EMERGING *ENTEROBACTERIACEAE* INFECTIONS: ANTIBIOTIC RESISTANCE AND NOVEL TREATMENT OPTIONS

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EMERGING *ENTEROBACTERIACEAE* INFECTIONS: ANTIBIOTIC RESISTANCE AND NOVEL TREATMENT OPTIONS

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Enterobacteriaceae are spread worldwide and the diseases they cause may be fatal especially in immunocompromised patients. Moreover, the high prevalence of ESBL producing Salmonella and Shigella species diseases worldwide suggests major underlying safety issues. According to the World Health Organization (WHO), 2015, approximately 220 million children contract diarrhoeal diseases every year and 96 000 die. As a result, the increase in single or multi drug-resistant foodborne bacterial pathogens is of major public health concern. Moreover, resistance to antimicrobials was found among Salmonella spp and Campylobacter spp from animals and food, and since fluoroquinolones became licensed for use in animal foods, especially for poultry, the rate of fluoroquinolone resistant Salmonella spp and Campylobacter spp in animals and human food, and then in human infections, rapidly increased. To that purpose, the findings of the conducted studies in the book chapters, 1) highlight surveillance studies reporting the occurrence and distribution of resistance to antimicrobial agents, namely, to third generation cephalosporins, carbapenems and fluoroquinolones, 2) describe the mechanisms of transmission of resistance determinants from animals, food products and clinical specimens, that allow implementation of appropriate measures to control their spread and adopt appropriate therapeutic measures, and 3) provide treatment options, useful to medical practice.

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EDITORIAL
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Editorial: Emerging *Enterobacteriaceae* Infections: Antibiotic Resistance and Novel Treatment Options

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Keywords: ***Enterobacteriaceae*, resistance, ESBLs, carbapenemases, treatment**

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Editorial on the Research Topic

Emerging *Enterobacteriaceae* Infections: Antibiotic Resistance and Novel Treatment Options

The high prevalence of diseases caused by organisms resistant to antibiotics in different regions of the world indicates an alarming global problem that requires rapid adequate action. Resistance to antimicrobials has been reported with a notable frequency in species belonging to the family *Enterobacteriaceae*, among other bacterial families, in sources including animals and food. For instance, the rate of fluoroquinolone-resistant *Salmonella* has rapidly increased in animals, food products, and human infections during the past few years. This has been correlated with the licensing of fluoroquinolone use in animal feed. This highlights the interconnectedness between various ecologies and supports the voices driving the global One Health initiative. In attempting to monitor current resistance trends in *Enterobacteriaceae*, the studies in the book at hand include surveillance information and patterns of transmission of resistant organisms to humans. This is in addition to providing possible guidelines that cater to particular agents, resistance mechanisms, and patient groups.

A study carried out over a period of 10 years by Hanna-Wakim et al. aimed to provide a comprehensive view of the epidemiological characteristics of urinary tract infections (UTIs) in hospitalized children, examine the risk factors of UTIs caused by extended spectrum beta-lactamase (ESBL)-producing organisms, and determine the resistance patterns in the isolated organisms.

The contribution by Moghnieh et al. focused on evaluating the epidemiology of bacteremia in cancer patients and emphasizing antibiotic resistance and risk factors of bacteremia associated with multi-drug-resistant organisms. They showed that emergence of resistance to third-generation cephalosporins and carbapenems in Gram-negative isolates has to be seriously considered in our local guidelines for empiric treatment of febrile neutropenia.

Liu et al. investigated the prevalence, resistance, and probable gene type of ESBL-producing *Escherichia coli* in China. The study further confirmed that ESBL producers, which are common among hospital strains of *E. coli*, are resistant to multiple drugs in addition to cephalosporins in China. This highlights the importance of closely monitoring such strains and providing scientific evidence for the rational application of antibiotics.

A study by Gao et al. evaluated the impact of ESBL-positive bacteria from animal manure on agricultural fields in rural regions of Taiwan, China. The study concluded that the application of

animal manure carrying drug-resistant bacteria is a likely contributor to the spread of antibiotic resistance genes.

Zhang et al. attempted to understand the prevalence of CTX-M type ESBL-harboring *Enterobacteriaceae* and to analyze risk factors related to fecal carriage in healthy rural residents of Taiwan, China. The study concluded that the prevalence of fecal carriage of CTX-M ESBL-producing *Enterobacteriaceae* among healthy rural humans in Taiwan was high, and recent antibiotic use and hospitalization history may be important contributors.

A study done by Liao et al. focused on third-generation cephalosporins resistance in bacteria; they determined the characteristics and distribution of $bla_{CTX\text{-}M\text{-}14}$, which encodes an ESBL, in *E. coli* harbored by food-producing animals in China. The study also suggested possible transmission of $bla_{CTX\text{-}M\text{-}14}$ between animals and humans and proposed that the resistance gene context continues to evolve in *E. coli* of food-producing animals.

Animal to human dissemination of resistant bacteria, via the food chain or as a result of direct contact, has often been reported. Hence, Kilani et al. studied the occurrence of $bla_{CTX\text{-}M\text{-}1}$, qnrB1, and virulence genes in avian ESBL-producing *E. coli* isolates from Tunisia. The authors also determined the antimicrobial resistance profile and the genomic variability of the isolates. They then concluded that plasmids harboring ESBL genes could be involved in the dissemination of certain resistance phenotypes.

Tokajian et al. also studied the molecular characteristics of an ESBL-producing *Klebsiella pneumoniae* strain, LAU-KP1, isolated from a stool sample from a patient admitted for a gastrointestinal procedure, by whole-genome sequencing. The entire plasmid content was also investigated. The study also questioned the potential role of *K. pneumoniae* as a reservoir for ESBL genes and other resistance determinants.

AmpC β-lactamases can hydrolyze broad- and extended-spectrum cephalosporins and are usually not inhibited by β-lactamase inhibitors such as clavulanic acid. Therefore, Hsieh et al. conducted a study that identified DHA-23, a novel plasmid-mediated and inducible AmpC β-lactamase obtained from *Enterobacteriaceae* isolates. They went on to characterize DHA-23 and compare it with other DHA molecules.

Insights into the resistance of carbapenems in two Gram-negative bacteria, *Raoultella ornithinolytica* and *Leclercia adecarboxylata*, are provided by Sun et al. They determined the nucleotide sequence of conjugative plasmids that encode the production of the enzyme New Delhi Metallo-beta-lactamase-1 (NDM-1), which is rarely isolated in these bacteria, from patients in China. The study also identified elements that facilitate transposition and mobilization of $bla_{NDM\text{-}1}$ gene contexts.

Chen et al. studied carbapenem resistance in a specific carbapenem-non-susceptible *Enterobacter aerogenes* strain, 3-SP, isolated from a human case of pneumonia in a Chinese teaching hospital. The study aimed to determine the plasmid (pNDM-BJ0-like conjugative plasmid) that encodes for the NDM-1 carbapenemase production, termed p3SP-NDM. The plasmid was fully sequenced and compared with other similar pNDM-BJ0-like plasmids. All reported pNDM-BJ01-like plasmids are exclusively found in members of the genus *Acinetobacter*, whereas this is the first report of identification of a pNDM-BJ01-like plasmid in a member of the *Enterobacteriaceae*.

Malek et al. attempted to study the topic of emergence of resistance from a different perspective; they investigated the presence and distribution of class I and class II integrons and the characteristics of the gene cassettes they carry in *Enterobacteriaceae* from nosocomial infections at a University Hospital in Egypt. They determined their impact on resistance and identified risk factors for the existence of integrons. The study deduced that integrons carrying gene cassettes encoding antibiotic resistance are significantly present among *Enterobacteriaceae* isolates causing nosocomial infections.

Antimicrobials are usually avoided in the treatment of Shiga-toxin-producing *E. coli* (STEC) infections since they are believed to induce bacterial cell lysis and the release of stored toxins. The review by Rahal et al. summarizes the approaches to treatment of emerging STEC infections. The review presents alternative treatment approaches including the administration of toxin-directed antibodies, toxin-adsorbing polymers, probiotic agents, and natural remedies. The review also discusses how the use of antimicrobial agents in treating STEC infections has been reconsidered in recent years with certain modalities showing promise.

Small cationic peptides such as host defense peptides (HDPs) play a vital role in innate immunity response and immunomodulatory stimulation. In the study by Yacoub et al., the antimicrobial activities of β-defensin peptide-4 (sAvBD-4) and -10 (sAvBD-10) derived from chickens against pathogenic organisms including bacteria and fungi were investigated. The study then indicated that synthetic avian peptides exhibit strong antibacterial and antifungal activity. The authors concluded that future research should be carried out to better understand the mechanisms of action of these peptides and their potential use in the pharmaceutical industry.

In conclusion, it is rather evident that antibiotic resistance in members of the family *Enterobacteriaceae* is on the rise. The consistent stream of publications reporting resistant strains and sometimes novel resistance mechanisms has made it evident that infectious agents have become a threat to society yet again. The aforementioned reports are not constrained to a particular geographical location, and with the current ease of travel and global migration, the spread of resistant agents is a graver hazard than ever before. This is hence a worldwide problem that can only be curbed via diligent monitoring of arising resistance in various global regions, communication among the scientific community, formulating, and implementing appropriate guidelines, in addition to public education on the dangers of antibiotic misuse. With this in mind, the research topic at hand aimed at examining the current situation in geographical locations within the Far East, the Middle East,

and North Africa. Lessons learned from this area of the world and observations in this global locale have often been different from those in the Western world, yet of mutual value. The articles encompassed by this topic also provide recommended guidelines for particular patient groups and infectious agents. Moreover, with a One Health mantra in mind, papers have included examination of soil and animal samples, hence stressing the relevance of arising resistance in such samples and its bearing on human health.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Epidemiology and characteristics of urinary tract infections in children and adolescents

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Background: Urinary tract infections (UTIs) are among the most common infections in the pediatric population. Over the last two decades, antibiotic resistance is increasing significantly as extended spectrum beta lactamase (ESBL) producing organisms are emerging. The aim of this study is to provide a comprehensive view of the epidemiologic characteristics of UTIs in hospitalized children, examine the risk factors of UTIs caused by ESBL-producing organisms, and determine the resistance patterns in the isolated organisms over the last 10 years.

Methods: Retrospective chart review was conducted at two Lebanese medical centers. Subjects were identified by looking at the following ICD-9 discharge codes: "Urinary tract infection," "UTI," "Cystitis," and/or "Pyelonephritis." Children less than 18 years of age admitted for UTI between January 1st, 2001 and December 31st, 2011 were included. Cases whose urine culture result did not meet our definition for UTI were excluded. Chi-square, Fisher's exact test, and multivariate logistic regression were used to determine risk factors for ESBL. Linear regression analysis was used to determine resistance patterns.

Results: The study included 675 cases with a median age of 16 months and female predominance of 77.7% (525 cases). Of the 584 cases caused *by Escherichia coli* or *Klebsiella* spp, 91 cases (15.5%) were found to be ESBL-producing organisms. Vesico-ureteral reflux and previous antibiotics use were found to be independent risk factors for ESBL-producing *E. coli* and *Klebsiella* spp. ($p < 0.05$). A significant linear increase in resistance to all generations of Cephalosporins ($r^2 = 0.442$) and Fluoroquinolones ($r^2 = 0.698$) was found.

Conclusion: The recognition of risk factors for infection with ESBL-producing organisms and the observation of increasing overall resistance to antibiotics warrant further studies that might probably lead to new recommendations to guide management of UTIs and antibiotic use in children and adolescents.

Keywords: ESBL, urinary tract infection, risk factors, children, antibiotic resistance

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Introduction

Urinary tract infections (UTIs) are among the most commonly encountered infections in the pediatric age group both in the community and hospital settings (Ronald et al., 2001; Stamm and Norrby, 2001; Nicolle, 2002; Hooton et al., 2004). Several studies from the United States of America estimate the direct and indirect cost of acute pyelonephritis in adults to 2.14 billion US dollars (year 2000 values) which is 2.19 billion US dollars in 2013 (Brown et al., 2005; Foxman, 2014). It is estimated that 150 million UTIs occur yearly worldwide, resulting in more than 6 billion dollars in direct healthcare cost (Stamm and Norrby, 2001).

Over the last two decades, the resistance to antibiotics in members of Gram-negative Enterobacteriaceae rose tremendously worldwide; highlighted by the emergence of extended spectrum beta-lactamase (ESBL) producing organisms (Ena et al., 2006; Pitout and Laupland, 2008; Ben-Ami et al., 2009; Soraas et al., 2013). Whereas the initial spread was in hospital settings, eventually these pathogens emerged in community-onset UTIs (Pitout and Laupland, 2008). In many countries of the Middle East, where unregulated prescription of antibiotics is prevalent, the resistance patterns of frequently observed uropathogens are alarming (Kanafani et al., 2005; Topaloglu et al., 2010; Daoud and Afif, 2011; Araj et al., 2012; Kizilca et al., 2012; Pourakbari et al., 2012; Al-Assil et al., 2013; Dayan et al., 2013; Dotis et al., 2013; Megged, 2014). However, there is limited data concerning the antibiotic resistance pattern of pediatric UTIs in the region of the Middle East.

Few studies have investigated the prevalence and risk factors associated with hospital and community-acquired UTIs secondary to ESBL-producing bacteria in the pediatric age group.

Previous studies have shown that renal abnormalities, septicemia, systemic disease such as metabolic diseases and malignancies, hospitalization within the previous 3 months preceding the onset of UTI, age less than 1 year, high recurrence rate of UTI, and recent antibiotic treatment are independent risk factors for UTI secondary to community-acquired ESBL-producing organisms (Topaloglu et al., 2010; Kizilca et al., 2012; Dayan et al., 2013; Dotis et al., 2013; Giardino et al., 2013; Megged, 2014). Those risk factors have not been well established in our region.

Accordingly, the purpose of this retrospective study is to provide a comprehensive review of the epidemiology and characteristics of UTIs in hospitalized children and adolescents, determine the risk factors of UTIs caused by ESBL-producing organisms of *E. coli* and *Klebsiella* spp, and examine the resistance patterns in the isolated organisms over the last 10 years.

Materials and Methods

Study Design

A multi-center retrospective cohort study was conducted in two major Lebanese hospitals both in Beirut; the American University of Beirut Medical Center (AUBMC) and Makassed General Hospital (MGH). The study was approved by the institutional review board (IRB) at each center (the AUB IRB approval number was PED.RW.01, the MGH IRB approval number was dated 12 June 2013).

All patients were identified retrospectively through medical records at AUBMC and MGH by looking at the following ICD-9 codes for discharge diagnosis: "Urinary tract infection," "UTI," "Cystitis," and/or "Pyelonephritis." Children less than 18 years of age with one of the above discharge diagnosis, admitted to the hospital between January 1st, 2001 and December 31st, 2011 were included in the study. We excluded patients whose urine culture result did not meet the definition for UTI established according to the clinical practice guidelines issued in 2011 for the diagnosis and management of UTI (Roberts, 2011). Accordingly, this included significant bacteriuria with recovery of at least 100,000 CFU/mL (colony forming unit per milliliter) of a single uropathogen from a clean catch specimen; or at least 50,000 CFU/mL of a single uropathogen from a catheterized specimen; or any uropathogenic bacteria from a suprapubic aspirate.

Data Collection

Data from each reviewed chart was documented on a case report form (CRF). Data collected included the following information: basic demographics (age, gender), hospital stay information (ICU/regular floor admission, length of stay), past medical history (underlying diseases, genitourinary disorders, immunosuppression), previous antibiotic use, recent hospitalizations, past surgical history, method of urine collection, laboratory information (urinalysis, urine culture, antimicrobial susceptibility patterns, complete blood count, basic metabolic panel), imaging results, clinical course and outcomes.

Analysis and Reporting of Results

The Statistical Package for Social Sciences (SPSS) program, version 22.0 for Windows was used for data analysis (IBM, Armonk, NY). Simple descriptive statistics were used to describe patients' demographics and characteristics of UTI. Bivariate and multivariate analyses of risk factors for ESBL were first analyzed by Pearson's Chi-square test or Fisher's exact test (when number of subjects in a subgroup was less than 5). Continuous risk factors were analyzed with student *t*-test. Statistical significance was considered below a type-1 error threshold (alpha level) of 0.05. Following that a multivariate logistic regression model comprised of significant risk factors was constructed and reported.

Linear regression analysis was conducted to determine resistance patterns to different classes of antibiotics over the past 10 years (correlation coefficient r^2 and *p*-value for regression analysis were reported).

Results

Number of Cases

Cases were identified and included in our study as shown in **Figure 1**. Our search retrieved 1650 cases: 719 at AUBMC and 931 at MGH. Only 666 records at AUBMC and 915 records at MGH were available for review. Of these, 211 cases at AUBMC and 394 cases at MGH were excluded for either having missing urine culture data or a negative urine culture result (no growth). The remaining cases were reviewed. Of these, 182 cases at

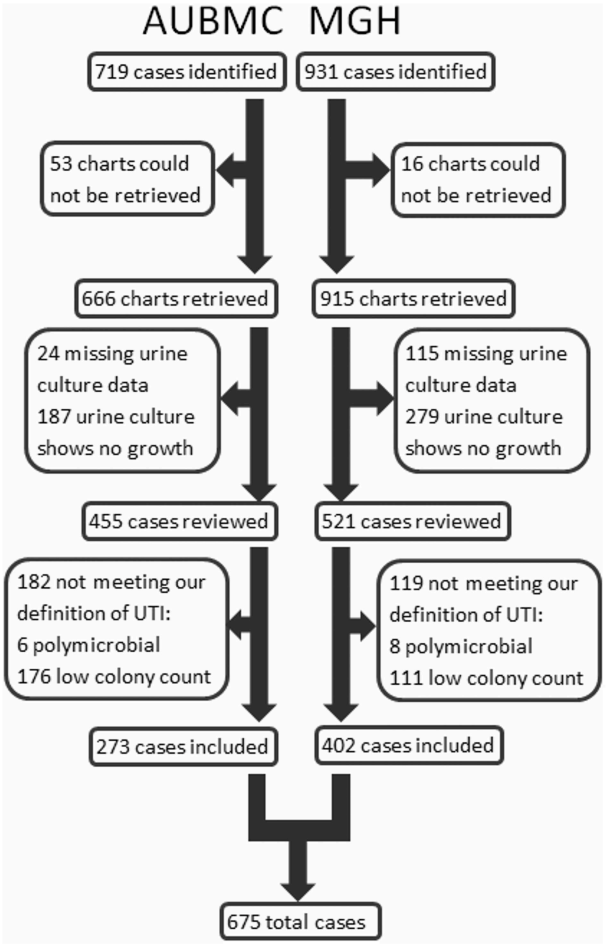

FIGURE 1 | Identification and inclusion of cases into the study.

AUBMC and 119 cases at MGH did not meet our definition of UTI and were excluded from the study. Therefore, a total of 675 cases were included in the analysis.

Demographic Characteristics

Our study population was divided into three groups, (less than 2 months, between 2 months and 2 years, and more than 2 years), which had a distribution of 9.5, 49.6, and 40.9% respectively. Overall, females were predominant (77.7%). However, in infants less than 2 months, 60.9% were males (**Table 1**). The median age was 16 months. The majority of males were circumcised (66%).

Isolated Microorganisms

E. coli was the most common pathogen isolated among all age groups, followed by Klebsiella and Proteus species, accounting for 79.4, 7.9 and 3.9% respectively. Other organisms such as *Pseudomonas, Enterococcus, and Enterobacter* were relatively rare (**Table 2**).

Imaging and Laboratory Findings

Renal ultrasound was performed in 69.8% of cases, 24.8% of the results were abnormal. Dilation of the pelvicalyceal system was the most common finding, representing 41.9% of abnormalities. Voiding cystourethrogram (VCUG) was performed in 53.4% of the cases with an abnormal ultrasound. Vesicoureteral reflux (VUR) was identified in 51% of these cases. In addition, 49.3% of the cases with normal ultrasound findings underwent VCUG, of which 19% had VUR (**Figure 2**).

Out of 273 cases from AUBMC, 103 underwent Dimercaptosuccinic acid (DMSA) radio-nucleotide scans, of which 60 cases had scarring or foci of pyelonephritis. No DMSA data was reported from MGH, as this procedure was not available in this hospital. When comparing ESBL and non-ESBL-producing *E. coli* and *Klebsiella* spp, 25.5% of cases with abnormal DMSA results had ESBL positive UTI.

Urinalysis showed a high white blood cell (WBC) count of more than 5 per high-power field (HPF) in only 60.1% of cases. Leukocyte esterase and nitrites were positive in only 80.2 and 39% of our cases, respectively. Approximately one third of patients (34.9%) had all three positive findings present on urinalysis.

ESBL Risk Factors and Emergence Patterns

The ESBL-producing and non-ESBL-producing groups consisted of 91 (15.5%) and 493 (84.5%) cases, respectively. ESBL cases were significantly more frequent at AUBMC ($p < 0.05$). For 5 of the 589 cases caused by *E. coli* or *Klebsiella* spp, the data on ESBL status was missing.

Median age, gender, and age group distribution revealed no significant differences between the ESBL-producing and non-ESBL-producing groups. In the ESBL-producing group,

TABLE 1 | Gender distribution by age groups.

Gender	Age group *n* (%)			Total
	<2 months	2 months–2 years	>2 years	
Females	25 (39.1)	254 (75.8)	246 (89.1)	525 (77.7)
Males	39 (60.9)	81 (24.2)	30 (10.9)	150 (22.3)
Total	64 (9.5)	335 (49.6)	276 (40.9)	675

TABLE 2 | Isolated uropathogens by age groups.

Organisms	Total *n* (%)	Age groups *n* (%)		
		<2 months	2 months –2 years	>2 years
E. coli	536 (79.4)	39 (60.9)	266 (79.4)	231 (83.7)
Klebsiella spp.	53 (7.8)	18 (28.1)	26 (7.7)	9 (3.3)
Proteus spp.	26 (3.8)	1 (1.6)	12 (3.6)	13 (4.7)
Pseudomonas aeruginosa	14 (2.1)	1 (1.6)	5 (1.5)	8 (2.9)
Enterococcus spp.	14 (2.1)	0 (0)	8 (2.4)	6 (2.2)
Enterobacter spp.	10 (1.5)	2 (3.1)	6 (1.8)	2 (0.7)
Others*	22 (3.3)	3 (4.7)	12 (3.6)	7 (2.5)
Total	675 (100)	64 (100)	335 (100)	276 (100)

**Others: Candida albicans, Candida non-albicans, coagulase negative Staphylococcus, Group B Streptococcus, Providencia startii, Citrobacter freundii, viridans group streptococci, Serratia marcescens, Morganella morgani, Alcaligenes faecalis, Citrobacter diversus, Salmonella group C.*

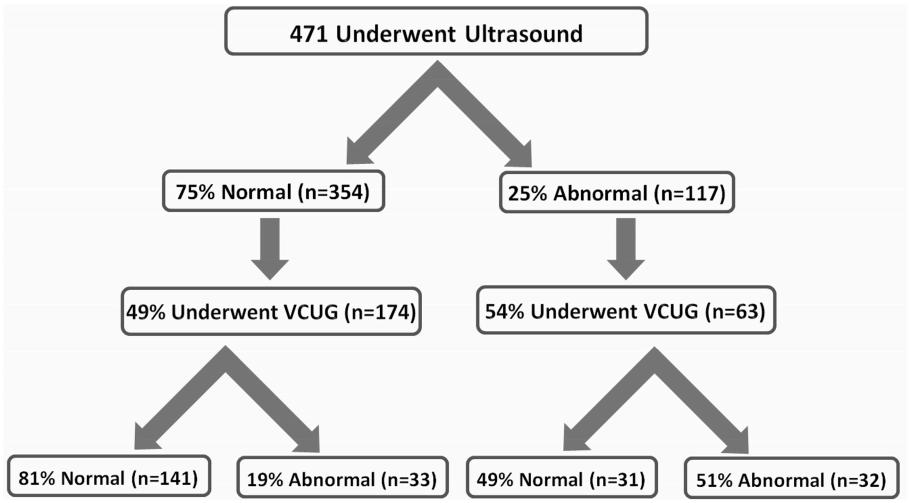

FIGURE 2 | Imaging results for ultrasound and voiding cystourethrogram (VCUG).

E. coli and *Klebsiella* isolates represented 82.4% and 17.6%, respectively; whereas, in the non-ESBL-producing group, *E. coli* and *Klebsiella* isolates represented 92.5 and 7.5%, respectively. Thus, *Klebsiella* spp. were more commonly found in the ESBL-producing group ($p = 0.02$). Additionally, longer duration of hospital stay was found to be statistically significant in cases with ESBL-producing organisms ($p < 0.001$) (**Table 3**).

Clinical Characteristics

When comparing ESBL and non-ESBL groups, there was no significant difference in the presenting symptoms regarding fever, vomiting, diarrhea, irritability, abdominal pain, flank pain and voiding dysfunction. However, enuresis was significantly higher in the ESBL group ($p < 0.05$).

Although liver, renal, and cardiac diseases were more prevalent in ESBL cases, the difference was not statistically significant ($p > 0.05$). On the other hand, VUR, recurrent UTI, and history of genitourinary (GU) surgery were found to be significantly higher in ESBL group ($p < 0.05$). Regarding circumcision, no significant difference was observed ($p = 0.703$) (**Table 4**).

A multivariate logistic regression analysis was performed with the above risk factors while controlling for age and gender. VUR and previous antibiotic use were found to be independent risk factors with odds ratio (OR) of 3.01 and 4.05, respectively.

Resistance to Different Classes of Antibiotics

When a linear regression was performed comparing ESBL pattern over the past 10 years, a significant association was found ($p = 0.004$) (**Figure 3**). There was a strong positive linear correlation ($r^2 = 0.783$), with a 2.08% yearly increase in UTIs secondary to ESBL-producing bacteria.

To observe the trends in UTI resistance to different classes of antibiotics in the period of 2001 to 2011, we plotted the yearly percentage of UTIs resistant to gentamicin, cephalosporins, fluoroquinolones, and trimethoprim-sulfamethoxazole (TMP-SMX). Resistance to first generation cephalosporins could not be evaluated because it is not tested for by the hospitals' laboratories included in this study. Since the trends in antibiotic resistance to second, third, and fourth generations of cephalosporins were very similar, we grouped the three generations of cephalosporins into one class. The fluoroquinolone class of antibiotics in our study included levofloxacin and ciprofloxacin; resistance to other fluoroquinolones is not tested by the hospitals' laboratories included in this study. The trends in antibiotic resistance to levofloxacin and ciprofloxacin were also similar.

Linear regression revealed statistically significant linear trends in antibiotic resistance to fluoroquinolones ($p = 0.001$) and

TABLE 3 | Characteristics of ESBL and Non-ESBL groups.

	ESBL (*N* = 91)	Non-ESBL (*N* = 493)	*p*-value†
Median age in months	23	15	
Mean age in months	38.8	38	0.883*
Female Gender, *n* (%)	77 (84.6)	395 (80.2)	0.318
AGE GROUPS, *N* (%)			
<2 months	8 (8.8)	48 (9.7)	0.368
2 months–2 years	40 (44)	251 (51)	
>2 years	43 (47.2)	194 (39.3)	
PREVALENCE BY LOCATION (%)			
MGH	12.1	87.9	0.004
AUBMC	21.1	78.9	
ORGANISMS, *N* (%)			
E. coli	75 (82.4)	456 (92.5)	0.020
Klebsiella spp.	16 (17.6)	37 (7.5)	
Mean LOS	14.33	6.48	<0.001*

LOS Length of hospital stay in days.
**Independent Sample-t test is used to compare means.*
†Pearson's Chi-Square test was used (no expected count less than 5).

TABLE 4 | Possible risk factors for ESBL cases.

Risk factors	ESBL *n* (%)	Non-ESBL *n* (%)	*p*-value†
Systemic diseases**	4 (4.4)	18 (3.7)	0.763*
Renal disease	8 (8.8)	20 (4.1)	0.062*
PUV	3 (3.3)	1 (0.2)	**0.013***
VUR	13 (14.3)	14 (2.8)	**<0.001***
Recurrent UTI	36 (39.6)	76 (15.4)	**<0.001**
GU surgery	13 (14.3)	15 (3)	**<0.001***
Circumcision	10 (76.9)	69 (82.1)	0.703*
Urinary catheter	7 (7.7)	13 (2.6)	**0.024***
Non-GU surgery	18 (19.8)	48 (9.7)	**0.005**
Previous antibiotic use	34 (37.4)	46 (9.3)	**<0.001**
Use of suppressive antibiotic	15 (16.5)	12 (2.4)	**<0.001***
Previous hospitalization	19 (20.9)	26 (5.3)	**<0.001**
Immunocompromised***	12 (13.2)	22 (4.5)	**0.001**
Chemotherapy	11 (12.1)	7 (1.4)	**<0.001***
Radiation therapy	3 (3.3)	3 (0.6)	0.051*
Diarrhea in the preceding week	15 (16.5)	94 (19.1)	0.561
Constipation	6 (6.6)	21 (4.3)	0.411
Toilet training: urine	28 (30.8)	156 (31.6)	0.869
Toilet training: stool	29 (31.9)	157 (31.8)	0.997
CVC or Arterial line	14 (15.4)	20 (4.1)	**<0.001**
ETT	2 (2.2)	4 (0.8)	0.237*

PUV, Posterior urethral valve; CVC, Central venous catheter; ETT, Endotracheal tube. Significant differences for p-values are indicated in bold.
†Pearson Chi-Square was used (no expected count less than 5).
**Fisher's exact test was used (at least one expected count less than 5).*
***Systemic Diseases: Cardiac disease, liver disease, pulmonary disease and diabetes.*
****Primary or secondary to malignancy (lymphoma, leukemia, or solid tumor).*

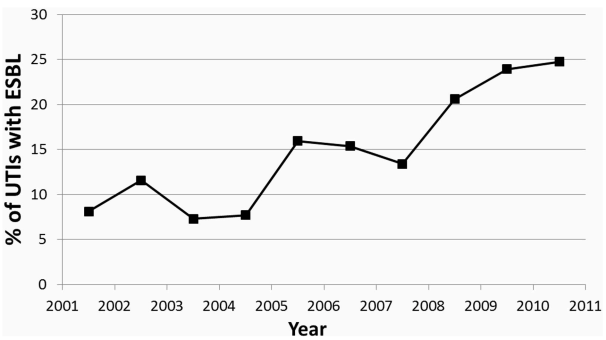

FIGURE 3 | Trend showing percentage of UTIs caused by ESBL-producing organisms over the 10 year period of the study.

cephalosporins ($p = 0.026$). No significant linear association was found for resistance to penicillin derivatives ($p = 0.09$), amikacin ($p = 0.444$), TMP-SMX ($p = 0.684$), and gentamicin ($p = 0.065$) across the years 2001–2011. Significant trends plotted are shown in **Figure 4**.

There was a strong linear association for resistance to fluoroquinolones across time ($r^2 = 0.698$), such that there was a 1.62% yearly increase in UTIs resistant to fluoroquinolones. There was a moderate linear association for resistance to

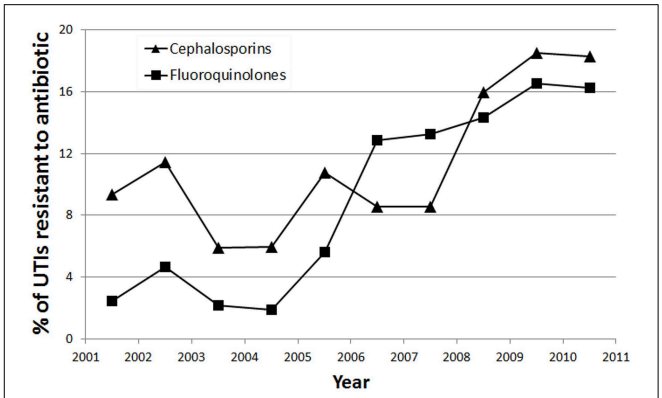

FIGURE 4 | Trend showing percentage of UTIs resistant to Cephalosporins (▲) and Fluoroquinolones (■) over the 10 year period of the study.

cephalosporins across time ($r^2 = 0.442$), such that there was a 1.21% yearly increase in UTIs resistant to cephalosporins.

Antibiotic Usage

The most common class of antibiotics used for inpatient treatment of UTIs in our study was cephalosporins, with 76.9% of patients with UTIs having used at least one cephalosporin during inpatient treatment. The most commonly used cephalosporins were third generation cephalosporins, used in 67.5% of cases. Second generation cephalosporins were used in 7.1% of cases, whereas fourth generation cephalosporins were used in 2.5% of cases. A penicillin derivative, most commonly ampicillin or amoxicillin-clavulanic acid, were used for inpatient treatment in 16% of cases. Aminoglycosides (amikacin or gentamicin) were used in 12.3% of cases. Carbapenems, glycopeptides, and fluroquinolones were used in 10.5, 3.3, and 1% of cases respectively. Simultaneous use of two or more antibiotics was practiced in 10.1% of cases; whereas 22.2% of cases required sequential use of multiple antibiotics.

Regarding antibiotic use on discharge, 66.2% of cases were discharged on at least one antibiotic with oral third generation cephalosporins used in 43.65% of cases.

Discussion

Epidemiologic Characteristics of UTI

UTIs are among the most frequent infections encountered in the pediatric population. To our knowledge this is the first Lebanese retrospective pediatric study that reviews the epidemiology of UTIs in hospitalized children, and outlines the risk factors and outcomes of UTIs caused by ESBL-producing *E. coli* and *Klebsiella* spp.

The epidemiology of pediatric UTIs depends on several factors such as age, gender, and genitourinary malformations. Our study showed an overall female predominance; this could be related to circumcision as 78 % of males in our study were circumcised. This is presumed to be the result of geographic location and religious affiliation. In addition, the shorter female urethra is a plausible

explanation of the increased prevalence of UTIs in females. Zorc et al. mentioned that UTI is prevalent in all children in their first year but after 1 year of age, it tends to decrease in males due to multiple factors such as circumcision (Circumcision, 1999).

Approximately, one-fifth of our patients (19.7%) with normal renal ultrasound had VUR, with approximately one-fourth (24.1%) being grade V. These findings were similar in comparison with other studies. Juliano et al. reported that despite normal renal bladder ultrasound, 24% of their patients had dilating vesicoureteral reflux (Juliano et al., 2013). Another study showed that the sensitivity of renal ultrasound in suggesting VUR was only 40% (Mahant et al., 2002). DiPietro et al found that renal ultrasound was unreliable in excluding VUR in children aged 5 years or older who were being evaluated for a UTI (Dipietro et al., 1997). Our study showed that approximately 5% of the patients who had normal renal ultrasound had VUR grade V needing surgical management, which means if VCUG is deferred, according to the last published guidelines in 2011(Roberts, 2011), parents should be counseled regarding this risk. Thus, if our findings are confirmed in other similar studies, the guidelines may have to be revisited.

The absence of pyuria in children with UTIs was found in a high percentage of cases (40%). This discordance was higher than that reported in previous studies (27%) (Roberts, 2011). There was no significant difference in the presence of pyuria between immunocompromised and non-immunocompromised patients (64.5% of immunocompromised patients had positive urinalysis WBC compared to 68% of non-immunocompromised patients). Nitrites were positive in only 39% of cases of UTI documented by urine culture. This is lower than the reported sensitivity in previous studies (53%) (Roberts, 2011). Leucocyte esterase was positive in 80.2% of cases of UTI documented by urine culture. This is similar to the reported sensitivity of leucocyte esterase in various studies (83%) (Roberts, 2011), making it a reliable test for predicting UTIs in young febrile children.

Risk Factors for ESBL

Our study revealed that *E. coli* is the most common pathogen in UTIs among all age groups, whether ESBL or non-ESBL producers. This finding is compatible with other studies (Calbo et al., 2006; Topaloglu et al., 2010; Kizilca et al., 2012; Dotis et al., 2013). Among *E. coli* and *Klebsiella* spp, the proportion of ESBL-producing bacteria was higher in *Klebsiella* spp. This was also reported by Kiziliza et al. suggesting that if the isolated microorganism of UTI belongs to *Klebsiella* spp, production of ESBL may be expected more frequently (Kizilca et al., 2012).

Lautenbach et al. revealed no association between hospital location and infection with ESBL-producing *E. coli* or *K. pneumonia* (Lautenbach et al., 2001). However, in our study, we found that ESBL infection is more common at AUBMC than MGH. This can be attributed to notable differences between the two hospitals. AUBMC is Lebanon's largest medical center, and has referral units for pediatric cardiology, oncology, neurosurgery and intensive care patients. Although both hospitals are tertiary care centers, MGH is a smaller center with fewer facilities than AUBMC. As such, the cases referred to AUBMC tend to be more complicated than cases admitted to MGH. As for the association with immunosuppression: immunosuppression secondary to malignancy and chemotherapy places the patient at higher risk of acquiring resistant bacteria (as ESBL producing bacteria) due to the multiple visits/stays in hospitals and the use of broad spectrum antibiotics.

Except for enuresis, there were no statistically significant differences in the presenting symptoms between ESBL and non-ESBL UTI. This is in contrast to a previous study (Dotis et al., 2013) that showed children with ESBL UTI presented clinically with more symptoms than children with non-ESBL UTI. In fact, our results indicate that UTIs secondary to ESBL-producing organisms are not associated with a more severe clinical picture.

Some of the risk factors for ESBL UTI that our study showed to be significant as recurrent UTIs, previous urological abnormalities, previous antibiotic use, previous hospitalization, and malignancies were found to be significant in other studies (Lautenbach et al., 2001; Topaloglu et al., 2010; Kizilca et al., 2012; Megged, 2014). However, only previous antibiotic use was found to be an independent risk factor. This is in contrast to the study by Topaloglu et al. (2010), that found previous antibiotic use was not an independent risk factor.

The mean length of hospital stay was significantly longer for patients with UTI caused by ESBL-producing bacteria than that for patients with UTI caused by non-ESBL-producing bacteria. This may be attributed to the fact that ESBL treatment in children necessitates parenteral antibiotic administration for 7–10 days; as opposed to adults, where oral Ciprofloxacin is a treatment option for ESBL UTI. Other studies also found that longer hospitalization periods are needed for the treatment of ESBL infections (Lautenbach et al., 2001; Megged, 2014). As quinolones become more routinely used in children and adolescents, this trend for longer hospitalizations with ESBL infections may be reversed but the rising quinolone resistance might cancel this hope.

Resistance Trends and Usage of Different Classes of Antibiotics

In our study over a 10-year period, there was an alarming increase in the incidence of ESBL-producing UTIs in hospitalized children, from 8% in 2001 to 25% in 2011 (**Figure 3**). A Lebanese study by Araj et al. on bacterial susceptibility patterns in all infections among all ages found nearly similar results within the same time period (Araj et al., 2012). Araj et al. demonstrated an increase of ESBL-producing *E. coli* and *Klebsiella* spp, from 4 and 12% in 2000, to 30 and 28% in 2011, respectively (Araj et al., 2012). A comparable finding was also demonstrated at another center in Lebanon by Daoud et al. (Daoud and Afif, 2011), who observed an increase in UTIs caused by ESBL-producing organisms from 2.3% in 2000 to 16.8% in 2009 among patients of all ages. Both studies attributed this finding to uncontrolled antimicrobial usage in Lebanon (Daoud and Afif, 2011; Araj et al., 2012).

Although percentage of UTIs resistant to TMP-SMX was considerably more than resistance to any other antibiotic, there has been no increase in resistance to TMP-SMX across the

years. One study in the United States showed that TMP-SMX is a poor empirical choice in many areas due to high resistance rates (Edlin et al., 2013). Our study revealed that in our setting, TMP-SMX still has a role in the treatment of UTIs; however it is usually reserved for use upon discharge and is infrequently used as inpatient treatment for hospitalized patients.

The trend in increasing resistance to fluoroquinolones has been well established in other studies. A study examining the epidemiology and resistance in uncomplicated UTI among adult females in Europe and Brazil found a significant increase in quinolone resistance of community-acquired urinary *E. coli* (Schito et al., 2009). One review attributed this trend in increasing fluoroquinolone resistance to overuse of this class of antibiotics (Nickel, 2007). While the results on antibiotic usage within our cohort reveal relatively controlled usage of fluoroquinolones, one must keep in mind that our results only shed light on antibiotic practice in treating UTI in hospitalized children. It is likely that there is in fact overuse of fluoroquinolones in our population's healthcare system, especially among adults in outpatient settings, leading to community-acquired resistance. In addition, as a study conducted in Canada has shown (Pepin et al., 2009), risk factors for the development of UTIs resistant to ciprofloxacin included the use of aminoglycosides in the last 12 months. Therefore, the trend of increasing resistance of uropathogens to fluoroquinolones may also be due to cross-reactivity from overuse of other antibiotics.

We also observed a significant trend of increasing resistance to cephalosporins over the study's 10 year period (**Figure 4**). This is likely due to overuse of antibiotics in our community, as our study has shown that the majority (76.9%) of hospitalized UTIs were treated with cephalosporins, specifically third generation cephalosporins. Our dependence on cephalosporins in light of increasing resistance will pose a challenge in the future, and emphasizes the need to find alternative antibiotics.

Limitations

Although our study is the first to investigate the epidemiology of UTIs and risk factors for ESBL in the pediatric population in Lebanon, it is important to note certain limitations. Our study only looked at hospitalized children with UTI, and therefore our findings may not apply to the general pediatric population. Furthermore, the number of cases included in our study was restricted by our criteria for defining UTI. A significant number of cases excluded due to negative or low colony count on urine culture are likely the result of over-the-counter antibiotic use before the culture was taken. A study examining the value of semi-quantitative bacterial counts in establishing the diagnosis of UTI caused by group B streptococcus in adults demonstrated occurrence of UTI at values as low as 100 CFU/ml (Tan et al., 2012). Similarly, in abiding by the cutoffs mentioned in our study we may have missed a substantial number of UTI cases with subthreshold colony counts. Finally, our study is not immune to the inherent limitations of a retrospective chart review; the cases included in our study were limited by the availability of data and incomplete medical records, such as missing laboratory results for urine cultures or other studies that were done in outside hospitals.

Conclusion

In conclusion, the resistance to commonly used antibiotics for UTIs has been increasing over the last 10 years, as ESBL-producing organisms are emerging. The recognition of the epidemiology and risk factors for ESBL-producing bacteria in the pediatric population may affect our management and therapeutic approach. In keeping with our finding that previous antibiotic use was the most important independent risk factor for emergence of ESBL-producing bacteria, further studies and new recommendations that guide management of UTIs and antibiotic use are warranted.

