubation at 26°C for 24 h, the minimum of attached cells of LM19047 isolates was 5.39 Log CFU/cm^2, and the maximum of attached cells of 60 LM19060 isolates was 6.50 Log CFU/cm^2, which were statistically different ($p < 0.05$) (**Figure 3**). However, with increasing biofilm age, the attached cells of LM19047 isolate increased up to 6.21 Log CFU/cm^2, and that of LM19060 decreased to 5.69 Log CFU/cm^2. The ability of biofilm forming of other isolates at different time was different (**Figure 3**).

TABLE 2 | Hygiene levels in different production units within the meat establishment.

Samples source	Hygiene level indicators	
	APC	Coliforms
Raw and accessory materials	6.15×10^5 CFU/g	<10 CFU/g
End product	4.6×10^5 CFU/g	3.5×10^2 CFU/g
Processing facility	–	2.4×10^3 CFU/cm^2
Air samples in processing rooms	33 CFU/plate	–

DISCUSSION

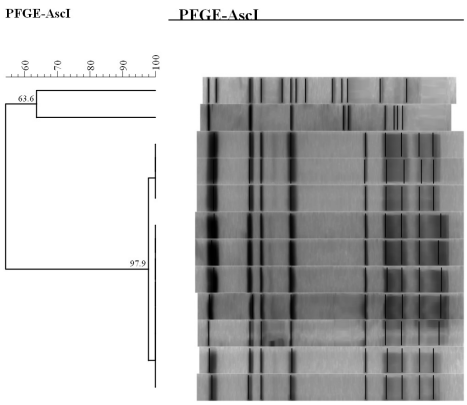
Hygiene indicators (APC and coliforms) are used to reflect specific food establishment practices and temporal influences (Weatley et al., 2014). APC was confirmed in only one processing facility, while only one end product was confirmed with APC and coliform (**Table 2**). Furthermore, the mean level of APC in air samples in RTE processing rooms was 33 CFU/plate, which can be permitted in the food processing environment. These results indicated that good hygiene practices were implemented in this plant.

Contrarily, 8 of the 12 *L. monocytogenes* isolates were confirmed in 8 processing facilities, which presented the predominant contamination scenarios. These findings were similar to a previous study that was conducted on *L. monocytogenes* colonized under these scenarios in a meat establishment (Nastasijevic et al., 2017). The confirmation of *L. monocytogenes* was irrespective of the observed hygiene levels (for example, in contrast with the confirmation of *L. monocytogenes* in processing facilities, only one processing facility was confirmed with APC). Therefore, it was still difficult to remove *L. monocytogenes* isolates in this plant although good hygienic practices were implemented.

L. monocytogenes isolates were confirmed in accessory materials, intermediate products, and an end product, but not in raw materials, which suggested that *L. monocytogenes* isolates might be introduced to these products from processing facilities by cross-contamination. A similar contamination model reported that when *L. monocytogenes* entered into food processing plants, recontamination and persistence frequently occurred (Chambel et al., 2007). *L. monocytogenes* isolates were able to survive in niche areas of the facilities and could adapt to stress factors such as low temperature and low pH (Melo et al., 2015). Furthermore, *L. monocytogenes* isolates could persist in processing equipment for a long time (Unnerstad et al., 1996). As a result, contamination could exist for an extended period.

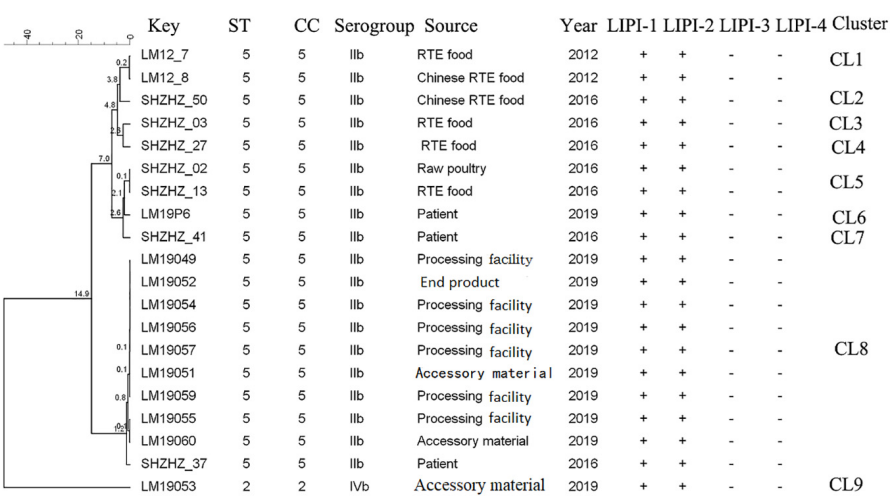
The epidemiological data showed that 12 *L. monocytogenes* isolates were detected in the same RTE meat processing plant at the same time, 10 of which shared an indistinguishable PT (PT3 and PT4, respectively) (**Figure 1**). Furthermore, the PFGE pattern of *L. monocytogenes* isolates in PT3 was highly similar to those in PT4, which might suggest the same ancestor. These results indicated clone transmission of *L. monocytogenes* isolates occurred in processing facilities in this plant.

Though PFGE is a useful tool for the characterization of the subtypes of *L. monocytogenes* isolates, it lacks discriminatory power to distinguish among closely related bacterial strains, which is essential for source tracking (Lomonaco and Nucera, 2014). Thus, WGS was used for obtaining better



Key	Source	PT
LM19055	Processing facility	PT1
LM19053	Accessory material	PT2
LM19058	Processing facility	PT3
LM19060	Accessory material	PT3
LM19059	Processing facility	PT3
LM19047	Processing facility	PT4
LM19049	Processing facility	PT4
LM19051	Accessory material	PT4
LM19052	End product	PT4
LM19054	Processing facility	PT4
LM19056	Processing facility	PT4
LM19057	Processing facility	PT4

FIGURE 1 | Relationships of the *L. monocytogenes* isolates based on PFGE. The 12 *L. monocytogenes* isolates were analyzed by PFGE using *Asc*I.



Key	ST	CC	Serogroup	Source	Year	LIPI-1	LIPI-2	LIPI-3	LIPI-4	Cluster
LM12_7	5	5	IIb	RTE food	2012	+	+	-	-	CL1
LM12_8	5	5	IIb	Chinese RTE food	2012	+	+	-	-	
SHZHZ_50	5	5	IIb	Chinese RTE food	2016	+	+	-	-	CL2
SHZHZ_03	5	5	IIb	RTE food	2016	+	+	-	-	CL3
SHZHZ_27	5	5	IIb	RTE food	2016	+	+	-	-	CL4
SHZHZ_02	5	5	IIb	Raw poultry	2016	+	+	-	-	
SHZHZ_13	5	5	IIb	RTE food	2016	+	+	-	-	CL5
LM19P6	5	5	IIb	Patient	2019	+	+	-	-	CL6
SHZHZ_41	5	5	IIb	Patient	2016	+	+	-	-	CL7
LM19049	5	5	IIb	Processing facility	2019	+	+	-	-	
LM19052	5	5	IIb	End product	2019	+	+	-	-	
LM19054	5	5	IIb	Processing facility	2019	+	+	-	-	
LM19056	5	5	IIb	Processing facility	2019	+	+	-	-	
LM19057	5	5	IIb	Processing facility	2019	+	+	-	-	CL8
LM19051	5	5	IIb	Accessory material	2019	+	+	-	-	
LM19059	5	5	IIb	Processing facility	2019	+	+	-	-	
LM19055	5	5	IIb	Processing facility	2019	+	+	-	-	
LM19060	5	5	IIb	Accessory material	2019	+	+	-	-	
SHZHZ_37	5	5	IIb	Patient	2016	+	+	-	-	
LM19053	2	2	IVb	Accessory material	2019	+	+	-	-	CL9

FIGURE 2 | Minimum spanning tree of cgMLST data for 20 *L. monocytogenes* isolates. The multiplication by 10 in the tree represents the number of different alleles among isolates. The corresponding data, including the name of the isolate (key), MLST type (ST), MLST clonal complex (CC), source, year, *Listeria* pathogenicity island (LIPI-1, LIPI-2, LIPI-3, and LIPI-4), and cluster are shown alongside the dendrogram to the right.

information about the genetic similarity between isolates. The indistinguishable *L. monocytogenes* isolates with the same PFGE pattern could be differentiated by cgMLST (**Figure 2**), which has a higher discriminatory power. cgMLST analysis of *L. monocytogenes* isolates in three PTs (PT1, PT2, and PT3) showed 0–8 allelic differences (**Figure 2**), these isolates could be identified as the same clone (Ruppitsch et al., 2015). Furthermore, seven of them were from processing facility samples, two from accessory materials, and one from an end product, which confirmed the clone transmission of *L. monocytogenes* isolates in the processing environment. Furthermore, in-house evolution occurred in *L. monocytogenes* isolates with one or eight alleles, which suggested that these *L. monocytogenes* isolates might have existed for a long period and also might be persistent in processing environments.

WGS data showed that most *L. monocytogenes* isolates (11/12) were ST5, CC5, 1/2b (**Table 2**). Our previous study indicated that the predominant *L. monocytogenes* isolates from both food and clinical isolates were ST5 in Shanghai, China (data unpublished). Similarly, ST5 was the most predominant ST in RTE meat product in Nanjing, China (Wang et al., 2015). Wang et al. reported that the ST5 had been identified as an important ST in China (Wang et al., 2012). There is no obvious difference between the distribution of the frequency of CC5 in foods and patients in France (Maury et al., 2016). The ST5 strains have been globally disseminated in geographically distant areas, e.g., Austria, Canada, Australia, Switzerland, and Finland (Schmid et al., 2014; Buchanan et al., 2017; Meier et al., 2017). Several outbreaks caused by *L. monocytogenes* have been linked to ST5 isolates (Buchanan et al., 2017). It is worth noting that 1 clinical isolate has 12 alleles with *L. monocytogenes* isolates obtained in this study belonging to CL8, which were from the RTE meat processing plant. Although there is a lack of epidemiological data confirming the relationship between them, the results

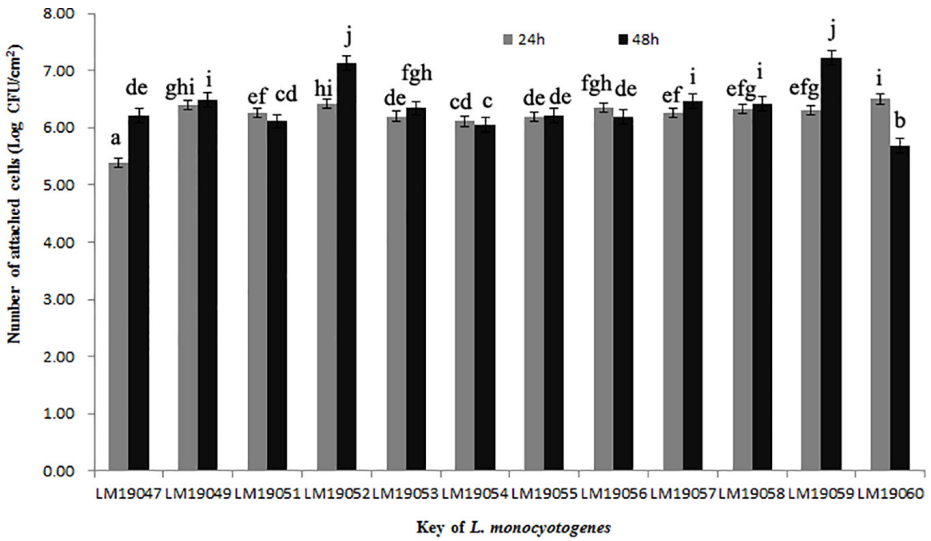


FIGURE 3 | Population (Log CFU/cm^2) of 12 *L. monocytogenes* isolates biofilms at 26°C on 24 and 48 h. Mean values at the different incubation time and different isolates with different lowercase letters are statistically different ($p < 0.05$).

suggested the potential risk of pathogenicity that these isolates pose to the consumers. Further studies are needed to uncover the pathogenicity of ST5.

ST5 isolates have been previously reported to be dominant in heavily contaminated food processing environments even after efforts on intensifying hygienic measures (Muhterem-Uyar et al., 2018). A further study indicated that ST5 plasmids harbored an efflux pump system (*bcrABC* cassette) and heavy metal resistance genes, which possibly provide a higher tolerance to disinfectants (Muhterem-Uyar et al., 2018). These *L. monocytogenes* isolates might exist in biofilm formation. *L. monocytogenes* biofilms could be formed on many different surfaces during food processing operations and provide a protective environment for bacterial survival and thereby increase the risk of subsequent contamination (Colagiorgi et al., 2017). Once established, *L. monocytogenes* biofilms act as permanent sources of contamination and dispersal in the environment and can lead to cross-contamination (Markkula et al., 2005). In this study, 12 *L. monocytogenes* isolates from the plant could form biofilm stainless steel coupons, which presented typical food-contact surfaces in food processing plants (**Figure 3**). These findings suggested that *L. monocytogenes* isolates in the processing equipment could not be cleaned due to biofilms formation, and *L. monocytogenes* biofilms might be persistent in the plant. Therefore, continuous surveillance and prevention strategies against *L. monocytogenes* should be implemented in this RTE meat processing plant to ensure food safety.

CONCLUSION

In this study, cross-contamination of *L. monocytogenes* in an RTE meat plant has occurred. The food processing facilities were heavily contaminated by ST5 (1/2b) isolates even though good hygienic measures had been implemented in this plant. The molecular typing and epidemiological data confirmed that clone transmission occurred in the plant. Furthermore, the clone had a strong ability to form biofilm in food-contact surfaces, which might be the reason that it could not be eradicated in the processing facilities. Furthermore, the ST5 (1/2b) isolates in this study had potential pathogenicity for having 12 alleles with clinical isolate. Therefore, continuous surveillance and effective measures to eradicate *L. monocytogenes* should be taken to ensure food safety.

DATA AVAILABILITY STATEMENT

We have uploaded completely the WGS data of *L. monocytogenes* in this study in Genbank of NCBI.

AUTHOR CONTRIBUTIONS

HZ, FQ, and BX collected the samples, analyzed the samples, performed the WGS, and analyzed the data. HZ drafted the manuscript. XZ and HL designed the study and revised this manuscript. LS tested the formation of biofilm. YZ and WC performed the PFGE. YY was involved in the collection of isolates. QD revised this manuscript. All authors contributed to the article and approved the submitted version.

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mcr-1 Identified in Fecal *Escherichia coli* and Avian Pathogenic *E. coli* (APEC) From Brazil

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Colisitin-associated resistance in bacteria of food producing animals has gained significant attention with the *mcr* gene being linked with resistance. Recently, newer variants of *mcr* have emerged with more than nine variants currently recognized. Reports of *mcr* associated resistance in *Escherichia coli* of poultry appear to be relatively limited, but its prevalence requires assessment since poultry is one of the most important and cheapest sources of the world's protein and the emergence of resistance could limit our ability to treat disease outbreaks. Here, 107 *E. coli* isolates from production poultry were screened for the presence of *mcr 1–9*. The isolates were collected between April 2015 and June 2016 from broiler chickens and free-range layer hens in Rio de Janeiro, Brazil. All isolates were recovered from the trachea and cloaca of healthy birds and an additional two isolates were recovered from sick birds diagnosed with colibacillosis. All isolates were screened for the presence of *mcr-1* to *9* using PCR and Sanger sequencing for confirmation of positive genes. Additionally, pulse field gel electrophoresis (PFGE) analysis, avian fecal *E. coli* (APEC) virulence associated gene screening, plasmid replicon typing and antimicrobial resistance phenotype and resistance gene screening, were also carried out to further characterize these isolates. The *mcr-1* gene was detected in 62 (57.9%) isolates (61 healthy and 1 APEC) and the *mcr-5* gene was detected in 3 (2.8%) isolates; *mcr-2, mcr-3, mcr-4, mcr-6, mcr-7, mcr-8*, and *mcr-9* were not detected in any isolate. In addition, *mcr 1* and *5* positive isolates were phenotypically resistant to colistin using the agar dilution assay (> 8ug/ml). PFGE analysis found that most of the isolates screened had unique fingerprints suggesting that the emergence of colistin resistance was not the result of clonal dissemination. Plasmid replicon types *IncI2, FIB, and B/O* were found in 38, 36, and 34% of the *mcr* positive isolates and were the most prevalent replicon types detected; *tetA* and *tetB* (32 and 26%, respectively) were the most prevalent antimicrobial resistance genes detected and *iutA*, was the most prevalent APEC virulence associated gene, detected in 50% of the isolates. Approximately 32% of the isolates examined could be classified as APEC-like, based on the presence of 3 or more genes of APEC virulence associated path panel (*iroN, ompT, hlyF, iss, iutA*). This

study has identified a high prevalence of *mcr-1* in poultry isolates in Brazil, suggesting that animal husbandry practices could result in a potential source of resistance to the human food chain in countries where application of colistin in animal health is practiced. Emergence of the *mcr* gene and associated colisitin resistance in production poultry warrants continued monitoring from the animal health and human health perspective.

Keywords: *mcr*, colistin, *Escherichia coli*, antimicrobial resistance, poultry *E. coli*, broiler, layer

INTRODUCTION

In 2019, poultry was the most consumed meat worldwide, representing 38.6% of the world's production (OECD, 2021). The United States is one of the largest producers and consumers of chicken meat, responsible for 19.9% of the world's production and 17.0% of world's consumption; Brazil, is the third largest poultry producer at 13.7%, and the fourth largest consumer at 10% (USDA, 2019).

To meet the demand for chicken, developments in production including genetic improvement of stock, nutrition, poultry health, and handling have contributed to market expansion, resulting in the exponential growth of the poultry sector (FAO, 2013).

Escherichia coli is a Gram negative mesophilic member of the enteric microbiota of mammals and most birds. Pathogenic strains of *E. coli* are divided into groups according to clinical symptoms and mechanisms of pathogenicity, that vary in their incubation periods and duration of the disease (Kaper et al., 2004).The production of virulence factors and the mechanisms by which these factors lead to disease, allow the classification of pathogenic *E. coli* strains into groups or pathotypes that include intestinal strains (InPEC) and extra-intestinal (ExPEC) strains. In birds, extra-intestinal disease associated with the avian pathogenic *E. coli* (APEC) pathotype has been defined (Kaper et al., 2004; Nolan et al., 2020).

APEC is the etiologic agent of colibacillosis, and the disease can present itself two forms: acute, which is characterized by septicemia and high mortality, and subacute, being characterized by hepatitis, pericarditis, airsacculitis, salpingitis, and egg yolk peritonitis in layers (Barbieri et al., 2017; Nolan et al., 2020). It is estimated that 15 to 20% of the isolates from the poultry microbiota can be considered potentially pathogenic because they harbor certain virulence factors capable of causing disease (Knöbl and Ferreira, 2009). In addition to having a high prevalence, colibacillosis causes high rates of mortality and carcass condemnation at slaughter, leading to great losses for the poultry industry in Brazil. Ferreira and colleagues (Ferreira et al., 2012), analyzed data from the Animal Products Inspection Coordination, and identified colibacillosis (19.8%) as the primary cause of bird carcass condemnations in 2010 in South Brazil (Ferreira et al., 2012).

In Brazil as elsewhere, antimicrobial resistance among *E. coli* has gained significant attention especially in light of production losses and the potential exposure of consumers to AMR strains. Poultry farms have been reported as sources of isolates harboring extended spectrum beta lactamase (ESBL) resistance (Mesa et al., 2006; Smet et al., 2008); high rates of resistance to tetracycline (Miles et al., 2006; Barbieri et al., 2013), quinolones (Bezerra et al., 2016), and trimethoprim/sulfonamides (Braga et al., 2016; Bezerra et al., 2016). In order to reduce the potential risks of AMR- associated with animal production, the use of β-lactams, sulfonamides and tetracycline for farm animal use in Brazil were banned as feed additives, and only approved for therapeutic purposes with prescription (MAPA IN-26, July 9, 2009) (MAPA, 2009).

Colistin is a broad-spectrum antimicrobial member of the polymyxin family that act on Gram negative bacteria, including many species of *Enterobacteriaceae*. The two polymyxins used therapeutically include polymyxin B and polymyxin E. Colistin was widely used as a growth promoter in Brazil until 2016 (MAPA, 2017). However, it also has important human impact because of the emergence of *Enterobacteriaceae* producing carbapenemase enzymes that has resulted in reliance on colistin human treatment (CDDEP, 2015).

Since the first report of *mcr-1* (colistin) associated resistance in *E. coli* from animals and humans in China (Liu et al., 2016). Researchers worldwide have assessed historical isolates to identify potential emergence dates for *mcr* and associated colisitin resistance and current reports have identified isolates as far back as 1980 may have harbored the gene (Shen et al., 2016). *mcr* associated resistance has been identified in a range of *Enterobacteriaceae* from humans and animals (Fernandes et al., 2016a; Haenni et al., 2016; Irrgang et al., 2016; Nordmann et al., 2016; Teo et al., 2016; Veldman et al., 2016).

For many years, resistance to colistin was not considered a problem because clinical resistance was chromosomal and restricted to hospitals. In 2015, Liu and colleagues (Liu et al., 2016) found *mcr-1* was localized to an IncI2 a plasmid identified as pHNSHP45, that demonstrated high *in vitro* transmission capacity between *E. coli* and other *Enterobacteriaceae* including *E. coli* ST131, *Klebsiella pneumoniae* ST11, and *Pseudomonas aeruginosa*.

It is believed that, regardless of selection pressure, plasmid containing *mcr-1* will likely be maintained in Enterobacteriaceae populations, facilitating ease of dissemination to the human population. The high prevalence of the *mcr-1* gene in *Escherichia coli* from meat cuts (14.9%) and birds (4.9% to 28%) suggests that the gene is widely disseminated in farm animals where colisitin is used and can be subsequently transmitted to man, because colisitin as an antimicrobial is rarely used in humans (Liu et al., 2016).

Escherichia coli -related virulence factors include adhesins, invasins, toxins, iron uptake systems (siderophores), which are involved in colonization, and survival in the host (Kaper et al., 2004). The use of molecular techniques for detecting these genes

has allowed the characterization of bacterial virulence (Johnson et al., 2008; Barbieri et al., 2015).

Johnson and colleagues (Johnson et al., 2008), studied the prevalence of 46 virulence genes in APEC and avian fecal *E. coli* (AFEC) (fecal commensal avian) strains, and found that the siderophore Salmochelin receptor virulence gene (*iroN*), a gene encoding in the episomal external outer membrane protein (*ompT*), gene encoding hemolysin (*hlyF*), the increased serum survival gene (*iss*), and aerobactin siderophore receptor gene (*iutA*) had a significantly greater prevalence in APEC compared to AFEC strains. Most APEC harbored three or more of these genes, demonstrating their presence can be used to identify potentially pathogenic strains for birds (Johnson et al., 2008). Other virulence factors of APEC include acquisition of iron through siderophores and other means that appear to play an important role in the pathogenicity of strains, especially in septicemia associated organisms allowing APEC to survive in serum where the iron concentration is extremely low (Janssen et al., 2001; Caza et al., 2008) directly influencing their pathogenesis (Gao et al., 2012). The genetic determinants involved in the pathogenicity of the APEC strains are, however, not yet fully understood (Maluta et al., 2016).

The overall goal of this study was to assess *E. coli* isolates recovered from the feces and trachea of healthy broilers in Brazil for the presence of colistin-associated resistance by *mcr* and other resistance determinants and to characterize all isolates for virulence and associated resistance traits. In addition, PFGE was performed to determine any potential genetic relatedness though DNA fingerprint analysis.

MATERIALS AND METHODS

Isolate Collection

The analysis consisted of one hundred and seven *E. coli* isolates collected between April 2015 and March 2016 from two broiler and one layer farms located in the Rio de Janeiro area of Brazil. All isolates were recovered from the trachea and cloaca of healthy birds. An additional two isolates were recovered from sick birds (Blepharitis B46; celoma cavity B157). All isolates were recovered from 30 to 41-day-old broiler chickens and free-range layer hens at 62 weeks-of age as detailed in **Supplementary Table 1**.

A total of 120 swab samples, 60 from cloaca and 60 from trachea, were collected on two broiler chicken farms (1 and 2) and 30 samples from the laying hens at farm 3.

Swab samples were collected from the cloaca or trachea of healthy birds and placed in Stuart media (Absorve® Jiangsu, China) for transportation. At the lab, all swabs were plated on MacConkey (MAC, HiMedia®, Mumbai, India) agar and Eosin Methylene Blue agar (EMB, HiMedia®) with incubation at 37°C for 18–24h. All suspect colonies (1 colony per sample) were confirmed as *E. coli* using MALDI-TOF MS (LT Microflex Bruker, Bruker, Germany). *E. coli* positive strains for MALDI-TOF were confirmed using a polymerase chain reaction (PCR) targeting the 16S DNA as described previously by Lamprecht et al. (Lamprecht et al., 2014). All strains were stored at −80°C in Luria-Bertani (LB) (BD Difco™, Sparks, United States) broth with 20% glycerol until use.

mcr PCR Analysis

All isolates were screened for the presence of the *mcr-1* to 9 gene using protocols recently described elsewhere (**Supplementary Table 2**) (Liu et al., 2016; Xavier et al., 2016; Borowiak et al., 2017; Carattoli et al., 2017; Yin et al., 2017; Kieffer et al., 2019; Yang et al., 2019). DNA was extracted from all strains using the boil prep method and PCR reaction preparation as described previously (Barbieri et al., 2013). All PCR amplifications were carried out under the following conditions 94°C for 10 min followed by 30 cycles of 94°C for 30 sec; 58°C for 30 sec and 72°C for 2 min; with a final extension of 72°C for 10 min.

Polymerase chain reaction products generated were subjected to electrophoresis in 2% (w/v) agarose gels (LE Agarose, Lonza, GA, United States) in 1X TAE buffer and run at 120V for 2 h. A Hi-Lo molecular weight marker (100 bp; Minnesota Molecular, MN) was used as the size standard; we used a laboratory strain (7-49-1) as a positive control for *mcr-1* (Barbieri et al., 2017) and DNAse/RNAse free water was used as the negative control. Gels were stained in 0.25% ethidium bromide (Fisher Scientific, Asheville, NC), and bands corresponding to each gene present were recorded using a UV Imager (Omega Fluor, Aplegen, Pleasanton, CA).

Sixty-two PCR products positive for the gene *mcr-1* and 3 PCR products positive for *mcr-5* were selected for sequencing. The full gene PCR product was treated with ExoSAP-IT® (Affymetrix, Santa Clara, CA) to remove primer and remaining DNTPs following manufacturer's protocols and submitted to Iowa State University's DNA facility for Sanger sequencing of the forward and reverse strands. Sequences generated were imported into Geneious® software and aligned to compare across the isolates positive for the fragment.

Antimicrobial Resistance Analysis

The antimicrobial susceptibility of all *E. coli* isolates was examined using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017), using *Escherichia coli* strain ATCC 25922 as a control. The 8 antimicrobial agents tested included: amoxicillin (AMO; 25 μg), ceftazidine (CAZ; 30 μg), cefoxitin (CFO; 30 μg), cefotaxime (CTX; 30 μg), aztronam (ATM; 30 μg), imipenem (IPM; 10 μg), cefepime (CPM; 30 μg), and a combination of amoxicillin and clavulanic acid (AMC; 20 + 10 μg).

The breakpoints used were obtained from CLSI (CLSI, 2017) for all antimicrobials (**Supplementary Table 3A**).

Colistin Antimicrobial Susceptibility Analysis

To assess the role of colistin resistance in strains positive for the *mcr* gene all strains were subjected to antimicrobial susceptibility analysis to colistin sulfate (Alfa Aesar, Ward Hill, MA) using the agar dilution assay. Overnight cultures of each strain were grown on Tryptone Soya Agar (TSA) plates and colonies selected were adjusted to an OD 0.5 Mc Farland in sterile water using an

nephelometer (Sensititre); then 10 μl of the suspension was added to 11 μl of Mueller Hinton (MH) broth and mixed well using a vortex. 10 μl of this suspension was used to spot inoculate the agar dilution plates (Turlej-Rogacka et al., 2018).

The agar dilution plates tested antimicrobial resistance to colistin in doubling dilutions at the following dilution range 0.5 to 32 μg/ml. Once all plates were inoculated as appropriate, they were allowed to dry and incubated at 37°C for 18h. Plates were observed for growth and minimum inhibitory concentrations (MIC's) were defined as the lowest concentration of antimicrobial to inhibit growth of the test strains.

Antimicrobial Associated Resistance Genes Screening

All isolates were tested for the presence of the antimicrobial associated resistance genes: *silP; intI1; pcoD; sulI; ISEc12; aad; aac3-VI; qacEΔ1; blaTEM; aac3-VI; tetB; tetA; groEL; aph*(3)*IA; dfr17* (Zhao et al., 2001; Brinas et al., 2002; Maynard et al., 2004; Grobner et al., 2009) using multiplex PCR and primers described in previous studies of our lab (**Supplementary Table 2**).

Plasmid Replicon Detection

Plasmids potentially associated with virulence and/or resistance in these test isolates were assessed using the plasmid replicon typing protocols as described by Carattoli et al. and Johnson and Nolan (Carattoli et al., 2005; Johnson and Nolan, 2009); in addition, *IncI2* (Zhao and Zong, 2016) using standard multiplex PCR protocols and primers as described previously (see **Supplementary Table 2**).

Genotyping Avian *E. coli* for *iroN, ompT, hlyF, iss,* and *iutA*

Escherichia coli strains were genotyped by multiplex PCR as previously described (Johnson et al., 2008). Reactions were performed as follows: denaturation for 2 minutes at 94°C; 25 cycles of 30 s at 94°C, 30 s at 63°C and 3 min at 68°C, followed by a final extension step of 10 min at 72°C. PCR products were run on a 2% agarose gel as described above. APEC O1 strain was used as the positive control and sterile water in place of DNA for the negative control.

Phylogenetic Typing

Samples of the DNA stock from each strain were also subjected to phylogenetic typing using the revised protocols described by Clermont et al. (Clermont et al., 2013). Here, a 25 μl PCR reaction volume as described above with the following PCR conditions: denaturation for 4 minutes at 94°C followed by 30 cycles of 5s at 94°C; 30s at 64°C (group E), or 63°C (quadruplex) or 66°C (group C) and 30s at 72°C with a final extension at 72°C for 5 min. Polymerase chain reaction products were run on a 1.5% agarose gel as described above.

Pulsed Field Gel Electrophoresis Analysis

All strains were subjected to molecular subtyping using PFGE. Isolates were analyzed using the method of Ribot et al. and Hussein et al. (Ribot et al., 2006; Hussein et al., 2013). Preparation, lysis, washing of plugs, and *Xba*I restriction were performed according to the PulseNet protocol. *Salmonella Braenderup* H9812 was used as the size standard. Macrorestriction patterns were compared using the BioNumerics Fingerprinting software (Ver 6.6, Applied Math, Austin, TX). The similarity index was calculated using the Dice coefficient, with a band position tolerance of 1% and an optimization of 0.5%.

Statistical Analysis

Data was analyzed using non-parametric tests due to asymmetry in the distribution of genes or other traits used for analysis.

For analysis of the association between the presence of two single genes or antimicrobial resistance traits (**Supplementary Table 4A**) were tested by use of the chi-square test.

For the analysis of virulence and resistance genes harbored by strains examined in the study the number of genes were treated as quantitative variables and the data was analyzed using non-parametric tests also due to asymmetry in the distribution of these genes. Direct comparisons (where possible) between two groups (**Supplementary Tables 4B,C**) were made using the Mann-Whitney *U* test.

All statistical analysis was performed using GraphPad Prism (Version 7.0d) for MAC OS X (GraphPad, La Jolla, CA) or IBM SPSS Statistics (Version 26.0) for MAC OS X (IBM Corp., Armonk, NY). Statistical significance was accepted when $p < 0.05$.

RESULTS

Isolate Distribution

A total of 175 suspect *E. coli* strains were isolated from farm 1, 2, and 3; 80/175 from farm 1 (45.7%), 83/175 (47.4%) from farm 2, and 12/175 (6.5%) from farm 3.

Escherichia coli isolates were confirmed using the MALDI TOF MS technique for 107 isolates, with scores of 2.0 to 2.495. *E. coli* positive strains for MALDI-TOF were confirmed using a PCR targeting the 16S DNA. A total of 107 *E. coli* isolates were used for the study. Based on distribution profile by collection site, it was observed that in farm 1, a total of 26/107 (24.3%) of the isolates came from the cloaca, while 20/107 (18.7%) were recovered from the trachea. On farm 2, 27/107 (25.3%) of the isolates were isolated from the cloaca and 21/107 (19.6%) came from the trachea. On farm 3, 8/107 (7.5%) of the isolates came from the cloaca while 3/107 (2.8%) were recovered from the trachea. Two additional *E. coli* isolates were included in this analysis recovered from sick birds diagnosed with colibacillosis on necropsy (Blepharitis B46 farm 1; celoma cavity B157 farm 3) (**Figure 1** and **Supplementary Table 1**).

Antimicrobial Resistance Analysis

Data from resistance analysis based on the disk diffusion assay are shown in **Figure 1** and **Table 1**. Data are presented based on source of origin (cloaca vs. trachea) and farm (**Figure 2A**).

On farm 1, 100% (26/26) of the *E. coli* strains recovered from the cloaca showed phenotypic resistance to colistin (COL),

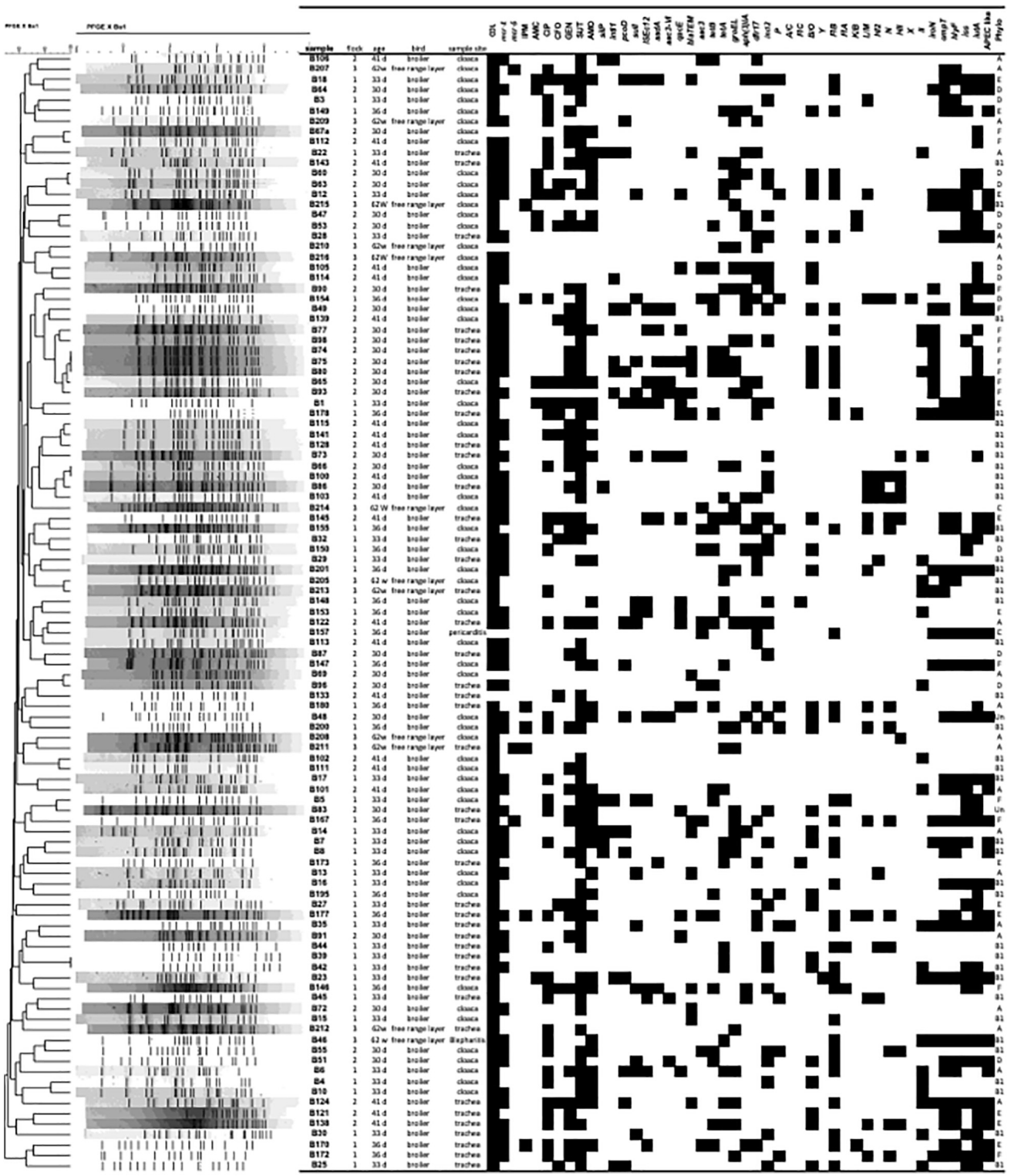

FIGURE 1 | Pulsed-field gel electrophoresis (PFGE) profile of 107 avian *Escherichia coli* isolates. The PFGE dendrogram was constructed by the unweighted-pair group method with arithmetic averages. The scale indicates levels of similarity within this set of isolates based upon *Xba*I enzyme restriction digestion of total bacterial DNA. The column Sample shows isolate designation; the column flock, indicates the farm the isolate came from **(1–3)**; the column Age, days or weeks old of the bird; bird shows type of bird; Sample Site, site of bacterial isolation; the subsequent columns depict the antimicrobial resistance and PCR results for virulence genes (VAGs) tested, with presence indicated in black and absence indicated in white; Phylo, phylogenetic group.

TABLE 1 | Prevalence of genes tested in all samples and in *mcr* positive samples.

		All samples		*mcr* positive	
		n	**%**	***n***	**%**
Antimicrobial Resistance					
Colistin	COL	102	95.33	62	100.00
Imipenem	IPM	7	6.54	3	4.84
Amoxicillin	AMC	10	9.35	4	6.45
Ciprofloxacin	CIP	41	38.32	22	35.48
Cefoxitin	CFO	16	14.95	7	11.29
Gentamicin	GEN	45	42.06	28	45.16
Sulphonamides and trimethoprim	SUT	76	71.03	45	72.58
Amoxicillin and clavulanic acid	AMO	40	37.38	23	37.10
Antimicrobial Associated Resistance Genes					
Colistin resistance	*mcr1*	62	57.94	62	100.00
Silver resistance	*silP*	7	6.54	6	9.68
Integrase	*intI1*	11	10.28	8	12.90
Copper resistance	*pcoD*	15	14.02	10	16.13
Sulfa resistance	*sulI*	17	15.89	9	14.52
Transposase	*ISEc12*	14	13.08	6	9.68
Aminoglycoside resistance	*aadA*	12	11.21	7	11.29
Gentamicin resistance	*aac3-VI*	8	7.48	6	9.68
Quarternary amonium resistance	*qac delta1*	17	15.89	12	19.35
Ampicillin resistance	*blaTEM*	16	14.95	9	14.52
Gentamicin resistance	*aac3-VI*	13	12.15	9	14.52
Tetracycline resistance	*tetB*	24	22.43	16	25.81
Tetracycline resistance	*tetA*	30	28.04	20	32.26
Chaperone	*groEL*	30	28.04	21	33.87
Gentamicin resistance	*aph(3)IA*	22	20.56	12	19.35
Trimethoprim resistance	*dfr17*	29	27.10	16	25.81
APEC Minimal Predictors					
Salmochelin siderophore receptor gene	*iroN*	24	22.43	15	24.19
Episomal outer membrane protease gene	*ompT*	36	33.64	19	30.65
Putative avian hemolysin F	*hlyF*	33	30.84	17	27.42
Episomal increased serum survival gene	*iss*	42	39.25	23	37.10
Aerobactin siderophore receptor gene	*iutA*	53	49.53	31	50.00
3 or more predictors		33	30.84	20	32.26
Plasmid Replicon Genes					
Plasmid replicon typing	*incI2*	34	31.78	23	37.10
Plasmid replicon typing	*T*	0	0.00	0	0.00
Plasmid replicon typing	*P*	11	10.28	4	6.45
Plasmid replicon typing	*A/C*	1	0.93	0	0.00
Plasmid replicon typing	*FIC*	2	1.87	0	0.00
Plasmid replicon typing	*B/O*	30	28.04	21	33.87
Plasmid replicon typing	*Y*	1	0.93	0	0.00
Plasmid replicon typing	*FIB*	39	36.45	22	35.48
Plasmid replicon typing	*FIA*	3	2.80	1	1.61
Plasmid replicon typing	*FIIA*	0	0.00	0	0.00
Plasmid replicon typing	*W*	0	0.00	0	0.00
Plasmid replicon typing	*K/B*	5	4.67	2	3.23
Plasmid replicon typing	*L/M*	16	14.95	10	16.13
Plasmid replicon typing	*HI2*	8	7.48	6	9.68
Plasmid replicon typing	*N*	13	12.15	9	14.52
Plasmid replicon typing	*HII*	8	7.48	7	11.29
Plasmid replicon typing	*X*	1	0.93	0	0.00
Plasmid replicon typing	*II*	24	22.43	14	22.58
Phylogenetic Typing					
Phylotype group	A	21	19.62	12	19.35
Phylotype group	B1	41	38.32	22	35.48
Phylotype group	B2	0	0.00	0	0.00
Phylotype group	C	2	1.87	1	1.61
Phylotype group	D	13	12.15	9	14.52
Phylotype group	E	12	11.21	6	9.68
Phylotype group	F	16	14.95	10	16.13

73% (19/26) to Sulfa-trimethoprim (SUT), 57% (15/26) to ciprofloxacin (CIP), 57% (15/26) to AMO, 30% (8/26) to gentamicin (GEN), 15% (4/26) to CFO, 11% (3/26) to AMC and 7% (2/26) to imipenem (IMP). Of the 21 strains of *E. coli* from trachea 95% (20/21) showed phenotypic resistance to COL, 76% (16/21) to SUT, 38% (8/21) to AMO, 28% (6/21) to CIP, 28% (6/21) to GEN, 19% (4/21) to CFO, 14% (3/21) to IMP and 4% (1/21) to AMC (**Figure 2A**).

On farm 2, 92% (25/27) of *E. coli* strains from cloaca were resistant to COL, 77% (21/27) to SUT, 51% (14/27) to GEN, 37% (10/27) to AMO, 22% (6/27) to CIP, 22% (6/27) to AMC and 18% (5/27) to CFO. Of the 21 strains originating from trachea 95% (20/21) showed resistance to COL, 85% (18/21) to SUT, 80% (17/21) to GEN, 33% (7/21) CIP, 28% (6/21) AMO and 14% (3/21) CFO (**Figure 2A**).

On farm 3, 87% (7/8) of the *E. coli* from the cloaca were resistant to COL, 25% (2/8) to CIP, 12% (2/8) to SUT, while in strains from trachea 100% (4/4) showed phenotypic resistance to COL, 75% (3/4) to CIP, 25% (1/4) to IMP, 25% (1/4) to SUT (**Figure 2A**).

Phenotype analysis of multidrug resistance found 18.7% (Nordmann et al., 2016) of the isolates were resistant to five antimicrobial agents or more; 19.6% (Haenni et al., 2016) were resistant to 4; 27.1% (Caza et al., 2008) were resistant to 3; 17.8% (Shen et al., 2016) were resistant to 2 and 14.0% (MAPA, 2009) were resistant to 1 and only 2.8% (FAO, 2013) isolates were susceptible to all agents tested (**Table 2**, and **Supplementary Table 3**). Of note, however, 3 strains was found to be resistant to 7 different antimicrobials (**Table 2**). The most frequent profile of resistance were COL, present in 15 isolates; COL, SUT present in 11 isolates and COL, SUT, GEN present in 12 isolates. No isolates were resistant to all eight antimicrobials tested (**Table 2**, **Supplementary Table 3**).

Among the strains of *E. coli* that displayed resistance to AMC, AMO, and CFO in the disk diffusion test, 52/107 (48.59%) were assessed for β-lactamase activity. 12/52 (23%) strains that presented phenotypic profiles compatible with those of ESBL producers, representing 11% of the total evaluated strains (12/107). In addition, two strains showed an AmpC production profile (2/52; 3%) and five (5/52; 9%) showed results compatible with the coproduction of both ESBL and AmpC enzymes, representing 2% (2/107) and 4% (5/107), of all strains examined. When the prevalence per farm was assessed, 27%

FIGURE 2 | Gene prevalence separated by organ and farm **(A)** Prevalence of Antimicrobial Resistance; **(B)** Prevalence of Genes Encoding Antimicrobial Resistance; **(C)** Prevalence of Genes Encoding Virulence Factors; **(D)** Prevalence of Plasmid Replicon Genes.

TABLE 2 | Antimicrobial resistance profiles among isolates examined.

Profile	Number of Strains
COL, SUT, GEN, CIP, AMO, CFO, AMC	3
COL, SUT, GEN, CIP, AMO, CFO	3
COL, SUT, GEN, CIP, AMO, IPM	2
COL, SUT, GEN, CIP, AMO	5
COL, SUT, GEN, CIP, CFO	1
COL, SUT, GEN, CIP, IPM	1
COL, SUT, GEN, CIP	4
COL, SUT, GEN, AMO, CFO, AMC	1
COL, SUT, GEN, AMO	1
COL, SUT, GEN, AMO	3
COL, SUT, GEN, CFO	3
COL, SUT, GEN, AMC	1
COL, SUT, GEN	12
COL, SUT, CIP, AMO, CFO, AMC	1
COL, SUT, CIP, AMO, CFO	1
COL, SUT, CIP, AMO, AMC	1
COL, SUT, CIP, AMO	6
COL, SUT, CIP, IPM	1
COL, SUT, CIP	6
COL, SUT, AMO, IPM	1
COL, SUT, AMO	5
COL, SUT, CFO	1
COL, SUT	11
COL, GEN, CIP	2
COL, GEN, AMO, CFO, AMC	1
COL, GEN, AMO, AMC	1
COL, CIP, AMO	1
COL, CIP	3
COL, AMO, AMC	1
COL, AMO	2
COL, IPM	2
COL	15
SUT, GEN, AMO	1
SUT, CFO	1
Susceptible	3

amoxicillin (AMO), ceftazidine (CAZ), cefoxitin (CFO), cefotaxime (CTX), aztronam (ATM), imipenem (IPM), cefepime (CPM), and a combination of amoxicillin and clavulanic acid (AMC).

(13/47) of the *E. coli* strains from farm 1 showed beta-lactamase production, 61% (8/13) ESBL, 15% (2/13) AmpC, and 23% (3/13) co-production of both enzymes, while on farm 2 10% (5/48) showed beta-lactamase production, with 60% (3/5) ESBL only and 40% (2/5) displaying enzyme coproduction. On farm 3, 8% (1/12) displayed an ESBL production profile only.

Using the Mann-Whitney *U* test, to compare antimicrobial resistance prevalence with farm of isolation we found some significant relationships ($p < 0.05$) between certain groups, including antimicrobials such as GEN and SUT in cloaca vs. trachea; IPM, GEN, SUT, and AMO in broiler vs. free range; IPM, CIP, and GEN in farm 1 vs. farm 2; GEN, SUT, and AMO in farm 1 vs. farm 3; IMP, GEN, and SUT in farm 2 vs. farm 3 (**Supplementary Table 4B**). Similarly, using the chi-square test (**Supplementary Table 3A**), that allows comparison between the presence of two antimicrobial resistances analyzed in all strains, significant associations were observed for some specific antimicrobials such as AMO and a number of other antimicrobials including AMC, CIP, CFO, and SUT ($p < 0.05$).

Colistin Antimicrobial Susceptibility Analysis and *mcr* Analysis

Among the 107 *E. coli* strains evaluated, 102 (95.33%) were resistant to colistin using the agar dilution assay (> 8ug/ml). We found that the *mcr-1* gene was detected in 62 (57.94%) isolates (61 healthy and 1 APEC); and the *mcr-5* gene was detected in 3 (2.8%) isolates; *mcr -2, 3, 4, 6, 7, 8,* and *9* were not detected in any isolate. However, 35% (37/102) displayed phenotypic resistance to colisitin without genotype confirmation of the presence of *mcr-1* (**Figure 1** and **Table 1**).

Using the Mann-Whitney *U* test, to compare *mcr* prevalence with farm of isolation we found significant relationships ($p < 0.05$) between certain groups, including *mcr-1* detection on farm 1 vs farm 2 and farm 2 vs. farm 3 (**Supplementary Table 4B**).

The sequence analysis of the 62 isolates harboring *mcr-1* (61 healthy and 1 APEC) found that 54 isolates had the exact same sequence compared with *mcr-1* in GenBank (KU886144.1) and 8 isolates have an amino acid change (H452Y) at position 452 (NG_052663.1) (**Figure 3**). The sequence analysis of the 3 isolates with *mcr- 5* have the identical sequence to *mcr-5* in GenBank (NG055658.1) (**Figure 4**).

Antimicrobial Associated Resistance Genes Screening

Among the 107 *E. coli* strains evaluated, 94 (88%) harbored some antimicrobial resistance-associated gene. One antimicrobial resistance gene was detected in 18% (19/107) of the *E. coli* examined, two genes were detected in 17% (18/107); three genes in 16% (17/107); four genes in 15% (16/107); five genes in 7% (8/107); six genes in 6% (6/107); seven genes in 5% (5/107); eight genes in 2% (2/107); nine genes in 2% (2/107) and ten antimicrobial resistance genes in 0.9% (1/107) of strains examined (**Figure 1** and **Table 1**).

Regarding resistance to the aminoglycoside gentamicin, there was a correlation between phenotypic and genotypic resistance in 40% (18/45) of the strains evaluated. The *aadA* gene was significantly ($p < 0.05$) associated with the phenotypic resistance observed with 75% (9/12) of the strains that had the gene, expressed resistance in the disk diffusion assay, but resistance was not statistically associated with the *aac3-VIb* genes with 50% (4/8) positive, *aph*AI (FAO, 2013) with 50% (11/22) positive and *aac3-VIa* with 38% (5/13) positive. It was also noted that some isolates harbored more than one gene associated with gentamicin (aminoglycoside) resistance (**Figure 2B** and **Supplementary Table 4A**).

Sulfa-trimethoprim was the second antimicrobial with the highest prevalence of phenotypic resistance in the disk diffusion assay (71%; 76/107), with phenotypic and genotypic correlation in 15.78% (12/76) of the strains evaluated. 12/17 (71%) strains that had the *sul1* gene and 20/28 (71%) of the strains that had the *dfr-17* gene showed phenotypic resistance to SUT. However, 64% (49/76) showed phenotypic resistance and but did not harbor either of the two genes (**Figure 1** and **Table 1**).

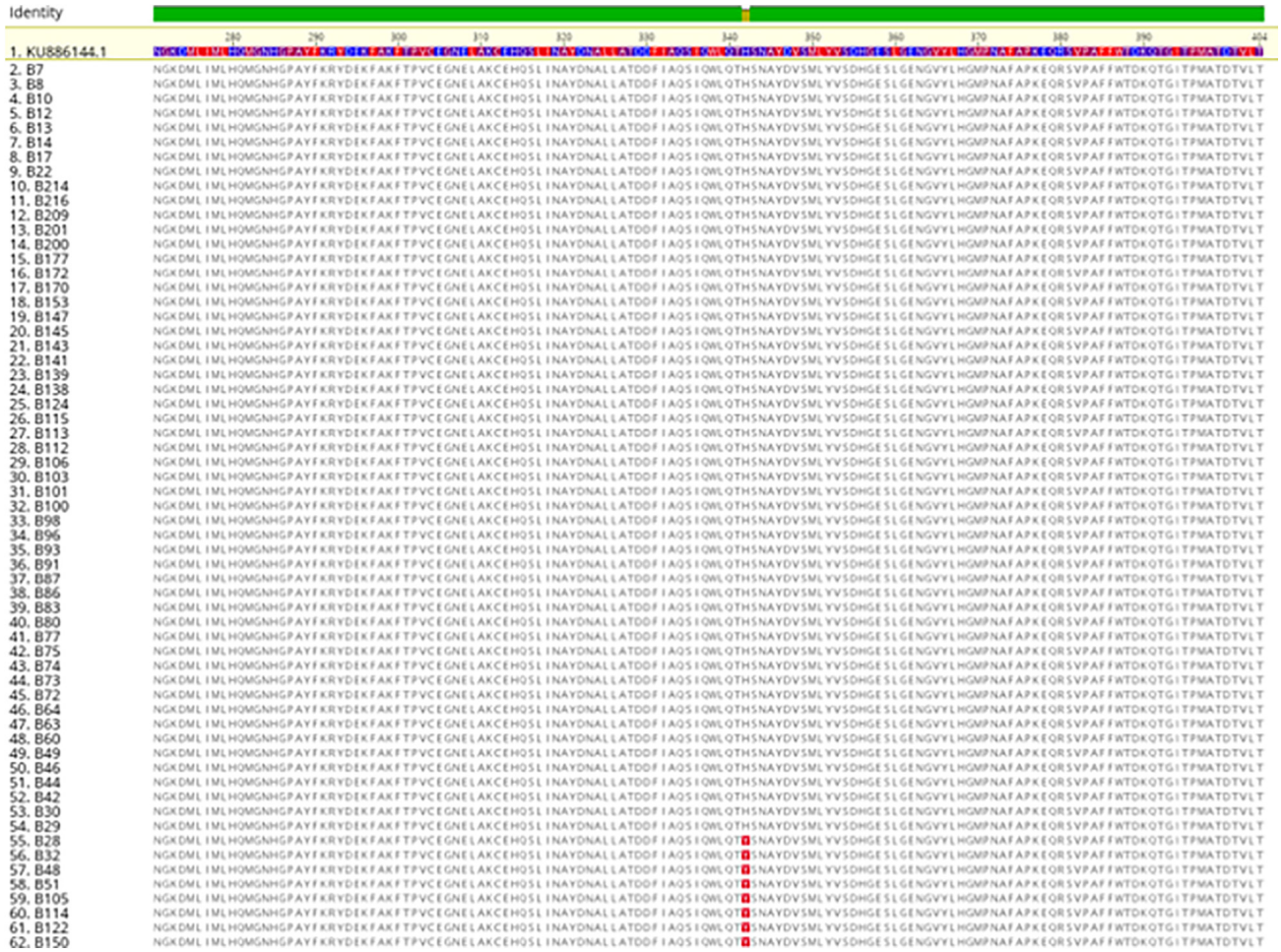

FIGURE 3 | Protein Alignment of *mcr-1* positive strains examined in this study.

The *blaTEM* gene was detected in 15% (16/107) of *E. coli* strains examined, however, only 25% (4/16) of strains that had the gene were considered to produce ESBL in phenotypic tests, this can be explained because there are extended spectrum variants of the gene that were not assayed by the PCR assays used in our analysis. The prevalence of resistance-related genes is shown in **Table 1**.