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Third generation cephalosporin resistant *Enterobacteriaceae* and multidrug resistant gram-negative bacteria causing bacteremia in febrile neutropenia adult cancer patients in Lebanon, broad spectrum antibiotics use as a major risk factor, and correlation with poor prognosis

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Introduction: Bacteremia remains a major cause of life-threatening complications in patients receiving anticancer chemotherapy. The spectrum and susceptibility profiles of causative microorganisms differ with time and place. Data from Lebanon are scarce. We aim at evaluating the epidemiology of bacteremia in cancer patients in a university hospital in Lebanon, emphasizing antibiotic resistance and risk factors of multi-drug resistant organism (MDRO)-associated bacteremia.

Materials and Methods: This is a retrospective study of 75 episodes of bacteremia occurring in febrile neutropenic patients admitted to the hematology-oncology unit at Makassed General Hospital, Lebanon, from October 2009-January 2012.It corresponds to epidemiological data on bacteremia episodes in febrile neutropenic cancer patients including antimicrobial resistance and identification of risk factors associated with third generation cephalosporin resistance (3GCR) and MDRO-associated bacteremia.

Results: Out of 75 bacteremias, 42.7% were gram-positive (GP), and 57.3% were gram-negative (GN). GP bacteremias were mostly due to methicillin-resistant coagulase negative staphylococci (28% of total bacteremias and 66% of GP bacteremias). Among the GN bacteremias, *Escherichia coli* (22.7% of total, 39.5% of GN organisms) and *Klebsiella pneumoniae*(13.3% of total, 23.3% of GN organisms) were the most important causative agents. GN bacteremia due to 3GC sensitive (3GCS) bacteria represented 28% of total bacteremias, while 29% were due to 3GCR bacteria and 9% were due to carbapenem-resistant organisms. There was a significant correlation between bacteremia with MDRO and subsequent intubation, sepsis and mortality. Among potential risk factors, only broad spectrum antibiotic intake >4 days before bacteremia was found to be statistically significant for acquisition of 3GCR bacteria. Using carbapenems or piperacillin/tazobactam>4 days before bacteremia was significantly associated with the emergence of MDRO ($p < 0.05$).

Conclusion: Our findings have major implications for the management of febrile neutropenia, especially in breakthrough bacteremia and fever when patients are already on broadspectrum antibiotics. Emergence of resistance to 3GCs and, to a lesser extent, to carbapenems in GN isolates has to be considered seriously in our local guidelines for empiric treatment of febrile neutropenia, especially given that their occurrence was proven to be associated with poorer outcomes.

Keywords: febrile neutropenia, bacteremia, 3GCR gram-negative bacteria, MDR gram-negative bacteria, Lebanon

INTRODUCTION

The progress of anticancer therapy with aggressive supportive care for patients with malignancies and patients undergoing hematopoietic stem cell transplantation (HSCT) have recently improved patient prognosis (Trecarichi and Tumbarello, 2014). However, these advances, resulting in a prolonged and profound level of immunosuppression, neutropenia in particular, along with the extensive use of implantable medical devices, have also increased the risk of severe infections (Trecarichi and Tumbarello, 2014). Different types of infections may occur in cancer patients, but bloodstream infections (BSIs) are the most common severe infectious complications; the reported prevalence of BSIs ranges from 11 to 38%, and the crude mortality rate reaches up to 40% (Wisplinghoff et al., 2003a,b; Tumbarello et al., 2012; Montassier et al., 2013).

The type of microorganisms isolated on blood culture from febrile neutropenic patients varies with time and place (Jones, 1999; Dettenkofer et al., 2003; Wisplinghoff et al., 2003b; Irfan et al., 2008; Freifeld et al., 2011). Data from the Middle East and North Africa (MENA) region is scarce.

At the beginning of the use of cytotoxic chemotherapy in the 1960s and 1970s in cancer patients, gram-negative bacteria (GNB) were the most common organisms causing bacteremia in febrile neutropenic patients (Jones, 1999; Irfan et al., 2008); however, at the turn of the century, the most common bacterial pathogens isolated from blood cultures were coagulase-negative staphylococci (Dettenkofer et al., 2003; Wisplinghoff et al., 2003b; Freifeld et al., 2011).

However, in recent years several studies have demonstrated a clear trend in the epidemiology of BSIs, showing a shift of prevalence from gram-positive to gram-negative bacteria (Wisplinghoff et al., 2003b; Pagano et al., 2012; Montassier et al., 2013). There is an emergence of drug-resistant GNB such as multidrug resistant (MDR) *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, extended-spectrum beta-lactamase (ESBL)-producing GNB, and carbapenemase-producing GNB (Zinner, 1999; Wisplinghoff et al., 2003b; Ramphal, 2004; Freifeld et al., 2011; Pagano et al., 2012; Wu et al., 2012; Montassier et al., 2013). The issue of antimicrobial resistance has become a significant problem worldwide, where treatment of infections due to MDR bacteria represents a clinical challenge because the therapeutic options are often very limited. Risk factors of bacteremia due to MDR GNB in febrile neutropenic cancer patients vary depending on the type of organism, duration of hospitalization, and antibiotic therapy (Gudiol and Carratala, 2014).

So, the fact that the epidemiology of pathogens is dynamic makes contemporary local data extremely important, and identification of pathogens locally recovered from blood cultures of febrile neutropenic patients and the patterns of their antibiotic susceptibilities are essential in making therapeutic decisions (Sigurdardottir et al., 2005).

The purpose of this study is to evaluate the epidemiology of bacteremia occurring during neutropenia in adult cancer chemotherapy patients in a university hospital in Lebanon, with a special emphasis on the prevalence, susceptibility profile and risk factors associated with bacteremia caused by third generation cephalosporin-resistant (3GCR) and carbapenem-resistant bacteria.

MATERIALS AND METHODS

SETTING, PATIENTS AND STUDY DESIGN

This is retrospective study performed at Makassed General Hospital, a 200-bed university hospital in Beirut, Lebanon. The hospital's Institutional Review Board approved the study, and an informed consent was waived since it was observation. Medical records of patients admitted between October 2009 and January 2012 were reviewed. Seventy-five episodes of bacteremia occurring in 70 hospitalized neutropenic adult patients were recorded.

Adult cancer patients with fever and neutropenia, including those undergoing Hematopoietic Stem Cell Transplantation (HSCT), with positive blood cultures were selected. All positive results of blood cultures with the corresponding antibiogram were checked and recorded from the Microbiology Laboratory log books and computerized laboratory records. Information regarding these episodes were collected and recorded in a specific database from patients' medical records.

Patient characteristics were identified, including age, gender, type of cancer, risk level, recurrence of admissions, duration of neutropenia prior to bacteremia, hospital stay prior to bacteremia, presence of a focus of infection, presence of a central line or an implantable venous access, outcome, sepsis, intubation, HSCT, antibiotic and antifungal intake in the hospital setting. Univariate analysis of these characteristics was used to identify risk factors for3GCR and multi-drug resistant (MDR) organism-associated bacteremia.

DEFINITIONS AND INCLUSION CRITERIA

Patients were included if they met all three of the following inclusion criteria:

(1) Fever, defined as a single oral temperature of 38.3°C or an oral temperature of 38°C lasting 1 h or more.
(2) Neutropenia, defined as a neutrophil count of <500 cells/mm^3, or a count of <1000 cells/mm^3 with a documented decrease to <500 cells/mm^3 within the following 48–72 h.
(3) Receipt of chemotherapy prior to the episode of febrileneutropenia.

Patients who had fever and neutropenia as a result of their underlying disease without having received chemotherapy were excluded.

In Lebanon, anaerobic cultures are performed only in reference laboratories. Hospital-based laboratories perform only aerobic cultures. According to hospital policy, one set of blood cultures consists of two bottles of aerobic cultures taken from two different draws at the same time, with no bottles for anaerobic culture. Bacteremia is defined as isolation of the same bacterial or pathogen from at least one set of blood cultures (2 bottles taken at the same time). Bacteremia is considered polymicrobial if at least two organisms from the same blood culture on two occasions are isolated or more than one organism each in at least two separate blood cultureswithin 48 h (Reuben et al.,

1989). Bacteremia occurring more than 14 days after a previous episode and separated by repeatedly negative blood cultures was considered a separate episode. Each separate hospital admission for febrile neutropenia was defined as one episode. Subsequent hospital admissions for febrile neutropenia in the same patient were included as separate cases.

Bacteremia caused by a potential skin contaminant (such as coagulase-negative staphylococci, *Bacillus*, or *Corynebacterium* species) was considered significant only if it met the following criteria:

(1) Growth of the same bacterial strainin two blood cultures taken from two different sites at the same time.
(2) Growth of the same bacterial strain in one blood culture and in one other sterile site (urine, cerebrospinal fluid, ascetic fluid, pleural fluid, joint fluid).
(3) Growth of the same bacterial strain in one peripheral blood culture and one blood culture taken from an intravenous catheter where both cultures were taken at the same time.

Interpretive criteria (breakpoints) for susceptible, intermediate, and resistant bacterial isolates were those included in the Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute, 2010).

The 3GCR *Enterobacteriaceae* phenotypes included all isolates not susceptible to one or more of five agents including aztreonam, cefotaxime, ceftizoxime, ceftazidime, and ceftriaxone (Clinical and Laboratory Standards Institute, 2010). These isolates were, however, susceptible to imipenem.

Many different definitions for multidrug resistance are used in the medical literature to characterize different patterns of resistance in healthcare-associated, antimicrobial-resistant bacteria (Magiorakos et al., 2012).However, generally speaking, MDR-Gram negative bacteria are resistant to key antimicrobial agents (Siegel et al., 2007; Hidron et al., 2008). Third and fourth generation cephalosporins, along with fluoroquinolones, aminoglycosides, and carbapenems, constitute the major therapeutic options in treatment guidelines of febrile neutropenia in adult cancer patients (Freifeld et al., 2011; Averbuch et al., 2013). In this study, gram-negative bacteria were considered MDR when resistant to third and fourth generation cephalosporins, fluoroquinolones, aminoglycosides and carbapenems, including *S. maltophilia*, carbapenem-resistant *P. aeruginosa* and *Acinetobacter baumannii*, or carbapenem-resistant 3GCR *Enterobacteriaceae*.

STATISTICAL ANALYSIS

Data were reported as the mean standard deviation (SD) or number of patients (percentage). *T*-tests (two-tailed), Fisher's exact tests and Chi-square tests were used to assess any significant differencesamong the groups. $P < 0.05$ were considered statistically significant.

RESULTS

During the study period, 75 episodes of bacteremia occurring in 70 hospitalized neutropenic adult patients with hematological malignancies, including those undergoing HSCT, were recorded. Epidemiological and clinical characteristics of the patients, antibiotic treatment, and patient outcomes are shown in **Table 1**.

Of the 75 bacteremias, 42.7% were due to gram-positive organisms, and the remaining 57.3% were gram-negative.

Table 1 | Epidemiological and clinical characteristics of all episodes of bacteremia.

Characteristic	Total [*n* = 75(%)]
AGE (YEARS)	
0–18	5 (6.67)
18–65	62 (82.67)
>65	8 (10.67)
GENDER	
Male	36 (48)
Female	39 (52)
TUMOR TYPE	
Leukemia	56 (74.67)
Lymphoma	15 (20)
Solid	4 (5.33)
MASCC score	
>21	5 (6.67)
<21	70 (93.33)
NEUTROPENIA DURATION PRIOR TO BACTEREMIA	
<7 days	26 (34.67)
>7 days	26 (34.67)
Unknown	23 (30.67)
HOSPITAL STAY PRIOR TO BACTEREMIA	
<2 days	21 (28)
Between 2 and 7 days	4 (5.33)
>7 days	48 (64)
Unknown	2 (2.67)
RECURRENT ADMISSION	
Yes	67 (89.33)
No	8 (10.67)
FOCUS OF INFECTION	
No/Unknown	17 (22.67)
Pneumonia	3 (4)
Gastroenteritis	13 (17.33)
Urinary tract infection	7 (9.33)
Skin and soft tissue infection	3 (4)
Central line associated	30 (40)
Portal catheter associated	2 (2.67)
PLACEMENT OF CENTRAL VENOUS CATHETER	
Yes	52 (69.33)
<10 days	18 (24)
>10 days	34 (45.33)
No	23 (30.67)
OUTCOME	
Death	7 (9.33)
Recovery	68 (90.67)
Sepsis	8 (10.67)
Intubation	7 (9.33)
Hematopoietic stem cell transplantation	41 (54.67)

N.B. MASCC, Multinational Association of Supportive Care in Cancer.

Gram-positive bacteremias were mostly due to methicillin-resistant coagulase negative staphylococci, which represented 66% of gram-positive bacteremias and 28% of total bacteremias. No methicillin-resistant *Staphylococcus aureus*-related bacteremia was detected and only one episode of methicillin-sensitive *Staphylococcus aureus*-related bacteremia was observed. (Refer to **Tables 2**, **3**).

Among the gram-negative bacteremias, *Escherichia coli* (22.7% of total, 39.5% of gram-negative) and *Klebsiella pneumonia* (13.3% of total, 23.3% of gram-negative)were the most important causative agents. Out of the 17 bacteremias caused by *E. coli*, eight were due to 3GCR resistant strains (10.7% of total bacteremias and 47% of *E. coli* strains). From those caused by *K. pneumoniae* (10 bacteremias), five cases were due to 3GCR strains (6.7% of total bacteremia and 50% of *K. pneumoniae*). In general, 28% of the total bacteremias were due to 3GC sensitive gram-negative bacteria and 29.3% of the total bacteremias were caused by 3GC resistant gram-negative bacteria. Concerning carbapenem susceptibility in the 3GC resistant category, seven cases were carbapenem-resistant, representing 9.3% of total bacteremias. (Refer to **Tables 2**, **4**).

Results of the univariate analysis of factors potentially associated with 3GCR bacteremia, including baseline and demographic characteristics, major disease and risk, hospitalization prior to bacteremia, recurrent admissions, neutropenia prior to bacteremia, presence of CVC, and focus of infection, did not show any statistical significance, suggesting that the former factors were not risk factors. However, history and duration of antibiotic intake before the episode of bacteremia was majorly implicated in the occurrence of 3GCR bacteremia. The type of broad spectrum antibiotic use did not affect the results; butduration of intake did affect results. The use of carbapenems, piperacillin/tazobactam, or 3rd or 4th GC ± aminoglycosides for more than 4 days prior to the bacteremic episode was significantly associated with 3GCR bacteremia compared with all other types of bacteremias. ($P < 0.01$) A worse outcome, defined by need for intubation and the occurrence of sepsis in bacteremic patients, was also statistically significant in the 3GCR group compared with the other groups. ($P < 0.03$) (Refer to **Tables 5**, **6**).

Similarly, infection with an MDR strain was associated with significantly higher rates of subsequent intubation, sepsis, and mortality. ($P < 0.03$)The history and duration of antibiotic intake before the episode of bacteremia was significantly associated with the occurrence of MDR bacteremia as well. The use of cephalosporins ± aminoglycosides was not significantly associated with MDR bacteremia, while the use of carbapenems or piperacillin/tazobactam for more than 4 days prior to MDR-bacteremia was significantly associated with its occurrence. ($P < 0.04$) (Refer to **Tables 5**, **6**).

Table 2 | Causative organisms of all episodes of bacteremia.

Causative organisms	Number of episodes [$n = 75$(%)]
gram-positive Bacteria	32 (42.67)
Staphylococcus aureus	1 (1.33)
Coagulase-negative staphylococci (CNS)	28 (37.33)
Methicillin resistant CNS	21 (28)
Streptococcus pneumoniae	1 (1.33)
Enterococcus faecium	1 (1.33)
Aerococcus viridans	1 (1.33)
gram-negative Bacteria (GNB)	43 (57.33)
Escherichia coli	17 (22.67)
3GCR -*Escherichia coli*	8 (10.67)
Pseudomonas aeruginosa	3 (4)
Pseudomonas putida	1 (1.33)
Pseudomonas stutzeri	1 (1.33)
Klebsiella pneumoniae	10 (13.33)
3GCR- *Klebsiella pneumoniae*	5 (6.67)
Klebsiella oxytoca	1 (1.33)
Proteus mirabilis	3 (4)
Enterobacter cloacae	2 (2.67)
Acinetobacter baumannii	2 (2.67)
Stenotrophomonas maltophilia	2 (2.67)
Salmonella species	1 (1.33)
3GC sensitive GNB	21 (28)
3GCR GNB	22 (29.33)
3GCR carbapenem-sensitive GNB	15 (20)
3GCR carbapenem-resistant GNB	7 (9.33)

N.B. 3GCR, third generation cephalosporin-resistant.

DISCUSSION

Data from Lebanon in neutropenic patients is scarce, and the available literature reveals the dynamic nature of the etiology of bacteremias with time. Previous studies from Lebanon have shown gram-negative organisms to be the predominant agents in febrile neutropenic patients (Hamzeh et al., 2000; Kanafani et al., 2007). In addition to the subdivision between gram-positive and gram-negative organisms, we investigated antibiotic susceptibility patterns and the risk factors associated with bacteremia caused by antibiotic-resistant gram-negative organisms.

In this study, the distribution of the 75 cases was almost equal between gram-positive and gram-negative organisms (42.7 vs. 57.3%). Yet, a study by Kanafani et al. (2007) in 2007 from Lebanon showed gram-negative predominance in febrile neutropenia bacteremic episodes. Gram-negative organisms were responsible for 78.8% of bloodstream infections compared with 33.3% gram-positive organisms (Kanafani et al., 2007).

In fact, the shift from a preponderance of gram-negative bacteria causing bloodstream infections in febrile neutropenic patients to gram-positive bacteria has been observed worldwide. The widespread use of indwelling catheters, early-generation fluoroquinolone prophylaxis, and broad spectrum empirical anti-gram-negative antibacterial therapy led to an increase in the incidence of gram-positive pathogens in the 1980s and 1990s (Zinner, 1999; Ramphal, 2004; Freifeld et al., 2011). Thereafter, the most common bacterial etiologic agent isolated from blood cultures in most centers was reported to be coagulase-negative staphylococci (Dettenkofer et al., 2003; Wisplinghoff et al., 2003b; Freifeld et al., 2011). In our study, coagulase-negative staphylococci were the most common among gram-positive organisms (37.3% of total cases, 87.5% of gram-positive cases), where methicillin-resistant

Table 3 | Antibiotic susceptibility profile of isolated gram-positive bacteria.

Antibiotic	No of susceptible gram-positive isolates (%)					
	CNS [*n* = 28(%)]	***S. aureus* [*n* = 1(%)]**	***S. pneumoniae* [*n* = 1(%)]**	***E. faecium* [*n* = 1(%)]**	***A. viridans* [*n* = 1(%)]**	**Total [*n* = 32(%)]**
Oxacillin	7 (25)	1 (100)	1 (100)	0 (0)	0 (0)	9 (28.1)
Ampicillin	–	1 (100)	1 (100)	0 (0)	0 (0)	10 (31.3)
Rifampin	24 (85.7)	1 (100)	1 (100)	0 (0)	0 (0)	26 (81.3)
Clindamycin	16 (57.1)	1 (100)	1 (100)	0 (0)	1 (100)	19 (59.4)
Teicoplanin	28 (100)	1 (100)	1 (100)	1 (100)	1 (100)	32 (100)
Vancomycin	28 (100)	1 (100)	1 (100)	1 (100)	1 (100)	32 (100)

N.B. A.viridans, Aerococcus viridans; CNS, Coagulase Negative Staphylococci; E. faecium, Enterococcus faecium; S. aureus, Staphylococcus aureus; S. pneumoniae, Streptococcus pneumonia.

Table 4 | Antibiotic susceptibility profile of isolated gram-negative bacteria.

Antibiotic	No. of susceptible gram-negative isolates (%)								
	***E. coli* [*n* = 17(%)]**	***Klebsiella* spp. [*n* = 11(%)]**	***Pseudomonas* spp. [*n* = 5(%)]**	***P.mirabilis* [*n* = 3(%)]**	***Enterobacter* spp. [*n* = 2(%)]**	***S. maltophilia* [*n* = 2(%)]**	***A. baumannii* [*n* = 2(%)]**	***Salmonella* spp. [*n* = 1(%)]**	**Total [*n* = 43(%)]**
Amox/Clav	5 (29.4)	5 (45.5)	0 (0)	3 (100)	0 (0)	0 (0)	0 (0)	1 (100)	11 (25.6)
Amikacin	15 (88.2)	10 (90.9)	5 (100)	3 (100)	2 (100)	0 (0)	1 (50)	1 (100)	37 (86)
Cefepime	5 (29.4)	6 (54.5)	3 (60)	3 (100)	1 (50)	0 (0)	0 (0)	1 (100)	19 (44.2)
Ceftazidime	8 (57.1)	5 (45.5)	4 (80)	3 (100)	0 (0)	1 (50)	1 (50)	1 (100)	23 (53.5)
Ceftriaxone	8 (57.1)	6 (54.5)	2 (40)	3 (100)	0 (0)	0 (0)	0 (0)	1 (100)	19 (44.2)
Imipenem	14 (82.4)	11 (100)	4 (80)	3 (100)	2 (100)	0 (0)	1 (50)	1 (100)	36 (83.7)
Pip/Tazo	13 (76.5)	7 (63.6)	5 (100)	3 (100)	0 (0)	0 (0)	1 (50)	1 (100)	30 (69.8)
Tigecycline	17 (100)	10 (90.9)	1 (20)	3 (100)	2 (100)	2 (100)	2 (100)	1 (100)	40 (93)
Colistin	17 (100)	11 (100)	4 (80)	3 (100)	2 (100)	0 (0)	2 (100)	1 (100)	40 (93)
Quinolones	7 (41.2)	7 (63.6)	4 (80)	1 (33.3)	0 (0)	2 (100)	1 (50)	0 (0)	22 (51.2)

N.B. A.baumannii, Acinetobacter baumannii; Amox/Clav, Amoxicillin/Clavulanate; E. coli, Escherichia coli; Pip/Tazo, Piperacillin/Tazobactam; P.mirabilis, Proteus mirabilis; S. maltophilia, Stenotrophomonas maltophilia; spp, species.

coagulase-negative staphylococciisolatesrepresented 28% of the total cases and 65.6% of gram-positive cases.

Our results showed that *E. coli* and *K. pneumoniae* were the most prevalent gram-negative organisms, representing 22.7 and 13.3%, respectively, of total cases. A systematic literature review conducted by Trecarichi and Tumbarello (2014) from January 2007 to August 2013 examined the recent trends in epidemiology and antimicrobial resistance in gram-negative bacteria recovered from cancer patients, with a particular emphasis on the impact of antimicrobial resistance on the clinical outcome of severe infections caused by such microorganisms. The gram-negative bacterial species most frequently isolated were *E. coli*, whose frequency ranged from 10.1 to 53.6% (mean 32.1%), and *K. pneumoniae*, which was isolated with a frequency ranging from 4.1 to 44.6% (mean 19.5%) (Trecarichi and Tumbarello, 2014).

In our study, 3GCR strains of *E. coli* and *K. pneumoniae* caused 10.7 and 6.7% of total bacteremias, respectively. 3GCR-*Enterobacteriaceae* colonization or infection of patients with febrile neutropenia has been reported with increased frequency during the last decade. Bloodstream infections due to 3GCR *E. coli* isolates have been reported with a frequency ranging from 12 to 75% (mean 35%) in cancer patients. 3GCR *K. pneumoniae* isolates causing BSIs in neutropenic patients have been reported with a frequency ranging from 3 to 66.6% (mean 37.8%) (Trecarichi and Tumbarello, 2014).

We found that 9.3% of episodes of bacteremia in cancer patients were caused by MDR gram-negative bacteria. This finding is in line with recent studies, which report an increase in antibiotic resistance among gram-negative bacteria in immunocompromised hosts. One study performed in Italy by Gudiol et al. (2011) reported an incidence of 13.7% of MDR gram-negative associated bacteremia, andin another study performed in Pakistan by Irfan et al. (2008), the emergence of carbapenem resistance was reported in *Pseudomonas* species (20.7% of the isolates) and in *Acinetobacter* species (65.4% of the isolates). In another study by Trecarichi et al. (2011) from Italy, among 38 patients diagnosed with *P. aeruginosa* bacteremia, 27 were MDR species (71.1%). The percentages of *in vitro* resistance to major antimicrobial classes were the following: carbapenems (imipenem and meropenem) 60%, antipseudomonal cephalosporins

Table 5 | Baseline and demographic characteristics, clinical features, and outcome of patients with 3GCR-bacteremia compared with non-3GCR-bacteremia and MDR-bacteremia compared with non-MDR-bacteremia.

Characteristic	3GCR-Bacteremia [*n* = 22(%)]	Non-3GCR-Bacteremia [*n* = 53(%)]	*P*-value	MDR Bacteremia [*n* = 7(%)]	Non-MDR Bacteremia [*n* = 68(%)]	*P*-value
AGE (YEARS)						
0–18	1 (4.5)	4 (7.5)		0 (0)	5 (7.4)	
18–65	19 (86.4)	43 (81.1)		6 (85.7)	56 (82.4)	
>65	2 (9.1)	6 (11.3)	0.846	1 (14.3)	7 (10.3)	0.734
GENDER						
Male	9 (40.9)	27 (50.9)		3 (42.9)	33 (48.5)	
Female	13 (59.1)	26 (49.1)	0.428	4 (57.1)	35 (51.5)	0.775
TUMOR TYPE						
Leukemia	17 (77.3)	39 (73.6)		6 (85.7)	50 (73.5)	
Lymphoma	4 (18.2)	11 (20.8)		0 (0)	15 (22.1)	
Solid	1 (4.5)	3 (5.7)	0.944	1 (14.3)	3 (4.4)	0.243
MASCC SCORE						
>21	0 (0)	5 (9.4)		0 (0)	5 (7.4)	
<21	22 (100)	48 (90.6)	0.136	7 (100)	63 (92.6)	0.458
NEUTROPENIA DURATION PRIOR TO BACTEREMIA						
<7 days	8 (36.4)	15 (28.3)		3 (42.9)	20 (29.4)	
>7 days	7 (31.8)	19 (35.8)		1 (14.3)	25 (36.8)	
Unknown	7 (31.8)	19 (35.8)	0.789	3 (42.9)	23 (33.8)	0.485
HOSPITAL STAY PRIOR TO BACTEREMIA						
<2 days	7 (31.8)	14 (26.4)		2 (28.6)	19 (27.9)	
Between 2 and 7 days	2 (9.1)	2 (3.8)		1 (14.3)	3 (4.4)	
>7 days	13 (59.1)	35 (66)		4 (57.1)	44 (64.7)	
Unknown	0 (0)	2 (3.8)	0.586	0 (0)	2 (2.9)	0.700
RECURRENT ADMISSION						
Yes	2 (9.1)	6 (11.3)		0 (0)	8 (11.8)	
No	20 (90.9)	47 (88.7)	0.776	7 (100)	60 (88.2)	0.337
FOCUS OF INFECTION						
No/Unknown	3 (13.6)	14 (26.4)		1 (14.3)	16 (23.5)	
Pneumonia	1 (4.5)	2 (3.8)		1 (14.3)	2 (2.9)	
Gastroenteritis	0 (0)	3 (5.7)		0 (0)	3 (4.4)	
UTI	6 (27.3)	7 (13.2)		2 (28.6)	11 (16.2)	
SStI	8 (36.4)	22 (41.5)		2 (28.6)	28 (41.2)	
Central line associated	1 (4.5)	1 (1.9)		0 (0)	2 (2.9)	
Portal catheter associated	3 (13.6)	4 (7.5)	0.507	1 (14.3)	6 (8.8)	0.702
PLACEMENT OF CVC						
Yes	7 (31.8)	16 (30.2)		2 (28.6)	21 (30.9)	
<10 days	6 (27.3)	12 (22.6)		3 (42.9)	15 (22.1)	
>10 days	9 (40.9)	25 (47.2)		2 (28.6)	32 (47.1)	
No	7 (31.8)	16 (30.2)	0.866	2 (28.6)	21 (30.9)	0.442
OUTCOME						
Death	5 (22.7)	2 (3.8)		4 (57.1)	3 (4.4)	
Recovery	17 (77.3)	51 (96.2)	0.010	3 (42.9)	65 (95.6)	<0.0001
Sepsis	5 (22.7)	3 (5.7)	0.029	4 (57.1)	4 (5.9)	<0.0001
Intubation	5 (22.7)	2 (3.8)	0.010	4 (57.1)	3 (4.4)	<0.0001
HSCT	13 (59.1)	29 (54.7)	0.728	4 (57.1)	38 (55.9)	0.949

N.B. MASCC, Multinational Association of Supportive Care in Cancer, UTI, Urinary tract infection; SStI, Skin and soft tissue Infection; CVC, Central Venous Catheter; HSCT, Hematopoietic stem cell transplantation.

(ceftazidime and cefepime) 42%, and piperacillin 24% (Trecarichi et al., 2011).

The emergence of 3GCR *Enterobacteriaceae* and MDR gram-negative organisms causing bacteremia in cancer patients is very critical in terms of empiric therapy for febrile neutropenia. Third and fourth generation cephalosporins remain the first line option for primary therapy in febrile neutropenia (Freifeld et al., 2011; Averbuch et al., 2013). In this group of patients, appropriate initial empirical antibiotic therapy is essential, (Gudiol and Carratala, 2014) and empiric antibiotic therapy in febrile

Table 6 | Antimicrobial history and duration prior to bacteremia in all cases, in patients with 3GCR- bacteremia compared with non-3GCR-bacteremia and in MDR-bacteremiacompared with non-MDR-bacteremia.

Antimicrobial duration before bacteremia	Total $n = 75$ (%)	3GCR-Bacteremia [$n = 22$(%)]	Non-3GCR-Bacteremia [$n = 53$(%)]	*P*-value	MDR-Bacteremia [$n = 7$(%)]	Non-MDR-Bacteremia [$n = 68$(%)]	*P*-value
Carbapenem (Group1) <4 days	8 (10.67)	1 (4.5)	7 (13.2)	0.269	1 (14.3)	7 (10.3)	0.745
Carbapenem>4 days	14 (18.67)	8 (36.4)	6 (11.3)	0.011	4 (57.1)	10 (14.7)	0.006
No Carbapenem	55 (73.33)	1 (4.5)	4 (7.5)	0.635	1 (14.3)	4 (5.9)	0.396
PIP/TAZ (Group2) <4 days	5 (6.67)	5 (22.7)	2 (3.8)	0.010	2 (28.6)	5 (7.4)	0.066
PIP/TAZ >4 days	7 (9.33)	3 (13.6)	12 (22.6)	0.375	3 (42.9)	12 (17.6)	0.112
No PIP/TAZ	65 (86.67)	6 (27.3)	5 (9.4)	0.047	2 (28.6)	9 (13.2)	0.275
3rd or 4th GC ± Amikacin (Group3) <4 days	15 (20)	3 (13.6)	12 (22.6)	0.375	3 (42.9)	12(17.6)	0.112
3rd or 4th GC ± Amikacin>4 days	11 (14.67)	8 (36.4)	6 (11.3)	0.011	3 (42.9)	11 (16.2)	0.085
No 3rd or 4th GC ± Amikacin	51 (68)	2 (9.1)	11 (20.8)	0.224	2 (28.6)	11 (16.2)	0.409
Group 1 or 2 or 3 <4 days	15 (20)	7 (31.8)	5 (9.4)	0.016	3 (42.9)	9 (13.2)	0.042
Group 1 or 2 or 3 >4 days	14 (18.67)	2 (9.1)	5 (9.4)	0.963	2 (28.6)	5 (7.4)	0.066
No group 2 or 3 or 4	49 (65.33)	4 (18.2)	5 (9.4)	0.288	2 (28.6)	7 (10.3)	0.156
Carbapenem or PIP/TAZ <4 days	13 (17.33)	1 (4.5)	7 (13.2)	0.269	1 (14.3)	7 (10.3)	0.745
Carbapenem or PIP/TAZ >4 days	12 (16)	8 (36.4)	6 (11.3)	0.011	4 (57.1)	10 (14.7)	0.006
No Carbapenem or PIP/TAZ	52 (69.33)	1 (4.5)	4 (7.5)	0.635	1 (14.3)	4 (5.9)	0.396
Antifungal other than Fluconazole <4 days	7 (9.33)	5 (22.7)	2 (3.8)	0.010	2 (28.6)	5 (7.4)	0.066
Antifungal other than Fluconazole >4 days	9 (12)	3 (13.6)	12 (22.6)	0.375	3 (42.9)	12 (17.6)	0.112
No Antifungal other than Fluconazole	62 (82.67)	6 (27.3)	5 (9.4)	0.047	2 (28.6)	9 (13.2)	0.275

N.B. PIP/TAZ, Piperacillin/Tazobactam; 3rd or 4th GC, Third or Fourth Generation Cephalosporin.

neutropenic patients has reduced mortality rates from approximately 21% (Viscoli et al., 2005) to 2–10%, (Vidal et al., 2004; Toussaint et al., 2006) depending upon the underlying diagnosis, degree of cancer control, duration of severe neutropenia and type of infection. Inappropriate empiric therapy is defined, in context, as not including at least one antibiotic active *in vitro* against the infecting microorganism(s) (Freifeld et al., 2011; Averbuch et al., 2013), and the emergence of 3GCR and MDR organisms in febrile neutropenia renders 3rd or 4th GCs inappropriate choices in certain situations.

Our results showed that the use of broad spectrum antibiotics, including carbapenems, piperacillin/tazobactam, and 3rd or 4th GC ± aminoglycosides, for more than 4 days prior to bacteremia was significantly associated with 3GCR bacteremia. ($P < 0.01$) However, in the case of carbapenem-resistant 3GCR bacteremia (MDR), the use of carbapenems or piperacillin/tazobactam, but not cephalosporins, for more than 4 days prior to MDR-bacteremia was significantly associated with its occurrence. ($P < 0.04$) (Refer to **Table 6**) Previous antibiotic therapy has been recognized as a major risk factor for the development of bacterial resistance. In prospective study involving13 Brazilian HSCT centers (Oliveira et al., 2007), 22% of 91 episodes of bacteremia were MDR-gram-negative in origin. Previous exposure to third generation cephalosporins either as prophylaxis or empirical therapy and belonging to one of the HSCT centers were associated with an increased risk for ESBL-producing *Enterobacteriaceae* (Oliveira et al., 2007).Another retrospective case-control study involving HSCT recipients (Garnica et al., 2009) showed by univariate analysis that previous use of a third or fourth-generation cephalosporin ($P = 0.005$ and 0.02, respectively) and duration of antibiotic use ($P < 0.001$) were among the factors associated with bacteremia due to MDR-gram-negative isolates including *K. pneumonia* and *P. aeruginosa*. In another study performed in the United States by Rangaraj et al. (2010), the use of multiple broad spectrum antibiotics compared with no antimicrobial agents was significantly associated with isolation of MDR *P. aeruginosa* (8.2 vs. 0.7%, $p < 0.005$).This finding is consistent with a more recent study in 2013 by Satlin et al. (2013), where exposure to any broadspectrum antibacterial agent may be sufficient to increase the risk of carbapenem-resistant *Enterobacteriaceae* acquisition and cause bloodstream infections in patients with hematologic malignancies.

Our results showed that patient outcome was influenced significantly by antimicrobial resistance, and the risk ofsubsequent

intubation, sepsis and mortality were high in the 3GC-resistant bacteremia group and in the MDR-bacteremia group compared with patients having other bacteremias($P < 0.03$).(Refer to **Table 5**) Other studies indicated that a dramatic increase in the detection rate of MDR gram-negative bacteremia compared with previous periods was associated with increased morbidity, mortality, and cost, especially in patients with hematological diseases (Lodise et al., 2007). Moreover, mortality was independently associated with inadequate initial antimicrobial treatment in the case of antibiotic-resistant bacteremia (Giske et al., 2008). Thus, local monitoring of bacterial isolates is recommended to adapt initial empiric antibiotic therapy based on the local prevalence of MDR strains (Caselli et al., 2010).

Our study has at least two major limitations. The first is that the samples were collected from a single medical center; therefore, results could not be generalized to other medical centers in Lebanon because the microbial ecology differs from one center to another. The second limitation is that the small sample size did not allow us to perform a multivariate analysis and limited our statistical analysis to a univariate model.

CONCLUSION

In conclusion, our data showed equal occurrence of gram-negative and gram-positive organisms causing bacteremia in febrile neutropenic cancer patients in our center. We found that bacteremia caused by gram-negative antimicrobial resistant strains is common among cancer patients, especially in those exposed to antibiotic pressure. Emergence of resistance to third and fourth generation cephalosporins and, to a lesser extent, to carbapenems, in gram-negative isolates has to be considered seriously in our local guidelines for empiric treatment of febrile neutropenia, especially given that their occurrence was associated with poorer clinical outcomes.

The empiric use of broad spectrum antibiotics in febrile neutropenia is very critical. On the one hand, it is crucial to decrease mortality during the febrile episode; on the other hand, it is a risk factor for emergence bacteremia with resistant organisms. In our therapeutic guidelines for the management of febrile neutropenia, we should include coverage for MDR bacteria in patients who have persistent or relapsing fever after 4 days of initial empiric therapy.

AUTHOR CONTRIBUTIONS

All authors have contributed equally to the analysis and interpretation of the study data as well as to the drafting of the article, but Rima Moghnieh made the primary contribution to the conception and design of the study and revising the draft critically for important intellectual contentand Nour Estaitieh to the acquisition and collection of data. All authors gave the final approval of the article to be sent for publication and agreed to be accountable for all aspects of the paper in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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The prevalence of *Escherichia coli* strains with extended spectrum beta-lactamases isolated in China

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The extended-spectrum-lactamases-producing Escherichia coli has rapidly spread worldwide. Escherichia coli has been becoming much more resistant to β-lactam antibiotics and other commonly available antimicrobials. We investigated the prevalence, resistance, and probable gene type of extended spectrum beta-lactamases (ESBLs) using minimum inhibitory concentrations (MICs) testing and polymerase chain reaction (PCR). We have collected 289 single-patient E. coli Isolates based on samples of China from July 2013 to August 2014. This article explored that the prevalence of ESBL-producing Isolates showed multi-resistant to antimicrobials such as fluoroquinolones, trimethoprim, tetracycline and aminoglycosides, and so on. The frequencies of resistance in Isolates were as follows: Ciprofloxacin, 74%, gentamicin, 69.5%, levofloxacin, 63%, tobramycin, 39%, and minocycline, 7.9%. According to our results, 197(68.2%) of the total 289 Isolates were ESBL-producing strains; further, 172 (87.3%) producers contained genes encoding CTX-M enzymes and 142(72.1%) producers contained genes encoding TEM enzymes. Most ESBL-producing Escherichia coli has produced more than one type of β-lactamase. Nucleotide sequence analysis has revealed the diversity of ESBLs types: CTX-M -15 is in the majority and TEM-135, CTX-M-3, CTX-M-98, CTX-M-14, CTX-M-142, CTX-M-65, CTX-M-55, CTX-M-27, and CTX-M-123 have been recovered. The results confirm that ESBL producers which are common in hospital strains of Escherichia coli are resistant to cephalosporins and other antibiotics in China. It is important to monitor such strains closely and provide scientific evidence of rational application of antibiotics to prevent their spread.

Keywords: extended-spectrum-lactamase, *Escherichia coli*, multi-drug resistant, PCR, TEM, CTX-M

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Introduction

The resistance of ESBL-producing *Enterobacteriaceae* has currently become one of the most important nosocomial resistance problems around the world and it has been found that there are much more community-acquired ones (Bradford, 2001; Valverde et al., 2004). *Escherichia coli* has been a universal commensal bacterium causing infections in humans and animals and often leads to urinary tract infections (UTI) and bacteremia for humans (Paterson and Bonomo, 2005). ESBL-producing *Enterobacteriaceae* were firstly reported in the middle of 1980s in Germany, since then a steady increase of these strains have been reported worldwide (Bradford, 2001).

Recently, researchers have paid much more attention to extended spectrum beta-lactamases (ESBLs). As well known, the production of β-lactamase is the most common mechanism of bacterial resistance to β-lactam antibiotics; with the introduction of extended-spectrum cephalosporins, ESBLs has spread across the world (Medeiros, 1997; Yagi et al., 2000; Saladin et al., 2002; Yong and Toleman, 2009). ESBLs could inactivate oxyimino-β-lactams like third-generation cephalosporins and aztreonam, but not hydrolyze the carbapenems, which are highly susceptible to inhibition by clavulanic acid and tazobactam (Livermore, 1995). ESBL-producing strains are not only highly resistant to β-lactam antibiotics but also resistant to aminoglycosides, quinolones, sulfonamides, and so on (Ho et al., 2007).

The epidemic situation, resistance and genotype distribution of ESBL-producing strains have varied with different countries, regions and even hospitals. ESBL strains have extended all over the world stage by stage, while detectable rates have shown significant difference in different hospitals or areas of the same country. Previous researches have indicated the detectable rate of ESBL-producing strains from 5 to 8% of *Escherichia coli* Isolates in Korea, Japan, Malaysia, and Singapore, from 12 to 24% in Thailand, Taiwan, Philippines, and Indonesia (Paterson and Bonomo, 2005), from 62 to 100% in India (Mathai et al., 2009). By contrast, the rate was almost up to 60% in China (Xiao et al., 2011).

Much more genetic subtypes of ESBLs have been found since 1980s, which has exceeded 200 so far. The vast majority of ESBLs belong to three types, including TEM, SHV, and CTX-M types (Bauernfeind et al., 1990; Barthelemy et al., 1992; Bradford, 2001; Paterson and Bonomo, 2005; Livermore et al., 2007; Cantón et al., 2012). Previous researches exploring TEM-type has mainly involved the predominant genotype (particularly, TEM-10, TEM-12, and TEM-26) in north America (Rasmussen et al., 1993; Urban et al., 1994); 92% ESBL-positive *E. coli* Isolates expressed a CTX-M-type enzyme from 2001 to 2006 and CTX-M-1 being the predominant group in Sweden (Fang et al., 2008); the CTX-M-15 enzyme was the most prevalent enzyme in United Kingdom (Baraniak et al., 2005; Livermore et al., 2007). In China, the CTX-M β-lactamases was the most prevalent ESBLs, mainly including CTX-M-3, −9, −14, and −15 (Xiong et al., 2002; Yu et al., 2007; Liu et al., 2009). In short, there exists tremendous variability in antimicrobial resistance amongst pathogens in different regions or in different hospitals located the same regions.