The gene that encodes the integrase enzyme (*intl1*) was detected in 10% (11/107) of *E. coli* strains and 13% (8/62) of *mcr-1* positive strains. The presence of the gene encoding the transposase enzyme (*ISEc12*) was also detected in 13% (14/107) of *E. coli* strains and 10% (6/62) of the positive *mcr-1* strains. *tetA* and *tetB* (32 and 25%) were the most prevalent antimicrobial resistance genes in *mcr-1* positive isolates.

Using the Mann-Whitney *U* test, to compare antimicrobial resistance prevalence with farm of isolation we found some significant relationships ($p < 0.05$) between certain groups, including antimicrobial resistance genes such as *dfr17* on farm 1 vs. farm 2; (**Supplementary Table 4B**). Similarly, using the chi-square test (**Supplementary Table 4A**), that allows comparison between the presence of two antimicrobial resistances analyzed in all strains, significant associations were observed for some specific antimicrobial resistance genes such as *qacΔ1* and several other antimicrobial resistance genes including *sulI*, *ISEc12,* and *aadA* ($p < 0.05$).

Plasmid Replicon Detection

Among the seventeen plasmid incompatibility groups tested, the three most prevalent included *IncI2*, *B/O*, and *FIB* with 32% (34/107), 28% (30/107), and 36% (39/107) prevalence, respectively. (**Table 1** and **Figure 2D**). When the prevalence of these plasmids was correlated with the presence of the *mcr-1* gene, a greater occurrence of this resistance gene was observed in the strains where these plasmids were detected separately and concomitantly (**Supplementary Table 4A**). Significant associations were observed for the detection of *mcr-1* and the *HII* plasmid replicon ($p < 0.05$).

IncI2, FIB, and *B/O* (37, 35, and 34%) were the most prevalent replicon types detected in the *mcr-1* positive isolates. We could not confirm that the *mcr-1* gene was contained on the plasmid, integron or transposon as this analysis was beyond the scope of the current study, however, the occurrence of these elements in positive *mcr-1* strains can be considered a risk factor, since

FIGURE 4 | Protein Alignment of *mcr-5* positive strains examined in this study.

the *mcr-1* gene can move to these mobile genetic elements, facilitating the dispersion of the gene. Such characteristics were observed in the B48 strain, where the genes *mcr-1*, *intl1*, *ISEc 12*, and plasmids *IncI2*, and *FIB* were detected simultaneously.

Using the Mann-Whitney *U* test, to compare plasmid replicon prevalence with farm of isolation we found some significant relationships ($p < 0.05$) between certain groups, including plasmid replicon such as *II* in the cloaca vs. trachea; *IncI2*, *FIB*, and *B/O* in broiler vs. free range birds; *P*, *FIB*, and *HII* on farm 1 vs. farm 2; *IncI2* and *FIB* on farm 1 vs. farm 3; *IncI2* on farm 2 vs. farm 3 (**Supplementary Table 4B**). Similarly, using the chi-square test (**Supplementary Table 4A**), that allows comparison between the presence of two plasmid replicons analyzed in all strains, significant associations were observed for some plasmid replicons such as *HII* and a few other plasmid replicons including *P*, *HI2* and *L/M* ($p < 0.05$).

Genotyping Avian *E. coli* for *iroN, ompT, hlyF, iss,* and *iutA*

Within the group of virulence genes used to characterize strains such as APEC, the gene with the highest prevalence was *iutA*, followed by *iss*, *ompT*, *hlyF*, *iroN* with 49, 39, 33, 30, and 22%, respectively (**Table 1** and **Figure 2C**). Of the 107 strains of *E. coli* analyzed, 31% (33/107) were characterized as APEC-like, as they harbored three or more virulence genes of the path panel. These strains, however, were not isolated from lesions of diseased birds but rather healthy birds.

The highest prevalence of strains characterized as APEC-like occurred on farm 1 (40%; 19/47), 38% (10/26) of cloaca and 42% (9/21) of trachea, on farm 2, 25% (12/48) of the strains were considered APEC-like, 22% (6/27) of the cloacal strains and 28% (6/21) of the trachea (**Figure 2C**). On farm 3, 16% (2/12) of the strains were considered potentially pathogenic, with a greater proportion detected in the trachea (25%; 1/4) compared with the cloaca (12%; 1/8). *iutA*, was the most prevalent APEC virulence associated gene in *mcr-1* positive isolates, present in 50% of the isolates and just considering *mcr-1* positive isolates, 32% could be classified APEC-like (**Figure 2C**).

Phylogenetic Typing

Most strains were classified as belonging to phylogenetic group B1 (38%; 41/107), followed by group A (20%; 21/107), group F (15%; 16/107), group D (12%; 13/107), group E (11%; 12/107) and group C (2%; 2/107). None of the isolates were identified as phylogenetic group B2 (**Table 1**).

Thirty-four strains were considered APEC-like, with 9 (26%) and 7 (20%) distributed in phylogenetic groups B1 and F, respectively. The *mcr-1* gene was detected in 62 strains, 22 (35%) belonging to phylogenetic group B1 and 10 (16%) belonging to phylogenetic group F.

The prevalence of other phylogenetic groups and the correlation between the characterization as APEC-like and the presence of the *mcr-1* gene can be seen in **Table 1** and **Supplementary Table 4D**.

Pulsed Field Gel Electrophoresis Analysis

When evaluating the results obtained by Pulsed-field Gel analysis, there was great genetic diversity within the strains of *E. coli* examined. However, there was 100% identify between isolates B74, B75, B80, between B66 and B100 and between B39 and B42 (**Figure 1**).

Strains B74, B75, and B80, were isolated from trachea of birds on farm 2, were resistant to COL, GEN, SUT, and positive for the resistance genes *mcr-1*, *blaTEM*, *tetA*, *tetB*. Variability was observed in the presence of the genes *intl-1*, *pcoD*, *sulI*, *aadA*, *aac3-VIb*, *qacE*Δ. As for plasmids, all were positive for the replicons *B/O* and *FIB*, and only the B80 strain was positive for *I1*. As for the presence of virulence genes, *iroN* and *iutA* were detected in the three strains. Regarding the classification of the phylogenetic group, all three were classified as phylogenetic group F (**Figure 1**).

Strains B66 and B100 were isolated from the cloaca of birds on farm 2, both showed phenotypic resistance to COL and SUT, with only strain B66 showing resistance to CIP. The B66 strain harbored the *tetA* resistance gene, while the *mcr-1* gene was detected only in B100. As for the detection of plasmid replicons, *IncI2*, *L/M*, *HI2*, *N*, *HII* were detected only in the B100 strain. Virulence genes were not detected in either strain and both strains classified as phylogenetic group B1 (**Figure 1**).

Evaluating the profile of strains B39 and B42, it was found that both were isolated from the trachea of birds on farm 1 and presented phenotypic resistance to COL, however, the *mcr-1* gene was detected only in B42, while the *tetA* gene was detected in both. The *IncI2* plasmid and *B/O* plasmid replicons were detected in both strains, however, plasmid replicons *FIB* and *II* were present only in B42. As for virulence genes, *iss* and *iutA* were detected only in the B42 strain and both strains classified as phylogenetic group B1 (**Figure 1**).

DISCUSSION

When analyzing the phenotypic resistance against the tested antimicrobials, it was found that *E. coli* from all three farms studied showed a high prevalence of colistin resistance (100% farm 1 cloaca, 95% farm 1 trachea, 92% farm 2 cloaca, 95% farm 2 trachea, 87% farm 3 cloaca, 100% farm 3 trachea). When evaluating *E. coli* from a Vietnamese broiler farm, Nguyen et al. (Nguyen et al., 2016) found 22% of isolates were resistant to colistin. Similarly, Fernandes et al. (Fernandes et al., 2016b) who, when evaluating a collection of *E. coli* strains collected from broiler chickens between 2000 and 2016 in Brazil, found that 40% were resistant to colistin. In a recent study carried out in Iran by Azizpour & Saeidi (Azizpour and Saeidi Namin, 2018) it was observed that 68.5% were resistant to colistin. Thus, our data appears to show that the colisitin resistance in poultry continues to persist in Brazil.

High levels of resistance were also found to the antimicrobial SUT on farms 1 (73% cloaca, 76% trachea) and 2 (77% cloaca and 85% trachea), we believe that such high levels may be related the historical context of a high density of breeders and the previous use of this class of antimicrobials in poultry flocks in the mountain region of Rio de Janeiro. However, the values observed on farm 3 (12% cloaca, 25% trachea) were considerably lower when compared to farms 1 and 2, which may be related to the low density of poultry producers and the recent trend toward organic and free-range poultry breeders in the northern region of the state. High levels of resistance to SUT (80%) were also observed by Azizpour & Saeidi (Azizpour and Saeidi Namin, 2018).

E. coli strains also showed resistance to gentamicin. Isolates from farm 1 displayed resistance in 31% of cloaca strains and 28% in trachea strains and farm 2, 52% in those that were present in the cloaca and 81% in trachea strains, however, on farm 3, no resistance was observed to this drug. These levels are also similar to those reported by Nguyen and collaborators (Nguyen et al., 2016) where 42% of *E. coli* from broilers were resistant. An additional survey carried out during visits to the farms of the current study found that the growth promoter enramycin was verified as in use on farms 1 and 2 and colistin on farm 3, enramycin is a polypeptide antibiotic. Thus, the resistance verified in phenotypic tests could be explained by the use of enramycin as a growth promoter, as observed by Costa et al. (Costa et al., 2017).

On examination of ciprofloxacin resistance data, the following results were noted 58% and 28% of the strains (cloaca and trachea, respectively) from farm 1 were resistant, on farm 2, 22% of the cloaca strains and 33% of trachea, in addition to 25% of cloaca and 75% of trachea in strains belonging to farm 3. Nguyen and collaborators (Nguyen et al., 2016) found that 73% of 90 isolates examined displayed resistance to ciprofloxacin when studying *E. coli* of avian origin in Vietnam. Abdi-Hachesoo et al. (Abdi-Hachesoo et al., 2017), when researching resistance to quinolones in broiler chicken farms in Iran, found 80% of strains of *E. coli* isolated were resistant from 30-day-old broilers, results similar to those were observed by Azizpour & Saeidi (Azizpour and Saeidi Namin, 2018) who noted a prevalence of 77% in broilers. Ciprofloxacin belongs to the quinolone class, as does enrofloxacin, which is widely used in therapeutic and prophylactic forms in the field. In the survey carried out on the farms during visits, farm 1 and farm 3 had a history of recent use of enrofloxacin. Farm 1 used the antimicrobial in the production batch prior to the survey and on farm 3, the use of the drug occurred in the last three months prior to collection, on both farms (farms 1 and 3) it was used to contain an outbreak of colibacillosis. This antimicrobial has, however, seen considerably limited use in some regions of the world for example in the US where it is no longer approved for use in poultry (FDA, 2005) and this is also the case for the European Union (EU) (EU, 2018).

Considerable levels of resistance to amoxicillin were also observed, with *E. coli* strains from cloacal isolates from farm 1 showing the highest prevalence (57%) followed by trachea strains on farm 1 (38%), cloaca on farm 2 (37%), trachea from farm 2 (28%) and trachea from farm 3 (25%), no resistance was observed in the cloacal strains of farm 3. This resistance may be related to the production of beta-lactamase as it was observed that 52/107 (48%) strains of *E. coli* were suspected of producing this enzyme based on the results of the disk diffusion test.

The most prevalent gene detected in this study was *mcr-1* which was detected in 58% (62/107) of all strains examined, all of which showed phenotypic resistance to colistin, but 39% (40/102) were resistant in the phenotypic test and did not show the presence of any *mcr*-associated gene when examined genotypically, demonstrating the need for future studies regarding the genetic variability of *mcr* and other potential causes of resistance (Yin et al., 2017).

The implementation of IN-45 as of 22 November 2016 in Brazil, prohibits the use of colistin as an additive in feed, is an attempt by the Ministry of Agriculture, Livestock and Supply to reduce the levels of resistance to colistin found in the field. However, it is too early to say whether such a measure will have an effect, since the use of the therapeutic form is currently still approved for use.

Transposons are genetic elements that move in the genome through the action of the enzyme transposase. This movement can occur both within the chromosome and between chromosome and plasmid. Transposons can contain integrons, facilitating the transmission of resistance genes between bacteria. The detection of the *intl1* gene is correlated with the presence of integrons, which are genetic elements that contain a site-specific recombination system capable of integrating, expressing specific DNA elements, called gene cassettes (Hall and Collis, 1995). Integrons consist of three elements: the gene encoding tyrosine recombinase (integrase, encoded by the *intl* gene), required for recombination specific site of the gene cassettes within the integron, the site specific recombination site *attI* and an open reading frame (Gillings, 2014) *qacE*Δ*1* and sulfonamides.

The presence of the gene encoding the transposase enzyme (*iseC12*) was also detected in 13% (14/107) of the *E. coli* strains examined and 10% (6/62) of the positive *mcr-1* strains. The transposons are recognized by integrase; and the promoter (Pc) located upstream of the integration site, is necessary for efficient transcription and expression of the gene cassette present in the integron. Most cassettes present in integrons already described encode resistance determinants, and these genetic elements appear to play an important role in the spread of antimicrobial resistance in Gram negative bacteria (Ploy et al., 2000).

In the strains B75 and B93, which were positive for the *intl1* gene, the concomitant presence of the resistance genes to quaternary ammonia (*qacE*Δ*1*) and sulfonamides (*sulI*) was observed, such characteristics are related to the presence of the class 1 integron, which is conserved in its region downstream the referred genes (Recchia and Hall, 1997).

The high prevalence of APEC-like strains observed on farm 1 could be associated with the occurrence of a collibacilosis case, due to omphalitis, in the first week of life of the animals that made up the batch analyzed in the second collection. The disease was controlled with antimicrobial treatment and disposal of the carcasses of dead animals. Despite these management approaches, it was found that 31.1% (19/61) of cloaca isolates were classified as APEC-like strains (**Table 1** and **Figure 2C**). This data contrasts with another study of *E. coli* cloaca isolates from Brazil (de Oliveira et al., 2015) where 53% of the isolates were classified as APEC-like strains.

When the results of phylogenetic group analysis were compared – most of the isolates in the current study classified as B1 (38%) and A (20%). These results are considerably different from those observed by Rocha et al. (Rocha et al., 2017) who examined APEC and UPEC strains and found that the most common phylogenetic group in APEC was phylogenetic group D (31%) and phylogenetic B2 was most prevalent in the UPEC strains (53%). In the studies by Rocha et al. (Rocha et al., 2017) phylogenetic group B1 was present in only 6% of the UPEC strains. In studies of APEC from Brazil (Barbieri et al., 2015; Braga et al., 2016), they found the majority of their collections classified as phylogenetic groups D with only 7.6% (Barbieri et al., 2015) and 13.3% (Braga et al., 2016) classified as B1. In a study of AFEC (cloacal swabs) from Egypt the most frequently detected phylogenetic groups were A (46.6%) with 33.3% of isolates examined classifying as B1 (Hussein et al., 2013). In a study of retail meat *E. coli* from 2013 from Brazil the most frequent phylogenetic group found was B1 (37.2%) while a 2007 study found phylogenetic group D was most common with a prevalence of 34.5% (Koga et al., 2015).

When PFGE data was assessed, we found significant diversity in the fingerprint profiles of all *E. coli* examined. These data are comparable to other works (Bergeron et al., 2012; Hussein et al., 2013; Barbieri et al., 2015; Braga et al., 2016; de Oliveira et al., 2020) showing that often disease outbreaks are linked to more than one strain of organism. Pulsed field gel electrophoresis is known as a standard tool for pathogen subtyping and has significant application in the identification of outbreak strains, but it is generally found not to be useful for APEC because of the great diversity of strains linked with disease, and in particular the diversity of strains on a single farm that are linked with disease make it difficult to use in tracing the source of the outbreak. Of note in this study, however, strains with the same profile were found between isolates B74, B75, B80 that were isolated from trachea of different birds on farm 2; between B66 and B100 that were isolated from the cloaca of different birds on farm 2; and between B39 and B42 were isolated from trachea of different birds on farm 1 (**Figure 1**).

CONCLUSION

The evaluation of the bacterial microbiota present in samples of cloaca and trachea of broilers found a high prevalence of *E. coli* in both the cloacal sample and tracheal swabs of birds at the various farms.

When assessing antimicrobial resistance in isolated strains, it was noted that the resistance profile varied according to the breeding system and history of antimicrobial use on each farm.

E. coli strains were found with phenotypes suggestive of ESBL and AmpC beta-lactamase production.

Phenotypic resistance to colistin was the most prevalent trait among the *E. coli* isolates examined, which was accompanied by a high prevalence of detection of the *mcr-1* gene. Correlations were also observed between the presence of the *mcr-1* gene and the plasmids *HII, IncI2, B/O*, and *FIB*. Although it cannot be confirmed that the *mcr-1* gene is located on plasmids, the occurrence of both in the same individual isolate is considered a risk factor, since plasmids can carry the resistance gene and favor its dispersion.

High genetic variability of *E. coli* strains was observed with prevalence of the phylogenetic group B1, related to commensal strains. However, the analysis of the virulence profile detected a high number of APEC-like strains, highlighting the importance of monitoring, cleaning, and disinfecting the environment, control of people and vehicles and the sanitary condition of the sheds between flocks, in order to avoid future infections, occurrence of colibacillosis and consequent economic losses.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

NB design the study, carried out the research, data analysis, and drafting of the manuscript. RP performed sampling collection, analysis of farms and farm visits. DM performed analysis for genes. LN provided assistance in drafting the manuscript and provided supplies for the study. MS provided assistance for sampling collection and provided supplies for the study. CL helped design the study, draft the manuscript, and provided materials for the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.659613/full#supplementary-material

Supplementary Table 1 | Source of isolates examined in this study.

Supplementary Table 2 | Primers used in this study.

Supplementary Table 3A | Zone Diameter and MIC Breakpoints used on this study.

Supplementary Table 3B | Multi Drug Resistance Among Isolates Examined.

Supplementary Table 3C | Antimicrobial resistance profiles.

Supplementary Table 4A | Chi-square analysis of the association between the presence of two single genes or antimicrobial resistance traits.

Supplementary Table 4B | Statistical analysis based on site of isolation.

Supplementary Table 4C | Prevalence analysis based on site of isolation.

Supplementary Table 4D | All data.

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Molecular Characterization of Multidrug-Resistant *Yersinia enterocolitica* From Foodborne Outbreaks in Sweden

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The foodborne pathogen *Yersinia enterocolitica* causes gastrointestinal infections worldwide. In the spring of 2019, the Swedish Public Health Agency and Statens Serum Institut in Denmark independently identified an outbreak caused by *Yersinia enterocolitica* 4/O:3 that after sequence comparison turned out to be a cross-border outbreak. A trace-back investigation suggested shipments of fresh prewashed spinach from Italy as a common source for the outbreak. Here, we determined the genome sequences of five *Y. enterocolitica* clinical isolates during the Swedish outbreak using a combination of Illumina HiSeq short-read and Nanopore Technologies' MinION long-read whole-genome sequencing. WGS results showed that all clinical strains have a fully assembled chromosome of approximately 4.6 Mbp in size and a 72-kbp virulence plasmid; one of the strains was carrying an additional 5.7-kbp plasmid, pYE-tet. All strains showed a high pathogen probability score (87.5%) with associated genes for virulence, all of which are closely related to an earlier clinical strain Y11 from Germany. In addition, we identified a chromosomally encoded multidrug-resistance cassette carrying resistance genes against chloramphenicol (*catA1*), streptomycin (*aadA1*), sulfonamides (*sul1*), and a mercury resistance module. This chromosomally encoded Tn*2670* transposon has previously been reported associated with IncFII plasmids in *Enterobacteriaceae*: a *Shigella flexneri* clinical isolate from Japan in 1950s, a *Klebsiella pneumoniae* outbreak from Australia in 1997, and *Salmonella enterica* serovar Typhimurium. Interestingly, we identified an additional 5.7-kbp plasmid with *tet*B (encoding an ABC transporter), *Rep*, and its own ORI and ORIt sites, sharing high homology with small *tet*B-*Rep* plasmids from *Pasteurellaceae*. This is the first time that Tn*2670* and *Pasteurellaceae* plasmids have been reported in *Y. enterocolitica*. Taken together, our study showed that the Swedish *Y. enterocolitica* outbreak strains acquired multi-antibiotic and metal-resistance genes through horizontal gene transfer, suggesting a potential reservoir of intraspecies dissemination of multidrug-resistance genes among foodborne pathogens. This study also highlights the concern of food-chain contamination of prewashed vegetables as a perpetual hazard against public health.

Keywords: ***Yersinia enterocolitica*, WGS, AMR, Tn*2670*, *tet*B**

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INTRODUCTION

Yersinia enterocolitica is a foodborne zoonotic bacterium of global importance, able to cause severe gastrointestinal infections among people of all ages (Scallan et al., 2011; Batz et al., 2012; Bancerz-Kisiel and Szweda, 2015). *Yersinia* is grouped under *Yersiniaceae* where *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Y. enterocolitica* are recognized as important human pathogens (Smego et al., 1999; Adeolu et al., 2016). *Y. enterocolitica* is divided into six biotypes (1A/B-5) depending on physio- and biochemical properties and additionally into approximately 50 serotypes depending on antigenic variation of the lipopolysaccharides (Jagielski et al., 2002).

While yersiniosis historically has been associated with consumption of undercooked pork products or un-pasteurized milk, the growing trend of ready-to-eat (RTE) vegetables in industrialized countries has been linked to sporadic outbreaks of *Y. enterocolitica* (Lee et al., 2004; Sakai et al., 2005; Much et al., 2009; Macdonald et al., 2011; Rahman et al., 2011). As discussed during the amplified implementation of the Hazard Analysis and Critical Control Point (HACCP) system in the 1990s, in the raw food industry, RTE products were highlighted as products without any pathogen elimination step (Motarjemi and Käferstein, 1999). Even without pathogen elimination, HACCP remains a good resource to reduce risks in food production, but intensification and mass distribution have nonetheless permitted quick nationwide spread of introduced contaminants. International trade has correspondingly introduced longer food-supply chains and countless wholesalers, increasing the risk of breaking the cold chain with consequential growth of foodborne pathogens (Motarjemi and Käferstein, 1999; Söderqvist et al., 2017a,b). Wholesalers in the European Union are required to regularly sample RTE vegetables for *Escherichia*, *Listeria*, and *Salmonella*, but *Yersinia* remains continuously absent from routine testing (EU commission 2073/2005) and its AMR surveillance occurs only through rare *ad hoc* reports (Fàbrega et al., 2015).

In the spring (February–April) of 2019, the Swedish Public Health Agency (PHAS) and Statens Serum Institut (SSI) in Denmark independently identified an outbreak with the same genotypic cluster of *Y. enterocolitica* 4/O:3. Whole-genome sequencing (WGS) comparisons were made using the respective outbreak sequences, resulting in a cross-border outbreak being declared. The outbreak reached 57 positive cases, mainly in a younger age group (15–39) (National Veterinary Institute (SVA), 2019). A Danish case–control study recognized fresh spinach as the cause behind the outbreak, and a traceback of common producers identified an Italian supplier behind shared market batches (Espenhain et al., 2019). Following the first outbreak, a second outbreak (April–May) occurred with an additional 30 cases. *Y. enterocolitica* in the second outbreak was of identical bioserotype and sequence type (ST) type but clustered separately on single nucleotide polymorphism (SNP) analysis. No food-related origin could be established for the latter outbreak (National Veterinary Institute (SVA), 2019).

Whole-genome sequencing is now routinely used in outbreak investigations to identify ST types, clusters, pathogenomics, and resistance genes, but typically this high-throughput resource is limited to short-read data (Lynch et al., 2016; Nutman and Marchaim, 2019; Adzitey et al., 2020). Short-read libraries are precise and cost-efficient but do rarely provide long enough contigs for molecular epidemiological identification of moveable genetic elements and small plasmids (Koren and Phillippy, 2015). Long-read sequencing technologies can now be used in combination with short-read data to provide high-quality full-genome assemblies (Jain et al., 2016; George et al., 2017; Lemon et al., 2017). To provide insight in resistance and adaptive traits in *Y. enterocolitica* causing foodborne outbreaks, we combined Illumina HiSeq and Oxford Nanopore technologies' MinION to generate and close whole genomes for five clinical strains, isolated in Sweden during the time of the two consecutive outbreaks in 2019. This is the first report of multidrug-resistant *Y. enterocolitica* identified from imported fresh spinach, raising concerns on food-chain contamination.

MATERIALS AND METHODS

Bacterial Isolation and Identification

A total of five clinical isolates from the spring of 2019 were characterized. PHAS received isolates from the Swedish clinical microbiological laboratories for epidemiological typing, and three of these were used in the study (Espenhain et al., 2019). Y30 was derived from the spinach-related outbreak (March), Y108 was isolated from the second outbreak (April), and Y72 was collected during the outbreak periods (April) but did not cluster with any of the outbreak strains (SNP analysis, PHAS). Two additional uncharacterized isolates were collected from the Clinical Microbiology Lab at Uppsala University Hospital from the same period, Y_Mar (March) and Y_May (May). All five isolates are in this study referred to as outbreak strains. One well-characterized strain Y11 (chromosome: FR729477, plasmid: FR745874) was received from the Leibniz Institute (DSM 13030) for comparative use. The Swedish strains were delivered in transport swabs with amies medium charcoal (SARSTEDT), streaked onto Cefsulodin Irgasan Novobiocin (CIN) agar with *Yersinia* supplement and incubated for 24 h at 26°C. A one-µl streak of confirmed *Yersinia* colonies was cultivated in Trypticase Soy Broth (TSB) for 24 h in shaking incubators at 26°C. Aliquots were saved in TSB-DMSO (10%) and stored in −80°C freezers until further use. Ethical approval was not required as the investigation was performed under a mandate of PHAS in its remit to undertake outbreak investigations regarding national communicable disease control in the interest of public health.

Whole-Genome Sequencing

Stocks were cultured on Trypticase Soy Agar (TSA) and in one ml TSB at 26°C overnight. Genomic DNA was extracted from overnight cultures with the MasterPure DNA purification kit (Epicentre, Lucigen) according to the manufacturer's instructions. Purified DNA was analyzed and quantified with 2100 NanoDrop Spectrophotometer and Qubit 2.0 (Thermo Fisher Scientific). DNA short-read libraries were prepared and sequenced using an Illumina HiSeq 2500 platform (Illumina)

by Novogene. Long-read libraries were prepared using an in-house MinION sequencer by Oxford Nanopore Technologies, run for 24+ hours with base calling in the MinKNOW software on standard settings. Assembly was performed in CLC Genomics Workbench 20.0.4 (**Supplementary Table 1**), and all Illumina data was deposited at PRJEB42815 (ENA). Obtained MinION raw reads from one strain (Y72) were fully *de novo* assembled into three separate contigs representing the chromosome, the virulence plasmid, and one additional plasmid. The Nanopore sequence from Y72 was corrected using the trimmed Illumina reads for the same strain. The corrected Y72 assembly was used as a reference for mapping of trimmed Illumina reads for the remaining four strains (**Supplementary Table 2**).

Preparing Reference Genomes

Illumina sequences were *de novo* assembled, and contigs were sorted by length. The five longest contigs (280–111 kbp) along with one additional 5.7-kb plasmid and a resistance cassette in Y72 were compared with genomic data available on NCBI using BLAST. As only a small set of WGS *Y. enterocolitica* genomes are available, the *Yersinia* EnteroBase V.1.1.2[1] was used to identify available sequence data for *Y. enterocolitica* biotype 4 (Zhou et al., 2020). A total of 24 scaffold sets for different *Yersinia* 4/O:3 chromosomes, mainly based on a previous assortment (PRJEB2116 and PRJEB2117) (Reuter et al., 2014), were collected to increase phylogenetic resolution of the outbreak strains and comparative power of the metadata (**Supplementary Table 3**). Metadata included biotype, serotype, collection year, isolation country, isolation host, McNally ST, Achtman ST, wgMLST, and cgMLST + HierCC V1 (**Supplementary Table 3**). Based on high-score BLAST hits, an additional 17 different sequences of chromosomal and plasmid origin were collected for cassette comparisons (**Supplementary Table 4**) along with a set of three different alignment matches for the 5.7-kb plasmid.

Functional Annotation

Molecular characterization for the outbreak strains and Y11 (six strains) were assessed with regard to pathogenicity potential, restriction–modification systems, and known virulence factors. Antimicrobial resistance genes were characterized in outbreak strains and the generated comparison platform (24 additional strains). Pathogenicity potential was assessed using PathogenFinder 1.1[2] with the automatic model selection for assembled genomes (Cosentino et al., 2013). Antimicrobial resistance was assessed using ResFinder 4.1[3] for acquired antimicrobial resistance genes among "other" species for assembled genomes (Zankari et al., 2017; Bortolaia et al., 2020). Restriction–modification systems were identified using Restriction-ModificationFinder 1.1[4] for type I–IV restriction enzymes including putative genes (Roer et al., 2016). Virulence was assessed using the Virulence Factor Database VFDB[5] for *Y. enterocolitica* (Chen et al., 2005, 2016; Yang et al., 2008; Liu et al., 2019).

Phylogenetic Analysis

The 29 strains of *Y. enterocolitica* biotype 4 and one strain of biotype 2 (outgroup) were selected and analyzed on EnteroBase (Zhou et al., 2020). Enterobase's cgMLST scheme was used to retrieve all SNPs from all strains. In total, 12,800 positions were polymorphic, of which about 3,600 were polymorphic in the ingroup. The alignment of all SNPs was used to infer a maximum-likelihood tree with IQ-TREE 2.1.2 (Minh et al., 2020). An extended range of substitution models was tested, and TVMe+ASC+R3, which had the lowest log likelihood according to Bayesian Information Criterion (BIC), was selected. This model assumes different rates for transitions and transversions, but with equal base frequencies (TVMe); it includes an ascertainment bias correction (ASC), which is appropriate in the case of SNP data (Lewis, 2001); it also includes a FreeRate model of rate heterogeneity across sites, with three categories (R3). A thousand ultrafast bootstraps (UFBoot) were drawn (Hoang et al., 2018).

All sequences matching the identified cassette were aligned to the known common ancestor *Shigella flexneri* R100 plasmid (Womble and Rownd, 1988), using the HOXD scoring-based Whole Genome Alignment plugin in CLC on standard settings (Chiaromonte et al., 2002). Only sequence regions overlapping with the *Yersinia* cassette were extracted (Extract Multiple Sequence Alignment tool) which corresponded to the R-det-Tn*2670* region of the R100 plasmid. As the 22 sequences shared different percentages of R-det coverage and the purpose of the comparison was to see the overall relationship among the transposon modules, a pairwise comparison (Create Average Nucleotide Identity Comparison) was generated with Alignment Percentage (AP) on standard settings. Based on the AP pairwise comparison, an AP similarity tree was constructed using Neighbor Joining (NJ), which joins clusters close to each other and far from other clusters, suitable for sequences with differential rates of evolution (Saitou and Nei, 1987). The AP NJ tree was rooted in the *Shigella flexneri* R100 plasmid. Both trees were later combined with collected metadata in CLC, and figure segments for **Figures 2**, **3** were combined in Serif's Affinity Designer 1.9.1.979.

Antimicrobial Susceptibility Testing

Minimum inhibitory concentration (MIC) tests were performed in biological duplicates with microdilutions in microtiter 96-well plates, where the antibiotic was added to the first well and diluted 1:2 per well in 10 consecutive wells. One column per plate was used as a positive control (no antibiotic), and one was used as a negative control (no bacteria). Bacterial concentrations of 0.5 McFarland standard units were added as 50 µl to each well. The starting concentrations were set to 2 × the clinical breakpoint (CB) for Enterobacterales (EUCAST). If no CB was available, E-tests were used as indicators for MIC and later adapted to broth microdilutions. All broth microdilutions were carried out in standardized EUCAST settings in Mueller Hinton media, incubated at 37°C for 16–20 h. Results were only accepted if

[1]https://enterobase.warwick.ac.uk/species/index/yersinia

[2]https://cge.cbs.dtu.dk/services/PathogenFinder/

[3]https://cge.cbs.dtu.dk/services/ResFinder/

[4]https://cge.cbs.dtu.dk/services/Restriction-ModificationFinder/

[5]http://www.mgc.ac.cn/VFs/main.htm

controls were functional and the duplicates did not deviate more than one dilution.

Growth Rate Measurements

Growth rate was used as an indicator for bacterial fitness and measured using the Bioscreen C (Oy Growth Curves Ab Ltd.) turbidity monitoring system. The bioscreen was performed in 300 μl TSB (1:1,000 dilutions from overnight culture) in biological triplicates with technical duplicates. Experiments were run at 26°C, with continuous shaking at medium amplitude and 195 rpm for 24 h. Measurement was done using a 600-nm brown filter every 4 min and shaking stop 5 s prior to measurement. Generation time was derived from the slope of the exponential growth phase ($0.02 < OD_{600} < 0.12$) where $R^2 \geq 0.98$ per sample and SD ≤ 0.001 (slope) across replicates. Data were analyzed and presented using GraphPad Prism 9.

RESULTS

Comparative Genomics

A total of five time-representative isolates were obtained from PHAS (Y30, Y72, Y108) and Uppsala University Hospital (Y_Mar, Y_May) during the time of the two outbreaks. A well-established clinical strain of the same bioserotype was used as a control (Y11).

Five strains were assembled to complete genomes, molecularly typed, and assessed together with Y11 with regard to pathogenicity and virulence. The genomes from the outbreaks had an average estimated probability score for human pathogenicity of 0.875 (chromosome) and were found matching 95–99 different families. Clinical strains carried similar known and putative Restriction-Modification systems (n = 4) as the Y11 strain, with two Type II restriction enzymes and two methyltransferases. Virulence factors were shared across the collected strains and included genes for adherence (*psa*, *yap*), invasion (*ail, inv*), proteases (*pla*), and chromosomal secretion systems. There were eight open reading frames (ORFs) for the O-antigen and 42 ORFs for the cluster I flagella (**Supplementary Table 5**). The Swedish outbreak strains differentiated from the Y11 strain by the presence of a chromosomal Type II Secretion System protein (*yts1O*) and genes for antimicrobial resistance (**Table 1** and **Figure 1**).

Assembled genomes were phylogenetically analyzed based on maximum likelihood from all polymorphic positions identified by cgMLST in EnteroBase and rooted using an isolate from biovar 2/O:9 (IP26766, France, 2002) as an outgroup. The Swedish outbreak strains clustered together, having the closest shared node with a swine isolate from the UK (YE213/02, 2002) and a bovine isolate from Germany (YE-150, unknown collection year) (**Figure 1**). On a larger scale, the Swedish cluster grouped with European isolates from Germany, the UK, and France, including Y11. As inferred by McNally ST typing, the most common ST-type for 4/O:3 was ST18, but Hierarchical Clustering of cgMLST (HierCC) confirmed high similarity multilevel clustering for the Swedish outbreak strains. Results from ResFinder moreover demonstrated the global and time-independent presence of previously well-studied *Y. enterocolitica vat*(*F*) and *blaA* resistance genes, conferring resistance against streptogramin and β-lactam antibiotics (Pham et al., 1991; Stock et al., 1999; Seoane and García Lobo, 2000). The Swedish outbreak isolates also displayed a previously unreported multidrug resistance pattern (**Figure 1**).

Resistance Cassette

We report the fully sequenced genome of Y72, showing the presence of a chromosomal multidrug resistance cassette harboring determinants against quaternary ammonium compounds (*qacE-delta1*), heavy metal mercury (*mer* operon), phenicols (*catA1*), streptomycin (*aadA1*), and folate pathway antagonists (*sul1*) (PRJEB42815). This cassette was shown to be a known variant of the Tn*2670* transposon, originally identified in the resistance segment (R-det) on a *S. flexneri* R100 plasmid in the 1950s (Nakaya et al., 1960). The average coverage of the transposon was approximately half of the chromosome in all strains but Y72 where it remained 100% (**Supplementary Table 2**). To investigate if carrying Tn*2670* might come at a fitness cost, growth rates were measured to estimate generation time, but no difference could be observed when grown in TSB compared to Y11 at 26°C (**Supplementary Figure 1**). Tn*2670* is a self-transmissible transposon flanked by direct repeats IS1*a* and IS1*b* with two overlapping open-reading frames (ORFs) that create a transposase after translational frameshifting (Hanni et al., 1982; Womble and Rownd, 1988; Escoubas et al., 1994; Partridge and Hall, 2004; Partridge, 2011). As demonstrated in **Figure 2C**, Tn*2670* carries the identified *catA1* (chloramphenicol acyltransferase) and *ybjA* (acyl-CoA acyltransferase) as well as the entire movable Tn*21* transposon (Sun et al., 2016). Tn*21* is an independent transposon lined with two indirect imperfect

TABLE 1 | Strain characterization.

	Outbreak	Biovar/serotype	Pathogenicity score (families)	Resistance genes	Chromosomal secretion systems
Y11	NA	4/O:3	0.89 (96)	*vat*(F), *blaA*	T3SS (21 ORFs)
Y30	First	4/O:3	0.875 (98)	*sul1*, *aadA1*, *catA1*, *vat*(F), *blaA*	T2SS (*yts1O*), T3SS (21 ORFs)
Y72	Unknown	4/O:3	0.875 (99)	*sul1*, *aadA1*, *catA1*, *vat*(F), *blaA*, *tetB*	T2SS (*yts1O*), T3SS (21 ORFs)
Y108	Second	4/O:3	0.872 (95)	*sul1*, *aadA1*, *catA1*, *vat*(F), *blaA*	T2SS (*yts1O*), T3SS (21 ORFs)
Y_Mar	First[a]	4/O:3	0.875 (98)	*sul1*, *aadA1*, *catA1*, *vat*(F), *blaA*	T2SS (*yts1O*), T3SS (21 ORFs)
Y_May	Unknown	4/O:3	0.875 (98)	*sul1*, *aadA1*, *catA1*, *vat*(F), *blaA*	T2SS (*yts1O*), T3SS (21 ORFs)

[a]*As identified by this study.*

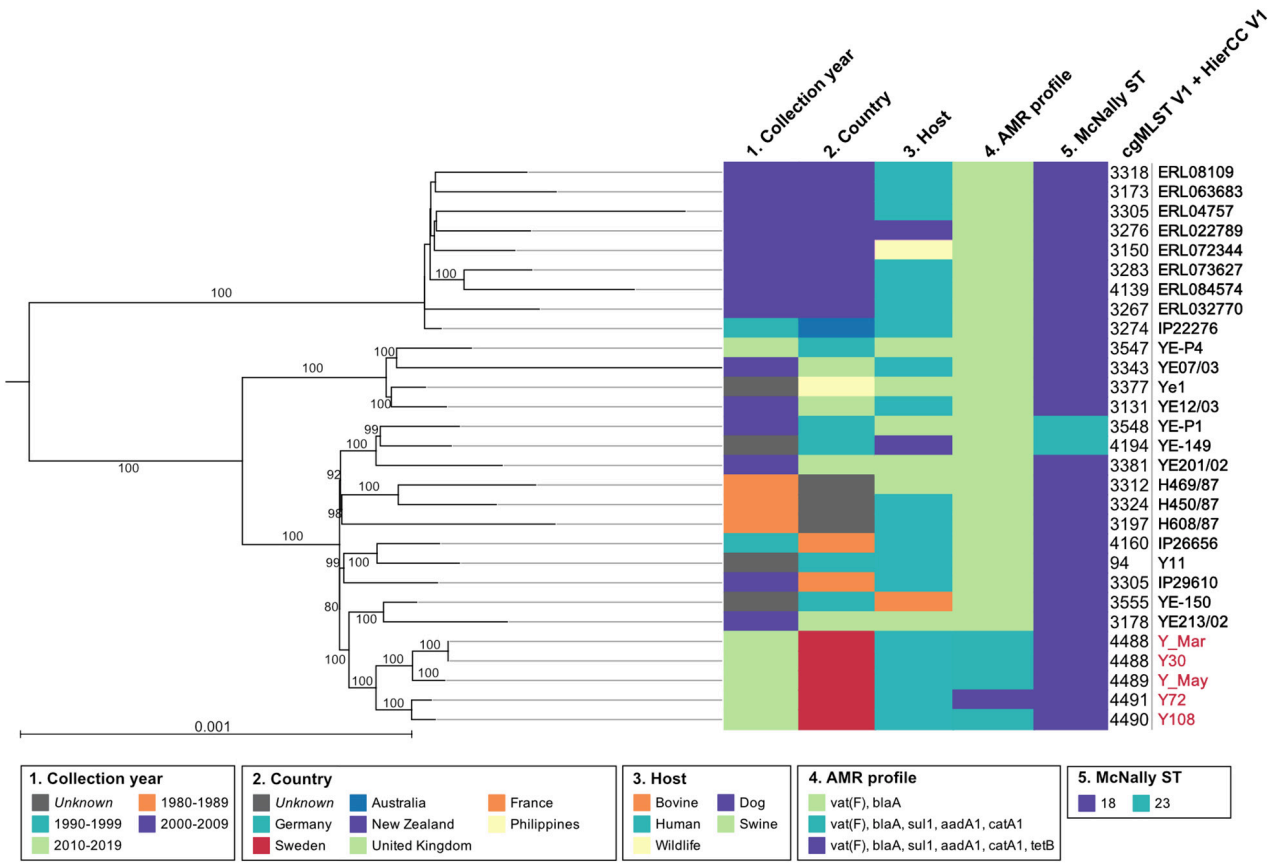

FIGURE 1 | Phylogeny of the 29 *Y. enterocolitica* 4/O:3. The maximum-likelihood tree is based on all polymorphic positions identified by cgMLST in 30 genomes, including and rooted in an isolate from biovar 2 used as an outgroup, and not shown in the tree. Numbers over branches are the percentage of ultrafast bootstraps supporting that branch; support values <80 are not represented. The scale represents the average number of substitutions per site. Metadata for collection year, country, host, AMR profile, McNally ST, and cgMLST V1 + HierCC V1 were presented using CLC Genomics Workbench.

repeats, IR_{tnp} (*tnp* genes for transposition) and IR_{mer} (*mer* operon for mercury resistance) (De la Cruz and Grinsted, 1982; Liebert et al., 1999). Inside Tn*21* lies the class I integron In2, bordered by the indirect imperfect repeats IR_i and IR_t, which on its own is not mobile due to a truncated *tniB* and lack of additionally required *tni* genes (Stokes and Hall, 1989; Brown et al., 1996). In2 can in turn be subdivided into three distinct regions: the integron as a unit, the "*aadA1* cassette," and an insertion sequence from a previous integron (IS*1326*). In2 can no longer transpose, but the integrase (*intI1*) can theoretically incorporate new genes at the *attI1* site, the location for adenyl transferase (*aadA1*), and dihydropteroate synthase (*sul1*) for streptomycin and sulfonamide resistance, respectively (Collis et al., 1993, 1998). The variant of Tn*2670* described here lacks IS*1353*, otherwise reported to locate inside IS*1326* (Grinsted and Brown, 1984; Brown et al., 1996) (**Figure 2C**).

The Tn*2670* transposon was compared with available sequences in NCBI using BLAST and analyzed using alignment percentage neighbor joining (**Figures 2A,B**). The transposon from Swedish strains clustered alone, but surrounded by *Escherichia* and *Shigella* carrying both plasmid-borne and chromosomally located sequence hits, mainly from plasmid types IncFII and IncB/O/K/Z. The cassette mainly matched sequences found in species of enteric bacteria, indicating interspecies spread and active dissemination of these determinants among *Enterobacteriaceae*.

TetB Plasmid

Along with the multidrug resistance cassette, our analysis revealed an additional plasmid carrying tetracycline resistance in strain Y72 with a coverage approximately 16 × that of the chromosome (**Supplementary Table 2**). The plasmid, hereafter termed pYE-tet (**Figure 3**), has a size of 5,681 bp and sporadically shares an approx. 5-kb segment with smaller plasmids identified in the family of *Pasteurellaceae*. The smaller plasmids have specifically been reported in *Actinobacillus pleuropneumoniae* (plasmid p780, MH457196.1), *Haemophilus parasuis* (plasmid pHPS1019, HQ622101.1), and *Pasteurella multocida* (plasmid pB1001, EU252517.1). These three plasmids were aligned with pYE-tet in **Figure 3**. The segment carries a suggested ORI transfer site (*traJ-II*), an ORI with four 21-bp iterons (4 × TTATACGACTAGAAATTTCCTG), a replication protein (*repA*), a tetracycline resistance gene (*tet*B), and five hypothetical proteins (San Millan et al., 2009; Li et al., 2018).

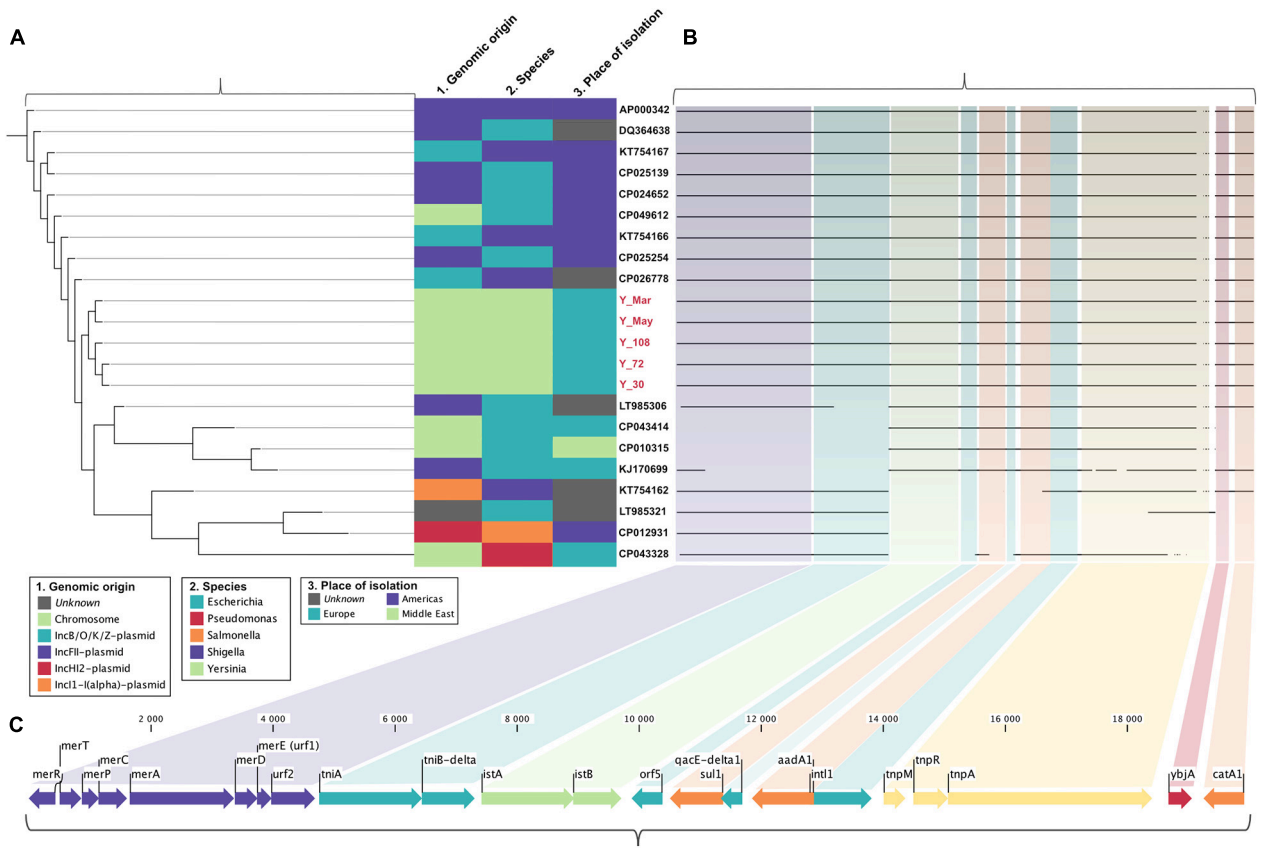

FIGURE 2 | Schematic representation of the multidrug-resistant Tn*2670* transposon in clinical *Y. enterocolitica* and of similar mobile elements identified through BLAST. Segments were generated in CLC Genomics Workbench and combined in Affinity Designer. **(A)** Alignment Percentage Neighbor Joining relationship tree based on pairwise comparison of sequence alignments. **(B)** Sequence alignment of the Tn*2670* transposon indicating missing segments. **(C)** The Tn*2670* transposon depicted as situated in the chromosome of *Y. enterocolitica* Y72. Arrows indicate the orientation of the ORFs. Antibiotic resistance determinants are portrayed in orange and the mercury resistance operon in purple.

The hypothetical protein nucleotide sequences were run through BLASTx and gave hits on another replication protein (*repB*) (qq: 100%, id: 100%, AXF94983.1), a vbH antitoxin (qq: 100%, id: 100%, WP_119774164.1), and a gene with partial agreement to the *E. coli* zeta toxin (qq: 94%, id: 36%, EFC6518787.1). An approx. 700-bp segment was identified as a pseudogene for an IS*1ab InsB* transposase shared with several families within the order Enterobacterales, including *Morganella morganii* (CP026651.1), *Shigella dysenteriae* (CP026778.1), *Klebsiella pneumoniae* (CP047701.1), *S. enterica* (CP049986.1), *Proteus vulgaris* (CP047346.1), *Citrobacter freundii* (CP047247.1), and *Enterobacter hormaechei* (LC590026.1). The *tet*B sequence was shared between both enteric bacteria and *Pasteurellaceae*.

Phenotypic Analysis of *Y. enterocolitica* Clinical Strains

Minimum inhibitory concentrations were assessed through broth microdilutions (**Table 2**). All outbreak strains were resistant to fusidic acid (>256 μg/ml), rifampicin (8 μg/ml), ampicillin (32 μg/ml), and erythromycin (64–128 μg/ml). Matching the characterized genotype was high-level resistance against chloramphenicol (>32 μg/ml), streptomycin (375–750 μg/ml), sulfamethoxazole (500 μg/ml), and tetracycline (32 μg/ml for Y72).

DISCUSSION

In the spring of 2019, the Swedish Public Health Agency and Statens Serum Institut in Denmark independently identified an outbreak caused by *Y. enterocolitica* 4/O:3. Sequence comparison and epidemiological investigation confirmed this cross-border outbreak, which was associated with imported fresh spinach. Here we determined the genome sequences, predicted virulence and pathogenicity factors, and charted the antimicrobial resistance profile of five *Y. enterocolitica* clinical isolates appearing during the time of the Swedish outbreaks. In comparison, we included a well-characterized clinical strain (Y11) isolated in Germany.

Of the five isolates, all expressed the virulence plasmid of *Yersinia* (pYV) along with genes commonly associated with infection, including the *myf* operon, *yadA*, *ail*,

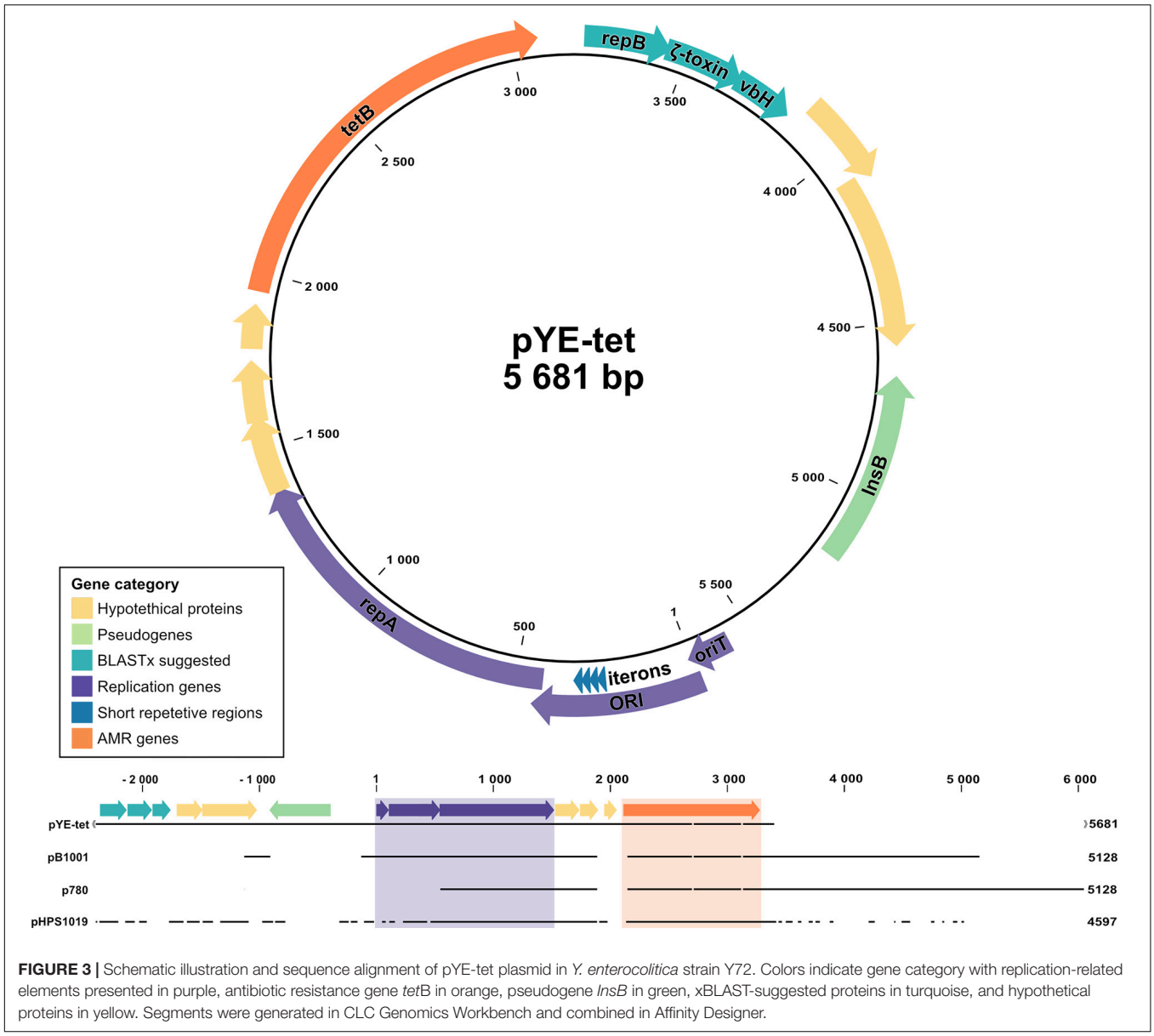

FIGURE 3 | Schematic illustration and sequence alignment of pYE-tet plasmid in *Y. enterocolitica* strain Y72. Colors indicate gene category with replication-related elements presented in purple, antibiotic resistance gene *tet*B in orange, pseudogene *lnsB* in green, xBLAST-suggested proteins in turquoise, and hypothetical proteins in yellow. Segments were generated in CLC Genomics Workbench and combined in Affinity Designer.

TABLE 2 | Minimum inhibitory concentrations (μg/ml) against routinely used antibiotics.