Given few wide-ranging survey researches in Shandong province, China, we aimed to explore ESBLs epidemiology, resistance and resistant genotypes of 289 *E. coli* strains Isolated from three hospitals in that area. In addition, we have collected clinical data about the patients to carry on further research. The study helps us reveal their distributions and local constitutions in that area, thus providing guidance for clinical treatment, and then contributing to avoiding delay the conditions of patients and medical resources waste.

Materials and Methods

Bacterial Strains

We have collected 289 clinical Isolates of *E. coli* from patients in three typical hospitals of Shandong province in, China, from July 2013 to August 2014. There exist no replicate strains Isolated from one patient in our samples. Additional information has been collected with each Isolate, including isolation date, origin of the specimen, demographic details. Our experiment has been approved by Medical Ethics Committee of Shandong University School of Medicine (Grant No. 201401048).

ESBL Identification and Antimicrobial Susceptibility Testing

Initially, all Isolates were tested using the Kirby Bauer disk diffusion method according to the Clinical Laboratory Standards Institute (2013). *E. coli* ATCC25922 was used as control strains. The tested antibiotics included: ampicillin, ampicillin/sulbactam, amoxicillin/clavulanate, ticarcillin, piperacillin, piperacillin/tazobactam, cephalothin, cefuroxime, cefoxitin, ceftriaxone, cefotaxime, ceftazidime, cefepime, meropenem, and aztreonam (Mast Diagnostics, Merseyside, UK). co-trimoxazole, gentamicin, nalidixic acid, ciprofloxacin, imipenem, meropenem, tetracycline, and nitrofurantoin (Oxoid Ltd., Basingstoke, UK). MIC values were extrapolated by the BIOMIC automated reading system and software package (Giles Scientific, New York, NY, USA).

The disk diffusion method was applied to assess ESBL production in all the Isolates with cefotaxime (30 ug) and ceftazidime (30 ug) alone and in combination with clavulanic acid (10 ug) as recommended by the Clinical and Laboratory Standards Institute (CLSI).

Nucleic Acid Extractions

DNA had been extracted from the Isolates by using boiling methods. Each strain was suspended in 500 ul of distilled water at concentration of MacFarland 0.5 and boiled at 100°C for 10 min. Then spined it in a centrifuge and kept the supernatant fluid in the −20°C freezer.

Polymerase Chain Reaction Detection and Sequencing of ESBLs

To determine the genotype of ESBLs, we performed polymerase chain reaction (PCR) amplification with the TEM and CTX-M. ESBLs Isolates were amplified with primers (Essack et al., 2001): 5′-ATGAGTATTC AACATTTCCGTG-3 and 5′-TTACCAATGCTTAATCAGTGAG-3, which have been designed to amplify TEM gene (length, 840 bp). Cycling conditions were as following: initial denaturation at 95°C for 3 min; 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min; and a final elongation at 72°C for 5 min. We applied CTX-M gene primers: C1 (5′-SCSATGTGCAGYACCAGTAA-3) and C2 (5′-CCGCRAT ATGRTTGGTGGTG-3) to detect CTX-M gene (length, 554 bp). Cycling conditions were as follows: initial denaturation at 94°C for 3 min; 35 cycles of 94°C for 30S, 55°C for 30s and 72°C for 45s; and a final elongation at 72°C for 5 min. PCR reactions for each strain were repeated at least three times.

Sequencing of TEM and CTX-M genes

The PCR products were purified with a QIA quick PCR purification kit (Qiagen, Hilden, Germany) and sequenced on an ABI

Prism 377 automated sequencer (Perkin-Elmer, Norwalk, CT, USA). The primers for sequencing were the amplification primer. All sequences were confirmed by two independent determinations and analyzed by Basic Local Alignment Search Tool.

Results

Bacterial Strains

In our samples, these strains were Isolated mainly from patients in urinary surgery ward 110 (38.1%) and ICU 63 (21.8%). The rest in turn were derived from Respiratory Medicine, 27 (9.3%), Neurosurgery 22 (7.6%), Thoracic Surgery and General Surgery 21 (7.3%), Oncology 11 (3.8%), Orthopedic Surgery 9 (3.1%), Cardiovascular Medicine 9 (3.1%), Gastroenterology 8 (2.8%), Endocrinology 7 (2.4%), Neurology 2 (0.7%). A total of 197 ESBL-producing Isolates were detected phenotypically using the CLSI criteria for ESBL screening and disk confirmation test, the detection rate being 68.2% (197/289) (See **Table 1**). We found that much more ESBL-producing Isolates exited in urinary surgery ward and ICU and the detection rate were higher than the other wards. The cohort of 197 patients had a mean age of 53.8, which ranges from 20 to 88 (See **Table 2**). Of all Isolates, 109 (55.3%) patients were over 60. According to statistical results by age, we concluded that we should further enhance elderly patients' management and treatment dynamics, completes the prevention and treatment work. The main sources of ESBL- producing *E-coil* were unire 96 (48.73%, of total ESBL-producing Isolates), wounds 27 (13.71%), sputa 26 (13.20%), genital secretion 16 (8.12%), hydrothorax and ascite 15 (7.61%), blood 12 (6.09%), bile 5 (2.54%) (See **Table 3**).

Antimicrobial Susceptibility

The ESBL-producing Isolates were often multidrug-resistant. ESBL producers have shown much higher rates of resistance than those non-ESBL to ciprofloxacin (ESBL versus non-ESBL, 74% versus 30%, $P < 0.01$), gentamicin (69.5% versus 28.5%, $P < 0.01$), co-trimoxazole (75.8% versus 36%, $P < 0.01$), aztreonam (79% versus 20.5%, $P < 0.01$), and levofloxacin (63% versus 40%, $P < 0.01$). The great majority of Isolates were susceptible to Minocycline (ESBL versus non-ESBL, 82% versus 92.1%). All Isolates were susceptible to amikacin and imipenem (χ^2 test for all groups).

Characteristics of the ESBL-producing Isolates

All 197 Isolates with an ESBL phenotype tested positive for blaCTX-M and blaTEM using the consensus primers. We found 172 (87.3%) Isolates were positive for blaCTX-M, while 142(72.1%) Isolates were positive for blaTEM and 11 non-TEM-CTX-M-type Producers. In addition, most Isolates appeared to produce both TEM and CTX-M enzymes (128). Using nucleotide sequence analysis we found that the TEM enzymes detected two types which were TEM-1(138) and TEM-135(4) β-lactamase. Unlike the TEM enzymes, the CTX-M enzymes with a variety of types included CTX-M-3(16), CTX-M-14(77), CTX-M-15(64), CTX-M-55(9), CTX-M-64(1), CTX-M-65(2) and CTX-M-98(3) and the genes of CTX-M-14 and CTX-M-15 were in the majority.

Discussion

In the article, 289 *E. coli* Isolates were collected from patients from July 2013 to August 2014; the detection rate of ESBL producers was 68.2% which was significantly higher than previous researches (Xiao et al., 2011). Therefore, we should pay more attention to the problem and try to take appropriate prevention and remedial measures. We found that much more ESBL-producing Isolates in urinary surgery ward and ICU and the detection rate were higher than the other wards. Because a majority of patients in ICU acquired serious diseases lowering their immunity, and involved heavy use of antibiotics for a long time, both of which contributing to higher detection rates. And for patients in urinary surgery, higher detection rates were related to their selves characteristics, one of which was mainly urinary tract obstruction in favors of bacteria propagation, in addition to urethral catheterization, further increasing opportunities for infection. Besides, we found most ESBL-producing strains occur in old man or woman, because the elderly themselves have low immunity. And in the patients, one patient over 60 repeatedly infected with the ESBL-producing *E. coli*. As a long duration of extended-spectrum β-lactamase-producing *Enterobacteriaceae* carriage after discharge

TABLE 1 | Distribution of strains in different wards.

Wards	Total	ESBLs	Detection rate
Urology	110	74	67.27%
Intensive care unit	64	54	84.38%
Respiratory medicine	27	17	62.96%
Neurosurgery	22	13	59.09%
Thoracic surgery and general surgery	21	13	61.90%
Oncology	11	7	63.64%
Cardiovascular medicine	9	3	33.33%
Orthopedic surgery	9	5	55.56%
Endocrinology	8	5	62.50%
Gastroenterology	6	4	66.67%
Neurology	2	1	50.00%
Total	289	197	68.2%

TABLE 2 | Frequency of ESBL Isolates by age.

Gender \ Age	20–40	40–60	60–80	>80	Total
Male	15	24	43	14	96
Female	21	28	40	12	101
Total	36	52	83	26	197

TABLE 3 | Distribution of *E. coli* Isolates by type of specimen.

Specimen type	Total	ESBLs	Non-ESBLs
Unire	126	96	30
Wounds	42	27	15
Sputa	38	26	12
Genital secretion	28	16	12
Hydrothorax and ascite	26	15	11
Blood	20	12	8
Bile	9	5	4
Total	289	197	92

(Birgand et al., 2013), we concluded that those patients underlying chronic illnesses which had lowered their immunity were apt to be infected with ESBL-producing *E. coli*. Therefore, we should initiate active interventions and treatments.

The susceptibility test data showed that the ESBL producers which were resistant to most-lactams and frequently resistant to the non-β-lactam antibiotics, for example, fluoroquinolones and aminoglycosides. If patients are infected by ESBL producers, cefmetazole, or imipenem will be chosen before results of antibiotic susceptibility test having been done. But if patients are in a critical condition, we should make choice of carbapenems (Paterson, 2000). ESBL producers have shown much higher rates of resistance than those non-ESBL. Therefore, we should avoid antibiotics abuse in outpatient clinic and community hospitals, reducing opportunities for emergence of ESBLs.

TEM is the main type of β-lactamase, the TEM-1 group being the most common ones. CTX-M enzymes was a new group of plasmid-mediated ESBLs which has become the predominant ESBLs reported in Europe from last decade and has increased dramatically in many countries (Bonnet, 2004; Cantón and Coque, 2006; Livermore et al., 2007; Pitout and Laupland, 2008). In different geographic areas, the antibiotic consumption and dissimilar risk factors might have also contributed to the current epidemiology of CTX-M enzymes. Our study found that the CTX-M-14 and CTX-M-15 were in the majority, Dolejska et al. (2011) and Hiroi et al. (2012) coincided with that.

Even though we have explored ESBLs epidemiology, resistance and resistant genotypes in three hospitals of Shandong province, with limitation of small samples, we cannot provide enough theoretical and practical guidance for clinical treatment. In the future research, we should base upon more large survey samples from perspective of increasing patients, hospitals and regions. On the other hand, because of much more ESBL producers resistant to carbapenems, to conduct a targeted research, we will focus on carbapenem-resistant *Enterobacteriaceae* (Centers for Disease Control and Prevention, 2013; Asakura et al., 2014). Given carbapenems being the first-line agent for patients with ESBL-producing bacteria, the occurrence of resistant organisms could be related to carbapenems, and then we will explore whether non-carbapenems plays more efficient role in infections with ESBL-producing *Enterobacteriaceae*.

Generally speaking, much more ESBL producers are detected among *E. coli* in China. The drug resistance rate of *E. coli* is high, with the tendency getting higher. Attaching more importance to monitor the drug resistance of bacterium and reasonable application and control abuse of antibiotics in clinic. Based on local database, our research have clarified the clinical and epidemiological features in Shandong province, which contributes to provide guidance for identifying appropriate treatment plans according to patients characteristics, especially for patients underlying chronic illnesses. We should take measures appropriately, and then contributing to avoiding delay the conditions of patients and medical resources waste.

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Application of swine manure on agricultural fields contributes to extended-spectrum β-lactamase-producing *Escherichia coli* spread in Tai'an, China

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The prevalence of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* (*E. coli*) is increasing rapidly in both hospital environments and animal farms. A lot of animal manure has been directly applied into arable fields in developing countries. But the impact of ESBL-positive bacteria from animal manure on the agricultural fields is sparse, especially in the rural regions of Tai'an, China. Here, we collected 29, 3, and 10 ESBL-producing *E. coli* from pig manure, compost, and soil samples, respectively. To track ESBL-harboring *E. coli* from agricultural soil, these isolates of different sources were analyzed with regard to antibiotic resistance profiles, ESBL genes, plasmid replicons, and enterobacterial repetitive intergenic consensus (ERIC)-polymerase chain reaction (PCR) typing. The results showed that all the isolates exhibited multi-drug resistant (MDR). CTX-M gene was the predominant ESBL gene in the isolates from pig farm samples (30/32, 93.8%) and soil samples (7/10, 70.0%), but no SHV gene was detected. Twenty-five isolates contained the IncF-type replicon of plasmid, including 18 strains (18/32, 56.3%) from the pig farm and 7 (7/10, 70.0%) from the soil samples. ERIC-PCR demonstrated that 3 isolates from soil had above 90% genetic similarity with strains from pig farm samples. In conclusion, application of animal manure carrying drug-resistant bacteria on agricultural fields is a likely contributor to antibiotic resistance gene spread.

Keywords: ESBL-producing *E. coli*, animal manure, ERIC-PCR, agricultural fields, antibiotic resistance gene spread

Introduction

The rapid increase of extended-spectrum beta-lactamases (ESBLs)-producing *Enterobacteriaceae* has attracted worldwide concern. ESBLs are enzymes that make bacteria, especially *Escherichia coli* and *Klebsiella pneumoniae*, resistant to beta-lactam antibiotics including 3rd and 4rd generation cephalosporins to decrease antibiotics available in clinical practices

(Bradford, 2001). ESBL-producing *E. coli* were widely found in hospital environments and farms (Edelstein et al., 2003; Smet et al., 2008) and they are able to survive in various natural environments such as feces, soils, and water bodies (Koczura et al., 2012; Blaak et al., 2014; Haque et al., 2014), so ESBL-producing *E. coli* is usually regarded as an indicator bacterium to trace the spread of antibiotic resistance gene (Gao et al., 2014). At present, numerous studies have focused on the occurrence and spread of ESBL genes in hospital environments, waste water treatment plants, water bodies, and food-producing animals (Edelstein et al., 2003; Agerso et al., 2012; Blaak et al., 2014). ESBL-producing *E. coli* have been shown to be able to transmit from animal farms to surrounding environments, even rural water reservoirs (Laube et al., 2014; Von Salviati et al., 2015; Zhang et al., 2015).

In rural areas of China, animal manure has been used to feed agricultural fields as fertilizers in the production of crops, fruits, and vegetables (Huang et al., 2014). But animal feces contain large amounts of antimicrobial-resistant bacteria, including certain pathogenic bacteria such as *Listeria monocytogenes* and *Salmonella* spp. and certain drug-resistant bacteria, such as ESBL-producing *E. coli*, vancomycin-resistant *Enterococcus* and methicillin-resistant *Staphylococcus aureus* (Guber et al., 2007). Animal manure fertilization has been found to increase the abundance of drug-resistant bacteria and the frequency of antibiotic resistance genes in soils (Venglovsky et al., 2009; Marti et al., 2013). Zhu et al. (2013) found diverse and abundant antibiotic resistance genes in compost and soil treated with manure. When animal manure is added into soil in the form of solid or liquid, antibiotic resistance genes can transfer between the same species and even different ones through genetic elements, especially plasmids (D'Costa et al., 2006; Guber et al., 2007; Heuer et al., 2011a). Moreover, antibiotic resistance genes or bacteria in soils could enter into other environments and food chain through various ways to threaten public health. Researchers have found polluted soil could contaminate surface water by over land flow or rainfall (Tate et al., 2000; Curriero et al., 2001). There are also reports about the contamination of resistance gene in vegetables (Ruimy et al., 2010; Reuland et al., 2014). Therefore, investigations on the influence of animal manure application on agriculture are of great significance.

In China, a large number of arable soils have been amended with commercial fertilizers and animal manures (Ju et al., 2007). But, up to date, little information about whether the fertilization model enhances the dissemination of drug-resistant bacteria of animal origins was provided. Previous studies have shown that manure application contributed to the accumulation of resistance genes in soil, mainly sulfonamide resistance genes (Sengeløv et al., 2003; Zhou et al., 2010; Heuer et al., 2011b). In the last decade, the prevalence of ESBL genes has increased rapidly. Meanwhile, ESBL genes that were found to be located on mobile genetic elements, and often associated with other resistance genes, could transfer horizontally between bacteria (Allen, 2014). Thus, this study was performed to assess the dissemination of ESBL-producing *E. coli* of animal origins into agricultural fields.

Materials and Methods

Sampling Sites and Collection of Samples

Manure samples and soil samples were collected from a pig farm with an intensity of 5000 pigs and its surrounding lands fed with manure between May and July 2014. The farm is located in rural region of Tai'an, China, far away from villages, surrounded by farm land where crops (corn and beans) were planted. Pig manure was piled up and then used as compost into the soil or directly fed the soil by local farmers instead of chemical fertilizer. The soil samples were collected from the cropland which has been receiving compost for at least 3 years.

The pig farm was visited four times. Each time, 10 fecal samples, five composts were collected. At the same time, 20 soil samples were collected from different locations in the surrounding field. Forty soil samples collected from soil treated with chemical fertilizer were used as control. All samples were immediately transported to the lab with an ice box and processed in 6 h.

Isolation and Confirmation of ESBL-producing *E. coli*

About 0.5 g sample soil/feces was dissolved in 5 ml phosphate buffered saline and homogenized. Then 1 ml solution was mixed with 9 ml BHI broth (Haibo, Qingdao, China) for enrichment at 37°C overnight under aerobic conditions. Hundred microliters soil enrichment or 1 loop of the fecal samples enrichment solution was spread onto MacConkey agar (Oxoid, Basingstoke, England) plates with 2 mg/l cefotaxime and then incubated overnight at 37°C.

A presumptive ESBL-producing *E. coli* colony with bright pink or red color was identified by traditional biochemical test including indole, Methyl red-Voges-Proskauer, and citrate biochemical testing and API 20E (bioMerieux, Marcy l'Etoile, France) (Chang et al., 2015).

The screened ESBL-producing *E. coli* isolates were further confirmed by phenotypic confirmatory tests using cefotaxime (30 μg), cefotaxime + clavulanic acid (30 μg/10 μg), ceftazidime (30 μg), ceftazidime + clavulanic acid (30 μg/10 μg) (CLSI, 2013).

Antimicrobial Susceptibility

The confirmed ESBL-producing *E. coli* were subjected to antimicrobial susceptibility tests according to the Clinical and Laboratory Standards Institute (CLSI, 2013) guidelines. Disk diffusion method was used to determine resistance profile of the isolates on Mueller-Hinton agar (Haibo, Qingdao, China). Eight drug classes containing 14 antibiotics (Oxoid, England) were included representing the most frequently used antibiotics in animals. Penicillins were represented by amoxicillin (AML). Cephalosporins included ceftiofur (CET), cephalothin (KF), cefotaxime (CTX), and ceftriaxone (CRO). Carbopenems were represented by imipenem (IPM). Aminoglycosides were represented by gentamicin (GM), kanamycin (K), and amikacin (AK). Quinolones included nalidixic acid (NA) and ciprofloxacin (CIP). Tetracyclines were represented by tetracycline (TE). Chloramphenicol (C) and florfenicol (FFC) were also included. The standard *E. coli* ATCC 25922 was used as quality control strain

(Zhang et al., 2014). *E. coli* isolates resistant to three or more categories were regarded as multi-drug resistant (MDR), resistant to one or two categories regarded as extensively drug-resistant (XDR) and resistant to all antimicrobial categories considered as pandrug-resistant (PDR) (Magiorakos et al., 2012).

Detection of ESBL Genes

Major β-lactamase genes detected in food-producing animals in China, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{CMY-2}, and bla_{OXA}, were amplified by PCR (Zhao et al., 2001; Monstein et al., 2007; Shaheen et al., 2011). Groups of CTX-M-positive *E. coli* were further determined using specific group primers for CTX-M-1, CTX-M-2, and CTX-M-9 (Batchelor et al., 2005). The PCR products were purified using a PCR purification kit (TianGen, Beijing, China) and then sequenced bi-directionally with the same primers by ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA) (Zhang et al., 2014). All gene sequences were subjected to BLASTn analysis in GenBank (http://www.ncbi.nlm.nih.gov/) or β-lactamase classification system (http://www.lahey.org/studies/webt.asp) to confirm subtypes of ESBL-encoding genes (Zheng et al., 2012).

Plasmid Replicon Typing

The plasmid replicon types of the ESBL-producing *E. coli* were detected using the inc/rep PCR method (a PCR method based on replicons of major plasmid incompatibility groups) (Carattoli et al., 2005). Multiplex- and simplex-PCR were used to recognize 18 plasmid incompatibility groups among *Enterobacteriaceae* including FIA, FIB, FIC, HI1, HI2, I1-Ig, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA and performed as previously described (Carattoli et al., 2005).

ERIC-PCR Analysis

Twenty-one ESBL-producing *E. coli* isolates from pig farm samples and seven isolates from treated soil samples were selected randomly and subjected to enterobacterial repetitive intergenic consensus sequence (ERIC-PCR) to analyze the similarity of the strains from manure, compost and treated soil. The PCR procedure and conditions were performed as previously described (Koczura et al., 2012). The amplified products were separated in 2% agarose gel by electrophoresis. The presence or absence of the amplified bands on the gel were recorded as 1 or 0 for further analysis. NTSYS-pc (Version 2.10) was used to do cluster analysis based on Dice's similarity coefficient with a 1% position tolerance and the unweighted pair group method using arithmetic averages (UPGMA). Isolates of different origins or the same origin with above 90% similarity were treated as the same strain (Edelstein et al., 2003; Yuan et al., 2010).

Results

Isolation of ESBL-producing *E. coli*

A total of 42 ESBL-producing *E. coli* were isolated from the 180 samples. There were 32 isolates from pig farm samples, including 29 from manure and three from compost samples. From the soil samples treated with compost or manure, 10 ESBL-producing *E. coli* were isolated. However, no ESBL-producing isolate was obtained from soil treated with chemical fertilizer (**Table 1**).

TABLE 1 | Isolation of ESBL-producing *E. coli* from manure, compost, treated soil, and untreated soil.

Origin	No. of samples	No. of ESBL-producer	Isolation rate (%)
Manure	40	29	72.5
Compost	20	3	15.0
Treated soil	80	10	12.5
Untreated soil	40	0	0
Total	180	42	23.3

Antimicrobial Susceptibility

All the 42 ESBL-producing *E. coli* from manure, compost, and treated soil showed multiple drug resistance to antibiotics tested and the isolates from different origins had similar drug-resistant spectrum against the 14 antibiotics. Forty-two isolates were all 100% resistant to AML and KF, but susceptible to IMP. Additionally, isolates from different sources had above 60% resistance to CET, K, TE, C, and FFC (**Table 2** and Table S1).

Characterization of β-Lactamase Genes

Among the 42 ESBL-producing *E. coli*, 37 isolates carried bla_{CTX-M}(37/42, 86.0%), including 30 isolates from the pig farm (30/32, 93.8%), and seven from treated soil (7/10, 70.0%). Thirty-one isolates carried $bla_{CTX-M+TEM}$ gene, including 25 isolates (25/32, 78.1%) from the pig farm and six (6/10, 60.0%) from treated soil. The other three genes (SHV, CMY-2 and OXA) were all not detected.

Sequence and blast results showed that the most prevalent ESBL gene in pig farm samples was $bla_{CTX-M-15}$, which was found in 12 isolates, followed by $bla_{CTX-M-27}$ and $bla_{CTX-M-65}$ respectively detected in seven isolates. Five isolates from the pig farm carried $bla_{CTX-M-14}$ and one isolate harbored $bla_{CTX-M-13}$. Two isolates from fecal samples carried both $bla_{CTX-M-15}$ and $bla_{CTX-M-65}$ gene. Both $bla_{CTX-M-15}$ and $bla_{CTX-M-14}$ genes were the common ESBL genes in isolates from soil samples and respectively detected in three strains as shown in **Table 2**.

Five isolates of the 42 ESBL-producing *E. coli* did not carry ESBL genes (three from soil samples and two from pig farm samples). Three isolates from soil samples and one from manure only carried bla_{TEM-1} gene. No β-lactamase gene was found in one isolate from manure.

Plasmid Replicon Type

Among the 42 ESBL-producing *E. coli* of different origins, IncF was the most prevalent replicon type in the isolates from the pig farm (18/32, 56.3%) and treated soil samples (7/10, 70%). IncN was detected in 16 ESBL-producers from pig farm samples and one from soil samples. IncFIB was found in nine isolates from pig farm samples and two from soil samples. Four isolates were not typed including both two from pig farm samples and soil samples as shown in **Table 2**.

Isolates from pig farm samples showed a higher diversity of inc/rep than that from soil samples. IncK and Y were detected in

TABLE 2 | Characteristics of the 42 ESBL-producing *E. coli* from different samples including β-lactamase genes, replicon type, and resistance profile.

Isolates	TEM	CTX-M	Replicon	AM	CET	KF	CTX	CROO	IMP	GM	K	AK	CIP	NA	TE	C	FFC
T1	TEM-1		F				I	I				I					
T2	TEM-1	CTX-M-14						I									
T3	TEM-1	CTX-M-15	F														
T4	TEM-1	CTX-M-14			I		I	I									
T5	TEM-1		F														
T6	TEM-1	CTX-M-15	F, N														
T7		CTX-M-14	FIB				I										
T8	TEM-1	CTX-M-65	F, FIB				I				I	I					
T9	TEM-1	CTX-M-15	F														
T10	TEM-1		F									I	I				
F1			N				I										I
F2	TEM-1	CTX-M-27	N, Y				I	I				I					
F3	TEM-1	CTX-M-15	F														
F4	TEM-1	CTX-M-15	F, N, Y, K				I							I			
F5	TEM-1	CTX-M-14	F, N, K									I					
F6	TEM-1	CTX-M-27	N		I						I						
F7	TEM-1	CTX-M-27	N, Y		I		I	I									
F8	TEM-1	CTX-M-14					I	I		I		I					
F9		CTX-M-27	N, Y, K		I		I	I				I					
F10	TEM-1	CTX-M-15	F, N, FIB				I										
F11		CTX-M-65	K														I
F12	TEM-1	CTX-M-65	Y, K				I	I		I							
F13	TEM-1	CTX-M-15	F														
F14	TEM-1	CTX-M-15	F, FIB														
F15		CTX-M-13					I	I									I
F16	TEM-1	CTX-M-27	N, Y		I		I	I				I					
F17	TEM-1	CTX-M-15	F, N, FIB														
F18	TEM-1		Y		I		I	I	I		I	I					
F19	TEM-1	CTX-M-15	F, N, K														
F20	TEM-1	CTX-M-15	F, FIB, K				I					I					
F21		CTX-M-65	Y, K				I										
F22	TEM-1	CTX-M-65	F, Y, K		I		I	I									
F23	TEM-1	CTX-M-14	F, FIB, Y, K		I		I	I									
F24		CTX-M-27	K		I		I	I		I	I						
F25	TEM-1	CTX-M-27	N, Y				I	I			I						
F26	TEM-1	CTX-M-15	F, N, FIB														
F27	TEM-1	CTX-M-14	F, Y, K				I										
F28	TEM-1	CTX-M-15/65	F, N, FIB, Y, K							I		I					
F29	TEM-1	CTX-M-15/65	F, N, FIB, Y, K											I			
C1	TEM-1	CTX-M-15	F, FIB, Y, K									I					
C2	TEM-1	CTX-M-14	F, N, Y, K				I										
C3	TEM-1	CTX-M-65	F, Y		I												

T, ESBL-producing E. coli isolates from soil; F, ESBL-producing E. coli from feces; C, ESBL-producing E. coli from compost.

The gray color represents resistant, the white color means susceptible and "I" represents intermediate. Different classifications of antibiotics were distinguished by bold lines. CET, ceftiofur; AML, amoxicillin; KF, cephalothin; CTX, cefotaxime; IPM, imipenem; CRO, ceftriaxone; GM, gentamicin; K, kanamycin; AK, amikacin; NA, nalidixic acid; CIP, ciprofloxacin; TE, tetracycline; C, chloramphenicol; FFC, florfenicol.

16 and 17 isolates from pig farm samples respectively, while none of them was found in soil samples.

Similarity Analysis of ESBL-producing *E. coli* from Manure, Compost, and Treated Soil

The similarity between ESBL-producing *E. coli* from pig farm and treated soil was between 70 and 100%. Three isolates (T2, T4, and T7) from treated soil had above 90% similarity with those from pig manure and compost samples (F8 and F1) (**Figure 1**), which suggested that they might come from the same strain.

Discussion

Animal wastes without composting process have been widely used in agricultural fields, especially in developing countries to improve the soil fertility and structure (Kumar et al., 2005; Hu et al., 2010). However, animal original bacteria carrying antibiotic resistance genes can regrow to high levels under favorable condition (Ghosh and LaPara, 2007; Marti et al., 2013; Chen and Jiang, 2014). Wide application of animal manure to agricultural fields has raised a concern about antibiotic resistance genes spread. Once antimicrobial-resistant bacteria from animal feces enter into arable soils, mobile genetic elements can transmit between the same or different species of bacteria under some conditions, especially between *Enterobacteriaceae* (Rensing et al., 2002; Heuer and Smalla, 2007). Additionally, these bacteria can spread to other environments through rainwater or other routes (Khaleel et al., 1982; Curriero et al., 2001).

Soil harbored a vast diversity of microorganisms, which was considered as a potential reservoir for antibiotic resistance (Forsberg et al., 2012). When drug-resistant bacteria of animal origins enter into this community, they could exchange resistance genes with soil-dwelling organisms. In this study, we isolated ESBL-producing *E. coli* from one pig farm as an indicator to better understand the pollution of ESBL-producing *E. coli* from animal manure on agricultural fields. We found that ESBL producers from manure, compost, and soil showed a remarkable similarity in terms of resistance phenotypes, ESBL genes, plasmid replicon type, and genomic characterization.

Previous studies have demonstrated that animal feces carrying drug-resistant bacteria can lead to antibiotic resistance genes spread, which attracted wide concern worldwide (Heuer et al., 2011a). In China, a large of arable soils have been amended with commercial fertilizers and animal manure (Huang et al., 2014), therefore it is of importance to investigate whether this fertilization model can enhance the dissemination of drug-resistant bacteria of animal origins can disseminate into the surrounding soil. In this study, we isolated ESBL-producing *E. coli* from pig farm samples (manure and compost) and soil treated with pig manure, but no isolate was detected in control soil samples only treated with chemical fertilizer. These results indicated that pig manure may be a likely contributor of drug-resistant bacteria or genes, and antibiotic selection may be another reason. Additionally, the presence of ESBL-producers in soils may be related with the sampling time and the frequency of fertilization (Jensen et al., 2001; Sengeløv et al., 2003; Riber et al., 2014), which is required to be further studied.

The 42 ESBL-producing *E. coli* all showed multi-drug resistance to 14 antibiotics commonly used in the local clinics, and the isolates both from pig farm samples and treated soil had the similar characteristics of resistance to a large extend. The close relatedness also indicated that application of pig manure to agricultural fields may play an important role in

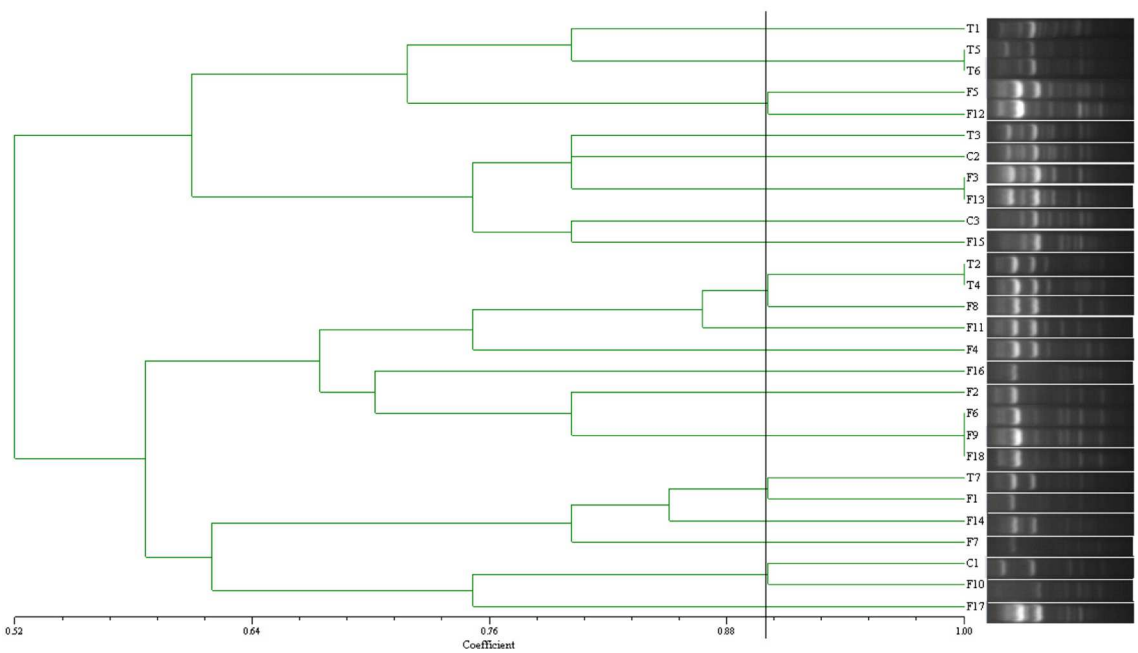

FIGURE 1 | Dendrogram of ERIC-PCR results of 28 ESBL-producing *E. coli* originating from manure (F), compost (C), and soil (T).

the dissemination of drug-resistant bacteria or genes of animal origins. ESBL-producers from different sources showed high resistance to the β-lactam antibiotics. It may be related with the use of ceftiofur that was allowed to be used in veterinary medicine in China (Guo et al., 2014).

CTX-M gene was the predominant ESBL genes in pig farm samples, which was also the main ESBL gene detected in soil samples as previous studies (Pitout and Laupland, 2008; Ewers et al., 2012). CTX-M-15, CTX-M-14, and CTX-M-65 genes were found in ESBL-producing *E. coli* of different origins. CTX-M-15 was detected in 12 isolates in pig farms accounting for 37.5%, similar prevalence 30% was observed in treated soil samples. IncF was the dominant replicon type in samples from pig farms and soil samples detected in 18 isolates from pig farm samples and seven from soil. Additionally, ERIC-PCR of the ESBL-producing *E. coli* showed four isolates from treated soil exhibited above 90% similarity with fecal isolates. The similar characteristics suggested that isolates in soil may mainly come from the manure or compost. The result showed that pig manure may be a likely contributor of drug-resistant bacteria or genes, including ESBL producers. However, isolates from the pig farm showed a high diversity of replicon or ESBL genes, which may be due to the different evolution and selective pressure of those isolates.

The bacteria community of arable soil was closely related with organic fertilizer applications. Previous studies have demonstrated that drug-resistant genes were found in the arable soil amended with manures (Sengeløv et al., 2003; Heuer et al., 2011a) and the repeated application of manure could increase the abundance of sulfonamide resistance gene (Heuer et al., 2011b). ESBL-producing *E. coli* have been detected on the surface of the ground in the vicinity of the animal farms (Walsh and Duffy, 2013; Laube et al., 2014).

In summary, ESBL-producing isolates from compost, treated soil, and manure showed high overlaps in terms of resistance phenotypes, ESBL genes, plasmid replicon type, and genomic backbone characterization, which implies the dissemination of resistance bacteria or genes of animal origins to soil that treated with animal manure.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00313/abstract

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High prevalence and risk factors of fecal carriage of CTX-M type extended-spectrum beta-lactamase-producing Enterobacteriaceae from healthy rural residents of Taian, China

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The study was carried out to understand the prevalence of CTX-M type extended-spectrum beta-lactamase (ESBL)-harboring Enterobacteriaceae and to analyze risk factors related with fecal carriage in healthy rural residents in Taian, China. A total of 620 stool samples were collected from rural residents. The ESBL-positive Enterobacteriaceae was screened using ChromID ESBL agar, and then further confirmed by double-disk diffusion. The CTX-M genes were determined using polymerase chain reaction. The risk factors associated with fecal carriage of CTX-M-positive isolates were analyzed using the standard statistic methods. 458 isolates carrying CTX-M gene (458/620, 73.9%) were obtained from different individuals, and the most dominant genotype was CTX-M-9 group (303/458, 66.2%). The dominant species were *Escherichia coli* (*E. coli*; 403/458, 88.0%) and *Klebsiella pneumoniae* (*K. pneumoniae*; 26/458, 5.7%) among the isolates carrying CTX-M genes. All the CTX-M producers were resistant to ampicillin, cefazolin, cefuroxime, and ceftriaxone, but were all susceptible to biapenem, imipenem, and meropenem. The results of multivariate logistic regression model identified the enrollment in formal education (OR 2.321; 95% CI 1.302–3.768; $P = 0.039$), the hospitalization history within the last 6 months (OR 1.753; 95% CI 1.127–2.584; $P = 0.031$) and the antibiotics use within the last 6 months (OR 1.892; 95% CI 1.242–2.903; $P = 0.034$). The three variables were significantly associated with carriage of CTX-M ESBL producers ($x^2 = 21.21$; $df = 3$; $P < 0.001$). The prevalence of fecal carriage of CTX-M ESBL-producing Enterobacteriaceae among healthy rural humans in Taian was high, and the recent antibiotic use and hospitalization history may be the important contributors.

Keywords: CTX-M type extended-spectrum beta-lactamase (ESBL), healthy rural residents, Enterobacteriaceae, risk factors, Taian

Introduction

The main resistance mechanism of Enterobacteriaceae against beta-lactam antibiotics is the production of extended-spectrum beta-lactamases (ESBL). The enzyme can hydrolyse penicillins, cephalosporins, and aztreonam, but they can be inhibited by clavulanic acid, sulbactam, and tazobactam (Bush et al., 1995). The global epidemic ESBL mainly includes SHV, TEM, and CTX-M types. During the past decade, the CTX-M type has been recognized as the predominant beta-lactamase in Enterobacteriaceae throughout the world (Bell et al., 2002; Livermore et al., 2007; Doi et al., 2013; Zhang et al., 2014).

A number of studies have shown that CTX-M type ESBL-producing Enterobacteriaceae not only cause hospital-acquired infections but also are the main cause of community-onset bloodstream infections. In addition, The ESBLs are plasmid-encoded enzymes, and the plasmids have the potential to transfer between Enterobacteriaceae, which further aggravate the public concerns (Bingen et al., 1993; Ben-Ami et al., 2006; Rodríguez-Baño et al., 2006; Pitout and Laupland, 2008). In China, the prevalence of ESBL-producing Enterobacteriaceae causing nosocomial infections in tertiary hospitals exhibited a high increase from less than 20.0 to 72.2% between 2000 and 2011 (Xiao et al., 2011, 2012). Another investigation of Chinese county hospitals carried out during 2010–2011 showed that the incidence of ESBL-producing Enterobacteriaceae causing community-onset infections varied from 30.2 to 57.0% in different regions (Zhang et al., 2014). It is noteworthy that the two nationwide investigations on ESBL-producing Enterobacteriaceae showed that the majority of ESBL-producing Enterobacteriaceae carried $bla_{\mathrm{CTX-M}}$ genes.

CTX-M type ESBL-producing Enterobacteriaceae has been no longer limited to community-onset or hospital-acquired infections. Fecal carriage of CTX-M type ESBL-producing Enterobacteriaceae from the healthy individuals has been noted in many regions across the world (Guimarães et al., 2009; Kader and Kamath, 2009; Vinué et al., 2009; Herindrainy et al., 2011; Janvier et al., 2011). But little information about the characterization of CTX-M type ESBL-producing Enterobacteriaceae isolated from healthy rural residents in Chinese villages is very little.

It is well-known that most Chinese people live in the rural areas, and meanwhile there are high prevalence of CTX-M ESBL-producing Enterobacteriaceae among hospital-acquired and community-onset infections. Therefore it is of utmost importance to investigate the prevalence of CTX-M ESBL-producing Enterobacteriaceae isolated from healthy rural humans in China and analyze its relatedness with risk factors.

Materials and Methods

Ethics Statement

This present study was approved by the Ethics Committee of Shandong Agricultural University. Written informed consent was obtained from each subject participating in the study.

TABLE 1 | Characteristics of the study participants.

Characteristics	Locations		*p*-value[a]
	Xintai, *N* = 320	Ningyang, *N* = 300	
Age (years), median (range)	42 (20–80)	45 (20–80)	0.565
Female gender	159 (49.7%)	145 (48.3%)	0.738
No formal education	15 (4.7%)	35 (11.7%)	<0.001
Admitted to a hospital within the last 6 months	102 (31.9%)	45 (15.0%)	<0.001
Was prescribed antibiotics within the last 6 months	201 (62.7%)	196 (65.3%)	0.652
Used antibiotics without a prescription within the last 6 months	220 (68.8%)	109 (36.3%)	<0.001

[a]*Calculated by x^2 test or Mann–Whitney U test as appropriate.*

TABLE 2 | Detection of CTX-M-type extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae.

Locations	Participants	ESBL phenotype[a]		CTX-M gene[b]		CTX-M group[c]							
						CTX-M-1[d]		CTX-M-2[e]		CTX-M-8[f]		CTX-M-9[g]	
Xintai	320	271	84.7%	251	78.4%	88	35.1%	0	0.0%	6	2.4%	170	67.7%
Ningyang	300	241	80.3%	207	69.0%	58	28.0%	0	0.0%	3	1.4%	133	64.3%
Total	620	512	82.6%	458	73.9%	146	31.9%	0	0.0%	9	1.9%	303	66.2%

[a]*ESBL phenotype was determined according to CLSI recommendations.*
[b]*CTX-M gene was determined by PCR.*
[c]*Genotype of CTX-M genes was determined by PCR.*
[d]*CTX-M-1 group includes CTX-M-1 and several other variants, such as CTX-M-3 and CTX-M-15.*
[e]*CTX-M-2 group includes CTX-M-2 and several other variants.*
[f]*CTX-M-8 group includes CTX-M-8 and several other variants.*
[g]*CTX-M-9 group includes CTX-M-9 and several other variants, such as CTX-M-14.*

Sample Collection and Questionnaires

Sampling was performed between October, 2013 and February, 2014 in two counties (Xintai and Ningyang) of Taian, China. The participants were selected by random door-to-door sampling. A total of 650 people aged >18 years (Xintai:330; Ningyang: 320) were approached to participate in this study, of whom 30 humans refused (Xintai:10; Ningyang: 20).

Before the stool sampling, participants were interviewed and the related information was recorded. The contents of questionnaires included: age, gender, education, antibiotic usage in the previous 6 months, and admission to hospital in the previous 6 months.