	FUS	RIF	AMP	CHL	STM	SMX	ERY	TET	CTX	CAZ	FOF	PIP	CIP	CST	GEN
Y11	>256	8	4	<8	<24	<8	4	<4	<2	<8	<32	<16	<0.5	<8	<4
Y30	>256	8	32	>32	375	500	64	<4	<2	<8	<32	<16	<0.5	<8	<4
Y72	>256	8	32	>32	375	500	128	32	<2	<8	<32	<16	<0.5	<8	<4
Y108	>256	8	32	>32	375	500	64	<4	<2	<8	<32	<16	<0.5	<8	<4
Y_Mar	>256	8	32	>32	750	500	64	<4	<2	<8	<32	<16	<0.5	<8	<4
Y_May	>256	8	32	>32	375	500	64	<4	<2	<8	<32	<16	<0.5	<8	<4

FUS: fusidic acid, RIF: rifampicin, AMP: ampicillin, CHL: chloramphenicol, STM: streptomycin, SMX: sulfamethoxazole, ERY: erythromycin, TET: tetracycline, CTX: cefotaxime, CAZ: ceftazidime, FOF: fosfomycin, PIP: piperacillin, CIP: ciprofloxacin, CST: colistin, GEN: gentamicin.

and *invA* (Bancerz-Kisiel et al., 2018). A predicted pathogenicity score averaged at 87.5% for chromosomally encoded genes when compared to 95–99 available families. All strains carried a Type II restriction enzyme (M.YenYEP1ORF12551P/M.YenY11ORF26101P) and a Type II methyltransferase (YenY11ORF26101P) along with a putative Type II restriction enzyme (M.SmaB3R3ORF2440P) and putative methyltransferase (Yen002ORF2900P). Our strains from 2019,

but not Y11, carried a prepilin-like peptidase (aspartic hydrolase), Yts1O, from the Type II Secretion System Yts1 (occasionally termed as Yst). The Yts1 Type II Secretion cluster has previously been linked to increased pathogenicity in *Y. enterocolitica*, but interestingly our outbreak strains only carried *yts1O*, without any of the remaining operon coding units for the Yts secreton. The Yts1 and Yts2 clusters have only recently been studied, and little is known about the function and molecular mechanisms related to the operons (Iwobi et al., 2003; Shutinoski et al., 2010; von Tils et al., 2012; Rusak et al., 2017). Prepilin peptidases share high sequence homology among bacteria and are known transmembrane proteins allocated to a wide set of functions including pilus biogenesis and Type II Secretion (Schreiber and Donnenberg, 2002; Dupuy et al., 2013). The exact role of Yts1O in the Yts1 cluster, and specifically here as a single enzyme, remains a pressing question for further investigation.

All five strains showed antimicrobial resistance to chloramphenicol (>32 μg/ml), streptomycin (375–750 μg/ml), and sulfamethoxazole (500 μg/ml), and strain Y72 additionally carried clinically relevant resistance against tetracycline (32 μg/ml). All outbreak strains, but not Y11, showed resistance against ampicillin (32 μg/ml) and erythromycin (64–128 μg/ml). While there is resistance against ampicillin and erythromycin which are two of the more commonly reported AMR phenotypes in 4/O:3, tetracycline resistance is only rarely reported (Fàbrega et al., 2015; Gkouletsos et al., 2019). Resistance against chloramphenicol, streptomycin, and sulfamethoxazole correlated well with the presence of *catA1*, *aadA1*, and *sul1* resistance genes situated on the chromosomally located Tn*2670* transposon (**Figure 2**). Moreover, resistance genes other than *vat*(*F*) and *blaA* appear rare incidents among 4/O:3 *Y. enterocolitica* (**Figure 1**; Pham et al., 1991; Stock et al., 1999; Seoane and García Lobo, 2000). This transposon, originally derived from the *S. flexneri* R100 plasmid (belonging to IncFII incompatibility group), has been shown transferable between plasmids *in vitro* by P7 phages (Nakaya et al., 1960; Hanni et al., 1982; Partridge and Hall, 2004). The internal transposon Tn*21* has been described widely spread among soil bacteria in both mercury-polluted and unpolluted sites and among clinically relevant Gram-negative bacteria, but to the best of our knowledge never before in *Yersinia* (Pearson et al., 1996; Liebert et al., 1999; Turner et al., 2003; Herrero et al., 2008; Partridge et al., 2018). Although both excision and loss rates are higher for composite transposons like Tn*2670* compared to isolated insertion sequences, it remains intriguing that the transposon coverage is about half that of the chromosome for all strains but one (Wagner, 2006; Sun et al., 2016). The fact that this known resistance cassette has never been reported in *Yersinia*, while found in all five independent isolates in this study, may indicate a new trend of antimicrobial resistance genes in European foodborne *Y. enterocolitica*.

The tetracycline resistance observed in strain Y72 was accompanied by a small *tet*B plasmid, pYE-tet, sharing genes with small plasmids from *Pasteurellaceae*. This pattern of genes on a small *tet*B plasmid was first reported in multidrug-resistant *P. multocida* (pB1001) from diseased pigs in Spain 2002–2005 (San Millan et al., 2009), later in *H. parasuis* (pHPS1019) from Chinese pigs in 2010 (Liu and He, 2010), and most recently in *A. pleuropneumoniae* (p780) from Brazilian pigs collected between 2006 and 2011 (Pereira et al., 2016; Li et al., 2018). Except for the more diverse zoonotic *P. multocida*, the previous carriers of such plasmids have all been bacteria restricted to swine. All three species colonize the upper respiratory tract of pigs where they share the same environmental niche as *Yersinia* (Oliveira and Pijoan, 2004; Reiner et al., 2010; Wilson and Ho, 2013). The *tet*B gene found on pYE-tet, pB1001, pHPS1019, and p780 has previously been speculated to originate from Gram-negative enteric bacteria, strengthened by previous horizontal gene transfer events of AMR genes from enterobacteria to *Pasteurellaceae* plasmids (Li et al., 2018; Michael et al., 2018). Both pB1001 and p780 have been described to lack standard mobilization genes (*mob*), and even though non-selectively and stably replicated in *Escherichia coli* following electroporation, no actual conjugation has been observed (San Millan et al., 2009; Li et al., 2018). Detailed work on the p780 plasmids revealed a 22-bp iteron Rep protein-binding site in ORI; although slightly different and one nucleotide shorter, this was also identified in pYE-tet (4 × TTATACGACTAGAAATTTCCTG). Preceding the ORI is, similar to p780, an ORI transfer site with a speculated secondary sRNA *traJ-II* structure with a *nic* cleavage site, suggesting specificity to TraJ-RP4 relaxomes (**Figure 3**). None of the plasmids encode their own relaxomes, and RP4 was previously illustrated insufficient for conjugation of the *Pasteurellaceae* plasmid in *E. coli*, leaving the mechanistic accounts behind plasmid transfer unexplained (Li et al., 2018).

Y30, isolated from the spinach-related outbreak, shared the same cgMLST as Y_Mar, which might well be related to the same outbreak. These two strains are in turn more similar to Y_May than to the two strains Y108 and Y72, supporting the previous notion that these in fact do not share the same spinach-related origin (**Figure 1**; National Veterinary Institute (SVA), 2019). Y72 carries the additional pYE-tet plasmid with a strong phylogenetic tie to species naturally occurring in the upper respiratory tract of swine, suggesting that this strain might be derived from an animal source instead. This proposition remains speculative, but further investigations on the stability (losing rate) and fitness cost of pYE-tet could provide valuable insight on time since parting with the HGT niche. The Swedish isolates may be of different origins, but all carry the Tn*2670* multidrug-resistant transposon, making it of even greater concern on food safety and public health. Although harboring an active large cassette (all strains) and the pYE-tet plasmid with a coverage approximately 16 × that of the chromosome (Y72), no growth rate differences could be observed as compared to Y11, which lacks all the mentioned elements. Our results suggest that these mobile elements do not come at a fitness cost at 26°C.

Neither the Tn*2670* transposon nor the small *tet*B resistance plasmid has been reported in foodborne *Yersinia* or in isolates derived from RTE products. The results presented here suggest horizontal gene transfer events in environment, agriculture, or animal husbandry, permitting *Y. enterocolitica* to be an additional foodborne carrier of multi-antibiotic and metal-resistance determinants. Prospective studies are needed to elucidate the mechanistic conjugative properties of pYE-tet, the

stability and potential mobility of the Tn*2670* transposon, and the prevalence of these elements in clinical and food-related *Yersinia*. Our study highlights the concerns of food-chain contamination as potential reservoir for transmission and dissemination of AMR, raising concerns on food safety and public health.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

PK and HW: conceptualization and writing—original draft. CJ, ET, PK, and HW: data curation. PK, LG, and HW: formal analysis. HW: funding acquisition. PK, CJ, and HW: investigation. PK, RH, LG, and HW: methodology. HW: project administration. CJ, ET, LG, JJ, and HW: validation. ET, CJ, RH, LG, JJ, and HW: writing—review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.664665/full#supplementary-material

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A Nitric Oxide-Responsive Transcriptional Regulator NsrR Cooperates With Lrp and CRP to Tightly Control the *hmpA* Gene in *Vibrio vulnificus*

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Nitric oxide (NO) is an important antimicrobial effector produced by the host innate immune system to counteract invading pathogens. To survive and establish a successful infection, a fulminating human pathogen *Vibrio vulnificus* expresses the *hmpA* gene encoding an NO dioxygenase in an NO-responsive manner. In this study, we identified an Rrf2-family transcriptional regulator NsrR that is predicted to contain the Fe-S cluster coordinated by three cysteine residues. Transcriptome analysis showed that NsrR controls the expression of multiple genes potentially involved in nitrosative stress responses. Particularly, NsrR acts as a strong repressor of *hmpA* transcription and relieves the repression of *hmpA* upon exposure to NO. Notably, *nsrR* and *hmpA* are transcribed divergently, and their promoter regions overlap with each other. Molecular biological analyses revealed that NsrR directly binds to this overlapping promoter region, which is alleviated by loss of the Fe-S cluster, leading to the subsequent derepression of *hmpA* under nitrosative stress. We further found that a leucine-responsive regulatory protein (Lrp) negatively regulates *hmpA* in an NsrR-dependent manner by directly binding to the promoter region, presumably resulting in a DNA conformation change to support the repression by NsrR. Meanwhile, a cyclic AMP receptor protein (CRP) positively regulates *hmpA* probably through repression of *nsrR* and *lrp* by directly binding to each promoter region in a sequential cascade. Altogether, this collaborative regulation of NsrR along with Lrp and CRP enables an elaborate control of *hmpA* transcription, contributing to survival under host-derived nitrosative stress and thereby the pathogenesis of *V. vulnificus*.

Keywords: *Vibrio vulnificus*, gene regulation, transcriptional regulator, nitric oxide dioxygenase, nitric oxide, nitrosative stress, stress response

INTRODUCTION

Nitric oxide (NO) is a highly reactive, toxic, and membrane-permeable radical gas. As one of the major components of the host innate immune system, NO is produced by inducible NO synthase (iNOS) which is expressed in phagocytes and epithelial cells under infectious conditions (Fang, 2004; Wang et al., 2010). NO produced by iNOS can subsequently be converted into other toxic

reactive nitrogen species (RNS) such as nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$), and dinitrogen trioxide (N_2O_3) which impose the nitrosative stress on pathogens (Fang, 2004; Stern and Zhu, 2014). Furthermore, intestinal commensals can reduce nitrate (NO_3^-) in the diet to nitrite (NO_2^-), which interacts with gastric acid, resulting in RNS that act as antimicrobial barriers against ingested enteric pathogens (Sobko et al., 2005; Tiso and Schechter, 2015). RNS can cause damage to cellular components, including the metal centers of proteins, membrane lipids and nucleotide bases, and thereby inhibit respiration and interfere with the DNA replication of pathogens (Fang, 2004). Therefore, pathogens have evolved sophisticated mechanisms to sense the increased level of RNS and express the proper genes to overcome nitrosative stress in a host (Bang et al., 2006; Stern et al., 2012).

To understand the NO-responsive gene expression in pathogens, numerous transcriptional regulators have been characterized (Spiro, 2007). Among them, two transcriptional regulators, NorR and NsrR, are known to have focused functions on sensing NO in a wide range of bacteria (Stern and Zhu, 2014). NorR directly recognizes NO using its non-heme iron center and controls the expression of genes for NO detoxification: *norVW* in *Escherichia coli* and *hmpA* and *nnrS* in *Vibrio cholerae* (D'Autreaux et al., 2005; Stern et al., 2012). Meanwhile, NsrR uses an iron-sulfur (Fe-S) cluster as a cofactor to directly sense NO and regulates a variety of genes involved in NO detoxification and NO damage repair, particularly *hmpA* in *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Streptomyces coelicolor* (Bang et al., 2006; Spiro, 2007; Tucker et al., 2008). The NO-responsive Fe-S cluster is coordinated to NsrR by three cysteine residues which are widely conserved in various bacterial NsrR (Tucker et al., 2010). A recent study showed that both [2Fe-2S] and [4Fe-4S] clusters can be coordinated to *S. coelicolor* NsrR (Crack et al., 2015). Upon exposure to NO, the Fe-S cluster is nitrosylated, forming the iron-nitrosyl species such as dinitrosyl iron complex (DNIC), Roussin's Red Ester (RRE), and Roussin's Black Salt (RBS) (Serrano et al., 2016; Crack and Le Brun, 2019). The resulting apo-NsrR lacking an intact Fe-S cluster shows a distinct protein conformation from that of holo-NsrR, leading to loss of DNA-binding activity and the subsequent derepression of its regulons (Crack et al., 2015; Volbeda et al., 2017). NsrR, as a homodimer, binds to the consensus NsrR-binding site consisting of inverted repeats of two 11 bp motifs (AAxATGCATTT; x, any nucleotide) separated by 1 bp spacing (Partridge et al., 2009; Crack et al., 2015).

The opportunistic human pathogen *Vibrio vulnificus* is a causative agent of foodborne diseases from mild gastroenteritis to primary septicemia (Jones and Oliver, 2009; Baker-Austin and Oliver, 2018). During infection, *V. vulnificus* exploits various transcriptional regulators to sense host-derived signals and modulate the expression of its virulence genes (Miller et al., 1989; Fang et al., 2016). Particularly, a leucine-responsive regulatory protein (Lrp) and a cyclic AMP receptor protein (CRP) are widely conserved and well-characterized global transcriptional regulators in bacteria (Cho et al., 2008; Manneh-Roussel et al., 2018). Lrp controls diverse cellular functions including amino acid metabolism, stress resistance, and virulence (Jeong et al., 2003; Rhee et al., 2008; Lee et al., 2020). The regulatory activity of Lrp on its regulons can be enhanced, reversed, or unaffected by the binding of a small effector molecule leucine (Cho et al., 2008). CRP is a central regulator of carbon and energy metabolism that forms a complex with cyclic AMP (cAMP) (Kim et al., 2011; Lee et al., 2020). In the absence of glucose, the intracellular cAMP level is increased by adenylate cyclase and the resulting cAMP-CRP complex binds DNA to regulate gene expression (Manneh-Roussel et al., 2018). In this way, Lrp and CRP coordinate the expression of genes involved in metabolism and pathogenesis in response to changing environmental conditions such as nutrient availability.

Like many other enteropathogenic bacteria, *V. vulnificus* is inevitably exposed to host-derived nitrosative stress in the course of infection. We recently reported that a multidomain NO dioxygenase HmpA is highly expressed in *V. vulnificus* exposed to NO (Kim et al., 2019). HmpA belongs to the flavohemoglobin family composed of the N-terminal heme-binding globin domain and the C-terminal NAD- and FAD-binding oxidoreductase domain, and detoxifies NO by oxidizing it to a less toxic NO_3^- under aerobic conditions (Bonamore and Boffi, 2008; Forrester and Foster, 2012; Kim et al., 2019). Because the *in vitro* NO-decomposition activity of *V. vulnificus* is mostly dependent on HmpA, it has a significant role in the survival and pathogenesis of *V. vulnificus* under nitrosative stress in a host (Kim et al., 2019). Nevertheless, definitive regulatory mechanisms and transcriptional regulators, by which *V. vulnificus* senses NO and induces HmpA, have not been yet elucidated in detail. In this study, we newly identified NsrR in *V. vulnificus* as an NO-responsive transcriptional regulator. The transcriptome analysis of the wild-type and isogenic *nsrR*-deletion mutant (Δ*nsrR*) strains revealed that NsrR controls the expression of 47 genes. Notably, *hmpA* was the most highly induced gene by the *nsrR* deletion, indicating that NsrR acts as a strong repressor of *hmpA*. To investigate the exact mechanism by which NsrR regulates *hmpA* expression, the *hmpA* transcript levels were compared in the wild-type and Δ*nsrR* strains under nitrosative stress *in vitro* and *ex vivo*. Furthermore, the combined effect of NsrR, Lrp, and CRP on *hmpA* expression was analyzed at the molecular level. In conclusion, this study suggests that NsrR tightly regulates *hmpA* transcription in response to nitrosative stress together with Lrp and CRP, contributing to the survival and overall success of *V. vulnificus* during host infection.

RESULTS

Genome and Transcriptome Analyses Identified NsrR in *V. vulnificus*

We previously reported that NO-induced HmpA encoded by VVMO6_RS01375 is crucial for survival under host-derived nitrosative stress and pathogenesis of *V. vulnificus* during infection (Kim et al., 2019). Notably, we further found that the expression of VVMO6_RS01380, which is divergently transcribed from *hmpA* (**Figure 1A**), is also induced by NO (Kim et al., 2019). VVMO6_RS01380 encodes an Rrf2-family transcriptional regulator showing an amino acid sequence homology to *E. coli* NsrR, *S.* Typhimurium NsrR, and *S. coelicolor*

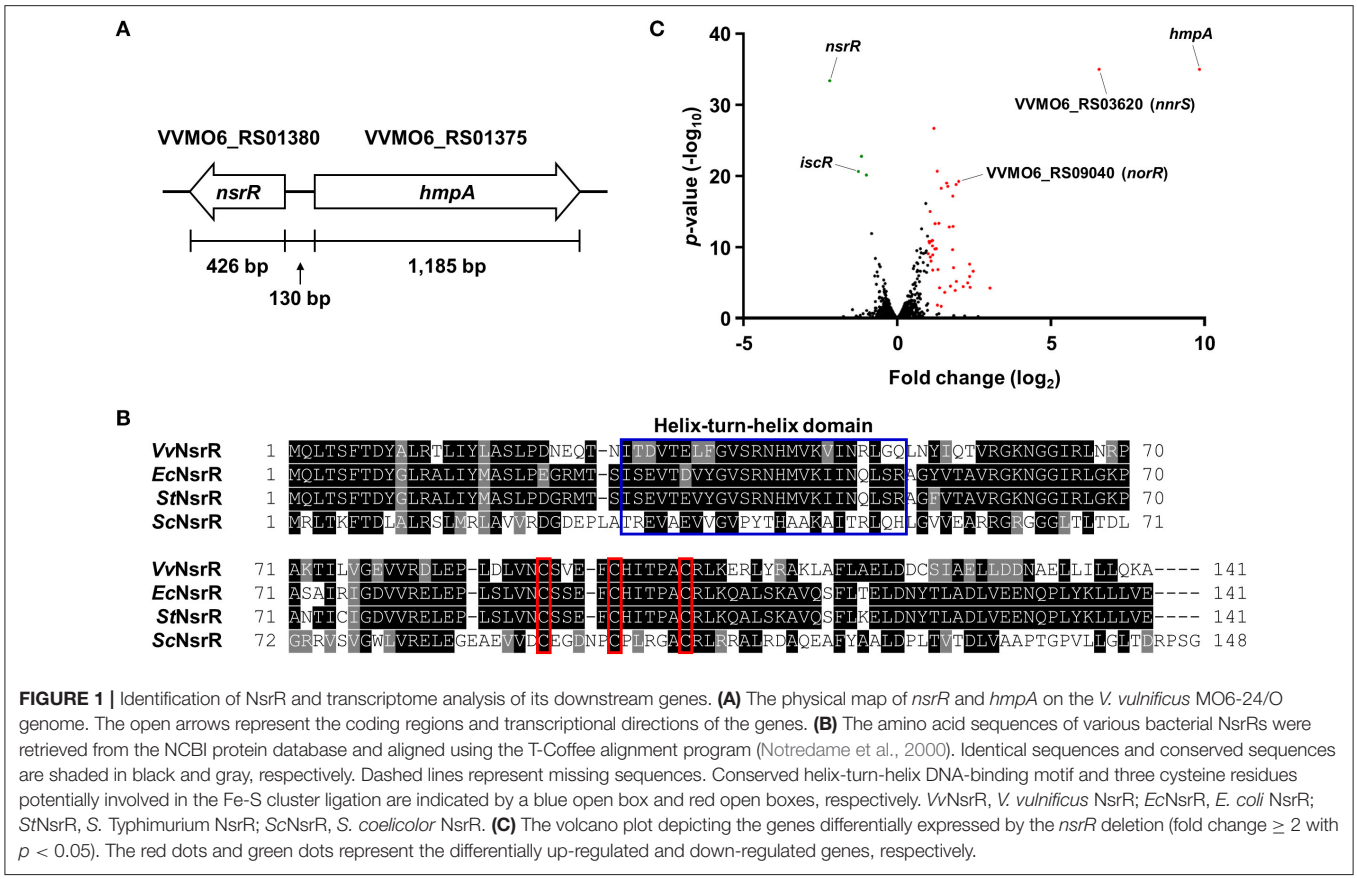

FIGURE 1 | Identification of NsrR and transcriptome analysis of its downstream genes. **(A)** The physical map of *nsrR* and *hmpA* on the *V. vulnificus* MO6-24/O genome. The open arrows represent the coding regions and transcriptional directions of the genes. **(B)** The amino acid sequences of various bacterial NsrRs were retrieved from the NCBI protein database and aligned using the T-Coffee alignment program (Notredame et al., 2000). Identical sequences and conserved sequences are shaded in black and gray, respectively. Dashed lines represent missing sequences. Conserved helix-turn-helix DNA-binding motif and three cysteine residues potentially involved in the Fe-S cluster ligation are indicated by a blue open box and red open boxes, respectively. *Vv*NsrR, *V. vulnificus* NsrR; *Ec*NsrR, *E. coli* NsrR; *St*NsrR, *S.* Typhimurium NsrR; *Sc*NsrR, *S. coelicolor* NsrR. **(C)** The volcano plot depicting the genes differentially expressed by the *nsrR* deletion (fold change ≥ 2 with $p < 0.05$). The red dots and green dots represent the differentially up-regulated and down-regulated genes, respectively.

NsrR (61, 62, and 35% identity, respectively) (**Figure 1B**). Moreover, the protein encoded by VVMO6_RS01380 contains three conserved cysteine residues, C91, C96, and C102, which are known to be essential for the Fe-S cluster ligation of Rrf2-family transcriptional regulators (**Figure 1B**) (Volbeda et al., 2017). This observation led us to designate the VVMO6_RS01380 gene product as an Fe-S cluster-containing transcriptional regulator NsrR.

For the comprehensive identification of NsrR-regulated genes in *V. vulnificus*, the transcriptomes of the wild-type and Δ*nsrR* strains were compared by RNA-seq. The transcriptome analysis revealed that, in total, 47 genes were differentially expressed by the *nsrR* deletion: 44 genes were up-regulated and 3 genes were down-regulated (**Figure 1C**, **Supplementary Table 1**). The overall fold changes of the up-regulated genes were greater than those of the down-regulated genes. This result implies that NsrR serves mainly as a repressor rather than as an activator. Intriguingly, the up-regulated genes included several genes that are predicted to encode proteins involved in the defense against nitrosative stress such as NO dioxygenase HmpA, NO detoxification protein NnrS, NO_2^- reductase large subunit, NO_2^- reductase small subunit, cytochrome *c* NO_2^- reductase subunit c_{552} NrfA, and NO reductase transcriptional regulator NorR (Stern et al., 2012; Kim et al., 2019). Among them, *hmpA* was the most highly up-regulated gene in the Δ*nsrR* strain (**Figure 1C**), suggesting that NsrR is a strong repressor of *hmpA* expression. Meanwhile, the down-regulated genes, *iscR*, *iscS*, and *iscU*, constitute the *isc* operon (*iscRSUA-hscBA-fdx*) encoding proteins required for the biogenesis of the Fe-S cluster (Lim and Choi, 2014). Taken together, this result shows that NsrR controls the expression of multiple genes involved in nitrosative stress responses, especially *hmpA*.

hmpA Transcription Is Derepressed by NsrR in Response to NO

To validate the RNA-seq results and examine whether NsrR mediates the induction of *hmpA* in response to NO, the *hmpA* transcript levels in the wild-type and Δ*nsrR* strains were compared under nitrosative stress *in vitro* and *ex vivo*. The *hmpA* transcript level in the wild-type strain was significantly elevated upon exposure to an *in vitro* NO donor, NO/PPNPs (NO-releasing poly(lactic-*co*-glycolic acid)-polyethylenimine nanoparticles) (**Figure 2A**) (Nurhasni et al., 2015). This result confirms our previous observation that *hmpA* is induced by NO (Kim et al., 2019). Additionally, the *hmpA* transcript level was dramatically increased in the Δ*nsrR* strain compared with that in the wild-type strain even in the absence of NO/PPNPs (**Figure 2A**), verifying that NsrR negatively regulates *hmpA*. Strikingly, the *hmpA* transcript level in the Δ*nsrR* strain was not affected by the addition of NO/PPNPs (**Figure 2A**), indicating that NsrR recognizes NO and alleviates the repression of *hmpA* expression *in vitro*.

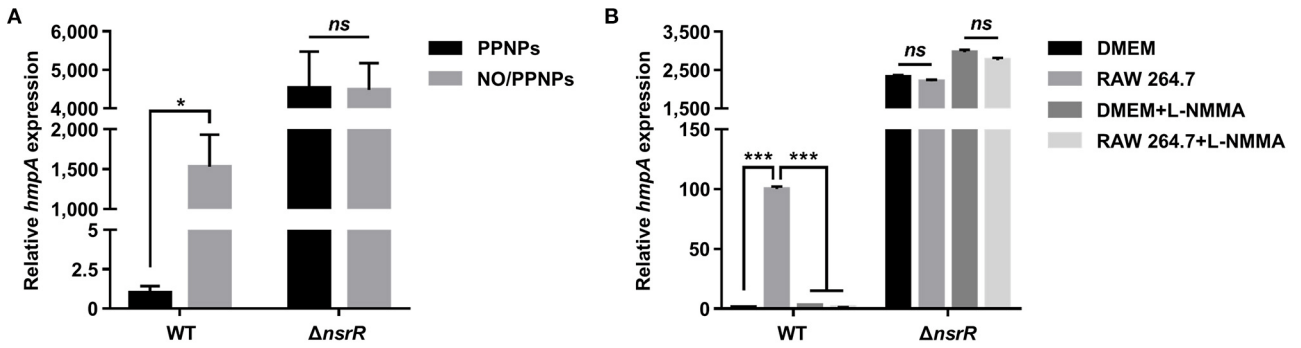

FIGURE 2 | The effect of nitrosative stress and the *nsrR* mutation on *hmpA* transcription *in vitro* and *ex vivo*. The wild-type and Δ*nsrR* strains were grown aerobically to an A_{600} of 0.5, and then exposed to 0.15 mg/ml NO/PPNPs *in vitro* **(A)** or NO-producing RAW 264.7 cells *ex vivo* in the presence or absence of L-NMMA **(B)** for 10 min. The *hmpA* transcript levels were determined by qRT-PCR, and the *hmpA* transcript levels in the wild-type strain exposed to PPNPs **(A)** or DMEM without L-NMMA **(B)** were set to 1. Error bars represent the SD. Statistical significance was determined by the Student's *t*-test (*$p < 0.05$; ***$p < 0.0005$; *ns*, not significant). WT, wild type; Δ*nsrR*, *nsrR*-deletion mutant.

The role of NsrR in *hmpA* expression was further investigated *ex vivo* using NO-producing murine macrophage RAW 264.7 cells. As shown in **Figure 2B**, the *hmpA* transcript level in the wild-type strain exposed to NO-producing RAW 264.7 cells was considerably elevated compared with that exposed to Dulbecco's modified Eagle's medium (DMEM; negative control). The extent of the increase in the *hmpA* transcript level upon exposure to the RAW 264.7 cells diminished by the addition of the NO synthase inhibitor L-N^G-monomethyl arginine citrate (L-NMMA) (**Figure 2B**). This result suggests that the *hmpA* induction upon exposure to RAW 264.7 cells is attributable to NO produced by the murine macrophages. In contrast, the highly increased *hmpA* transcript level in the Δ*nsrR* strain was not altered by the RAW 264.7 cells and L-NMMA (**Figure 2B**), confirming that NsrR mediates the derepression of *hmpA* under nitrosative stress derived from host immune cells. The combined results show that NsrR has a critical role to sense NO and to induce the *hmpA* expression both *in vitro* and *ex vivo*.

Then, we examined whether the introduction of recombinant *nsrR* can reduce the increased *hmpA* transcript level in the Δ*nsrR* strain. Introduction of a *nsrR*-expressing plasmid significantly decreased the *hmpA* transcript level, although it was not comparable with that in the wild-type strain (**Supplementary Figure 1A**). One possible explanation for this lack of complementation is that the recombinant NsrR expressed from the exogenous plasmid is less functional for unknown reasons. On the other hand, ectopic expression of *nsrR* on the chromosome effectively reduced the *hmpA* transcript level comparable with that in the wild-type strain (**Supplementary Figure 1B**). Similarly, the HmpA protein levels in the Δ*nsrR* strain were highly increased compared with those in the wild-type strain and significantly decreased by complementation (**Supplementary Figures 1C,D**). Altogether, the results suggest that NsrR is a major transcriptional regulator that recognizes NO and regulates *hmpA* expression mainly at the transcription level.

Three Conserved Cysteine Residues Are Essential for NsrR to Regulate *hmpA* and *nsrR*

As shown in **Figure 1B**, *V. vulnificus* NsrR contains three conserved cysteine residues (C91, C96, and C102) that are predicted to act as ligands of the NO-responsive Fe-S cluster (Tucker et al., 2008; Volbeda et al., 2017). To investigate the role of these three cysteine residues, three different strains were constructed: a parent strain GR204 chromosomally encoding 3×FLAG-tagged NsrR ($NsrR^{FLAG}$), an isogenic *nsrR*-deletion mutant, and an isogenic $nsrR_{3CS}$ mutant chromosomally encoding apo-locked $NsrR^{FLAG}$ ($NsrR^{FLAG}_{3CS}$) (see see Materials and Methods for a detailed description). The *hmpA* transcript and HmpA protein levels in the Δ*nsrR* strain were highly elevated compared with those in the parent strain (**Figures 3A,B**), indicating that $NsrR^{FLAG}$ in the parent strain is still functional as a repressor of *hmpA*. Notably, the *hmpA* transcript and HmpA protein levels in the $nsrR_{3CS}$ strain were comparable with those in the Δ*nsrR* strain (**Figures 3A,B**). Moreover, a similar effect of the mutation in the three cysteine residues and the *nsrR* deletion on *hmpA* expression was observed in the wild-type background (**Supplementary Figure 1E**). These results that $NsrR_{3CS}$ cannot repress the *hmpA* transcription reveal that coordination of the Fe-S cluster by the three cysteine residues is essential for the NsrR activity to repress *hmpA*.

Furthermore, the $NsrR^{FLAG}_{3CS}$ protein level in the $nsrR_{3CS}$ strain was significantly elevated compared with the $NsrR^{FLAG}$ protein level in the parent strain (**Figure 3B**). This observation prompted us to examine the activity of the *nsrR* promoter (P_{nsrR}, determined in **Figures 4B,C**) in the wild-type, Δ*nsrR*, and $nsrR_{3CS}$ strains using the P_{nsrR}-*luxCDABE* transcriptional fusion reporter. The P_{nsrR} activity in the Δ*nsrR* strain was higher than that in the wild-type strain (**Figure 3C**), demonstrating that NsrR represses its own transcription. Additionally, the increased P_{nsrR} activity in the $nsrR_{3CS}$ strain was comparable with that in the Δ*nsrR* strain (**Figure 3C**). This result suggests that NsrR relieves the repression of its own transcription by the mutation in the

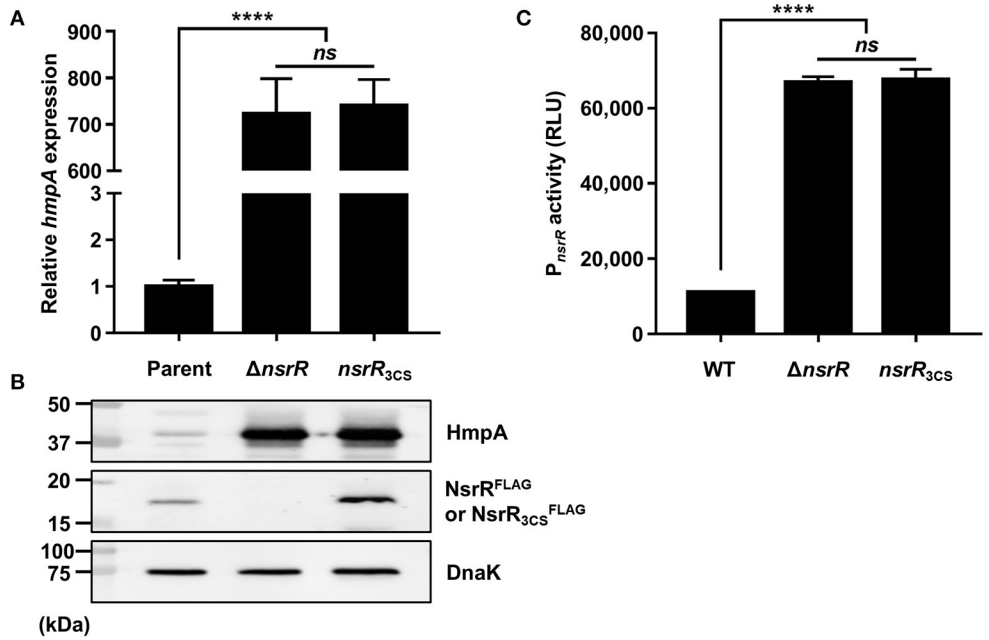

FIGURE 3 | The role of the three cysteine residues in NsrR on *hmpA* and *nsrR* transcription. **(A,B)** Total RNA and proteins were isolated from the parent and mutant strains grown aerobically to an A_{600} of 0.5. **(A)** The *hmpA* transcript levels were determined by qRT-PCR, and the *hmpA* transcript level in the parent strain was set to 1. **(B)** The cellular HmpA, $NsrR^{FLAG}$ or $NsrR^{FLAG}_{3CS}$, and DnaK (internal control) protein levels were determined by Western blot analysis. Molecular size markers (Bio-Rad) are shown in kDa. Parent, parent strain; Δ*nsrR*, *nsrR*-deletion mutant; $nsrR_{3CS}$, strain expressing apo-locked $NsrR^{FLAG}$. **(C)** A PCR fragment carrying the P_{nsrR} was cloned into pBBR-lux to create a reporter plasmid, pGR2025. The wild-type and mutant strains containing pGR2025 were grown aerobically to an A_{600} of 0.5, and then used to measure the cellular luminescence. Error bars represent the SD. Statistical significance was determined by the Student's *t*-test ($^{****}p < 0.00005$; *ns*, not significant). RLU, relative luminescence unit; WT, wild type; Δ*nsrR*, *nsrR*-deletion mutant; $nsrR_{3CS}$, strain expressing apo-locked NsrR.

three cysteine residues and the consequent loss of the Fe-S cluster. Combined with the previous data (**Figure 2**), we propose a model in which holo-NsrR containing the Fe-S cluster represses both *hmpA* and *nsrR* transcription, shifts to the clusterless apo-form under nitrosative stress, and then alleviates the repression of *hmpA* and *nsrR* transcription.

P_{hmpA} and P_{nsrR} Overlap Divergently With Each Other

To map the *hmpA* promoter, the transcription start site (TSS) of *hmpA* was determined by primer extension analysis. A single reverse transcript was produced from the primer extension of RNA isolated from the Δ*nsrR* strain grown to an A_{600} of 0.5 (**Figure 4A**). This reverse transcript observed in the Δ*nsrR* strain was not detected in the wild-type strain (**Figure 4A**), confirming that the *hmpA* transcription is strongly repressed by NsrR. The 5′-end of *hmpA* was located 57-bp upstream of the translation start codon of *hmpA*. Next, the TSS of *nsrR* was determined in a similar way. A single reverse transcript was produced from the primer extension of RNA isolated from the wild-type strain grown to an A_{600} of 0.5 (**Figure 4B**). The 5′-end of *nsrR* was located 18-bp upstream of the translation start codon of *nsrR*. The putative promoters constituting the TSSs were named P_{hmpA} and P_{nsrR} to represent the *hmpA* promoter and the *nsrR* promoter, respectively. The sequences for putative −10 and −35 regions of each promoter were assigned based on the similarity to the consensus sequences of *E. coli* σ^{70} promoters (**Figure 4C**). Strikingly, these results show that P_{hmpA} and P_{nsrR} overlap with each other. This overlapping promoter region was termed the *nsrR-hmpA* regulatory region for our further research.

NsrR Directly Binds to the *nsrR-hmpA* Regulatory Region to Repress *hmpA* and Its Own Expression

To investigate whether NsrR directly binds to the *nsrR-hmpA* regulatory region, electrophoretic mobility shift assays (EMSAs) were performed. The addition of NsrR to 6-carboxyfluorescein (6-FAM)-labeled DNA probe encompassing the *nsrR-hmpA* regulatory region resulted in a single retarded band in an NsrR concentration-dependent manner (**Figure 5A**). The same unlabeled DNA fragment competed for NsrR binding in a dose-dependent manner (**Figure 5A**), confirming the specific binding of NsrR. Then, the binding of $NsrR_{3CS}$ to the *nsrR-hmpA* regulatory region was compared with that of NsrR. The amount of the retarded band of the DNA-$NsrR_{3CS}$ complex was reduced compared with that of the DNA-NsrR complex (**Figure 5B**). This result implies that the DNA-binding affinity of $NsrR_{3CS}$ is considerably lower than that of NsrR, which leads to the derepression of *hmpA* and *nsrR* under nitrosative stress.

To determine the precise location of NsrR-binding site(s) in the *nsrR-hmpA* regulatory region, DNase I protection assays were performed using the same DNA probe. When NsrR was added

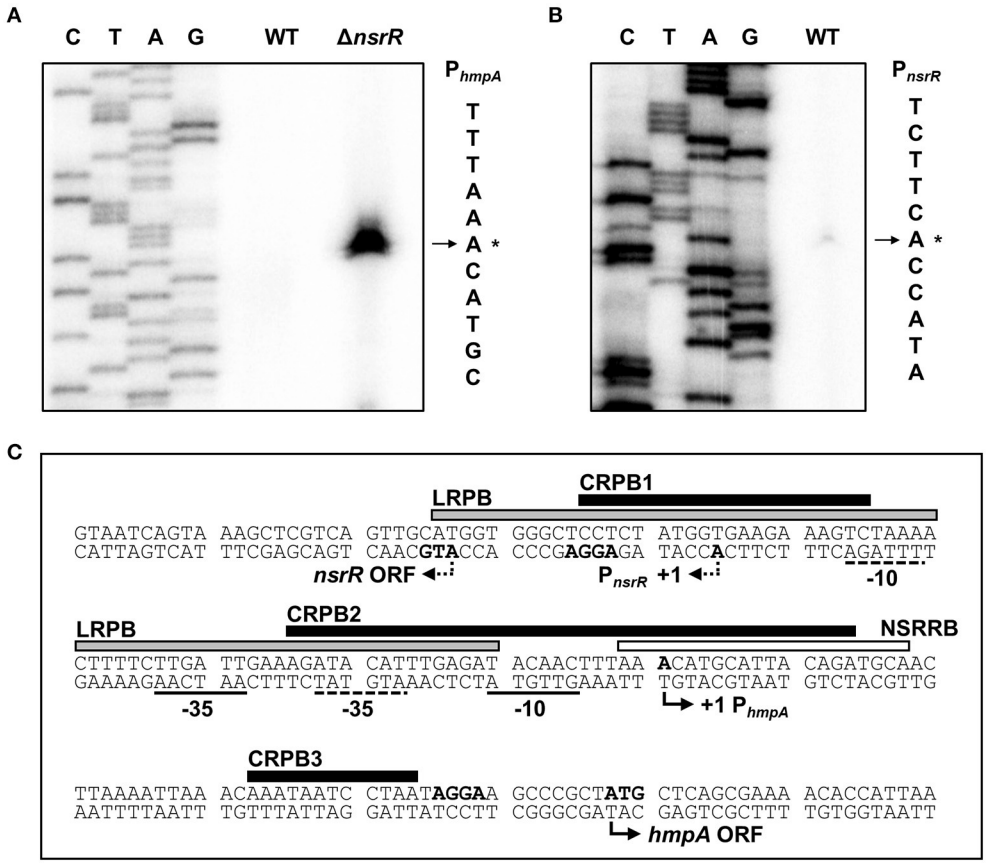

FIGURE 4 | Sequence analysis of the *nsrR-hmpA* regulatory region. **(A,B)** The TSSs of *hmpA* **(A)** and *nsrR* **(B)** were determined by the primer extension of RNA isolated from the wild-type and Δ*nsrR* strains grown aerobically to an A_{600} of 0.5. Lanes C, T, A, and G represent the nucleotide sequencing ladders. The asterisks indicate the TSSs. WT, wild type; Δ*nsrR*, *nsrR*-deletion mutant. **(C)** Double-stranded DNA sequence of the *nsrR-hmpA* regulatory region is shown. The TSS and putative translation start codon of *nsrR* are indicated by dashed bent arrows, and those of *hmpA* are indicated by solid bent arrows. The putative −10 and −35 regions are underlined with dashed lines for P_{nsrR} and solid lines for P_{hmpA}. The putative ribosome-binding sites (AGGA) are boldface. The binding sequences of NsrR (NSRRB; a white box), Lrp (LRPB; a gray box), and CRP (CRPB1, CRPB2, CRPB3; black boxes) were determined in the later parts of this study.

to the DNA probe, NsrR protected a single region extending from −2 to +18 (NSRRB, centered at +8.5 from the TSS of *hmpA*) from DNase I digestion (**Figures 4C**, **5C**). The sequence of NSRRB showed about 87% similarity to the 11-1-11 bp consensus NsrR-binding sequence in *E. coli* (Bodenmiller and Spiro, 2006; Partridge et al., 2009). Combined with the EMSA data (**Figure 5A**), these results indicate that NsrR concurrently represses *hmpA* and its own transcription by directly binding to the single specific sequence in the *nsrR-hmpA* regulatory region.

Lrp Represses *hmpA* in an NsrR-Dependent Manner by Directly Binding to the *nsrR-hmpA* Regulatory Region

To determine other factors involved in the *hmpA* regulation, we further explored various known transcriptional regulators in *V. vulnificus*. Among them, the role of Lrp in the *hmpA* regulation was evaluated. The *hmpA* transcript and HmpA protein levels in the *lrp*-deletion mutant (Δ*lrp*) were significantly increased compared with those in the parent strain and restored by complementation (**Figures 6A,B**). The *hmpA* transcript level in the wild-type strain was not altered by exogenous leucine (**Supplementary Figure 2A**), suggesting that Lrp negatively affects the *hmpA* transcription in a leucine-independent manner. To investigate the regulatory relationship between NsrR and Lrp, the *lrp*-deleted $nsrR_{3CS}$ mutant ($nsrR_{3CS}\Delta lrp$), in which both NsrR and Lrp are not functional, was constructed from the parent strain. Interestingly, the *hmpA* transcript and HmpA protein levels in the $nsrR_{3CS}\Delta lrp$ strain were comparable with those in the $nsrR_{3CS}$ strain (**Figures 6C,D**). The observation that Lrp was not able to affect *hmpA* transcription in the absence of functional NsrR indicates that the negative effect of Lrp on *hmpA* is mediated by NsrR. This result led us to examine whether Lrp positively regulates the cellular level of NsrR to repress *hmpA*. However, both the P_{nsrR} activity and $NsrR^{FLAG}$ protein level were not affected by the *lrp* deletion (**Supplementary Figure 2B**, **Figure 6B**).

Next, EMSAs were performed to investigate whether Lrp directly binds to the *nsrR-hmpA* regulatory region. The addition of Lrp to the DNA probe resulted in a single retarded band

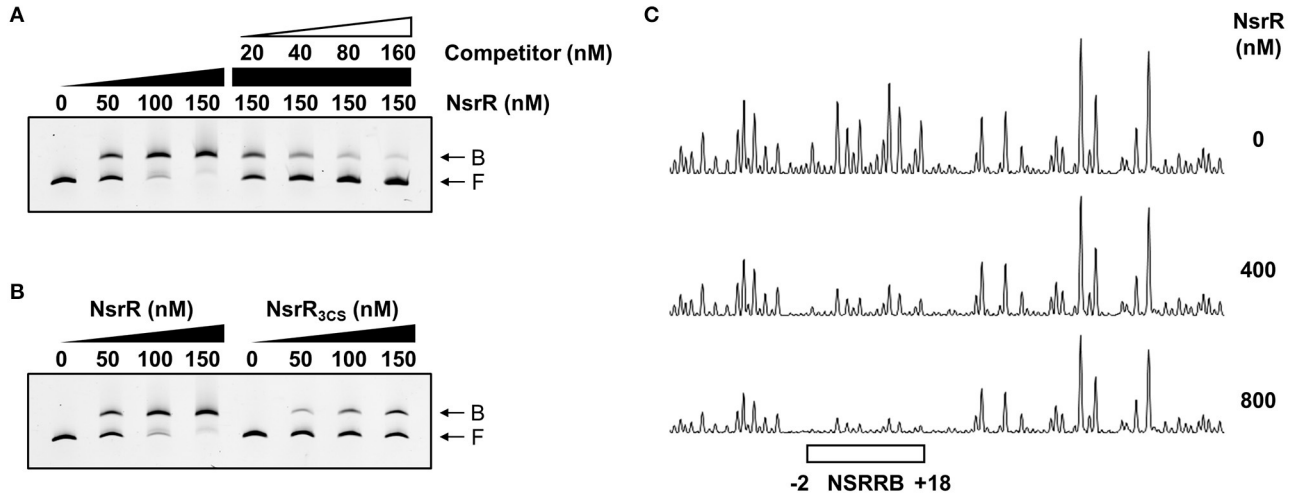

FIGURE 5 | Specific binding of NsrR to the *nsrR-hmpA* regulatory region. **(A,B)** A 393-bp DNA fragment of the *nsrR-hmpA* regulatory region (10 nM) was labeled with 6-FAM, and then incubated with increasing amounts of NsrR **(A,B)** or $NsrR_{3CS}$ **(B)** as indicated. For the competition analysis, various amounts of the unlabeled DNA fragment were added as a self-competitor. B, bound DNA; F, free DNA. **(C)** The same DNA probe (40 nM) was incubated with increasing amounts of NsrR as indicated, and then digested with DNase I. The region protected by NsrR is indicated by a white box (NSRRB). Nucleotide numbers shown are relative to the TSS of *hmpA*.

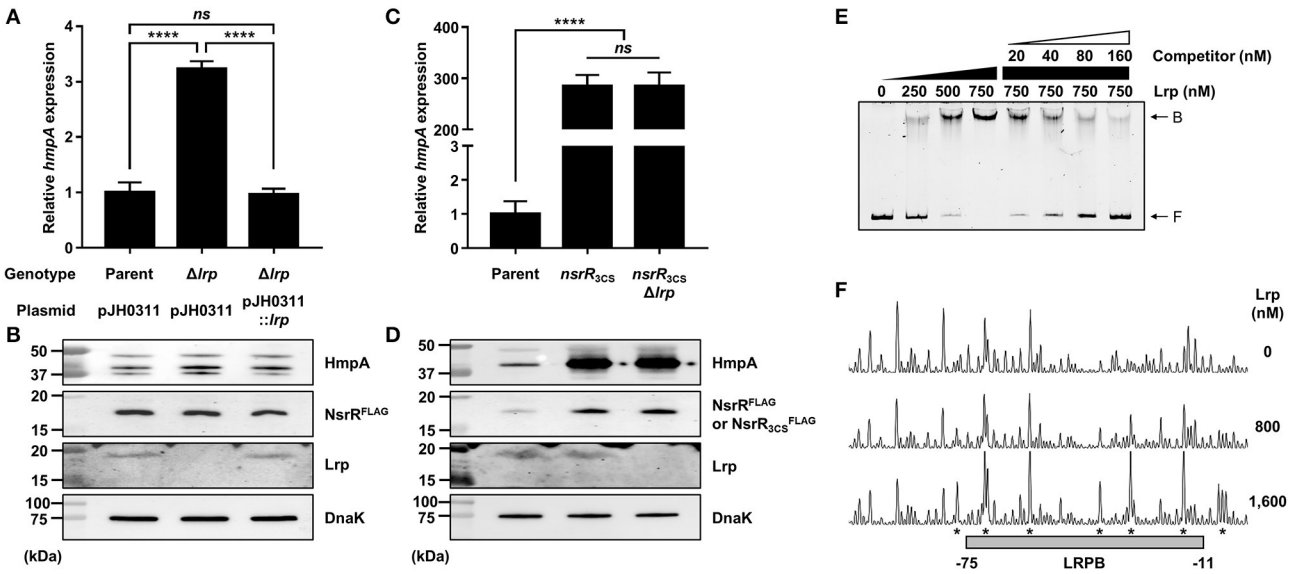

FIGURE 6 | The effect of the *lrp* mutation on *hmpA* transcription and the specific binding of Lrp to the *nsrR-hmpA* regulatory region. **(A–D)** Total RNA and proteins were isolated from the parent and mutant strains grown aerobically to an A_{600} of 0.5. **(A,C)** The *hmpA* transcript levels were determined by qRT-PCR, and the *hmpA* transcript levels in the parent strain were set to 1. Error bars represent the SD. Statistical significance was determined by the Student's *t*-test ($^{****}p < 0.00005$; *ns*, not significant). **(B,D)** The cellular HmpA, $NsrR^{FLAG}$ or $NsrR_{3CS}^{FLAG}$, Lrp, and DnaK (internal control) protein levels were determined by Western blot analysis. Molecular size markers (Bio-Rad) are shown in kDa. Parent, parent strain; Δ*lrp*, *lrp*-deletion mutant; pJH0311, broad-host-range vector; pJH0311::*lrp*, pJH0311 carrying the *lrp* gene (pZW1818); $nsrR_{3CS}$, strain expressing apo-locked $NsrR^{FLAG}$; $nsrR_{3CS}$Δ*lrp*, *lrp*-deletion mutant expressing apo-locked $NsrR^{FLAG}$. **(E)** A 393-bp DNA fragment of the *nsrR-hmpA* regulatory region (10 nM) was labeled with 6-FAM, and then incubated with increasing amounts of Lrp as indicated. For the competition analysis, various amounts of the unlabeled DNA fragment were added as a self-competitor. B, bound DNA; F, free DNA. **(F)** The same DNA probe (40 nM) was incubated with increasing amounts of Lrp as indicated, and then digested with DNase I. The region protected by Lrp is indicated by a gray box (LRPB). The nucleotides showing enhanced cleavage are indicated by asterisks. Nucleotide numbers shown are relative to the TSS of *hmpA*.

in an Lrp concentration-dependent manner (**Figure 6E**). The same unlabeled DNA fragment showed competition for Lrp binding in a dose-dependent manner (**Figure 6E**), demonstrating the specific binding of Lrp. DNase I protection assays revealed that Lrp largely protected a single region extending from −75 to −11 (LRPB, centered at −43 from the TSS of *hmpA*) from DNase I digestion (**Figures 4C**, **6F**). Combined with the EMSA data (**Figure 6E**), these results indicate that Lrp binds

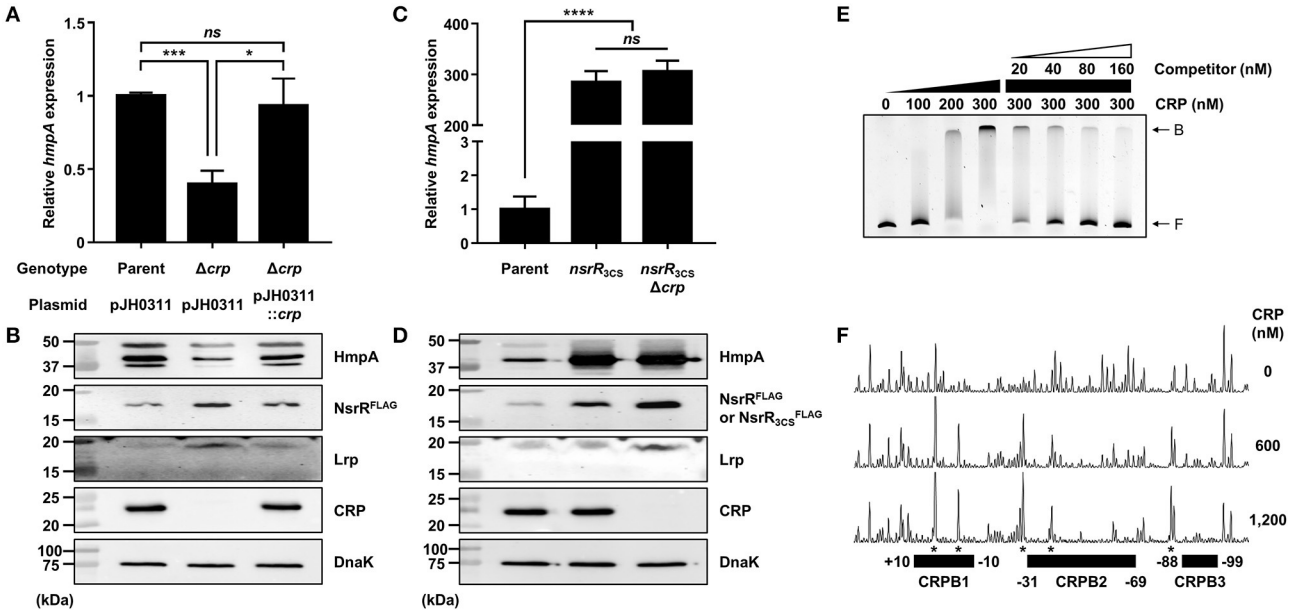

FIGURE 7 | The effect of the *crp* mutation on *hmpA* and *nsrR* transcription, and the specific binding of CRP to the *nsrR-hmpA* regulatory region. **(A–D)** Total RNA and proteins were isolated from the parent and mutant strains grown aerobically to an A_{600} of 0.5. **(A,C)** The *hmpA* transcript levels were determined by qRT-PCR, and the *hmpA* transcript levels in the parent strain were set to 1. Error bars represent the SD. Statistical significance was determined by the Student's *t*-test (*$p < 0.05$; ***$p < 0.0005$; ****$p < 0.00005$; *ns*, not significant). **(B,D)** The cellular HmpA, $NsrR^{FLAG}$ or $NsrR^{FLAG}_{3CS}$, Lrp, CRP, and DnaK (internal control) protein levels were determined by Western blot analysis. Molecular size markers (Bio-Rad) are shown in kDa. Parent, parent strain; Δ*crp*, *crp*-deletion mutant; pJH0311, broad-host-range vector; pJH0311::*crp*, pJH0311 carrying the *crp* gene (pKK1502); $nsrR_{3CS}$, strain expressing apo-locked $NsrR^{FLAG}$; $nsrR_{3CS}$Δ*crp*, *crp*-deletion mutant expressing apo-locked $NsrR^{FLAG}$. **(E)** A 393-bp DNA fragment of the *nsrR-hmpA* regulatory region (10 nM) was labeled with 6-FAM, and then incubated with increasing amounts of CRP as indicated. For the competition analysis, various amounts of the unlabeled DNA fragment were added as a self-competitor. B, bound DNA; F, free DNA. **(F)** The same DNA probe (40 nM) was incubated with increasing amounts of CRP as indicated, and then digested with DNase I. The regions protected by CRP are indicated by block boxes (CRPB1, CRPB2, and CRPB3). The nucleotides showing enhanced cleavage are indicated by asterisks. Nucleotide numbers shown are relative to the TSS of *nsrR*.

directly and specifically to the *nsrR-hmpA* regulatory region. Notably, within the region protected by Lrp, a periodic pattern of reduced cleavage followed by short regions of enhanced cleavage was observed (**Figure 6F**). This pattern known as phased hypersensitivity implies DNA bending by a multimeric Lrp (Pul et al., 2007), suggesting that the Lrp multimer induces a conformation change of the *nsrR-hmpA* regulatory region. Moreover, EMSA with both NsrR and Lrp showed that NsrR and Lrp simultaneously bind to the *nsrR-hmpA* regulatory region, rather than displace each other (**Supplementary Figure 2C**). Altogether, the combined results propose that direct binding of Lrp to the *nsrR-hmpA* regulatory region does not alter the *nsrR* transcription but represses *hmpA* transcription presumably through the modification of the DNA conformation enhancing the *hmpA* repression by NsrR.