Isolation and Confirmation of ESBL-Producing Enterobacteriaceae

The fresh stool samples were collected by the subjects using a nylon flocked ESwab 480CE (Copan, Brescia, Italy), and then the swab was spread onto the ChromID ESBL agar (BioMerieux, Marcy l'Etoile, France). After the incubation at 37.8°C for 24 h, the confirmation of ESBL phenotype was carried out by the disk diffusion method according to the recommendation of CLSI (2011). The positive isolates were further identified using conventional biochemical tests and the API 20E system (Sysmex-bioMerieux, Tokyo, Japan).

TABLE 3 | Species composition of CTX-M-type ESBL-producing Enterobacterioceae.

Strains	No. (X/N)	Percentage (%)
Escherichia coli	403 (226/177)	88.0
Klebsiella pneumoniae	26 (19/7)	5.7
Enterobacter cloacae	12 (7/5)	2.6
Enterobacter aerogenes	10 (6/4)	2.2
Citrobacter freundii	7 (4/3)	1.5
Total	458	100.0

X, Xintai county; N, Ningyang county.

Antimicrobial Susceptibility Testing

The disk diffusion method was used in this study to test the susceptibility of all the ESBL-producing Enterobacteriaceae to 12 commonly used antibiotics in clinical practices, including ampicillin, cefazolin, cefuroxime, ceftriaxione, cefoxitin, biapenem, imipenem, meropenem, piperacillin, gentamicin, amikacin, and fosfomycin (Chinese National Institute for the Control of Pharmaceutical and Biological Products). *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 700603) were used as the quality control strains in this study (CLSI, 2011).

Identification of Bla_{CTX-M} Gene

The extraction of DNA was conducted by boiling suspensions of the ESBL-producing isolates, and then the identification of bla_{CTX-M} genes were performed by multiple polymerase chain reactions (PCR; Monstein et al., 2007). According to the previous reference, four primer sets were used to amplify group-specific bla_{CTX-M} genes (CTX-M-1, CTX-M-2, CTX-M-8 and CTX-M-9; Pitout et al., 2004).

Statistical Analysis

Mann–Whitney U test and x^2 test were used to compare the continuous data and the categorical data, respectively. The univariate and multivariate logistic regression was used to analyze the risk factors associated with the fecal carriage of ESBL-producing Enterobacteriaceae. The results were presented as OR with 95% CI. Significance was set at $P < 0.05$. All the data were analyzed by SPSS version 18.0 software (The Predictive Analytics Company, Chicago, IL, USA).

Results

There were 320 and 300 stool samples collected in Xintai and Ningyang counties, respectively. The information about demographics and hospitalization history and antibiotics use within 6 months was recorded (**Table 1**). Better education and higher month income were found in the rural subjects from Xintai county.

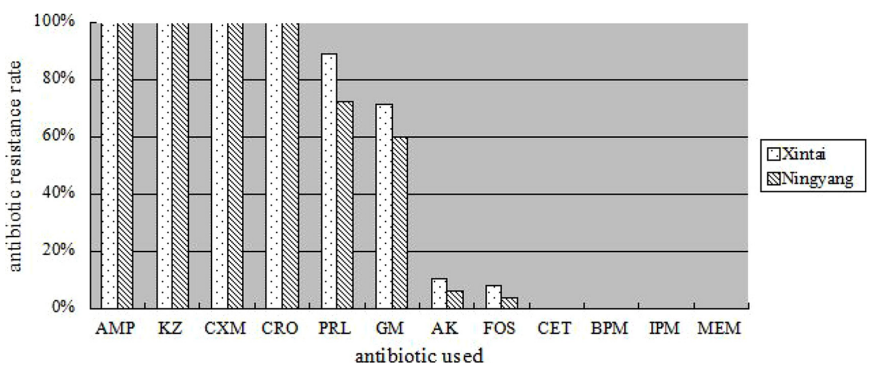

FIGURE 1 | Resistance rates of CTX-M extended-spectrum beta-lactamase (ESBL)-producers to 12 antibiotics. AMP, ampicillin; KZ, cefazolin; CXM, cefuroxime; CRO, ceftriaxone; PRL, piperacillin; GM, gentamicin; AK, amikacin; FOS, fosfomycin; CET, cefoxitin; BPM, biapenem; IPM, imipenem; MEM, meropenem.

There were 458 CTX-M ESBL-producing Enterobacteriaceae (458/620, 73.9%) confirmed by PCR in this survey (**Table 2**), and the isolates were from different subjects. Compared with Ningyang county, Xintai county had a significant higher isolation rates of CTX-M-type ESBL-producing Enterobacteriaceae ($P < 0.001$). The genotyping results of bla_{CTX-M} gene demonstrated that 303 CTX-M-carrying isolates belonged to the CTX-M-9 group (303/458, 66.2%), followed by the CTX-M-1 group (146/458, 31.9%), and CTX-M-8 group (9/458, 1.9%). But there was no isolates belonging to the CTX-M-2 group. The dominant species among CTX-M ESBL-producing Enterobacteriaceae were *E. coli* (403/458, 88.0%) and *K. pneumoniae* (26/458, 5.7%). In addition, *Citrobacter* and *Enterobacter* were found among the CTX-M-type ESBL producers (**Table 3**).

Among the CTX-M-type ESBL-producing isolates, they were all resistant to ampicillin, cefazolin, cefuroxime, and ceftriaxione, but were all susceptible to cefoxitin, biapenem, imipenem, and meropenem (**Figure 1**). The drug-resistance rate of CTX-M ESBL producers isolated from Xintai county to piperacillin, gentamicin, amikacin, and fosfomycin was significantly higher than from Ningyang ($P = 0.013$).

The multivariate logistic regression model used in this study identified the enrollment in formal education (OR 2.321; 95% CI 1.302–3.768; $P = 0.039$), the hospitalization history within the last 6 months (OR 1.753; 95% CI 1.127–2.584; $P = 0.031$), and the antibiotics use within the last 6 months (OR 1.892; 95% CI 1.242–2.903; $P = 0.034$). The three variables were significantly associated with carriage of CTX-M ESBL producers ($x^2 = 21.21$; $df = 3$; $P < 0.001$).

Discussion

Although there were several reports in China about the prevalence of ESBL-carrying Enterobacteriaceae in healthy people, the subjects were from city residents (Yu et al., 2007; Hu et al., 2013). Up to date, there is little information about isolation rate of ESBL-carrying Enterobacteriaceae in the rural residents in China, especially its risk factors associated with fecal carriage. The result of this present study demonstrated that high prevalence of CTX-M type ESBL-producing Enterobacteriaceae existed in the healthy rural residents in Taian, China (73.9%). The result showed a more or less similar prevalence with previous surveys in China and other country (Yu et al., 2007; Tian et al., 2008; Luvsansharav et al., 2012; Hu et al., 2013; Sun et al., 2014). Moreover, among CTX-M-positive isolates, the most dominant genotype was CTX-M-9 group (66.2%) and the dominant species were *E. coli* (88.0%). The results were in agreement with previous investigations about ESBL-producing Enterobacteriaceae isolated hospitals in China (Munday et al., 2004; Zhang et al., 2014).

The results of antimicrobial resistance testing revealed that all the CTX-M producers isolated from the rural residents were resistant to ampicillin, cefazolin, cefuroxime, and ceftriaxone, but all isolates were susceptible to biapenem, imipenem, and meropenem. The drug-resistant characteristics of CTX-M producers isolated from the rural residents are similar with those of ESBL-positive isolates from Chinese hospitals (Zhang et al., 2014).

The risk factor analyses in this study showed that the use of antibiotics and the hospitalization history within the past 6 months exhibited higher risks for carrying CTX-M ESBL-producing Enterobacteriaceae in this study. The result was consistent with the previous researches (Tian et al., 2008; Luvsansharav et al., 2012). Additionally, the rural residents in Xintai had a relatively better education and more frequent use of antibiotics, and they had a higher prevalence of CTX-M type ESBL-producing Enterobacteriaceae compared with humans from Ningyang. The result may be related the fact that humans with good economic income in the rural area of China are more likely to take some nonprescription drugs (Sun et al., 2014).

There were some limitations in this study. Fecal sampling was carried out only in two counties in Taian, so the results may not be representative of the whole area. The risk factor analysis was conducted according to the self-reporting of subjects, so recall bias such as the past drug use, may occurred. Most of the antibiotics tested were beta-lactams, but antibiotics of other classes were relatively less. Further sequence analysis of the CTX-M genes was not conducted.

In summary, the healthy rural residents in Xintai and Ningyang counties had high fecal carriage of CTX-M-type ESBL-producing Enterobacteriaceae. It is very important for the public health-care departments to educate the rural residents to rationally take over-the-counter drugs, control hospital infection strictly, and guide doctors to prescribe proper antibiotics for rational duration.

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Characterization of CTX-M-14-producing *Escherichia coli* from food-producing animals

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Bacterial resistance to the third-generation cephalosporin antibiotics has become a major concern for public health. This study was aimed to determine the characteristics and distribution of $bla_{CTX-M-14}$, which encodes an extended-spectrum β-lactamase, in *Escherichia coli* isolated from Guangdong Province, China. A total of 979 *E. coli* isolates isolated from healthy or diseased food-producing animals including swine and avian were examined for $bla_{CTX-M-14}$ and then the $bla_{CTX-M-14}$ –positive isolates were detected by other resistance determinants [extended-spectrum β-lactamase genes, plasmid-mediated quinolone resistance, *rmtB,* and *floR*] and analyzed by phylogenetic grouping analysis, PCR-based plasmid replicon typing, multilocus sequence typing, and plasmid analysis. The genetic environments of $bla_{CTX-M-14}$ were also determined by PCR. The results showed that fourteen CTX-M-14-producing *E. coli* were identified, belonging to groups A (7/14), B1 (4/14), and D (3/14). The most predominant resistance gene was bla_{TEM} ($n = 8$), followed by *floR* ($n = 7$), *oqxA* ($n = 3$), *aac(6′)-1b-cr* ($n = 2$), and *rmtB* ($n = 1$). Plasmids carrying $bla_{CTX-M-14}$ were classified to IncK, IncHI2, IncHI1, IncN, IncFIB, IncF or IncI1, ranged from about 30 to 200 kb, and with insertion sequence of IS*Ecp1*, IS*26,* or ORF513 located upstream and IS*903* downstream of $bla_{CTX-M-14}$. The result of multilocus sequence typing showed that 14 isolates had 11 STs, and the 11 STs belonged to five groups. Many of the identified sequence types are reported to be common in *E. coli* isolates associated with extraintestinal infections in humans, suggesting possible transmission of $bla_{CTX-M-14}$ between animals and humans. The difference in the flanking sequences of $bla_{CTX-M-14}$ between the 2009 isolates and the early ones suggests that the resistance gene context continues to evolve in *E. coli* of food producing animals.

Keywords: *Escherichia coli*, CTX-M-14, plasmids, MLST, cephalosporin

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INTRODUCTION

Enterobacteria with resistance to third and fourth generation cephalosporin antibiotics, especially *Escherichia coli* bearing extended-spectrum β-lactamase genes (ESBLs), have been detected in a wide range of food-producing animals. In 1989, CTX-M-type β-lactamases, whose carriers are highly resistant to cefotaxime but sometimes susceptible to ceftazidime, were considered as a novel group of class-A β-lactamases with extended-spectrum properties. This family of

enzymes are well inhibited by clavulanate and tazobactam (Tzouvelekis et al., 2000). Since then, the CTX-M family of ESBLs has become prominent and is common in *E. coli* with many infections occurring in human patients in the community (Livermore and Hawkey, 2005). In addition, the occurrence and prevalence of CTX-M in food-producing animals were also reported frequently (Hammerum et al., 2012; Reich et al., 2013). Rapid dissemination of $bla_{\text{CTX-M}}$ genes involved plasmids and mobile genetic elements as well as epidemic spread of particular strains (Eckert et al., 2006). IS*Ecp1*-like insertion sequences (ISs) have repeatedly been observed upstream of open reading frames (ORFs) encoding members belonging to the CTX-M-1, CTX-M-2, and CTX-M-9 clusters. ISs such as IS*10,* IS*26*, and IS*903* have also been observed flanking the ORF region of $bla_{\text{CTX-M}}$ genes (Arduino et al., 2002).

Of the CTX-M enzymes, the CTX-M-1 and CTX-M-9 clusters have been the most frequently reported worldwide, and although in some places CTX-M-2 group remains endemic, the emergence of new CTX-M groups (mainly CTX-M-1 and CTX-M-9) has been documented (D'Andrea et al., 2013). In addition, the CTX-M-14 enzyme is, besides CTX-M-9, the most widespread enzyme of the CTX-M-9 group (Valverde et al., 2009), especially in China (Li et al., 2010; Zheng et al., 2012). CTX-M-14 was first isolated from hospital in China in 1997 (Chanawong et al., 2002). It is a member of the CTX-M-9 cluster and differs from $bla_{\text{CTX-M-9}}$ only by the substitution Ala 231⟶Val (Ma et al., 2002), and it has spread almost all over the world (Canton and Coque, 2006). Reports showed that IS*Ecp1*, IS*26*, ORF513, IS*903*, and ORF1005 located upstream and downstream of $bla_{\text{CTX-M-14}}$, respectively, which might have contributed to its widespread transmission (Izumiya et al., 2005; Eckert et al., 2006; Bae et al., 2007; Navarro et al., 2007). Moreover, most of the literature indicates that novel CTX-M enzyme genes were often derived from or recombinated with CTX-M-14-like β-lactamase (Djamdjian et al., 2011; He et al., 2013; Tian et al., 2014), indicating that $bla_{\text{CTX-M}}$ genes evolve by homologous recombination between members of different groups, especially with $bla_{\text{CTX-M-14}}$ (Tian et al., 2014).

Due to the importance of $bla_{\text{CTX-M-14}}$ in antimicrobial resistance and its limited information in food producing animals, we examined the characteristics and distribution of $bla_{\text{CTX-M-14}}$ in *E. coli* of food-producing animals in China in this study.

MATERIALS AND METHODS

Bacterial Isolates and CTX-M-14 Detection

From 2002 to 2009, a total of 979 *E. coli* isolates, including 455 isolates in 2002, 119 isolates in 2003–2004, 76 isolates in 2007 and 329 isolates in 2009 were isolated from healthy or diseased food-producing animals from Guangdong Province in China. Samples were collected from rectal swabs of healthy animals, or the liver, heart, lung, or muscle samples of diseased or dead animals. Samples were seeded on MacConkey agar at 37°C, and one colony with typical *E. coli* morphology was selected from each sample. Each isolate was from an individual animal. The bacterial strains were identified by classical biochemical methods and confirmed using the API-20E system (bioMérieux). All confirmed *E. coli* isolates were stored at −80°C in the Luria–Bertani broth medium containing 30% glycerol.

Cefotaxime-resistant *E. coli* isolates were selected on MacConkey agar containing cefotaxime at 2 μg/mL and then the $bla_{\text{CTX-M-14}}$ gene was detected by PCR analysis with the primer described previously (Yu et al., 2007), the primers and PCR programs were listed in Supplementary Table S1. PCR products were directly sequenced, and then made comparison in the GenBank nucleotide database.

Antimicrobial Susceptibility Testing and Genes Characterization

The minimum inhibitory concentrations (MICs) of quinolones (nalidixic acid), fluoroquinolones (ciprofloxacin, enrofloxacin, and levofloxacin), third-generation cephalosporins (ceftiofur, cefotaxime, and ceftazidime) and other antimicrobials (olaquindox, ampicillin, trimethoprim-sulfamethoxazole, tetracycline, gentamicin, amikacin, chloramphenicol, and florfenicol) in $bla_{\text{CTX-M-14}}$ positive isolates were determined by the agar dilution method following the CLSI guidelines. The breakpoints for individual antimicrobial were used as recommended by the CLSI (M100-S19), CLSI (Vet01-A4/Vet01-S2), and DANMAP 98 (olaquindox). *E. coli* ATCC25922 was used as a quality control strain. All $bla_{\text{CTX-M-14}}$ positive isolates were tested for $bla_{\text{CTX-M-1G}}$, $bla_{\text{CTX-M-2G}}$, $bla_{\text{CTX-M-8G}}$, bla_{TEM}, and bla_{SHV}, bla_{OXA} and bla_{CMY-2} by PCR and then confirmed by sequencing. At the same time, plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *aac(6′)-Ib-cr,* and *oqxA*), *rmtB* and *floR* were also detected. The PCR programs and primer sequences were described previously (Yu et al., 2007; Yue et al., 2008; Veldman et al., 2011; Li et al., 2013; Liu et al., 2013, 2014). The primers and PCR programs were listed in Supplementary Table S1. All PCR products were directly sequenced, and the results were compared with those in the GenBank nucleotide database.

Clonal Relatedness

A multiplex PCR methodology was employed to assign isolates harboring $bla_{\text{CTX-M-14}}$ to one of the four phylogenetic groups (A, B1, B2, or D). Primers and methodology have been described previously (Doumith et al., 2012). For isolates carrying $bla_{\text{CTX-M-14}}$, multilocus sequence typing (MLST) was performed using seven conserved housekeeping genes (*adk*, *purA*, *recA*, *mdh*, *icd*, *gyrB,* and *fumC*; Wirth et al., 2006). The internal fragments of all loci were sequenced. Allelic profiles and sequence type (ST) determinations were performed according to the *E. coli* MLST website[1] scheme. MLST data were analyzed by using the eBURST program (version 3[2]), which assesses the relationship within clonal complexes. The set minimum group allele number was five. The UPGMA method of START program (version 2) was used to construct phylogenetic grouping tree. The genetic distance is 0.1.

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

[2]http://eburst.mlst.net

TABLE 1 | Characteristics of the 14 *Escherichia coli* isolates carrying $bla_{CTX-M-14}$.

Isolates	Origin	Year	Drug-resistant spectrum	Group	Resistant genes	MICs (μg/ml)			Replicon typing	Genetic environment
						CTF	CTX	CTR		
ZLP20	Pig	2002	AMP/CTX/CTR/CTF/KAN/DOX/TET/NAL/ENR/CIP/FLF	A	$bla_{CTX-M-14a}$, *oqxA*, *floR*	64	64	64	F, Y	
ZLP20-D			AMP/ CTR/CTF/CTX		$bla_{CTX-M-14a}$	32	64	64	F	IS*Ecp1*, IS*903*
ZLP19	Pig	2002	AMP/CTX/CTR/CTF/KAN/DOX/TET/NAL/ENR/CIP/FLF	A	$bla_{CTX-M-14a}$, *floR*	64	64	128	F, Y	
ZLP19-D			AMP/ CTX /CTR/CTF		$bla_{CTX-M-14a}$	32	64	64	F	IS*Ecp1*, IS*903*
ZLP21	Pig	2002	AMP/CTX/CTR/CTF/KAN/GEN/DOX/TET/NAL/ENR/CIP/FLF	A	$bla_{CTX-M-14a}$	32	32	64	HI1, N	
ZLP21-D			AMP/ CTX /CTR/CTF		$bla_{CTX-M-14a}$	32	32	32	HI1, N	IS*Ecp1*, IS*903*
ZLP25	Pig	2002	AMP/CTX/CTR/KAN/DOX/TET/NAL/ENR/CIP/FLF	A	$bla_{CTX-M-14a}$, *aac(6')-1b-cr*	64	32	64	HI1, N	
ZLP25-D			AMP/ CTX /CTR/CTF/TET		$bla_{CTX-M-14a}$	16	32	16	HI1, N	IS*Ecp1*, IS*903*
HN428	Duck	2005	AMP/CTR/CXT/CTF/KAN/SM/DOX/TET/NAL/ENR/CIP/FLF	D	$bla_{CTX-M-14a}$, bla_{TEM-1}	64	32	64	FIB, FIC, I1, F	
HN428-D			AMP/ CTX /CTR /TET		$bla_{CTX-M-14a}$, bla_{TEM-1}	4	16	8	FIB, F	IS*Ecp1*, IS*903*
a88	Duck	2007	AMP/CTR/CTF/KAN/SM/TET/NAL/ENR/CIP/ FLF	D	$bla_{CTX-M-14a}$, bla_{TEM-1}	64	32	64	F, Y, K	
a88-D			AMP/ CTX /CTR		$bla_{CTX-M-14a}$	4	16	16	F	IS*Ecp1*, IS*903*
14	Duck	2009	AMP/CTX/CTR/CXT/KAN/GEN/DOX /TET/NAL/ENR/CIP/FLF	B1	$bla_{CTX-M-14a}$, $bla_{CTX-M-79}$	128	256	256	I1, K	
14-D			AMP/ CTX /CTR/CTF/TET		$bla_{CTX-M-14a}$	128	256	128	K	IS*Ecp1*, IS*903*
16	Duck	2009	AMP/CTF/CTX/CTR/KAN/GEN/DOX/TET/NAL/ENR/CHL/CIP/FLF	B1	$bla_{CTX-M-14a}$, $bla_{TEM-135}$, *oqxA*, *floR*	64	128	128	HI2, FIB, K	
16-D			AMP/CTF/CTX/CTR/CHL/CIP/FLF		$bla_{CTX-M-14a}$	64	128	32	K	ORF513, IS*903*
40	Duck	2009	AMP/CTX/CTR/KAN/GEN/DOX/TET/NAL/ENR/CIP/FLF	A	$bla_{CTX-M-14a}$, bla_{TEM-1}, *oqxA*, *floR*	16	64	64	HI2, FIA, F, FIB	
40-D			AMP/CTF/CTX/CTR/GEN/FLF		$bla_{CTX-M-14a}$, *floR*	16	32	8	HI2, F	IS*Ecp1*, IS*903*
103	Goose	2009	AMP/CTX/CTR/CXT/CTF/KAN/GEN/DOX/TET/NAL/ENR/CIP/FLF	A	$bla_{CTX-M-14b}$, bla_{TEM-1}, *aac(6')-1b-cr*, *rmtB*	64	64	128	I1, FIB, F, K	
103-D			AMP/CTF/CTX/CTR/CHL/CIP/FLF		$bla_{CTX-M-14b}$, *rmtB*	64	64	64	I1	IS*Ecp1*, ORF513, IS*903*
132	Goose	2009	AMP/CTX/CTF/CTR/KAN/DOX/TET/NAL/ENR/CIP/FLF	B1	$bla_{CTX-M-14a}$, *oqxA*, *floR*	64	256	256	P, F, K	
132-D			AMP/CTX/CTF/CTR		$bla_{CTX-M-14a}$	64	128	32	F	IS*903*
156	Pig	2009	AMP/CTF/CTX/CTR/KAN/GEN/DOX/TET/NAL/ENR/CIP/FLF	D	$bla_{CTX-M-14a}$, bla_{TEM-1}, *floR*	128	64	128	FIA,P, F, K	
156-D			AMP/CTF/CTX/CTR/KAN/GEN/DOX/TET/NAL/ENR/CIP/FLF		$bla_{CTX-M-14a}$, bla_{TEM-1}, *floR*	128	256	128	K	IS*Ecp1*,IS*903*
173	Pig	2009	AMP/CTX/CTF/CTR/CTF/KAN/GEN/DOX/TET/NAL/ENR/CIP/FLF	A	$bla_{CTX-M-14a}$, bla_{TEM-1}, *floR*	64	64	64	FIB, Y, F, K	
173-D			AMP/CTX/CTF/CTR		$bla_{CTX-M-14a}$, *floR*	64	64	64	F	*IS26*, IS*Ecp1*, IS*903*

(Continued)

TABLE 1 | Continued

Isolates	Origin	Year	Drug-resistant spectrum	Group	Resistant genes	MICs (μg/ml)			Replicon typing	Genetic environment
						CTF	CTX	CTR		
187	Pig	2009	AMP/CTX/CTF/CTR/KAN/GEN/DOX/TET/NAL/ENR/CIP/FLF	B1	$bla_{CTX-M-14a}$, bla_{TEM-1b}	32	64	64	FIB, I1, Y, F, K	
187-D			AMP/CTX/CTF/CTR/KAN/GEN/CIP		$bla_{CTX-M-14a}$	16	64	16	I1	IS*903*

D, corresponding conjugants or transformants; AMP, Ampicillin; CTX, Cefotaxime; CTF, Ceftiofur; CXT, Cefoxitin; CTR, Ceftriaxone; CTZ, Ceftazidime; STR, Streptomycin; GEN, Gentamycin; KAN, Kanamycin; FLF, Florfenicol; TET, Tetracycline; NAL, Nalidixic acid; CIP, Ciprofloxacin; ENR, Enrofloxacin; DOX, Doxycycline.

Transfer of the $bla_{CTX-M-14}$ Gene and Plasmid Analysis

Transferability of the identified $bla_{CTX-M-14}$ genes was determined by conjugation using streptomycin-resistant *E. coli* C600 as the recipient strain (Zheng et al., 2012). Transconjugants were selected on MacConkey agar plates supplemented with cefotaxime (2 μg/mL) and streptomycin (2000 μg/mL). For those isolates that failed in conjugation experiments, plasmid DNA was extracted by QIAGENPrep Plasmid Midi Kit (Qiagen, Germany), and electroporated into electrocompetent *E. coli* DH5α (TaKaRa Biotechnology, Dalian, China) using a Gene Pulser apparatus (Biorad Laboratories). Transformants were selected on MacConkey agar plates supplemented with cefotaxime (2 μg/mL). The presence of $bla_{CTX-M-14}$ was confirmed by PCR. Plasmids were preliminarily classified according to their incompatibility group by using the PCR-based replicon typing (PBRT) scheme described previously (Carattoli et al., 2005). PFGE with S1 nuclease (TakaRa Biotechnology, Dalian, China) digestion of whole genomic DNA was performed for all 14 transconjugants and transformants as described previously (Barton et al., 1995). After Southern transfer to a Hybond-N^+ membrane (GE Healthcare, Little Chalfont, UK), the plasmids were probed with the $bla_{CTX-M-9G}$ gene (DIG High Prime DNA Labeling and Detection Starter Kit I, Roche Applied Science, Mannheim, Germany).

Genetic Environment of $bla_{CTX-M-14}$

Detection of the ISs including IS*Ecp1*, IS*26*, ORF513, IS*903*, and ORF1005, which are located upstream or downstream of $bla_{CTX-M-14}$, were performed by PCR according to the methods in previous reports (Izumiya et al., 2005; Eckert et al., 2006; Bae et al., 2007; Navarro et al., 2007; Barlow et al., 2008).

RESULTS

Antimicrobial Susceptibility and Detection of Resistance Genes

Among the 979 *E. coli* isolates surveyed, fourteen isolates harbored $bla_{CTX-M-14}$, among which four were isolated from swine in 2002, two from duck in 2005 and 2007, and the other eight were isolated from swine and duck in 2009. All the 14 $bla_{CTX-M-14}$-positive isolates were multidrug resistant (resistant to three or more classes of antimicrobials) and all of them were resistant to ampicillin, cefotaxime, ceftiofur, ceftriaxone, florfenicol, tetracycline, kanamycin, and ciprofloxacin. In addition, the resistance of the $bla_{CTX-M-14}$-positive isolates to gentamicin and doxycycline were 64 and 86%, respectively (**Table 1**). Among the fourteen isolates harboring $bla_{CTX-M-14}$, only one isolate was confirmed as $bla_{CTX-M-14b}$-carrying strain, the other thirteen were $bla_{CTX-M-14a}$. In addition, the fourteen isolates were also subjected to the detection of ESBLs, PMQR genes and other resistance genes (*rmtB* and *floR*). The most predominant gene was bla_{TEM} ($n = 8$), including six bla_{TEM-1}, one bla_{TEM-1b}, and one $bla_{TEM-135}$, followed by *floR* ($n = 7$), *oqxA* ($n = 3$), *aac(6′)-1b-cr* ($n = 2$), and *rmtB* ($n = 1$) (**Table 1**).

Clonal Relatedness and Transfer of $bla_{CTX-M-14}$

Phylogenetic group analysis showed that group A (7/14) was dominant amongst the isolates that produced the CTX-M-14 enzymes, followed by group B1 (4/14) and group D (3/14). None of them belonged to group B2 (**Table 1**). The result of MLST showed that the 14 isolates have 11 ST, among which ST2929 and ST2962 were newly discovered (Supplementary Table S2). The MLST results belonged to five groups. ST10, ST206, ST2929, ST2930, and ST2962 belonged to Group 1 (**Figure 1A**), while ST155, ST224, and ST602 were classified into Group 2 (**Figure 1B**). Furthermore, ST648, ST359, and ST405 belonged to Group14, Group16, and Group17, respectively. Phylogenetic grouping tree suggested that ST10, ST2929, ST206, and ST2930 were close in one branch, while ST224, ST602, ST155, ST359, and ST2962 were clustered in another branch. ST648 and ST405 were separated from others (**Figure 2**). Eight transconjugants and six transformants were successfully obtained by conjugation/transformation experiments. Co-transfer of bla_{TEM-1} or *rmtB* or *floR* genes were also detected. The $bla_{CTX-M-14}$-positive strain isolated in 2005 co-transferred with bla_{TEM-1}. Among the 2009 isolates, one had co-transfer of *rmtB*, two had co-transfer of *floR*, and another one had co-transfer of both bla_{TEM-1} and *rmtB*. MICs of cefotaxime, ceftiofur, and ceftriaxone increased two–fourfold compared with the recipients.

Plasmids and Genetic Environment of $bla_{CTX-M-14}$

Plasmids containing $bla_{CTX-M-14}$ were predominately belonging to IncF ($n = 5$), IncK ($n = 3$), and IncI1 ($n = 2$). Additionally, 2 of the 14 were positive for two replicons (IncHI1 and IncN), another one was positive for IncFIB and IncF, and the

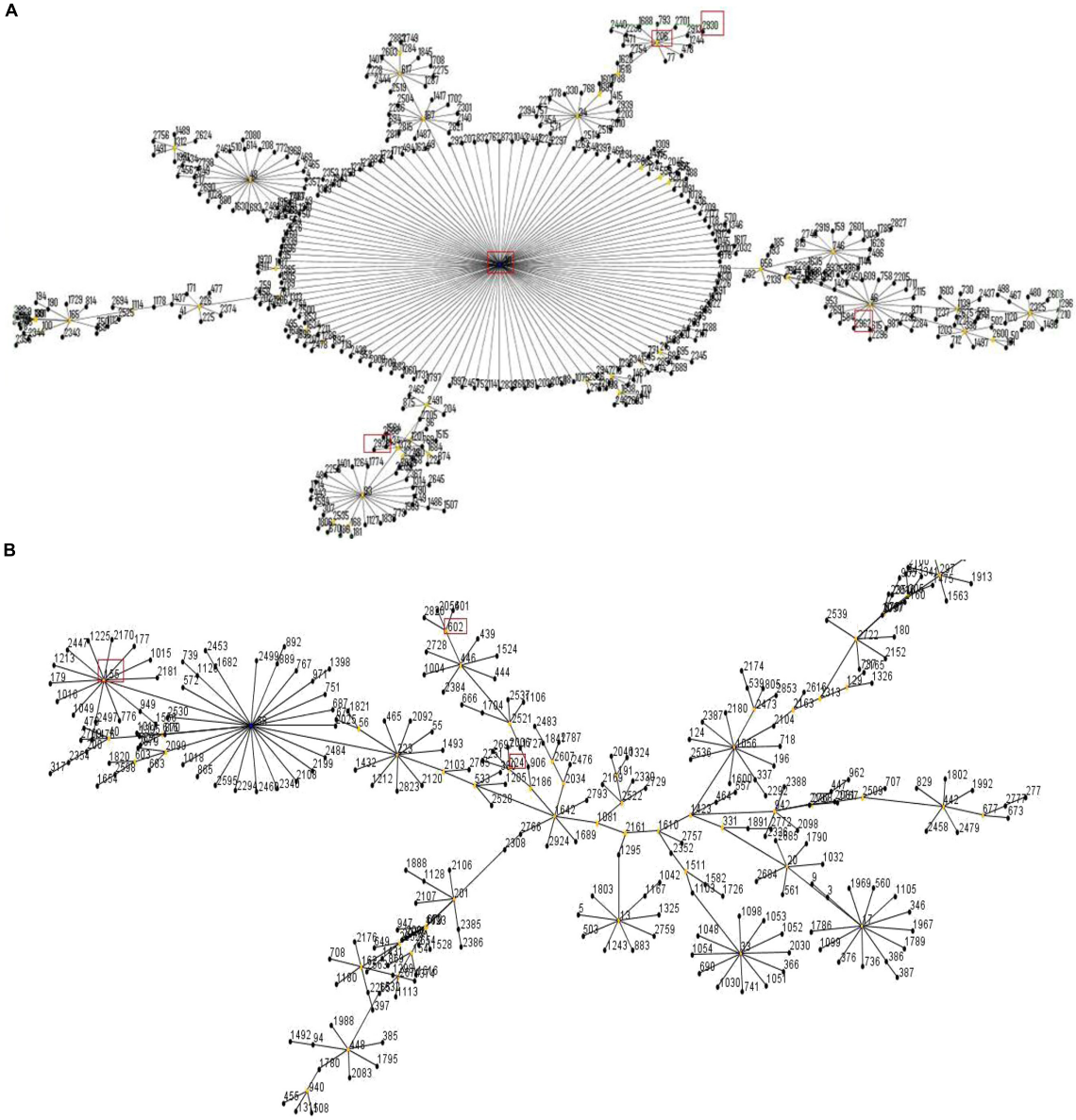

FIGURE 1 | (A) eBRUST results of Group 1. The red boxes indicate that the corresponding five sequence types (STs ; ST206, ST10, ST2929, ST2930, and ST2962) we detected in Group 1. **(B)** eBRUST results of Group 2. The red boxes indicate that the corresponding three STs (ST155, ST224, and ST602) we detected in Group 2.

remaining one belonged to IncHI2 and IncF. The size of the plasmids ranged from about 30 to 200 kb (Supplementary Figure S2). IS*Ecp1* and IS*903* were found upstream and downstream, respectively, of the $bla_{CTX-M-14}$-positive isolates isolated from 2002 to 2007(Supplementary Figure S1). Among the eight positive isolates isolated in 2009, four were detected with IS*Ecp1* and IS*903* upstream and downstream, respectively, one harbored IS*Ecp1* upstream, and the other three carried IS*903* downstream. In addition, IS*26* was confirmed in one strain of 2009, and ORF513 positive isolates were also found in this year. None of the isolates harbored ORF1005.

DISCUSSION

CTX-M-type ESBLs, with 150 variants, have recently been the most widespread ESBLs in *E. coli*. CTX-M variants can be divided into six clusters: the CTX-M-1, −2, −8, −9, −25, and KLUC groups. Additionally, the most frequently reported groups are CTX-M-1 and CTX-M-9, and CTX-M-14 is the most variant within the latter group (D'Andrea et al., 2013).

In this study, phylogenetic group analysis showed that group A (7/14) was dominant amongst the isolates that produced the CTX-M-14 enzymes, followed by group B1 (4/14) and group

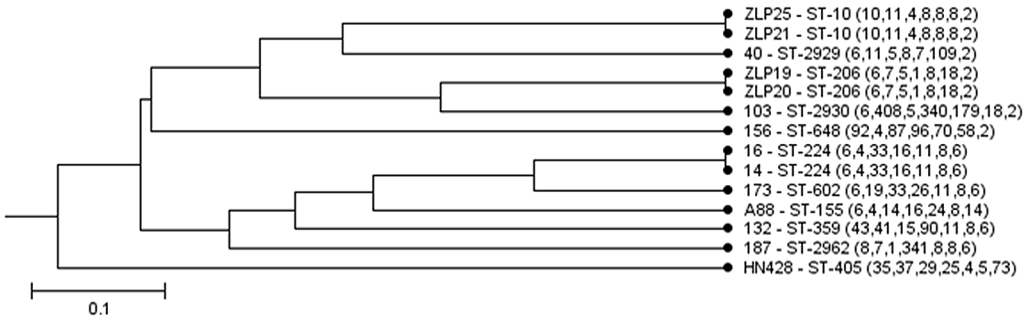

FIGURE 2 | Evolutionary branches of 14 *bla*$_{CTX-M-14}$-positive *Escherichia Coli.*

D (3/14), which was consistent with the reports in Portugal, Spain, and China (Valverde et al., 2009; Zheng et al., 2012). Previous studies showed that most *E. coli* strains responsible for urinary tract infections and other extraintestinal infections in humans belong to group B2 or, to a lesser extent, to group D (Johnson and Stell, 2000; Yang et al., 2012). Investigation of urinary *E. coli* isolates from 20 widely dispersed tertiary Chinese hospitals revealed although phylogroups D and B2 were most frequently observed, phylogroups A and B1 were also found in *bla*$_{CTX-M-14}$-producing *E. coli* isolates (Cao et al., 2011).

According to recent reports, replicon types of *bla*$_{CTX-M-14}$-positive plasmids belonged to IncF, IncFIB, IncI1, IncA/C, IncN, IncFII, and IncI1-Iγ (Millan et al., 2011; Song et al., 2011; Tamang et al., 2011). In this study, *bla*$_{CTX-M-14}$-carrying plasmids predominately belonged to IncF and IncK. The spread of *bla*$_{CTX-M-14}$ in *E. coli* in Spain is reported to be mediated by IncK plasmids (Valverde et al., 2009), while in Korea and France *bla*$_{CTX-M-14}$ is mostly carried on IncF plasmids (Marcade et al., 2009). IncF plasmids were found frequently to be associated with CTX-M enzyme genes of *E. coli* (Matsumura et al., 2013; Mnif et al., 2013). IncK plasmids may facilitate the ability of *E. coli* to colonize the intestine and, consequently, enhance the pathogenic profile of specific clones or clonal groups (Oshima et al., 2008). Besides, reports showed that the acquisition of IncK plasmids containing *bla*$_{CTX-M-14}$ by group A and B1 *E. coli* clones could have enhanced their ability to colonize the urinary tract in patients exposed to antibiotics (Valverde et al., 2009). IncHI1, IncHI2 and IncN plasmids were rarely reported in *bla*$_{CTX-M-14}$-producing *E. coli*.

In this study, 11 different STs (including two new STs) were detected among 14 *bla*$_{CTX-M-14}$-producing *E. coli* isolates. The findings indicate that no ST predominates in CTX-M-14-producing *E. coli* from food-producing animals of Guangdong. ST10 and ST648 were common in *E. coli* isolated from human and animals (Shabana et al., 2013; Maluta et al., 2014; Xia et al., 2014; Jamborova et al., 2015). ST155 was once found in human, duck, and bovine (Ben Sallem et al., 2012; Sváb et al., 2013; Maluta et al., 2014). ST359 was once reported in human and duck (Maluta et al., 2014). ST405, a global clonal group associated with the global increase of ESBLs, was mainly reported in human origin as well as once reported in rooks and food origins (Jouini et al., 2013; Matsumura et al., 2014; Jamborova et al., 2015). ST602 in *E. coli* of cats was once reported (Nebbia et al., 2014). ST224 was detected in *E. coli* of human, dogs and buffalo origin (Mshana et al., 2011; Dahmen et al., 2013; Aizawa et al., 2014), while ST224 was found in duck origin in 2009 in this study[3]. Moreover, recent reports revealed that *E. coli* of human origin, especially *E. coli*-producing ESBLs associated with urinary tract infection, mainly belonged to the ST10 complex. In Portugal, Spain, and Brazil, ST155 and ST359 were found rising in patients suffering from urinary tract infection (Canton and Coque, 2006). In this study, the STs we have found were mainly reported in human, suggesting that *bla*$_{CTX-M-14}$ could transfer between human and food-producing animals.

Insertion sequences played an important role in the transfer of *bla*$_{CTX-M-14}$. In this study, IS*Ecp1* was detected 42 nucleotides upstream of both *bla*$_{CTX-M-14a}$ and *bla*$_{CTX-M-14b}$. It is of interest to note that an identical 42-bp region has also been detected upstream of different genes encoding ESBLs of the CTX-M-9 cluster, such as CTX-M-9, −14, −16, and −17 (Barlow et al., 2008), which means this subtype may have the same origin as *bla*$_{CTX-M-14}$. From 2002 to 2007, the genetic environment of *bla*$_{CTX-M-14}$-positive isolates was the same, with IS*Ecp1* and IS*903* found upstream and downstream, respectively, while *bla*$_{CTX-M-14}$-positive isolates in 2009 showed diversity of the genetic platform. IS*26* and ORF513 were both found in 2009. It is important to note that ORF513 located upstream of *bla*$_{CTX-M-14a}$ in strain 16-D was the same as *bla*$_{CTX-M-14b}$ of strain 103-D. This showed that resistant genes of incompatible plasmids have the possibility to transfer and then recombine.

Extended-spectrum β-lactamase genes genes were often found to be strongly associated with PMQR or 16S rRNA methyltransferase (16S-RMTase) genes, and some were often found to be located on the same plasmid, both in human and animals (Carattoli, 2009; Liu et al., 2013). In this study, *bla*$_{CTX-M-14}$ of the isolates isolated from 2002 to 2007 tended to conjugate alone, while co-transfer with *bla*$_{TEM-1}$, *rmtB,* or *floR* on the same plasmid were common in the 2009 isolates. Co-existence or co-spread of ESBLs with PMQR, *rmtB* or *floR* suggests that the resistant isolates could be selected by

[3]http://mlst.ucc.ie/mlst/dbs/Ecoli/GetTableInfo.html

different classes of antibiotics. The fourteen isolates carrying $bla_{CTX-M-14}$ were found to be multidrug resistant and showed resistance to more than two non-β-lactam antimicrobial agents, including kanamycin, tetracycline, doxycycline, nalidixic acid, ciprofloxacin, enrofloxacin, and florfenicol. Some of them were also resistant to other cephalosporins, including ceftiofur, cefoxitin, and ceftriaxone, but remained susceptible to ceftazidime fortunately. In addition, although the $bla_{CTX-M-14}$-positive isolates showed resistance to kanamycin and gentamycin, most of them (13/14) remain susceptible to amikacin (data not shown), which indicated amikacin might be effective for treating $bla_{CTX-M-14}$-positive *E. coli* infection.

CONCLUSION

The evolution of $bla_{CTX-M-14}$ gradually became diversified in food-producing animals of Guangdong, China, from 2002 to 2009. Findings from this study and previous publications by others suggest that antibiotics, especially the third- and fourth-generation cephalosporins, should be used more prudently in food-producing animals.

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SUPPLEMENTARY MATERIAL

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Occurrence of *bla*$_{CTX-M-1}$, *qnr*B1 and virulence genes in avian ESBL-producing *Escherichia coli* isolates from Tunisia

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Avian ESBL-producing *Escherichia coli* isolates have been increasingly reported worldwide. Animal to human dissemination, via food chain or direct contact, of these resistant bacteria has been reported. In Tunisia, little is known about avian ESBL- producing *E. coli* and further studies are needed. Seventeen ESBL-producing *Escherichia coli* isolates from poultry feces from two farms (Farm 1 and farm 2) in the North of Tunisia have been used in this study. Eleven of these isolates (from farm 1) have the same resistance profile to nalidixic acid, sulfonamides, streptomycin, tetracycline, and norfloxacine (intermediately resistant). Out of the six isolates recovered from farm 2, only one was co-resistant to tetracycline. All isolates, except one, harbored *bla*$_{CTX-M-1}$ gene, and one strain co-harbored the *bla*$_{TEM-1}$ gene. The genes *tetA* and *tetB* were carried, respectively, by 11 and 1 amongst the 12 tetracycline-resistant isolates. Sulfonamides resistance was encoded by *sul1*, *sul2,* and *sul3* genes in 3, 17, and 5 isolates, respectively. The *qnrB1* was detected in nine strains, one of which co-harbored *qnrS1* gene. The search for the class 1 and 2 integrons by PCR showed that in farm 1, class 1 and 2 integrons were found in one and ten isolates, respectively. In farm 2, class 1 integron was found in only one isolate, class 2 was not detected. Only one gene cassette arrangement was demonstrated in the variable regions (VR) of the 10 *int2*-positive isolates: *dfrA1- sat2-aadA1*. The size of the VR of the class 1 integron was approximately 250 bp in one *int1*-positive isolate, whereas in the second isolate, no amplification was observed. All isolates of farm 1 belong to the phylogroup A (sub-group A0). However, different types of phylogroups in farm 2 were detected. Each of the phylogroups A1, $B2_2$, $B2_3$ was detected in one strain, while the D2 phylogroup was found in 3 isolates. The virulence genes *iut*A, *fimH*, and *traT* were detected in 3, 7, and 3 isolates, respectively. Two types of gene combination were detected: *iutA*+*fimH*+*traT* in 3 isolates and *iutA*+*fimH* in one isolate. The isolates recovered in farm 1 showed the same profile of PFGE macro-restriction, while isolates of farm 2 presented unrelated PFGE patterns. We conclude that these avian ESBL-producing *E. coli* isolates show homo- and heterogenic genetic background and that plasmids harboring ESBL genes could be involved in the dissemination of this resistance phenotype.

Keywords: ***Escherichia coli*, poultry, *bla*$_{CTX-M-1}$, *qnr*B1, integrons, clonality**

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Introduction

Escherichia coli is a commensal bacterium in humans and animals and considered an indicator of fecal contamination of food. Antimicrobial resistant isolates and resistance genes of *E. coli* can be transferred to humans through the food chain. This transfer represents a potential risk for public health (Alexander et al., 2010; Cortés et al., 2010; Canton et al., 2012; Ryu et al., 2012). The use of broad-spectrum cephalosporins in animals, such as ceftiofur and cefquinome, has been recognized a major driving force for the selection and spread of extended spectrum beta-lactamases (ESBL) (Dutil et al., 2010). In both reservoirs, high prevalence of genes belonging to CTX-M group dominates. Numerous data highlight the extent to which certain ESBL genes, ESBL plasmids or ESBL-producing clones are shared between animals and humans (Naseer and Sundsfjord, 2011).

In recent years, there has been increasing concern in the scientific community about the emergence and dissemination of *E. coli* strains producing ESBLs, especially of the CTX-M class, which are very frequently associated with community infections (Eckert et al., 2004; Livermore et al., 2007; Pitout and Laupland, 2008). Recently, different reports have indicated the dissemination of ESBL-positive *E. coli* strains among the intestinal microbiota of healthy humans (Vinué et al., 2009), in food producing animals, and in food products (Brinas et al., 2005; Blanc et al., 2006; Girlich et al., 2007; Jouini et al., 2007; Li et al., 2007; Smet et al., 2008). These resistant bacteria could be transferred to humans through the food chain. This transfer represents a problem for public health. Comparison of human and animal ESBL-producing isolates is important in enhancing the knowledge of the potential routes of transfer of these bacteria and resistance genes in different ecosystems. In Tunisia, ESBL-producers were initially reported from food samples such as raw chicken meat (Jouini et al., 2007; Ben Slama et al., 2010). More recently, ESBL-producing *E. coli* were described in healthy food animals at farm level (Ben Sallem et al., 2012b, 2013; Mnif et al., 2012). In Tunisia, ESBL-producing bacteria were found in chickens and a dromedary, suggesting that poultry constitutes a major reservoir of ESBL genes. The dominant ESBL gene found was $bla_{\text{CTX-M-1}}$, which was mainly detected with *Inc*I1 replicons (Mnif et al., 2012). A recent study also demonstrated that the $bla_{\text{CTX-M-1}}$ IncI1/ST3 plasmid was dominant in Tunisian chickens and pets (Grami et al., 2013). Finally, recent data showed that 7.3% of Tunisian healthy humans were fecal carriers of CTX-M-1-producing *E. coli*. This finding suggests that foodstuff of poultry origin may contribute to the transmission of the $bla_{\text{CTX-M-1}}$ gene from animals to humans (Ben Sallem et al., 2012b).

The aim of this study was to characterize ESBL producing *E. coli* isolates recovered from feces of healthy chickens in Tunisia by investigating genes encoding ESBL, resistance to tetracycline, sulfonamides and fluoroquinolones as well as content of virulence genes, integrons, and genetic clonality using PFGE.

Materials and Methods

Bacterial Strains

Sixty-five fecal samples were collected from healthy chickens in 2013. Of these samples, 45 came from 58-week-old chickens in a farm located at Sidi Thabet in the North of Tunisia (Farm 1) and 20 from 7-week-old chickens in a farm located at Morneg region (Farm 2). These samples were cultivated on Mac-Conkey agar containing 2 mg/L of cefotaxime and incubated overnight at 37°C. For each sample, one colony with typical *E. coli* trait was picked and re-isolated on Mac-Conkey agar and the phenotypic identification result was confirmed using Api20E (Bio-Mérieux, France).

Antimicrobial Susceptibility Testing and ESBL Identification

Antimicrobial susceptibility testing was carried out using the agar disk diffusion method on Mueller–Hinton agar plates in accordance with the Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute, 2012). The following antimicrobial agents were tested (μg/disk): ampicillin (10), amoxicillin (25), amoxicillin-clavulanic acid (20/10), ceftazidime (30), cefotaxime (30), gentamicin (10), kanamycin (30), streptomycin (10), amikacin (30), trimethoprim-sulfamethoxazol (1.25/23.75), tetracycline (30), nalidixic acid (30), ciprofloxacin (5), sulfonamides (200) and chloramphenicol (50). The Double-Disk Synergy Test (DDST) with cefotaxime or ceftazidime in the proximity to amoxicillin-clavulanic acid was used for the screening of ESBL (Clinical and Laboratory Standards Institute, 2012). *E. coli* ATCC25922 was used as ESBL negative and *Klebsiella pneumoniae* 700603 was used as ESBL positive reference strain.

Resistance Genotype

All primers used to characterize the resistance genotype are presented in **Table 1**. The presence of genes encoding TEM, SHV, CTX-M implicated in the beta-lactam resistance was analyzed by PCR (Sáenz, et al., 2004; Batchelor et al., 2005). Amplified DNA fragments were sequenced on both strands and the nucleotide and their deduced amino acid sequences were compared with those included in the Gen-Bank database as well as with those deposited at the website http://www.lahey.org/Studies/ in order to confirm the specific type of β-lactamase gene (Sáenz, et al., 2004; Batchelor et al., 2005). Genes encoding resistance to tetracycline (*tetA*, *tetB*, and *tetC*), sulfonamide (*sul1*, *sul2*, and *sul3*), and quinolones (*qnrA*, *qnrB*, and *qnrS*) were investigated by PCR as reported previously (Sáenz, et al., 2004; Wang et al., 2008; Rocha-Gracia et al., 2010). For positive isolates, PCR products of *qnrB* and *qnrS* were sequenced. The presence and characterization of integrons were studied by PCR of the class 1 and 2 integrase encoding genes as well as the 3′ conserved region (*qacE*Δ1 + *sul1* genes) (**Table 1**) and by PCR and subsequent sequencing of the variables regions (VRs) of these integrons (Sáenz, et al., 2004).

TABLE 1 | Primers of genes encoding resistance genes and integrons used in PCR of resistance genotype.

Primer name	Sequence (5′-3′)	Target gene or region	PCR products (bp)	References
intI1-F	GGGTCAAGGATCTGGATTTCG	*intI1*	483	Sáenz, et al., 2004
intI1-R	ACATGCGTGTAAATCATCGTCG			
Int-F	GGCATCCAAGCAGCAAG	Class 1 integron variable region	Variable	Sáenz, et al., 2004
Int-R	AAGCAGACTTGACCTGA			
intI2-F	CACGGATATGCGACAAAAAGGT	*intI2*	788	Sáenz, et al., 2004
intI2-R	GTAGCAAACGAGTGACGAAATG			
Hep-F	CGGGATCCCGGACGGCATGCACGATTTGTA	Class 2 integron variable region	Variable	Sáenz, et al., 2004
Hep-R	GATGCCATCGCAAGTACGAG			
Qac-F	GGCTGGCTTTTTCTTGTTATCG	*qacE*Δ1-*sul1* region	1125	Sáenz, et al., 2004
SUL1-R	GCGAGGGTTTCCGAGAAGGTG			
SUL1-F	TGGTGACGGTGTTCGGCATTC	*sul1*	789	Sáenz, et al., 2004
SUL1-R	GCGAGGGTTTCCGAGAAGGTG			
SUL2-F	CGGCATCGTCAACATAACC	*sul2*	722	Sáenz, et al., 2004
SUL2-R	GTGTGCGGATGAAGTCAG			
SUL3-F	CATTCTAGAAAACAGTCGTAGTTCG	*sul* 3	990	Sáenz, et al., 2004
SUL3-R	CATCTGCAGCTAACCTAGGGCTTTGGA			
TetA-F	GTAATTCTGAGCACTGTCGC	*tetA*	937	Sáenz, et al., 2004
TetA-R	CTGCCTGGACAACATTGCTT			
TetB-F	CTCAGTATTCCAAGCCTTTG	*tetB*	416	Sáenz, et al., 2004
TetB-R	CTAAGCACTTGTCTCCTGTT			
TetC-F	TCTAACAATGCGCTCATCGT	*tetC*	570	Sáenz, et al., 2004
TetC-R	GGTTGAAGGCTCTCAAGGGC			
aac(6')-Ib-F	TTGCGATGCTCTATGAGTGGCTA	*aac(6′)-Ib*	482	Rocha-Gracia et al., 2010
aac(6')-Ib-R	CTCGAATGCCTGGCGTGTTT			
QepA-F	GGACATCTACGGCTTCTTCG	*qepA*	671	Rocha-Gracia et al., 2010
QepA-R	CAACTGCTTGAGCCCGTAG			
QnrA-F	GGGTATGGATATTATTGATAAA	*qnrA*	580	Rocha-Gracia et al., 2010
QnrA-R	CTAATCCGGCAGCACTATTA			
QnrBnew-F	GATCGTGAAAGCCAGAAAGG	*qnrB*	468	Wang et al., 2008
QnrBnew-R	ACGATGCCTGGTAGTTGTCC			
QnrS-F	AGTGATCTCACCTTCACCGC	*qnrS*	550	Rocha-Gracia et al., 2010
QnrS-R	CAGGCTGCAATTTTGATACC			
TEM-F	ATTCTTGAAGACGAAAGGGC	*bla*TEM	1150	Sáenz, et al., 2004
TEM-R	ACGCTCAGTGGAACGAAAAC			
SHV-F	CACTCAAGGATGTATTGTG	*bla*SHV	885	Sáenz, et al., 2004
SHV-R	TTAGCGTTGCCAGTGCTCG			
CTXM-Univ-F	CGATGTGCAGTACCAGTAA	*bla*CTXM	585	Batchelor et al., 2005
CTXM-Univ-R	TTAGTGACCAGAATCAGCGG			

Virulence Genotyping

The presence of 30 virulence genes (*fimA, TartT, iutA, MaIX, Ibe, FyuA, BmaE, papGalleleIII, papC, colV, cdtB, papG alleleI, nfaE, SfaS, iha, iss, ire, ehxA, sxt1, sxt2, eltA, fasA, estII, aggC, esat1, cdt, ipah, hly, cnf1*, and *bfp*) was determined by using PCR in all ESBL-positive *E. coli* strains (Chapman et al., 2006; Wu et al., 2007).

Clonal and Phylogenetic Analysis of *E. coli* Isolates

Chromosomal DNA was prepared as previously described using the restriction enzyme *XbaI* (Amersham Life Sciences, Uppsala, Sweden) (Kaufmann, 1998). DNA fragments were separated by electrophoresis in 1.2% agarose gels (pulsed-field agarose certified; Bio-Rad, Hemel Hempstead, United Kingdom) and 0.5 X Tris-borate-EDTA buffer using a contour-clamped homogeneous electric field (CHEF-DRIII system; Bio-Rad) under the following electrophoresis conditions: 12°C at 6 V/cm for 27 h with pulse times ranging from 10 to 40 s. Clonal relationships were established following Tenover criteria (Tenover et al., 1995). *E. coli* Isolates were allotted to phylogenetic groups A, B1, B2, or D, using a triplex PCR assay targeting the *chuA*, *yjaA* genes and the DNA fragment TSPE4.C2 (Clermont et al., 2000). Strains were sub-grouped according to Escobar-Paramo et al. (2006): subgroup A0: *chuA*-, *yjaA*-, and TspE4.C2-; subgroup A1: *chuA*-, *yjaA*+, and TspE4.C2-;

group B1: *chuA*-, *yjaA*+/-, and TspE4.C2+; subgroup $B2_2$: *chuA*+, *yjaA*+, and TspE4.C2-; subgroup $B2_3$: *chuA*+, *yjaA*+, and TspE4.C2+; subgroup D1: *chuA*+, *yjaA*-, and TspE4.C2-; subgroup D2: *chuA*+, *yjaA*, and TspE4.C2+. Appropriate positive and negative controls were included in the assay.

Results

Occurrence of ESBL-producing *E. coli* Isolates and Antibiotic Susceptibility

Eleven (24%) and 6 (30%) ESBL-producing *E. coli* isolates were detected in the 45 and 20 fecal samples collected in farm 1 and in farm 2, respectively. In addition to ESBL production, isolates in farm 1 have the same profile of resistance to nalidixic acid, norfloxacine (intermediate), trimethoprim-sulfamethoxazole, sulfonamides, streptomycin and tetracycline. But, they remained susceptible to imipenem, gentamicin, tobramycin and chloramphenicol. With the exception of one strain that was co-resistant to tetracycline, isolates, from farm 2, were susceptible to all non beta-lactam antibiotics (**Table 2**).

Gene Coding for the Production of ESBL

PCR and sequencing showed that all strains, except one, harbored the $bla_{\text{CTX-M-1}}$ genes. One strain co-harbored $bla_{\text{TEM-1}}$ gene. The bla_{SHV} gene was not detected.

Occurrence of Class 1 and 2 Integrons

In farm 1, class 1 and 2 integrons were found in one and ten isolates, respectively. In farm 2, class 1 integron was found in only one isolate, while class 2 was not detected. Amplification of the VRs of class 2 integrons showed identical DNA fragments with an approximate size of 2000 bp. All these VRs contained a unique gene cassette arrangement, being *dfrA1- sat2-aadA1*, encoding resistance for trimethoprim, streptothricin and streptomycin, respectively. In one isolate, the VR of class 1 integron was amplified and yielded a DNA fragment of ca. 250 bp, whereas no amplification was observed in the second *int1*-positive isolate.

Determination of Phylogroups and Virulence Factors

In farm 1, all isolates were found to belong to phylogroup A (subgroup A0); however, different phylogroups were detected in farm 2 (A1, $B2_2$, and $B2_3$, each in one isolate, and D_2, in 3 isolates). The virulence genes *iut(A), fimH*, and *traT* were detected in 3, 7, and 3 isolates, respectively. The other genes were not detected in our collection. Two types of gene combination were detected: *iutA+fimH+traT* (in 3 isolates); *iutA+fimH* (in one isolate).

Genes Encoding Tetracycline-, Sulphonamide, and Fluroquinolones Resistance

Amongst the 12 tetracycline-resistant isolates, eleven and one carried *tetA* and *tetB*, respectively. Sulfonamides resistance was encoded by *sul1*, *sul2* and *sul3* genes in 3, 17 and 5 isolates, respectively. Gene *qnrB1* was detected in 9 isolates, one of them co-harbored *qnrS1* gene.

PFGE Typing

All isolates from farm 1 were clonally related, while isolates of the farm 2 were genetically unrelated (**Figure 1**).

TABLE 2 | Phenotypic and molecular characteristics of the 17 ESBL-producing *E. coli* isolates.

E. coli strain	Resistance profil*	Phylogenetic group	β-Lactamase (s)	Int**	VR** (bp)	*tet*	*sul*	PMQR**	Virulence factors	PFGE type
Ec139	NA, Nor[I], Sxt, S, Tet	Ao	CTX-M-1	2	2000	A	*sul 2*	*qnrB1*	*iut A*	A
Ec149	NA, Nor[I], Sxt, S, Tet	Ao	CTX-M-1	2	2000	A	*sul 1-2*	-	*iut A*	A
Ec174	NA, Nor[I], Sxt, S, Tet	Ao	CTX-M-1	2	2000	A	*sul 2*	-	*iut A*	A
Ec143	NA, Nor[I], Sxt, S, Tet	Ao	CTX-M-1	2	2000	A	*sul 2*	*qnrB1*	*iut A*	A
Ec147	NA, Nor[I], Sxt, S, Tet	Ao	CTX-M1	2	2000	A	*sul 2*	-	*iut A*	A
Ec154	NA, Nor[I], Sxt, S, Tet	Ao	CTX-M1	2	2000	A	*sul 2*	*qnrB1*	*iut A*	A
Ec146	NA, Nor[I], Sxt, S, Tet	Ao	CTX-M1	2	2000	A	*sul 1-2*	-	*iut A*	A
Ec172	NA, Nor[I], Sxt, S, Tet	Ao	CTX-M-1+ TEM-1	1	250	A	*sul 2-3*	*qnrB1*	*iut A, traT, fimH*	A
Ec156	NA, Nor[I], Sxt, S, Tet	Ao	CTX-M-1	2	2000	A	*sul 2*	*qnrB1, qnrS1*	-	A
Ec151	NA, Nor[I], Sxt, S, Tet	Ao	CTX-M-1	2	2000	A	*sul 2*	*qnrB1*	*iut A*	A
Ec173	NA, Nor[I], Sxt, S, Tet	Ao	CTX-M-1	2	2000	A	*sul 2*	*qnrB1*	*iut A*	A
Ec67	-	A1	CTX-M-1	1	-	-	*sul 1-2*	-	*fimH*	B
Ec68	TET	$B2_2$	-	-	-	B	*sul 2-3*	-	*iut A, traT, fimH*	C
Ec69	-	D_2	CTX-M-1	-	-	-	*sul 2*	*qnrB1*	*iut A, traT, fimH*	D
Ec71	-	$B2_3$	CTX-M-1	-	-	-	*sul 2-3*	*qnrB1*	*iut A, fimH*	E
Ec 72	-	D_2	CTX-M-1	-	-	-	*sul 2-3*	-	*fimH*	F
Ec 76	-	D_2	CTX-M-1	-	-	-	*sul 2-3*	-	*fimH*	G

*Resistance to other antibiotic in addition to ESBL production, NA, nalidixic acid; Nor, Norfloxacine; Sxt, trimethoprim-sulfamethozole; S, streptomycin; Tet, tetracycline.
**Int, integron class; VR, variable region; PMQR, Plasmid Mediated Quinolone Resistance.

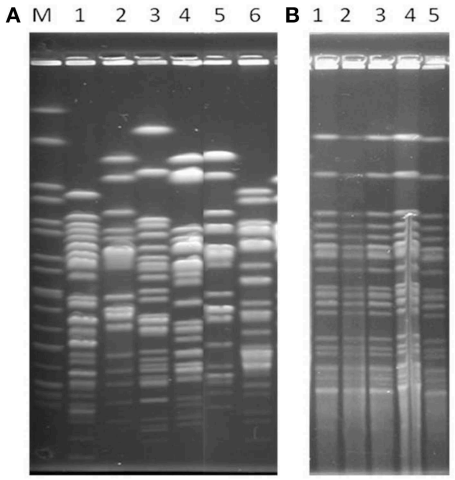

FIGURE 1 | *Xba*I-PFGE profiles of representative *E. coli* isolates. (**A**, Unrelated isolates) Lanes M, *Xba*I-digested DNA of *Salmonella enterica* serovar Braenderup H9812, used as size standard; Lanes 1–6, Ec 67, Ec68, Ec69, Ec71, Ec72; (**B**, Clonal isolates) Lanes 1 to5, EC139, EC149, Ec 174, Ec143, Ec147, respectively.

Discussion

We collected 65 samples of feces from 45 reproductive 58-weeks-old chickens in a public farm in the region of Sidi Thabet (30 Km Nord-West of Tunis, Tunisia) (Farm1) and 20 samples of 7-weeks-old breeder chickens in the region of Morneg (Farm 2). Seventeen samples (26.1%) contained cefotaxime-resistant isolates, 11 (24%) and 6 (30%) positives samples were observed in farms 1 and 2, respectively. The frequency of cefotaxime-resistant isolates is similar to those reported by other authors worldwide (2%; 31.7%) (Dierikx et al., 2013; Randall et al., 2014), but lower than those found by other studies in Tunisia (42% and 45, 5%) (Ben Sallem et al., 2012b; Mnif et al., 2012). In our study, all isolates from farm 1 exhibited the same resistance profile to nalidixic acid, trimethoprim-sulfamethoxazole, sulfonamides, streptomycin, tetracycline and norfloxacine (intermediately resistant). These resistance markers are often reported in previous Tunisian studies (Ben Slama et al., 2010; Mnif et al., 2012). All isolates were susceptible to imipenem, gentamicin, kanamycin and chloramphenicol. Indeed, low rates of resistance to these antibiotics have been reported worldwide (Grami et al., 2013). However, the 6 isolates from farm 2, except one that was co-resistant to tetracycline, were susceptible to all antibiotics tested. Worldwide, the CTX-M group has been emerged as the predominant determinant encoding ESBL production in human and animal *Enterobacteriaceae*, especially *E. coli* (Girlich et al., 2007; Coque et al., 2008; Nicolas-Chanoine et al., 2008; Dahmen et al., 2012, 2013). The $bla_{\text{CTX-M-1}}$ gene was amplified in 16 out of 17 isolates; in addition, one isolate co-harbored the $bla_{\text{TEM-1}}$ gene. This result is in agreement with those of other Tunisian findings (Jouini et al., 2007, 2009; Ben Slama et al., 2010; Ben Sallem et al., 2012b, 2013; Mnif et al., 2012). The high rate occurrence of *E. coli* harboring $bla_{\text{CTX-M-1}}$ in poultry and other food products of animal origin can contribute to the transmission of this gene or these strains to humans. Indeed, it was recently demonstrated that *E. coli* containing CTX-M-1 was identified in 7.3% of healthy Tunisians (Ben Sallem et al., 2012a). Identical or closely related isolates from humans and animals have been previously reported in Netherlands, suggesting a likely transmission of ESBL-*E. coli* isolates from poultry to human, most probably via the food chain (Overdevest et al., 2011). More recently, Chinese data showed a high occurrence of CTX-M-14 in isolates from animals where this enzyme is very widespread in human isolates (Zheng et al., 2012). In the EC68 strain, no *bla* gene was detected. This strain could harbor a gene not investigated in our study. Further investigation must be undertaken. Antibiotic resistance, especially the multiresistance, has been mainly linked to the dissemination of linked genes encoding resistance inserted in mobile genetic elements, mainly integrons. Integron of class 1 was found in two isolates while integron of class 2 in ten isolates. Our results are not in agreement with other findings, which showed the dominance of integron of class 1 in animal-derived *E. coli* or in animal products as well as human isolates (Machado et al., 2005; Soufi et al., 2009, 2011; Ben Slama et al., 2010; Cergole-Novella et al., 2010). Two hypotheses could explain our finding: firstly, the real absence of class 1 integrons in the integron-free isolates, as reported by other authors (Jouini et al., 2007; Ben Sallem et al., 2012b). Secondly, the presence of insertion sequences, such as *IS*26, truncating the *intI* and thus, leading to inhibition of PCR amplification. In the case of truncated *intI* gene by *IS*26, the use of other primers was proposed (Marchant et al., 2013). Owing to a number of financial limitations, we could not realize these PCRs actually. The then *int2*-positive isolates presented an identical gene cassette array in their VRs: *dfrA-sat2-aadA1*, being frequent in other studies (Soufi et al., 2009, 2011). The integrase Int2 is not functional and thus unable to integrate new gene cassettes into the variable region, other than those already present (Partridge et al., 2009). The variable region of the class 2 integron mainly carries *dfrA1* (encoding trimethoprim resistance), *sat1* (encoding streptothricin resistance) and *aadA1* (encoding streptomycin/spectinomycin resistance) (Partridge et al., 2009; Soufi et al., 2009, 2011). The size of the VR of class 1 integron in EC172 was approximately 250 bp, a fragment that cannot correspond to any gene cassette of the known ones (Ravi et al., 2014). The VR of class 1 integron in EC67 was not amplified; this might suggest an empty VR or mismatches of used primers.

Tetracycline resistance was encoded by *tetA* and *tetB* genes in 11 and one tetracycline-resistant isolates, respectively. This finding was also reported by other studies (Koo and Woo, 2011; Xibiao et al., 2011), while the *tetC* gene is rarely reported (Skoèková et al., 2013). The *sul1*, *sul2* and *sul3* genes were detected in 3, 17, and 5 isolates, respectively, not only amongst sulfonamid resistant isolates, but also in the six-sulfonamide susceptible isolates. In other reports, *sul1* is most frequently reported followed by *sul2* gene while *sul3* gene is generally less so (Sköld, 2000; Perreten and Boerlin, 2003; Hammerum et al., 2006; Trobos et al., 2008). Resistance toward quinolone and fluoroquinolones is mainly due to target mutations in quinolone

resistance determining region (QRDR) of DNA gyrase (*gyrA* and *gyrB*) and topoisomerases IV (*parC* and *parE*) (Hawkey, 2003). However, Plasmid Mediate Quinolone Resistance (PMQR) genes such as *qnrA, qnrB, qnrC, qnrD, qnrS, qepA,* and *aac(6′)Ib-cr* have been increasingly reported in bacterial pathogens since 2000 (Strahilevitz et al., 2009). In our study, among 11 isolates resistant to nalidixic acid and intermediately susceptible sensibility to norfloxacine, seven isolates carried *qnrB1* gene and one also co-heberged *qnrS1* gene. These two genes were often reported by other authors worldwide (Ben Sallem et al., 2013, 2014; Ferjani et al., 2014) in *E. coli* producers of CTX-M from human and animal origin. In our study, we did not consider *qepA* and *aac (6′)-Ib-cr* genes, while other authors reported their presence in *E. coli* from animal origin (Ma et al., 2009; Xie et al., 2014). The *qnr* gene types have no big effect on the increase of minimal inhibitory concentration (MIC) of fluoroquinolones (ciprofloxacine and norfloxacine) and were generally not detected by disk diffusion method. Indeed, the resistance toward quinolones in 7 amongst the 11 nalidixic acid resistant isolates could be explained by these *qnr* genes but it could also certainly explained by the presence of chromosomal mutations in QRDR of GyrA and/or ParC (Abbassi et al., 2010). The quinolone-susceptible isolate containing *qnrB1* gene confirmed the low level expression of this resistance (in term of increase of MIC).

Concerning the distribution of pathogenic *E. coli* strains according to phylogroups, it is well-known that pathogenic strains producing extra-intestinal infections (ExPEC) belong mainly to the B2 group and less to the D group. They are responsible for meningitidis, abscess, peritonitis, septicemia and urinary tract infections (Le Gall et al., 2007; Nandanwar et al., 2014); while, groups A and B1 *E. coli* strains are considered non-virulent commensal strains (Ewers et al., 2007). All the strains of farm 1 were found to belong to the phylogroup A (sub-type A0), while, the strains of farm 2 to phylogroups A (A1, 1 isolate), D (D2, 3 isolates) and B2 ($B2_2$, 1 isolate; $B2_3$, 1 isolate). In the literature, the majority of animal *E. coli* isolates producing ESBL belonged to phylogroup A and B1 (Mnif et al., 2012; Huber et al., 2013) contrary to human isolates, which mainly belonged to B2 phylogroup. The low number of virulence genes detected was in relation with the appurtenance of our isolates (12/17) to the phylogroup A. Strains of phylogroups B2 and D were somewhat distinguished from strains of phylogroup A by the occurrence of the fimbrial *fimH* gene and the serum resistance-associated outer membrane (*traT*). The low number of genes detected could be explained by fitness notion or the competitively of strains. Indeed, these avian intestinal *E. coli* isolates were under selective presser by antibiotics. This selective pressure enhances the expansion and maintenance of antibiotic-resistant strains rather than virulent ones.

PFGE showed that the 11 isolates of the farm 1 were indistinguishable, while isolates of farm 2 were unrelated. The cloanlity of these isolates would evoke a strong power of dissemination of clonal isolates in this poultry breeding. The difference in genetic contents in isolates of farm2 (*sul* genes, *qnr* genes and virulence genes) might be due to independent acquisition of genes carried by plasmids or integrons.

Conclusion

Despite the limited samples analyzed in this study, that might not reflect the real epidemiological situation of avian ESBL-producing *E. coli* in these two farms, our study showed two typical epidemiologic characteristics of ESBL-producing bacteria. Firstly, clonal isolates are disseminated within the same farm. Secondly, "singleton" isolates occur with limited ability of spread, evoking the potential horizontal transfer of ESBL genes between different *E. coli* populations. Plasmids or integrons might be implicated in the mobilization of $bla_{\text{CTX-M-1}}$ genes among avian isolates. Further studies should be performed in the future to track the evolution of ESBL types and their frequencies in different ecosystems.

Author Contributions

HK, MA, RM, SH, NC, and IB: Conceiving and designing the study, collecting and interpreting the data, and writing the article. SS, RS, IJ, and SF: interpreting the data, revising the article.

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Whole genome sequencing of extended-spectrum β-lactamase producing *Klebsiella pneumoniae* isolated from a patient in Lebanon

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Objective: The emergence of extended-spectrum β-lactamase (ESBL)-producing bacteria is now a critical concern. The ESBL-producing *Klebsiella pneumoniae* constitutes one of the most common multidrug-resistant (MDR) groups of gram-negative bacteria involved in nosocomial infections worldwide. In this study we report on the molecular characterization through whole genome sequencing of an ESBL-producing *K. pneumoniae* strain, LAU-KP1, isolated from a stool sample from a patient admitted for a gastrointestinal procedure/surgery at the Lebanese Amrican University Medical Center-Rizk Hospital (LAUMCRH) in Lebanon.

Methods: Illumina paired-end libraries were prepared and sequenced, which resulted in 4,220,969 high-quality reads. All sequence processing and assembly were performed using the A5 assembly pipeline.

Results: The initial assembly produced 86 contigs, for which no scaffolding was obtained. The final collection of contigs was submitted to GenBank. The final draft genome sequence consists of a combined 5,632,663 bases with 57% G+C content. Automated annotation was performed using the RAST annotation server. Sequencing analysis revealed that the isolate harbored different β-lactamase genes, including $bla_{\text{oxa}-1}$, $bla_{\text{CTX}-\text{M}-15}$, $bla_{\text{SHV}-11}$, and $bla_{\text{TEM}-1\text{b}}$. The isolate was also characterized by the concomitant presence of other resistance determinants most notably *acc(6′)-Ib-cr* and *qnrb1*. The entire plasmid content was also investigated and revealed homology with four major plasmids pKPN-IT, pBS512_2, pRSF1010_SL1344, and pKPN3.

Conclusions: The potential role of *K. pneumonia* as a reservoir for ESBL genes and other resistance determinants is along with the presence of key factors that favor the spread of antimicrobial resistance a clear cause of concern and the problem that Carbapenem-non-susceptible ESBL isolates are posing in hospitals should be reconsidered through systematic exploration and molecular characterization.

Keywords: ESBL, *Klebsiella pneumoniae*, whole genome sequencing, CTX-M-15, SHV-11

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Introduction

Extended-spectrum β-lactamases (ESBL) are enzymes produced by many bacterial species as a means for defense against β-lactam drugs with the genes encoding for those enzymes being mainly located on mobile genetic elements (Pfeifer et al., 2010). ESBL enzymes confer antimicrobial resistance to a wide spectrum of β-lactams such as penicillins, aztreonam, as well as first, second, and third generation cephalosporins (Jacoby and Medeiros, 1991; Bradford, 2001). β-lactamases are widespread and were detected in a wide range of *Enterobacteriaceae* (Bradford, 2001) predominantly in *Escherichia coli,* and *Klebsiella pneumoniae*, as well as in other non-enteric microorganisms, such as *Pseudomonas aeruginosa, Acinetobacter baumanni* (Naas et al., 1999; Spanu et al., 2002; Goossens and Grabein, 2005; Paterson and Bonomo, 2005), and *Capnocytophaga ochracea* (Rosenau et al., 2000). In addition, *Shigella* species have been increasingly reported to be resistant to β-lactams including third-generation cephalosporins (Levesque et al., 1995).

K. pneumoniae is an important human pathogen causing nosocomial infections (Podschun and Ullmann, 1998). ESBL-producing *K. pneumoniae* is one of the most common multidrug-resistant (MDR) groups of gram-negative bacteria worldwide (Breurec et al., 2013). Infections caused by ESBL-producing *K. pneumoniae* are often associated with the urinary and respiratory tracts along with epidemic clones causing outbreaks in intensive care units (Peirana et al., 2012).

There are four molecular classes of β-lactamases. Classes A, C, and D. β-lactamases possess an active-site serine, while class B β-lactamases are metalloenzymes requiring Zn molecule for their activity (Ambler et al., 1991; Bush et al., 1995). CTX-M is a dominant ESBL family in *K. pneumoniae* strains, but TEM and SHV enzymes in addition to members of the three classes of β-lactamases (B, C, D) are also common (Breurec et al., 2013). Carbapenems are usually used for the treatment of infections caused by ESBL producing *Enterobacteriaceae.* Increasing rates of ESBL-producing isolates has led to the overuse of carbapenems creating a pressure and triggering resistance. Resistance to carbapenems may be associated with the production of carbapenemases including class A KPCs, class B metallo-β-lactamases (VIM, IMP, or NDM-1) or the class D OXA-type enzymes (Queenan and Bush, 2007; Matar et al., 2008; Nordmann et al., 2009).

The aim of this study is to characterize an ESBL-producing *K. pneumoniae* isolated from a stool sample from a patient admitted for a gastrointestinal procedure/surgery in a Lebanese hospital using whole genome sequencing (WGS).

Materials and Methods

Ethical Approval

The University's Institutional Review Board approved the study (UMCRH.AF.12/Dec/2012).

Study Design and Bacterial Isolate

Samples were collected from the University Medical Center-Rizk Hospital (UMC-RH). All patients included in the study were screened for the presence of ESBL-producing *Enterobacteriaceae* by means of three consecutive rectal swab or stool cultures, as per regular screening for ESBL detection. The first fecal sample was collected at the same time as the other preoperative tests. The second and third samples were collected later, latest day 2-post surgery. *K. pneumoniae* LAU-KP1 sequenced in this study was isolated from a 62-year-old woman working as practitioner nurse or nurse's assistant. She was admitted for radical resection of an abdominal tumor and repair of abdominal hernia. The patient had other than her cancer, recurrent urinary tract infections. She had been using amoxicillin-clavulanate, metronidazol, and cefriaxon in the 3 months prior to presentation. She had been admitted to hospital in the year prior to surgery, but was not known to be a carrier of multidrug resistant organism and no one in her household was known to be a carrier of multidrug resistant organisms.

Screening for ESBL

The standard disk diffusion method with combined discs (ceftazidime/clavulanate and cefotaxime/clavulanate) was used for screening. The standard *E*-test method using double sided strips containing on one side ceftazidime or cefotaxime and on the other the same antibiotic combined with clavulanate was used to assess for screening and confirming ESBL production in all strains according to the recommendations of the CLSI. *E. coli* ATCC25922 and *K. pneumoniae* ATCC700603 were used as the quality control strains.

Antimicrobial Testing

Antimicrobial susceptibility test by the disk diffusion method was performed to determine the resistance patterns of the isolates to 17 antibiotics: cefotaxime, ceftriaxone, ceftazidime, aztreonam, ceftazidime/clavulanate, cefotaxime/clavulanate, cephalothin, cefuroxime sodium, cefepime, gentamicin, amikacin, ciprofloxacin, tetracycline, ampicillin, amoxicillin/clavulanate, piperacillin, piperacillin/tazobactam, imipenem, meropenem, ertapenem, and sulfamethoxazole/trimethoprim [Oxoid, England; disc contents according to Clinical and Laboratory Standards Institute (CLSI) guidelines]. All antimicrobial testing was performed on Mueller-Hinton agar by the flooding technique and data interpreted according to the CLSI guidelines.

DNA Isolation and Sequencing

Bacterial DNA was extracted using the Nucleospin kit (Macherey-Nagel) and following the manufacturer's instructions.

Genome Sequencing

Genomic DNA (gDNA) was used as input for library preparation using the Illumina TruSeq DNA library preparation kit (Illumina). Bioruptor® NGS was used to sonicate 1 μg of sample DNA (100 μl final sonication volume in TE buffer) for 8 min of 30 s on/off cycles at 4°C. Sheared DNA was used as input for library preparation using the Illumina TruSeq DNA library preparation kit (Illumina). The gDNA was subjected to end-repair, A-tailing, ligation of adaptors including sample-specific barcodes as recommended in the manufacturer's protocol. After ligation of the adaptors, and to reduce the number of fragments

smaller than 500 bp, fragments between 500 and 1000 bp were selected using a Pippin Prep™ DNA size selection system (Sage Science). The resulting library was quantified by quantitative PCR in triplicate at 1:1000 and using the Kapa library quantification kit (Kapa Biosystems, Woburn, MA) and as recommended by the manufacturer's protocol. The resultant library size was assessed using an Agilent Bioanalyzer with the High Sensitivity DNA Kit. The library was multiplexed, clustered, and sequenced on an Illumina MiSeq with paired-end 500 cycles protocol to read a length of 250 bp.

Genome Assembly

Genome assembly was performed *de novo* using A5 with default parameters (Tritt et al., 2012). This pipeline automates the processes of data cleaning, error correction, contig assembly, scaffolding and quality control.

Genome Analysis

The assembly was uploaded and annotated using RAST server (http://rast.nmpdr.org). The service identified protein-encoding, rRNA and tRNA genes, assigned functions to the genes, and predicted which subsystems are represented in the genome (Larsen et al., 2012).

The multi-locus sequence type for the isolate was determined from the WGS data. The presence of known acquired resistance genes was determined by mapping the data from the isolate to an online database. The ResFinder web server (www.genomicepidemiology.org) was used to identify acquired antimicrobial resistance genes in the WGS data, using a threshold of 98.00% identity (ID) (Zankari et al., 2012). ResFinder detects the presence of whole resistance genes, but not functional integrity and expression or resistance due to acquired variation in housekeeping genes. Based on the ResFinder results, a predicted phenotype was determined using phenotypes from original published studies of the genes found.

A *K. pneumoniae* concatenated marker gene maximum-likelihood tree was constructed using all available assembled *K. pneumoniae* genomes on the NCBI ftp server. The genomes were first processed with PhyloSift (Darling et al., 2014) using –besthit and –isolate options to get alignments to concatenated conserved housekeeping genes. The tree was made using FastTree (Price et al., 2010). The tree was edited and visualized using Dendroscope (Huson and Scornavacca, 2012). Clades with zero branch lengths were collapsed to a single representative. For visualization of LAU-KP1, the assembly was aligned against the reference strain *K. pneumoniae* Ecl8's (GCF_000315385) using progressiveMauve (Darling et al., 2010). Ring representation was obtained using BRIG 0.95 (Alikhan et al., 2011). Ecl8 is the *K. pneumoniae* isolate which was closest to LAU-KP1 in the concatenated marker gene tree that had a complete genome.

Nucleotide Sequence Accession Number

The draft sequence of the *K. pneumoniae* LAU-KP1 have been deposited at DDBJ/EMBL/GenBank under the accession no. AYQE00000000.1.

Results and Discussion

To the best of our knowledge we report for the first time the isolation of a CTX-M-15, SHV-11, KPC-producing *K. pneumoniae* belonging to ST336 in Lebanon. This clone belongs to CG17 and was isolated from a 62-year-old woman working as practitioner nurse or nurse's assistant. She was admitted for radical resection of an abdominal tumor and repair of abdominal hernia. The patient had other than her cancer, recurrent urinary tract infections.

In *K. pneumoniae* MLST is based on genetic variation in seven housekeeping genes (*rpoB, gapA, mdh, pgi, phoE, infB,* and *tonB*) (Diancourt et al., 2005). ST allele profile of ST336 is: 2-1-1-1-72-4-4, while ST258, being the most common KPC-producing isolate (Woodford et al., 2011), is: 3-3-1-1-1-1-79 (http://www.pasteur.fr/mlst). Screening patients for rectal carriage by Baraniak et al. (2013) revealed the prevalence of CG17 in France, highlighting the role of human mobility in spreading MDR *K. pneumoniae* clones.

LAU-KP1 was found to be resistant to the following drugs: ciprofloxacin, cefotaxime, aztreonam, and ceftazidime. It was intermediate to amoxicillin clavulanic acid, and sensitive to imipenem and gentamycin. Analysis of the data extracted from the draft genome sequence however, revealed that LAU-KP1 was characterized by the presence of multiple resistance determinants, most notably was the concomitant presence of *acc(6′)lb-cr*, *qnr*, SHV-11, and CTX-M-15. CTX-M-15 detection was consistent with the recent predominance of CTX-M and CTX-M-15 in particular (Rossolini et al., 2008), which additionally was found to be more common in *K. pneumoniae* than in *E. coli* in rehabilitation units (Baraniak et al., 2013). Rodrigues et al. (2014) reported recently that the increase in the incidence of CTX-M-15 and diverse SHV ESBL types observed in a Portuguese hospital was associated with the increase of MDR *K. pneumoniae* epidemic clones (ST15, ST147, ST336). Harbouring the *qnr*B1 (quinolone resistance) was another important finding that additionally distinguished LAU-KP1. Previously Breurec et al. (2013) revealed that *qnr*B was predominant among strains from Africa while *qnr*S was mainly detected in strains from Vietnam.