CRP Activates *hmpA*, but Represses *nsrR* by Directly Binding to the *nsrR-hmpA* Regulatory Region

The role of CRP in the *hmpA* regulation was also explored. The *hmpA* transcript and HmpA protein levels in the *crp*-deletion mutant (Δ*crp*) were considerably decreased compared with those in the parent strain and restored by complementation (**Figures 7A,B**). In addition, the *hmpA* transcript level in the wild-type strain was decreased by exogenous glucose while that in the Δ*crp* strain was not affected (**Supplementary Figure 3A**). These results indicate that CRP has a positive effect on the *hmpA* transcription which is relieved in the presence of exogenous glucose. Then, we compared the *hmpA* transcript and HmpA protein levels in the parent strain, the $nsrR_{3CS}$ strain, and the *crp*-deleted $nsrR_{3CS}$ mutant ($nsrR_{3CS}$Δ*crp*). Similar to Lrp, the *hmpA* transcript and HmpA protein levels in the $nsrR_{3CS}$Δ*crp* strain were comparable with those in the $nsrR_{3CS}$ strain (**Figures 7C,D**), suggesting that the positive effect of CRP on the *hmpA* transcription is also mediated by NsrR. Thus, we further examined whether the effect of CRP on *hmpA* expression results from the increased cellular level of NsrR. Notably, the P_{nsrR} activity and $NsrR^{FLAG}$ protein level were significantly increased by the *crp* deletion (**Supplementary Figure 3B**, **Figure 7B**), showing that CRP acts as a repressor of *nsrR* transcription. Moreover, the Lrp protein level in the Δ*crp* strain was elevated compared with that in the parent strain as we observed previously (**Figure 7B**) (Lee et al., 2020). Accordingly, we hypothesized that CRP indirectly activates *hmpA* through the repression of both *nsrR* and *lrp* in a sequential manner.

To investigate whether CRP directly binds to the *nsrR-hmpA* regulatory region, EMSAs were performed. As shown in **Figure 7E**, the addition of CRP to the DNA probe resulted in a single retarded band in a CRP concentration-dependent

manner. The same unlabeled DNA fragment competed for CRP binding in a dose-dependent manner (**Figure 7E**), confirming the specific binding of CRP. DNase I protection assays determined three regions protected by CRP extending from −10 to +10 (CRPB1, centered at −0.5 from the TSS of *nsrR*), −69 to −31 (CRPB2, centered at −50 from the TSS of *nsrR*), and −99 to −88 (CRPB3, centered at −93.5 from the TSS of *nsrR*) from DNase I digestion (**Figures 4C**, **7F**). Combined with the EMSA data showing a single retarded band by CRP (**Figure 7E**), this result implies that CRP binds to CRPB1, CRPB2, and CRPB3 with similar DNA-binding affinities. Taken together, the combined results propose that CRP directly and specifically binds to the *nsrR-hmpA* regulatory region to repress *nsrR* as well as *lrp*, and consequently induces the *hmpA* transcription in a sequential cascade. In conclusion, the results in this study suggest that NsrR tightly regulates the *hmpA* transcription in response to NO, which could be elaborated by Lrp and CRP.

DISCUSSION

In this study, we newly identified and characterized an NO-responsive transcriptional regulator NsrR in *V. vulnificus* (**Figure 1**). The transcriptome analysis discovered that 44 genes are negatively regulated and 3 genes are positively regulated by NsrR (**Supplementary Table 1**). Notably, our previous transcriptome analysis of the wild-type strain revealed that 42 of the 44 genes repressed by NsrR are significantly induced upon exposure to NO (**Supplementary Figure 4A**) (Kim et al., 2019). Among the 42 genes, we further identified that the induction of *nnrS*, in addition to *hmpA*, is mediated by NsrR in response to NO (**Figure 2**, **Supplementary Figure 4B**). Accordingly, it is possible to propose that NsrR also regulates the expression of various genes other than *hmpA* and *nnrS* by sensing nitrosative stress. Meanwhile, although *iscR*, *iscS*, and *iscU* were positively regulated by NsrR (**Supplementary Table 1**), NsrR did not directly bind to the promoter region of the *isc* operon under the conditions tested (**Supplementary Figure 5**), indicating that NsrR controls the *isc* operon indirectly. Considering that functional NsrR requires the intact Fe-S cluster, up-regulation of the *isc* operon would be advantageous for NsrR to control its regulons effectively.

Besides *nsrR*, we found that the expression of *norR*, encoding another putative NO-responsive transcriptional regulator NorR, is induced by NO (Kim et al., 2019). In *V. cholerae*, NorR activates *hmpA* and *nnrS* by sensing NO, contributing to NO detoxification and the sustained colonization of host intestines (Stern et al., 2012). However, NorR did not affect the *hmpA* transcription in *V. vulnificus* under our experimental conditions (**Supplementary Figure 6**). Meanwhile, NorR in *E. coli* is known to activate the *norVW* genes encoding a flavorubredoxin to detoxify NO (D'Autreaux et al., 2005), but we could not find *norVW* homologs in the *V. vulnificus* genome. Although NorR-regulated genes and their role require further studies, NsrR appears to be the major transcriptional regulator for *V. vulnificus* to respond against nitrosative stress so far.

Figure 8A depicts the regulatory network comprising NsrR, Lrp, and CRP for the *hmpA* transcription proposed by this study. NsrR relieves the direct repression of *hmpA* losing its Fe-S cluster and DNA-binding affinity under nitrosative stress (**Figures 3**, **5**). The strong repression of *hmpA* by NsrR could allow *V. vulnificus* to prevent unnecessary waste of cellular components such as heme, NAD, and FAD as cofactors of HmpA (Kim et al., 2019). On the other hand, it could facilitate the rapid and strong induction of *hmpA* when the repression by NsrR is abolished, which may ensure an effective response against nitrosative stress (Alon, 2007). Thus, it is tempting to suppose that NsrR has evolved to regulate *hmpA* transcription by a derepression mechanism rather than simple activation.

Furthermore, Lrp and CRP elaborate the *hmpA* regulation by functional NsrR. Lrp directly binds to the *nsrR-hmpA* regulatory region but is not able to repress *hmpA* in the absence of functional NsrR (**Figure 6**). As one of the bacterial nucleoid-associated proteins, Lrp can modulate gene expression by remodeling the DNA structure (Dillon and Dorman, 2010). Thus, one possible explanation for the NsrR-dependent *hmpA* repression by Lrp is that the formation of a multicomponent complex containing Lrp multimers and the resulting conformation change of DNA enhance the ability of holo-NsrR to repress *hmpA*. Meanwhile, this study further demonstrated that CRP acts as a repressor of *nsrR* by directly binding to the *nsrR-hmpA* regulatory region (**Figure 7**, **Supplementary Figure 3B**). In addition, we confirmed our previous report that CRP directly represses *lrp* by binding to its promoter (**Figure 7B**) (Lee et al., 2020). These results led us to propose that CRP activates *hmpA* by the repression of *nsrR* and *lrp* as a sequential cascade.

Particularly, we showed that CRP upregulates the *hmpA* transcription in response to low levels of glucose (**Supplementary Figure 3A**). During conditions of intestinal inflammation, NO produced by host cells is rapidly decomposed to less toxic NO_3^- by diverse detoxifying enzymes of enteric pathogens including HmpA. The accumulated NO_3^- in the intestinal lumen can be utilized as an electron acceptor for anaerobic respiration of pathogens in hypoxic environments (Vazquez-Torres and Baumler, 2016; Bueno et al., 2018). In the NO_3^-/NO_2^- respiration, NO_3^- is converted to NO_2^- that is harmful to bacteria. Thus, NO_2^- is subsequently reduced to ammonia (NH_3) by an NO_2^- reductase, which can generate NO as a by-product (Spiro, 2007; Tiso and Schechter, 2015). Intriguingly, it has been reported that CRP activates the NO_3^-/NO_2^- respiration under nutrient-poor or low-oxygen conditions in *E. coli* and *Shewanella oneidensis* (Stewart et al., 2009; Dong et al., 2012). Accordingly, we could assume that CRP induces *hmpA* as well as activates NO_3^-/NO_2^- respiration under low-glucose conditions to scavenge the low levels of endogenous NO during NO_3^-/NO_2^- respiration. Since the utilization of host-derived NO_3^- enhances the growth and fitness of pathogens (Vazquez-Torres and Baumler, 2016), NsrR and CRP might coordinate nitrosative stress defense systems and energy production in *V. vulnificus* for survival during infection. Altogether, the collaborative regulation by NsrR along with Lrp and CRP enables the tight and precise tuning of *hmpA* transcription by integrating various signals including nitrosative

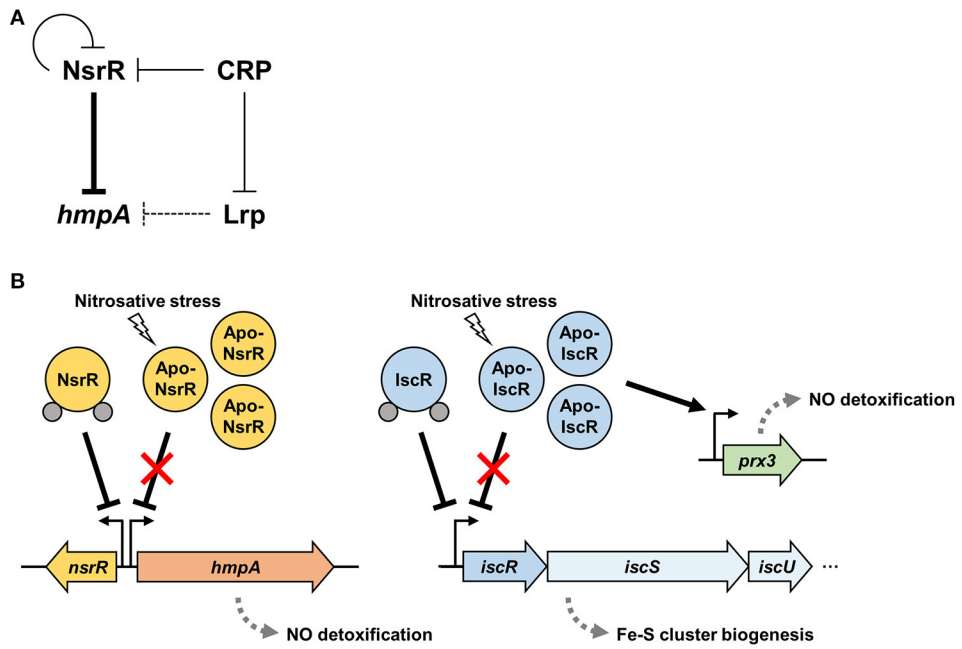

FIGURE 8 | A regulatory network controlling *hmpA* transcription and nitrosative stress defense systems in *V. vulnificus*. **(A)** A regulatory network comprising transcriptional regulators NsrR, Lrp, and CRP controls the *hmpA* transcription. NsrR directly represses *hmpA* and *nsrR* itself. Lrp indirectly represses *hmpA* presumably by enhancing the repression activity of NsrR through DNA structure remodeling. CRP indirectly activates *hmpA* possibly through the repression of *nsrR* and *lrp* in a sequential cascade. **(B)** Apo-NsrR alleviates the repression of *hmpA* and *nsrR* upon exposure to nitrosative stress. On the other hand, apo-IscR relieves the repression of the *isc* operon, and the resulting increased apo-IscR directly activates *prx3* encoding 1-cysteine peroxiredoxin. The induced NO-decomposition proteins, HmpA and Prx3, would facilitate the survival of *V. vulnificus* under host-derived nitrosative stress. The gray dots represent the Fe-S cluster.

stress and nutrient availability, thereby contributing to the fitness and pathogenesis of *V. vulnificus* within the host.

Our current understanding of the nitrosative stress defense systems in *V. vulnificus* is summarized in **Figure 8B**. In addition to NsrR, we previously demonstrated that *V. vulnificus* IscR, another Rrf2-family [2Fe-2S] containing transcriptional regulator, also turns to an apo-form lacking the Fe-S cluster under nitrosative stress (Lim and Choi, 2014; Choi et al., 2020). Apo-IscR dissociates from the promoter of the *isc* operon to express the *isc* operon and to facilitate the biogenesis of the Fe-S cluster (Lim et al., 2014b). In addition, the resulting increased apo-IscR further activates the expression of *prx3* encoding 1-cysteine peroxiredoxin with an NO-decomposition activity by directly binding to the *prx3* promoter region (P_{prx3}) (Lim et al., 2014a; Ahn et al., 2018). The regulatory characteristic of IscR on P_{prx3} is distinguishable from that of NsrR on P_{hmpA} in which IscR can bind to P_{prx3} in the apo-form, and the increased apo-IscR protein level results in *prx3* activation. Taken together, these assorted nitrosative stress defense systems would provide *V. vulnificus* with the benefit of having inclusive modulation of various NO-detoxifying gene expression and the consequent survival under host-derived nitrosative stress during infection.

MATERIALS AND METHODS

Strains, Plasmids, and Culture Conditions

The strains and plasmids used in this study are listed in **Supplementary Table 2**. Unless otherwise noted, the *V. vulnificus* strains were grown aerobically in Luria-Bertani (LB) medium supplemented with 2% (w/v) NaCl (LBS) at 30°C, and their growth was monitored spectrophotometrically at 600 nm (A_{600}). When required, 3 μg/ml chloramphenicol was added to the media. To visualize the cellular NsrR protein levels, *V. vulnificus* GR204, which carries 3×FLAG-coding sequence fused to the 3′-end of *nsrR* ORF on the chromosome, was constructed as a parent strain (**Supplementary Table 2**). The parent strain and its isogenic mutants were used to quantify the cellular NsrR protein levels. The murine macrophage RAW 264.7 cells were grown in DMEM containing 10% fetal bovine serum (VWR, Radnor, PA) and the antibiotics [100 units/ml penicillin G and 100 μg/ml streptomycin (Gibco-BRL, Gaithersburg, MD)] in air supplemented with 5% CO_2 at 37°C. To induce NO production, the RAW 264.7 cells were suspended in fresh DMEM containing 500 ng/ml *E. coli* O111:B4 lipopolysaccharide (Sigma, St. Louis, MO) and 1 mM L-arginine (Sigma) (Walker et al., 1997; Choi et al., 2020).

Generation and Complementation of the Mutants

For construction of the isogenic deletion mutants, target genes were inactivated *in vitro* by deletion of each ORF using the PCR-mediated linker-scanning mutation method as described previously (Jang et al., 2016; Choi et al., 2020). Briefly, the deleted ORF fragment was amplified by PCR

with appropriate primer pairs (**Supplementary Table 3**), and the resulting fragment was ligated into SphI-SpeI-digested pDM4 (Milton et al., 1996). *E. coli* S17-1 λ*pir* (Simon et al., 1983) containing pDM4 with the desired insert was used as a conjugal donor to an appropriate *V. vulnificus* strain to generate the deletion mutant (**Supplementary Table 2**). The conjugation and isolation of the transconjugants were conducted using a method described previously (Choi et al., 2020). The *lrp*-deletion mutant ZW181 and the *crp*-deletion mutant DI0201 were constructed previously (Choi et al., 2002; Lee et al., 2020).

For construction of the parent strain GR204 encoding $NsrR^{FLAG}$ on the chromosome, the 3×FLAG-coding sequence was fused to the 3'-end of *nsrR* ORF by PCR using the primer pairs NSRR01-F and NSRR01F-R, or NSRR02F-F and NSRR02-R (**Supplementary Table 3**). The amplified fragment was cloned into pDM4, resulting in pGR2008 (**Supplementary Table 2**). *E. coli* S17-1 λ*pir* containing pGR2008 was used as a conjugal donor to the Δ*nsrR* strain as described above to generate GR204 (**Supplementary Table 2**).

The three cysteine residues in NsrR (C91, C96, and C102) were replaced with serine to examine their regulatory function with the minimal structural change of NsrR. For construction of the $nsrR_{3CS}$ strain DY192, three cysteine residues were substituted with serine *in vitro* by using the QuikChange® site-directed mutagenesis kit (Agilent Technologies, Loveland, CO) (Bang et al., 2012; Lim et al., 2014a). The complementary mutagenic primers listed in **Supplementary Table 3** were used to create pDY1907 carrying the $nsrR_{3CS}$ gene on pDM4 (**Supplementary Table 2**). *E. coli* S17-1 λ*pir* containing pDY1907 was used as a conjugal donor to the Δ*nsrR* strain as described above to generate DY192, and the $nsrR_{3CS}$ mutation in DY192 was confirmed by DNA sequencing. For construction of the 3×FLAG-tagged $nsrR_{3CS}$ strain GR217 (**Supplementary Table 2**), a similar method was adopted except using pGR2016 carrying 3×FLAG-coding sequence fused to the 3′-end of $nsrR_{3CS}$ ORF on pDM4 instead of pDY1907.

To complement the *nsrR* mutation with a plasmid-based system, the *nsrR* gene was amplified by PCR using the primer pair NSRRC-F and -R (**Supplementary Table 3**). The amplified fragment was cloned into the broad-host-range vector pJH0311 (Goo et al., 2006) to create pDY1702 (**Supplementary Table 2**). To complement the *lrp* and *crp* mutation, pZW1818 and pKK1502 carrying the *lrp* and *crp* gene on pJH0311, respectively, were used in this study (**Supplementary Table 2**) (Jang et al., 2017; Lee et al., 2020). The plasmids were transferred into appropriate mutants by conjugation as described above.

To complement the *nsrR* mutation by ectopic expression of *nsrR* on the chromosome, the *nsrR* regulatory region and its ORF was integrated into a cryptic *lacZ* gene by PCR using specific primer pairs listed in **Supplementary Table 3** (Hall, 1999; Chodur et al., 2017). The amplified fragment was cloned into pDM4, resulting in pGR2007 (**Supplementary Table 2**). *E. coli* S17-1 λ*pir* containing pGR2007 was used as a conjugal donor to the Δ*nsrR* strain as described above to generate GR203 (**Supplementary Table 2**).

RNA-seq and Transcriptome Analysis

To analyze the effect of the *nsrR* deletion on the *V. vulnificus* transcriptome, total RNA was isolated from biological duplicates of the wild-type and Δ*nsrR* strains, grown aerobically to an A_{600} of 0.5 in M9 minimal media supplemented with 0.4% (w/v) glucose (M9G) and then exposed to PPNPs for 10 min (Nurhasni et al., 2015; Kim et al., 2019). The RNA was further purified by removing DNA using TURBO DNase (Ambion, Austin, TX), and mRNA was selectively enriched by depleting rRNA using a Ribo-Zero rRNA removal kit (Epicenter, Madison, WI) according to the manufacturer's instructions. Strand-specific cDNA libraries were constructed and sequenced using HiSeq 2500 (Illumina, San Diego, CA) as described previously (Lee et al., 2019). The raw sequencing reads were mapped to the *V. vulnificus* MO6-24/O reference genome (GenBank™ accession numbers: CP002469 and CP002470, www.ncbi.nlm.nih.gov), and the expression level of each gene was calculated as a reads per kilobase of transcript per million mapped sequence reads (RPKM) value using EDGE-pro v1.3.1 (Estimated Degree of Gene Expression in PROkaryots) (Magoc et al., 2013). The RPKM values were normalized and analyzed statistically using DeSeq2 v1.26.0 to identify the differentially expressed genes (fold change ≥ 2 with $p < 0.05$) (Love et al., 2014). A heat map was generated by the Matplotlib python package using the RPKM-fold change for each gene (Hunter, 2007).

qRT-PCR and Primer Extension Analysis

Relative transcript levels in the total RNA isolated from the *V. vulnificus* strains grown under various environmental conditions were determined by quantitative RT-PCR (qRT-PCR). In detail, *V. vulnificus* was grown to an A_{600} of 0.5 in M9G and then exposed to either 0.15 mg/ml PPNPs (negative control) or NO/PPNPs for 10 min (Nurhasni et al., 2015; Kim et al., 2019). Additionally, *V. vulnificus* grown to an A_{600} of 0.5 in LBS was exposed to DMEM (negative control) or RAW 264.7 cells at a multiplicity of infection 10 for 10 min in the presence or absence of 500 μM L-NMMA (Sigma), which is a known NO synthase inhibitor (Nathan and Hibbs, 1991; Choi et al., 2020). When necessary, *V. vulnificus* was grown to an A_{600} of 0.5 in LBS with various amounts of L-leucine (Sigma) or 1% glucose (Sigma). Total RNA from the *V. vulnificus* cells was isolated and quantified using a RNeasy® Mini Kit (Qiagen, Valencia, CA) and a NanoDrop One^c Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), respectively. cDNA was synthesized from 500 ng of the total RNA with the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR amplification of the cDNA was performed with the Chromo 4 real-time PCR detection system (Bio-Rad) and specific primer pairs (**Supplementary Table 3**) as described previously (Jang et al., 2017). Relative expression levels were calculated with the 16S rRNA expression level as an internal reference for normalization (Jang et al., 2017).

For primer extension analysis, primers HMPAUP-R and NSRRUP-R (**Supplementary Table 3**) complementary to the coding region of *hmpA* and *nsrR*, respectively, were end-labeled with [γ-32P]-ATP and added to the RNA. The primers were extended with SuperScript II reverse transcriptase (Invitrogen,

Carlsbad, CA). The cDNA products were purified and resolved on a sequencing gel alongside sequencing ladders generated from pDY1706 and pDY1707 (**Supplementary Table 2**) with the same primers, respectively. The plasmid pDY1706 was constructed by cloning the 219-bp *hmpA* upstream region extending from −120 to +99, amplified by PCR using a primer pair HMPAUP-F and -R (**Supplementary Table 3**), into pGEM-T Easy (Promega, Madison, WI). Similarly, pDY1707 carrying the 198-bp *nsrR* upstream region extending from −113 to +85 on pGEM-T Easy was constructed using a primer pair NSRRUP-F and -R (**Supplementary Table 3**). The primer extension product was visualized with the Typhoon FLA 7000 phosphorimager (GE healthcare, Menlo Park, CA).

Protein Purification and Western Blot Analysis

To overexpress NsrR and $NsrR_{3CS}$, each ORF of *nsrR* and $nsrR_{3CS}$ was amplified by PCR using specific primer pairs (**Supplementary Table 3**). The amplified fragments were cloned into pET-28a(+) (Novagen, Madison, WI) to create pEJ1902 and pEJ1903, respectively (**Supplementary Table 2**). The resulting His_6-tagged NsrR and $NsrR_{3CS}$ were expressed in *E. coli* BL21 (DE3) and purified by affinity chromatography according to the manufacturer's instructions (Qiagen). The buffers used for NsrR and $NsrR_{3CS}$ are as follows: 20 mM Tris-Cl (pH 8.0), 500 mM NaCl, and 5 mM β-mercaptoethanol; additional 10% glycerol for a lysis buffer; additional 20 mM imidazole for a wash buffer; additional 250 mM imidazole for an elution buffer; additional 50% glycerol for a dialysis buffer. To overexpress Lrp and CRP, pZW1903 carrying the *lrp* gene on pET-28a(+) and pHK0201 carrying the *crp* gene on pRSET A (Invitrogen) were used in this study (**Supplementary Table 2**) (Choi et al., 2002; Lee et al., 2020). The His_6-tagged Lrp and CRP were purified as described previously (Lee et al., 2020).

For Western blot analysis, *V. vulnificus* cells were lysed using B-PER™ Bacterial Protein Extraction Reagent with Enzymes (Thermo Fisher Scientific), and residual cell debris was removed by centrifugation to obtain clear cell lysates. The protein levels of HmpA, Lrp, CRP, and DnaK in the clear cell lysates were determined as described previously (Kim et al., 2019; Lee et al., 2020). Similarly, cellular $NsrR^{FLAG}$ protein was detected using Monoclonal ANTI-FLAG® M2 antibody produced in mouse (Sigma).

Construction of P_{nsrR}-*luxCDABE* Transcriptional Fusion

A 393-bp *nsrR-hmpA* regulatory region (−262 to +131 from the TSS of *nsrR*) was amplified with the primer PnsrR-F carrying a SacI restriction site and PnsrR-R carrying a SpeI restriction site (**Supplementary Table 3**). The resulting DNA fragment was cloned into the SacI-SpeI-digested pBBR-lux carrying the promoterless *luxCDABE* genes to create pGR2025 (**Supplementary Table 2**) (Lenz et al., 2004). pGR2025 was transferred into the *V. vulnificus* strains by conjugation as described above. The cellular luminescence and growth (A_{600}) of each strain grown to an A_{600} of 0.5 in LBS were measured using a microplate reader (Infinite™ microplate reader, Tecan, Männedorf, Switzerland), and RLUs were calculated by dividing the luminescence with the A_{600} (Lee et al., 2019).

EMSA and DNase I Protection Assay

For the EMSAs, a 393-bp *nsrR-hmpA* regulatory region (−186 to +207 from the TSS of *hmpA*, equivalent to −262 to +131 from the TSS of *nsrR*) was amplified by PCR using 6-FAM-labeled PnsrRhmpA-F and -R as primers (**Supplementary Table 3**). Similarly, a 321-bp *isc* operon regulatory region [−194 to +127 from the TSS of *isc* operon (Lim et al., 2014b)] was amplified by PCR using 6-FAM-labeled Pisc-F and -R as primers (**Supplementary Table 3**). The 6-FAM-labeled DNA probe (10 nM) was then incubated with purified NsrR or CRP for 30 min at 30°C in a 20-μl reaction mixture containing 1× NsrR binding buffer (10 mM Tris-Cl (pH 8.0), 10 mM KCl, 1 mM DTT, and 100 μg BSA; additional 1 mM cAMP only for CRP) and 0.1 μg of poly(dI-dC) (Sigma) as a non-specific competitor. Similarly, the DNA probe was incubated with purified Lrp or both NsrR and Lrp for 30 min at 30°C in a 20-μl reaction mixture containing 1× Lrp binding buffer (50 mM Tris-Cl (pH 8.0), 20 mM KCl, 1 mM DTT, and 100 μg BSA, and 10% glycerol) and 0.1 μg of poly(dI-dC) (Sigma) as a non-specific competitor. For the competition analysis, various concentrations of unlabeled DNA fragment were added as a self-competitor to the reaction mixture before incubation. Electrophoretic analysis of the DNA-protein complexes was performed as described previously (Lee et al., 2020).

The same 393-bp *nsrR-hmpA* regulatory region was amplified by PCR using unlabeled PnsrRhmpA-F and 6-FAM-labeled PnsrRhmpA-R as primers for the DNase I protection assays (**Supplementary Table 3**). The binding of NsrR, Lrp, and CRP to the DNA probe (40 nM) was performed as described above, and DNase I digestion of the DNA-protein complexes followed the procedures described previously (Jang et al., 2017). The digested DNA products were precipitated with ethanol and eluted in sterilized H_2O, and then analyzed using an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA) with Peak Scanner™ Software v1.0 (Applied Biosystems) (Hwang et al., 2019).

Data Analysis

Average and standard deviation (SD) values were calculated from at least three independent experiments. Statistical analysis was performed by the Student's *t*-test using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA).

DATA AVAILABILITY STATEMENT

The raw data of the RNA-seq analysis can be found in the NCBI BioProject database–PRJNA704465; https://www.ncbi.nlm.nih.gov/bioproject/PRJNA704465.

AUTHOR CONTRIBUTIONS

GC, DK, and SC designed the research. GC and DK performed the experiments. GC and SC wrote the manuscript. All authors analyzed, interpreted the data, reviewed the results, and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.681196/full#supplementary-material

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Epidemiologic Associations Vary Between Tetracycline and Fluoroquinolone Resistant *Campylobacter jejuni* Infections

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Campylobacter jejuni is the leading cause of bacterial gastroenteritis and antibiotic resistant *C. jejuni* are a serious threat to public health. Herein, we sought to evaluate trends in *C. jejuni* infections, quantify resistance frequencies, and identify epidemiological factors associated with infection. *Campylobacter jejuni* isolates ($n = 214$) were collected from patients via an active surveillance system at four metropolitan hospitals in Michigan between 2011 and 2014. The minimum inhibitory concentration for nine antibiotics was determined using microbroth dilution, while demographic and clinical data were used for the univariate and multivariate analyses. Over the 4-year period, a significant increase in the recovery of *C. jejuni* was observed ($p \leq 0.0001$). Differences in infection rates were observed by hospital and several factors were linked to more severe disease. Patients residing in urban areas, for instance, were significantly more likely to be hospitalized than rural residents as were patients over 40 years of age and those self-identifying as non-White, highlighting potential disparities in disease outcomes. Among the 214 *C. jejuni* isolates, 135 (63.1%) were resistant to at least one antibiotic. Resistance was observed for all nine antibiotics tested yielding 11 distinct resistance phenotypes. Tetracycline resistance predominated ($n = 120$; 56.1%) followed by resistance to ciprofloxacin ($n = 49$; 22.9%), which increased from 15.6% in 2011 to 25.0% in 2014. Resistance to two antibiotic classes was observed in 38 (17.8%) isolates, while multidrug resistance, or resistance to three or more classes, was observed in four (1.9%). Notably, patients with ciprofloxacin resistant infections were more likely to report traveling in the past month (Odds Ratio (OR): 3.0; 95% confidence interval (CI): 1.37, 6.68) and international travel (OR: 9.8; 95% CI: 3.69, 26.09). Relative to patients with only tetracycline resistant infections, those with ciprofloxacin resistance were more likely to travel internationally, be hospitalized and have an infection during the fall or summer. Together, these findings show increasing rates of infection and resistance and highlight specific factors that impact both outcomes. Enhancing understanding of factors linked to *C. jejuni* resistance and more severe infections is critical for disease prevention,

particularly since many clinical laboratories have switched to the use of culture-independent tests for the detection of *Campylobacter*.

Keywords: *Campylobacter*, antibiotic resistance, epidemiology, ciprofloxacin resistance, tetracycline resistance, risk factor

INTRODUCTION

Campylobacter spp. are a leading cause of bacterial gastroenteritis infections worldwide (1) and represent the most common cause of foodborne infections in the U.S. since 2013 (2). While *C. jejuni* causes a vast majority of human infections, other species including *C. coli*, *C. upsaliensis*, *C. lari*, *C. fetus*, *C. insulaeingrae*, and *C. hyointestinalis*, are also important (3). Collectively, these pathogens were estimated to cause 1.5 million infections in the U.S. each year, contributing to 13,000 hospitalizations and 120 deaths (4). In 2018, the Centers for Disease Control and Prevention (CDC) estimated the incidence of campylobacteriosis to be 19.6 cases per 100,000 individuals, which had increased from 12.0 cases per 100,000 in 2015–2017, among sites participating in the Foodborne Diseases Active Surveillance Network (FoodNet) (2).

Clinical manifestations of campylobacteriosis include fever, abdominal pain, vomiting, weight loss, chills, fatigue, myalgia, malaise, and acute watery or bloody diarrhea (1). The incubation period is typically 1–4 days after exposure, and the severity of symptoms tends to vary by bacterial density and strain (5). Post-infectious immune sequalae such as Guillain-Barré Syndrome, Miller-Fisher syndrome, and reactive arthritis, have been linked to *Campylobacter* infection as have inflammatory bowel disease, esophageal and colo-rectal cancers, and extra-intestinal infections like bacteremia and meningitis (6). Although most infections are self-limiting, antibiotics are often needed for immunocompromised patients or those with more severe or persistent infections (7).

Water, poultry and livestock are common reservoirs for *C. jejuni* (8). Transmission to humans typically occurs via consumption of contaminated food products, and direct contact with animal or environmental reservoirs (9). According to a meta-analysis of 72 studies, the key risk factor for campylobacteriosis was international travel, yet consumption of undercooked chicken and direct exposure to *Campylobacter* from the environment or farm animals were also important (10). Regardless, it is important to note that risk factors often vary by geographic location even across the U.S., with different FoodNet sites reporting considerable variation in the frequency of infections (11). In addition, the FoodNet sites were not selected to be representative of the U.S. population and were shown to have an unequal representation of all racial and ethnic groups and contained fewer individuals living below the poverty level (12, 13).

Campylobacter jejuni has also been designated a serious antibiotic resistant threat resulting in 448,400 resistant infections and 70 deaths each year (14). Resistance to ciprofloxacin, a fluoroquinolone used to treat more severe human infections, increased in the U.S. from 13% in 1997 to 25.3% in 2015 (14, 15). *Campylobacter jejuni* resistance to multiple drug classes has also increased over time (16) and resistant isolates have been linked to more severe infections requiring lengthier hospitalizations (17). Because NARMS does not utilize data from each state and the Midwest region only receives a subset of *Campylobacter* isolates from the Minnesota FoodNet site for testing (18), these resistance frequencies and trends may not be representative of those in other locations. Additionally, many clinical laboratories have shifted to the use of culture-independent tests to detect *Campylobacter* infections, which can obscure actual rates of resistance circulating within patient populations and prevent the identification of risk factors for resistant infections. Indeed, a 2019 FoodNet report noted that 42% of *Campylobacter* infections were detected using a culture-independent test (2). This shift is concerning and highlights the need for more culture-based studies to better define the epidemiology of and resistance phenotypes in this common foodborne pathogen.

Herein, we sought to describe the susceptibility profiles for 214 *C. jejuni* isolates cultured from patients with campylobacteriosis during surveillance activities in Michigan (2011–2014) and to identify risk factors for both susceptible and resistant infections. We also sought to make comparisons to national data available through NARMS since *Campylobacter* resistance is not monitored in Michigan via NARMS (19). Studies such as these highlight the importance of using culture-based diagnostic tests to more accurately monitor resistance phenotypes and frequencies in distinct geographic locations to identify potential exposures and risk factors that may be state and/or region specific.

MATERIALS AND METHODS

Strain Source and Speciation

Campylobacter isolates were recovered from stools of patients with campylobacteriosis between 2011 and 2014 via an active surveillance system at four metropolitan hospitals located in Detroit, Grand Rapids, Ann Arbor, and Lansing, Michigan. Isolates were transported to the Michigan Department of Health and Human Services (MDHHS) and stored in 10% skim milk at −80°C until use.

Isolates were thawed and cultured on Tryptone Soy Agar (TSA) containing 5% sheep blood and cefoperazone (20 μg), amphotericin B (4 μg/mL), and vancomycin (20 μg/mL) in microaerophilic conditions (20). DNA was extracted and multiplex PCR was performed to classify the species of each *Campylobacter* isolate using a previously described protocol (21). Briefly, the Kapa2G Taq (Kapa Biosystems; Wilmington, MA) was used for PCR amplification using the following conditions: denaturation at 95°C for 15 min followed by 25 cycles of 95°C

for 30 s and 58°C for 1 min and 30 s and 72°C for 8 min. Roughly 91 of the 214 (43%) *C. jejuni* isolates included in the analysis were characterized previously (22).

Antimicrobial Susceptibility Profiling

The minimum inhibitory concentration (MIC) was determined for nine antibiotics using microbroth dilution utilizing Sensititre™ Campylobacter Campy AST plates (ThermoFisher; Waltham, MA) according to the manufacturer's protocols. The antibiotics (classes) were: ciprofloxacin (fluoroquinolone), nalidixic acid (quinolone), azithromycin (macrolide), erythromycin (macrolide), tetracycline, florfenicol (phenicol), telithromycin (ketolide), clindamycin (lincomycin), and gentamicin (aminoglycoside). *Campylobacter jejuni* ATCC 33560 was used a control. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards were used, as per the current NARMS protocol, for classifying isolates as resistant or susceptible (19). NARMS data were extracted from isolates collected in the same time period for comparison. Isolates with any ciprofloxacin resistance and any tetracycline resistance were counted; these two categories were not mutually exclusive as some isolates had resistance to both drugs. A subset of data submitted from NARMS Region 5 representing the Midwest (Ohio, Indiana, Michigan, Illinois, Wisconsin, and Minnesota and 34 federally recognized tribes) were also included in this analysis for comparison.

Epidemiological Variables and Data Analysis

Demographics, exposures, and clinical data were extracted from the Michigan Disease Surveillance System (MDSS), an online database containing epidemiological data for notifiable infections. The sample collection date was used to classify the season as follows: spring (March, April, May), summer (June, July August), fall (September, October, and November), and winter (December, January, and February). Cases reporting a history of travel in the past month were classified as traveling domestically (within the U.S.) or internationally. Michigan counties were designated as urban or rural based on data presented in a National Center for Health Statistics report (23); all but 10 Michigan counties were considered rural. Cattle densities per county were obtained from a 2019 report (24), and the high vs. low categories were developed based on the average number of cattle in all Michigan herds with data available.

Chi-square tests were used for dichotomous variables to identify associations between the dependent and independent variables, while the Mantel-Haenszel Chi-square test was used to examine trends. Differences in proportions were evaluated using the Chi-square test for equal proportions and for variables with small sample sizes, or less than five per cell, the Fisher's exact test was used. A $p \leq 0.05$ was considered significant for each test, however, all variables yielding a $p \leq 0.20$ in the univariate analysis were included in the multivariate analyses. Potential confounders such as age, sex, and residence location, were also included in the forward logistic regression analyses to identify predictors of each outcome. Odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated to describe the magnitude of each association. SAS version 9.4 (SAS Institute, Cary, NC, USA) and Epi Info™ version 7 were used.

RESULTS

Recovery of *Campylobacter* in Michigan

In all, 277 *Campylobacter* isolates were recovered from Michigan residents diagnosed with campylobacteriosis at four large metropolitan hospitals between January 2011 and December 2014. Approximately 234 (84.5%) of the isolates were viable and could be speciated using PCR. Among these, 217 (92.7%) were classified as *C. jejuni*, while 15 (6.0%) were *C. coli*; two isolates (0.9%) were characterized as *C. upsaliensis*. Given that *C. jejuni* was the most common species, the analysis was restricted to these isolates and cases. Three additional isolates from residents living outside of Michigan were also excluded from the analysis.

Significant variation in the recovery of *C. jejuni* was observed across hospitals ($p \leq 0.0001$), with most isolates ($n = 174$; 82.1%) coming from two sites; the hospital location was missing for three isolates. The frequency of *C. jejuni* at each site was 42.9% ($n = 91$), 39.2% ($n = 83$), 7.6% ($n = 16$), and 9.9% ($n = 21$). A significant difference in the recovery of *C. jejuni* isolates was also observed over time with 57.5% ($n = 123$) of the infections occurring in 2013 and 2014 ($p \leq 0.0001$). Differences were also observed by season since more isolates were recovered in the summer and fall months ($n = 158$; 73.8%) compared to the winter and spring ($n = 56$; 26.2%) ($p \leq 0.0001$). Moreover, a greater proportion of cases resided in urban ($n = 119$; 62.0%) vs. rural ($n = 73$; 38.0%) areas ($p = 0.0009$).

Demographics and Exposure History of *C. jejuni* Cases

Among the 214 *C. jejuni* cases from Michigan residents, 110 (54.4%) were male and 53.1% ($n = 113$) were between the age of 19 and 65 years; the age was missing for one case (**Supplementary Table 1**). Sixty-five (30.5%) cases represented children between 1 day and 9 years of age. Twenty (30.8%) of these children were ≤1 year old and 33 (50.8%) were between 1 and 5 years of age. Among the 17 elderly patients over 65 years, over half ($n = 11$) were between 70 and 87 years of age. Significantly more cases self-identified as White/Caucasian ($n = 137$; 79.7%), though a subset self-identified as Black/African American ($n = 17$; 9.8%), Asian ($n = 6$; 3.5%), or another race ($n = 13$; 7.5%). Thirteen (8.8%) cases self-identified as Hispanic/Latino and 25 (19.1%) self-identified as Arab, however, up to 83 (38.8%) cases did not indicate their ethnicity.

The majority ($n = 88$; 59.9%) of cases did not travel in the month prior to infection compared to 40.9% of cases who did. Among 59 of the 61 cases reporting their travel location, 18.4% ($n = 27$) traveled internationally and 22.4% ($n = 33$) reported domestic travel. While more of these cases traveled during the summer ($n = 30$; 44.4%) and fall ($n = 18$; 29.5%) as opposed to the winter and spring ($n = 13$; 21.3%), the difference was not significant ($p = 0.26$).

Despite the greater proportion of cases reporting animal contact prior to illness onset ($n = 96$; 64.0%), multiple animal species were reported. Among these cases, 88 (91.7%)

cases reported contact with domestic animals, 13 (13.5%) with livestock and 11 (11.5%) with birds or poultry. Seventeen cases (17.7%) reported contact with other animals and three (3.1%) had contact with reptiles. Furthermore, most ($n = 199$; 81.5%) cases drank municipal and/or bottled water and consumed poultry ($n = 115$; 87.8%) up to a week prior to symptom onset. Food history data, however, was not available for up to 38.8% of the cases.

Because significantly more cases lived in urban areas, we also sought to determine whether any factors were associated with urban residence (**Supplementary Table 2**). Importantly, the odds of hospitalization for urban residents was significantly greater ($n = 34$; 73.9%) than rural residents ($n = 12$; 26.1%), yet no differences in symptoms were observed. Urban patients were also significantly less likely to report any travel in the past month, either domestic or international, and were less likely to be between 19 and 40 years of age than rural patients. Indeed, 83.6% ($n = 46$) of children < 10 years of age resided in an urban area compared to 16.4% ($n = 9$) for rural children. Although exposure to livestock and Arab ethnicity were significantly associated with rural and urban residence, respectively, the sample sizes were small for each variable and many records had missing data.

Clinical Symptoms and Association with More Severe Infections

Among the subset of cases reporting symptoms, diarrhea (95.0%) was the most common followed by abdominal pain (69.7%), nausea (41.3%), and fatigue (40.9%). Only 36.2% of cases reported the presence of bloody diarrhea, while 34.5, 28.7, and 26.6% reported chills, body aches, and headaches, respectively. In all, 46 (25.3%) patients were hospitalized ranging from 1–11 days with an average of 3 days.

In addition to urban residence, several other factors were associated with hospitalization, a marker for more severe disease, in the univariate analysis (**Supplementary Table 3**). An increasing odds of hospitalization was observed as age increased. Compared to adults between years, adult patients between 41 and 65 years and the elderly over 65 years were significantly more likely to be hospitalized. The same was true when children < 9 years was used as the reference group. Patients self-reporting nausea and fatigue were also more likely to be hospitalized as were patients self-identifying as non-White. By contrast, patients reporting domestic or international travel in the month prior to symptom onset were significantly less likely to be hospitalized. No association was observed for sex, season, source of drinking water, or any other symptoms, and no differences were detected when the analysis was limited to only those individuals without a recent history of international travel.

Controlling for potential confounders such as residence type (urban vs. rural), sex, season, and age, multinomial logistic regression identified the oldest age groups, 41–65 years (adjusted OR: 6.1; 95 CI: 2.37, 15.70) and >65 years (adjusted OR: 10.5; 95 CI: 2.63, 42.19), to be predictors of hospitalization relative to the younger age groups. International travel in the past month (adjusted OR: 0.3; 95% CI: 0.07,0.94), non-White race (adjusted OR: 4.8; 95% CI: 1.62, 14.01), and nausea (adjusted OR: 2.8; 95% CI: 1.15, 6.68) were also independently associated with hospitalization.

Antibiotic Resistance Phenotypes and Frequencies

Resistance was detected in 63.1% ($n = 135$) of the 214 *C. jejuni* isolates and at least one isolate was resistant to each of the nine antibiotics tested. Tetracycline resistance ($n = 120$; 56.1%) predominated followed by resistance to ciprofloxacin ($n = 49$; 22.9%) (**Figure 1A**). Fewer than five isolates had resistance to clindamycin, azithromycin, and telithromycin, and only one isolate was resistant to gentamicin and another to phenicol. All isolates with ciprofloxacin resistance were also resistant to nalidixic acid. Among the 135 resistant isolates, 93 (43.5%) were resistant to one class of antibiotics, whereas 38 (17.8%) were resistant to two. In all, 11 different *C. jejuni* resistance phenotypes that varied in frequency (**Figure 1B**). Five of these phenotypes included tetracycline resistance, six included ciprofloxacin resistance, and three phenotypes included both. The predominant phenotypes were tetracycline resistance alone ($n = 82$; 38.3%) and in combination with ciprofloxacin ($n = 35$; 16.4%). Multidrug resistance (MDR), which is defined as resistance to three or more antibiotic classes, was observed in four (1.9%) isolates.

Fluctuations in resistance frequencies were observed by year. Although no significant increase in any resistance or MDR was observed over the 4-year period, notable trends were observed for some phenotypes (**Figure 2**). For instance, a significant decrease in the frequency of isolates with only tetracycline resistance was observed over time ($p = 0.04$), while a slight insignificant increase in ciprofloxacin resistance was observed alone ($p \leq 0.24$) and in combination with tetracycline resistance ($p \leq 0.50$). Despite the gradual increase in the frequency of any resistance to ciprofloxacin from 15.6% in 2011 to 25.0% in 2014, the change was not significant ($p \leq 0.31$). The same was true for any resistance to tetracycline, which decreased from 65.6% in 2011 to 52.5% in 2014 ($p = 0.17$).

Epidemiological Associations with Antibiotic Resistant *C. jejuni* Infections

Several notable associations were identified between epidemiological factors and the most common antibiotic resistant phenotypes, ciprofloxacin resistance and tetracycline resistance. These two predominant phenotypes were classified as the dependent variables to uncover associations with each phenotype relative to cases with either susceptible infections or infections with resistance to all other antibiotics.

Patients with ciprofloxacin resistance ($n = 49$) were more likely to travel in the month prior to infection (OR: 3.0; 95% CI: 1.37, 6.68) relative to all other cases (**Table 1**). More specifically, they were more likely to report international travel (OR: 9.8; 95% CI: 3.69, 26.09). Patients with tetracycline resistance ($n = 120$) were also more likely to report international travel in the past month, however, the difference was not significant (OR: 2.2; 95% CI: 0.85, 5.46). Patients with tetracycline resistance were also significantly less likely to have an infection during the

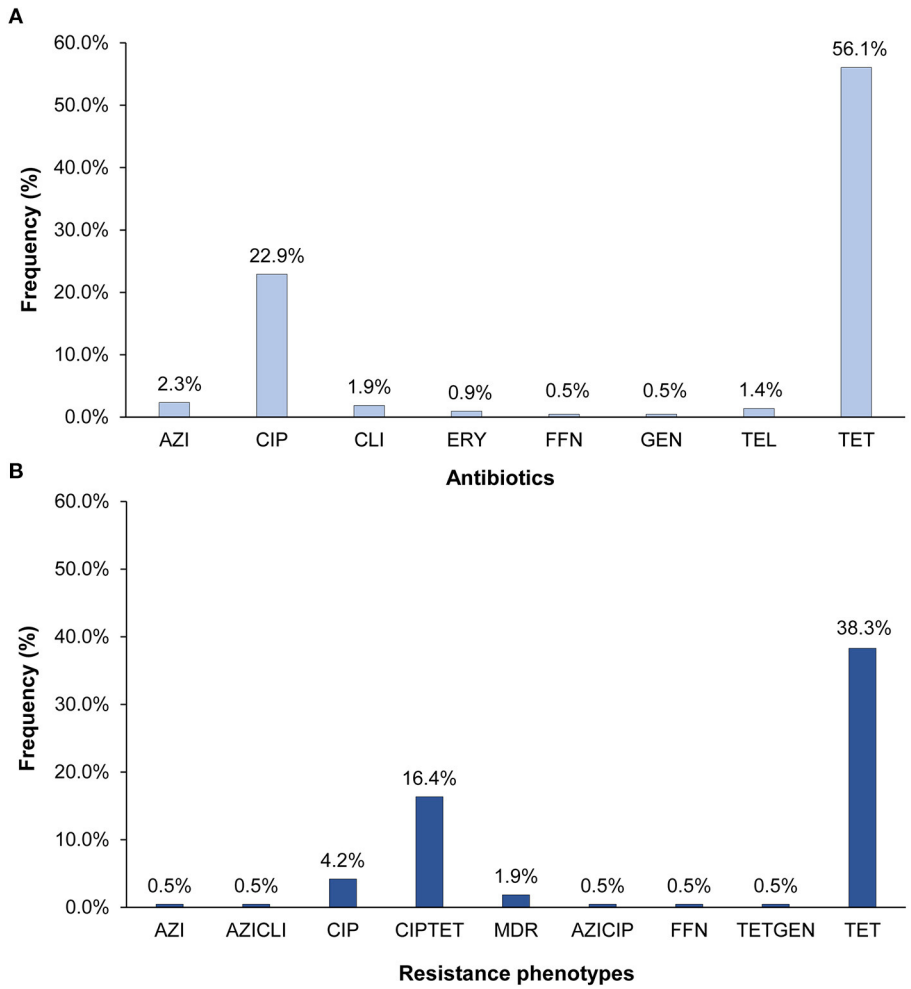

FIGURE 1 | Percentage of the 214 *Campylobacter jejuni* isolates with **(A)** resistance to eight different antibiotics and **(B)** distinct antibiotic resistance phenotypes. AZI, azithromycin; CIP, ciprofloxacin; CLI, clarithromycin; ERY, erythromycin; FFN, phenicol; GEN, gentamicin; TEL, telithromycin; TET, tetracycline. The multidrug resistant (MDR) phenotype includes isolates with resistance to CIPAZIERYCLITEL, CIPTETCLI, and CIPTETTEL. All CIP resistant isolates were also resistant to nalidixic acid.

summer or fall months (OR: 0.5; 95% CI: 0.27, 0.97) and to report contact with livestock (Fisher's exact test $p = 0.04$) or well water (OR: 2.3; 95% CI: 0.95, 5.75); the latter association was not significant. No association was observed between resistance to either antibiotic and domestic travel history, hospitalization, or clinical symptoms including body aches, diarrhea with blood, fatigue, fever, abdominal pain, and headache.

Because a subset of the isolates had both tetracycline and ciprofloxacin resistance, we created mutually exclusive categories to identify risk factors for each. In this analysis, ciprofloxacin resistance was defined as any resistance to ciprofloxacin even if resistance to other drugs including tetracycline was observed. Among the 135 resistant isolates, 49 (36.3%) had ciprofloxacin resistance. Tetracycline resistance was defined as any resistance to tetracycline but without the co-occurrence of ciprofloxacin resistance; 83 (61.5%) isolates had tetracycline resistance without ciprofloxacin resistance. Individuals with susceptible isolates and those representing different resistance profiles were excluded from the analysis. Compared to patients with tetracycline resistance, those with ciprofloxacin resistant infections were significantly more likely to report traveling in the past month (OR: 2.9; 95% CI: 1.20, 7.02) and specifically, international travel (Fisher's exact test $p < 0.0001$) (**Table 2**). Only four (19.1%) of the 21 patients who traveled internationally had tetracycline resistant infections compared to 17 (81.0%) of those with ciprofloxacin resistant infections. A difference was also observed for hospitalization, which was significantly more common in ciprofloxacin resistant infections (OR: 2.5, 95% CI: 1.02, 6.14), while contact with livestock was more common in tetracycline resistant infections (Fisher's exact test $p = 0.09$), yet the latter association was not significant. No association was observed for age, sex, race, ethnicity, residence types, season, water source, cattle density, poultry consumption, or clinical symptoms.

Multinomial logistic regression was performed to identify predictors of ciprofloxacin resistance relative to tetracycline resistance while controlling for age, sex, urban residence,

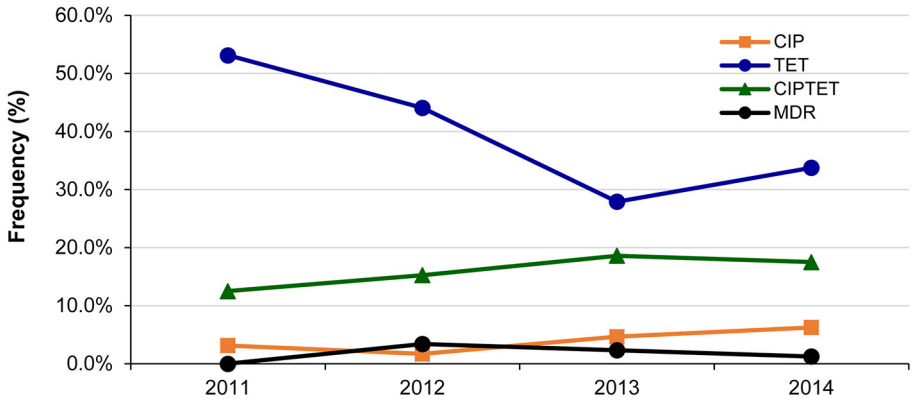

FIGURE 2 | Changes in the frequency of the most common antibiotic resistance phenotypes among 214 *Campylobacter jejuni* isolates over a 4-year period in Michigan. CIP, ciprofloxacin; TET, tetracycline. Multidrug resistant (MDR) isolates were resistant to CIPAZIERYCLITEL, CIPTETCLI, and CIPTETTEL.

season, and international travel. Notably, international travel in the past month (adjusted OR: 13.0; 95% CI: 3.71, 45.64) and infection during the summer or fall months were the only significant predictors of ciprofloxacin resistance (**Table 2**). Hospitalization was also more common in patients with ciprofloxacin resistance than tetracycline resistance, yet the association was not significant in the model (adjusted OR: 2.4; 95% CI: 0.86, 6.46), which could be due to the small sample size.