Resistance to carbapenems involves multiple mechanisms including alterations in outer membrane permeability mediated by the loss of porins, upregulation of efflux systems along with hyper production of AmpC β-lactamases or more commonly the production of carbapenemases (Chen et al., 2014). Class A carbapenemases include the *K. pneumoniae* carbapenemase (KPC). These enzymes hydrolyze all β-lactams including monobactams (El-Herte et al., 2012). Carbapenem resistant *Enterobacteriaceae have* spread in Northeastern USA and to other countries including Argentina, Greece, Italy, and China (Nordmann and Poirel, 2014). Transmission of the KPC gene can be mediated through horizontal gene transfer on mobile elements including transposons and plasmids (Munoz-Price and Quinn, 2009). Spread of the KPC-producing *K. pneumonia* has been linked to a major multi locus sequence type (MLST-ST) ST258 and its variants (Cuzon et al., 2010; Chen et al., 2014). LAU-KP1 harbored INCFII$_K$ plasmids and DNA comparison showed the presence of sequences with high homology to plasmid pKPN-IT

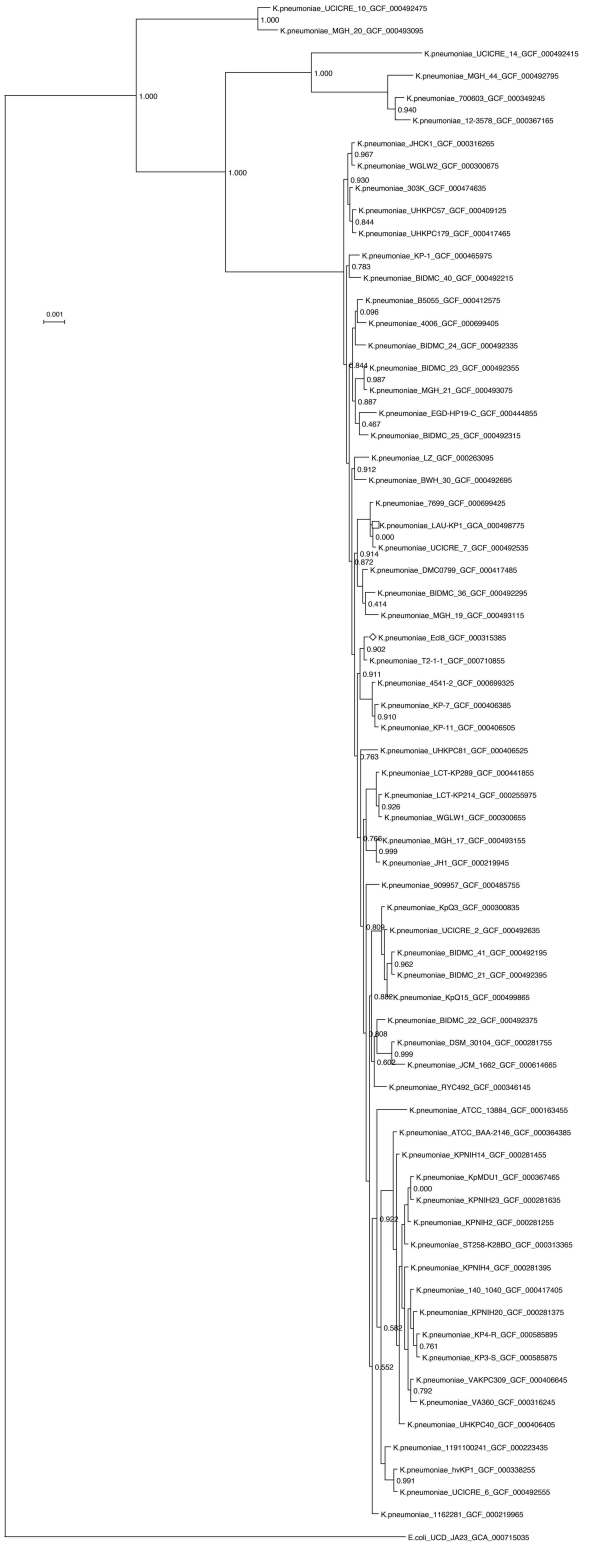

FIGURE 1 | Phylogenetic tree of all assembled *K. pneumoniae* genomes available on the NCBI ftp server as of 2-11-15, based on a concatenated alignment of 37 conserved genes obtained using PhyloSift (Darling et al., 2014). The tree was constructed using default *(Continued)*

FIGURE 1 | Continued
settings in FastTree (Price et al., 2010) and edited and visualized with Dendroscope v.3 (Huson and Scornavacca, 2012). Clades with zero branch lengths were collapsed to a single representative. *K. pneumoniae* LAU-KP1 is indicated by a square, and the reference genome used in **Figure 2** is indicated by a diamond.

and pKPN3. This was consistent with the finding that the dissemination of CTX-M-15 was primarily associated with $IncFII_K$ plasmid in *K. pneumoniae* isolates (Coelhoa et al., 2010). Earlier Garcia-Fernandez et al. (2012) detected in *K. pneumoniae* ST258 $IncFII_K$-FIB like plasmid and was named as pKPN-IT. This plasmid was highly related to plasmid pKPN3. The plasmid pKPN-IT was shown to also acquire Fec-like iron (III) dicitrate transport system and a class I integron carrying trimethoprim resistance, both of which were also detected in LAU-KP1.

β-lactamase class C, the presence of which renders bacteria to become resistant to most β-lactams including cephamycins and β-lactam/β-lactamase combinations (Doi and Paterson, 2007) was and along with macrolide-specific efflux pump MacAB-TolC important factors increasing the resistance of LAU-KP1. Periplasmic membrane fusion proteins (MFPs) are essential components of the type I protein secretion systems and drug efflux pumps in Gram-negative bacteria (Tikhonova et al., 2007). MacA is a peripheral membrane protein of the MFP family and a component of the MacAB macrolide efflux transporter complex. MacB on the other hand, is an integral membrane protein. Similar to other MFP-dependent transporters from *E. coli*, MacAB requires the outer membrane channel TolC for its function (Kobayashi et al., 2001; Tikhonova et al., 2007).

Additionally, a key feature of these MDR efflux systems is their ability to extrude a broad spectrum of substrates, including various antimicrobial agents (Bambeke et al., 2000; Putman et al., 2000; Poole, 2001). One important family of drug transporters that contribute to multidrug resistance (MDR) in gram-negative bacteria are the resistance-nodulation-cell division (RND) efflux systems, which consist of an inner membrane transporter, a periplasmic fusion protein, and an outer membrane protein (Zgurskaya and Nikaido, 2000). Detecting CmeABC operon in LAU-KP1 was an important feature. CmeABC is an energy-dependent efflux system contributing to the intrinsic resistance of *Campylobacter* to diverse antimicrobial agents. Disruption of the CmeABC resulted in MICs that were 8-fold lower for ciprofloxacin, 2-fold for norfloxacin and nalidixic acid, 256-fold for cefotaxime, and 32-fold for ampicillin (Lin et al., 2002).

In *K. pneumoniae* MLST is based on genetic variation in seven housekeeping genes (*rpoB, gapA, mdh, pgi, phoE, infB,* and *tonB*) (Diancourt et al., 2005). ST allele profile of ST336 is: 2-1-1-1-72-4-4, while ST258, being the most common KPC-producing isolate (Woodford et al., 2011), is: 3-3-1-1-1-1-79 (http://www.pasteur.fr/mlst). Screening patients for rectal carriage by Baraniak et al. (2013) revealed the prevalence of CG17 in France, highlighting the role of human mobility in spreading MDR *K. pneumoniae* clones.

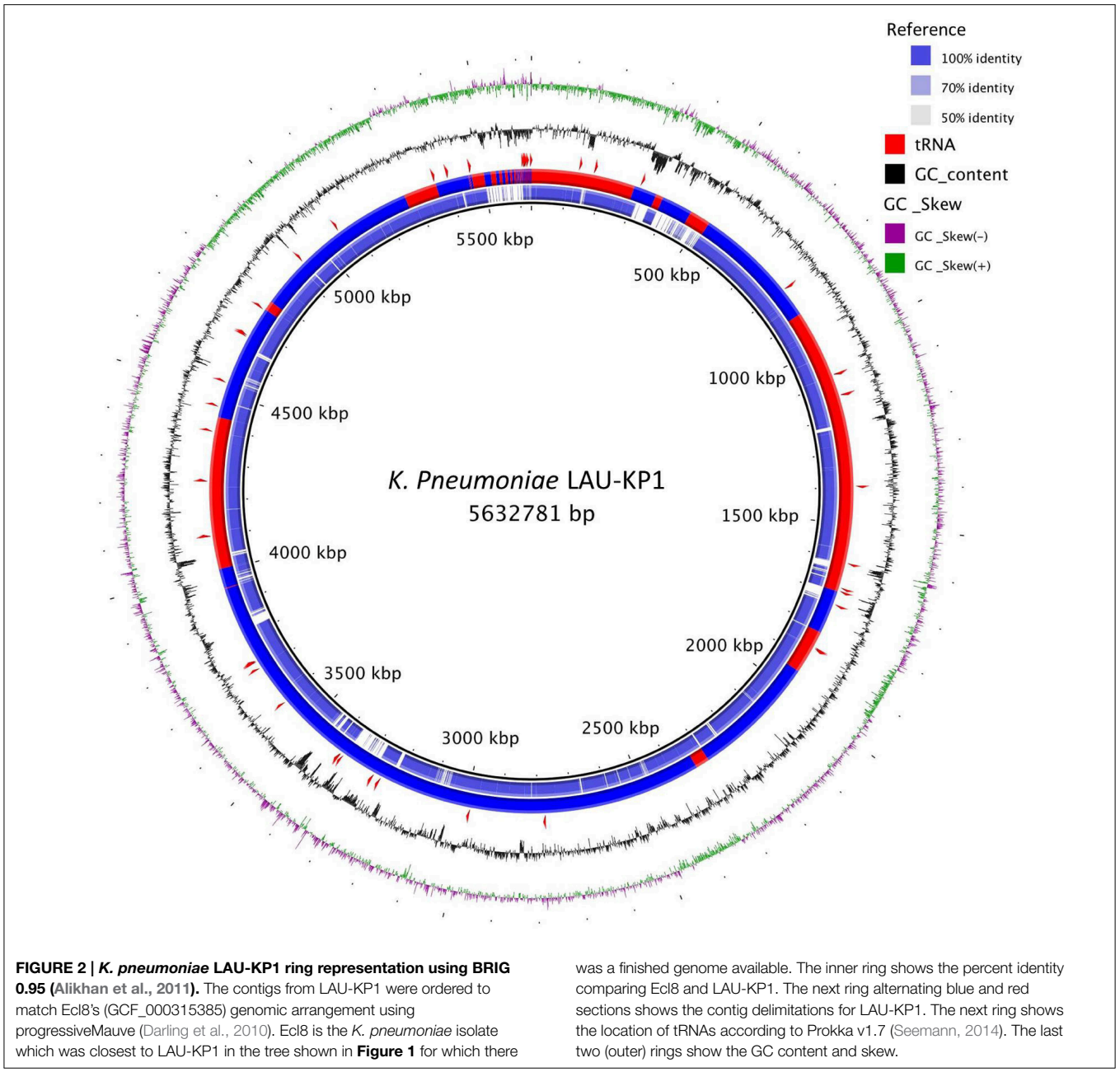

FIGURE 2 | *K. pneumoniae* LAU-KP1 ring representation using BRIG 0.95 (Alikhan et al., 2011). The contigs from LAU-KP1 were ordered to match Ecl8's (GCF_000315385) genomic arrangement using progressiveMauve (Darling et al., 2010). Ecl8 is the *K. pneumoniae* isolate which was closest to LAU-KP1 in the tree shown in **Figure 1** for which there was a finished genome available. The inner ring shows the percent identity comparing Ecl8 and LAU-KP1. The next ring alternating blue and red sections shows the contig delimitations for LAU-KP1. The next ring shows the location of tRNAs according to Prokka v1.7 (Seemann, 2014). The last two (outer) rings show the GC content and skew.

Furthermore, the phylogenetic analysis of LAU-KP1 showed that it falls within a clade of other known multidrug resistant *K. pneumoniae* strains and demonstrated that there is considerable genomic diversity among the included isolates (**Figure 1**). Within the closely related isolates was *K. pneumonia* 7699 previously cultured from a perianal swab of a patient admitted to the intensive care unit of the University of Maryland Medical Center (UMMC) in Baltimore, MD (Hazen et al., 2014). The isolate had a 167-kb IncA/C plasmid encoding the FOX-5 β-lactamase, a CARB-2 β-lactamase, other antimicrobial resistance genes, and heavy metal resistance genes. On the other hand, a considerable phylogenetic difference was detected between LAU-Kp1 and *K. pneumoniae* ATCC BAA-2146, which is the first U.S. isolate found encoding the gene for the broad-spectrum and carbapenem-active metallo-β-lactamase NDM-1 (Hudson et al., 2014). Additionally, we mapped our contigs to the phylogenetically closest *K. pneumoniae* isolate with a complete genome, Ecl8, in order to visualize the genomic organization (**Figure 2**).

The detection of such a multidrug resistant strain of *K. pneumoniae* emphasizes the potential for transmission of highly resistant gram-negative pathogens that are difficult to treat. This merits more detailed future studies directed toward characterization of molecular mechanisms, environmental factors, and selection pressures promoting the spread of such strains.

Detection of IncFII$_K$ a virulence plasmid (Dolejska et al., 2013) known to evolve quickly by replicon diversification and acquisition of antibiotic resistance traits (Coelhoa et al., 2010; Carattoli, 2013), increases the potential role of *K. pneumoniae* as a reservoir for ESBL genes and other resistance determinants. The spread of antimicrobial resistance in this region could be attributed to different factors including: uncontrolled consumption of antimicrobial agents through self-medication, inappropriate antibiotic prescription, the substandard quality of some drugs, and a lack of effective measures to prevent nosocomial infections. The virtual lack of physical barriers between the community and hospital settings in the countries in this region and the increasing pressure from war immigrants along with poor living conditions may be also important contributory factors. High-resolution genotyping studies of isolates collected over larger spatial and temporal scales will be necessary to decipher the dynamics of the emergence of MDR *K. pneumoniae* clonal groups.

Therefore, we urgently need well-designed epidemiological and molecular studies to understand the dynamics of transmission, risk factors, and reservoirs for *K. pneumoniae*. This will provide a better insight into the emergence and spread of these multidrug resistant strains and hopefully lead to information essential in preventing infections and limiting the spread of such organisms.

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Identification of DHA-23, a novel plasmid-mediated and inducible AmpC beta-lactamase from Enterobacteriaceae in Northern Taiwan

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Objectives: AmpC β-lactamases are classified as Amber Class C and Bush Group 1. AmpC β-lactamases can hydrolyze broad and extended-spectrum cephalosporins, and are not inhibited by β-lactamase inhibitors such as clavulanic acid. This study was conducted to identify DHA-23, a novel plasmid-mediated and inducible AmpC β-lactamase obtained from Enterobacteriaceae.

Methods: A total of 210 carbapenem-resistant Enterobacteriaceae isolates were collected from a medical center (comprising two branches) in Northern Taiwan during 2009–2012. AmpC β-lactamase genes were analyzed through a polymerase chain reaction using plasmid DNA templates and gene sequencing. The genetic relationships of the isolates were typed using pulsed-field gel electrophoresis following the digestion of intact genomic DNA by using *Xba*I.

Results: Three enterobacterial isolates (one *Escherichia coli* and two *Klebsiella pneumoniae*) were obtained from three hospitalized patients. All three isolates were resistant or intermediately susceptible to all β-lactams, and exhibited reduced susceptibility to carbapenems. These three isolates expressed a novel AmpC β-lactamase, designated DHA-23, approved by the curators of the Lahey website. DHA-23 differs from DHA-1 and DHA-6 by one amino acid substitution (Ser245Ala), exhibiting three amino acid changes compared with DHA-7 and DHA-*Morganella morganii*; three amino acid changes compared with DHA-3; four amino acid changes compared with DHA-5; and eight amino acid changes compared with DHA-2 (>97% identity). This AmpC β-lactamase is inducible using a system involving *ampR*.

Conclusion: This is the first report to address DHA-23, a novel AmpC β-lactamase. DHA-type β-lactamases are continuous threat in Taiwan.

Keywords: AmpC beta-lactamase, Enterobacteriaceae, antimicrobial resistance epidemiology

Introduction

AmpC enzymes are located in the bacterial periplasm, with the exception of the AmpC β-lactamase of *Psychrobacter immobilis*, which is secreted mainly into the external medium (Feller et al., 1997). They are active on cephalosporins, cephamycins (such as cefoxitin), oxyiminocephalosporins (such as ceftazidime and cefotaxime), and monobactams (such as aztreonam). AmpC β-lactamases are classified according to their Amber molecular structure as belonging to Class C, whereas according to function, they are classified into Group 1 (Bush and Jacoby, 2010). The sequence of the *ampC* gene differed from the sequence of penicillinase-type β-lactamases such as TEM-1 but similarly, had serine at its active site (Knott-Hunziker et al., 1982). AmpC β-lactamases, in contrast to extended-spectrum β-lactamases (ESBLs), can hydrolyze broad and extended-spectrum cephalosporins, and are not inhibited by β-lactamase inhibitors such as clavulanic acid. AmpC β-lactamases are assumed to be chromosomally mediated; however, *Klebsiella pneumoniae*, *K. oxytoca*, *Proteus mirabilis*, and *Salmonella* sp. lack a chromosomal bla_{AmpC} gene (Bergstrom et al., 1983; Bauernfeind et al., 1989). Described plasmid-mediated *AmpC* genes in a *K. pneumoniae* isolate from South Korea. There are various types of plasmid-mediated AmpC β-lactamases: CMY, MIR, MOX, LAT, FOX, DHA, ACT, ACC, and CFE (Jacoby, 2009). AmpR is a member of the LysR transcriptional regulator family. During normal growth, in the absence of β-lactam as an inducer, the AmpR regulator binds with a peptidoglycan precursor uridine pyrophosphoryl-*N*-acetyl muramyl-L-alanyl-D-glutamyl*meso*-diaminopimelic acid-D-alanyl-D-alanine (UDP-*N*-acetylmuramic acid peptide). AmpR- UDP-*N*-acetylmuramic acid peptide complex binds to the operator site between the *ampC* and *ampR* structural genes, leading to the repression of *ampC* expression. Displacement of the UDP-*N*-acetylmuramic acid peptides signals a conformational change in AmpR, which activates the transcription of *ampC*. AmpR mutations are less common but can also result in high-constitutive or hyperinducible phenotypes (Kaneko et al., 2005). Resistance caused by plasmid-mediated AmpC β-lactamase is less common than the production of ESBLs, but may be more difficult to detect. The purpose of this study was to identify DHA-23, a novel plasmid-mediated and inducible AmpC β-lactamase identified in clinical enterobacterial isolates that were obtained from a hospital in Northern Taiwan.

Materials and Methods

Isolates and Data Collection

A total of 210 carbapenem-resistant Enterobacteriaceae isolates were collected from a medical center (comprising two branches) in Northern Taiwan from 2009 to 2012, including 100 *K. pneumoniae*, 53 *Escherichia coli*, 41 *Enterobacter cloacae,* and 16 other isolates (one *K. oxytoca*, one *Citrobacter freundii*, two *Providencia rettgeri*, eight *Serratia* sp., and four *E. aerogenes*). The isolates were identified using the VITEK2 system (bioMérieux Vitek Systems Inc., Hazelwood, MO, USA).

Polymrase Chain Reaction Detection of Carbapenemase Genes and Insertion Sequences

The carbapenemase-encoding genes were detected using polymerase chain reaction (PCR) methods as previously suggested by Woodford et al. (2006) and Ellington et al. (2007). All primers used in this study, such as bla_{TEM}-type, bla_{SHV}-type, $bla_{\text{CTX-M}}$-type, bla_{CMY}-type, and bla_{DHA}-type primers, were as described previously (Tenover et al., 1995; Chung et al., 2011; Qi et al., 2011; Huang et al., 2012). Sequence similarity searches were conducted using the BLAST program[1].

Pulsed-Field Gel Electrophoresis

The isolates were compared using pulsed-field gel electrophoresis (PFGE; Seifert et al., 2005) following the digestion of intact genomic DNA by using *Xba*I (Biolabs, UK). The *Xba*I restriction profiles were initially compared using visual inspection according to the criteria of Tenover et al. (1995). A computer-assisted analysis was performed using BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium) software.

Conjugation and Electrotransformation Experiments

Plasmid DNAs were extracted using the Qiagen Plasmid Purification Midi Kits (Qiagen, Courtaboeuf, France). Plasmid

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

TABLE 1 | Minimum inhibitory concentrations (MICs) of three DHA-23 carrying enterobacterial isolates to antimicrobial agents.

Antimicrobial agent	MIC (mg/L)		
	KP11*	KP19*	EC56*
Ampicillin	≥32	≥32	≥32
Cefazolin	≥64	≥64	≥64
Cefoxitin	≥64	≥64	≥64
Cefuroxime	≥64	≥64	≥64
Cefotaxime	128	128	128
Ceftazidime	64	32	128
Flomoxef	≥64	≥64	≥64
Cefpirome	16	16	≥64
Ciprofloxacin	≥4	≥4	≥4
Moxifloxacin	≥8	≥8	≥8
Piperacillin-tazobactam	≥128	≥128	≥128
Trimethoprim-sulfamethoxazole	≥320	≥320	≥320
Amikacin	≥64	≥64	≥64
Gentamicin	≥16	≥16	≥16
Tigecycline	4	2	≤0.5
Colistin	≤0.5	≥16	≤0.5
Ertapenem	2	2	4
Meropenem	0.12	0.12	0.12
Imipenem	2	2	1
Doripenem	0.5	0.25	0.06

**KP11 and KP19 expressed TEM-1, SHV-11, CTX-M-14, and DHA-23, EC56 expressed TEM-1, CTX-M-14, CMY-2, and DHA-23.*

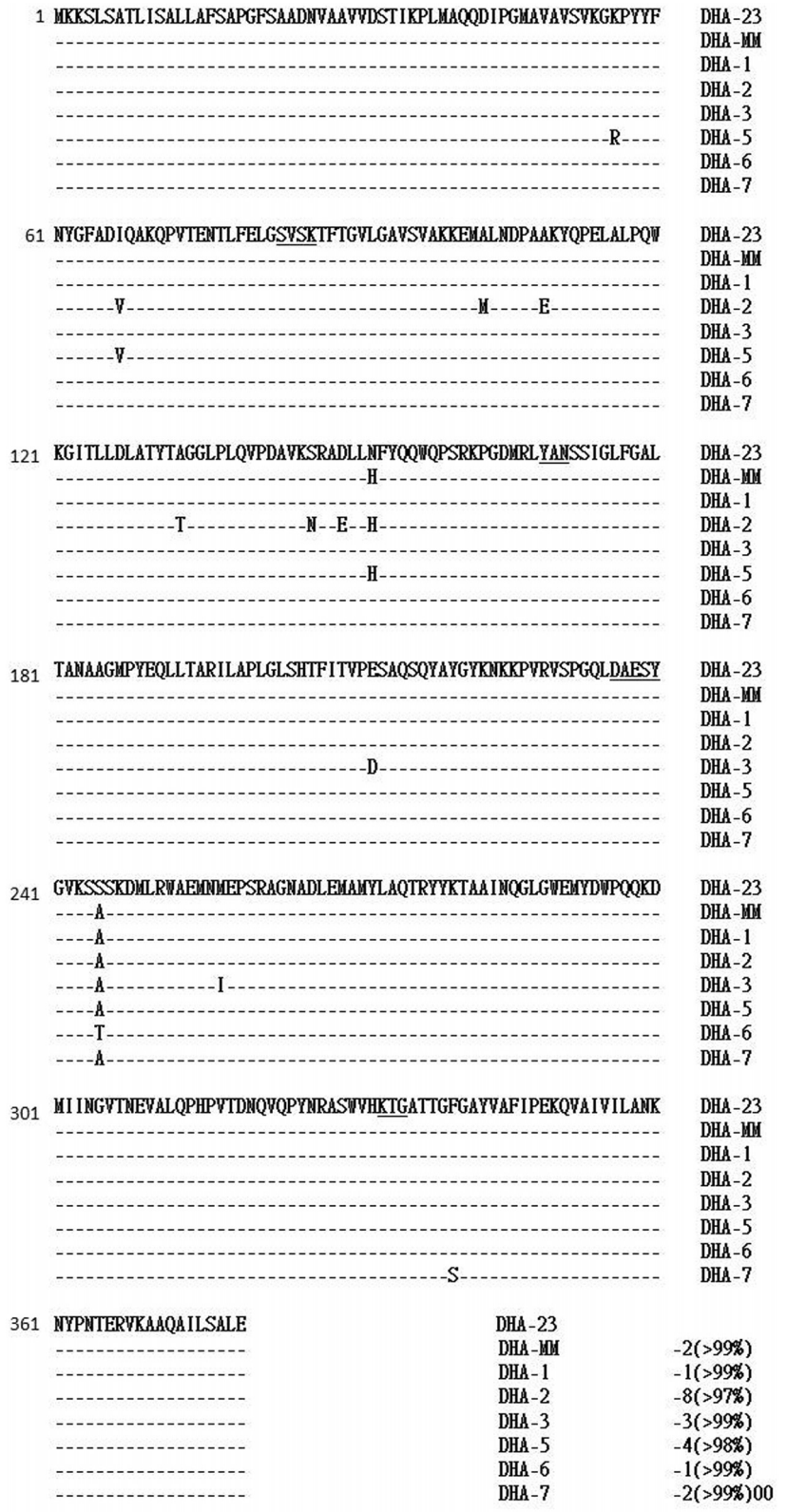

FIGURE 1 | Alignment of the deduced amino-acid sequences of DHA-23 with those of other DHA type AmpC enzymes. Identical amino-acids are marked with dashes. The underlined amino-acids are those that may be involved in the catalytic site of these AmpC enzymes, including the β-lactamase active site S–V–S–K and the conserved triad K–T–G. DHA-MM, DHA-*Morganella morganii*.

```
  1 MVRRYLPLNPLRAFEAAARHLSFTRAAIELNVTHAAVSQQVRALEEQLGCVLFTRVSRGL    DHA-23
    ------------------------------------------------------------    DHA-MM
    ------------------------------------------------------------    DHA-1
    ------------------------------------------------------------    DHA-2
    ----------------------L-------------------------------------    DHA-3

 61 VLTHEGEGLLPVLNEAFDRIADTLECFSHGQFRERVKVGAVGTFAAGWLLPRLAGFYDSH    DHA-23
    ------------------------------------------------------T-----    DHA-MM
    ------------------------------------------------------------    DHA-1
    ---------------------------W--------------------------------    DHA-2
    ------------------------------------------------------------    DHA-3

121 PHIDLHISTHNNHVDPAAEGHDYTIRFGNGAWHESDAELIFSAPHAPLCSPAIAEQLQQP    DHA-23
    ------------------------------------------------------------    DHA-MM
    ------------------------------------------------------------    DHA-1
    ------------------------------------------------------------    DHA-2
    ------------------------------------------------------------    DHA-3

181 DDVHRFTLLRSFRRDEWSRWLDCAGGTPPSPSQPVMVFDTSLAMAEAAQLGAGVAIAPVC    DHA-23
    ------------------------------------------------------------    DHA-MM
    ----------------------------T-------------------------------    DHA-1
    ------------------------------------------------L-----------    DHA-2
    ------------------------------------------------------------    DHA-3

241 MFSRLLQSGALVQPFAAEITLGGYWLTRLQSRTETPAMQQFARWLLNTAAA   DHA-23
    ------------------------------------------------------  DHA-MM  -1(>99%)
    ------------------------------------------------------  DHA-1   -1(>99%)
    --N---------------------------------------------------  DHA-2   -3(>98%)
    ----------------------R-------------------------------  DHA-3   -2(>99%)
```

FIGURE 2 | Alignment of the deduced amino-acid sequences of the AmpR protein of DHA-23 with those of other DHA type AmpC enzymes.

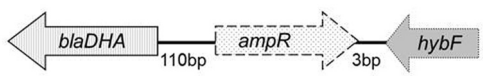

FIGURE 3 | Genetic environment of *bla*DHA-23.

conjugation experiments were performed using *E. coli* DH5α as the recipient (Poirel et al., 1999b). Transconjugants were selected on Luria–Bertani agar plates supplemented with sodium azide (100 mg/L) and cefotaxime (2 mg/L). In addition, cefoxitin (8 mg/L) was added to prevent the selection of ESBL-producing transconjugants. Electrotransformants were selected on agar containing cefotaxime (2 mg/L) or cefoxitin (8 mg/L).

Minimum Inhibitory Concentrations

Minimum inhibitory concentrations (MICs) were determined using the agar dilution method, which was repeated twice for each sample. MICs of tested antibiotics were interpreted according to Clinical and Laboratory Standards Institute guidelines (M7–A9, CLSI, 2012). Antibiotics were purchased from Sigma–Aldrich (St. Louis, MO, USA). Quality control was assured by testing *Staphylococcus aureus* ATCC 29213, *E. coli* ATCC25922, and *Pseudomonas aeruginosa* ATCC 27853.

Results

Novel AmpC β-Lactamase Discovered in Three Enterobacterial Isolates

One *E. coli* (EC56) and two *K. pneumoniae* (KP11 and KP19) isolates that exhibited resistance to cefoxitin, cefotaxime, and ceftazidime were isolated from 3 adult patients hospitalized in Northern Taiwan (**Table 1**). We had the designated DHA-23 approved by the curators of the Lahey website[2]. Using specific primers for bla_{DHA-1}, we obtained PCR fragments from plasmid DNA preparations of *E. coli* EC56 and *K. pneumoniae* KP11 and KP19. The deduced amino-acid sequence (**Figure 1**) indicated that DHA-23 exhibited only one amino-acid change compared with DHA-1 and DHA-6 (Ser245Ala), two amino acid changes compared with DHA-7 and DHA-*Morganella morganii* (DHA-MM; Barnaud et al., 1998), three amino acid changes compared with DHA-3 (Wu et al., 2005), four amino acid changes compared with DHA-5, and eight amino acid changes compared with DHA-2 (>97% identity; Fortineau et al., 2001).

ampR Gene Found Immediately Upstream from the *ampC* Gene

The 110-bp intercistronic region of *ampC* and *ampR* contained the promoter sequences for *ampC* and *ampR* expression. This region of $bla_{\mathrm{DHA-23}}$ was identical to the corresponding region of

[2]http://www.lahey.org/Studies/

bla_{DHA-1} (Barnaud et al., 1998) and was 97.3% identical to that in the chromosome of *M. morganii* (Poirel et al., 1999a). The *ampR* gene had an overlapping and divergently oriented promoter, as described for other *ampC–ampR* regulatory systems (Bennett and Chopra, 1993). Comparing the deduced amino-acid sequences of the AmpR of DHA-23 with those of DHA-1 and *M. morganii* indicated >99% identity, with only one amino-acid change in each case (**Figure 2**). A comparison of the AmpR of DHA-2 or the AmpR of DHA-3 revealed that they were at least >98% similar, exhibiting two and three amino acid changes, respectively. Similar to DHA-1, DHA-2, and DHA-3 (Barnaud et al., 1998; Fortineau et al., 2001; Wu et al., 2005), the sequences surrounding bla_{DHA} showed that *hybF* was found upstream from the *ampC* gene in DHA-1, DHA-2, and DHA-3, whereas *orf-1* was found downstream from the *ampC* gene only in DHA-1 (Barnaud et al., 1998). Subsequently, *hybF* and *orf-1* were investigated in the sequences surrounding bla_{DHA-23}. The *hybF* was identified downstream of *ampR*, but *orf-1* was not detected (**Figure 3**). The *hybF* was coded for HybF and shared sequence homologies with hydrogenase subunits of *E. coli* (Menon et al., 1994). Although *ampR* and bla_{DHA} were mobilized from the chromosome of *M. morganii* into a complex of In6–In7–*sulI*-type class-1 integrons in a strain of *Salmonella enterica* serovar Enteritidis (Verdet et al., 2000), an integron carrying bla_{DHA-23} was not detected in *E. coli* EC56 through PCR experiments employing 5′-CS and 3′-CS specific primers of class one integrons.

Clavulanic Acid Acts as An Inducer of DHA-23

E. coli EC56 contained 4 β-lactamases that corresponded to TEM-1, CTX-M-14, CMY-2, and DHA-23. *K. pneumoniae* KP11 and KP19 both contained β-lactamases that corresponded to TEM-1, SHV-11, CTX-M-14, and DHA-23. The genotyping of isolates was conducted using PFGE by using *Xba*I digestion. The results indicated that these two *K. pneumoniae* isolates had similar pulsotypes. Conjugation experiments were subsequently conducted, regardless of whether cefoxitin was used as a selecting agent, and the transfer of a plasmid coding the DHA-23 cephalosporinase into *E. coli* DH5α was not observed. The data suggested that the bla_{DHA-23} plasmid is not self-transferable. Electroporation experiments were then performed using plasmid DNA preparation of *E. coli* EC56 and *K. pneumoniae* KP11 and KP19. Amplification and gene sequencing were used to analyze the plasmid DNA, and the results revealed that the *E. coli* DH5α (pEC56) transformants contained two β-lactamases, TEM-1 and DHA-23. The *E. coli* DH5α (pKP11) and *E. coli* DH5α (pKP19) transformants contained only one β-lactamase, TEM-1. However, repeated electrotransformation experiments failed to obtain transformants carrying DHA-23 from KP11 and KP19. The MIC of ceftazidime (128 mg/L) for *E. coli* EC56 was not reduced by clavulanic acid. However, the MICs of cefotaxime (0.12 mg/L) and ceftazidime (1.0 mg/L) for *E. coli* DH5α (pEC56) increased in the presence of clavulanic acid, suggesting that clavulanic acid may act as an inducer of DHA-23 (**Table 2**).

Discussion

This study presented a novel plasmid-mediated and inducible AmpC β-lactamase obtained from Enterobacteriaceae, designated DHA-23 and approved by the curators of the Lahey website. There are currently 22 DHA variants listed on the site, most of which are assigned with no reference to the sequence.

In **Table 1**, MICs of the DHA-23 carrying enterobacterial isolates *E. coli* EC56 and *K. pneumoniae* KP11 and KP19 to antimicrobial agents show that cephamycins (such as cefoxitin) are not hydrolyzed by ESBLs, but are hydrolyzed by associated AmpC β-lactamase. Class-C enzymes hydrolyze cephamycins but do not hydrolyze extended-spectrum cephalosporins effectively. DHA-23 showed that the MICs did not substantially increase for cefotaxime and ceftazidime when DHA-23 was present in the transformants, whereas the MICs of cefoxitin increased to 32 mg/L (**Table 2**). Resistance to the carbapenems was variable. All strains were fully susceptible to meropenem and doripenem (**Table 2**). In general,

TABLE 2 | Minimum inhibitory concentrations of β-lactams for clinical isolate *Klebsiella pneumoniae* KP19, *Escherichia coli* EC56, electroporant *E. coli* DH5α (pPK19), *E. coli* DH5α (pEC56), and reference strain *E. coli* DH5α.

Antimicrobial agent	MIC (mg/L)				
	KP19	EC56	*E. coli* DH5α (pKP19)[#]	*E. coli* DH5α (pEC56)[†]	*E. coli* DH5α
Ampicillin	≥32	≥32	≥32	≥32	≤2
Cefoxitin	≥64	≥64	≤4	32	≤4
Cefotaxime	128	128	0.06	0.12	0.03
Ceftazidime	32	128	0.25	1	0.25
Ceftazidime-clavulanate*	32	≥128	0.25	2	0.25
Cefpirome	16	≥64	≤1	≤1	≤1
Ciprofloxacin	≥4	≥4	≤0.25	≤0.25	≤0.25
Ertapenem	2	4	≤0.5	≤0.5	≤0.5
Imipenem	2	1	≤1	≤1	≤1

*Clavulanate was tested at a fixed concentration of 4 mg/L.

[#]*E. coli DH5α (pKP19) expressed the plasmid-mediated TEM-1from K. pneumoniae 19.*

[†]*E. coli DH5α (pEC56) expressed the plasmid-mediated TEM-1 and DHA-23 from E. coli 56.*

carbapenems are regarded as the preferred agent for treatment. However, the production of AmpC β-lactamase significantly increased the MICs of carbapenems was reported by Bradford et al. with the ACT-1 β-lactamase (Bradford et al., 1997), and by Lee et al. (2010) with the DHA-1 β-lactamase. Based on the recombinant experiments, Martinez-Martinez et al. (1999) demonstrated that MICs of carbapenems increased significantly in the recombinant *K. pneumoniae* strain harboring over expressing AmpC β-lactamase and loss of porins. They proposed that the spread of strains that express the plasmid-mediated AmpC β-lactamases and lack porins may create serious therapeutic problems in the future. Furthermore, proteomic investigation of the inner-membrane fraction of carbapenem-resistant strain of *Acinetobacter baumannii* supported a model for the importance of upregulated AmpC β-lactamases and down-regulated OmpW production in the mediation of carbapenem resistance in *A. baumannii* (Tiwari et al., 2012).

Enterobacteriaceae isolates producing a DHA-1-like enzyme have been identified previously in Taiwan (Yan et al., 2002; Yu et al., 2004; Wu et al., 2005). We report that DHA-type β-lactamases remain a threat in this country. Further nationwide surveillance should be conducted, antibiotic stewardship should be advocated, and strict infection control measures should be enforced.

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Production of plasmid-encoding NDM-1 in clinical *Raoultella ornithinolytica* and *Leclercia adecarboxylata* from China

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Raoultella ornithinolytica YNKP001 and *Leclercia adecarboxylata* P10164, which harbor conjugative plasmids pYNKP001-NDM and pP10164-NDM, respectively, were isolated from two different Chinese patients, and their complete nucleotide sequences were determined. Production of NDM-1 enzyme by these plasmids accounts for the carbapenem resistance of these two strains. This is the first report of bla_{NDM} in *L. adecarboxylata* and third report of this gene in *R. ornithinolytica*. pYNKP001-NDM is very similar to the IncN2 NDM-1-encoding plasmids pTR3, pNDM-ECS01, and p271A, whereas pP10164-NDM is similar to the IncFII$_Y$ bla_{NDM-1}-carrying plasmid pKOX_NDM1. The bla_{NDM-1} genes of pYNKP001-NDM and pP10164-NDM are embedded in Tn*125*-like elements, which represent two distinct truncated versions of the NDM-1-encoding Tn*125* prototype observed in pNDM-BJ01. Flanking of these two Tn*125*-like elements by miniature inverted repeat element (MITE) or its remnant indicates that MITE facilitates transposition and mobilization of bla_{NDM-1} gene contexts.

Keywords: ***Raoultella ornithinolytica*, *Leclercia adecarboxylata*, NDM-1, plasmid, carbapenem resistance**

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Introduction

Raoultella ornithinolytica is widely found in aquatic environments, insects and fishes. *R. ornithinolytica* is able to convert histidine to histamine (scombroid toxin) and is thus known to cause fish poisoning. Symptoms primarily manifest with facial flushing, dizziness, vomiting, diarrhea, dyspnea, headache, urticarial, and generalized pruritus and commonly subside in a few hours (Kanki et al., 2002). Infections by *R. ornithinolytica* are exceedingly rare in humans and have been reported as bloodstream, urinary tract and soft tissue infections in adults and as fatal neonatal infections. Most adult cases are linked with underlying diseases, especially malignancies (Morais et al., 2009; Mau and Ross, 2010; Solak et al., 2011; Hadano et al., 2012; Haruki et al., 2014; Chun et al., 2015).

Raoultella ornithinolytica produces at least two different chromosomally encoded class A β-lactamases. Accordingly, *R. ornithinolytica* is resistant to ampicillin but commonly remains susceptible to cefotaxime and imipenem (Walckenaer et al., 2004). Notably, carbapenem-resistant

R. ornithinolytica has been reported due to the production of plasmid-encoding carbapenemase KPC-3 (Castanheira et al., 2009) or NDM-1 (Khajuria et al., 2013; Zhou et al., 2014).

Leclercia adecarboxylata is a ubiquitous organism that is rarely clinically isolated in humans. However, *L. adecarboxylata* has been recognized as an opportunistic pathogen in immunocompromised patients suffering from primary diseases and often depends on co-flora to cause polymicrobial infection (Shin et al., 2012; De Mauri et al., 2013; Garcia-Fulgueiras et al., 2014). *L. adecarboxylata* is isolated from various clinical specimens (e.g., blood, feces, sputum, urine, and wound pus) and causes bacteremia, endocarditis, sepsis, peritonitis, cellulitis, endocarditis, and cholecystitis (Shin et al., 2012; De Mauri et al., 2013; Garcia-Fulgueiras et al., 2014). *L. adecarboxylata*-induced monomicrobial infections (e.g., wound infection, pharyngeal abscess, and bacteremia) have also been reported in immunocompetent patients (Hess et al., 2008; Bali et al., 2013; Michael et al., 2013; Garcia-Fulgueiras et al., 2014; Keren et al., 2014).

Leclercia adecarboxylata is generally susceptible to commonly used antibiotics, but there are a few reports of *L. adecarboxylata* harboring different antibiotic resistance mechanisms. Cephalosporin- and carbapenem-resistant strains of *L. adecarboxylata* have been identified due to the production of extended-spectrum β-lactamase (ESBL) SHV-12 (Mazzariol et al., 2003) and carbapenemase KPC-2 (Geffen et al., 2013) or VIM-1 (Papagiannitsis et al., 2013), respectively. Notably, clinical isolates of multidrug-resistant *L. adecarboxylata* have been known to harbor multiple antibiotic resistance genes that are captured by class 1 integrons (Yao et al., 2011; Shin et al., 2012; Garcia-Fulgueiras et al., 2014).