Comparing Resistance Frequencies to National Data Reported *Via* NARMS

A comparison of resistance frequencies for the NARMS isolates recovered during the same time period uncovered region-specific differences for both ciprofloxacin and tetracycline resistance. A significantly greater proportion of the Michigan isolates were resistant to tetracycline when compared to the 3,457 isolates from all regions except Region 5. Although Region 5 covers Michigan and other midwestern states, the data were generated by examining only a subset of isolates recovered from the Minnesota FoodNet site (**Figure 3A**). No difference in tetracycline resistance frequencies was observed between Michigan and the Region 5 isolates ($n = 585$), or for any of the ciprofloxacin resistance frequencies. However, when our Michigan isolates ($n = 214$) were added to Region 5, differences were observed for both ciprofloxacin and tetracycline resistance across the NARMS regions (**Figure 3B**). Notably, Region 5 had significantly more tetracycline resistance than all other regions combined (OR: 1.6; 95% CI: 1.41, 1.92) as well as a significantly greater proportion of ciprofloxacin resistance than Regions 2 (OR: 1.6; 95% CI: 1.24, 2.03) and 6 (OR: 2.1; 95% CI: 1.50, 3.00). Relative to Region 1, however, the proportion of ciprofloxacin resistance was significantly lower than in Region 5 (OR: 0.6; 95% CI: 0.50, 0.79). No other differences were observed for ciprofloxacin resistance by region.

DISCUSSION

Through this study we have detected important trends in the prevalence of campylobacteriosis and antibiotic resistant *C. jejuni* isolated from Michigan patients between 2011 and 2014, further highlighting the importance of pathogen surveillance efforts using culture-based methods. Since campylobacteriosis was not classified as a notifiable infection until 2015 (25), data about disease frequencies and resistance profiles have been limited, particularly for states like Michigan that are not participating in FoodNet or NARMS. In addition, the widespread adoption of culture-independent tests has hampered the ability to routinely monitor important phenotypes such as antibiotic susceptibility profiles. Indeed, it was estimated that 42% of campylobacteriosis cases identified via FoodNet in 2019 were diagnosed by culture-independent tests and among these, culture for *Campylobacter* was only attempted for 63% of the positive samples (2).

In the four Michigan hospitals examined herein, we observed a significant increase in *C. jejuni* infections over time, which is similar to national trends (2) and could partly be due to improved sampling and detection capacity. Seasonal differences were also observed with a greater proportion (73.8%) of Michigan cases occurring during the summer and fall. Seasonal variation has been reported previously with several studies showing a peak incidence of *C. jejuni* infections during warmer months; climate, temperature, increased shedding from animal reservoirs, and/or seasonal-specific behaviors have all been suggested to contribute to seasonality (26–28).

Extracting epidemiological data from case records has also facilitated the identification of factors that increase risk of campylobacteriosis. Similar to our prior study of 7,182 campylobacteriosis cases reported in Michigan between 2004 and 2013 (29) and those from the FoodNet sites (25, 30), most infections affected children <10 (30.8%) or adults between 19 and 65 (53.1%) years of age. Despite this bimodal distribution, the likelihood of hospitalization increased with increasing age. Cases between 41 and 65 years were significantly more likely to be hospitalized than those between 19 and 40 years of age as were cases over 65. The link between older age and more severe disease has been reported for the FoodNet sites and in our prior population-based study (29, 31).

TABLE 1 | Univariate analysis to identify factors associated with any ciprofloxacin resistance (CIP) and any tetracycline resistance (TET) among 214 *Campylobacter jejuni* isolates from Michigan, 2011 to 2014.

	Any CIP resistance ($n = 49$)				Any TET resistance ($n = 120$)			
Characteristics[a]	**No.**	**(%)**	**OR (95% CI)**[b]	***p*-value**[c]	**No.**	**(%)**	**OR (95% CI)**[b]	***p*-value**[c]
Age (years)								
0–9 ($n = 65$)	14	(21.5)	0.7 (0.31, 1.65)	0.43	39	(60.0)	1.1 (0.53, 2.32)	0.77
10–18 ($n = 18$)	2	(11.1)	–	0.21	6	(33.3)	0.4 (0.12, 1.14)	0.08
19–40 ($n = 54$)	15	(27.8)	1.0	–	31	(57.4)	1.0	–
41–65 ($n = 59$)	16	(27.1)	1.0 (0.42, 2.21)	0.94	33	(55.9)	0.9 (0.45, 1.98)	0.87
≥65 ($n = 17$)	2	(11.8)	-	0.21	10	(58.8)	1.1 (0.35, 3.20)	0.92
Sex								
Male ($n = 110$)	23	(20.9)	1.0	–	58	(52.7)	1.0	–
Female ($n = 96$)	23	(24.0)	0.8 (0.44, 1.62)	0.60	54	(56.3)	1.2 (0.66, 2.00)	0.61
Self-reported race[d]								
White/Caucasian ($n = 137$)	32	(23.4)	1.0	–	76	(36.5)	1.0	–
Non-white/other ($n = 35$)	9	(25.7)	1.1 (0.48, 2.67)	0.77	17	(48.6)	0.8 (0.36, 1.59)	0.46
Arab ethnicity								
No ($n = 106$)	27	(25.5)	–	–	53	(50.0)	1.0	–
Yes ($n = 25$)	3	(12.0)	–	0.19	17	(68.0)	2.1 (0.84, 5.35)	0.10
Season								
Winter, Spring ($n = 56$)	12	(21.4)	1.0	–	38	(67.9)	1.0	–
Summer, fall ($n = 158$)	37	(23.4)	1.1 (0.54, 2.34)	0.76	82	(51.9)	0.5 (0.27, 0.97)	0.04
Any travel in the past month								
No ($n = 88$)	13	(14.8)	1.0	–	45	(51.1)	1.0	–
Yes ($n = 61$)	21	(34.4)	3.0 (1.37, 6.68)	0.005	37	(60.7)	1.5 (0.76, 2.86)	0.25
Type of travel in the past month								
None ($n = 88$)	13	(14.8)	1.0	–	45	(51.1)	1.0	–
Domestic ($n = 33$)	4	(12.5)	–	1.0	18	(56.3)	1.1 (0.51, 2.56)	0.74
International ($n = 27$)	17	(63.0)	9.8 (3.69, 26.09)	<0.0001	18	(69.2)	2.2 (0.85, 5.46)	0.10
Type of drinking water								
Municipal, bottled ($n = 119$)	25	(21.1)	1.0	–	60	(50.4)	1.0	–
Any well water ($n = 27$)	5	(18.5)	0.9 (0.29, 2.48)	0.77	19	(70.4)	2.3 (0.95, 5.75)	0.06
Poultry consumption								
No ($n = 16$)	4	(25.0)	–	–	10	(62.5)	1.0	
Yes ($n = 115$)	24	(20.9)	–	0.75	61	(53.0)	0.7 (0.23, 1.99)	0.48
Any animal contact								
No ($n = 54$)	12	(22.2)	1.0	–	30	(55.6)	1.0	-
Yes ($n = 96$)	20	(20.8)	0.9 (0.41, 2.07)	0.84	52	(54.2)	0.9 (0. 48, 1.85)	0.87
Contact with livestock								
No ($n = 137$)	31	(22.6)	–	–	71	(51.8)	–	–
Yes ($n = 13$)	1	(7.7)	–	0.30	11	(84.6)	–	0.04
Cattle density in resident county[e]								
Low <8,400 cattle ($n = 23$)	3	(13.0)	–	–	12	(52.2)	1.0	–
High ≥8,400 cattle ($n = 82$)	21	(25.6)	–	0.27	50	(61.0)	1.4 (0.56, 3.63)	0.45
Residence type								
Rural ($n = 73$)	18	(24.7)	1.0	–	45	(61.6)	1.0	–
Urban ($n = 119$)	25	(21.0)	0.8 (0.41, 1.62)	0.56	62	(52.1)	0.7 (0.37, 1.22)	0.20
Hospitalized								
No ($n = 136$)	27	(19.9)	1.0		80	(58.8)	1.0	–
Yes ($n = 46$)	14	(30.4)	1.8 (0.83, 3.76)	0.14	21	(45.7)	0.6 (0.30, 1.15)	0.12

[a] *Not all numbers add up to the total number of cases per category due to missing data for some variables or the exclusion of susceptible isolates.*
[b] *The 95% confidence interval (CI) for the odds ratio (OR) is presented; ORs were calculated separately for CIP and TET relative to all other isolates.*
[c] *The Fisher's Exact Test was used for variables with ≤5 in one cell; no ORs could be calculated.*
[d] *Self-reported race categories in the online Michigan Disease Surveillance System questionnaire were: Caucasian, African American, Asian, American Indian/Alaska Native, Hawaiian/Pacific Islander, Unknown, or Other.*
[e] *Cattle density was not known for multiple counties with high case counts.*

TABLE 2 | Epidemiological factors associated with any ciprofloxacin resistance (CIP) vs. only tetracycline resistance (TET) among 135 patients with resistant infections.

	Any CIP resistance ($n = 49$)		Only TET resistance ($n = 83$)			
Characteristics[a]	**No.**	**(%)**	**No.**	**(%)**	**OR (95% CI)**[b]	***p*-value**[c]
Age (years)						
0–40 ($n = 83$)	31	(37.4)	52	(62.5)	1.0	–
≥41 ($n = 48$)	18	(37.5)	30	(62.5)	1.0 (0.48, 2.10)	0.99
Sex						
Male ($n = 65$)	23	(35.4)	42	(64.6)	1.0	–
Female ($n = 59$)	23	(39.0)	36	(61.0)	1.2 (0.41, 1.78)	0.85
Residence type						
Rural ($n = 48$)	18	(37.5)	30	(37.5)	1.0	–
Urban ($n = 70$)	25	(35.7)	45	(64.3)	0.9 (0.43, 1.98)	0.84
Season						
Winter, spring ($n = 39$)	12	(30.8)	27	(69.2)	1.0	–
Summer, fall ($n = 93$)	37	(39.8)	56	(60.2)	1.5 (0.67, 3.30)	0.33
Any travel in the past month						
No ($n = 49$)	13	(26.5)	36	(73.5)	1.0	–
Yes ($n = 41$)	21	(51.2)	20	(48.8)	2.9 (1.20, 7.02)	0.02
Type of travel in the past month						
None ($n = 49$)	13	(26.5)	36	(73.5)	1.0	–
Domestic ($n = 19$)	4	(21.1)	15	(79.0)	–	0.76
International ($n = 21$)	17	(81.0)	4	(19.1)	–	<0.0001
Type of drinking water						
Municipal, bottled ($n = 67$)	25	(37.3)	42	(62.7)	1.0	–
Any well water ($n = 20$)	5	(25.0)	15	(75.0)	–	0.42
Poultry consumption						
No ($n = 16$)	4	(40.0)	6	(60.0)	–	–
Yes ($n = 115$)	24	(35.3)	44	(64.7)	–	0.77
Contact with livestock						
No ($n = 79$)	31	(39.2)	48	(60.8)	–	–
Yes ($n = 11$)	1	(9.1)	10	(90.9)	–	0.09
Hospitalized						
No ($n = 85$)	27	(31.8)	58	(68.2)	1.0	–
Yes ($n = 26$)	14	(53.9)	12	(46.2)	2.5 (1.02, 6.14)	0.04
Multivariate analysis[d]					Adjusted OR (95% CI)	*p*-value
Age					1.0 (0.98, 1.02)	0.87
Female					0.5 (0.17, 1.40)	0.18
Urban residence					1.0 (0.33, 2.83)	0.95
Summer or fall infection					3.7 (1.03, 13.47)	0.04
International travel only					14.9 (4.00, 55.57)	<0.0001
Hospitalized					3.0 (0.78, 11.19)	0.11
Well water					0.6 (0.16, 2.26)	0.44
Livestock contact					0.2 (0.02, 2.25)	0.21

[a] *Number of isolates may not add up to the total for some variables due to missing data; percentages were calculated using the number with each characteristic as the denominator.*
[b] *95% confidence interval for the odds ratio (OR). ORs were calculated for ciprofloxacin resistance relative to tetracycline resistance.*
[c] *The Fisher's Exact test was used for variables with fewer than 5 in one cell; no ORs could be calculated.*
[d] *Multivariate results were generated using forward stepwise logistic regression while controlling for variables with p-values ≤0.2 in the univariate analysis as well as potential confounders. A base model consisted of the following variables: age (continuous), female sex, urban residence, season (fall and summer), and international travel. Each additional variable was added separately to the base model. The Homer and Lemeshow Goodness-of-Fit test ($p > 0.05$) was examined to ensure support for each model. Adjusted ORs were calculated and the Wald Chi-Square test was used to determine significance with 95% Wald Confidence Limits.*

Although males and rural residents represented a greater proportion of the 7,182 campylobacteriosis cases in Michigan (29), similar distributions were not observed among the cases at the four hospitals. For instance, no difference was observed by sex and significantly more cases (62.0%) were from urban areas, suggesting that the four hospitals may not be entirely representative of the Michigan population of campylobacteriosis cases. Such differences are likely due to the structure of the

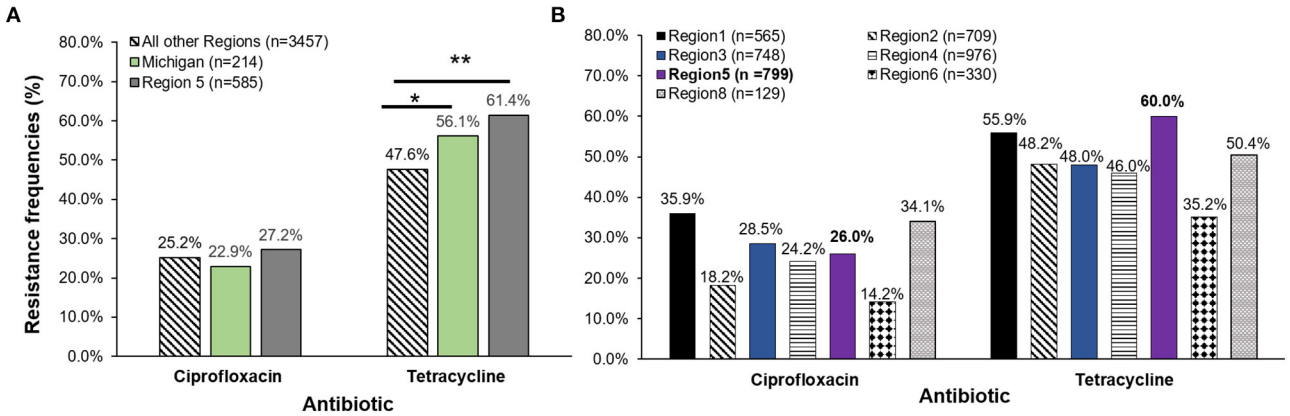

FIGURE 3 | (A) Antibiotic resistance frequencies of *Campylobacter jejuni* strains recovered from four Michigan hospitals ($n = 214$) in 2011–2014 as compared to the National Antimicrobial Resistance Monitoring System (NARMS) data for the same time period. Michigan frequencies were compared to NARMS data from Region 5 acquired from Minnesota (representing Ohio, Indiana, Michigan, Illinois, Wisconsin, and Minnesota and 34 federally recognized tribes) and the total national data (excluding Region 5). **(B)** Michigan frequencies were added to Region 5 national data ($n = 585$) leaving a total of 799 strains in the Midwest region for comparison to NARMS regions 1, 2, 3, 4, 6, and 8. $^{*}p \leq 0.05$, $^{**}p \leq 0.0001$; χ^2 test. The 10 FoodNet sites representing Connecticut, Georgia, Maryland, Minnesota, New Mexico, Oregon, Tennessee, California, Colorado, and New York send data captured by the state public health laboratories to NARMS to represent the different regions. Data from Region 1 (Connecticut, Maine, Massachusetts, New Hampshire, Rhode Island, and Vermont), Region 2 (New Jersey, New York, Puerto Rico, and the Virgin Islands), Region 3 (Delaware, District of Columbia, Maryland, Pennsylvania, Virginia, and West Virginia), Region 4 (Alabama, Florida, Georgia, Kentucky, Mississippi, North Carolina, South Carolina, and Tennessee), Region 6 (Arkansas, Louisiana, New Mexico, Oklahoma, and Texas), and Region 8 (Colorado, Montana, North Dakota, South Dakota, Utah and Wyoming) were included in the analysis. Regions 7, 9, and 10 did not have data available for *Campylobacter jejuni* from 2011 to 2014 for comparison.

surveillance system since we utilized four of the largest health care systems. Despite having wide catchment areas, each hospital is in a metropolitan location that can result in differences in access to health care, particularly for rural residents, supporting the suggestion that geography as well as patient-specific and cultural factors can impact care seeking behaviors (11). Indeed, we observed a lower likelihood of hospitalization among rural residents in this and our prior study (29), although this association was not significant after controlling for race, sex, and age.

Because the likelihood of hospitalization was significantly greater for cases self-identifying as non-White, urban areas should be an important focus for reducing disparities in infections caused by *C. jejuni* and other enteric pathogens. Certainly, neighborhood and geographic barriers have previously been suggested to be important for the acquisition of foodborne disease (13). Although race, ethnicity, and other socially constructed categorizations such as socio-economic status, are not typically collected for foodborne disease surveillance systems, prior studies have document increased frequencies of gastroenteritis in minority and low-socioeconomic populations globally (32–34). Additional studies are needed, however, to identify specific risk factors, exposures and causal factors within urban environments that may explain these relationships. Use of previously reported proxies and markers of poverty such as urban residence, and social constructs like self-reported race as we have used, have complex interactions with social determinants of health (35, 36). We therefore cannot describe causal factors for hospitalization of *C. jejuni* without addressing these shortcomings. We also cannot rule out the possibility that different strain populations with distinct pathogenic traits are circulating in the different areas and are partly responsible for the differences observed.

Since most hospital laboratories in Michigan have switched to the use of culture-independent tests to detect *C. jejuni*, viable isolates are not typically recovered for characterizing important phenotypic or genotypic traits. Hence, our assessment of resistance frequencies and trends in the four hospitals over this 4-year period yielded notable results. Resistance was detected in 63.1% of the 214 isolates and to all nine antibiotics comprising 11 distinct resistance profiles. The overall predominance of tetracycline (56.1%) and ciprofloxacin (22.9%) resistance was similar in our prior study of 94 isolates recovered in 2011 and 2012 (22). The inclusion of 120 additional isolates recovered from the same hospitals in 2013–2014, however, allowed for the detection of several important changes over time, including an increase in the frequency of fluoroquinolone resistance. This gradual increase is concerning given that fluoroquinolones are commonly used to treat human infections and the Food and Drug Administration (FDA) banned use of these drugs in poultry in 2005 (37). Point mutations in chromosomal genes such as *gyrA*, which is critical for DNA replication and transcription, have been linked to fluoroquinolone resistance (38, 39). Given that these mutations do not halt transcription, there is no impact on bacterial survival and hence, these resistant bacterial populations can persist in the absence of antibiotic selection (40, 41). This increasing frequency of ciprofloxacin resistance in *C. jejuni* is consistent with national trends for older strain sets recovered via culture-based detection methods (25). Because of the increased use of culture-independent methods to detect *Campylobacter*,

however, actual rates of ciprofloxacin resistance among clinical isolates in different parts of the U.S. are not well-established. Additionally, despite the critical role that FoodNet and NARMS have played in the detection of resistant foodborne pathogens, neither system is entirely representative of the U.S. population (12). To represent the entire Midwest (Region 5) that includes Michigan, for instance, NARMS only receives a subset of isolates from Minnesota for testing (18). It is therefore important to note that the frequencies and trends reported by NARMS may not accurately reflect those observed in other locations with distinct geographic features and population traits.

Furthermore, fluoroquinolone resistant *C. jejuni* infections have also been reported to increase the duration of illness (42, 43). Mutations in *gyrA* have been tied to changes in DNA supercoiling, which can lead to enhanced colonization of the chicken gut and an increase in virulence properties such as motility, biofilm formation and invasion of intestinal epithelial cells *in vitro* (44–47). These studies establish a mechanism by which fluoroquinolone resistant mutations enhance virulence and support prior associations between resistance and a lengthier illness duration. Additional support comes from our finding that patients with ciprofloxacin resistant infections were twice as likely to be hospitalized than patients with tetracycline resistant infections, suggesting that the former may be more severe. It is not clear, however, if a unique patient population, differential treatment regimens, or distinct bacterial factors account for the difference in the hospitalization rates observed.

Significant differences in the frequency of tetracycline resistance, which was highest in this population of Michigan patients despite the gradual decrease in tetracycline resistance over time, were also observed. These data indicate that unique regional factors may impact resistance rates, yet those factors that contribute to variation across locations are not clear. The tetracyclines have been used to treat zoonotic and rickettsial diseases in human medicine (48) and have been used extensively in livestock and poultry production worldwide. In the U.S., the FDA reported that tetracyclines were the predominant drug class used in food-producing animals at the time of this study (2009–2014), representing an average of 42% of all antibiotics used (49). Continuous use of tetracycline has selected for resistant strains and resistance genes that can persist in reservoir hosts and the environment. For example, TetO has been shown to mediate resistance to tetracycline in *C. jejuni* by offering ribosomal protection by binding to an unoccupied site (39). This protein is encoded by *tet*(O), which is commonly carried on the pTet plasmid but has also been detected in the chromosome (50, 51). Given the high transmissibility rates of these resistance plasmids within bacterial populations even in the absence of tetracycline use (52, 53), it is clear that *C. jejuni* serves as an important reservoir for these and other resistance genes. In our prior study, we demonstrated that tetracycline resistance was more common in strains belonging to multilocus sequence type (ST)-982, a lineage that was also common in Michigan cattle (22, 54) and has been linked to livestock in other locations (55, 56). Together, these data show the importance of clonal expansion of resistant lineages and highlight the role that mobile genetic elements play in dispersion and maintenance of tetracycline resistance.

Although we observed a significant association between livestock contact and tetracycline resistance, the number of cases ($n = 13$) reporting this exposure was low. It is noteworthy, however, that only one of the cases reporting contact with livestock had a ciprofloxacin resistant infection compared to 11 (84.6%) with tetracycline resistant infections. While increasing frequencies of ciprofloxacin resistant *C. jejuni* have been recovered from feedlot cattle throughout the U.S. (57), our data suggest that different factors are important for the acquisition of ciprofloxacin vs. tetracycline resistant infections. Consistent with prior studies (25, 30, 42, 58), we have demonstrated that international travel in the month prior to infection is the strongest predictor of ciprofloxacin resistance in this sample of Michigan patients. Infection during the summer or fall months was also independently associated with ciprofloxacin resistance, but we did not observe an association with poultry consumption as was described in other studies (42, 58, 59). This difference could be due to the low number of cases reporting no poultry consumption a week before symptom onset or the high frequency of missing data since many patients failed to answer the food history questions, a common problem with long-term epidemiological studies (60). In general, however, the identification of risk factors that have also been described in other studies is encouraging and indicates that these factors are likely important regardless of the geographic location.

Collectively, the data presented herein demonstrate the importance of monitoring antibiotic resistance phenotypes and frequencies using culture-based methods in multiple geographic locations. The significant difference that we observed in NARMS Region 5 relative to other regions after including our Michigan data with those from Minnesota, illustrates the need for more comprehensive testing and highlights the variation across different geographic locations. Future studies are still needed, however, to link resistance profiles and patient data to epidemiological data to identify those exposures and risk factors that are unique to specific states or regions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Boards at Michigan State University (10-735SM), the Michigan Dept. of Health and Human Services (842-PHALAB) and each of the four participating hospitals. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SDM, WC, and JAR designed the study. JTR, DWN, HS, PL, and WK organized sample collection at each site. REM and WC isolated pathogens and extracted epidemiological data. JAR and WC performed the experiments. JAR, SM, and SDM managed the data and conducted analyses. JAR developed the first manuscript draft. All authors contributed and approved the manuscript content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.672473/full#supplementary-material

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Monitoring of Antimicrobial Resistance to Aminoglycosides and Macrolides in *Campylobacter coli* and *Campylobacter jejuni* From Healthy Livestock in Spain (2002–2018)

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Antimicrobial resistance (AMR) in *Campylobacter* spp. (*Campylobacter coli* and *Campylobacter jejuni*) is a concern due to its importance in public health, particularly when it involves aminoglycosides and macrolides, drugs of choice for treatment of human cases. Co-resistance to these two antimicrobial classes involves transfer of genetic elements and/or acquisition of mutations in different genetic loci, which can in turn spread through vertical or horizontal gene transfer (HGT) phenomena, with each route having different potential implications. This study aimed at evaluating the association between the presence of phenotypic resistance to these two antimicrobial classes in *C. coli* and *C. jejuni* recovered from livestock at slaughterhouses in Spain (as part of the AMR surveillance program), and at assessing the genetic heterogeneity between resistant and susceptible isolates by analysing the "short variable region" (SVR) of the *flaA* gene. Over the 2002–2018 period, antimicrobial susceptibility test results from 10,965 *Campylobacter* isolates retrieved from fecal samples of broilers, turkeys, pigs and cattle were collected to compare the proportion of resistant isolates and the Minimum Inhibitory Concentrations (MICs) against six antimicrobials including gentamicin (GEN), streptomycin (STR), and erythromycin (ERY). AMR-associated genes were determined for a group of 51 isolates subjected to whole genome sequencing, and the *flaA* SVR of a subset of 168 isolates from all hosts with different resistotypes was used to build a Neighbor-Joining-based phylogenetic tree and assess the existence of groups by means of "relative synonymous codon usage" (RSCU) analysis. The proportion of antimicrobial resistant isolates to both, aminoglycosides and macrolides,

varied widely for *C. coli* (7–91%) and less for *C. jejuni* (all hosts 0–11%). Across hosts, these proportions were 7–56% in poultry, 12–82% in cattle, and 22–91% in pigs for *C. coli* and 0–8% in poultry and 1–11% in cattle for *C. jejuni*. Comparison of the MIC distributions revealed significant host-specific differences only for ERY in *C. jejuni* (p = 0.032). A significant association in the simultaneous presentation of AMR to both antimicrobial classes was observed across hosts/bacterial species. The *flaA* gene analysis showed clustering of isolates sharing resistotype and to a lesser degree bacterial species and host. Several resistance markers associated with resistance to aminoglycosides and macrolides were found among the sequenced isolates. The consistent association between the simultaneous presentation of AMR to aminoglycosides and macrolides in all hosts could be due to the persistence of strains and/or resistance mechanisms in *Campylobacter* populations in livestock over time. Further studies based on whole genome sequencing are needed to assess the epidemiological links between hosts and bacterial strains.

Keywords: *Campylobacter*, antibiotics, antimicrobial resistance, aminoglycosides, macrolides, flagellin, genes

INTRODUCTION

Campylobacter coli and *Campylobacter jejuni*, thermophilic bacteria of the genus *Campylobacter* spp., are the most frequently notified human gastrointestinal zoonotic pathogens in the European Union (EU) since 2005 (EFSA, 2005, 2006, 2007). Traditionally, *C. jejuni* was more frequently isolated from human cases than *C. coli*, but in 2017, 24.1% of *Campylobacter* confirmed human infections in the EU were caused by *C. coli* versus 22.2% due to *C. jejuni*, thus suggesting this pattern may vary (EFSA-ECDC, 2019b). Poultry and poultry products are considered the main source of human campylobacteriosis, followed by ruminants (beef, dairy cattle, and their manure) and environmental sources (Ravel et al., 2017; Rosner et al., 2017; An et al., 2018; Thepault et al., 2018). Consumption of pig meat has been linked to human cases caused by *C. coli* (Rosner et al., 2017). Although the prevalence of *Campylobacter* spp. in pork is high, previous studies found it was only associated with 2% of all human cases (Kittl et al., 2013), while beef has been linked to 19% of human cases (Boysen et al., 2014).

Treatment of human cases, when necessary, may be hampered by the increasing threat of antimicrobial resistance (AMR) observed in recent years (Friedrich, 2019). Macrolides [erythromycin (ERY) and azithromycin] and fluoroquinolones [ciprofloxacin (CIP)] are the drugs of choice in human patients requiring antibiotic treatment, but the latter class is not recommended for children. When these drugs are ineffective, systemic administration of aminoglycosides is the only option left (Bolinger and Kathariou, 2017; World Health Organization (WHO), 2017). Levels of AMR to aminoglycosides and macrolides in clinical thermophilic *Campylobacter* from humans are increasing (Aarestrup and Wegener, 1999; Bolinger and Kathariou, 2017; EFSA-ECDC, 2019b), and a similar trend for macrolides has been observed in isolates from pigs and broilers (Moore et al., 2006; Wang et al., 2016). In Europe, the proportion of resistant *Campylobacter* spp. isolates from food producing animals and humans vary depending on the country. Isolates originating from Spain showed higher levels of resistance to aminoglycosides [gentamicin (GEN) and streptomycin (STR)], macrolides (ERY), quinolones [CIP and nalidixic acid (NAL)], and tetracycline (TET) in *C. coli* from broilers, turkeys and pigs, and *C. jejuni* from broilers (EFSA, 2005, 2006, 2007; EFSA-ECDC, 2016, 2017, 2018a, 2019b). *C. coli* has traditionally shown higher levels of AMR to most antimicrobials compared with *C. jejuni* from both humans and animals (EFSA-ECDC, 2017, 2018a, 2019b). Therefore, the AMR problem may intensify if the importance of *C. coli* as a human pathogen keeps increasing. *C. coli* is now as prevalent as *C. jejuni* in broilers in some countries (Wieczorek and Osek, 2013), thus, monitoring of *C. coli* and *C. jejuni* AMR levels, particularly to macrolides and aminoglycosides, is equally important (EFSA-ECDC, 2018a, 2019b).

Resistance to aminoglycosides and macrolides can be mediated by multiple mechanisms, including chromosomal mutations and horizontal gene transferable elements (Davies and Wright, 1997; Saenz et al., 2000; Moore et al., 2006; Luangtongkum et al., 2009; Wieczorek and Osek, 2013; Bolinger and Kathariou, 2017). Antibiotic modifying enzymes (AMEs) are commonly involved in resistance to aminoglycosides (Saenz et al., 2000; Wieczorek and Osek, 2013; Garneau-Tsodikova and Labby, 2016), whereas ribosome methyltransferases (RMTs) are frequently related to resistance to macrolides (Saenz et al., 2000; Aarestrup, 2005). Co-resistance to both aminoglycosides and macrolides, as well as multidrug resistance (MDR) to additional antimicrobial classes, can be acquired through several mechanisms such as 16S rRNA RMTs (RmtB, ArmA) encoded in multi-drug resistance genomic islands (MDRGIs) carrying *erm* genes in *C. coli* (Aarestrup, 2005; Garneau-Tsodikova and Labby, 2016; Bolinger and Kathariou, 2017) and 23S rRNA RMTs (Saenz et al., 2000). Additional mechanisms can involve transferable genomic islands carrying multiple aminoglycoside resistance genes encoding AMEs in *C. coli* (Davies and Wright, 1997; Luangtongkum et al., 2009; Qin et al., 2012; Wieczorek and Osek, 2013) and multidrug macrolide efflux pumps (including

the resistance enhancing *CmeABC* in *C. jejuni*), alone or in combination with target gene mutations (Aarestrup, 2005; Wieczorek and Osek, 2013; Garneau-Tsodikova and Labby, 2016; Bolinger and Kathariou, 2017). Although several of these mechanisms were first discovered in *C. coli*, evidence of transfer to *C. jejuni* was shown thereafter (EFSA-ECDC, 2018a).

Epidemiological studies complemented with genetic analyses are essential to help understand the mechanisms by which co-resistance and MDR to aminoglycosides and macrolides may be emerging in thermophilic *Campylobacter* from animals and humans (Luangtongkum et al., 2009; Wieczorek and Osek, 2013). Here, data from the national surveillance program on AMR in *Campylobacter* spp. from broilers, turkeys, pigs and cattle in Spain were analyzed to assess the prevalence of *Campylobacter* species in different animal hosts and the patterns of phenotypic AMR co-resistance to macrolides and aminoglycosides over the years. The results of this research can contribute to better explain AMR co-selection/MDR phenomena between these two antimicrobial classes in *Campylobacter* from livestock.

MATERIALS AND METHODS

Study Population

The data analyzed here is based on sample collection, culture and antimicrobial susceptibility testing (AST) work carried out during 2002–2018 on isolates retrieved through the Spanish national veterinary AMR monitoring program for *Campylobacter* spp. in poultry (broilers and turkeys), pigs and cattle, according to EU legislation (EC, 2013). Samples for each animal species, originating from multiple farms, were collected at slaughterhouses covering 60% of the national throughput (**Supplementary Figures 1**, **2**). Broiler samples were retrieved every year from 2002 to 2014 and every 2 years thereafter, turkey samples every 2 years from 2014 to 2018, pig samples every year from 2002 to 2013 and every 2 years thereafter, and cattle samples every year from 2007 to 2013 and every 2 years thereafter. Pooled samples collected every year for each host species ranged between 76 and 500 (mean = 228) for broilers, 467 and 500 (mean = 485) for turkeys, 171 and 384 (mean = 268) for pigs, and 163 and 384 (mean = 261) for cattle (**Supplementary Table 1**).

In relation to sampling and culture, pools made of samples from animals belonging to the same farm (from 10 caeca in poultry or from the caecum content of 2 animals in pigs and cattle) were collected at the slaughterhouse and transported refrigerated to the laboratory, where they were processed within 24 h after collection according to ISO 10272-2006-1. Single colonies with morphology compatible with *Campylobacter* spp. were identified as "*C. coli*," "*C. jejuni*," or "*C.* spp." using API strips (up to 2010) and a multiplex PCR (2010 onward) (Ugarte-Ruiz et al., 2012).

Regarding AST, the AMR phenotype of *Campylobacter* spp. isolates was determined using the two-fold broth micro-dilution reference method (calculating Minimum Inhibitory Concentrations – MICs, according to ISO Norm 20776-1:2006) or diffusion technique (calculating Inhibition Zone Diameters – IZDs) (Ugarte-Ruiz et al., 2015).

Antimicrobial susceptibility testing results for the six antimicrobials listed on the AMR surveillance programs for *Campylobacter* spp. in the EU (EC, 2013) were available for isolates from all species and years: CIP, TET, NAL, STR, ERY, and GEN. For ERY in broilers, IZDs were used up to 2004 (included) and MICs were used thereafter. For STR in broilers and pigs, IZDs were used up to 2005 (included) and MICs were used thereafter.

Samples in which information on culture result, molecular identification and/or AMR typing was missing were excluded from the analysis. The following information was available for all samples in the study: host species, *Campylobacter* growth result, *Campylobacter* species, year and AST result. Isolates were classified as "susceptible" (wild-type strains) or "not susceptible" (resistant strains) according to epidemiological cut-off points (ECOFFs) provided by the "European Committee on Antimicrobial Susceptibility Testing" (EUCAST)[1] (**Table 1**). Proportions of resistant isolates for each bacterial and host species and period were defined as very low (<1%), low (1.1–10%), moderate (10.1–20%), high (20.1–50%), very high (50.1–70%), and extremely high (70.1–100%), as recommended by EFSA (EFSA-ECDC, 2019b).

Statistical Analyses

Proportions of resistant *C. coli* and *C. jejuni* isolates to each antimicrobial from the different hosts were compared using Z-tests, adjusted for multiple comparisons by the Holm-method. Cochrane-Armitage logistic regressions were used to test for trends of AMR phenotypic resistance in *C. coli* and *C. jejuni* per antimicrobial and host species, and the relative change in the proportion of resistant isolates per year was computed along with its 95% confidence interval. The association in the simultaneous presentation of phenotypic resistance to STR/ERY, GEN/ERY, and GEN/STR over the whole study period and in different time periods (2002–2006, 2007–2012, and 2013–2018) was further

[1]EUCAST-European Society of Clinical Microbiology and Infectious Diseases, MIC and zone distributions and ECOFFs http://www.eucast.org/mic_distributions_and_ecoffs/ (accessed 14/02/2020).

TABLE 1 | Epidemiological cut-offs (ECOFFs) used for interpretation of MICs in *Campylobacter* spp. (Source EUCAST).

Antimicrobial	*C. coli* MIC (>) Micro-dilution	*C. coli* DIAM (<) Difusion	*C. jejuni* MIC (>) Micro-dilution	*C. jejuni* DIAM (<) Difusion
Gentamicin (GEN)	2	NA	2	20
Streptomycin (STR)	4	13	4	13
Erythromycin (ERY)	8	24	4	22
Ciprofloxacin (CIP)	0,5	26	0,5	26
Nalidixic acid (NAL)	16	NA	16	NA
Tetracycline (TET)	2	30	1	30

evaluated for each bacterial and host species using relative risks and chi-squared and Fisher's exact tests.

In order to evaluate differences in the distribution of MICs values in *C. coli* and *C. jejuni* from the four host species, available data were represented as "squashtograms." The existence of statistical differences in MICs distributions in susceptible and not-susceptible (here referred to as "resistant") isolates depending on bacterial species (for a given host) or on host (for a given bacterial species) was evaluated using Mann–Whitney *U* or Kruskal–Wallis tests followed by Dunn's *post hoc* tests, correcting for multiple comparisons by the Benjamini-Hochberg method.

Molecular Characterization Based on *flaA* Sequencing

A subset of 125 isolates including all combinations of hosts, bacterial species, year of recovery and AMR phenotype, randomly chosen within each category, was used to assess their genetic relatedness by comparing the flagellin *flaA* short variable region (SVR) gene sequence as described by other authors (Ugarte-Ruiz et al., 2013; Zhang et al., 2018). Selected isolates were classified into two categories: isolates with simultaneous phenotypic resistance to aminoglycosides and ERY (n = 53, "cases") and isolates not presenting this simultaneous resistance (n = 72, "controls"). Amplification of the *flaA* SVR gene sequence by PCR was performed as previously described (Ugarte-Ruiz et al., 2013), and the obtained amplicons were sequenced. Additionally, the *flaA* sequence of fifty-one isolates (8 "cases" and 43 "controls") previously subjected to whole genome sequencing (GenBank accession codes SRX5575129 to SRX5587545) was extracted (along with information on the presence of resistance genes) using a homemade Python script. The resulting 176 sequences were then aligned using MUSCLE (Edgar, 2004) and a Neighbour-Joining (NJ) phylogenetic tree with 1,000 bootstraps was built to evaluate the phylogenetic relationship between isolates. The *flaA* gene of the NCTC 1168 *C. jejuni* strain (1719 nucleotides-long, bacterial chromosome positions 1269232 to 1270950) was used as an external reference. A multiple correspondence analysis (MCA) of the relative synonymous codon usage (RSCU) values categorized as > 1 (positive bias) or < 1 (negative bias) was performed as described before (Meinersmann et al., 2005). The MCA included, along with the RSCU of variable codons, other available covariates (bacterial species, host species, resistance to GEN, ERY, and STR, and clade as determined in the NJ phylogenetic tree).

Microsoft Access was used for data handling and database initial analyses. Data were further handled with Microsoft Excel and imported into "R" version 3.6.3 (R Core Team, 2020). The R packages "FSA" (Ogle et al., 2020), "plyr" (Wickham, 2011), and "ggplot2," "dplyr," "reshape2," and "tidyr" (Wickham et al., 2019) were used for the analysis and visual representation of the data. Information on resistance-associated markers from the sequenced strains was extracted using ResFinder (Bortolaia et al., 2020). MEGA-X (Kumar et al., 2018) and DnaSP6 (Rozas et al., 2017) were used on imported DNA sequences for the preparation and analysis of sequence alignments. R packages "BiocManager" ("coRdon") (Morgan and Ramos, 2019) and "seqinr" (Charif and Lobry, 2007) were used for the calculation of RSCUs. "Corrplot" (Wei et al., 2017), "FactoMineR" (Lê et al., 2008), "factoextra" (Kassambara and Mundt, 2020), and "ggtheme" were used for the MCA analysis. All Figures were generated using R except **Figure 1** (Excel) and **Figure 6** (MEGA-X).

RESULTS

In total 3,413 independent samples from broilers, 1,455 from turkeys, 3,750 from pigs and 2,347 from cattle were included in the analysis, of which 2,000 (58.6%), 1,090 (74.9%), 2,218 (59.2%), and 1,273 (54.3%) resulted in the isolation of *Campylobacter* spp., respectively (**Table 2**). The number of samples analyzed, isolates recovered, and isolates subjected to AST varied depending on year and host species (**Supplementary Table 2**).

Over the entire study period, *C. coli* was the most frequently isolated species in pig (88.7%; 1,968/2,218) and turkey (74.8%; 815/1,090) samples, while *C. jejuni* was the most frequent species in cattle (84.4%; 1,074/1,273). In broilers, the proportion of *C. coli* and *C. jejuni* was very similar (51.2%; 1,023/2,000 and 47.8%; 957/2,000, respectively) (**Table 2**).

Although there were differences depending on the year, the proportion of positive samples to *C. coli* and *C. jejuni* remained relatively constant over the whole study period in pigs and cattle, with one bacterial species being more prevalent than the other one, while the situation was more variable in broilers (**Figure 1**). Significant increasing trends were observed in the proportion of positive samples for *C. jejuni* in broilers and turkeys, with annual-biannual rates of increase of 9.7% (95%CI: 6.16–13.33%) and 26.2% (95%CI: 15.81–37.43%), respectively (**Figure 1**).

The overall proportion of isolates resistant to CIP, NAL, and TET was extremely high (>80%) in both *C. coli* and *C. jejuni* from all host species (**Table 3** and **Figures 2**–**5**), with yearly values exceeding 70% throughout the study period except in *C. jejuni* from cattle (**Figure 5**). Still, there were significant differences in the proportion of resistant isolates to these three antimicrobials depending on the host (**Table 3**). The proportion of CIP and NAL-resistant *C. coli* isolates was significantly lower in cattle compared with broilers and turkeys ($p < 0.05$) (and in pigs compared with turkeys for CIP, $p < 0.001$). In the case of TET, *C. coli* isolates from pigs were significantly more resistant than *C. coli* from cattle and broilers ($p < 0.001$), although resistance was still above 95% in all species (**Table 3**). In the case of *C. jejuni*, cattle isolates were significantly less resistant to the three antimicrobials compared with isolates from poultry ($p < 0.05$).

The proportion of resistance to the remaining three antimicrobials (STR, ERY, and GEN) was much more variable (**Table 3** and **Figures 2**–**5**). In the case of STR, extremely high (80–91%) or high to very high (∼55%) levels were found in *C. coli* from cattle and pigs and from broilers and turkeys, respectively, with significant differences between all hosts species except between broilers and turkeys (pigs > cattle > poultry). In contrast, values <11% were observed in *C. jejuni* from all three host species

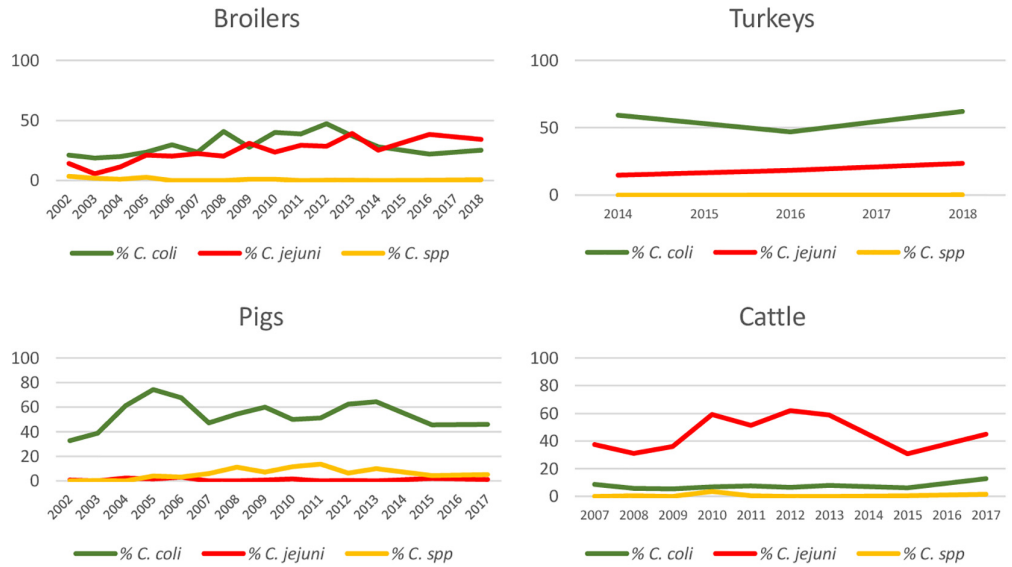

FIGURE 1 | Percentage of *Campylobacter* isolates over total sampled, identified as *C. coli*, *C. jejuni*, and *C.* spp. for each host species throughout study period.

from which this bacterial species was recovered (broilers, turkeys, and cattle). Although levels of resistance in *C. jejuni* were always significantly lower than in *C. coli* for any given host species, no significant differences between host species were observed.

The proportion of resistance to ERY in *C. coli* was very high (67%) for pigs, high (35%) for broilers and turkeys, and moderate (19%) for cattle (pigs > poultry > cattle) (**Table 3** and **Figures 2–5**). Overall values in *C. jejuni* from all host species were < 3% and significantly lower than those from *C. coli*, again with no significant differences across hosts.

Finally, the proportion of GEN resistant-isolates was low (<25%) in *C. coli* from all species (**Table 3** and **Figures 2–5**), although the proportion in pigs was again significantly higher than that observed in other host species ($p < 0.001$). Resistance levels in *C. jejuni* were lower (<2%) and significantly different from those observed in *C. coli* from the same host and, once more, no significant differences between hosts were observed.

Significant ($p < 0.05$) trends in the proportion of cattle resistant isolates were observed associated to increasing annual rates for ERY and *C. coli* (29.9% although with a wide 95%CI: −31.36 to 145.77) and STR and *C. jejuni* (16.7%, 95%CI: 2.89–32.28) (**Figure 5**). Significant ($p < 0.001$) trends in the proportion of resistant *C. jejuni* isolates recovered yearly from broilers were also observed for two antimicrobials, in both cases associated to decreasing annual rates: STR (−9.9%, 95%CI: −16.13 to −3.14) and ERY (−27.5%, 95%CI: −44.60 to −5.10) (**Figure 2**).

Other significant ($p < 0.001$) trends were found associated with modest annual rates of increase in *C. jejuni* in cattle for CIP (5.22%, 95%CI: 3.91–6.54) and NAL (4.11%, 95%CI: 2.20–6.06), in *C. coli* in broilers for TET (1.61%, 95%CI: 0.93–2.29), and in *C. coli* in pigs for CIP (0.64%, 95%CI: 0.20–1.09). For the rest of antimicrobials, host and bacterial species no significant trends were detected (**Figures 2–5**).

For any given bacterial species, an analysis of the quantitative AST results across hosts species revealed significant differences ($p = 0.032$) in the distribution of MIC values between "susceptible" and "not susceptible" isolates only for *C. jejuni* strains from turkeys "not susceptible" to ERY (MIC ≥ 256 mg/L) (**Supplementary Excel File 1**). In contrast, for any given host species, no significant differences were observed between MIC distributions of "susceptible" and "not susceptible" *C. coli* vs. *C. jejuni* isolates.

Co-resistance and MDR Phenotypic Profiles

The main resistance profiles observed in each bacterial and host species are shown on **Supplementary Table 3**. Of all *C. coli* isolates from all host species, >85% were resistant to three (CIP-TET-NAL, TET-ERY-STR) or more antimicrobials and >60% were resistant to three or more antimicrobial classes (MDR). The most common resistance profiles for *C. coli* from each host were CIP-TET-NAL and CIP-TET-NAL-STR in broilers and turkeys (18–28% of all isolates in each host species for each profile), CIP-TET-NAL-STR in cattle (∼50% of all *C. coli* isolates) and CIP-TET-NAL-STR-ERY in pigs (∼40% of all *C. coli* isolates). The proportion of pan-susceptible isolates for *C. coli* in all host species

TABLE 2 | Collection period, number of fecal samples and positive samples for *Campylobacter* isolation from each host species included in the study.

Host species	Broilers	Turkeys	Pigs	Cattle
Years	2002–2018	2014–2018	2002–2017	2007–2017
Sample size	3,413	1,455	3,750	2,347
C. coli	1,023 (30.0%)	815 (56.0%)	1,968 (52.5%)	183 (7.8%)
C. jejuni	957 (28.0%)	273 (18.8%)	33 (0.9%)	1,074 (45.8%)
C. spp.	20 (0.6%)	2 (0.1%)	217 (5.8%)	16 (0.7%)

TABLE 3 | Percentage of *Campylobacter* isolates not susceptible (resistant) to each antimicrobial in the four host species throughout the studied period.

Antimicrobials		Tetracycline	Nalidixic acid	Ciprofloxacin	Streptomycin*	Erythromycin*	Gentamicin
		Coli (MIC > 2)	*Coli/Jejuni* (MIC > 16)	*Coli/Jejuni* (MIC > 0.5)	*Coli/Jejuni* (MIC > 4; DIAM < 13)	*Coli* (MIC > 8; DIAM < 24)	*Coli/Jejuni* (MIC > 2)
		Jejuni (MIC > 1)				*Jejuni* (MIC > 4; DIAM < 22)	
C. coli (%)	Broilers (*n* = 634)	95.9^{a}	93.3^{a}	94.5^{a,b}	54.7^{a}	34.8^{a}	14.7^{a}
	Turkeys (*n* = 279)	97.5^{a,b}	95.3^{a}	98.2^{b}	55.9^{a}	36.6^{a}	7.5^{b}
	Pigs (*n* = 1,692)	99.1^{b}	91.7^{a,b}	91.7^{a,c}	90.6^{b}	66.6^{b}	22.2^{c}
	Cattle (*n* = 149)	95.3^{a}	86.7^{b}	87.3^{c}	82.0^{c}	19.3^{c}	12.0^{a,b}
C. jejuni (%)	Broilers (*n* = 772)	83.1^{a}	88.5^{a}	91.1^{a}	7.7^{a}	2.9^{a}	1.0^{a}
	Turkeys (*n* = 231)	83.1^{a}	86.1^{a}	88.7^{a}	6.1^{a}	2.6^{a}	0.0^{a}
	Cattle (*n* = 828)	74.1^{b}	63.1^{b}	63.8^{b}	10.2^{a}	1.7^{a}	1.4^{a}

Different superscripts indicate significant differences between hosts for each bacterial species
**Diffusion technique: streptomycin in broilers and pigs (2002–2005) and erythromycin in broilers (2002–2004).*

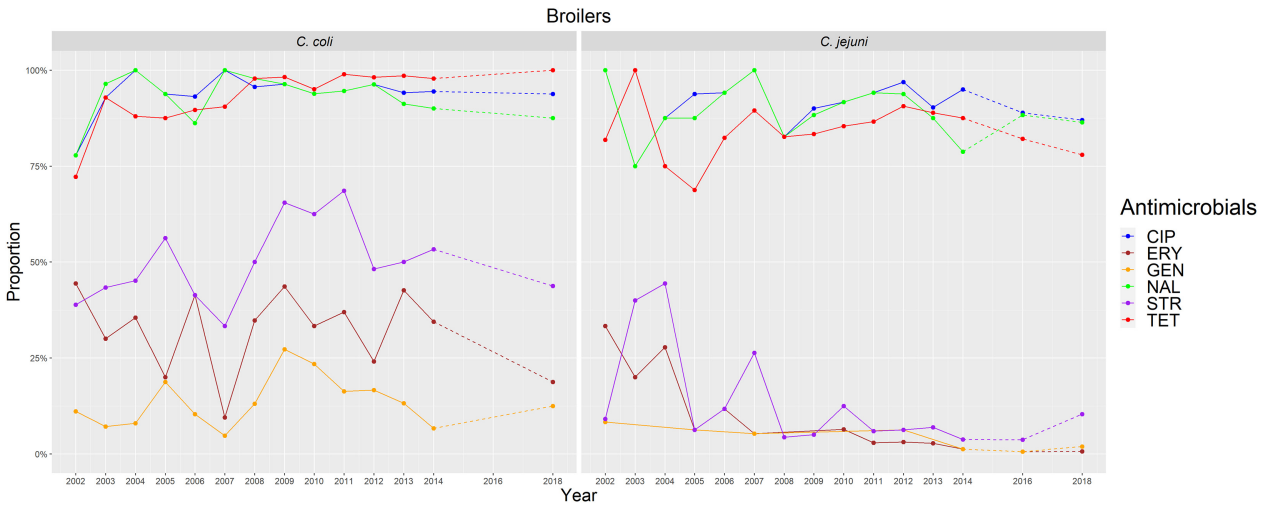

FIGURE 2 | Graphical representation of AMR proportions to each antimicrobial in *C. coli* and *C. jejuni* from broilers for 2002–2018. Years in which AST was performed are indicated in the X axis; dashed lines indicate periods in which AST was not performed every year (no AST performed in *C. coli* in 2016).

was low (0–4%), and lower than the proportion of resistant isolates to all six antimicrobials (0–16%).

In comparison, the proportion of *C. jejuni* isolates from all hosts resistant to three or more antimicrobials was 54–76% whereas the MDR proportion was 6–9%. The most common resistance profile for *C. jejuni* from all hosts was CIP-TET-NAL, amounting to between ∼45% of all cattle and 60–70% of all broiler and turkey isolates. In this case, the proportion of pan-susceptible isolates (5–15%) was higher than that of resistant isolates to the six antimicrobials in all species (<1%).

Association Between Resistance to GEN, STR, and ERY

Overall, a significant association between the occurrence of phenotypic resistance to aminoglycosides and macrolides was observed, so that *C. coli* and *C. jejuni* from all host species (except *C. coli* in pigs and turkeys and *C. jejuni* in turkeys) resistant to one of the two aminoglycosides (or to both) were more likely to be also resistant to ERY (**Table 4**). This association was stronger in *C. jejuni* and/or cattle isolates.

Analysis of *flaA* and AMR Genes

Over 300 bp (including the 267 bp-long SVR used in the analysis) of the *flaA* gene sequence were correctly determined in 168 of the 176 chosen isolates (all except 2 "cases" and 6 "controls"). **Figure 6** displays the phylogenetic tree constructed using the selected final 168 isolates (59 "cases" and 109 "controls") plus the reference strain. Overall, a total of 127 single nucleotide polymorphisms (SNPs) located in 100 polymorphic sites were found, leading to 73 unique *flaA* SVR gene sequences. The haplotype diversity (Hd – probability that two randomly selected sequences are different) was 0.975, and every two sequences differed on average by 27 SNPs with an overall mean evolutionary distance (d) between the two sequences of 0.09.

The 168 isolates were classified into five groups based on the topology of the tree: group 1, including the majority of the

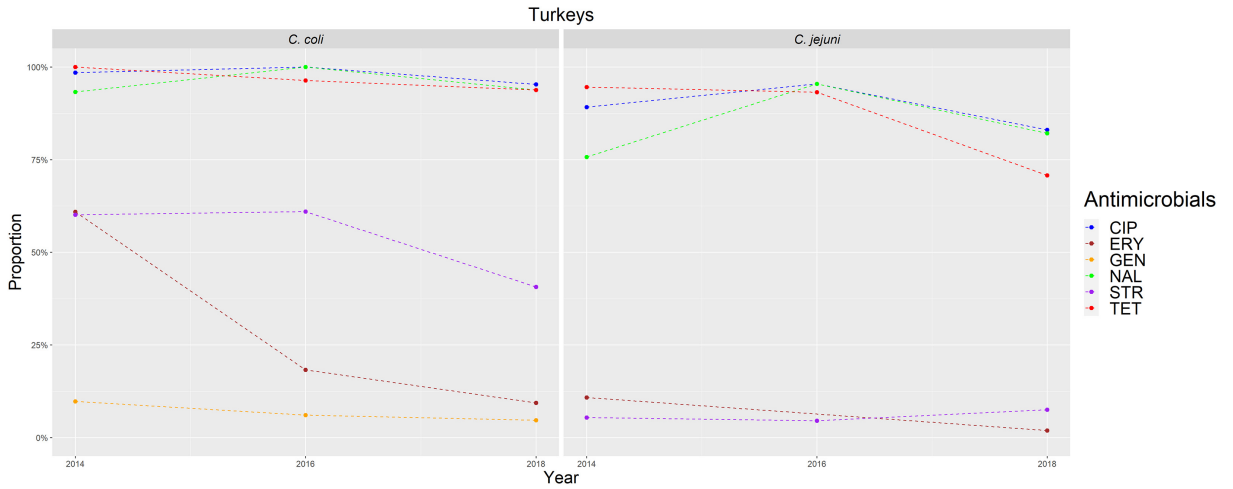

FIGURE 3 | Graphical representation of AMR proportions to each antimicrobial in *C. coli* and *C. jejuni* from turkeys for 2014–2018. Years in which AST was performed are indicated in the *X*-axis; dashed lines indicate periods in which AST was not performed every year.

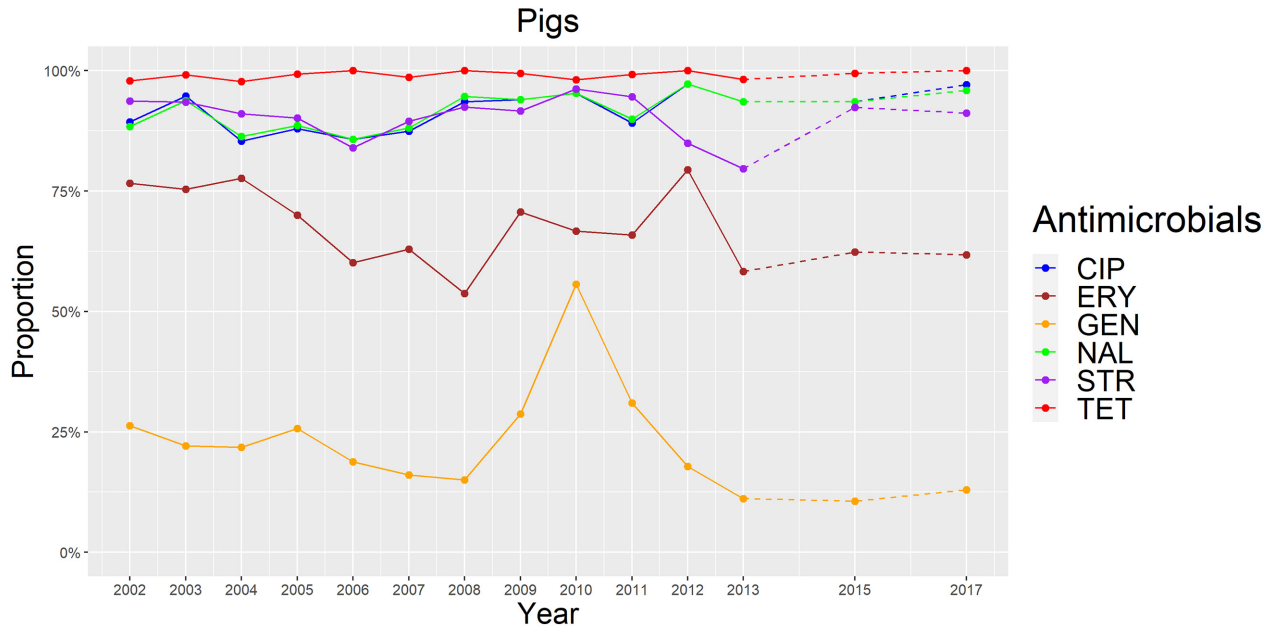

FIGURE 4 | Graphical representation of AMR proportions to each antimicrobial in *C. coli* from pigs for 2002–2017. Years in which AST was performed are indicated in the *X*-axis; dashed lines indicate periods in which AST was not performed every year.

sequenced isolates (n = 100 isolates), group 2 (n = 31 isolates), group 3 (n = 12 isolates), group 4 (n = 17 isolates), and group 5 (n = 8 isolates) (**Figure 6**). Groups 2–5 formed separate clades from group 1 (bootstrap > 60). Groups 2 and 3, consisting mainly of *C. coli* strains (28/31 and 10/12, respectively) predominantly from poultry (>60%), showed similar proportions of isolates resistant to aminoglycosides and macrolides ("cases") and of "controls" (18/31 and 6/12, respectively) (**Table 5**). Groups 4 and 5 showed a higher proportion of *C. jejuni* isolates (13/17 in group 4 and 8/8 in group 5) from cattle (9/17 and 8/8), and the frequency of isolates with simultaneous resistance to both antimicrobial classes ("cases") in these groups was much lower (1/17 and 1/8, respectively) (**Table 5**).

Only complete RSCU values from the 20 variable codons among the 168 isolates were included in the MCA analysis. The first two dimensions identified in the MCA explained 38% of the total variability observed. Isolates included in each of the five groups identified in the phylogenetic tree were also clustered according to the first two dimensions of the MCA (**Figure 7**).

Different resistance markers involved in AMR against macrolides and aminoglycosides were found in the isolates subjected to WGS. Several genes involved in the CmeABC

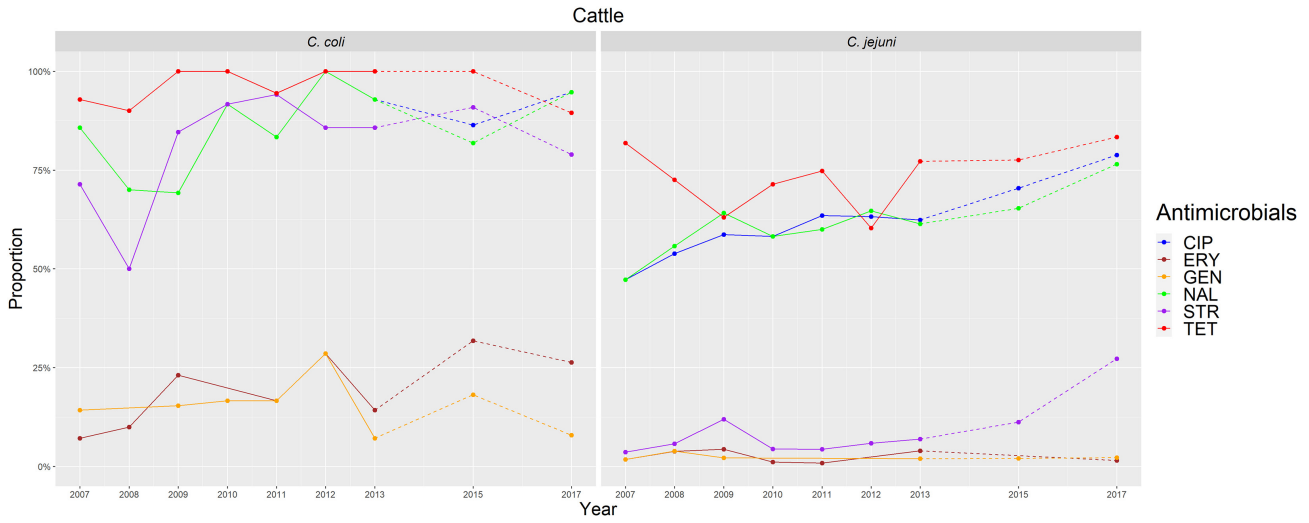

FIGURE 5 | Graphical representation of AMR proportions to each antimicrobial in *C. coli* and *C. jejuni* from cattle for 2007–2017. Years in which AST was performed are indicated in the *X*-axis; dashed lines indicate periods in which AST was not performed every year.

TABLE 4 | Association between phenotypic resistance to gentamicin, streptomycin, and erythromycin in *C. coli* and *C. jejuni* isolates from livestock.

Host	Bacterial species	*N*	Erythromycin-R (%)	Antimicrobial	Resistance (%)	Resistance among EryR (%)	*p*-value	RR*
Broilers	*C. coli*	634	34.5	Streptomycin	54.4	68.9	<0.001	1.86
				Gentamicin	14.7	24.7	<0.001	1.90
	C. jejuni	772	2.5	Streptomycin	7.6	31.6	<0.001	5.58
				Gentamicin	1.0	15.8	<0.001	17.90
Pigs	*C. coli*	1692	66.7	Streptomycin	90.7	90.7	0.953	1.00
				Gentamicin	22.0	25.0	<0.001	1.18
Turkeys	*C. coli*	279	36.6	Streptomycin	56.0	66.7	0.008	1.58
				Gentamicin	7.5	10.8	0.156	1.48
	C. jejuni	231	2.6	Streptomycin	6.0	33.3	0.045	7.75
				Gentamicin	0.0	0.0	1	17.85
Cattle	*C. coli*	149	19.5	Streptomycin	82.6	96.6	0.028	5.92
				Gentamicin	12.1	31.0	0.002	3.27
	C. jejuni	828	1.7	Streptomycin	10.3	78.6	<0.001	32.05
				Gentamicin	1.4	28.6	<0.001	27.20

**Relative risk of presenting resistance to erythromycin in isolates resistant to streptomycin/gentamicin.*

efflux pump (*cmeB*, *cmeC*, and *cmeR*) were present in 39–40/40 sequenced *C. jejuni* isolates and missing in 10–11/11 *C. coli* strains, while the *cmeA* gene was present in 34/40 and 4/11 of the *C. jejuni* and *C. coli* strains, respectively, but their presence was not associated with ERY resistance (**Supplementary Excel File 2**). Among the 12 ERY-resistant isolates, three presented mutations associated with macrolide-resistance in the 23S rRNA encoding gene and other three carried the *erm*(B) gene, while no resistance marker was found in the remaining six isolates. Regarding aminoglycoside-resistance associated genes, seven different genes were found in one or more strains (between 0 and 3 per strain), and their presence was associated with resistance to STR and/or GEN except in one susceptible *C. jejuni* strain from cattle (**Supplementary Excel File 2**). No apparent association between a specific *flaA* gene group and the presence of any of the resistance markers was observed.

DISCUSSION

Antimicrobial resistance is becoming a major problem for the treatment of diseases caused by zoonotic bacteria such as thermophilic *Campylobacter*. The mechanisms by which AMR can spread in a bacterial population (vertically or horizontally) has enormous implications, since it can determine the speed at which AMR phenotypes disseminate. Of particular concern are genetic traits conferring MDR (Magiorakos et al., 2012), particularly when transmitted together. Hence, it is of paramount importance to explore the genetic mechanisms implicated in AMR in *C. coli* and *C. jejuni* from the different hosts involved in the epidemiology of infection in humans (EFSA-ECDC, 2020). As described elsewhere, this study of isolate-based phenotypic data versus aggregated data has also proven to be a reliable means of gaining insight into such mechanisms (Alvarez et al., 2020),

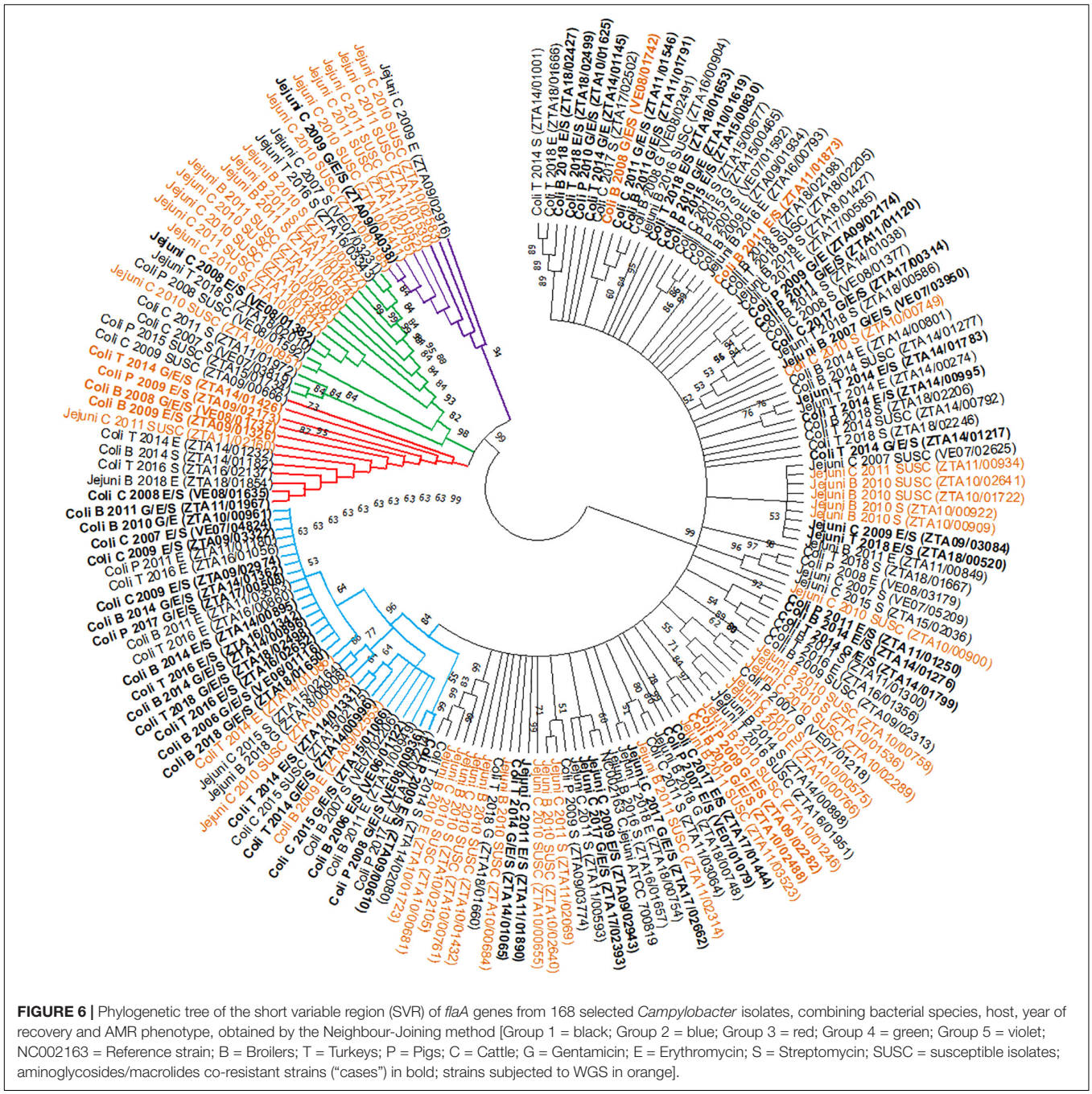

FIGURE 6 | Phylogenetic tree of the short variable region (SVR) of *flaA* genes from 168 selected *Campylobacter* isolates, combining bacterial species, host, year of recovery and AMR phenotype, obtained by the Neighbour-Joining method [Group 1 = black; Group 2 = blue; Group 3 = red; Group 4 = green; Group 5 = violet; NC002163 = Reference strain; B = Broilers; T = Turkeys; P = Pigs; C = Cattle; G = Gentamicin; E = Erythromycin; S = Streptomycin; SUSC = susceptible isolates; aminoglycosides/macrolides co-resistant strains ("cases") in bold; strains subjected to WGS in orange].

and the assessment of phenotypic susceptibility patterns found here can guide the genetic analysis in a "top-down" approach (Sheppard and Maiden, 2015).

The annual proportion of *Campylobacter* positive samples found in our samples from broilers collected over a 17-year period (ranging from 26.2 to 76.7%) was higher than values reported by EFSA from EU member states (26%) (EFSA-ECDC, 2019a) and mostly higher than values reported in other regions of the world such as China (30.2%) (Tang et al., 2020). The proportion of *Campylobacter* positive samples in cattle from 2007 to 2017 (37–69.5%) was also much higher than values reported by EFSA for 10 EU countries (1.5–3.5%) (EFSA-ECDC, 2018b, 2019a), although country-specific studies in Finland (Hakkinen et al., 2007), and Lithuania (Ramonaite et al., 2013) reported values more similar to the ones found here (39.6 and 80%, respectively). Similarly, the percentage of pig samples from which *Campylobacter* isolates were retrieved in our study (33.4–80%) was in the range of results reported for Greece (49.1%) (Papadopoulos et al., 2020), much higher than previously reported by EFSA for 8 EU countries (2–7%) (EFSA-ECDC, 2018b, 2019a) and lower than reported in a Danish study (92%) (Boes et al., 2005). However, the percentages we found in turkeys

TABLE 5 | Numbers and proportions of isolates included in each of the groups formed from the phylogenetic analysis based on phenotypical AMR susceptibility, bacterial species, host species, and case/control categories.

Group #	1	2	3	4	5	Total
N	(*n* = 100)	(*n* = 31)	(*n* = 12)	(*n* = 17)	(*n* = 8)	(*n* = 168)
GEN-R	22 (22.0%)	**11 (35.5%)**	**3 (25.0%)**	0 (0.0%)	1 (12.5%)	37 (22.0%)
ERY-R	45 (45.0%)	**26 (83.9%)**	**8 (66.7%)**	1 (5.9%)	2 (25.0%)	82 (48.8%)
STR-R	58 (58.0%)	**19 (61.3%)**	**8 (66.7%)**	10 (58.8%)	1 (12.5%)	96 (57.1%)
Coli	54 (54.0%)	**28 (90.3%)**	**10 (83.3%)**	4 (23.5%)	0 (0.0%)	96 (57.1%)
Jejuni	46 (46.0%)	3 (9.7%)	2 (16.7%)	**13 (76.5%)**	**8 (100.0%)**	72 (42.9%)
Broilers	37 (37.0%)	**12 (38.7%)**	**5 (41.7%)**	4 (23.5%)	0 (0.0%)	58 (34.6%)
Cattle	27 (27.0%)	7 (22.6%)	3 (25.0%)	**9 (52.9%)**	**8 (100.0%)**	54 (32.1%)
Pigs	**14 (14.0%)**	**4 (12.9%)**	1 (8.3%)	2 (11.8%)	0 (0.0%)	21 (12.5%)
Turkeys	22 (22.0%)	**8 (25.8%)**	**3 (25.0%)**	2 (11.8%)	0 (0.0%)	35 (20.8%)
Cases	33 (33.0%)	**18 (58.1%)**	**6 (50.0%)**	1 (5.9%)	1 (12.5%)	59 (35.1%)
Controls	67 (67.0%)	13 (41.9%)	6 (50.0%)	**16 (94.1%)**	**7 (87.5%)**	109 (64.9%)

In bold = predominant proportions within each group and category.

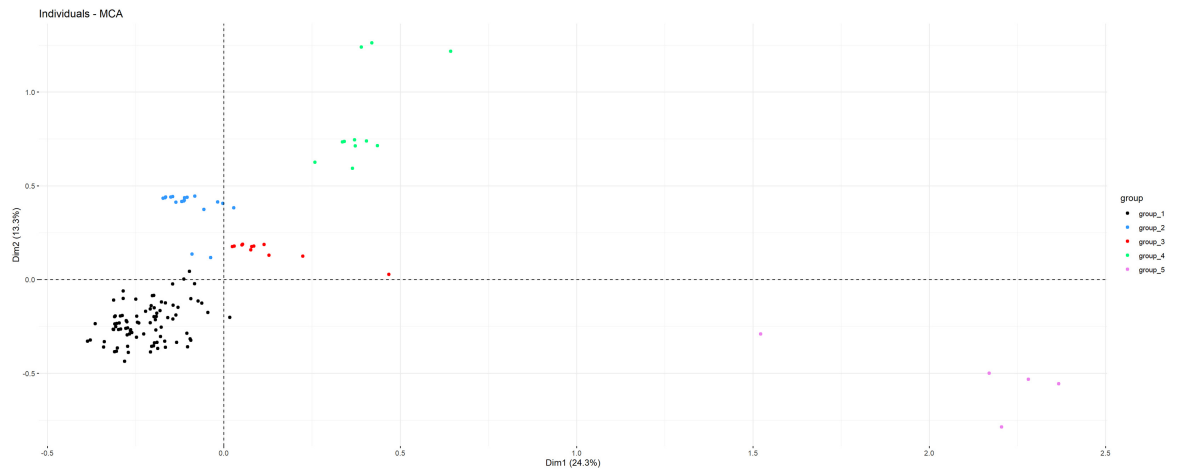

FIGURE 7 | Distribution of the 168 isolates subjected to *flaA* gen sequencing according to the first two dimensions of a multiple correspondence analysis (MCA) performed considering information on the RSCU of variable codons, bacterial and host species, resistance to gentamicin, erythromycin, and streptomycin, and clade as determined in the NJ phylogenetic tree (observations are colored according to the clade).

(65.4–85.9%) were similar to an EU report comprising 5 countries (71.6%) (EFSA-ECDC, 2019a) but lower than found in a German study (90–100%) (Ahmed et al., 2016).

As expected, the host species were strongly associated with the *Campylobacter* species retrieved in positive samples, although proportions found for each bacterial species may vary depending on isolation protocols used. The predominance of *C. coli* in pig samples found in our study is in agreement with previous studies from Denmark (Boes et al., 2005). However, and even though this bacterial species has been traditionally associated with pigs, it is becoming more common in poultry (Miller et al., 2006). In our collection, *C. coli* was in fact the predominant species in turkey, while a more balanced distribution between *C. coli* and *C. jejuni* was reported in turkey samples from Germany (Ahmed et al., 2016). In broilers, a close to 50/50 distribution for *C. coli*/*C. jejuni*, as the one found here, was also observed in samples from China (Tang et al., 2020). However, EFSA reported a predominance of *C. jejuni* with 2,452 *Campylobacter* positive samples from 16 countries (EFSA-ECDC, 2019a). In cattle, a study from Denmark (Nielsen et al., 1997) found similar proportions for each bacterial species (6.8% *C. coli*, 90.9% *C. jejuni*, and 2.3% *C.* spp.) than our study (14.4% *C. coli*, 84.4% *C. jejuni*, and 1.2% *C.* spp.).

As presumed, the level of resistance to the antimicrobials used in our study was closely linked with the *Campylobacter* bacterial species found, with higher levels of resistance in *C. coli* than in *C. jejuni* in agreement with previous research (Pergola et al., 2017; Alvarez et al., 2020). The lack of barriers to horizontal gene transfer (HGT) in *C. coli* may explain the higher levels of MDR observed in this bacterial species compared to *C. jejuni* (Pearson et al., 2015).

Out of the six antimicrobials assessed here, high to extremely high levels of resistance were found for three of them (CIP, NAL, and TET) in *C. coli*, while in *C. jejuni* they ranged between medium to very high. For CIP and NAL these levels were consistently higher than those described for isolates from food animals in other European countries with the exception of

C. jejuni in turkeys (equal levels to Italy, Poland and Portugal at 70%) and cattle (equal levels to Italy at 80%) (EFSA-ECDC, 2020). For TET in *C. jejuni* in cattle, levels in Spain (85%) were between levels reported in Austria, Denmark and the Netherlands (60%) and levels reported in Italy (95%) (EFSA-ECDC, 2020).

Resistance levels to the remaining three antimicrobials analyzed in this study were much more variable, yet again consistently higher than in other European countries across hosts and bacterial species. The exceptions were *C. jejuni* from turkeys (15% ERY in Portugal vs. 2.6% in Spain; 20% STR in Poland vs. 6.1% in Spain) and cattle (10% ERY in Italy vs. 1.7% in Spain) (EFSA-ECDC, 2020).

Overall, a significant association between the presentation of phenotypic resistance to ERY (macrolide) and STR and GEN (aminoglycosides) was consistently found for both *C. coli* and *C. jejuni* from most host species (**Table 3**). When the association between antimicrobial pairs was analyzed stratifying by time-periods (2002–2006, 2007–2012, and 2013–2018) certain categories were not significantly associated, probably due to being smaller sample sizes (data not shown). Unsurprisingly, STR-resistant isolates had a significantly higher probability of being also resistant to GEN, which was expected given that they belong to the same antimicrobial class (aminoglycosides) and therefore share resistance mechanisms, mostly based on natural transformation, homologous recombination and sharing of MGEs (Davies and Wright, 1997; Luangtongkum et al., 2009; Qin et al., 2012; Wieczorek and Osek, 2013).

Campylobacter is considered a high-risk pathogen in terms of AMR due to the high levels of HGT and the association of AMR genes in MDRGIs. Some authors argue that the transfer of MDRGIs is likely to lead to co-selection phenomena after their genetic mobilization. This could explain why *Campylobacter* adapts so quickly in its interaction with the host, constantly obtaining improved phenotypes (Sheppard and Maiden, 2015).

The *erm*(B) gene, previously described only in Asia (Qin et al., 2014) and possibly originating from Gram-positive bacteria, was found in Spain in one *C. coli* from broiler in 2015 (Florez-Cuadrado et al., 2016) and two *C. coli* from turkeys in 2017 (Florez-Cuadrado et al., 2017). This was the first European report of this gene, associated with other genes in MDRGIs bearing resistance to ERY, CIP, TET, and NAL, and involved in AMR to STR (and present in isolates that may be susceptible to GEN). The three *erm*(B)-carrying strains, included in this study, were found in isolates showing simultaneous resistance to aminoglycosides and were clustered in different clades (1, 2, and 3). However, given the very limited number of ERY-resistant sequenced strains no conclusions can be drawn regarding their association with specific genetic populations. The inclusion of *erm*(B) genes in plasmids encoding additional resistance genes to other antibiotics in *C. coli* from food animals could pave the way to rapid dissemination of macrolide resistance (EFSA-ECDC, 2018a, 2019b). Besides, reported resistance levels to ERY in humans have been consistently higher for *C. coli* than for *C. jejuni* (EFSA-ECDC, 2018a), and similar reports have been made in poultry (Pergola et al., 2017) in agreement with our findings. Since macrolides are one of the three "Critically Important Antimicrobial" classes used for the treatment of human campylobacteriosis (along with fluoroquinolones and aminoglycosides) (World Health Organization (WHO), 2017), a more in-depth knowledge into their resistance mechanisms is warranted.

The increasing rates of resistance to ERY in *C. coli* and to STR in *C. jejuni* of cattle origin described here suggest this host species could play an increasingly important role in the epidemiology of AMR in *Campylobacter*. A nationwide case-control study carried out in Luxembourg identified beef consumption as an important source of infection for *C. coli* (Mossong et al., 2016), thus suggesting that cattle may be a relevant reservoir for this foodborne pathogen.

MICs values in isolates classified as "susceptible" or "not susceptible" may indicate the presence or absence of different AMR determinants in the bacterial genome. The significantly higher MICs values observed in this study for ERY in turkey resistant isolates (**Supplementary Excel File 1**) could indicate the presence of the transferable *erm*(B) gene. However, out of the 12 ERY-resistant isolates subjected to WGS, only three carried the *erm*(B) gene (and had MICs ranging between 32 and 256 ug/ml), and additionally, mutations in the 23S rRNA encoding gene were found in just three isolates (**Supplementary Excel File 2**). This suggests that other mechanisms may be involved in the observed increased MICs in certain isolates, such as mutational resistance affecting the expression of the CmeABC efflux pump in *C. jejuni* (Zhang et al., 2017). This, linked with the high proportion of ERY-resistant isolates found in *C. coli* from turkeys in other European countries (EFSA-ECDC, 2017, 2020) further highlights the need of clarifying the resistance mechanisms present in resistant isolates from this host. In fact, EFSA recommends investigating the molecular mechanisms of macrolide resistance, especially in isolates resistant to high concentrations of ERY, in order to detect chromosomal mutations or the presence of the transferable *erm*(B) gene (EFSA-ECDC, 2019b). Furthermore, these same guidelines recommend searching for ERY resistant genes, not only in resistant strains presenting concomitant resistance to aminoglycosides or a MDR phenotype, but also in susceptible isolates. Thus, an in-depth characterization of resistant isolates would be needed to confirm this hypothesis. The integration of phenotypic and genomic analyses may allow predicting differences in resistance levels beyond resistance thresholds (Bolinger and Kathariou, 2017; EFSA-ECDC, 2019b).

Phylogenetic studies based on *flaA* SVR gene sequencing have been used in the past to study the epidemiology of *Campylobacter* spp. from different sources (Zhang et al., 2018). Previously, studies based on the *flaA* gene sequence had not found a relationship between AMR and specific genotypes (Corcoran et al., 2006). However, in our strain collection five distinct groups were identified, two of which were associated with an increased proportion of simultaneous resistance to aminoglycosides and macrolides (groups 2 and 3), predominantly formed by *C. coli* isolates from broilers and turkeys (**Table 5**). In contrast, isolates in groups 4 and 5 were primarily *C. jejuni* of cattle origin. The existence of "cattle specialist *C. jejuni* lineages" has been previously speculated, implying that adaptation of *C. jejuni* to cattle could be associated with the presence of genetic

elements favoring its survival in the intestine of cattle (Sheppard and Maiden, 2015) and with a significant gene gain and loss (Mourkas et al., 2020). Interestingly, cattle *C. jejuni* showed the strongest association between resistance to aminoglycosides and macrolides (with RR > 25), but only 2 co-resistant isolates (out of 7 co-resistant *C. jejuni* cattle isolates sequenced) were classified into groups 4 and 5.

Among its limitations, the sample size used in the first part of this long study period (2002–2008) was relatively small. Furthermore, AST in the first years (2002–2005) was based on determining IZDs and MICs depending on the antimicrobial considered. Therefore, conclusions based on data from that period must be interpreted carefully. In addition, only 1.5% (168) of the total number of isolates were subjected to the *flaA* gene analysis, and only 51 of them were analyzed by WGS.

Nevertheless, our findings confirm that high resistance levels in *Campylobacter* spp. from food producing animals were consistently observed, and that resistance to macrolides and aminoglycosides was strongly associated across hosts and bacterial species. Further studies based on WGS would be needed in order to determine the genetic determinants behind this resistance and the possible existence of more prevalent lineages.

In this study, *C. coli* isolates, more prevalent in pigs and poultry (especially turkeys), showed significantly higher resistance levels than *C. jejuni* strains. The significant association in the simultaneous presentation of phenotypic resistance to aminoglycosides and macrolides, confirmed in *C. coli* isolates independently from host species of origin, suggests the possible circulation of resistance genes against both antimicrobial classes. Such resistance mechanisms could have been transmitted together, or else, have disseminated via resistant clones in the studied livestock species. The genetic analysis revealed the presence of some isolates more genetically related with resistant phenotypes in poultry and others with susceptible phenotypes in cattle. In order to test these hypotheses it would be necessary to characterize the resistance mechanisms present in isolates from the different species by means of a thorough molecular analysis of their whole genome.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required because animals included in this study were sampled in the slaughterhouse during routine processing of livestock and were not subjected to any additional handling of any kind. Samples were collected in the frame of official monitoring programs according to EU and national legislation.

AUTHOR CONTRIBUTIONS

VL-C, LD, and JA: conceptualization. VL-C and JA: investigation, writing – original draft preparation, methodology, software, formal analysis, and validation. JA: Funding acquisition and project administration and supervision. JS, CF, TS, IP, MU-R, CB, and MG: data supply. MU-R, CB, and MG: data curation. VL-C and AO: laboratory work. VL-C, MU-R, CB, AO, MG, JS, IP, MM, LD, and JA: writing – review and editing. All authors contributed to the article and approved the submitted version.

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Organic Acid Exposure Enhances Virulence in Some *Listeria monocytogenes* Strains Using the *Galleria mellonella* Infection Model

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Prior research has suggested that the use of organic acids in the food industry may unintentionally enhance pathogenicity of *Listeria monocytogenes* strain N1-227 and R2-499. This study explored the connection between habituation to L-lactic acid or acetic acid and virulence in *L. monocytogenes* strains N1-227 and R2-499 using selected gene expression analysis and the *in vivo Galleria mellonella* wax worm model for infection. Expression of transcription factors (*sigB* and *prfA*) and genes related to acid resistance (*gadD2, gadD3,* and *arcA*) and bile resistance (*bsh* and *bilE*) or to virulence (*inlA, inlB, hly, plcA, plcB, uhpT,* and *actA*) was investigated by quantitative real-time PCR (qRT-PCR), while *in vivo* virulence was assessed by following the lethal time to 50% population mortality (LT_{50}) of *G. mellonella* larvae after injection of untreated and habituated *L. monocytogenes.* Twenty minutes of habituation to the organic acids at pH 6.0 significantly increased expression of key acid and bile stress response genes in both strains, while expression of virulence genes was strain-dependent. The expression of transcription factor *sigB* was strain-dependent and there was no significant change in the expression of transcription factor *prfA* in both strains. Habituation to acid increased virulence of both strains as evidenced by decreased LT_{50} of *G. mellonella* larvae injected with *Listeria* habituated to either acid. In summary, habituation of both *L. monocytogenes* strains to organic acids up-regulated expression of several stress and virulence genes and concurrently increased virulence as measured using the *G. mellonella* model.

Keywords: organic acid, acid resistance, bile resistance, virulence, gene expression, *Listeria monocytogenes, Galleria mellonella*

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INTRODUCTION

The genus *Listeria* is comprised of Gram-positive, non-spore-forming, rod-shaped, facultative anaerobic bacteria which can be found ubiquitously in the environment (Mélanie et al., 2006; Gahan and Hill, 2014; Lani and Hassan, 2016). Among *Listeria* species, only *L. monocytogenes* and *L. ivanovii* are pathogenic (Robinson and Batt, 1999); *L. ivanovii* primarily infects animals while

L. monocytogenes shows pathogenicity toward both humans and animals (Liu, 2006). During food production, *L. monocytogenes* can experience several stresses such as low pH and high salt. The ability of *Listeria* to adapt to these adverse conditions plays a crucial role in food contamination and food-borne infection (Lani and Hassan, 2016).

In response to stress, *L. monocytogenes* may induce an acid tolerance response and other stress responses mechanisms that allow it to overcome these hurdles (Glass et al., 1995; Silva et al., 2012; Melo et al., 2015). *L. monocytogenes* is able to utilize a variety of regulators (over 100 different transcriptional regulators have been identified) to survive and grow in different environments (Glaser et al., 2001; Gaballa et al., 2019). Among those regulators, the alternative sigma factor B (σ^B) and the listeriolysin positive regulatory factor A (PrfA) are two essential transcriptional regulators for stress response and for host infection.

σ^B, encoded by *sigB*, is a general stress responsive transcription sigma factor in *L. monocytogenes* and many other Gram-positive bacteria (Kazmierczak et al., 2005; Chaturongakul et al., 2008). In *L. monocytogenes*, σ^B regulates numerous genes that are associated with acid, bile and other physiological stressors (Sue et al., 2004; Zhang et al., 2011; Smith et al., 2012; Melo et al., 2015). The acid stress response systems in *L. monocytogenes* include the glutamate decarboxylase (GAD) system and an arginine deiminase (ADI) system. The GAD system, which involves genes encoding three glutamate decarboxylase enzymes (*gadD1, gadD2* and *gadD3*) and two gamma aminobutyric acid (GABA) antiporters (*gadT1* and *gadT2*), plays a significant role in pH homeostasis in *L. monocytogenes* (Cotter et al., 2001; Melo et al., 2015). Expression of the GAD system results in the decarboxylation of glutamate into γ-aminobutyrate with consumption of intracellular protons (Cotter et al., 2001; Karatzas et al., 2012). Additionally, the arginine deiminase (ADI) system also contributes to the stabilization of the bacterial cytoplasmic pH (Melo et al., 2015). The ADI pathway involves the enzymes arginine deiminase, ornithine carbamoyltransferase and carbamate kinase, which are encoded by *arcA*, *arcB,* and *arcD*, respectively (Melo et al., 2015). With respect to bile resistance, one of the most important mechanisms in *L. monocytogenes* involves the ability to detoxify individual conjugated bile acid through bile salt hydrolase (BSH) (Dussurget et al., 2002; Begley et al., 2005). Another novel bile resistance system in *L. monocytogenes* is the bile exclusion system (BilE), which acts to exclude bile from bacterial cells (Sleator et al., 2005).

The listeriolysin positive regulatory factor A (PrfA), encoded by *prfA*, is a bacterial transcription factor that controls and coordinates the expression of key virulence genes in *L. monocytogenes* associated with cell invasion and the intracellular infection cycle (Kazmierczak et al., 2006; Scortti et al., 2007; de las Heras et al., 2011). Cell invasion is mediated by two surface proteins, internalin A and B (InlA and InlB); after entering the cell, *L. monocytogenes* are entrapped in a phagocytic vacuole from which they escape by lysing the membrane of the vacuole through the combined actions of the pore-forming toxin listeriolysin O (LLO, encoded by *hly*) and two phospholipases, PlcA and PlcB (Mélanie et al., 2006). Multiplication and invasion within host cells can then occur with the involvement of the permease UhpT (a hexose phosphate transporter) and the surface protein ActA (propel bacteria through the cytoplasm) (Chico-Calero et al., 2002; Mélanie et al., 2006; Cossart and Toledo-Arana, 2008).

Acid stress resistance has been well studied and observed in various microorganisms such as *Escherichia coli* (Goodson and Rowbury, 1989) and *Salmonella* (Foster and Hall, 1990). Prior research by our group has suggested that the use of organic acids in the food industry may unintentionally enhance virulence of some *L. monocytogenes* strains (Zhang et al., 2014). Those results showed that habituation of two *L. monocytogenes* strains, N1-227 and R2-499, to organic acid under mildly acidic conditions (pH = 6.0) induced acid and bile resistance, which indicated these treatments could promote virulence by enhancing survival during passage through the gastrointestinal tract (Zhang et al., 2014). It also suggested the increased acid and bile resistance was specifically due to organic acid exposure rather than a decrease in environmental pH (Carpenter and Broadbent, 2009; Zhang et al., 2014). Similar responses were not observed in that study with other pathogenic strains of *L. monocytogenes* (Zhang et al., 2014), so R2-499 and N1-227 were selected for further study to explore the genetic basis for inducible acid and bile resistance, and to determine if it affected virulence in an *in vivo* model.

Virulence of *Listeria* spp. is frequently assessed using a murine model (Lecuit, 2007). However, this model has limitations for studying human pathogenicity of *L. monocytogenes* because the interaction between InlA and mouse E-cadherin (identified as InlA receptor in human) is poor, which makes *L. monocytogenes* entry into epithelial cells less efficient (Mengaud et al., 1996; Lecuit et al., 1999). The larvae of *Galleria mellonella* have also been used as a model for *L. monocytogenes* virulence (Joyce and Gahan, 2010; Mukherjee et al., 2010, 2013; Banville et al., 2012; Ramarao et al., 2012; Schrama et al., 2013). Compared to the mammalian model and other alternative models, the *G. mellonella* model offers several significant advantages, including structural and functional similarities with the mammalian immune system (Hoffmann et al., 1999; Strand, 2008). Additionally, the infection process can be performed over a range of temperatures (from 15°C to above 37°C), which enables use of the *G. mellonella* model to study the virulence of *L. monocytogenes* human pathogens at 37°C (Jones et al., 2010; Rejasse et al., 2012).

To better understand the molecular basis and potential consequences of induced acid and bile resistance in organic acid habituated strains, we used quantitative real time polymerase chain reaction (qRT-PCR) to measure the expression of key transcription factors and some of their target genes related to acid or bile resistance or virulence in *L. monocytogenes* strains N1-227 and R2-499 after habituation to lactic acid or acetic acid at pH 6.0. Additionally, the *G. mellonella* infection model was used to analyze the *in vivo* virulence of control and acid habituated *L. monocytogenes* strains.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Original cultures (**Table 1**) were stored as frozen stocks at −80°C in tryptic soy broth (TSB, pH 7.4; Becton, Dickinson and Company, Sparks, MD) supplemented with 20% v/v glycerol. Prior to use, cultures were first propagated on tryptic soy agar (TSA; Becton, Dickinson and Company, Sparks, MD) plate and incubated at 37°C for 24 h. A single colony from the TSA plate was transferred into TSB and incubated overnight at 37°C with shaking (220 rpm).

RNA Isolation

Overnight cultures of each strain were harvested by centrifugation (2,500 × g for 10 min; Sorvall RT1, Thermo Scientific, Germany) at 4°C, and then diluted to an optical density at 600 nm (OD_{600}) of 0.03 in TSB. Cells were acid habituated as described by Zhang et al. (2014). A 1% inoculum (v/v) of diluted overnight cultures was transferred into 50 mL of standard TSB (pH 7.4) and incubated at 37°C for 4 h with shaking (220 rpm) to reach mid-log phase as determined by Zhang et al. (2014). The cultures were collected by centrifugation (2,500 × g for 10 min) at 4 °C and then suspended in 50 mL of either standard TSB (pH 7.4, baseline control) or TSB without dextrose (pH 6.0 adjusted with HCl, Becton, Dickinson and Company, Sparks, MD) containing 0 (pH control) or 4.75 mM of either L-lactic acid (Sigma Chemicals, St. Louis, MO) or acetic acid (Johnson Matthey Company, Ward Hill, MA). The cultures were incubated at 37°C for 20 min with shaking (220 rpm). After incubation, 100 mL of RNAprotect Bacteria Reagent (Qiagen, Inc., Valencia, CA) was added to each sample. Cells were incubated at room temperature for 10 min then collected by centrifugation (9,500 × g for 10 min). The supernatant was discarded and cell pellets were suspended in 900 μL of lysozyme solution (Sigma-Aldrich, 20 mg/mL in Tris-EDTA buffer) that contained 20 units of mutanolysin (Sigma-Aldrich). Samples were incubated for 30 min at 37°C on a shaker incubator at 220 rpm, then 20 μL of proteinase K (Omega Bio-Tek Inc., Norcross, GA) (20 mg/mL) was added and the samples were returned to the shaker/incubator for 30 min. Total RNA was isolated using an Aurum total RNA mini kit (Bio-Rad, Hercules, CA) following the vendor's recommended procedures. Residual DNA was removed using The Ambion® DNA-free™ DNase Treatment and Removal Reagents. RNA samples were then purified using the GeneJET RNA Cleanup and concentration Micro Kit PCR purification kit (Thermo Fisher Scientific, Lithuania). The amount and quality of the RNA were measured using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, United States) and TapeStation System (Agilent, Santa Clara, CA), respectively.

TABLE 1 | Listeria monocytogenes strains used in this study.

Strain	Ribotype	Lineage	Serotype	Source
FSL R2-499	DUP-1053A	II	1/2a	Human isolate associated with the US outbreak linked to sliced turkey, 2000
FSL N1-227	DUP-1044A	I	4b	Food isolate associated with the US outbreak, 1998–1999

cDNA Synthesis and Real Time Quantitative PCR (qPCR)

cDNA was synthesized from total RNA using random primers (Invitrogen, Carlsbad, CA) and SuperScript II reverse transcriptase (Invitrogen). The qPCR was carried out using cDNA as template in an Opticon II thermal cycler (MJ Research, Reno, NV) using HotStart-IT™ SYBR Green qPCR Master Mix with UDG kit (Affymetrix, Inc.). Each reaction was performed in triplicate and the relative gene expression of targeted genes was calculated by the Pfaffl Method and normalized by the baseline control (Pfaffl, 2001). The primers used in this study are listed in **Table 2** and *rpoB* was used as a housekeeping gene to normalize the gene expression data (Bookout and Mangelsdorf, 2003; Tasara and Stephan, 2007). The amplification efficiency for each primer was tested by plotting the cycle threshold (Ct) value with different template concentrations and fitting the data to a regression line (Bookout and Mangelsdorf, 2003; Ruijter et al., 2009). The amplification efficiency for all the primers reached 90% or above (Li, 2020).

Galleria mellonella Wax Worm Model

The *in vivo* virulence of *L. monocytogenes* strains was determined using the *Galleria mellonella* wax worm model described by Ramarao et al. (2012). A 1% inoculum (v/v) of freshly prepared *L. monocytogenes* cells was transferred into 50 mL of either standard TSB (pH 7.4, baseline control) or TSB without dextrose (pH 6.0 with HCl) containing 0 (pH control) or 4.75 mM of either L-lactic acid or acetic acid and incubated at 37°C for 4 h with shaking (220 rpm). The mid-log phase cultures were collected by centrifugation (2,500 × g for 10 min) at 4°C. The bacterial cells were then re-suspended with sterile PBS solution (pH 7.4) and diluted to an optical density at 600 nm (OD_{600}) of 0.25. Ten microliters of 10^8 cfu/mL *L. monocytogenes*, either control or acid habituated, was injected into the haemocoel of the wax worms using an automated syringe pump (KDS 100, KD Scientific; 20 larvae per treatment; see **Figure 1** for schematic experimental design and injection order. Injection was done in two biological repetition). A PBS-only control injection was also included. The larvae were placed in petri dish (5 per dish) and incubated at 37°C. Larvae survival was evaluated every 24 h for 5 days after injection. The larvae were considered dead when they showed no movement in response to finger touch. Lethal times until 50% population mortality (LT_{50}) for each treatment were then determined by Probit analysis (Bliss, 1934, 1935).

Enumeration of *Listeria monocytogenes* in *Galleria mellonella* Wax Worms

L. monocytogenes in *G. mellonella* larvae was enumerated at 5, 10, 15, and 20 h after injection. At each time point, 5

TABLE 2 | Primers used in this study.

Protein	Function	Gene		Sequence (5′– > 3′)
General stress-responsive sigma factor B	Required for the expression of *L. monocytogenes* stress response factors	*sigB*	F	TGTTGGTGGTACGGATGATGG
			R	ACCCGTTTCTTTTTGACTGCG
Arginine deiminase	Catalyze L-arginine to L-citrulline	*arcA*	F	GCGTGATTGCGGAGGTTTTG
			R	CCCCATCATTCCACTGCTCT
Glutamate decarboxylase β	Convert glutamate to GABA	*gadD2*	F	ATCGATATGCGCGTTGTTCCA
			R	ATACCGAGGATGCCGACCACA
Glutamate decarboxylase γ	Convert glutamate to GABA	*gadD3*	F	TTCCGCATTGTTACGCCAG
			R	TCTTACTTGGGGACTTCGAC
Bile salt hydrolase	Detoxify conjugated bile acid	*Bsh*	F	TTTGTTGTTCCACCGAGCCTA
			R	GGGCGGAATTGGCTTACCTG
Bile exclusion protein	Exclude bile from cell	*bilE*	F	CATCAACGGAGCCTGTCGAA
			R	TCCAGATGACGCGCTAAGAA
Positive regulatory factor A	Required for the expression of *L. monocytogenes* virulence factors	*prfA*	F	CGATGCCACTTGAATATCCT
			R	CTTGGCTCTATTTGCGGTCA
Internalin A	Host cell invasion	*inlA*	F	CTATACCTTTAGCCAACCTGT
			R	GGTTGTTTCTTTGCCGTCCAC
Internalin B	Host cell invasion	*inlB*	F	CTGGACTAAAGCGGAAAACCTT
			R	TCCAGACGCATTTCTCACTCTT
Listeriolysin O	Phagosome lysis	*hly*	F	ATGCAATTTCGAGCCTAACC
			R	ACGTTTTACAGGGAGAACATC
Phosphatidylinositol-specific phospholipase C	Phagosome lysis	*plcA*	F	ACCGTATTCCTGCTTCTAGTT
			R	ACACAACAAACCTAGCAGCG
Phosphatidylcholine phospholipase C	Phagosome lysis	*plcB*	F	TAGTCAACCTATGCACGCCAA
			R	TTTGCTACCATGTCTTCCGTT
Actin assembly-inducing ptotein	Stimulates actin-based intracellular bacterial motility	*actA*	F	TTATGCGTGCGATGATGGTG
			R	TTCTTCCCATTCATCTGTGT
Hexose phosphate transporter	Intracellular bacterial growth	*uhpT*	F	TTCAGCACCACAGAACTAGG
			R	GCATTTCTTCCATCCACGAC
RNA polymerase beta subunit	Housekeeping gene	*rpoB*	F	CTCTAGTAACGCAACAACCTC

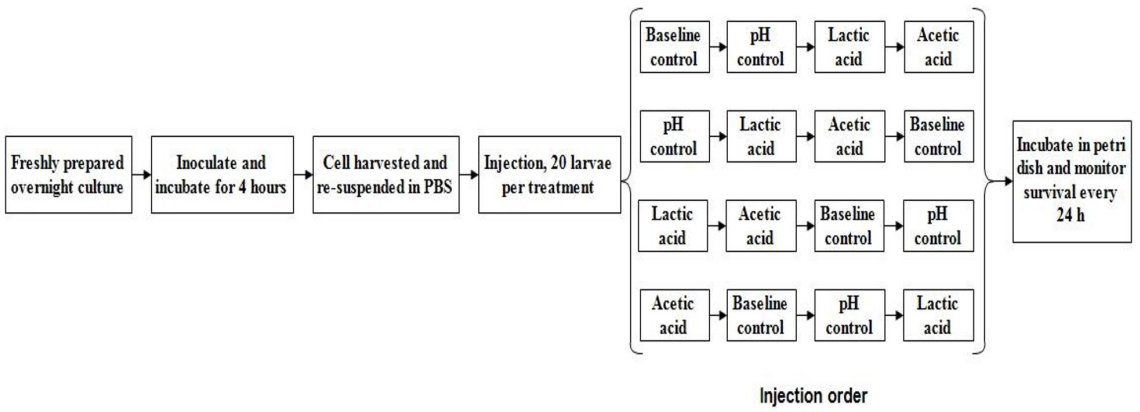

FIGURE 1 | *Galleria mellonella* schematic experimental design of one biological repetition.

larvae were collected and homogenized in 9 mL of sterile peptone physiological solution (PPS) in a stomacher. Serial dilutions were made by pipetting 1 mL of diluted sample into 9 mL PPS, then 100 μL of diluted samples was spread on Palcam agar (*L. monocytogenes* selective media; Oxoid Limited, Hampshire, United Kingdom). Plates were incubated at 37°C

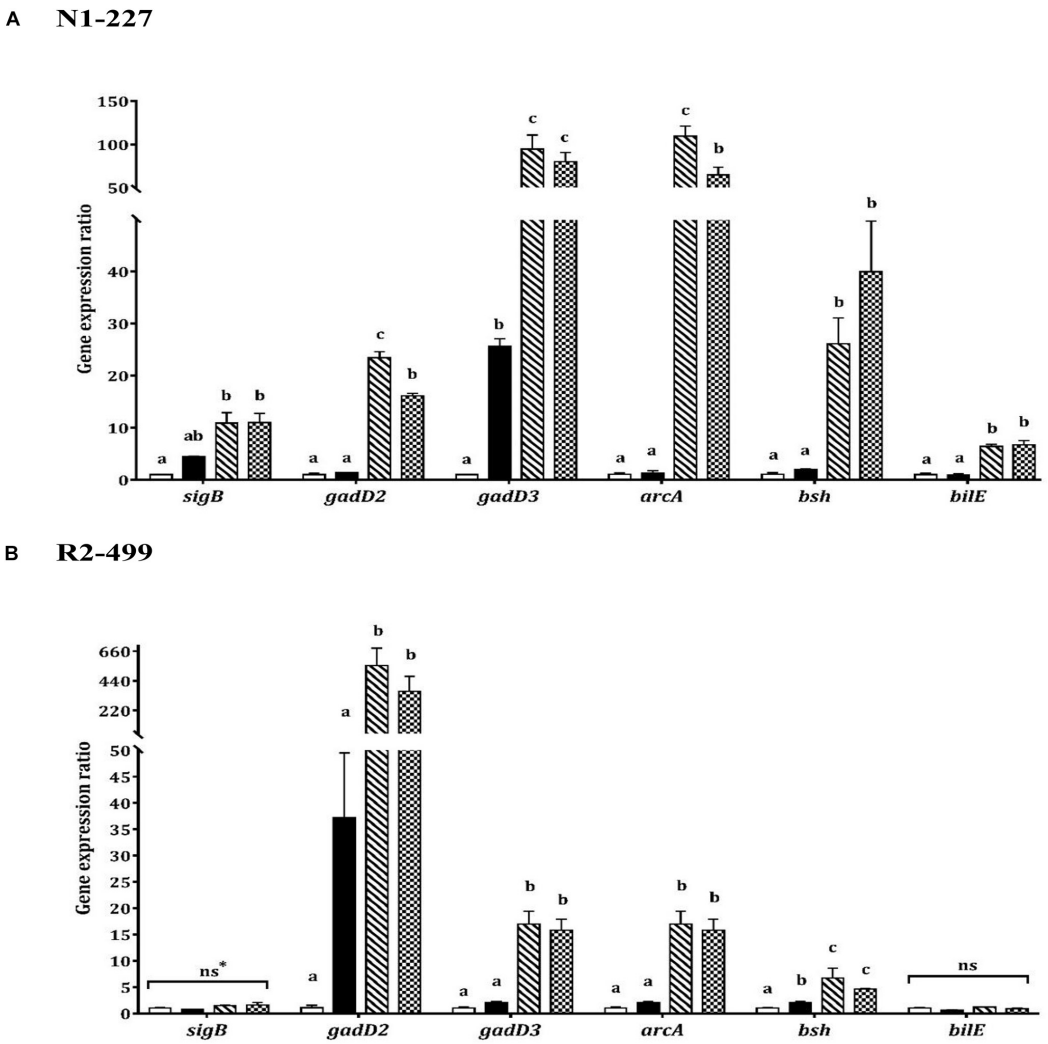

FIGURE 2 | Relative gene expression of acid and bile stress response related genes in 20 min habituated *Listeria monocytogenes* strains **(A)** N1-227 and **(B)** R2-499 cells in comparison with non-habituated cells (baseline control, TSB pH 7.4, ▭). Habituated treatments include as follows: TSB pH 6.0 (pH control, ■), TSB at pH 6.0 with 4.75 mM of acetic acid (▨) and TSB at pH 6.0 with 4.75 mM of L-lactic acid (▩). Error bars represent standard error of mean for two biological trials with three replicates for each trial. Different letters indicate that treatments are significantly different ($p < 0.05$) as determined by one-way ANOVA with Tukey's *post-hoc* tests; *ns, Non-significant.

for 48 h then *L. monocytogenes* colonies were enumerated. Microbiological count data were expressed as $\log_{10}$ of colony-forming units per larvae.

Statistical Analysis

The data collected in this study (relative expression ratio of target genes compared with reference genes in three biological repetitions, the survival rate of *G. mellonella* larvae and the enumeration of *L. monocytogenes* in *Galleria mellonella* wax worm in two biological repetitions) were continuous outcome variables for every categorical treatment variable (acidification treatments of *L. monocytogenes*). Significant differences in each outcome between treatments were assessed using one-way analysis of variance (ANOVA) followed by Tukey's test to compare means of the gene expression outcome variables between treatments. Differences were considered significant at $P < 0.05$.