NDM is an Ambler class B metallo-β-lactamase that confers resistance to nearly all β-lactam antibiotics, including carbapenems, and bla_{NDM} genes have been identified in a large array of *Enterobacteriaceae* species (Nordmann et al., 2011; Johnson and Woodford, 2013; Dortet et al., 2014). Both *R. ornithinolytica* and *L. adecarboxylata* are members of *Enterobacteriaceae*. In this study, we analyzed complete nucleotide sequences of two different NDM-1-encoding plasmids, pYNKP001-NDM, and pP10164-NDM, recovered from *R. ornithinolytica* YNKP001 and *L. adecarboxylata* P10164, respectively, of clinical origin in China.

Materials and Methods

Bacterial Strains and Identification

The use of human specimens and all related experimental protocols were approved by the Committee on Human Research of indicated institutions and carried out in accordance with the approved guidelines. Informed consent was obtained from the indicated patients. All bacterial strains were subjected to species identification by BioMérieux VITEK 2, Bruker MALDI Biotyper, and 16S rRNA gene sequencing. For 16S rRNA gene sequence determination, nearly the complete coding region of 16S rRNA gene was amplified by PCR with the universal primers 27f (AGAGTTTGATCCTGGCTCAG) and 1492r (TACCTTGTTACGACTT) (Frank et al., 2008). The major carbapenemase and ESBL genes (Table S1) were subjected to PCR detection. All PCR amplicons were sequenced on an ABI 3730 Sequencer with the same primers for PCR.

Plasmid Conjugal Transfer

Plasmid conjugal transfer experiments were carried out with *Escherichia coli* EC600 (rifampin-resistant) or TB1 (streptomycin-resistant) used as recipient and the bla_{NDM}-positive strain YNKP001 or P10164 as donor. Then, 3 ml of overnight culture of each of donor and recipient bacteria were mixed together, harvested and resuspended in 80 μl of brain heart infusion (BHI) broth (BD Biosciences). The mixture was spotted on a 1-cm^2 filter membrane placed on BHI agar (BD Biosciences) plates. The plates were incubated for mating at 37°C for 12–18 h. Bacteria were washed from filter membrane and spotted on Muller-Hinton (MH) agar (BD Biosciences) plates containing 1000 μg/ml rifampin or 250 μg/ml streptomycin and 200 μg/ml ampicillin for selection of bla_{NDM}-positive *E. coli* transconjugants.

Detection of Carbapenemase Activity

Activity of class A/B/D carbapenemases was determined via a CarbaNP test (Dortet et al., 2012) with modifications. Overnight bacterial cell culture in MH broth was diluted 1:100 into 3 ml of fresh MH broth, and bacteria were allowed to grow at 37°C with shaking at 200 rpm to reach an OD_{600} of 1.0–1.4. If required, ampicillin was used at 200 μg/ml. Bacterial cells were harvested from 2 ml of the above culture and washed twice with 20 mM Tris-HCl (pH 7.8). Cell pellets were resuspended in 500 μl of 20 mM Tris-HCl (pH 7.8) and lysed by sonication, followed by centrifugation at 10000 × g at 4°C for 5 min. Then, 50 μl of the supernatant (enzymatic bacterial suspension) was mixed with 50 μl of substrate I–V, followed by incubation at 37°C for a maximum of 2 h. Substrate I: 0.054% red phenol plus 0.1 mM $ZnSO_4$ (pH 7.8). Substrate II: 0.054% red phenol plus 0.1 mM $ZnSO_4$ (pH 7.8) and 0.6 mg/μl imipenem. Substrate III: 0.054% red phenol plus 0.1 mM $ZnSO_4$ (pH 7.8), 0.6 mg/μl mg imipenem, and 0.8 mg/μl tazobactam. Substrate IV: 0.054% red phenol plus 0.1 mM $ZnSO_4$ (pH 7.8), 0.6 mg/μl mg imipenem, and 3 mM EDTA (pH 7.8). Substrate V: 0.054% red phenol plus 0.1 mM $ZnSO_4$ (pH 7.8), 0.6 mg/μl mg imipenem, 0.8 mg/μl tazobactam, and 3 mM EDTA (pH 7.8).

Determination of Minimum Inhibitory Concentration (MIC)

The MIC values of the indicated bacterial strains were tested by VITEK 2 according to the manufacturer's instructions, and antimicrobial susceptibility was assessed by the Clinical and Laboratory Standards Institute (CLSI) standards.

Determination of Plasmid DNA Sequences

The chromosome DNA-free plasmid DNA was isolated from the cell cultures of indicated *E. coli* transconjugant using a Qiagen large construct kit and then sequenced using the whole-genome shotgun strategy in combination with Illumina HiSeq 2500 sequencing technology. The contigs were assembled with Velvet, and the gaps were filled through combinatorial PCR and

Sanger sequencing with an ABI 3730 Sequencer. The genes were predicted with GeneMarkS and further annotated by BLASTP against UniPort and NR databases.

Nucleotide Sequence Accession Numbers

The complete sequences of plasmids pP10164-NDM and pYNKP001-NDM were submitted to GenBank under accession numbers KP900016 and KP900017, respectively.

Results

Characterization of *R. ornithinolytica* YNKP001 and *L. adecarboxylata* P10164

Raoultella ornithinolytica YNKP001 was recovered in November 2010 from the blood specimens of a 4-year-old child with acute encephalitis, bronchitis and tympanitis from a hospital in Kunming City in China. *L. adecarboxylata* P10164 was isolated in August 2012 from the sputum specimens of a 43-year-old male with pneumonia admitted to a teaching hospital in Chongqing City in China. Both patients received empirical intravenous administration with ceftazidime for at least 1 week, but their symptoms did not improve. Subsequent antimicrobial susceptibility tests indicated that both strains were resistant to multiple β-lactams, including imipenem, and meropenem, but remained susceptible to fluoroquinolones. The patients then received intravenous administration with moxifloxacin and were cured and discharged approximately 10 days after initiating antimicrobial treatment.

Plasmids pP10164-NDM and pYNKP001-NDM could be readily transferred from P10164 and YNKP001 into *E. coli* EC600 and TB1, respectively, generating two corresponding *E. coli* transconjugants: P10164-NDM-EC600 and YNKP001-NDM-TB1. PCR detection of the major ESBL and carbapenemase genes (Table S1) indicated that P10164 harbored the bla_{NDM}, $bla_{\mathrm{CTX-M-1}}$ group, bla_{TEM}, and $bla_{\mathrm{OXA-1}}$ group genes, whereas YNKP001, P10164-NDM-EC600 and YNKP001-NDM-TB1 contained only bla_{NDM}, as confirmed by PCR amplicon sequencing. Class B carbapenemase activity was detected in P10164, YNKP001, P10164-NDM-EC600 and YNKP001-NDM-TB1 (**Figure S1**).

P10164 was resistant to all of the tested drugs, including penicillin, β-lactam inhibitor, cephalosporin, carbapenem, monobactam, fluoroquinolone, furane, and aminoglycoside (**Table 1**). YNKP001, P10164-NDM-EC600, and YNKP001-NDM-TB1 were resistant to penicillins, β-lactam inhibitors, cephalosporins, and carbapenems but remained susceptible to fluoroquinolones, aminoglycosides, aztreonam, and macrodantin (**Table 1**).

Comparative Genomics of pYNKP001-NDM

As revealed by high-throughput sequencing with a mean coverage fold of 143, plasmid pYNKP001-NDM was 41,190 bp in size with a mean GC content of 50.8% and contained 54 open reading frames (ORFs) (**Figure 1A**). The replication module present on pYNKP001-NDM belonged to the IncN2 incompatibility group (Poirel et al., 2011). This plasmid was mostly similar to another NDM-encoding IncN2 plasmid, pTR3 (Chen et al., 2012), with genetic differences in only three single nucleotide polymorphisms (SNPs).

Further linear genomic comparison (**Figure 2A**) was performed with all four NDM-encoding IncN2 plasmids, namely, pYNKP001-NDM, pTR3 (Chen et al., 2012), pNDM-ECS01 (Netikul et al., 2014), and p271A (Poirel et al., 2011). The former three plasmids essentially had the same genomic organization. The genome of each plasmid could be divided into the backbone and accessory module. The backbone was composed of genes responsible for plasmid replication (*repA*), stability (*stbABC*, *ssb*, *korB*, *klcA*, etc.) and conjugal transfer (*tra*). p271A lacked a 5.2 Kb region found in pYNKP001-NDM, pTR3, and pNDM-ECS01. This region corresponded to the CUP (conserved upstream repeat)-controlled regulon commonly found in the IncN1 and IncN2 plasmids, and the loss of this region might be due to recombination between CUPs (Partridge et al., 2012).

The four plasmids shared a single conserved accessory module, which was sequentially organized as an intact miniature inverted repeat element (MITE), a $bla_{\mathrm{NDM-1}}$-containing Tn*125*-like element, a MITE remnant, an intact IS*Sen4* element with 26 bp inverted repeats (IRs) at both sides, a truncated *aphA6* (aminoglycoside resistance) gene, and an intact Tn*5403* element with 39 bp IRs at both ends (**Figure 3A**). The Tn*125* prototype was sequentially organized as IS*Aba125*, $bla_{\mathrm{NDM-1}}$, ble_{MBL} (bleomycin resistance), Δ*trpF*, *dsbC*, *cutA*, Δ*groES*, *groEL*, *ISCR27,* and IS*Aba125*; Tn*125* was a typical composite transposon (Tn) containing two flanking copies of IS*Aba125* (each with 26 bp IRs at both ends); Tn*125* could be inserted into a site downstream of *aphA6*, leaving 3 bp direct repeats (DRs) at both ends, as observed in pNDM-BJ01 (Hu et al., 2012; Poirel et al., 2012) (**Figure 3A**). Compared with the Tn*125* prototype, the Tn*125*-like element in pYNKP001-NDM lacked the entire fragment of *dsbC*, *cutA*, Δ*groES*, *groEL*, *ISCR27,* and IS*Aba125*. In addition, the upstream copy of IS*Aba125* was a truncated version lacking the left IR and was interrupted by IS*Ec33* (with 17 bp IRs at both ends) (**Figure 3A**).

The flanking of a genetic context (e.g., class 1 integron) by two identical MITEs has been recently characterized as a mechanism for mobilizing antimicrobial resistance determinants via MITE-mediated transposition or homologous recombination (Poirel et al., 2009; Domingues et al., 2013; Zong, 2014). Notably, the Tn*125*-like element in pYNKP001-NDM was embedded between an intact 257 bp MITE and a 55 bp MITE remnant (**Figure 3A**). The intact MITE contained 39 bp IRs and could potentially form a long stem-loop RNA structure (Delihas, 2011), whereas the downstream MITE remnant corresponded to the 3′-terminal 55 bp fragment (including the right IR) of the intact MITE.

Comparative Genomics of pP10164-NDM

Plasmid pP10164-NDM was fully sequenced, with a mean coverage fold of 248. Moreover, it was 99,276 bp in size, with a mean GC content of 55%, and contained 101 ORFs (**Figure 1B**). pP10164-NDM was assigned to the $\mathrm{IncFII_Y}$ incompatibility group encoding two different replication proteins $\mathrm{IncFII_Y}$ (*repA*) and IncFIB (*repB*) (Villa et al., 2010). Comparative genomics

TABLE 1 | MIC values and antimicrobial susceptibility.

Category	Antibiotics	MIC (μg/ml)/antimicrobial susceptibility					
		P10164	P10164-NDM-EC600	EC600	YNKP001	YNKP001-NDM -TB1	TB1
Penicillins	Ampicillin	≥32/R	≥32/R	16/I	≥32/R	≥32/R	16/I
	Ampicillin/sulbactam	≥32/R	≥32/R	8/S	≥32/R	≥32/R	8/S
	Piperacillin	≥128/R	≥128/R	≤4/S	≥128/R	≥128/R	≤4/S
	Piperacillin/tazobactam	≥128/R	≥128/R	≤4/S	≥128/R	≥128/R	≤4/S
Cephalosporins	Cefazolin	≥64/R	≥64/R	≤4/S	≥64/R	≥64/R	≤4/S
	Cefuroxime sodium	≥64/R	≥64/R	16/I	≥64/R	≥64/R	16/S
	Cefuroxime axetil	≥64/R	≥64/R	16/I	≥64/R	≥64/R	16/S
	Cefotetan	≥64/R	≥64/R	≤4/S	≥64/R	≥64/R	≤4/S
	Ceftriaxone	≥64/R	≥64/R	≤1/S	≥64/R	≥64/R	≤1/S
	Ceftazidime	≥64/R	≥64/R	≤1/S	≥64/R	≥64/R	≤1/S
Carbapenems	Imipenem	≥16/R	≥16/R	≤1/S	≥16/R	≥16/R	≤1/S
	Meropenem	8/R	4/R	≤0.25/S	≥16/R	≥16/R	≤0.25/S
Monobactams	Aztreonam	16/R	≤1/S	≤1/S	≤1/S	≤1/S	≤1/S
Fluoroquinolones	Ciprofloxacin	≥4/R	≤0.25/S	≤0.25/S	≤0.25/S	≤0.25/S	≤0.25/S
	Levofloxacin	≥8/R	0.5/S	0.5/S	0.5/S	≤0.25/S	≤0.25/S
Furanes	Macrodantin	≥512/R	≤16/S	≤16/S	32/S	≤16/S	≤16/S
Aminoglycosides	Amikacin	≥64/R	≤2/S	≤2/S	≤2/S	≤2/S	≤2/S
	Gentamicin	≥16/R	≤1/S	≤1/S	≤1/S	≤1/S	≤1/S
	Tobramycin	≥16/R	≤1/S	≤1/S	≤1/S	≤1/S	≤1/S

analysis was performed with the only two characterized NDM-encoding IncFII$_Y$ plasmids: pP10164-NDM and pKOX_NDM1 (Huang et al., 2013) (**Figure 2B**). pP10164-NDM differed from pKOX_NDM1 in 214 SNPs, 11 single-nucleotide indels, and three large deletions (11285 bp, 180 bp, and 45 bp, respectively). pP10164-NDM and pKOX_NDM1 had very similar backbones composed of genes responsible for plasmid replication (*repA* and *repB*), stability (*pmaAB*, *psiAB*, *klcA*, *yub*, etc.) and conjugal transfer (*tra*); the 45 bp deletion (nucleotide position 102082 to 102126 in pKOX_NDM1; located within the *traD* gene) was the only observed structural difference between the backbones of pP10164-NDM and pKOX_NDM1 (**Figure 2B**).

pP10164-NDM contained a single accessory module that was 38,098 bp in size, in which the 11285 bp and 180 bp deletions (nucleotide positions 10620–21904 and 37157–37336 in pKOX_NDM, respectively) compared with the counterpart of pKOX_NDM1 were located (**Figure 3B**). The accessory module of pKOX_NDM1 harbored three copies of 256 bp MITEs highly similar to the above-mentioned 257 bp MITE, constituting a linear structure organized as the 11285 bp region (MITE plus 11029 bp region), MITE, Tn*125*-like element, and MITE (**Figure 3B**). Homologous recombination mediated by the first two copies of MITE appeared to lead to the insertion of the 11285 bp region into pKOX_NDM1 relative to pP10164-NDM (**Figure 3B**). In addition to the first copy of MITE, the 11285 bp region still contained *rmtC* (16S rRNA methylase for aminoglycoside resistance), IS*CR14* and an uncharacterized Tn (*tniB*, Δ*tniA*, and *tniA*) (**Figure 3B**).

The *bla*$_{NDM-1}$-harboring Tn*125*-like element was flanked by the second and third copies of MITE, indicating a similar mechanism of MITE-mediated insertion to the Tn*125*-like element (**Figure 3B**). Compared with the Tn*125* prototype in pNDM-BJ01, the Tn*125*-like element in pP10164-NDM (**Figure 3B**) exhibited absence of a downstream copy of IS*Aba125*, as well as truncation of *ISCR27* and an upstream copy of IS*Aba125*.

In addition, three potential composite Tns, with the characteristic of being flanked by two separate copies of IS*5* or IS*1*, were identified within the accessory module of pP10164-NDM or pKOX_NDM1, but none of the flanking DRs as target sites could be found.

Discussion

Horizontal transfer of plasmid-borne *bla*$_{NDM}$ genes enabled NDM enzymes to be rapidly spread in *Enterobacteriaceae*, less frequently in *Acinetobacter*, and rarely in *Pseudomonas* (Nordmann et al., 2011; Johnson and Woodford, 2013; Dortet et al., 2014). *Klebsiella pneumoniae* and *E. coli* are the most commonly described *bla*$_{NDM}$-carrying *Enterobacteriaceae* species, and *bla*$_{NDM}$ genes have been described in many other enterobacterial species, such as *K. oxytoca*, *K. ozaenae*,

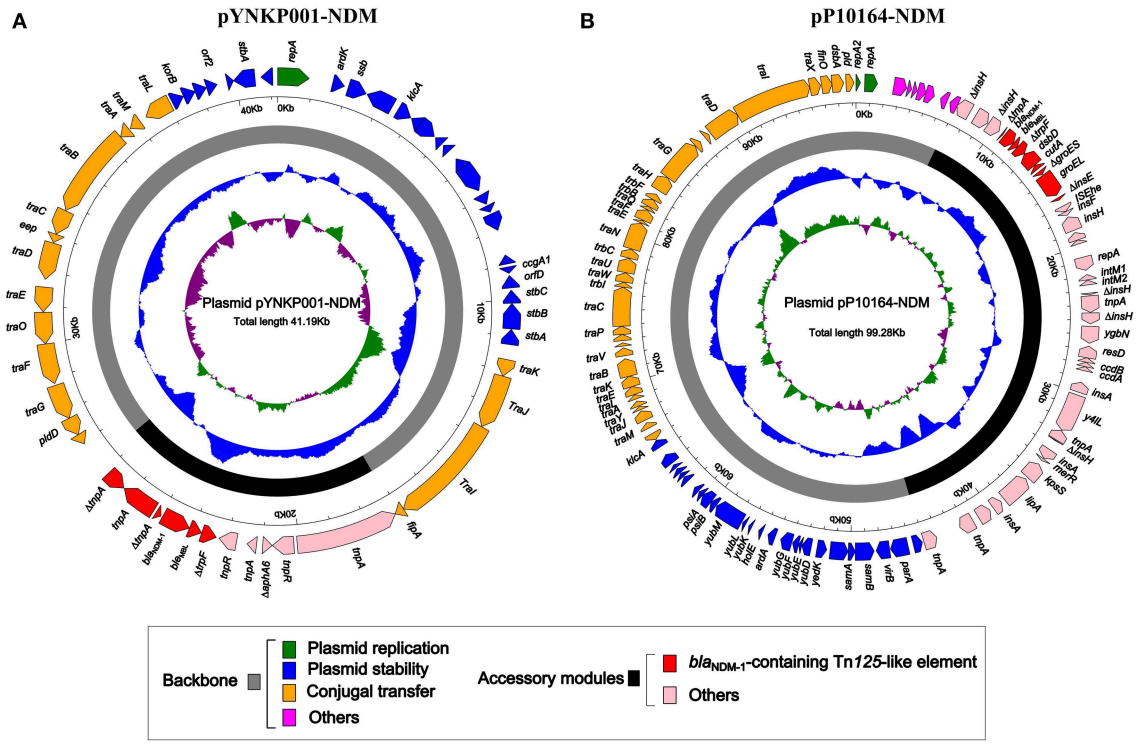

FIGURE 1 | Schematic maps of plasmids pYNKP001-NDM (A) and pP10164-NDM (B). Genes are denoted by arrows and colored based on gene function classification.The innermost circle presents GC-Skew [(G − C)/(G + C)] with a window size of 500 bp and step size of 20 bp. The blue circle presents GC content. Backbone and accessory module regions are also shown.

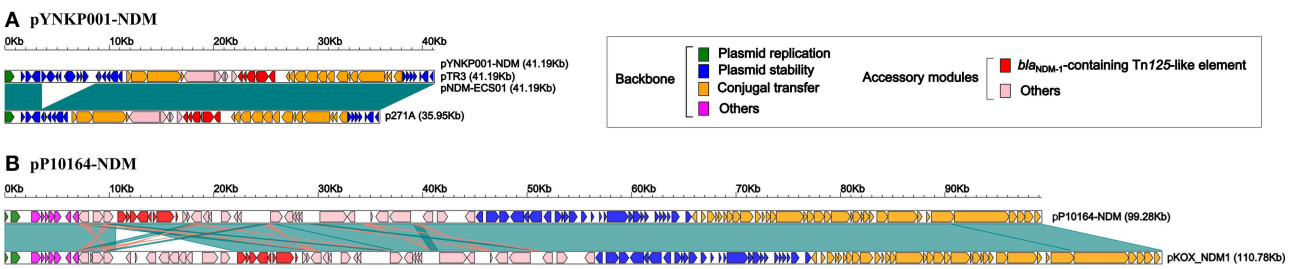

FIGURE 2 | Linear comparison of YNKP001-NDM (A) or pP10164-NDM (B) with its closely related plasmid. Genes are denoted by arrows and colored based on gene function classification. Dark green shading denotes shared regions of homology (>98% nucleotide similarity).

Citrobacter freundii, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Proteus mirabilis*, *Morganella morganii*, *Providencia spp.*, *Serratia marcescens*, and *Salmonella enterica* (Ou et al., 2014; Biedenbach et al., 2015). These NDM-producing bacteria have been shown to cause hospital- and community-acquired infections (Ou et al., 2014; Biedenbach et al., 2015). $bla_{\mathrm{NDM-1}}$-positive bacteria are frequently found in environmental settings, indicating environmental origins of bla_{NDM} genes in human pathogens, whereas use of antimicrobial drugs in an indiscriminate manner in some countries makes $bla_{\mathrm{NDM-1}}$-positive bacteria spread easily and pose a serious public health threat (Walsh et al., 2011; Isozumi et al., 2012).

This is the first report of bla_{NDM} in *L. adecarboxylata*. The clinical *L. adecarboxylata* isolate characterized herein harbors a conjugative $\mathrm{IncFII_Y}$ plasmid pP10164-NDM that encodes the NDM-1 enzyme. pP10164-NDM is highly similar to pKOX_NDM1, which is recovered from a nosocomial *K. oxytoca* strain from a patient following medical transfer from a hospital in Jiangxi to Taiwan, China (Huang et al., 2013). There are only two reports of bla_{NDM} in *R. ornithinolytica*, one from India (Khajuria et al., 2013) and the other from China (Zhou et al., 2014), both of which were confined to surveillance of bla_{NDM} genes. This work presents extended evidence that the production of NDM-1 by a conjugative IncN2 plasmid pYNKP001-NDM accounts for the carbapenem resistance of a clinical *R. ornithinolytica* isolate from China. pYNKP001-NDM is very similar to pTR3 (identified in *K. pneumoniae* from a Chinese patient) (Chen et al., 2012), pNDM-ECS01 (in *E. coli* from a Thai patient) (Netikul et al., 2014) and

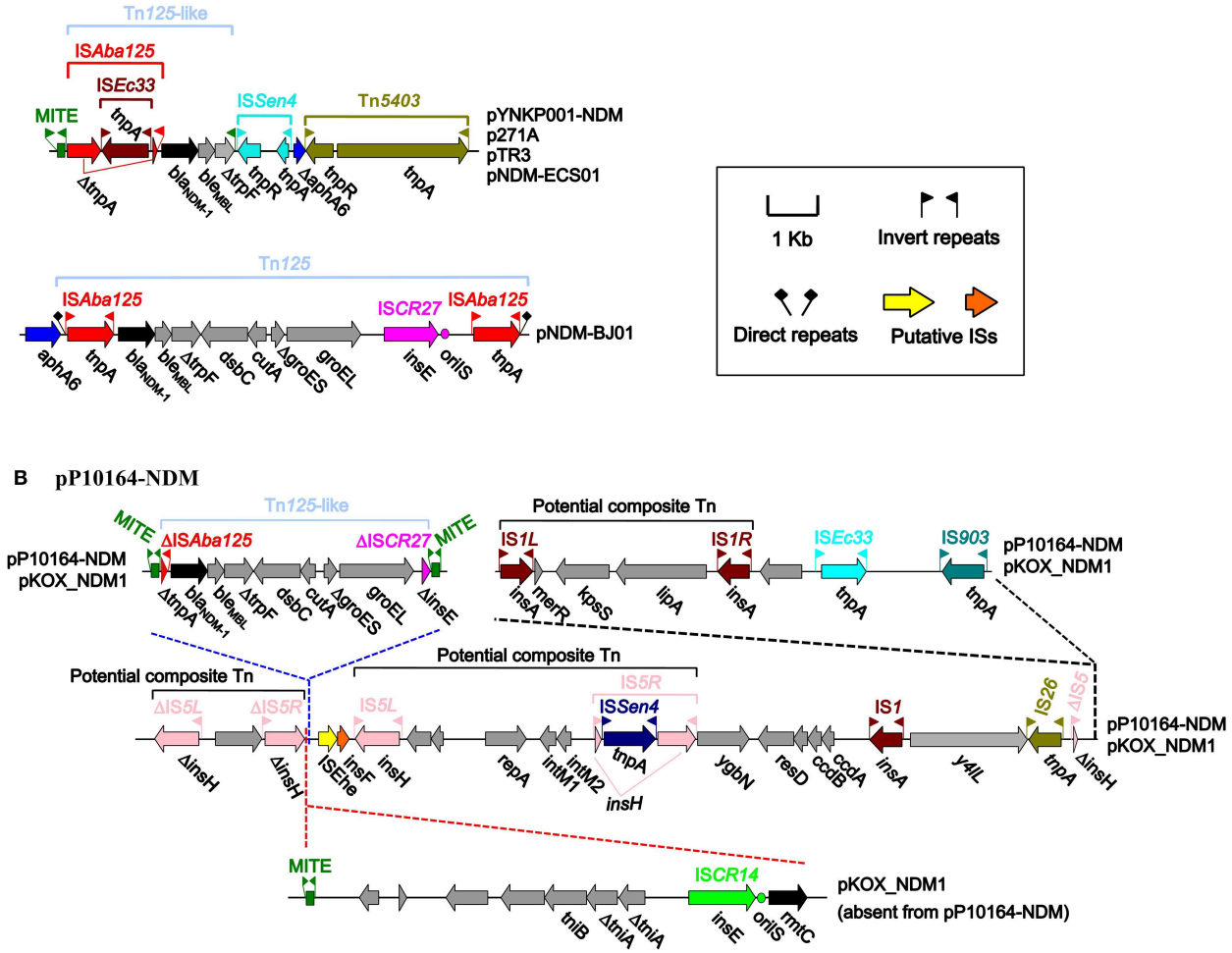

FIGURE 3 | Accessory modules of plasmids pYNKP001-NDM (A) and pP10164-NDM (B). Genes are denoted by arrows and colored based on gene function classification.

p271A (in *E. coli* from a patient following medical transfer from a hospital in Bangladesh to Australia) (Poirel et al., 2011).

The bla_{NDM-1} genes of pYNKP001-NDM and pP10164-NDM are embedded in Tn*125*-like elements, which are characterized as two distinct truncated versions of the prototype NDM-encoding Tn*125*, as observed in pNDM-BJ01 (Hu et al., 2012).

The Tn*125*-like elements with a large array of derivatives represent the major genetic platforms for bla_{NDM} genes across host bacteria (Bonnin et al., 2012; Mcgann et al., 2012; Partridge and Iredell, 2012; Zhang et al., 2013; Zong and Zhang, 2013; Fiett et al., 2014; Jones et al., 2014; Mataseje et al., 2014). Notably, flanking of Tn*125*-like elements by MITE or its remnant as observed herein indicates that MITE facilitates transposition and mobilization of bla_{NDM-1} gene contexts.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00458/abstract

Figure S1 | Detection of carbapenemase activity. In the presence of any carbapenemase, relevant carbapenems are hydrolyzed and transformed into its carboxylic form, leading to a pH decrease, which is detected by a color change of phenol red solution (red to yellow-orange). Ambler class A carbapenemases are at least partially inhibited by tazobactam, whereas class B carbapenemases (metallo-ß-lactamases) are inhibited by divalent cation chelators, such as EDTA. There is no available chemical inhibitor for class D carbapenemases. In this study, the bla_{NDM-1}-positive strains *R. ornithinolytica* YNKP001 and *L. adecarboxylata* P10164, *E. coli* YNKP001-NDM-TB1 and P10164-NDM-EC600, and *K. pneumoniae* ATCC BAA-2146 (positive control) (Rasheed et al., 2013) exhibited class B carbapenemase activity. As expected, *E. coli* EC600 and DH10B had no carbapenemase activity.

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NDM-1 encoded by a pNDM-BJ01-like plasmid p3SP-NDM in clinical *Enterobacter aerogenes*

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A carbapenem-nonsusceptible *Enterobacter aerogenes* strain named 3-SP was isolated from a human case of pneumonia in a Chinese teaching hospital. NDM-1 carbapenemase is produced by a pNDM-BJ01-like conjugative plasmid designated p3SP-NDM to account for carbapenem resistance of 3-SP. p3SP-NDM was fully sequenced and compared with all publically available pNDM-BJ01-like plasmids. The genetic differences between p3SP-NDM and pNDM-BJ01 include only 18 single nucleotide polymorphisms, a 1 bp deletion and a 706 bp deletion. p3SP-NDM and pNDM-BJ01 harbor an identical Tn*125* element organized as IS*Aba125*, bla_{NDM-1}, ble_{MBL}, Δ*trpF*, *dsbC*, *cutA*, Δ*groES*, *groEL*, *ISCR27*, and IS*Aba125*. The bla_{NDM-1} surrounding regions in these pNDM-BJ01-like plasmids have a conserved linear organization IS*Aba14*-*aphA6*-Tn*125*-unknown IS, with considerable genetic differences identified within or immediately downstream of Tn*125*. All reported pNDM-BJ01-like plasmids are exclusively found in *Acinetobacter*, whereas this is the first report of identification of a pNDM-BJ01-like plasmid in *Enterobacteriaceae*.

Keywords: *Enterobacter aerogenes*, NDM-1, Plasmid, p3SP-NDM

Enterobacter aerogenes is a Gram-negative bacterium widely found in the human gastrointestinal tract and in the environment, and generally non-pathogenic to healthy humans. Since 1990s, *E. aerogenes* has become an important opportunistic pathogen commonly affecting those with weakened immune systems to cause hospital-acquired infections such as pneumonia, bacteremia, urinary tract infection, surgical site infection, and meningitis (Georghiou et al., 1995; Davin-Regli et al., 1996; De Gheldre et al., 1997; Jalaluddin et al., 1998; Ronveaux et al., 1999).

E. aerogenes strains isolated from hospitalized patients generally exhibit high resistance to commonly used broad-spectrum antibiotics; in particular, the use of carbapenems imipenem and meropenem as the first-line antimicrobial agents for treating serious or refractory infections has led to considerable increase in prevalence of carbapenem-resistant *E. aerogenes* (De Gheldre et al., 1997; Chen et al., 2008; Lavigne et al., 2013). Carbapenem resistance of *E. aerogenes* is usually a result of production of plasmid-encoding carbapenemases such as KPC (Chen et al., 2014; Kuai et al., 2014; Luo et al., 2014; Qin et al., 2014), IMP (Biendo et al., 2008; Ding et al., 2014), VIM (Biendo et al., 2008; Souli et al., 2008) and NDM (Ho et al., 2012), decreased membrane permeability (due to altered porin expression or efflux pump overexpression) together with

production of AmpC-type cephalosporinase or extended-spectrum β-lactamase (ESBL) (Bornet et al., 2003; Lavigne et al., 2012, 2013), and lipopolysaccharide modification (Leying et al., 1991).

The 47.27 Kb plasmid pNDM-BJ01 is isolated from a clinical *A. lwoffii* strain in China in 2010 (Hu et al., 2012) and it cannot be assigned into any known incompatibility group. In this study, phenotypic and biochemical experiments combined with plasmid sequencing and comparative genomics analyses disclose that production of NDM-1 by a pNDM-BJ01-like conjugative plasmid p3SP-NDM accounts for carbapenem resistance of a clinical *E. aerogenes* isolate recovered from a human case of pneumonia in a Chinese teaching hospital.

Materials and Methods

Bacterial Strains and Identification

The use of human specimens and all related experimental protocols were approved by the Committee on Human Research of indicated institutions and carried out in accordance with the approved guidelines, and moreover the informed consent was obtained from indicated patient. All the bacterial strains in this study were subjective for species identification by BioMérieux VITEK 2, Bruker MALDI Biotyper, and 16s rRNA gene sequencing. For determination of 16S rRNA gene sequence, the almost complete coding region of 16S rRNA gene was amplified by PCR with the universal primers 27f (AGAGTTTGATCCTGGCTCAG) and 1492r (TACCTTGTTACGACTT) (Frank et al., 2008). The major carbapenemase and ESBL genes as listed in Table S1 were subjected to PCR detection. All PCR amplicons were sequenced on ABI 3730 Sequencer with the same primers for PCR.

Plasmid Transfer

Plasmid conjugal transfer experiments were carried out with the rifampin-resistant *E. coli* EC600 being used as recipient and the bla_{NDM}-positive strain 3-SP as donor. 3 ml of overnight culture of each of donor and recipient bacteria were mixed together, harvested, and resuspended in 80 μl of Brain Heart Infusion (BHI) broth (BD Biosciences). The mixture was spotted on a 1 cm^2 filter membrane that was placed on BHI agar (BD Biosciences) plate, and then incubated for mating at 37°C for 12–18 h. Bacteria were washed from filter membrane and spotted on Muller-Hinton (MH) agar (BD Biosciences) plate containing 750 μg/ml rifampin and 200 μg/ml ampicillin for selection of bla_{NDM}-positive *E. coli* transconjugants.

To prepare competent cells for plasmid electroporation, 200 ml of overnight culture of *E. coli* DH10B in Super Optimal Broth (SOB) at an optical density (OD_{600}) of 0.4–0.6 was washed three times with electroporation buffer (0.5 M mannitol and 10% glycerol) and concentrated into a final volume of 2 ml. 1 μg of plasmid DNA, which was isolated from 3-SP with QIAGEN Plasmid Midi Kit, were mixed with 100 μl of competent cells for electroporation at 25 μF, 200 Ω, and 2.5 Kv. The resulting cells were suspended in 500 μl of SOB, and an appropriate aliquot was spotted on SOB agar plate containing 200 μg/ml ampicillin for selection of bla_{NDM}-positive *E. coli* electroporants.

S1-PFGE and Southern Blot

Bacterial genomic DNA was prepared in agarose plugs and digested with S1 nuclease (Takara). The linearized plasmids and partially digested genomic DNA were separated through the CHEF-Mapper XA PFGE system (Bio-Rad). The DNA fragments were stained with ethidium bromide (EtBr), transferred to a Hybond N^+ membrane (GE Amersham Biosciences) and hybridized with a DIG-labeled probe specific to bla_{NDM} (Rasheed et al., 2013). Probe labeling and signal detection were carried out with DIG high primer DNA labeling and detection starter kit II according to the manufacturer's instructions (Roche Diagnostics).

Detection of Carbapenemase Activity

Activity of class A/B/D carbapenemases was determined by CarbaNP test (Dortet et al., 2012) with modifications. Overnight bacterial cell culture in MH broth was diluted 1:100 into 3 ml of fresh MH broth, and bacteria were allowed to grow at 37°C with shaking at 200 rpm to reach an OD_{600} of 1.0–1.4. If required, ampicillin was used at 200 μg/ml. Bacterial cells were harvested from 2 ml of the above culture, and washed twice with 20 mM Tris-HCl (pH 7.8). Cell pellets were resuspended in 500 μl of 20 mM Tris-HCl (pH 7.8), and lysed by soniation, followed by centrifugation at 10,000 ×g at 4 °C for 5 min. 50 μl of the supernatant (the enzymatic bacterial suspension) were mixed with 50 μl of substrate I to V, respectively, followed by incubation at 37°C for a maximum of 2 h. Substrate I: 0.054% phenol red plus 0.1 mM $ZnSO_4$ (pH 7.8). Substrate II: 0.054% phenol red plus 0.1 mM $ZnSO_4$ (pH 7.8), and 0.6 mg/μl imipenem. Substrate III: 0.054% phenol red plus 0.1 mM $ZnSO_4$ (pH 7.8), 0.6 mg/μl mg imipenem, and 0.8 mg/μl tazobactam. Substrate IV: 0.054% phenol red plus 0.1 mM $ZnSO_4$ (pH 7.8), 0.6 mg/μl mg imipenem, and 3 mM EDTA (pH 7.8). Substrate V: 0.054% phenol red plus 0.1 mM $ZnSO_4$ (pH 7.8), 0.6 mg/μl mg imipenem, 0.8 mg/μl tazobactam, and 3 mM EDTA (pH 7.8).

Determination of Minimum Inhibitory Concentration (MIC)

The MIC values of indicated bacterial strains were tested by using VITEK 2 according to manufacturer's instructions, and antimicrobial susceptibility was judged by Clinical and Laboratory Standards Institute (CLSI) standard.

Determination of Plasmid DNA Sequence

The chromosome DNA-free plasmid DNA was isolated from the cell cultures of indicated *E. coli* transconjugant using a Qiagen large construct kit, and then sequenced by using whole-genome shotgun strategy in combination with Illumina HiSeq 2500 sequencing technology. The contigs were assembled with Velvet, and the gaps were filled through combinatorial PCR and Sanger Sequencing on ABI 3730 Sequencer. The genes were predicted with GeneMarkS and further annotated by BLASTP against UniPort and NR databases.

Nucleotide Sequence Accession Numbers

The complete sequence of plasmid p3SP-NDM was submitted to GenBank under accession number KP900015.

Results

Carbapenem-nonsusceptible *E. aerogenes* 3-SP

In June 2012, an 86-year-old male with cough and fever visited a teaching hospital in Xi'an city of China. The patient had underlying sequelae of cerebral hemorrhage, and complained of recurrent pulmonary infection. The patient received oral administration with cefradine for a week, but his symptoms did not improve. The patient was subsequently hospitalized, and chest X-ray examination confirmed presence of bilateral pulmonary infection and he was accordingly diagnosed to have pneumonia. The sputum specimens were sampled on the same day of admission. On the next day, round bacterial colonies were observed after cultivation of sputum on MH agar, and the bacterial isolate designated 3-SP was identified as *E. aerogenes* by VITEK 2, Bruker MALDI Biotyper, and 16s rRNA gene sequencing. The antimicrobial susceptibility test using VITEK 2 indicated 3-SP was resistant to multiple β-lactam antibiotics including imipenem and meropenem but remained susceptible to fluoroquinolones. The patient accordingly received intravenous administration with levofloxacin, and his symptoms associated with pulmonary infection disappeared and he was discharged after 10 days of antimicrobial treatment.

NDM-producing Plasmid p3SP-NDM

PCR detection of the major ESBL and carbapenemase genes (Table S1) indicated presence of only bla_{NDM} in *E. aerogenes* 3-SP (**Figure S1**), which was confirmed by PCR amplicon sequencing. A bla_{NDM}-positive *E. coli* EC600 transconjugant named 3-SP-NDM-EC600 and a bla_{NDM}-positive *E. coli* DH10B electroporant designated 3-SP-NDM-DH10B were obtained. The S1-PFGE/southern hybridization assay detected a ∼48 kb plasmid in each of 3-SP, 3-SP-NDM-EC600 and 3-SP-NDM-DH10B, which could hybridize with a bla_{NDM}-specific probe (**Figure S2**). The modified CarbaNP test showed that 3-SP, 3-SP-NDM-EC600, and 3-SP-NDM-DH10B had Ambler class B carbapenemase activity (**Figure S3**). The antibiotic susceptibility test showed that 3-SP, 3-SP-NDM-EC600, and 3-SP-NDM-DH10B were highly resistant to all the penicillin, β-lactamase, monobactam, cephalosporin, and carbapenem drugs tested, but remained to be susceptible to fluoroquinolones, furanes, aminoglycosides, and sulfanilamide tested (**Table 1**). The above results indicated that 3-SP contained a conjugative NDM-encoding plasmid (designated p3SP-NDM), which accounted for carbapenem resistance of 3-SP and could be transferred into and mobilized in *E. coli* recipients.

TABLE 1 | MIC values and antimicrobial susceptibility.

Category	Antibiotics	MIC (μg/ml)/antimicrobial susceptibility				
		3-SP	3-SP-NDM-EC600	3-SP-NDM-DH10B	EC600	DH10B
Penicillin	Ampicillin	>=32/R	>=32/R	>=32/R	16/I	<=2/S
	Ampicillin/sulbactam	>=32/R	>=32/R	>=32/R	8/S	<=2/S
	Piperacillin	>=128/R	>=128/R	>=128/R	<=4/S	<=4/S
	Piperacillin/tazobactam	>=128/R	64/R	64/R	<=4/S	<=4/S
Monobactam	Aztreonam	>=64/R	>=64/R	>=64/R	<=1/S	<=1/S
Cephalosporin	Cefazolin	>=64/R	>=64/R	>=64/R	<=4/S	<=4/S
	Cefuroxime sodium	>=64/R	>=64/R	>=64/R	16/I	4/S
	Cefuroxime axetil	>=64/R	>=64/R	>=64/R	16/I	4/S
	Cefotetan	>=64/R	>=64/R	32/R	<=4/S	<=4/S
	Ceftriaxone	>=64/R	>=64/R	>=64/R	<=1/S	<=1/S
	Ceftazidime	>=64/R	>=64/R	>=64/R	<=1/S	<=1/S
Carbapenem	Imipenem	8/R	>=16/R	>=16/R	<=1/S	<=1/S
	Meropenem	8/R	4/R	8/R	<=0.25/S	<=0.25/S
Fluoroquinolone	Ciprofloxacin	2/I	<=0.25/S	<=0.25/S	<=0.25/S	<=0.25/S
	Levofloxacin	2/S	0.5/S	<=0.25/S	1/S	<=0.25/S
Furane	Macrodantin	64/I	<=16/S	<=16/S	<=16/S	<=16/S
Aminoglycoside	Amikacin	<=2/S	<=2/S	<=2/S	<=2/S	<=2/S
	Gentamicin	<=1/S	<=1/S	<=1/S	<=1/S	<=1/S
	Tobramycin	<=1/S	<=1/S	<=1/S	<=1/S	<=1/S
Sulfanilamide	Trimethoprim/sulfamethoxazole	<=20/S	<=20/S	<=20/S	<=20/S	<=20/S

S, sensitive; R, resistant; I, Intermediate.

Plasmid DNA was isolated from 3-SP-NDM-EC600, and the whole genome sequence of p3SP-NDM was determined to 46,570 bp in length with a 137 fold coverage, forming a circular DNA sequence with a total of 45 open reading frames annotated (**Figure 1**).