RESULTS

Influence of Acid Habituation on Expression of Acid and Bile Stress Response Genes

Increased expression of *gadD3* was observed for strain N1-227 in the pH control relative to the baseline control ($P < 0.05$, **Figure 2**). Additionally, acetic acid or lactic acid habituation resulted in significant upregulation of *gadD3* as compared to the pH control in both N1-227 and R2-499 ($P < 0.05$). No significant change of *gadD2* expression was observed for both strains in the

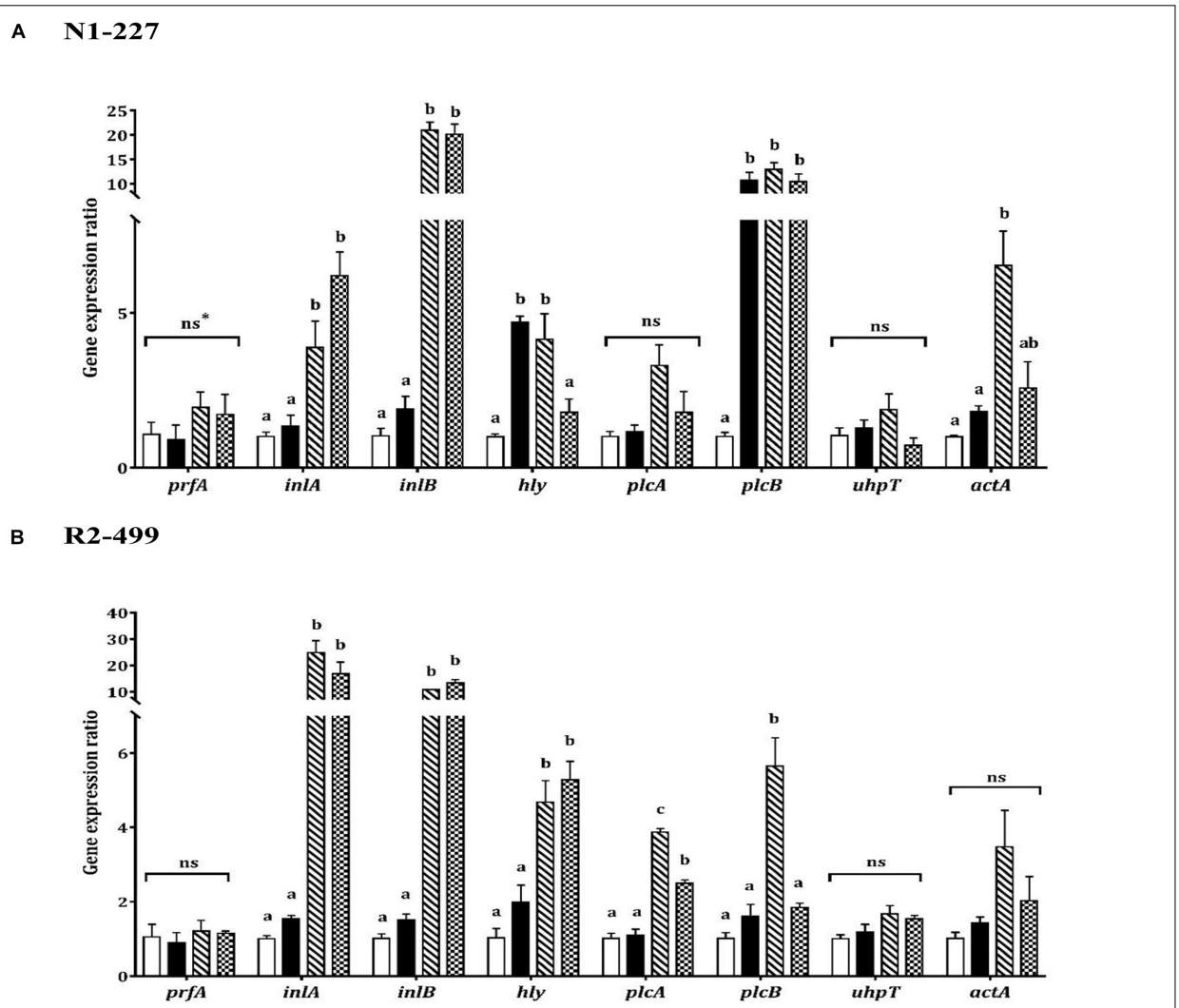

FIGURE 3 | Relative gene expression of virulence related genes in 20 min habituated *Listeria monocytogenes* **(A)** N1-227 and **(B)** R2-499 cells in comparison with non-habituated cells (baseline control, TSB pH 7.4, ▭). Habituated treatments include as follows: TSB at pH 6.0 (pH control, ■), TSB at pH 6.0 with 4.75 mM of acetic acid (▨) and TSB pH 6.0 with 4.75 mM of L-lactic acid (▩). Error bars represent standard error of mean for two biological trails with three replicates for each trail. Different letters indicate that treatments are significantly different ($p < 0.05$) as determined by one-way ANOVA with Tukey's *post-hoc* tests. *ns, Non-significant.

pH control compared to the baseline control. However, similar to *gadD3*, acetic acid or lactic acid habituation induced significant and dramatic expression of *gadD2* in comparison with pH control in both strains ($P < 0.05$). The qPCR results for both strains also showed no significant changes in the expression of gene encoding arginine deiminase (*arcA*) in the pH control relative to the baseline control, and that acetic acid or lactic acid habituation significantly increased *arcA* expression in both strains ($P < 0.05$, **Figure 2**).

In contrast, transcription of genes related to bile tolerance was variable between the strains. Habituation to lactic acid or acetic acid significantly increased *bsh* gene expression in comparison with the pH control for both strains ($P < 0.05$). However, the pH control had no significant effect on *bsh* expression relative to the baseline control in strain N1-227 (**Figure 2**). Changes in the expression of *bilE* were also strain-dependent. For strain N1-227, *bilE* was significantly overexpressed ($P < 0.05$) when cells were habituated to acetic or L-lactic acid, whereas no significant changes were observed in strain R2-499. Finally, qPCR data showed habituation to L-lactic acid or acetic acid significantly ($P < 0.05$) induced *sigB* expression in strain N1-227 cells compared to the baseline control (**Figure 2A**). However, no significant change on *sigB* expression was observed between treatments in strain R2-499 (**Figure 2B**).

Influence of Acid Habituation on Expression of Virulence Genes

As was observed with stress genes, qPCR results showed similarities and differences between strains with respect to virulence gene expression in response to organic acid habituation (**Figure 3**). The transcription level of *prfA* or *uhpT* was not

TABLE 3 | Lethal times until 50% population mortality (LT_{50}) for *Galleria mellonella* larva injected with *Listeria monocytogenes* strains habituated to various acid treatments.

Strain	Treatments	LT_{50} (Hours) (95% CI)
N1-227	Baseline	40.72 (32.58–50.90)[a]
	pH control	34.23 (26.28–44.59)[a]
	Acetic acid	19.76 (15.50–25.19)[b]
	L-lactic acid	17.14 (13.58–21.65)[b]
R2-499	Baseline	37.23 (31.22–44.39)[a]
	pH control	29.83 (22.79–39.04)[a]
	Acetic acid	17.14 (13.10–22.42)[b]
	L-lactic acid	14.01 (10.97–17.88)[b]

Different letters within a strain indicate that treatments are significantly different ($p < 0.05$) as determined by Probit analysis.

significantly impacted by pH or acid exposure in either strain. However, expression of *inlA, inlB* and *hly* increased in both strains when the pH was decreased. Both strains showed significantly ($P < 0.05$) increased expression of *inlA* and *inlB* in organic acid habituated cells compared to the baseline control or pH control (**Figure 3**). Furthermore, *hly* expression was significantly ($P < 0.05$) increased in R2-499 cells habituated to acetic acid or L-lactic acid relative to baseline control and pH control. However, significant ($P < 0.05$) overexpression of *hly* in strain N1-227 compared to baseline control was only observed with the pH control and acetic acid habituation treatment.

The qPCR results showed the expression profile for the other virulence genes (*plcA, plcB, actA*) was also strain-dependent (**Figure 3**). No significant changes were observed in *plcA* expression for strain N1-227 (**Figure 3A**), while organic acid habituation significantly ($P < 0.05$) increased expression of this gene in strain R2-499 compared to baseline control and pH control (**Figure 3B**). All three acid treatments (pH control and organic acid habituated cells) significantly ($P < 0.05$) induced *plcB* expression compared to the baseline control in strain N1-227, whereas significant induction in strain R2-499 was only observed with the acetic acid treatment. Conversely, no significant differences were recorded in *actA* expression for strain R2-499, and only acetic acid habituated N1-227 cells showed a significant ($P < 0.05$) increase in the expression level of this gene (**Figure 3**).

Effect of Habituation to Organic Acid on *Galleria mellonella* Survivability

The lethal time to 50% population mortality (LT_{50}) of each treatment for both strains (**Table 3**) was determined based on the survival of *G. mellonella* over 5 days post-injection (see **Supplementary Figure 1**). For both N1-227 and R2-499, the LT_{50} of larvae injected with *L. monocytogenes* habituated with HCl (pH control) was lower than that of larvae injected with non-habituated *L. monocytogenes* (baseline control) and LT_{50} values decreased considerably more when larvae were injected with organic acid habituated *L. monocytogenes* (**Table 3**). The shortest LT_{50} results were noted with organic acid habituated *L. monocytogenes* R2-499, which suggests this strain may be more virulent than N1-227.

To test whether the previous organic acid habituation affected the survival or growth of *L. monocytogenes* in *G. mellonella* larvae, post-injection bacterial cell numbers were determined over time. The number of *L. monocytogenes* cells showed a slight decrease for the first 5 h and then remained constant through the 20 h sampling period (see **Supplementary Figure 2**). Other researchers have also reported that *L. monocytogenes* cells decreased in number for the first 2 h post-injection (Joyce and Gahan, 2010; Schrama et al., 2013). No statistically significant differences were observed between treatments for either *L. monocytogenes* strain, indicating that the enhanced virulence observed in organic acid habituated cells is not due to enhanced survival or growth in the larvae.

DISCUSSION

The qPCR experiments showed organic acid habituation impacted the expression of genes encoding important acid and bile stress response mechanisms in both strains of *L. monocytogenes*. The GAD system serves as a key mechanism of *L. monocytogenes* survival in acid environments (Cotter et al., 2001; Melo et al., 2015). Karatzas et al. (2012) proposed a model wherein GAD-mediated acid resistance consists of two semi-independent systems: An intracellular system that involves GadD3 acting on intracellular glutamate and an extracellular system that involves GadD2 decarboxylation of glutamate imported by the antiporter GadT2. Interestingly, the differential induction of *gadD3* vs. *gadD2* in strains N1-227 and R2-499 suggests that *gadD3* may play a more prominent role in acid protecting in N1-227, while *gadD2* serves as primary defense mechanism in R2-499. Additionally, the fold-change in *bilE* expression was lower than that of *bsh* in both strains, which might be a consequence of cell growth phase. Sue et al. (2003) showed that *bilE* expression is growth phase-dependent, with highest expression level observed in stationary phase cells, and this study used cells collected at mid-log phase.

Infection of host cells by *L. monocytogenes* can be divided into three stages that require specific virulence factors: Initial cell invasion (InlA and InlB), escape from vacuole (Hly, PlcA, and PlcB) and cell-to-cell spread (ActA and UhpT) (Cossart et al., 1989; Mélanie et al., 2006; Schnupf and Portnoy, 2007; Joyce and Gahan, 2010; Hamon et al., 2012). It has been reported that *L. monocytogenes* is able to sense different environments and host cell compartments and regulate virulence gene expression accordingly (Freitag and Jacobs, 1999; Gaballa et al., 2019). Other researchers have found that *inlA* and *inlB* are induced prior to the cell invasion, while *hly*, *plcB,* and *plcA* are overexpressed within the phagosome and *uhpT* and *actA* are expressed in the cytosol (Bubert et al., 1999). In this study, *inlA* and *inlB* showed a similar expression pattern in both strains in response to acid exposure (**Figure 3**). Significant induction of other virulence genes in response to pH or acid was also observed but the patterns were strain-dependent. Additionally, although the transcription level of *prfA* was not significantly altered by acid exposure, *hly*

transcription is PrfA-dependent (Kazmierczak et al., 2006; Scortti et al., 2007; de las Heras et al., 2011). The observed induction of *hly* may therefore reflect post-transcriptional control of PrfA activity in these cells.

In summary, RT-qPCR demonstrated that habituation to L-lactic or acetic acids induces statistically significant increases in the expression of several genes associated with acid and bile stress resistance in two *L. monocytogenes* strains that are known human pathogens. While many of these changes were strain-specific, induction patterns for several stress and virulence genes, including *gadD2*, *arcA*, *bsh*, two internalin genes *ilnA* and *ilnB*, in response to acid habituation were similar between N1-227 and R2-499. Future studies might explore the role of nucleotide polymorphism in promoter sequences or in DNA binding motifs in gene expression patterns.

Organic acid habituation also enhanced *in situ* virulence of both *L. monocytogenes* strains as evidenced by a reduced the LT_{50} value in the *in vivo G. mellonella* infection model. Our finding that HCl or organic acid habituation enhanced virulence of both strains in the *G. mellonella* model stands in contrast with the report of Schrama et al. (2013), who observed acid or salt adaptation reduced the infectious ability of some *L. monocytogenes*. However, factors such as different strains and stressors may have contributed to this discrepancy. Our prior research suggests this difference is likely due to strain-specific variation among *L. monocytogenes* (Zhang et al., 2014), and illustrates the need for further study to determine how widespread this phenomenon is among pathogenic and non-pathogenic strains of *L. monocytogenes*.

Taken together, these results suggest that exposure to organic acids can increase the pathogenicity of some *L. monocytogenes* strains by enhancing their ability to survive passage through the gastrointestinal tract while simultaneously priming them for intracellular virulence. While our prior results indicate that this phenomenon may not be universally shared among strains of *L. monocytogenes* (Zhang et al., 2014), the fact that it does occur in pathogenic strains associated with foodborne outbreaks (**Table 1**) underscores the potential for organic acids to have unanticipated consequences on food safety and public health. To fully understand the broader impact, future studies are needed to determine how widespread this phenomenon is among additional strains of *L. monocytogenes*, including both known human pathogens and strains not currently recognized as pathogenic, and to examine the impact of food systems and conditions encountered during processing and storage such as refrigeration temperatures.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

CC and JB: conceptualization, data curation, funding, acquisition, project administration, supervision, validation, and writing—review and editing. ML: formal analysis, investigation, methodology, data collection, software, visualization, and writing—original draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.675241/full#supplementary-material

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REVIEW
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Reducing the Risk of Transmission of Critical Antimicrobial Resistance Determinants From Contaminated Pork Products to Humans in South-East Asia

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Antimicrobial resistance (AMR) is a critical challenge worldwide as it impacts public health, especially via contamination in the food chain and in healthcare-associated infections. In relation to farming, the systems used, waste management on farms, and the production line process are all determinants reflecting the risk of AMR emergence and rate of contamination of foodstuffs. This review focuses on South East Asia (SEA), which contains diverse regions covering 11 countries, each having different levels of development, customs, laws, and regulations. Routinely, here as elsewhere antimicrobials are still used for three indications: therapy, prevention, and growth promotion, and these are the fundamental drivers of AMR development and persistence. The accuracy of detection of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) depends on the laboratory standards applicable in the various institutes and countries, and this affects the consistency of regional data. *Enterobacteriaceae* such as *Escherichia coli* and *Klebsiella pneumoniae* are the standard proxy species used for indicating AMR-associated nosocomial infections and healthcare-associated infections. Pig feces and wastewater have been suspected as one of the hotspots for spread and circulation of ARB and ARG. As part of AMR surveillance in a One Health approach, clonal typing is used to identify bacterial clonal transmission from the production process to consumers and patients – although to date there have been few published definitive studies about this in SEA. Various alternatives to antibiotics are available to reduce antibiotic use on farms. Certain of these alternatives together with improved disease prevention methods are essential tools to reduce antimicrobial usage in swine farms and to support global policy. This review highlights evidence for potential transfer of resistant bacteria from food animals to humans, and awareness and understanding of AMR through a description of the occurrence of AMR in pig farm food

chains under SEA management systems. The latter includes a description of standard pig farming practices, detection of AMR and clonal analysis of bacteria, and AMR in the food chain and associated environments. Finally, the possibility of using alternatives to antibiotics and improving policies for future strategies in combating AMR in a SEA context are outlined.

Keywords: antibiotic resistance, alternatives to antibiotics, one-health, pig production, policy, slaughtering process, South East Asia

INTRODUCTION

Antimicrobial resistance (AMR) in bacterial species and strains is a critical global threat, and has been monitored in animals, humans and the environment through international collaborations aimed at AMR reduction (Gerbin, 2014; Lechner et al., 2020; Singh et al., 2021). The main factors contributing to the emergence of AMR are overuse and misuse of antimicrobials, and the lack of quality control of antimicrobials and active pharmaceutical ingredients in the market (Simba et al., 2016; Tangcharoensathien et al., 2018; Barroga et al., 2020). Also, the multi-faceted nature of the problem includes misconceptions about antibiotic types and AMR; lack of diagnostic facilities; limited availability of alternatives to antibiotics as a means to establish antibiotic-free farms; and insufficient training of veterinarians on AMR and antibiotic prescribing (Lekagul et al., 2021). Low- and middle-income countries (LMICs), including those in South East Asia (SEA) are crucial in the global response to AMR due to their diverse medicine regulation and access to antimicrobials. Furthermore, folk conceptions or unexpected practices around antimicrobials in these regions can complicate AMR communication efforts and entail unforeseen consequences (Haenssgen et al., 2019). Nevertheless, it is encouraging that countries such as Denmark and the Netherlands that both have massive pig production in recent years have achieved tremendous reductions in antimicrobial usage while sustaining peak production (Dall, 2019). Comparable results have been accomplished in Belgium, France, Sweden, and the United Kingdom (More, 2020). In Brazil, one of the biggest pork producers in the world, the increased demand for meat production has led to ongoing environmental problems, such as soil and water contamination by pathogenic and/or resistant microorganisms (Silva et al., 2015; Brisola et al., 2019). In Russia, relatively high levels of MDR *Escherichia coli* that are resistant to critically important antimicrobials such as colistin, cefotaxime, and ciprofloxacin have been recorded (Makarov et al., 2020). A study in New Zealand also found that the widespread use of oral antimicrobials within pig production was a significant risk factor for development of AMR and MDR *E. coli* (Riley et al., 2020). The rate of antibiotic resistance differs considerably from country to country, depending upon the amount of usage. In the EU, the lowest levels of AMR *E. coli* isolates were found in countries where lower antimicrobial usage was practiced, such as in Norway, Sweden, and Finland, whereas countries with high levels of use such as Spain, Portugal, and Belgium had relatively higher levels of AMR *E. coli* (Holmer et al., 2019).

Nosocomial infections or healthcare-associated infections fall under the political concerns of the World Health Organization (WHO). Nosocomial infections account for 7 and 10% of all infections in developed and developing countries, respectively (Khan et al., 2017; World Health Organization [WHO], 2019). *Enterobacteriaceae* such as *Escherichia coli* and *Klebsiella pneumoniae* have been identified as the most common resistant bacteria found in healthcare-associated infections (Malchione et al., 2019; Shrestha et al., 2019). The possibility that AMR colonization in humans can be related to livestock and environmental sources was shown in a recently published One Health AMR risk assessment (Opatowski et al., 2020). WHO emphasizes the importance of antimicrobial classes (agents) that are used in humans being targeted for antimicrobial susceptibility surveillance in livestock, including aminoglycosides (gentamicin), carbapenems (meropenem), cephalosporins (3rd, 4th, and 5th generation: ceftriaxone, cefepime, ceftaroline, ceftobiprole), glycopeptides (vancomycin), amoxicillin-clavulanic-acid, polymyxins (colistin), and quinolones (ciprofloxacin). Due to high prevalence and incidence of AMR rates in the pig industries in South-East Asian (ASEAN) countries, a regional database of AMR surveillance has been developed (Nguyen N. T. et al., 2016; Nhung et al., 2016; Lugsomya et al., 2018a,b; Mobasseri et al., 2019; Vounba et al., 2019; Khine et al., 2020; World Health Organization [WHO], 2020; Dawangpa et al., 2021). It is important to emphasize that SEA is a diverse region, where different levels of development, customs, laws, and regulations among different countries in the region. Pork is one of the most common protein sources and is widely produced for local consumption, with some export also occurring. Hence it is an important target for AMR surveillance.

Antimicrobial resistance surveillance through the pig production cycle, covering from the neonatal to the slaughtering periods has demonstrated the possibility of transmission of AMR bacteria in the food chain, and has provided a modicum of comparative evidence for nosocomial infection through genetic fingerprinting of isolates using multi-locus sequence typing (MLST) and whole-genome sequencing (Mather et al., 2018). A plasmid carrying *mcr-1* conferring colistin resistance was first identified in China in 2014 (Liu et al., 2016). To date, up to 10 types of the *mcr* gene family harbored in *Enterobacteriaceae* have been reported in clinical samples and in animals and human carriers (Xu et al., 2021). Colistin has been extensively used in pig production, often as a growth promoter until the 2017 prohibition of its use under regional policy. However, a high prevalence of colistin-resistant *Klebsiella pneumoniae* was

found in healthy people living in rural communities in Thailand, Vietnam, and Laos with no history of colistin therapy (Olaitan et al., 2014; Yamaguchi et al., 2020). A 10–22% prevalence of the *mcr-1* and *mcr-3* genes has been reported in *E. coli* isolated from pig and chicken farms in Vietnam and Thailand (Malhotra-Kumar et al., 2016; Khine et al., 2020) and in *Klebsiella pneumoniae* from farmed pigs in Malaysia (Mobasseri et al., 2019). The clonal similarity of a colistin-resistant *E. coli* isolate from a local pig with a human isolate was demonstrated in Laos (Olaitan et al., 2015). Overall, these occurrences confirm the interchangeability of colistin-resistant bacteria between humans and farm animals that use colistin as a feed additive. Cross-species transmission is a suspected course of distribution.

Similar clonal types of multidrug-resistant *Salmonella* Typhimurium and *E. coli* have been found in humans and pigs in SEA and in the United States (Oloya et al., 2009; Nhung et al., 2016; Mather et al., 2018; Sudatip et al., 2021). Pigs and the environment can be potential sources for the dissemination of AMR to humans; however, there have been a few longitudinal studies monitoring linkages of AMR strains among pigs, the environment, and consumers/patients. Genetic similarity between bacterial clones from farmed pigs and pork was not found in any comprehensive monitoring study in Thailand (Lugsomya et al., 2018b). To our knowledge, to date there has been no unequivocal direct evidence of transmission of AMR bacteria to human patients from farms or the slaughtering process in SEA.

This article aims to improve awareness and understanding of AMR through a description of the occurrence of AMR in pig farm and food chains under AMR management systems in SEA: this includes a description of standard farming practices; detection of AMR and clonal analysis of bacteria; AMR in the food chain, wastewater, and associated environments, the possibilities of AMR transfer from farming and slaughtering practices to humans. Finally, the possibility of reducing AMR by using alternatives to antibiotics and improving policies are discussed.

STANDARD FARMING SYSTEMS IN SEA

Asia is the largest producer of pigs in the world (58.4%), and within SEA the top three pig producing countries are Myanmar, Vietnam, and the Philippines. In contrast, Thailand exports the most pork raised under a high standard of farming and food safety conditions (FAOSTAT, 2019). Pig production systems in SEA vary greatly, from backyard pigs held in small-scale often peri-urban farms to semi-commercial units and large intensive units. For example, 80% of pig farms in Cambodia involve smallholders, while 80% in Thailand are intensive farms. However, even pig farming in peri-urban areas operates under a high standard of biosecurity similar to that in large commercial approved farms (World Organization of Animal Health, 2020).

According to the ASEAN Good Animal Husbandry Practice Guidelines (ASEAN GAHP), pig farms should be located in areas with an appropriate clean water source, with physical barriers to reduce the risk of contamination arising from visitors and animal access. Quarantine pens should be located in a separate area and must be prepared for sick pigs and pig carcass to limit pathogen spread. Housing and equipment providing good hygiene and ventilation with easy-to-clean pig manure systems are recommended, along with good personal hygiene management for workers, including a pre-entry farm protocol which includes facilities for showering and hair washing, and changing into protective farm clothes and footwear before accessing the animal housing. Wastewater and manure should be kept in a closed area well away from the animal house, and the farm should possess a treatment system that limits odor and hazards such as biogas. Moreover, a veterinarian must authorize a program of vaccination and antimicrobial and chemical use for disease prevention and treatment. Vaccination is a potential method to prevent certain infectious diseases and is established as an important alternative to antibiotics (Founou et al., 2016). In many countries the governments produce and promote vaccines against the Foot and Mouth Diseases virus and Classical Swine Fever virus, as these are very important pathogens. While commercial and autogenous vaccines against pathogenic bacteria are alternatives to control infections, their efficacy varies considerably. To date, vaccines against bacteria such as *E. coli, Lawsonia intracellularis,* and *Actinobacillus pleuropneumonia* have been commercialized and used worldwide (FAO, 2014; Founou et al., 2016).

In 2013 the total amount of antimicrobials used for food-producing animal in SEA was 2,950 tons: antimicrobial classes included penicillin (666 tons), tetracyclines (484 tons), quinolones (321 tons), sulfonamides (317 tons) and macrolides (281 tons). Thailand consumed the largest amount of antimicrobials (531 tons in 2013), and the amount has subsequently increased (3,816.3 tons in 2018), probably due to the growing number of food-producing animals (Van Boeckel et al., 2017; Ministry of Public Health, 2020). In practice, antimicrobials are used for three indications: therapy, prevention, and growth promotion. Therapeutic use involves a short duration of treatment with a high dose of antimicrobial given only to sick pigs. Prophylactic administration is used to prevent disease occurring where it is routinely expected to occur (e.g., to control diarrhea after weaning), and the same amount and duration of administration is applied to all susceptible pigs during this period. Lastly, growth promotor use involves administering a lower than recommended therapeutic dose, long-term for all pigs on-farm (FAO, 2014). The intention is to improve growth rate by controlling subclinical infections and improving digestive efficacy. Antimicrobial growth promotion use has been banned in Thailand and Vietnam (Coyne et al., 2019), while Indonesia, Myanmar, and Timor-Leste policies promote a decrease in antimicrobial use in response to WHO announcements (Cardinal et al., 2020). Furthermore, the use of colistin as a feed additive has been restricted in Malaysia and Thailand (Olaitan et al., 2021), and nitrofuran has been banned in the Philippines and Thailand (Islam et al., 2014).

Implementation of standard biosecurity and management systems in pig farms is an essential part of veterinary preventive medicine, and promotes improved pig production, and reduced antimicrobial consumption in pigs, leading to fewer AMR

bacteria on pig farms which otherwise might enter the food chain (Founou et al., 2016).

ANTIMICROBIAL USAGE AND RELATIONSHIP TO ANTIMICROBIAL RESISTANCE

The key driver of resistance in bacterial population is considered to be the extensive use of antimicrobial agents which created a selection pressure on susceptible bacteria (Aarestrup et al., 2008; Koningstein et al., 2010; Lunha et al., 2020). Frequency, amount, duration, and combined use of antimicrobial agents are important factors related to the emergence of both ARB and MDR bacteria (McEwen, 2006; Martinez and Baquero, 2009). Additionally, there have been many reports on a positive association between the amount of antimicrobial use and the prevalence of ARB in both animals and human (Lazarus et al., 2015). A recent study from Thailand reported the detection of high frequencies of ARB isolates from pigs, particularly resistance against tetracycline, which is also reported as an antimicrobial agent that has had long term and extensive use in livestock both in Thailand and throughout SEA (Van Boeckel et al., 2017; Lunha et al., 2020). Nevertheless, it is very important that monitoring of AMU using standardized systems for monitoring programs for AMR data is continued, and that the quantification and temporal trends in AMU are continued to be recorded in order to obtain evidence between the linkage of AMU and AMR so as to further support policy and decision makers to understand and fight against AMR (Aarestrup, 2005; Magouras et al., 2017).

DETECTION OF AMR AND CLONAL ANALYSIS

At present, the main approaches to detect antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARG) from bacteria in hosts and environments are primarily categorized into the conventional culture-based methods, and molecular biology-based methods. Clone typing by core-gene multilocus sequence typing (MLST) is used to determine the molecular epidemiology of bacteria in the family *Enterobacteriaceae*.

Conventional Culture-Based Methods

This approach requires isolating and growing the microorganisms of interest on a nutrient medium. For samples from wastewaters and associated environments the membrane filtration method is frequently applied along with selective media supplemented with the antibiotics of interest for isolation, and enumeration of individual ARB (Rizzo et al., 2013). The antibiotic resistance pattern of individual pure isolates can be further investigated by performing antibiotic susceptibility testing, which depends on the phenotypic traits of individual isolates. The frequently applied resistance susceptibility testing methods are of four types, including diffusion methods (Stokes method and Kirby-Bauer method), dilution methods (Minimum inhibitory concentration such as broth and agar dilution), diffusion and dilution methods (*E*-test method and gradient diffusion method), and automated instrument methods (such as Vitek 2 system by bioMérieux and Sensititre ARIS 2X by Trek Diagnostic Systems) (Jorgensen and Ferraro, 2009; McLain et al., 2016; Benkova et al., 2020). The results from antibiotic susceptibility testing should be interpreted following standardized values, which allow qualitative assessment distinction between susceptible, intermediate, and resistant isolates (Clinical & Laboratory Standards Institute: CLSI Guidelines; EUCAST: AST of bacteria; Jorgensen and Ferraro, 2009).

Nevertheless, the conventional culture-based methods are time-consuming and somewhat labor-intensive. Investigators are required to constantly attain a reliable and up-to-date interpretation of the resistance susceptibility testing. Besides, only a minority of bacteria are culturable and suitable for the conventional culture-based methods, which are presumed to be only a tiny fraction of the total bacteria present (Kummerer, 2004; Bouki et al., 2013; Rizzo et al., 2013).

Molecular Biology-Based Methods

This approach allows the identification of deoxyribonucleic acid (DNA) targets with and without the requirement to culture individual isolates. Methods such as Polymerase Chain Reaction (PCR) amplification and use of DNA probes are well known for detecting resistance genes and genetic elements. With the application of multiplex PCR, various ARGs can be screened and detected simultaneously (Luby et al., 2016; Anjum et al., 2017; Preena et al., 2020). In comparison, quantitative PCR (qPCR) has been applied as a quantifying method for tracking and tracing ARGs from various pathogenic bacteria in different environments from municipal wastewater to surface water. However, the nucleic acid extraction method and its product quality are critical for PCR gene detection, since wastewater and sludge samples contain much indeterminate substance (Obst et al., 2006; Lupo et al., 2012; Rizzo et al., 2013). Additionally, several typing methods are used for determining and characterizing the diversity of resistant strains and clones of individual isolates, such as pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), and amplified ribosomal DNA restriction analysis (ARDRA) (Hamelin et al., 2006; Faria et al., 2009; Li et al., 2009; Brooks et al., 2014; Papadopoulos et al., 2016). Unfortunately, there is limited availability of specific primer sets, especially for PCR and qPCR methods. These molecular techniques often encounter an absence of standardization and validation of interlaboratory tests (Rizzo et al., 2013).

Most recently, molecular high throughput (HPT) and next-generation sequencing (metagenomic analysis, transcriptome, and resistome) are among the techniques that facilitate studies of the microbial community and their diverse genomes and gene expression without a requirement for culture. Moreover, these techniques are very valuable since they can compute numerous target genes without knowledge of the bacteria or genes in the samples, are not limited by specific primer sets, and are even faster to perform than the conventional molecular methods such as PCR and qPCR (Rizzo et al., 2013; Limayem et al., 2019; Lira

et al., 2020). They also provide a deeper insight into antibiotic resistance conditions related to both vertical and horizontal gene transfer occurrences (Von Wintersdorff et al., 2016).

Therefore, molecular biology-based methods make possible the detection of ARB and ARG that cannot be cultured and express in individual isolates and provide faster detection of ARB and ARG with high specificity and sensitivity compared with conventional culture-based methods. Nevertheless, these methods cannot separate between living and non-living microorganisms. They are also less useful for identifying ARB and resistance patterns compared to the conventional culture-based methods.

Clonal Analysis of AMR Strains

Bacterial strains identified using DNA fingerprinting techniques can be assigned as clones representing the ancestor's phylogenetic root tree. In general, bacteria are highly diverse genetically, even at the species-level, because of inter-and intraspecies gene interchange leading to diversification of genotype in a particular niche of a bacterial clone (a clonal complex). For AMR, clonal relations are used as a tool to indicate the phenomenon of bacterial transmission or "spillover" between sources: humans to animals, animals to the environment, or environment to humans or vice versa (Spratt, 2004). The diversification of bacterial clones depends on the extent of gene recombination and varies by bacterial type. Some species, e.g., *Salmonella enterica* members, are stable clones, whereas in some species, e.g., *Helicobacter pylori*, these may be transient or genetical diverse without phenotypical change (Selander et al., 1990; Kennemann et al., 2011). Index genetic parameters are used to assign the clones and clonal complexes. In the 1980s to 2000s, the best results were achieved using pulsed-field gel electrophoresis and multilocus sequence typing (Schwartz and Cantor, 1984; Maiden et al., 1998). Since the Whole Genome Sequencing (WGS) era, WGS-based strain typing has been increasingly used to analyze bacterial pathogens and AMR bacteria in the public health field and for implementing disease control strategies (Snitkin et al., 2012; Harris et al., 2013; Walker et al., 2013). Core genome MLST (cgMLST) has become a standard tool for WGS-based strain typing as it has high accuracy and epidemiological concordance. Outcomes from cgMLST are analyzed by comparison of shared common genes sets from genomes within the same species, which can be used to determine the source and routes of transmission, trace cross-contamination of healthcare-associated pathogens, and identify antibiotic-resistant lineages or subpopulations (Dekker and Frank, 2016; Mellmann et al., 2016; Nadon et al., 2017). From each species-specific cgMLST scheme, there is the joint-calculated parameter called the "relatedness threshold." If cgMLST allelic mismatches between two bacterial stains are less than (or equal to) the relatedness threshold, there are clonal relations between them (Miro et al., 2020). The relatedness threshold is calculated using an algorithm in the Ridom SeqSphere + online package (Calibrating the cgMLST Complex Type Threshold[1]). The relatedness threshold of clonality from cgMLST diversity depends on insight of species-specific population genetics (i.e., the relative impact of mutation and recombination on genetic variation) (Schurch et al., 2018), as shown in **Table 1**.

For clonal relationships, the horizontal genetic transmission of mobile genetic elements (plasmids, integrative conjugative elements, transposons) and their relatedness are important parameters indicating the transmission of AMR characteristics (Conlan et al., 2014, 2016; Sheppard et al., 2016). There are not many reports of clonal typing during AMR surveillance in a One Health approach. Epidemiological data and sources of bacterial strains defining clonal relationships in Thailand and Vietnam are shown in **Table 2**; however, insights into bacterial clonal transmission from the production process to consumers and patients remain incomplete.

POSSIBILITY OF AMR TRANSFER FROM PIGS TO HUMANS

Humans can be exposed to antibiotic-resistant bacteria, both pathogenic and commensal bacteria, by direct transmission or through food or the environment. AMR problems are global issues since resistant bacteria can occur from any sector and spread intra-species, inter-species, and across borders (Kempf et al., 2013; Daniel et al., 2015). Certain antibiotics, particularly the Critically Important Antimicrobials to human medicine are applied in livestock farming. Inappropriate use of antibiotics in animals accelerates selective pressure and is a cause of antimicrobial cross-resistance in human medicine (Marshall and Levy, 2011). Moreover, the genes encoding antibiotic resistance in bacteria from animals can be transferred to bacteria that are pathogenic to humans (Archawakulathep et al., 2014). Many studies have reported the emergence of ARB from pigs and spread to humans; for example, quinolone resistant Salmonella spreading from pigs to human in Taiwan, and nourseothricin resistant *E. coli* from pigs to pig farmers, families, municipal communities, and patients with urinary tract infections (Witte, 1998; Aarestrup, 2005). Since livestock farms are a hotspot for resistant bacteria, workers on farms and in the food chain are potentially exposed to AMR bacteria from animals.

TABLE 1 | Relatedness criteria used for the cgMLST schemes for clinically relevant bacteria.

Species	Relatedness threshold cgMLST alleles	References
Acinetobacter baumannii	≤8	Higgins et al., 2017
Campylobacter coli, C. jejuni	≤14	Cody et al., 2013; Llarena et al., 2017
Enterococcus faecium	≤20	de Been et al., 2015
Escherichia coli	≤10	Dekker and Frank, 2016
Klebsiella pneumoniae	≤10	Snitkin et al., 2012
Salmonella enterica	≤4	Vincent et al., 2018
Staphylococcus aureus	≤24	Bartels et al., 2015

[1]https://www.ridom.de/u/Server_Database_Initialization.html

TABLE 2 | Lists of clonal relationships of AMR strains from South East Asia Countries in the One Health paradigm.

Year	Countries	Bacteria	Source and epidemiological data	Molecular typing	Conclusion of genetic relativeness	References
2018	Thailand	*E. coli*	Isolates from pig in the finishing period and pork from the same pigs	PFGE, MLST, plasmid replicon typing	Similar pulsotypes and sequence type (ST) from 2 pairs of strains from pig and pork.	Lugsomya et al., 2018b
					Similar plasmid Inc group (Frep-HI2 and FIB-FIC-Frep) from 2 pairs of strains from pig and pork.	
2019	Thailand	*E. coli*	Isolates from pigs and healthy humans from the same province	MLST	4 STs of ESBL producing *E. coli* shared between humans and pigs (ST10, ST70, ST117, ST685).	Seenama et al., 2019
2019	Thailand	*Salmonella* Rissen	Isolates from pigs, pork from slaughterhouses and markets from the same region	cgMLST,	A close similarity (percentage was not defined) from cgMLST between pig and pork sources from slaughterhouses and markets.	Prasertsee et al., 2019
2020	Thailand	*Salmonella* Weltevreden	Isolates from beef from a fresh market and isolates from patients in the same province	cgMLST, *in silico* plasmid typing	A high similarity from cgMLST between patient and beef sources.	Patchanee et al., 2020
					Same plasmid oriT type (MOB F) and plasmid incompatibility group (IncFII) of *mcr-1* plasmids from patient and beef strains.	
2021	Vietnam	*E. coli*	Isolates from pigs, chickens and patients	MLST, *in silico* plasmid typing	No evidence of clonal transmission between strains from a different host.	Nguyen et al., 2021
					The IS6 elements flanking ISEcp1–*bla*CTX-M–orf477/IS903B structures shared between strains from different hosts.	

AMR IN LIVESTOCK

The AMR situation is critical in LMICs, including in the SEA. Although there is social and economic progress in those regions, the average population is still in poverty (Singh, 2017). Moreover, inadequate sanitation, poor quality control in slaughterhouses, and conditions where people live adjacent to animals (backyard farming) accelerate the spreading of resistant pathogens and ARG (Cook et al., 2017). Self-medication is cheaper in those regions, and antibiotics are easily accessible over the counter, leading to an increase in antimicrobial use not only by farmers but also by people without proper diagnosis (Nguyen et al., 2013). The major potential pathways for spreading of resistant bacteria and/or genes between food animals and humans could be horizontal transmission or clonal transfer of resistant bacteria (Lipsitch et al., 2002; Chang et al., 2015). An association between clonal types of human cephalosporin-resistant *E. coli* with those from food animals linked through food products has been reported (Lazarus et al., 2015).

Additionally, a study in Denmark found the possible transmission of sulfonamide resistant *E. coli* via the food chain to a healthy human who did not receive antibiotic therapy (Hammerum et al., 2006; Wu et al., 2010). The occurrence of resistant bacteria carrying mobile genetic elements in livestock or food of animal origins is concerning. The spread of antibiotic-resistance genes via horizontal gene transmission could lead to acquisition of multidrug-resistant (MDR) bacteria. MDR infections in humans can cause treatment failure and high mortality, especially if resistance is to a drug of last resort. Worrisomely, there are growing cases of colistin-resistant *Enterobacteriaceae* and sporadic cases of carbapenem-resistant cases in pigs from some Asian countries.

A study in Vietnam found that almost half of the pig farms in Bac Ninh province had colistin-resistant *E. coli*, and farmworkers also harbored such bacteria (Dang et al., 2020). Moreover, colistin-resistant *E. coli* have been detected in humans from rural areas of Vietnam (5–71.4%) (Trung et al., 2017; Kawahara et al., 2019). The *mcr* genes are currently globally distributed and are mostly detected from livestock origins followed by from humans and meat products. In SEA, *mcr*-1 positive *E. coli* has been detected in Cambodia, Vietnam, Malaysia, Singapore, and Thailand (Olaitan et al., 2016; Paveenkittiporn et al., 2017; Runcharoen et al., 2017; Srijan et al., 2018). On the other hand, *K. pneumoniae* isolates harboring *mcr*-1 genes have been reported in Laos and Singapore (Rolain et al., 2016; Teo et al., 2016). Moreover, ESBL-producing *K. pneumoniae* clones ST307, ST2958 and ST2959 that were resistant to β-lactams, aminoglycosides, fluoroquinolones, macrolide, lincosamide and streptogramins, rifampicin, sulfonamides, trimethoprim, phenicols and tetracycline, have been observed to be shared between both pig and human-sources within and across abattoirs. *K. pneumoniae* has been suggested to be a potential

unheeded reservoir of resistance in the food chain that impacts on public health (Founou et al., 2018).

In addition to enteric bacteria, livestock-associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) is another resistant bacterium of substantial health concern. LA-MRSA has been found worldwide, predominantly among people involved in livestock farming (Smith et al., 2009; Frana et al., 2013). These bacteria can be transmitted to humans in proximity to MRSA-harboring animal skin, especially from pigs (Smith and Pearson, 2011). There are several reports regarding the occurrence of MRSA cases in Asian countries, including in Malaysia (5.5%), China (15%), and Northern Thailand (2.53%) (Patchanee et al., 2014). The spa type t4358-SCC*mec* V is the most abundant clone in Malaysia, whilst it is t337-SCCmec IX in Thailand. In northeastern Thailand patients were identified whose MRSA pulso type pattern (PFGE) was related to that of MRSA discovered in a diseased pig in the same area (Lulitanond et al., 2013). A similar case providing evidence for the potential for transmitting resistant clones from pigs to humans occurred where an isolate from a human and two isolates from pigs shared SCC*mec* IX-*S. haemolyticus* (Sinlapasorn et al., 2015). The occurrence of livestock associated ARB in SEA is shown in **Table 3**.

Unfortunately, the role of farm animals in the emergence and dissemination of resistant bacteria or genes to humans is still controversial (Marshall and Levy, 2011). Further studies focusing on genomic data of resistant bacteria and nation-wide epidemiological approaches are required to explore the transmission of AMR from animals to humans.

AMR IN THE FOOD CHAIN

As discussed, possible transmission routes of ARB and ARGs to humans may be from direct contact with colonized animals or from the consumption of foodstuffs that are contaminated with ARB/ARGs along the food chain (Founou et al., 2016; Thapa et al., 2020). There are different potential pathways for contamination of ARB and ARGs along the food chain. ARB may disseminate to crops and plants from the utilization of

TABLE 3 | Reported occurrence of livestock associated ARB in SEA.

Bacterial species	Antimicrobial resistance pattern	Livestock sources	Country	References
Escherichia coli	Tetracycline, trimethoprim/sulfamethoxazole, chloramphenicol, gentamicin, ampicillin, colistin	Swine	Thailand	Lugsomya et al., 2018b; Lunha et al., 2020
			Vietnam	Hounmanou et al., 2021; Nguyen et al., 2021
			Singapore	Guo et al., 2021
			China	Olaitan et al., 2016
Escherichia coli	Ampicillin, ciprofloxacin, gentamicin, colistin, ceftazidime	Poultry	Vietnam	Nguyen et al., 2021
Escherichia coli	Ceftazidime, cefotaxime, cefotaxime with clavulanic acid	Cattle	Malaysia	Kamaruzzaman et al., 2020
Klebsiella pneumoniae	Colistin, β-lactam, aminoglycosides, fluoroquinolones, phenicols, tetracycline, sulfonamides, trimethoprim, bleomycin	Swine	Laos	Rolain et al., 2016
			Singapore	Teo et al., 2016
			Malaysia	Mobasseri et al., 2021
Methicillin-resistant *Staphylococcus aureus*	Erythromycin, ceftriaxone, cefoxitin, ciprofloxacin, gentamicin, tetracycline, trimethoprim-sulfamethoxazole, clindamycin, quinupristin-dalfopristin, tigecycline, cephalexin, fusidic acid, oxytetracycline, penicillin	Swine	China	Cui et al., 2009
			Hong Kong	Guardabassi et al., 2009
			Malaysia	Neela et al., 2009; Khalid et al., 2009
			Thailand	Patchanee et al., 2014
Salmonella spp.	Ciprofloxacin, sulfisoxazole, phenicols, nalidixic acid, ampicillin, tetracycline, sulfamethoxazole trimethoprim, norfloxacin, ciprofloxacin, amoxicillin, chloramphenicol	Poultry	Malaysia	Goni et al., 2018
			Thailand	Prasertsee et al., 2019; Vidayanti et al., 2021
			Singapore	Zwe et al., 2018
			China	Xu et al., 2020
			Vietnam	Ha et al., 2012; Ta et al., 2014;
Salmonella spp.	Ampicillin, streptomycin, florfenicol, tetracycline, sulfamethoxazole/trimethoprim, gentamicin	Swine	China	Yi et al., 2017

contaminated irrigation water, mostly from manure discharges of both human and animals and from irrigated soil (Gatica and Cytryn, 2013). Whilst livestock products such as pork, beef and poultry can be contaminated with ARB during the slaughtering processes, aquaculture products may become contaminate with ARB and ARGs that have already been circulating in aquatic environments (Bridier et al., 2019; Amarasiri et al., 2020). The phenotype of AMR bacteria may be influenced by food preservation stresses such as decreasing pH to under 5.0, or increasing salt to more than 4.5%, which has been shown to increase phenotypic antibiotic resistance in *E. coli, S. enterica* serovar Typhimurium, and *S. aureus* (McMahon et al., 2007). In addition, contamination by ARB and ARGs may occur during transportation and distribution of food products, or occur as a post-contamination event after food processing, or from a cross-contamination as a result of inappropriate food handlings which are among the most common contamination pathways along the food chain (Verraes et al., 2013; Holzel et al., 2018).

AMR IN WASTEWATER AND ASSOCIATED ENVIRONMENTS

Alarmingly, there have been increasing reports about the existence of AMR in the environment, especially resulting from the activities of both humans and animal industries (Prestinaci et al., 2015). The concern focuses mainly on the increase in ARB and ARG resulting from the dissemination of antibiotics in the environment (Kraemer et al., 2019; Serweciñska, 2020). Wastewater has been suspected as being one of the hotspots for the spread and circulation of ARB and ARG (Rizzo et al., 2013; Pazda et al., 2019). Human and animal excreta have been demonstrated to contain large amount of antibiotics which are distributed to municipal wastewater where the antibiotics are absorbed into sewage sludge (Nagulapally et al., 2009; Singer et al., 2016). Sludge (biosolids) is produced from the wastewater treatment processes in the wastewater treatment plant, and contains a large amount of non-degraded fat, oil, and protein. This composition contributes to poor retrieval of water-soluble antimicrobial agents such as ciprofloxacin from sludge (Gerba and Pepper, 2009; Singer et al., 2016; Serweciñska, 2020). Therefore, it is no surprise that ARB and ARG have been detected mainly in samples from wastewater and associated environments such as groundwater and surface water (Barancheshme and Munir, 2018; Amarasiri et al., 2020; Sabri et al., 2020).

AMR IN THE SLAUGHTERING PROCESS

The risk of AMR bacterial contamination in the food chain is associated with AMR commensal bacteria circulating on carcasses at slaughterhouses (EFSA, 2016). In addition, the direct exposure of workers to resistant bacteria can happen throughout the production cycle to the slaughtering process and during food preparation (You et al., 2016). The exposure of slaughterhouse workers to extended spectrum beta lactamase (ESBL)-producing *Enterobacteriaceae* has received global attention (Wadepohl et al., 2020). Substantial data relating to the association between ESBL producing *Enterobacteriaceae* in pigs, farmworkers and slaughterhouse workers has been reviewed (Alali et al., 2008; Hammerum et al., 2014; Huijbers et al., 2014; Dohmen et al., 2015). In the Netherlands, workers were most likely to be exposed to ESBL producing *E. coli* with $bla_{\mathrm{CTX-M-1}}$ in the early slaughtering process (before chilling of carcasses), at a prevalence of 4.8% (Dohmen et al., 2017). In comparison, a 4.5% prevalence of ESBL/AmpC producers was found in residents living in the vicinity of livestock farms in the Netherlands (Wielders et al., 2017). Exposure to ESBL carriage is especially high in the slaughtering process, particularly during removal of visceral organs and handling of the throat area where there is massive colonization with enteric bacteria (Lowe et al., 2011; Lăpuşan et al., 2012). Moreover, a high percentage (50%, $n = 59$) of MDR *E. coli* have been recovered from slaughterhouse workers, where the rates in Nigeria were more elevated in butchers than in cleaners (Aworh et al., 2021). In slaughterhouses in Thailand, ESBL-producing *E. coli* were detected in 76.7% of pig feces, 33.3% of fresh pork samples, and 75% of workers (Boonyasiri et al., 2014). In a comparative study between Thailand and Cambodia, the prevalence of ESBL-producing *E. coli* in pig rectal and carcass swabs were similar, at only 5.3–5.6%. Among these, ESBL-producing *E. coli* containing $bla_{\mathrm{TEM-1}}$ and $bla_{\mathrm{CMY-2}}$ were found in both countries, but the $bla_{\mathrm{CTX-M-15}}$ gene was detected only in the Thai isolates (Trongjit et al., 2016).

The occurrence of multidrug-resistance bacteria circulating in slaughterhouses also has been reporting in many studies in SEA. *Salmonella* spp. are frequently used as an indicator of pathogen and AMR carriage, reflecting management standards. In Thailand, salmonella isolates with high levels of resistance to tetracycline, ampicillin, sulfonamide-trimethoprim, and streptomycin, at approximately 63–82%, can be found in pigs, workers, and the environment from farms to slaughterhouses (Tadee et al., 2015; Phongaran et al., 2019). *Salmonella* Rissen was found to be a common serovar in a comprehensive survey of pig farms, slaughterhouses, and retail outlets in Northern Thailand (Phongaran et al., 2019). Moreover, Wu et al. (2019) compared AMR salmonella contamination in pig rectal swabs and carcasses between slaughterhouses with and without a hazard analysis critical control point (HACCP) system. Both systems had a high level of indirect contamination, but the HACCP system decreased salmonella contamination in carcasses and reduced the number of AMR patterns (Wu et al., 2019). In a comparison between pig slaughterhouses in Thailand and Laos, the prevalence of salmonella contamination of pig carcasses in the two countries were 30.9 and 53.3%, respectively. *Salmonella* spp. from both countries were highly resistant to sulfonamide (98.3%), ampicillin (91%), and tetracycline (92.5%). These isolates contained class1 integrons carrying resistance cassette *dfrA12-aadA2*. Interestingly, five *Salmonella* isolates from Thai pig carcasses were producers of extended-spectrum □-lactamase (ESBL) and harbored $bla_{\mathrm{CTX-M-14}}$, whereas isolations from Laos were negative. The study reflected differences in the hygienic procedures of slaughterhouses and antibiotics uses in the two countries (Sinwat et al., 2016). In Vietnam, salmonella isolated from pork slaughterhouses and retail shops were

highly resistant to ampicillin, tetracycline, and chloramphenicol (Nguyen D. T. et al., 2016), and *Salmonella* Rissen isolated from a pig carcass in 2013 showed phenotypical colistin resistance, with a plasmid-mediated *mcr-1* gene (Gonzalez-Santamarina et al., 2020). In the Philippines, 46.7% of pig tonsil and jejunum samples from three below-standard slaughterhouses were positive for *Salmonella enterica*, rates higher than those from five national accredited slaughterhouses, although there was no significant difference in prevalence. Most isolates (n = 183) were resistant to nitrofurantoin (93.4%), ampicillin (67.8%), and sulfa-trimethoprim (80.3%) (Calayag et al., 2017), even though nitrofurantoin use in livestock had been banned for a decade. However, it needs to be determined whether the source of contamination was from farming practices or occurred in the slaughtering process. The occurrence of ARB in the slaughtering process is summarized in **Table 4**.

Overall, the risk of bacterial contamination in the slaughtering process and of pig products is critical, and there is still a lack of comprehensive monitoring throughout the system. Operation under HACCP programs and good manufacturing and sanitation practice (GMP) are intended to control meat safety (Samelis, 2006; Wu et al., 2019), but a reduction in salmonella and AMR bacteria prevalence is not relevant to its implementation in SEA. Decontamination applications post-slaughter aim to reduce bacterial contamination in pork, and include physical applications such as hot water washing, chilling, freezing, and high-pressure processing. Secondly, chemical applications such as treatment with organic acid solutions, or electrolyzed or ozonated water are recommended. Lastly, biological applications include bacteriophages and wrapping film with postbiotics containing lactic acid bacteria. However, all applications must take into account meat quality and the physiology of the end product (Bolumar et al., 2020; Li et al., 2020; Shafipour Yordshahi et al., 2020; Aydin Demirarslan et al., 2021).

ALTERNATIVES TO ANTIBIOTICS

The use of antibiotics on farms increase the possibility of the AMR transmission from livestock production systems to consumers. There have been many attempts to replace antibiotic use on farms. This review outlines two applications for reduction of antibiotic use, including for growth promotion and disease prevention. An overview of possible antibiotic alternatives for growth promotion derived from previous studies is presented in **Supplementary Table 1** (Cha et al., 2012; Kim et al., 2014; Yoon et al., 2014; Zeng et al., 2014; Devi et al., 2015; Gois et al., 2016; Hossain et al., 2016; Lee et al., 2016; Peng et al., 2016; Sbardella et al., 2016; Tang et al., 2016; Upadhaya et al., 2016; Wan et al., 2016; Lan et al., 2017; Lei et al., 2017; Ma et al., 2017; Pan et al., 2017; Wu et al., 2017; Huang et al., 2018; Lei et al., 2018; Samolińska et al., 2018; Sayan et al., 2018; Seo et al., 2018; Xu et al., 2018; Pei et al., 2019; San Andres et al., 2019; Wang et al., 2019; Duarte et al., 2020; Petry et al., 2020; Ren et al., 2020; Satessa et al., 2020; van der Peet-Schwering et al., 2020; Wei et al., 2020; Zhang et al., 2020; Sun et al., 2021; Wang et al., 2021). These vary considerably from chemical compounds, biologically active substances, and microbially derived products. These could perform several functions, including eliminating pathogenic microbes, modulating gut microbial communities, strengthening intestinal integrity, enhancing growth performances, or diminishing morbidity from other causes (Cheng et al., 2014; Liao and Nyachoti, 2017; Zeineldin et al., 2019).

Although these alternatives could be used as antibiotic replacements in terms of in-feed growth promotion, they are not suitable for therapy. In addition, proper disease prevention measures, including vaccination and enhanced biosecurity, are needed to help reduce antibiotic usage. Vaccines trigger a protective immunity that simulates the effects of a natural infection without the negative consequences. Vaccination has been successfully used in animals (Rose and Andraud, 2017). In livestock production, vaccination has been extensively used for disease prevention caused by pathogenic bacteria or viruses, and as such it is an important substitute for antibiotic usage (Meeusen et al., 2007). Remarkably, even protection against viral infections may reduce antibiotic use by decreasing the risk of misdiagnosis and treatment of secondary bacterial infections (Potter et al., 2008). For example, vaccination against Porcine Circovirus Type 2 (PCV-2), which is an immune-suppressive viral infection leading to secondary bacterial infections, resulted in a significant diminution of antibiotic use in swine farms (Raith et al., 2016). Similarly, vaccination against Porcine Reproductive and Respiratory Syndrome (PRRS) virus on pig farms reduced antibiotic consumption (Van Looveren et al., 2015). Amongst important bacterial pathogens of swine, vaccination against *Lawsonia intracellularis*, which causes severe ileitis, has been reported to reduce oxytetracycline medication in pigs (Bak and Rathkjen, 2009), whilst immunization against the respiratory pathogen *Actinobacillus pleuropneumoniae* also reduced administration of antibiotics (Kruse et al., 2015).

Biosecurity and farm management are essential parts of disease prevention that can enhance health status and significantly diminish the risk of exposure of pigs to pathogens (Postma et al., 2016b). These practices have been applied in varied species, production systems, and for different pathogens (Dahiya et al., 2006). Practices such as all-in all-out housing systems, reduced stocking rates, increased ventilation and improved waste management all help to improve the health status of farms. Improvements in biosecurity have been widely accepted as an effective tool for protecting from introduction of diseases into farms (Lewerin et al., 2015). Postma et al. (2016a) found that better biosecurity resulted in a lower antibiotic usage from birth to slaughter in pig herds. Similarly, Raasch et al. (2018) and Caekebeke et al. (2020) reported the same relationship between increased biosecurity in pig farms and reduced antibiotic use.

In summary, various means are available to reduce antibiotic use on farms. The addition of some alternatives and improved disease prevention in practice are important tools to reduce antimicrobial usage in swine farm and support global policy. Their increased use in the SEA region is to be encouraged.

TABLE 4 | Phenotypic AMR in organisms isolated from difference sources.

Reference	Country	Organism	Host	Source	No. sample	% positive of sample	Prevalence of AMR
Boonyasiri et al., 2014	Thailand	*ESBL producing E. coli*	Pig rectal swab	Farm	400	76.70%	
			Carcass swab	Slaughterhouse	18	33.30%	
			Pork	Market	15	61.50%	
			Stool of workers	Farm	30	77.30%	
			Stool of workers	Food-factory	544	75.50%	
Trongjit et al., 2016	Thailand	*Escherichia coli*	Carcass swab	Slaughterhouse	88	85.20%	AMP (87%), TRI (82%), SUL (67%), and 5.7% (10/175) was ESBL-producing *E. coli* that contained $bla_{CTX-M15}$, bla_{TEM-1}, bla_{CMY-2}
			Carcass swab/Pork	Fresh market	87	35.60%	
			Pig rectal swabs	Slaughterhouse	85	ND	
	Cambodia	*Escherichia coli*	Carcass swab	Slaughterhouse	20	55%	AMP (78%), TRI (64.3%), SUL (70.5%), and 4.5% (5/110) was ESBL-producing *E. coli* that some contained bla_{TEM-1}, bla_{CMY-2}
			Carcass swab/Pork	Fresh market	90	40%	
			Pig rectal swabs	Slaughterhouse	82	ND	
Tadee et al., 2015	Thailand	*Salmonella* spp.	Pigs	Farm	86	ND	TET (82.56%), AMP (81.40%), and STR (63.95%)
			Farm worker				
			Slaughterhouse worker	Slaughterhouse			
			Environment	Farm and slaughterhouse			
Phongaran et al., 2019	Thailand	*Salmonella* spp.	Pig feces	Slaughterhouse	562	37.54%	AMP (69.05%), TET(66.19%), and SXT (35.71%)
Wu et al., 2019	Thailand	*Salmonella* spp.	Pig rectal swab	Slaughterhouse	360	61.11% in HACCP, 17.22% in non-HACCP slaughterhouse	Rectal of HACCP; AMP (76.36%), CHL (11.82%), STR (60.00%), SXT (17.27%), TET (85.45%). Non-HACCP; AMP (61.29%), CHL (3.23%), STR (29.03%), SXT (38.71%), and TET (64.52%)
			Carcass swab		360	12.78% in HACCP, 36.67% in non-HACCP slaughterhouse	Carcass swab of HACCP;AMP (95.65%), STR (95.65%), TET(95.65%).Non-HACCP;AMP (69.70%), CHL (1.52%), STR (45.45%), SXT (15.15%), and TET (69.70%)
Sinwat et al., 2016	Thailand	*Salmonella* spp.	Pig rectal swab	Slaughterhouse	185	34%	AMP (100%), TET (91.4%), STR (74.3%), SPE (87.1%), SUL (100%), TRI (55.7%)
			Carcass swab	Slaughterhouse	184	30.90%	AMP (96.7%), TER (91.8%), STR (75.4%), SPE (86.9%), SUL (100%), TRI (52.5%) and 5 isolates was ESBL-producing strains with $bla_{CTX-M-14}$
			Carcass swab	Fresh market	180	60.50%	AMP (96.4%), TET (91.4%), STR (81.3%), SPE(80.6%), SUL(100%) and 8 isolates was ESBL-producing strains with blaCTX-M-14
			Worker	Slaughterhouse	52	13.40%	AMP (68%), TET (84%), STR (68%), SPE (88%), and SUL (100%)
			Butchers	Fresh market	50	4%	
			Patients	Hospital	78	17.90%	
	Laos	*Salmonella* spp.	Pig rectal swab	Slaughterhouse	129	38.70%	AMP (95%), TET (89.8%), STR (84.7%), SPE (57.6%), and SUL (91.5%)

(Continued)

TABLE 4 | Continued

Reference	Country	Organism	Host	Source	No. sample	% positive of sample	Prevalence of AMR
			Carcass swab	Slaughterhouse	137	53.30%	AMP (91.5%), TET (97.6%), STR (96.3%), SPE (67%), and SUL (100%)
			Carcass swab	Fresh market	112	72.30%	AMP (91.3%), TET (93.8%), STR (96.9%), SPE (69.8%), SUL (95.8%), and TRI (56.6%)
			Worker	Slaughterhouse	36	25%	AMP (62.5%), TET (100%), STR (87.5%), SPE (81.3%), SUL (100%), and TRI (62.5%)
			Butchers	Fresh market	8	0.00%	
			Patients	Hospital	36	11.10%	
Nguyen N. T. et al., 2016	Vietnam	*Salmonella* spp.	Pork	Slaughterhouse	30	69.70%	AMP (54.2%), TET(627%), and CHL (508%)
				Retail stores	69		
Gonzalez-Santamarina et al., 2020	Vietnam	*Salmonella* spp.	Pig carcass	Slaughterhouse	1	ND	AMP (blaTEM-1b), GEN and TOB [aac(6")-Iaa, aac(3)-IId, ant(3")-Ia], and COL (mcr-1)
Calayag et al., 2017	Philippines	*Salmonella* spp.	Pig tonsil	Slaughterhouse	240	46.7% of total, 44.0% of accredited slaughterhouse, 46.7% of locally registered slaughterhouse	NI (93.4%), AMP(67.8%), and SXT(80.3%)
			Jejunum		240		

ND, no data; AMP, ampicillin; AUG, amoxicillin-clavulanic acid; CHL, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; NA, nalidixic acid; NOR, norfloxacin; STR, streptomycin; SXT, sulfamethoxazole-trimethoprim; TE, tetracycline; SPE, spectinomycin; SUL, Sulfamethoxazole; TRI, Trimethoprim; GEN, gentamicin; TOB, tobramycin; COL, colistin; NI, Nitrofurantoin.

POLICY AND STRATEGIES ON AMR IN FOOD ANIMALS IN THE ASSOCIATION OF SOUTH EAST ASIAN NATION (ASEAN)[2]

The importance of AMR as a human security threat has been recognized worldwide since the 1990s. In response the World Health Organization arranged a series of consultative meetings and developed recommendations for action. The culmination of this work was the 2001 WHO global strategy for containment of AMR (Sack et al., 2001). Later, in 2015, the Global Action Plan on AMR (GAP-AMR) was adopted by WHO member states at the World Health Assembly, the Food and Agricultural Organization Governing Conference and the World Assembly of World Organisations for Animal Health (World Health Organization [WHO], 2015). Member States committed to develop multi-sectoral national action plans on AMR and endorsed a 'One Health' approach to facilitate collaboration among various sectors and actors in the defense of human, animal, and environmental health. Through global political declarations, a common direction to tackle AMR was seen across countries and sectors (United Nations [UN], 2016).

Most countries in the ASEAN region have taken action to tackle AMR through the development of their National AMR action plan. According to the Global Database for the 2019, Tripartite AMR Country Self-assessment Survey (TrACSS), all eleven countries in the ASEAN region have developed a National action plan (NAP) which the government has approved (World Health Organization [WHO], 2018). The One Health approach has been adopted as a key principle for multi-sectoral coordination, and it was enshrined in all NAPs (Chua et al., 2021). Other policies for controlling and optimizing the use of antimicrobials have been developed through legal provisions and program implementation, including surveillance of AMR and antimicrobial consumption in both humans and the animal sectors.

Reviews of policy interventions that address AMR in food animal production indicate that there are many existing regulations for controlling veterinary medicines, including antimicrobials, in ASEAN countries. However, they are relatively varied across countries (Goutard et al., 2017). Policies of banning antibiotics for growth promotion in food animals have been enforced in some countries, such as Singapore and Thailand (Chua et al., 2021) while others have yet to enforce the ban. Thailand has restricted the use of antimicrobials for growth promotion since 2015 (Sommanustweechai et al., 2018). In 2019, some antimicrobial classes were assigned the highest priority, with critically important antimicrobials for human medicine only being available for food animals through veterinary prescription (notably polymyxins, third and fourth generation cephalosporins, macrolides, and fluoroquinolones) (Scott et al., 2019). However, a few SEA countries, including Vietnam and Myanmar, have neither laws nor regulations on the

[2]The scope of this article covers 11 member countries of the Association of Southeast Asian Nations (ASEAN). They are: Brunei Darussalam, Cambodia, Indonesia, Lao PDR, Malaysia, Myanmar, the Philippines, Singapore, Thailand, and Vietnam.

prescription and sale of antimicrobials for animal use (World Health Organization [WHO], 2018). In terms of antimicrobial stewardship programs, NAPs in most countries in the Region cover both human and animal health sectors, except for Cambodia and Malaysia. Indonesia and Thailand set codes of practices for control of veterinary drug use. Likewise, Brunei has set up national guidelines for prudent use of antimicrobial in livestock (Goutard et al., 2017).

Surveillance on AMR and antimicrobial consumption is a vital component of GAP-AMR. The WHO Global Antimicrobial Resistance and Use Surveillance System (GLASS) and Organisation for Animal Health global database on antimicrobials in animals were established to help provide evidence on the emergence of resistance and trends in antimicrobial use globally (World Health Organization [WHO], 2017). The 2019 TrACSS reported that most countries in the ASEAN region have a national surveillance system for AMR in food animals, with only a few countries such as Laos and Myanmar having inadequate laboratory capacity to perform AMR surveillance. Only Thailand and Malaysia have established systems for monitoring antimicrobial consumption in animals, in which are recorded data on the total quantity of antimicrobials sold for/used in animals nationally, by antimicrobial class, by species, method of administration, and by type of use. Malaysia and Thailand share their reports with the OIE annually (Chua et al., 2021). Thailand has successfully published One Health Reports on AMR, antimicrobial consumption, and public knowledge and awareness on AMR in 2017 and 2018, contributing to an evidence-based policy decision (NSC, 2020a,b). The 2019 version will be launched in April 2021.

Despite existing national commitments, implementation of AMR policy remains a significant challenge, particularly with the monitoring of AMR and AMU in the human and animal sectors. The monitoring systems are hampered by lack of institutional capacity, inadequate investment in human resources, and the need to strength data platforms for routine monitoring. Significant financial boosts are required to support these areas.

CONCLUSION

This paper addressed the cross-species transmission of AMR and ARGs in the SEA. The importance of pig production in SEA has encouraged particular attention to this industry, but similar problems occur with other animal industries (chicken, fish etc.). Due to the limited number AMR studies, it is difficult to quantify the extent of the problem in the region, although some countries have better data available than others. Certainly, there is reason to believe that transmission of resistant bacteria and ARGs from pigs at the farm and through the slaughtering process provide risks to the human population, either through direct contact or via contaminated environments or pork products. Efforts should be made to reduce antimicrobial use at the farm level by improving farm hygiene and the use of alternatives to antibiotics. The AMR policy in the region has been implemented, although progress in this area has varied considerably between different countries due to different implementation's capacities. Additional funding to support AMR surveillance and improved control in SEA is required.

AUTHOR CONTRIBUTIONS

NP: original idea, study design, and supervision of writing. WS: AMR distribution in food chain, wastewater, and detection. PA and PP: alternatives to antibiotics and data analysis. AL: policy and strategy controlling AMR in SEA. IS and NK: risk of AMR in pig production cycle and slaughtering. KL: molecular epidemiology analysis. DH: study design, supervision, and proofreading of the writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.689015/full#supplementary-material

Supplementary Table 1 | Summary of available alternatives to antibiotics in swine production.

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Inhibition of Antimicrobial-Resistant *Escherichia coli* Using a Broad Host Range Phage Cocktail Targeting Various Bacterial Phylogenetic Groups

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Antimicrobial-resistant (AMR) commensal *Escherichia coli* is a major reservoir that disseminates antimicrobial resistance to humans through the consumption of contaminated foods, such as retail poultry products. This study aimed to control AMR *E. coli* on retail chicken using a broad host range phage cocktail. Five phages (JEP1, 4, 6, 7, and 8) were isolated and used to construct a phage cocktail after testing infectivity on 67 AMR *E. coli* strains isolated from retail chicken. Transmission electron microscopic analysis revealed that the five phages belong to the *Myoviridae* family. The phage genomes had various sizes ranging from 39 to 170 kb and did not possess any genes associated with antimicrobial resistance and virulence. Interestingly, each phage exhibited different levels of infection against AMR *E. coli* strains depending on the bacterial phylogenetic group. A phage cocktail consisting of the five phages was able to infect AMR *E. coli* in various phylogenetic groups and inhibited 91.0% (61/67) of AMR *E. coli* strains used in this study. Furthermore, the phage cocktail was effective in inhibiting *E. coli* on chicken at refrigeration temperatures. The treatment of artificially contaminated raw chicken skin with the phage cocktail rapidly reduced the viable counts of AMR *E. coli* by approximately 3 log units within 3 h, and the reduction was maintained throughout the experiment without developing resistance to phage infection. These results suggest that phages can be used as a biocontrol agent to inhibit AMR commensal *E. coli* on raw chicken.

Keywords: bacteriophage, cocktail, antimicrobial-resistant *Escherichia coli*, raw chicken, phage cocktail

INTRODUCTION

Escherichia coli is the most common enteric bacteria inhabiting the gastrointestinal tract of a wide range of animals and humans (Kaper et al., 2004). Due to the ubiquitousness in the intestines, commensal *E. coli* is likely to be exposed to orally ingested antibiotics and develops antimicrobial resistance in food-producing animals and may act as a donor and a recipient

of antimicrobial resistance genes (Poirel et al., 2018). Antimicrobial-resistant (AMR) commensal *E. coli* is frequently isolated from food-producing animals and their meat products (Szmolka and Nagy, 2013). Although commensal *E. coli* does not cause AMR infection in humans, AMR commensal *E. coli* is considered a major reservoir for disseminating antimicrobial resistance to humans. For instance, extended-spectrum β-lactamases (ESBL)-producing *E. coli* is highly prevalent in retail poultry (Saliu et al., 2017). ESBL are β-lactamase enzymes conferring resistance to all β-lactam drugs except carbapenem and mainly located on conjugative plasmids, enhancing their rapid spread in *E. coli* populations and pathogenic bacterial species in the Enterobacterales order (Rupp and Fey, 2003).

AMR bacteria in food-producing animals can be transmitted to foods during processing and subsequently to humans through the consumption of contaminated foods. According to the Centers for Disease Control and Prevention (CDC), approximately 1 in 5 AMR infections in the United States are associated with food and animals (CDC, 2013). Since meat and poultry are the major food commodity implicated in 35% of foodborne illnesses in the United States (Painter et al., 2013; Dewey-Mattia et al., 2018), antimicrobial resistance originating from food-producing animals poses a serious public health concern. Particularly, AMR bacteria are frequently isolated from retail raw chickens (do Monte et al., 2017; Schrauwen et al., 2017; Wang et al., 2017). Our previous studies also showed that ESBL-producing *E. coli* is highly prevalent in retail poultry (Park et al., 2019). In addition, we isolated from retail chicken a pan drug-resistant *E. coli* possessing a plasmid harboring *mcr-1*, which confers resistance to colistin, one of the last resort antibiotics to treat Gram-negative infections (Liu et al., 2016; Kim et al., 2019).

To mitigate the public health risk of antimicrobial resistance, it is important to control the sources that are involved in the spread of antimicrobial resistance. Especially, AMR commensal *E. coli* in chickens is an important target to control because it is highly prevalent and capable of transferring antimicrobial resistance to pathogenic bacteria, such as pathogenic *E. coli* and *Salmonella* (Nhung et al., 2017). Among non-antibiotic-based intervention measures for the control of AMR commensal *E. coli*, bacteriophages (phages) are considered an ideal antimicrobial alternative because phages specifically infect only target bacteria with completely different antimicrobial mechanisms from those of existing antibiotics (Sulakvelidze et al., 2001; Skurnik and Strauch, 2006; Endersen et al., 2014). Whereas antibiotics affect bacteria other than pathogens, moreover, phages can selectively infect only the target bacteria (Altamirano and Barr, 2019; Nogueira et al., 2019). However, the strict host specificity of phage infection is rather a disadvantage because the host range is generally too narrow to inhibit bacteria with wide genetic diversity (Bert et al., 2010; Talukdar et al., 2013; Park et al., 2019). To overcome the limitations, phages are generally used in a cocktail by mixing phages capable of infecting a range of different hosts (Nilsson, 2014). In this study, we isolated phages that preferentially infect the major phylogenetic groups of *E. coli* isolates from retail chickens and developed a phage cocktail that effectively inhibited AMR *E. coli* on chicken carcasses.

MATERIALS AND METHODS

Phage Isolation and Stock Preparation

Sixty-seven AMR *E. coli* strains (E1–E67) were isolated from retail raw chicken in our previous study (Park et al., 2019). The AMR *E. coli* strains and *E. coli* MG1655 were routinely cultured at 37°C in Luria-Bertani (LB) media (Difco, United States). Phages were isolated from food (retail chicken and duck carcasses), sewage, and animal (chicken and pig) feces as described previously (Kim and Ryu, 2011). Briefly, the samples were homogenized by vortexing in sodium chloride-magnesium sulfate (SM) buffer (100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, and 50 mM Tris·HCl, pH 7.5). After centrifugation at 10,000 × *g* for 5 min, the supernatant was filtered through a 0.22 μm pore sized filter (Millipore, United States). Five milliliters of filtered samples were mixed with the equal volume of 2 × LB broth and 100 μl overnight culture of the AMR *E. coli* strains. After incubation at 37°C overnight, the culture was centrifuged and filter-sterilized. To confirm the presence of phages, supernatants were serially diluted and spotted on 0.4% LB soft top agar containing an overnight culture of AMR *E. coli* strains. After incubation at 37°C overnight, a single plaque was picked and eluted with 1 ml SM buffer. This step was repeated at least three times for each plaque.

To propagate phages, the incubation time was determined based on the lysis activity of each phage. The purified lysate was added to the culture of exponentially grown propagation host strains (JEP1: *E. coli* MG1655, JEP4: *E. coli* E15, JEP6: *E. coli* E55), and the mixture was incubated at 37°C for 4 h (JEP1, 4, and 6) in LB broth. Also, the purified lysates of JEP7 and JEP8 were incubated with the overnight culture of propagation host strains (JEP7: *E. coli* E61, JEP8: *E. coli* E63) overnight in LB broth. Phage propagation was performed with three different culture volumes (4, 40, and 250 ml LB broth), and then the culture was centrifuged and filtered. Phage particles were precipitated by mixing with polyethylene glycol (PEG) 6000 (Junsei Chemical Co. Ltd., Japan) and 1 M NaCl. Finally, CsCl density gradient ultracentrifugation (Himac CP 100b, Hitachi, Japan) with CsCl step densities (1.3, 1.45, 1.5, and 1.7 g/ml) at 78,500 × *g* was conducted at 4°C for 2 h. After centrifugation, a blue band of viral particles was collected and dialyzed twice for 1 h in 1 L of SM buffer. The concentrated phage stocks were stored at 4°C until used.

Determination of Phage Host Range

A total of 67 strains of AMR *E. coli* were used to assess the host ranges of eight phage infections. Each strain was incubated at 37°C overnight with shaking (200 rpm), and then 100 μl of each bacterial culture was added to 5 ml of 0.4% LB soft agar and mixed. The mixture was overlaid on a 1.5% LB agar plate and dried at room temperature for 20 min. Subsequently, 10-fold serially diluted by SM buffer of each phage lysates were spotted onto a prepared bacterial lawn and incubated at 37°C for 12 h. After incubation, the formation of single plaques was recorded to determine the phage sensitivity of each strain. The efficiency of phage infection of each strain was compared to that of the host strain.

Transmission Electron Microscopy Analysis

The CsCl-purified phages were morphologically characterized with transmission electron microscopy (TEM) analysis. Briefly, 10 μl of purified phage (ca. 1×10^{10} PFU/ml) was placed on carbon-coated formvar/copper grids (200 mesh) and negatively stained with 2% aqueous uranyl acetate (pH 4.0) for 10 s. The phages were observed with energy-filtering TEM (LIBRA 120, Carl Zeiss, Germany) at 120-kV accelerating voltage at the National Instrumentation Center for Environmental Management (Seoul, South Korea). The phages were identified and classified using the International Committee on Taxonomy of Viruses (ICTV) classification (King et al., 2011).

DNA Purification and Whole-Genome Sequencing of Phages

To extract genomic DNA from phages, bacterial nucleic acids were removed by DNase I and RNase A (1 μg/ml each) at room temperature for 30 min. The virions were then lysed by incubating with a mixture [final concentration of 50 μg/ml proteinase K, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS)] at 56°C for 1 h. After lysis, DNA was purified by phenol-chloroform extraction and precipitated by ethanol. The library was constructed with the Illumina TruSeq DNA library prep kit using purified genomic phage DNA. It was sequenced using the Illumina Miseq platform (300 bp paired ended) and assembled with GS de novo assembler software (Roche, Switzerland) at Sanigen Inc., South Korea. Prediction of open reading frames (ORFs) was performed using the combination of Glimmer3 and GeneMarkS2 software. The complete genome sequences of JEP1, 4, 6, 7, and 8 were deposited in GenBank with the accession numbers of MT740314, MT740315, MT764206, MT764207, and MT764208, respectively. The presence of genes associated with antimicrobial resistance and virulence in the phage genomes was examined with ResFinder 4.1[1] and VirulenceFider 2.0[2], respectively.

Phylogenetic Analysis of Phages

Phylogenetic analysis of the five phages was performed in comparison with sixty *E. coli* phages in the *Myoviridae* family, which were reported in a previous study (Korf et al., 2019) using VICTOR[3] that performs based on genome-BLAST Distance phylogeny (GBDP) method. The phage sequences were obtained through the NCBI nucleotide databases[4]. All pairwise comparisons of the amino acid sequences were conducted using the GBDP method (Meier-Kolthoff et al., 2013) under the settings recommended for prokaryotic viruses (Meier-Kolthoff and Göker, 2017). The resulting intergenomic distances were used to infer a balanced minimum evolution tree with branch support via FASTME including SPR postprocessing for formulas D4 (Lefort et al., 2015). The branch support was inferred from 100 pseudo-bootstrap replicates each. Trees were rooted at the midpoint (Farris, 1972) and visualized with FigTree (Rambaut, 2006). The taxon boundaries at the species, genus, and family levels were estimated with the OPTSIL program (Göker et al., 2009) using the recommended clustering thresholds (Meier-Kolthoff and Göker, 2017) and an F value (fraction of links required for cluster fusion) of 0.5 (Meier-Kolthoff et al., 2014).

Phage Inhibition Assays

The infection efficiency of the phage cocktail was evaluated with mixed cultures of *E. coli* strains which were randomly selected from the major phylogenetic groups (A, B1, B2, and D), including mixed culture 1 (E20, E41, E55, and E59), mixed culture 2 (E3, E43, E55, and E59), mixed culture 3 (E17, E41, E52, and E59), and mixed culture 4 (E20, E45, E52, and E59). The each strain was incubated at 37°C with shaking (200 rpm) overnight. The mixed culture of the *E. coli* strains was prepared by transferring 1% (v/v) of each strain to fresh LB broth. Then, a single phage or the phage cocktail was added to the bacterial suspension at a multiplicity of infection (MOI) of 1. The optical density at 600 nm (OD_{600}) was measured with the SpectraMax i3 multimode microplate reader (Molecular Devices, Sunnyvale, CA, United States) for 12 h. The infection assay was also performed at 4 and 25°C. After cultivation to an OD_{600} of 0.5, the mixed culture of AMR *E. coli* strains (E20, E41, E55, and E59) was diluted in LB broth and added to 4 ml of LB broth at 10^5 CFU/ml. The infection was initiated by adding the 10 μl phage cocktail (approximately 10^8 PFU/ml; MOI 10^3), and the SM buffer was used as a control. The cultures were incubated with shaking (200 rpm) at food storage and handling temperatures (4 and 25°C), and samples were taken at 3, 6, and 24 h postinfection for bacterial counting. Viable counts were determined by 10-fold serial dilution in PBS and plating on LB agar plates.

Inhibition of AMR *E. coli* on Raw Chicken Skin by Phage Cocktail

Raw chicken skin samples were purchased from retail stores, and the skin was cut into a 2 cm × 2 cm square with a sterilized razor. For decontamination, the chicken skin samples were immersed in 70% ethanol overnight and UV-treated on both sides for 30 min in a biosafety cabinet. Mixed culture 1 (E20, E41, E55, and E59), which was used in the phage inhibition assay, was prepared as mentioned above and diluted to 8×10^6 CFU/ml in PBS. Then 50 μl of the mixed culture of the *E. coli* strains was spotted onto a 2 cm × 2 cm chicken skin to achieve the final inoculum level of approximately 5 log CFU/cm^2 on a chicken skin sample. The same volume of PBS was added as a negative control. Samples were dried in a biosafety cabinet for 30 min. Then 100 μl of the phage cocktail (MOI = 10^3) or SM buffer (control) were spotted onto chicken skin samples and incubated at 4 and 25°C. At 3, 6, and 12 h of incubation, each sample was mixed with 10 ml 0.1% buffered peptone water (BPW) and vortexed for 2 min in a 50 ml tube. After removal of the chicken skin, the mixture was centrifuged at 10,000 × *g* for 5 min, and pellets were resuspended with 10 ml of BPW. Viable counts were determined by 10-fold serial dilution in PBS and plating on LB agar plates.

[1] https://cge.cbs.dtu.dk/services/ResFinder/

[2] https://cge.cbs.dtu.dk/services/VirulenceFinder/

[3] https://victor.dsmz.de

[4] http://www.ncbi.nlm.nih.gov/nuccore

RESULTS

Determination of the Host Range of Phages Infecting AMR *E. coli* Isolates From Raw Chicken

A total of eight *E. coli* phages were isolated from meat, sewage, and animal fecal samples (**Table 1** and **Supplementary Table 1**) and used in phage infection assays with 67 AMR *E. coli* isolates from retail chicken (**Figure 1A** and **Supplementary Tables 2**, **3**). Among the eight *E. coli* phages, five phages (JEP1, 4, 6, 7, and 8) were selected for the construction of a cocktail mainly because of their host range (**Figure 1A** and **Table 2**). Importantly, the design to construct a phage cocktail was mainly based on the differential infection frequencies depending on the phylogenetic group of *E. coli* (**Figure 1B**). For instance, JEP4 phage infected 73.7% (28/38) of AMR *E. coli* strains in phylogroup A, whereas JEP1 and JEP7 phages effectively infected the strains in phylogroups B1 [69.3% (9/13) and 76.9% (10/13), respectively] (**Figure 1** and **Table 2**). The three phages (JEP1, JEP4, and JEP7) showed similar inhibition frequencies against *E. coli* strains in phylogenetic group D (**Figure 1** and **Table 2**). A cocktail was constructed to target various phylogenetic groups of *E. coli* based on the infection pattern dependent on the phylogenetic group. *E. coli* isolates in phylogenetic groups A, B1, B2, and D could be inhibited by JEP4, JEP1 & 7, JEP6, and JEP1, 4 & 7, respectively (**Figure 1B** and **Table 2**). In addition, JEP8 was included in the cocktail to inhibit *E. coli* isolates in the minor phylogenetic groups (i.e., E and F) (**Figure 1B** and **Table 2**). The cocktail consisting of the five phages infected 91.0% (61/67) of AMR *E. coli* strains isolated from retail chicken (**Figure 1** and **Table 2**).

Characterization of the Five *E. coli* Phages

The morphology and genome sequences of JEP1, 4, 6, 7, and 8 phages were analyzed. Based on the TEM analysis, the five phages exhibited the typical morphological features of the *Myoviridae* family, such as a big head and an inflexible/contractile tail (**Figure 2A** and **Table 1**). The phages had various genome sizes ranging from 39 kb (JEP4) to 170 kb (JEP6) (**Table 1**), and genes associated with antimicrobial resistance and virulence were not found in the phage genomes (data not shown). The phylogenetic association of the five phages was analyzed with previously reported *E. coli* phages in the *Myoviridae* family (Korf et al., 2019). JEP1 & JEP4 and JEP6 & JEP8 belonged to the same genus clusters, and JEP7 belonged to a different genus cluster (**Figure 2B**). At the species level, the five phages were clustered into different groups, indicating that the five phages are phylogenetically distinct from each other.

Inhibition of AMR *E. coli* With the Phage Cocktail

Mixed cultures of AMR *E. coli* strains were treated with the phage cocktail to evaluate infection efficiency because retail raw chicken is normally contaminated by multiple strains, not a single. *E. coli* strains were randomly selected from the major phylogenetic groups A, B1, B2, and D, combined in a single culture, and treated with each single phage or the phage cocktail. The treatment of mixed cultures with single phages did not, or only marginally, reduce the growth of mixed cultures of *E. coli*. However, the phage cocktail substantially inhibited the growth of the mixed cultures (**Figure 3**). In mixed cultures 2 (**Figures 3B**) and 4 (**Figure 3D**), for instance, single phages did not cause any growth reduction compared to the non-treated negative control, whereas the phage cocktail markedly reduced the growth of *E. coli* strains in mixed cultures. These results indicated the phage cocktail was effective at simultaneously inhibiting multiple strains of *E. coli* belonging to different phylogenetic groups.

Because raw chicken products are preserved normally at refrigeration temperatures and sometimes exposed to room temperatures during handling, we evaluated the infection frequency at 4 and 25°C. At 25°C, the treatment with the phage cocktail significantly reduced the level of AMR *E. coli* strains at the beginning of infection (approximately 0.35 log CFU/ml after 6 h; $P = 0.0378$), but further incubation did not make a difference in the viable counts of *E. coli* compared to the non-treated control (**Figure 4A**). However, the level of AMR *E. coli* was significantly reduced at 4°C within a few hours, and the reduction was maintained during the entire course of the experiment (**Figure 4B**). We examined the inhibition efficiency of the phage cocktail on raw chicken skin. To mimic the situation of food contamination, raw chicken skin was artificially contaminated with the mixed culture of AMR *E. coli* strains. Compared to LB media (**Figures 4A,B**), interestingly, the phage cocktail reduced AMR *E. coli* more significantly on chicken skin at both 4 and 25°C (**Figures 4C,D**). When raw chicken

TABLE 1 | Morphological and genomic features of the five phages used in the cocktail.

Phage	Isolation source	Morphological features (nm; n = 3)		Genomic features				
		Head	Tail	Size (bp)	GC (%)	ORF[a]	tRNA	Accession No.
JEP1	Retail chicken	79.6 ± 1.9	101.0 ± 3.8	143,610	43.54	223	5	MT740314
JEP4	Chicken feces	106.3 ± 5.5	102.9 ± 2.6	39,195	47.05	61	0	MT740315
JEP6	Pig feces	109.1 ± 1.7	110.3 ± 2.3	170,340	35.31	274	7	MT764206
JEP7	Retail duck	103.9 ± 5.2	95.2 ± 2.4	52,936	45.94	71	0	MT764207
JEP8	Retail chicken	96.1 ± 2.5	95.9 ± 3.5	165,295	40.47	272	0	MT764208

[a]*Open reading frame.*

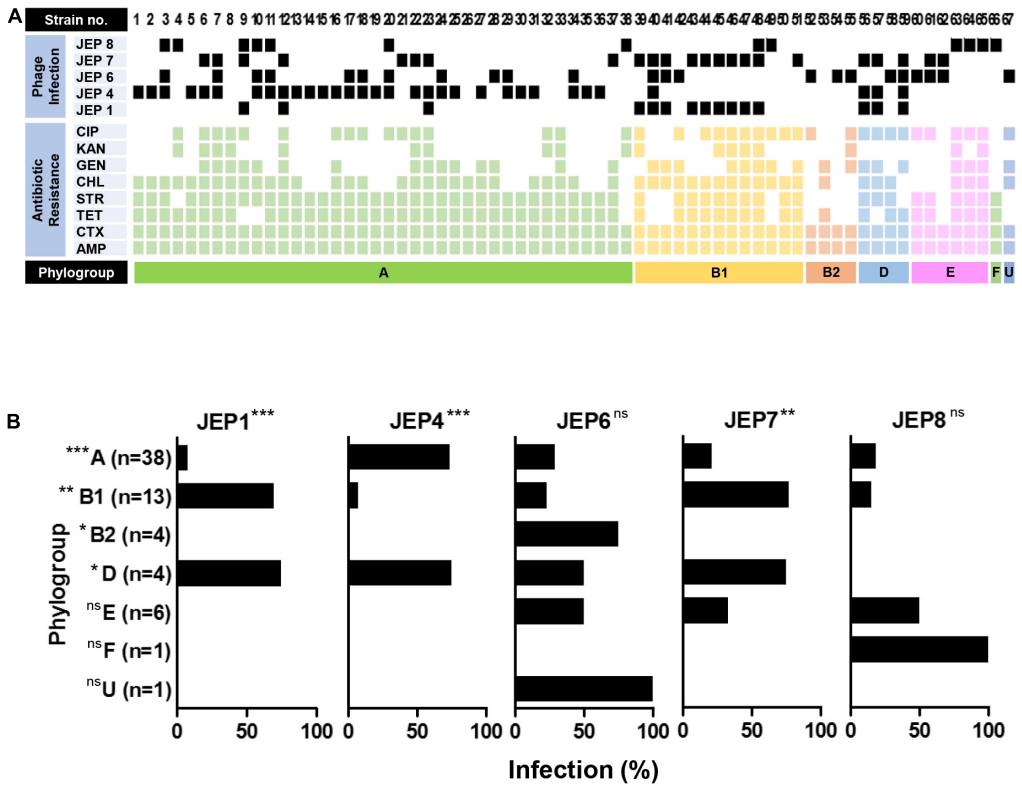


FIGURE 1 | Host range of the five phages. **(A)** The phage infection of 67 strains of AMR *E. coli* isolated from retail chicken. The antimicrobial resistance patterns of the *E. coli* strains and the infectivity of five phages are indicated. CIP, ciprofloxacin; KAN, kanamycin; GEN, gentamicin; CHL, chloramphenicol; STR, streptomycin; TET, tetracycline; CTX, cefotaxime; AMP, ampicillin; and phylogroup U, Unknown. **(B)** Association of the phylogenetic groups of AMR *E. coli* with the infection frequency of the five phages. The experiment was repeated three times. Statistical analysis was performed using the chi-square test with GraphPad Prism (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant).

TABLE 2 | Infection frequencies of the five phages used in the cocktail.

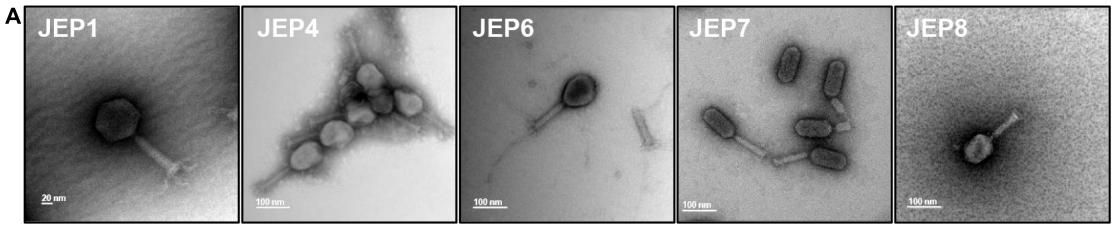
Phage	Phylogenetic group of ESBL-producing *E. coli*							Total (*n* = 67)
	A (*n* = 38)	B1 (*n* = 13)	B2 (*n* = 4)	D (*n* = 4)	E (*n* = 6)	F (*n* = 1)	U[a] (*n* = 1)	
JEP1	3 (7.9%)	9 (69.3%)	0 (0.0%)	3 (75.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	15 (22.4%)
JEP4	28 (73.7%)	1(7.7%)	0 (0.0%)	3 (75.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	32 (47.8%)
JEP6	11 (28.9%)	3 (23.1%)	3 (75.0%)	2 (50.0%)	3 (50.0%)	0 (0.0%)	1 (100.0%)	23 (34.3%)
JEP7	8 (21.1%)	10 (76.9%)	0 (0.0%)	3 (75.0%)	2 (33.3%)	0 (0.0%)	0 (0.0%)	23 (34.3%)
JEP8	7 (18.4%)	2 (15.4%)	0 (0.0%)	0 (0.0%)	3 (50.0%)	1 (100.0%)	0 (0.0%)	13 (19.4%)
Total	34 (89.5%)	12 (92.3%)	3 (75.0%)	4 (100.0%)	6 (100.0%)	1 (100.0%)	1 (100.0%)	61 (91.0%)

[a]*unknown.*

skin samples were treated with the phage cocktail at an MOI of 10^3 at 25°C, the mixed culture of AMR *E. coli* strains was rapidly reduced by 2.19 log CFU/cm^2 and 2.58 log CFU/cm^2 after 3 h and 6 h, respectively (**Figure 4C**). After that, the mixed culture of AMR *E. coli* strains continued to grow on chicken skin (**Figure 4C**). At 4°C, however, the treatment of raw chicken skin with the phage cocktail significantly reduced the level of AMR *E. coli* strains within 3 h and continued to reduce the viable counts of AMR *E. coli* by 3.17 CFU/cm^2 and 3.28 log CFU/cm^2 after 6 and 24 h, respectively (**Figure 4D**). The results showed that the phage cocktail is highly effective in inhibiting AMR *E. coli* on chicken carcasses especially at refrigeration temperatures.

DISCUSSION

Since bacteria develop phage resistance rapidly (Labrie et al., 2010), phages are normally treated in a cocktail using those that recognize different host receptors (Tanji et al., 2004; Gu et al., 2012; Yen et al., 2017). This is because, if bacteria develop resistance to one phage, another phage in the cocktail, which



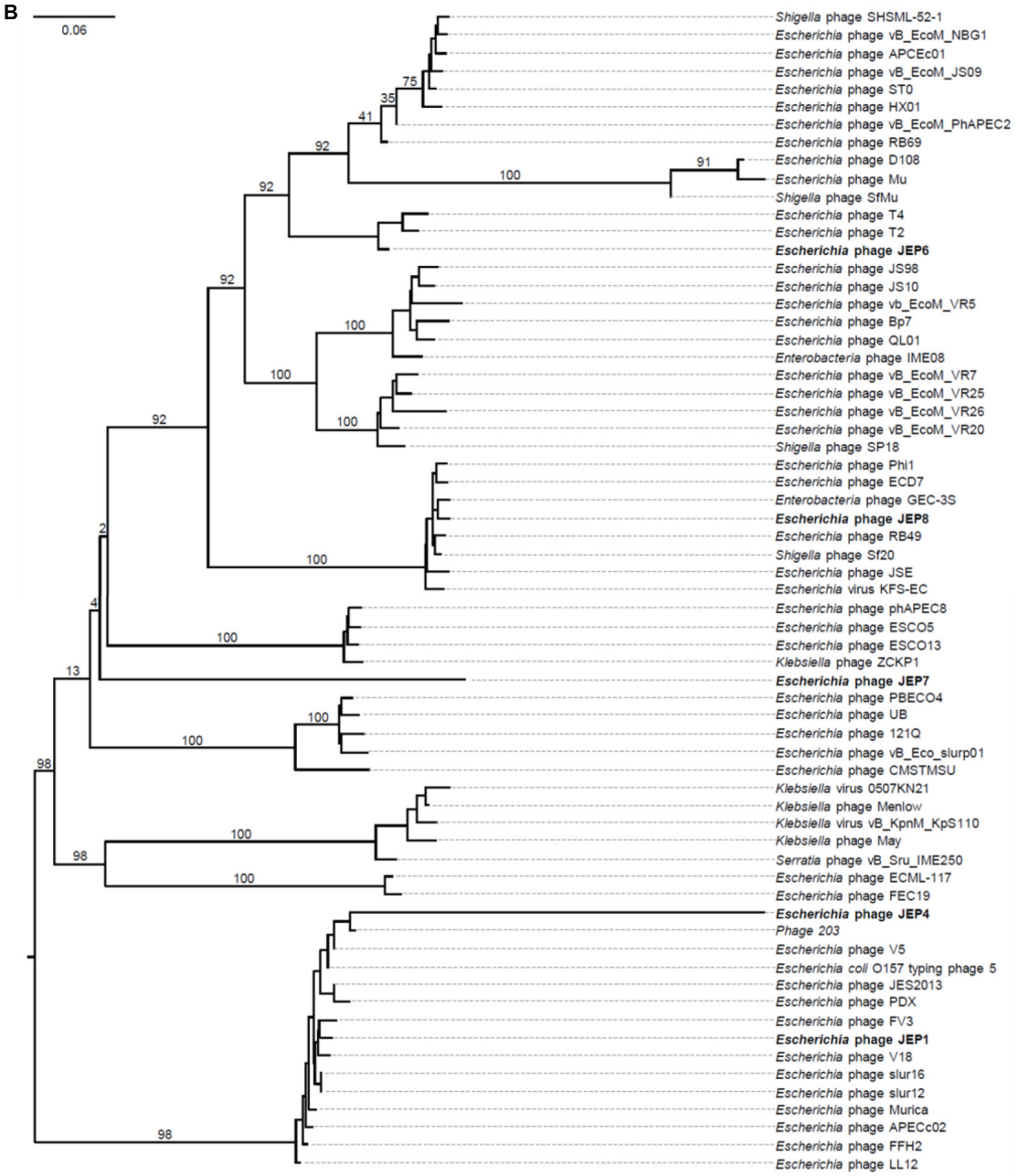


FIGURE 2 | The morphological and genomic features of phages. **(A)** Transmission electron microscopy (TEM) images of the five phages. **(B)** Phylogenetic analysis showing the relationship between the five phages and 60 *Myoviridae* family phages that were reported in a previous study (Korf et al., 2019).

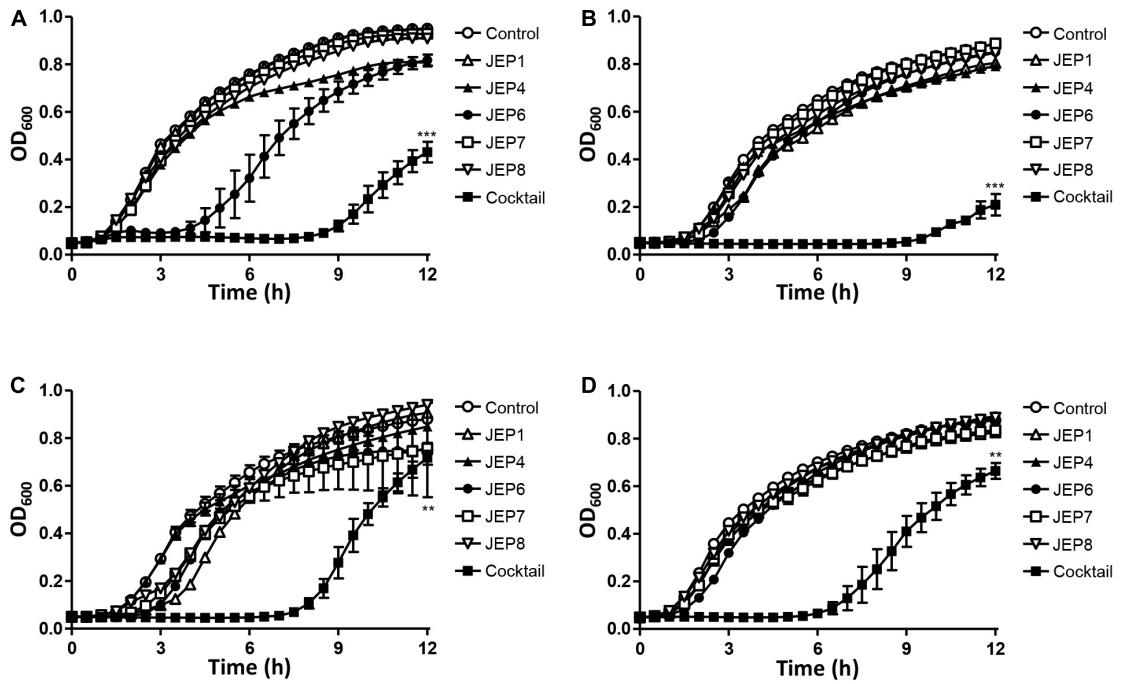


FIGURE 3 | Phage inhibition of mixed cultures of AMR *E. coli* strains in LB broth at 37°C. The four mixed cultures used in the assay included: **(A)** Mixed culture 1 consisting of AMR *E. coli* strains E20, E41, E55, and E59, **(B)** Mixed culture 2 consisitng of E3, E43, E55, and E59, **(C)** Mixed culture 3 consisiting of E17, E41, E52, and E59, and **(D)** Mixed culture 4 consisting of E20, E45, E52, and E59. The reduction in the OD_{600} of the mixed culture of AMR *E. coli* strains was measured after treatment with single phages or the phage cocktail. The data present the means and the standard errors of the mean (SEM) of the results of three experiments. Statistical analysis was performed using a Student's *t*-test compared to the control in the same sampling (12 h) with GraphPad Prism (**$P < 0.01$; ***$P < 0.001$).

recognizes a different receptor, still can infect the bacteria. However, the identification of the host receptor of a phage requires a series of experiments involving mutagenesis, which is often labor-intensive and time-consuming. Without identifying the host receptors of *E. coli* phages, in this study, we constructed a phage cocktail that can effectively infect *E. coli* isolates from retail chicken using phages that preferentially infect the major phylogenetic groups of *E. coli*. The Clermont phylotyping classifies *E. coli* into four major (A, B1, B2, and D) and two minor groups (E and F) (Clermont et al., 2013). The phylogenetic group of *E. coli* is related to certain pathotypes and the host origin. For instance, phylogroups A and B2 normally predominate in human strains, while *E. coli* isolates from chicken mostly belong to phylogroups A and B1 (Unno et al., 2009; Kluytmans et al., 2012; Xu et al., 2014; Coura et al., 2015). Consistently, in our previous study, AMR *E. coli* strains isolated from retail chicken dominantly belonged to phylogroups A and B1 (Park et al., 2019). In the cocktail, we included three phages (JEP1, JEP4, and JEP7) that infected the major phylogenetic groups of *E. coli* isolates from chicken (i.e., A and B1) (**Figure 1B** and **Table 2**), and the phage cocktail infected 91.0% of the tested AMR *E. coli* strains (**Figure 1A** and **Table 2**). The same approach can be used to construct phage cocktails to target other pathogenic bacteria demonstrating unique phylogenetic features. For example, extraintestinal pathogenic *E. coli* (ExPEC) predominates phylogroups B2 and D (Picard et al., 1999; Cortés et al., 2010). Based on this, cocktails can be constructed using *E. coli* phages that preferentially infect phylogroups B2 and D.

The association of phage infectivity with the phylogenetic group of *E. coli* may be related to the prevalence of phage receptors. Phages initiate infection by binding to host receptors on the bacterial surface. Phage receptors in *E. coli*, which have been reported thus far, include the ferrichrome outer membrane transporter FhuA (Raya et al., 2011), the major outer membrane protein OmpC (Morita et al., 2002), surface glycoconjugates (Kudva et al., 1999), and the O antigen of lipopolysaccharide (LPS) (Perry et al., 2009). Bacteria often develop phage resistance by hindering this critical step of infection using various mechanisms, such as the alteration of phage receptors through spontaneous mutations (Uhl and Miller, 1996), the removal of receptor genes by an insertion sequence (Kim and Ryu, 2011), and the interruption of phage access to host receptors (Kim and Ryu, 2012). The prevalence of host receptors can be related to the phylogenetic group of *E. coli* because the distribution of genes encoding outer membrane proteins, fimbriae, or capsular proteins is different depending on the phylogenetic group (Johnson et al., 2002). Also, the phylogenetic group of *E. coli* is related to the type of the core oligosaccharide of LPS, a common host receptor for phage infection (Amor et al., 2000; Dissanayake et al., 2008). At this stage, further studies are required to examine this possibility.

Phages have been used for the control of foodborne pathogenic bacteria on chicken carcasses. A previous study demonstrated

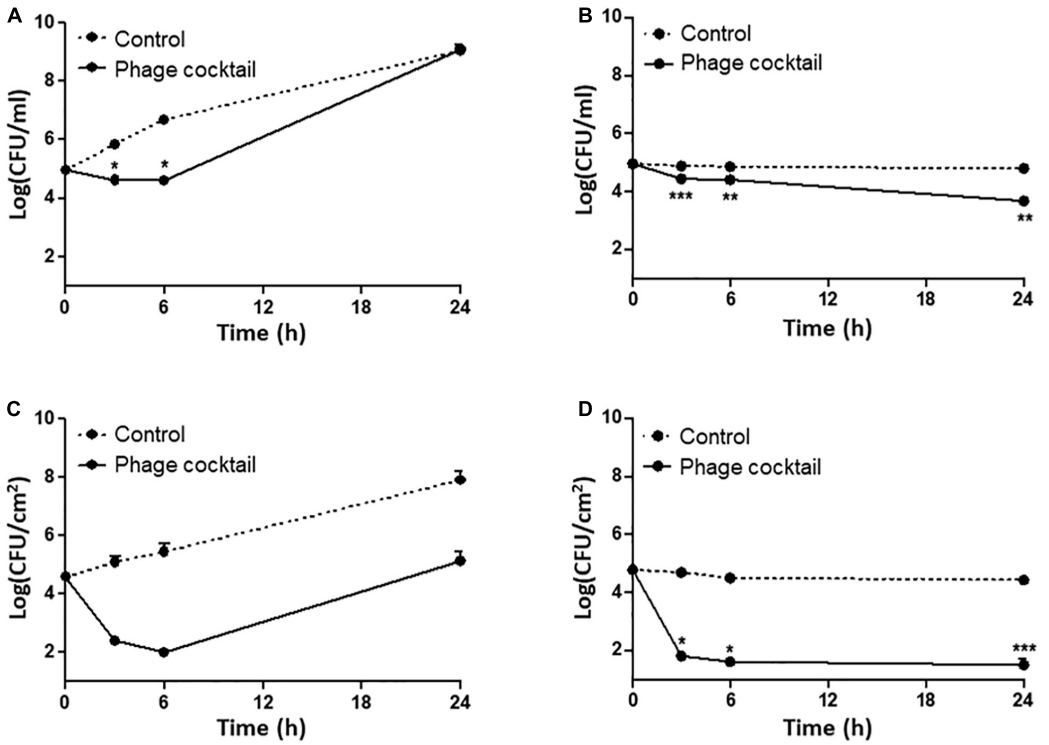


FIGURE 4 | Phage inhibition of the viability of a mixed culture of AMR *E. coli* isolates (E20, E41, E55, and E59) in LB broth **(A,B)** and on raw chicken skin **(C,D)**. The viable counts of a mixed culture of AMR *E. coli* strains in LB broth without (control) and with the phage cocktail at 25°C **(A)** and 4°C **(B)**. The levels of AMR *E. coli* isolates on chicken skin without (control) and with the phage cocktail at 25°C **(C)** and 4°C **(D)**. The data show the means and the standard errors of the mean (SEM) of the results of three experiments. Statistical analysis was performed using a Student's *t*-test compared to the non-treated control with GraphPad Prism (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

that the treatment with a phage cocktail significantly reduced the number of *Listeria monocytogenes* on chicken carcasses (Cufaoglu and Ayaz, 2019). Atterbury et al. reported that *Campylobacter jejuni* phages effectively infected *C. jejuni* on chicken skin at 4°C (Atterbury et al., 2003). The results in this study showed that phages effectively inhibited *E. coli* on chicken skin particularly at 4°C (**Figures 4C**, and **D**). For the control of bacterial contamination of food, temperatures are an important factor affecting the efficacy of phage infection (Seeley and Primrose, 1980; Tokman et al., 2016). The increased efficacy of phage infection at refrigeration temperatures is probably because low temperatures may reduce the emergence of phage-resistant bacteria due to the reduced growth rate of *E. coli* at 4°C compared to 25°C and the limited function of the restriction-modification systems involved in the degradation of phage DNA injected into the host (Dodds et al., 1987; Kim et al., 2012). Additionally, the temperature is a critical environmental factor that determines the viability of phages. Since phages stored at 4°C are more stable than those stored at ≥10°C (Olson et al., 2004), differential phage sensitivity at different temperatures may also affect phage infectivity at 4°C even though the phages were tested at refrigeration temperatures relatively for a short time (24 h) in this study. Regardless of the underlying molecular mechanisms, the increased lytic activity of the phage cocktail on foods (i.e., chicken) at low temperatures may enable the phages to inhibit AMR *E. coli* on raw chicken effectively because raw chicken products are distributed in the cold chain. Given this, the broad host range phage cocktail in this study can be used to control AMR commensal *E. coli* on retail chicken products. To achieve practical application of phages to food, additionally, further studies are needed to develop methods to make phages maintain their infectivity long enough in the food supply chain.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MT740314; https://www.ncbi.nlm.nih.gov/genbank/, MT740315; https://www.ncbi.nlm.nih.gov/genbank/, MT764206; https://www.ncbi.nlm.nih.gov/genbank/, MT764207; and https://www.ncbi.nlm.nih.gov/genbank/, MT764208.

AUTHOR CONTRIBUTIONS

JK, SR, and BJ designed the study, analyzed the data, and reviewed the manuscript. JK and HP performed the experiments.

JK and BJ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.699630/full#supplementary-material

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