Comparative Genomics of pNDM-BJ01-like Plasmids

p3SP-NDM is highly similar to pNDM-BJ01 with genetic differences including only 18 single nucleotide polymorphisms and an 1 bp deletion (Table S2) and a 706 bp deletion (**Figure S3**, see also below).

Linear structural comparison (**Figure 2**) was performed with whole genome sequences of p3SP-NDM, pNDM-BJ01 and all the six additional pNDM-BJ01-like plasmids pNDM-BJ02 (Hu et al., 2012), pNDM-40-1 (Jones et al., 2014), pNDM-AB (Zhang et al., 2013), pNDM-Iz4b (KJ547696), pAbNDM-1 (JN377410), and pM131_NDM1 (JX072963) (collected from GenBank on November 20, 2014). p3SP-NDM and pNDM-Iz4b essentially had the same genomic organization.

The above eight plasmids contain a highly conserved backbone composed of two separate regions of plasmid replication/transfer and one region of type VI section system, with only one structural polymorphism that a 706 bp fragment (nucleotide position 43,861–44,566 in pNDM-BJ01; located within the plasmid replication/transfer region and contains only one annotated gene encoding hypothetical protein) is deleted from p3SP-NDM and pNDM-Iz4b relative to all the other plasmids (**Figure 2**).

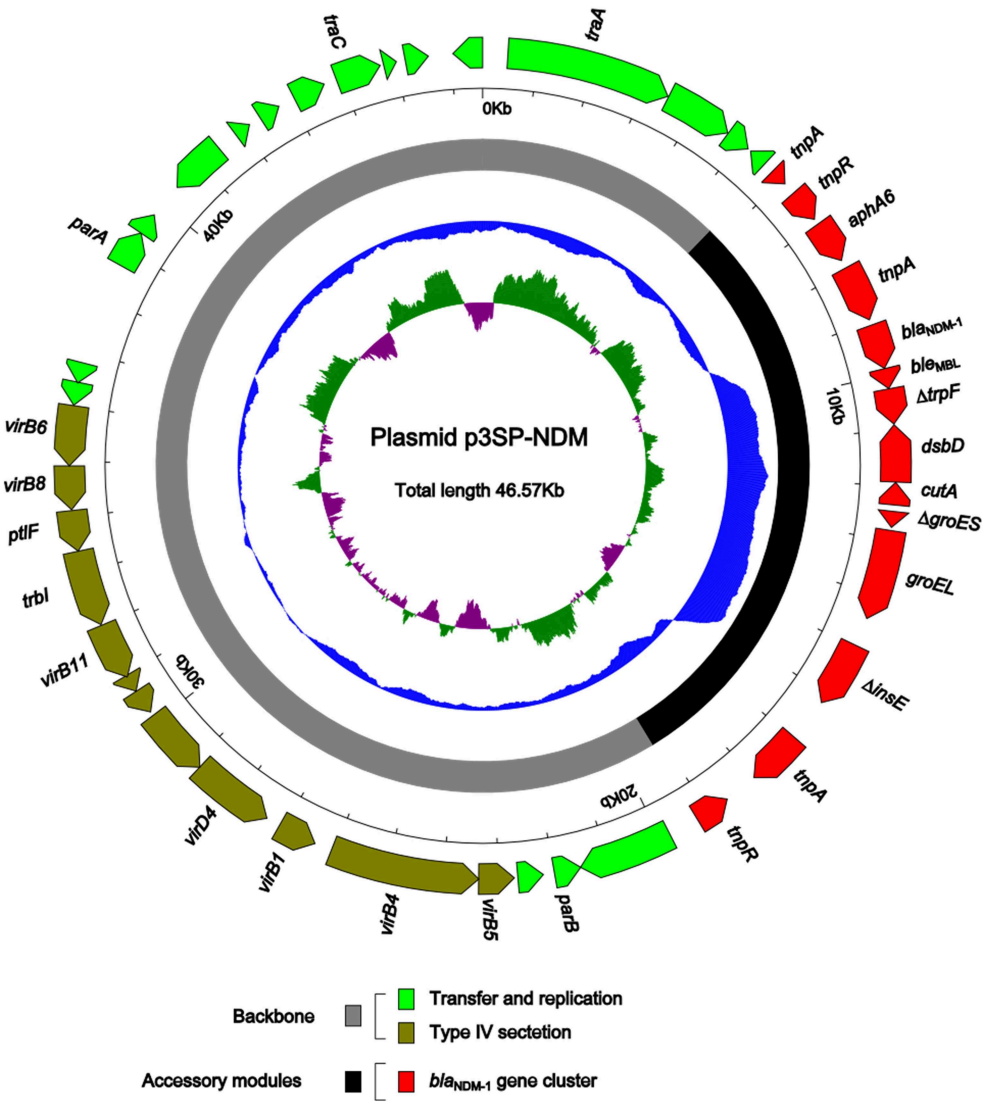

FIGURE 1 | Schematic map of p3SP-NDM. Genes are denoted by arrows and colored based on gene function classification. The innermost circle presents GC-Skew [(G − C)/(G + C)] with a window size of 500 bp and a step size of 20 bp. The blue circle presents GC content. Shown also are backbone and accessory module regions. All the gene organization figures in this work were drawn by using the Inkscape software (https://inkscape.org/).

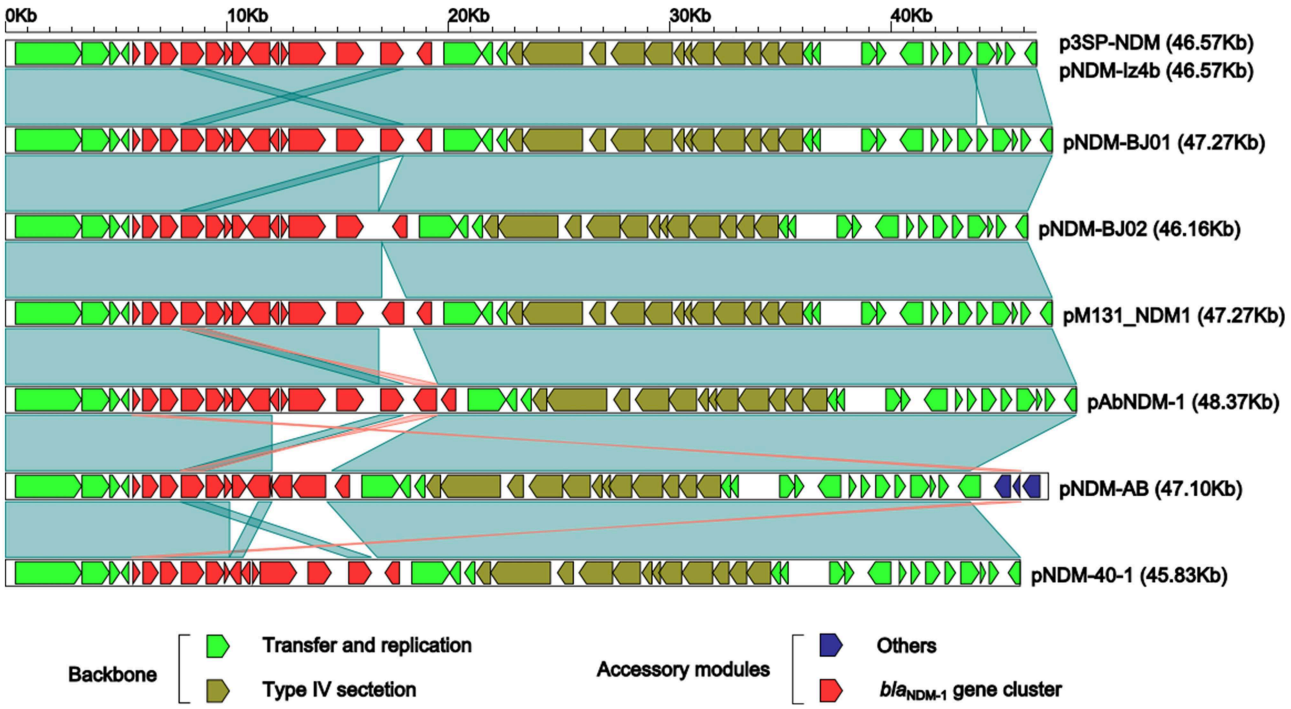

FIGURE 2 | Linear comparison of sequenced plasmids. Genes are denoted by arrows and colored based on gene function classification. Dark green shading regions denote regions of homology (>99% nucleotide similarity).

As for accessory modules (**Figure 2**), each of these eight plasmids contains a bla_{NDM-1} gene cluster located around nucleotide position 5685; in addition, pNDM-AB harbors an additional 3.5 Kb accessory region, which is located around nucleotide position 5570 and composed of an IS*Aba14* element and a gene encoding type I restriction-modification system methyl transferase subunit.

The bla_{NDM-1} gene clusters from the above eight plasmids show a conserved linear organization IS*Aba14-aphA6*-Tn*125*-unknown IS, and the IS*Aba14-aphA6* and unknown IS fragments are essentially identical structurally in these plasmids while structural differences occur within or immediately downstream of the composite transposon Tn*125* (**Figure 3**). pNDM-BJ01 and p3SP-NDM contain the prototype Tn*125*, which is sequentially organized as IS*Aba125*, bla_{NDM-1}, ble_{MBL} (bleomycin resistance), Δ*trpF*, *dsbC*, *cutA*, Δ*groES*, *groEL*, *ISCR27*, and IS*Aba125* (**Figure 3**); Tn*125* is inserted into a site downstream of *aphA6* (aminoglycoside resistance), which is evidenced by presence of GTT direct repeats at both ends, and the two copies of IS*Aba125* likely target bla_{NDM-1} surrounding sequences to promote formation and transposition of Tn*125* (Poirel et al., 2012).

Compared to the counterpart in pNDM-BJ01/p3SP-NDM, Tn*125* in pNDM-40-1 (**Figure 3**) is a truncated version with two deletions: a 1298 bp deletion within original ble_{MBL}-Δ*trpF-dsbC* to generate Δble_{MBL}-Δ*dsbC*, and a 150 bp deletion within IS*CR27* (Jones et al., 2014). A third copy of IS*Aba125* or an intact IS*Aba11* element is inserted immediately downstream of the intact Tn*125* of pAbNDM-1 or pM131_NDM1, respectively, while the downstream copy of IS*Aba125* is deleted from Tn*125* of pNDM-BJ02 (**Figure 3**). As for Tn*125* in pNDM-AB, *cutA*-Δ*groES-groEL-ISCR27* observed in pNDM-BJ01 is replaced by Δ*cutA-mph*(E)- *msr*(E), leading to absence of downstream GTT direct repeat (Zhang et al., 2013); by contrast, GTT direct repeats are intact in all other seven plasmids (**Figure 3**). The *mph*(E) and *msr*(E) genes confer macrolide/triamilide resistance (Michael et al., 2012).

Discussion

NDM, initially identified in *Klebsiella pneumoniae* in 2009, is a metallo-β-lactamase (MBL) capable of hydrolyzing almost all clinically used β-lactams (Tiwari and Moganty, 2013), and the bla_{NDM} genes have been found in a large collection of Gram-negative bacteria of clinical, environmental and animal origins, especially including *Acinetobacter, Enterobacteriaceae*, and *Pseudomonas* (Nordmann et al., 2011; Johnson and Woodford, 2013; Dortet et al., 2014). Fourteen NDM variants have been described, differing by several amino acid changes, and a few of them have been tested for their enzymatic kinetics, which denotes that amino acid substitution is a major source of MBL activity extension (Nordmann et al., 2012; Tada et al., 2013). Nevertheless, a systematic characterization of enzymatic kinetics of all the identified NDM variants is needed.

Intact IS*Aba125* has never been found in bacterial species other than *Acinetobacter*, and thus IS*Aba125* ought to originate from *Acinetobacter*. bla_{NDM-1} is most likely generated in an

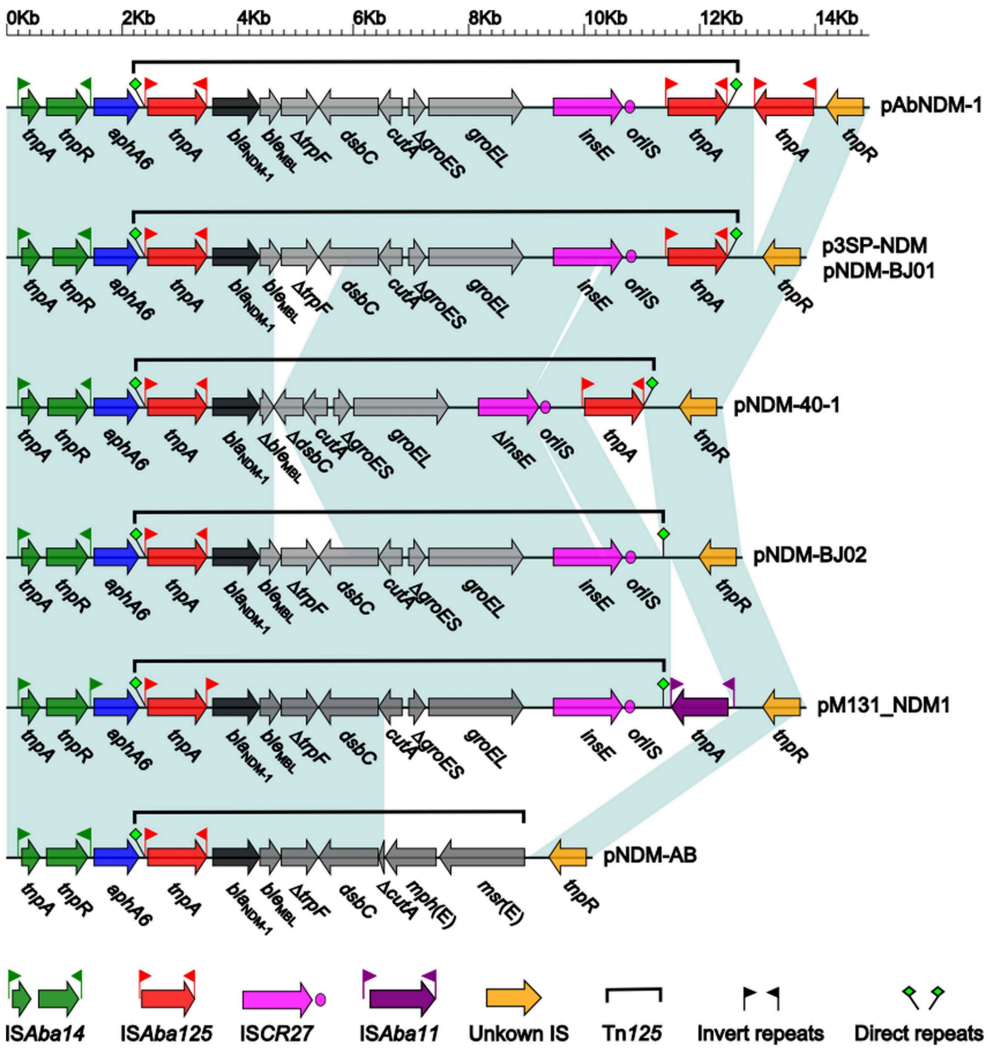

FIGURE 3 | Linear comparison of *bla*$_{NDM-1}$ gene clusters. Genes are denoted by arrows and colored. Dark green shading regions denote regions of homology (>99% nucleotide similarity).

Acinetobacter background by a fusion event between *aphA6* and an ancestral metallo-β-lactamase gene (Poirel et al., 2012; Toleman et al., 2012; Zong and Zhang, 2013). Insertion of various derivates of *bla*$_{NDM-1}$-carrying Tn*125* have been found within *Acinetobacter* chromosomes (Pfeifer et al., 2011; Bonnin et al., 2012; Partridge and Iredell, 2012; Poirel et al., 2012) and plasmids (Hu et al., 2012; Partridge and Iredell, 2012; Zhang et al., 2013; Zong and Zhang, 2013; Jones et al., 2014) at different locations, and moreover Tn*125* derivates also represent plasmid-borne *bla*$_{NDM-1}$ contexts in *Enterobacteriaceae* (Sekizuka et al., 2011; Mcgann et al., 2012; Partridge and Iredell, 2012; Fiett et al., 2014; Mataseje et al., 2014). These indicate emergency of *bla*$_{NDM-1}$in *Acinetobacter* and then dissemination among *Enterobacteriaceae*. In addition, the upstream copy of IS*Aba125*, either intact or interrupted by other mobile elements, of Tn*125* provides *bla*$_{NDM}$ with a strong promoter to drive high-level production of NDM enzymes (Poirel et al., 2011; Toleman et al., 2012).

At the time of writing this paper, there are at least eight additional pNDM-BJ01-like plasmids have been deposited in GenBank. All the above plasmids are exclusively found in *Acinetobacter* species including *A. lwoffii*, *A. baumannii*, *A. ereziniae*, *A. pittii,* and an unidentified *Acinetobacter* species from China, India, and Pakistan. This is the first report of identification of a pNDM-BJ01-like plasmid in *Enterobacteriaceae*, indicating spread of pNDM-BJ01-like plasmids from *Acinetobacter* to *Enterobacteriaceae*.

There is only one preliminary report describing detection of *bla*$_{NDM}$ in *E. aerogenes*, and this strain harbors a ∼50 Kb *bla*$_{NDM-1}$-encoding plasmid and is recovered from the stool sample of a 1-year-old infant with cough and intermittent fever in Hunan Province of China (Ho et al., 2012). This work presents extended evidence that NDM-1 is produced by a conjugative 46.57 Kb plasmid p3SP-NDM, and accounts for carbapenem resistance of clinical *E. aerogenes*; phenotypic and biochemical

experiments combined with plasmid sequencing and comparative genomics analyses give a deeper understanding of antibiotic resistance mechanism of this NDM-1-producing *E. aerogenes* strain.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00294/abstract

Figure S1 | PCR detection of *bla* genes. The major ESBL and carbapenemase genes (Table S1) were screened by PCR with strains 3-SP, 3-SP-NDM-EC600, 3-SP-NDM-DH10B, ATCC BAA-2146 (a NDM-1-producing reference strain of *K. pneumoniae*, Rasheed et al., 2013), EC600, and DH10B. Of all the *bla* genes detected, only *bla*$_{NDM}$ was shown to be present in each of 3-SP, 3-SP-NDM-EC600, and 3-SP-NDM-DH10B.

Figure S2 | S1-PFGE/Southern blot. The S1-digested genomic DNA samples was analyzed on an EtBr-stained PFGE gel **(A)**, and then subjected to Southern blot hybridization with a DIG-labeled probe specific to *bla*$_{NDM-1}$ **(B)**.

Figure S3 | Detection of carbapenemase activity. In the presence of any carbapenemase, relevant carbapenems are hydrolyzed and transformed into its carboxylic form, thus leading to a pH decrease which is detected by a color change of phenol red solution (red to yellow–orange). Ambler class A carbapenemases are, at least partially, inhibited by tazobactam, whereas class B carbapenemases (metallo-ß-lactamases) are inhibited by divalent cation chelators such as EDTA. There is no available chemical inhibitor for class D carbapenemases. In this study, the *bla*$_{NDM}$-psitive strains 3-SP, 3-SP-NDM-EC600, 3-SP-NDM-DH10B, and ATCC BAA-2146 had class B carbapenemase activity. As expected, *E. coli* EC600 and DH10B had no carbapenemase activity.

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Occurrence of classes I and II integrons in *Enterobacteriaceae* collected from Zagazig University Hospitals, Egypt

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Integrons are genetic units characterized by the ability to capture and incorporate gene cassettes, thus can contribute to the emergence and transfer of antibiotic resistance. The objectives of this study were: (1) to investigate the presence and distribution of class I and class II integrons and the characteristics of the gene cassettes they carry in *Enterobacteriaceae* isolated from nosocomial infections at Zagzig University Hospital in Egypt, (2) to determine their impact on resistance, and (3) to identify risk factors for the existence of integrons. Relevant samples and full clinical history were collected from 118 inpatients. Samples were processed; isolated microbes were identified and tested for antibiotic susceptibilities. Integrons were detected by polymerase chain reaction (PCR) and were characterized into class I or II by restriction fragment length polymorphism (RFLP). Integron-positive isolates were subjected to another PCR to detect gene cassette, followed by gene cassette sequencing. Risk factors were analyzed by logistic regression analysis. Seventy-six *Enterobacteriaceae* isolates were recognized, 41 of them (53.9%) were integron-positive; 39 strains carried class I and 2 strains carried class II integrons. Integrons had gene cassettes encoding different combinations and types of resistance determinants. Interestingly, *blaOXA129* gene was found and *ereA* gene was carried on class I integrons. The same determinants were carried within isolates of the same species as well as isolates of different species. The presence of integrons was significantly associated with multidrug resistance (MDR). No risk factors were associated for integron carriage. We conclude that integrons carrying gene cassettes encoding antibiotic resistance are significantly present among *Enterobacteriaceae* causing nosocomial infection in our hospital. Risk factors for acquisition remain to be identified.

Keywords: integron, *Enterobacteriaceae*, MDR, risk factors, sequencing

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Introduction

The widespread use of antibiotics with the intra- and inter-species transfer of resistant determinants mediated by plasmids, transposons and gene cassettes in integrons have contributed to the rapid transmission of drug resistance in bacterial pathogens, especially among members of the *Enterobacteriaceae* family (Cergole-Novella et al., 2011). Integrons are mobile DNA elements capable of detention and excision of genes, particularly those responsible for antibiotic resistance. Integrons achieve this by site-specific recombination (Hall and Collis, 1995). The different combinations of gene cassettes can contribute to the diverse genetic organization of integrons. There are five different classes of integrons, each encoding a distinct integrase gene (Mazel, 2006). Class I integrons are the most common type present in clinical isolates of the *Enterobacteriaceae* (DeLappe et al., 2003). Class II integrons are associated with the Tn7 transposon, whose transposition activity is directed at specific attachment sites on chromosomes or plasmids (Rodríguez-Minguela et al., 2009). Although the class II integrons share their cassettes pool with the class I integrons, they are distinguished by divergent integrase sequences (Gillings, 2014). Class I integron possess two conserved segments (5′-CS) and (3′-CS) separated by a variable region including the gene cassettes integrated with antibiotic resistant genes. The 5′-CS consists of (*intI*) gene codes for integrase, adjacent recombination site (*att1*) recognized by the integrase and acts as a receptor for gene cassettes, and the promoter (P) which controls the transcription of integrated resistance markers, as these genes do not have their own promoters (Mazel, 2006). The 3′-CS usually includes truncated *qacE* (*qacED1*) and *sul1* genes that confer resistance to quaternary ammonium compounds and sulfonamides, respectively (Paulsen et al., 1993). Gene cassettes typically comprise of a recombination site (attC) and a single-promoter-less gene, most of which encode antibiotic resistance factors (Partridge et al., 2009). Class I integron has been identified as the primary source of antimicrobial resistance genes and are suspected to serve as reservoirs and exchange platforms of resistant genes in a variety of Gram-negative bacteria (Ke et al., 2011).

During the last months, there were complaints from clinicians about the emergence and dissemination of MDR in the intensive care unit (ICU), the orthopedic unit, the neonatology unit and the chest unit. This resistance causes treatment failure, morbidity and sometimes mortality. Preliminary investigations pointed out the possibility of integrons (among other mechanisms of resistance). To the best of our knowledge, little is known about the integrons and their associated gene cassettes in *Enterobacteriaceae* isolates in our hospital specifically, in Egypt generally. In order to provide helpful base-line information for further comparison with follow up studies, we thus conducted the current work. The objectives were: (1) to investigate the presence and distribution of class I and class II integrons and the characteristics of the gene cassettes they carry in *Enterobacteriaceae* isolated from nosocomial infection cases at Zagzig University Hospital, (2) to determine their impact on resistance, and (3) to identify risk factors of the existence of integrons.

Materials and Methods

Subjects

Over a period of 23 months (May 2012–March 2014), 118 in-patients were enrolled from different clinical departments at Zagazig University Hospital. Patients were included only if they are suspected to have hospital-acquired infection (Horan et al., 2008) and the laboratory results reported Gram-negative bacilli from the infection sites. Clinical units selected were those reported to the Infection Control Unit as having increased incidence of infections caused by resistant-*Enterobacteriaceae*. The demographic and clinical data was collected from all patients.

Ethical Consideration

An informed written consent was taken from each individual after explaining the nature of investigation as well as the purpose of the study in accordance with the ethical standards of the responsible Regional Committee. Participants' data is confidential. Institutional approval was obtained from the Institutional Review Board (IRB) committee.

Study Design

Observational cross sectional study.

Clinical Samples

Clinical samples were collected by systematic random sampling. The origin and type of samples are shown in **Table 1**. Samples were collected and processed using standard Microbiologic

TABLE 1 | Origin and type of the strains included in the study.

Origin	No. and types of samples					Enterobactericae isolates	
	Endotracheal aspiration	Urine	Swabs from pus	Blood	Total		
ICU	8	21	11	12	52	42	(80.8%)
Orthopedic unit	–	9	4	–	13	8	(61.5%)
Neonatology unit	–	–	–	10	10	6	(60%)
Chest unit	5	4	4	2	15	8	(53.3%)
Surgery unit	–	17	11	–	28	12	(42.9%)
Total	13	51	30	24	118	76	(64.4%)

procedures (Collee and Marr, 1996; Raul and Melvin, 2001; Cheesbrough, 2004; Forbes et al., 2007); isolated microbes were identified by colonial characteristics, Gram stain and conventional biochemical tests and confirmed by the API 20E Identification System (bioMerieux, France).

Antibiotic Susceptibility

Antimicrobial susceptibility profiles of the isolates were determined using the disk diffusion method according to the standard procedures of the Clinical and Laboratory Standards Institute (CLSI, 2013). *Escherichia coli* (ATCC25922) (Microbiologics, USA) was used as the quality control strain. The following 17 antibiotic discs (Oxoid) were used: amikacin, amoxicillin+clavulanic acid, ampicillin+sulbactam, azithromycin, cefepime, cefixime, cefotaxime, cefoxitin, cefperazone, ceftazidime, ceftriaxone, chloramphenicol, gentamycin, imipenem, levofloxacin, meropenem, and trimethoprim-sulphamethexazole. MDR was defined according to the guidelines of the European Society of Clinical Microbiology and Infectious Diseases (Magiorakos et al., 2012).

Molecular Characterization of Isolated *Enterobacteriaceae*

Integrons Detection

DNA was extracted from overnight cultures grown on MacConkey's agar, using the QIAamp DNA Mini Kit (QIAGEN, USA), according to the manufacturer's instructions. The DNA was quantified following the recommendations of Surzycki (2000).

PCR Detection of Integrons

Integrons were detected by PCR with the degenerate primers designed to hybridize conserved regions of encoded integrase genes *intI1*, *intI2*, hep35 (5′-TGCGGGTYAARGATBTKGATTT-3′), and hep36 (5′-CARCACATGCGTRTARAT-3′) give a PCR product of 491 bp (White et al., 2001).

RFLP for Differentiation of Class I and Class II Integrons

Digestion of PCR Product

Using *Rsa I* restriction enzyme (White et al., 2001), PCR products were subjected to digestion with *Rsa I* as follow, 10 μl of the amplified gene segment, 2 μl of the l0x buffer supplied, 1 μl of the enzyme and complete reaction with sterile water to attain the final volume of 20 μl, which was incubated for 3 h at 37°C. Then, the total volume was loaded on the agarose gel. After digestion, integrase I gave rise to one fragment of 491 bp, while integrase II gave rise to two fragments of 334 and 157 bp, respectively.

Amplification of Gene Cassettes of Class I and Class II Integrons

Class I integron cassette structures were amplified using hep58 (5′-TCATGGCTTGTTATGACTGT3-3′) and hep59 (5′-GTAGGGCTTATTATGCACGC-3′) which bind 3′-CS and 5′-CS conserved segments, respectively. Class II integron cassette regions were amplified using hep74 (5′-CGGGATCCCGGACGGCATGCACGATTTGTA-3′), which binds to *attI2* and hep51 (5′-GATGCCATCGCAAGTACGAG-3′), which binds to *orfX* situated downstream of the cassette region within Tn*7* (GenBank accession number AJ002782) (White et al., 2001). PCR was performed for 30 cycles, each cycle consisted of 94°C for 30 s, 55°C for 30 s and extension at 72°C for 45 s for amplification of the integrase genes, or 4 min for amplification of the cassette region. Amplification cycles were performed with DNA thermal cycler (Biometra, Germany), as mentioned elsewhere (White et al., 2000). For each batch of PCR reactions, a positive and negative control was included. Positive control was an isolate confirmed as integrase positive by DNA sequencing. PCR products were analyzed in parallel with a DNA MW-marker (Fermentas) by electrophoresis on 2% agarose gel.

DNA Sequencing

Sequencing reactions were performed through using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). Each reaction mixture contained 8 μl of the BigDye Terminator ready reaction mixture and 3.2 pmol of primer in a 20 μl reaction mixture. The PCR products of inserted gene cassettes within class I intgrons were sequenced using primers hep58, 5′-TCATGGCTTGTTATGACTGT-3′ and hep59, 5′-GTAGGGCTTATTATGCACGC-3′. The PCR program for all sequencing reactions included initial denaturation at 96°C for 1 min, followed by 25 cycles of denaturation for 30 s at 96°C, primer annealing for 5 s at 55°C, and extension for 4 min at 60°C according to the BigDye Terminator v3.1 Cycle Sequencing Kit Protocol Manual. The resulting sequences were identified by partial nucleotide sequencing and compared with the sequences in the GenBank database of the National Center for Biotechnology Information via the BLAST network service (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Li et al., 2013). MEGABLAST model of BLAST program was used. The best Blast Hits on our Query nucleotide sequence was selected based on the highest identity in the gene bank database.

Statistical Analysis

Collected data were computerized and statistically analyzed using SPSS program (Statistical Package for Social Science) version 18.0. Qualitative data were represented as frequencies and relative percentages. Chi square test was used to calculate difference between qualitative variables. Quantitative data were expressed as mean ± SD (Standard deviation). Multivariate logistic regression analysis was used to illuminate the interrelation within and between significant predictors for specific variable. The level of significance for all statistical tests was determined. The threshold of significance is fixed at 5% level (*P*-value); $P > 0.05$ indicates non-significant results, *P*-value of <0.05 indicates significant results.

Results

Seventy-six *Enterobacteriaceae* isolates were studied. They were recovered from different clinical samples collected from 118 in-patients admitted to different departments at Zagazig University Hospitals (**Table 1**). The highest rate of isolating *Enterobacteriaceae* was from ICU (42 out of 76).

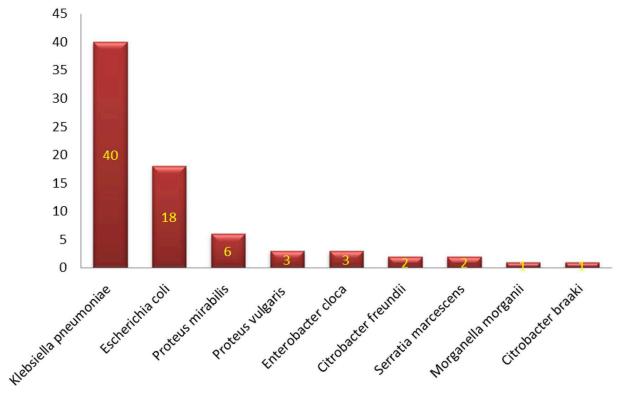

FIGURE 1 | Distribution of different species of Enterobacteriacae.

TABLE 2 | Detection of class I and class II integrons by PCR–RFLP.

***Enterobacteriaceae* isolates**		**Class I**	**Class II**	**Total**
Organism	**No.**	**No. (%)**	**No. (%)**	**No. (%)**
Klebsiella pneumonia	40	17 (42.5)	1 (2.5)	18 (45)
Escherichia coli	18	14 (77.8)	1 (5.5)	15 (83.3)
Enterobacter cloaca	3	1 (33.3)		2 (66.6)
Citrobacter freundii	2	1 (50)		1 (50)
Serratia marcescens	2	1 (50)		1 (50)
Morganella morgani	1	0 (0.0)		0 (0.0)
Proteus mirabialis	6	3 (50)		3 (50)
Proteus vulgaris	3	1 (33.3)		1 (33.3)
Citrobacter breakie	1	0 (0.0)		0 (0.0)
Total	76	39 (51.32)	2 (2.6)	41 (53.9)

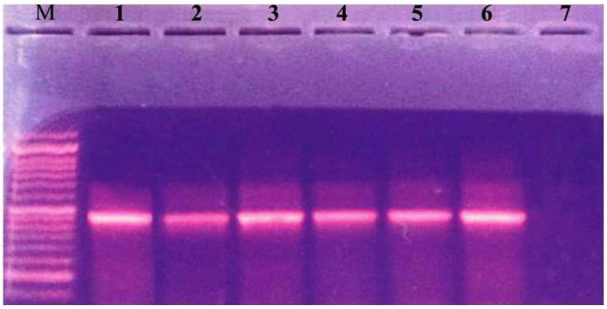

FIGURE 2 | Ethidium bromide-stained agrose gel showing +ve isolates for integrase gene. Lane M: molecular size marker (100–1000). Lane 1: positive control. Lanes 2–6: 491 (bp PCR product from positive strains). Lane 7: negative control.

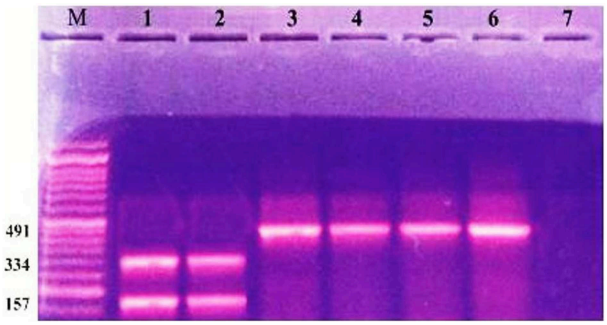

FIGURE 3 | Ethidium bromide stained agrose gel showing +ve class I and class II integron with RFLP by restriction enzyme digestion. Lane M: molecular size marker (100–1000). Lanes 1, 2: 2 segments (bands) 334, 157 bp, respectively. Lanes 3–6: 491 (bp PCR product class 1 positive strain). Lane 7: negative control.

Characterization of different species of *Enterobacteriaceae* is shown in **Figure 1**. *Klebsiella pneumoniae* was the most common organism isolated (50.6%). Integrons were identified in 41 (53.9%) out of the 76 strains studied (**Table 2**). Thirty-nine (39) carried class I integron and 2 strains carried class II integron **Figures 2**, **3**.

Sequencing of PCR product of gene cassettes carried on class I integron revealed different combination of gene cassettes encoding the different types of resistance determinants. Interestingly, *blaOXA*129 gene was found and *ereA* gene was detected on class I integrons. The same determinants were carried within isolates of the same species as well as isolates of different species (**Table 3**).

Isolates harboring integrons were more likely to be resistant to amikacin, cefepime, ceftazidime, ceftriaxone, chloramphenicol, gentamycin, levofloxacin, and trimethoprim-sulphamethexazole than those without integrons (**Table 4**). All *Enterobacteriaceae* isolates were sensitive to both imipenem and meropenem; therefore, carbapenems group was excluded from statistical analysis. Fifty-one isolates were found to be MD-resistant (**Table 5**). A significant statistical association was found between MDR and the presence of integrons (**Table 6**).

Preliminary analysis using Chi square test revealed admission to ICU, trauma and hospitalization more than 7 days to be significantly correlated with integron positivity (**Table 7**). However, logistic regression analysis revealed no significant association (**Table 8**).

Discussion

Members of the family *Enterobacteriaceae* are frequently identified as etiological agents of nosocomial infections (Holt et al., 1994; Obeng-Nkrumah et al., 2013). Hospitalized patients, especially those admitted to ICUs, are at high risk (Archibald et al., 1997; El-Bialy and Abu-Zeid, 2009; Elsharkawy et al., 2013). This could explain the highest rate of isolating *Enterobacteriaceae* from the ICU encountered in the current work (**Table 1**), which support findings of others (Obeng-Nkrumah et al., 2013). In our study *Klebsiella pneumoniae* was the most commonly isolated organism, followed by *Escherichia coli* (**Figure 1**). This agrees with Abdel-Hady et al. (2008), Obeng-Nkrumah et al. (2013) and Defife et al. (2009), but disagrees with Asencio et al. (2014). The differences can be due to variations in study population, sample

TABLE 3 | Occurrence and characterization of gene cassettes carried by of class I integrons.

Bacterial species	Gene cassette(s)	No.
K. pneumonia	*dfrA14-arr2-cmiA 5*	2
	bla$_{OXA-1}$-aac A6-dfrA 5	2
	dfrA5-aph 31a	2
	dfrA5-TEM_1	1
	OXA 129-dfrA5-aacA4	1
	dfrA	3
	aacC1-aadA1	1
	aadA1-dfrA1	3
	aadA2	2
	aadA-qac-e	2
E. coli	*aadB*	1
	aadA1-dfrA1	2
	AadA	3
	aac6 II-	2
	ere A2-dfr A5	2
	Not determined	2
P. mirabilis	*ere A2-dfr A5*	2
	Not determined	1
E. cloaca	*dfr A5*	1
	DfrA1-aadA2	1
S. marcescens	*blaP1-aadB*	1
C. freundii	*dfrA-aadA5-qac-e*	1
P. vulgaris	*OXA 129-dfrA5-accA4*	1

dfr, Dihydrofolate reductases; arr, rifampicin resistance; cmi, chloramphenicol resistance protein; OXA, oxasillinase; aac, aminoglycoside acetyltransferases; aph, 3-O-phosphotransferase; aad, aminoglycoside adenylyltransferases B; qac, quaternary ammonium compound; TEM, beta lactamase gene; bla P, beta lactamase gene.

TABLE 4 | Association between antibiotic resistance and integron positivity.

Antibiotic	Resistant		Integron +ve		*P*
	No.	(%)	No.	(%)	
Amikacin	22	31.5	18	81.8	0.0164
Amoxicillin+clavulanic	76	100	40	52.6	0.779
Ampicillin+sulbactam	66	86.8	35	35.03	0.7634
Azithromycin	64	84.2	35	54.7	0.646
Cefepime	47	61.8	33	70.3	0.0302
Cefixime	34	44.7	18	52.9	0.731
Cefotaxime	45	59.2	25	55.6	0.586
Cefoxitin	25	32.8	11	44.0	0.806
Cefperazone	51	67.1	36	70.6	0.169
Ceftazidime	44	57.8	29	65.9	0.0163
Ceftriaxone	53	69.7	39	73.6	0.006
Chloramphenicol	20	26.3	16	80.0	0.0293
Gentamycin	29	28.9	23	79.3	0.0108
Levofloxacin	11	14.4	10	90.9	0.0273
Trimethoprim-sulphamethexazole	48	63.1	39	81.3	0.001

Level of significance: $P < 0.05$ indicates significant results.

size, the presence of an epidemic as well as dissimilarities in antibiotic use.

The rate of integron-positive *Enterobacteriaceae* identified in this work is 53.9% compared to what was previously reported (White et al., 2001; Kor et al., 2013). We found the highest percentage of integrons in *Escherichia coli* (83.3%) which correlate with the report of Essen-Zandbergen et al. (2007), but not with Daikos et al. (2007) (**Table 2**). This could be attributed to diverse geographical distribution. The predominance of class I integrons among our isolates agree with many workers (Chang et al., 2000; White et al., 2001; Kor et al., 2013).

PCR product of cassette regions of class I integron were sequenced. The cassette regions of three class I integrons could not be amplified, possibly due to the lack of a 3′-conserved segment or due to presence of early stop codon (Ramírez et al., 2010). It is worth mentioning that the number and combination of genes found in gene cassettes of our isolate are different from that reported by many other researchers (White et al., 2001; Leverstein-van Hall et al., 2002; Kang et al., 2005), which prove the effect of geographic difference on integron distribution (Yu et al., 2004). Class II integons were excluded from sequencing analysis due to the very small sample size which limits the power of any conclusions (**Table 3**).

In this study, class I integrons harbored different cassette arrays conferring resistance to nearly every major class of antibiotics, with the remarkable exception of the quinolones (Daikos et al., 2007; Essen-Zandbergen et al., 2007). The most common types were those conferring resistance to trimethoprim antibiotic (*dfr*) a finding in agreement with Daikos et al. (2007) and Essen-Zandbergen et al. (2007). The high prevalence of *dfr* gene cassettes may be due to the wide use of trimethoprim as a first line therapy for the treatment of urinary tract infections, a clinical condition that is very common in both hospital and community settings. Another common types of cassette carried by class I integrons were those conferring resistance to streptomycin and spectinomycin (White et al., 2001). Aminoglycoside resistance genes (*aadB, aac,* and aph) encoding resistance to aminoglycosides other than streptomycin and spectinomycin (gentamicin, kanamycin, and amikacin) were found in 11 of our isolates. The selective pressure exerted by aminoglycosides intensively used in our hospitals might account for this finding (**Table 3**). Resistance to beta-lactam antibiotic was represented in this work by four cassettes (*bla OXA 129-OXA 1-TEM-blaP1*). To the best of our knowledge, *bla OXA 129* is detected for the first time in Egypt. Till date, only two reports are available on the presence of *OXA 129* in Gram-negative bacteria. The first was in 2008 from Brazil, from *Salmonella enterica* subsp. *enterica* serovar *Bredeney* porcine isolates (Michael et al., 2008), and the second was from China, in 2010 from *Pseudomonas aeruginosa* (Liu et al., 2010). The *blaOXA-1* gene was found in two of our isolates. All the genes for the *OXA-1*-like beta-lactamases were identified in the form of gene cassettes inserted into class I integrons (Naas and Nordmann, 1999; Aubert et al., 2001; Dubois et al., 2003; Poirel et al., 2004). Other cassettes detected were those for erythromycin (*ereA2*), and for rifampicin resistance. These two

TABLE 5 | Resistance profile of 51 MDR* isolate.

Bacterial isolates	Antimicrobial categories	MDR(51)	
		Integron+ve (33)	Integron −ve (18)
Klebsiella pneumoniae	Aminoglycosides, extended-spectrum cephalosporins; folate pathway inhibitors, penicillins+β-lactamase inhibitors, chloramphenicol, cephamycins	5	1
	Extended-spectrum cephalosporins; folate pathway inhibitors, penicillins+β-lactamase inhibitors, cephamycins	8	4
	Extended-spectrum cephalosporins; folate pathway inhibitors, penicillins+b-lactamase, quinolones, aminoglycosides	3	0
	Penicillins+b-lactamase inhibitors, chloramphenicol, cephamycins	1	1
	Extended-spectrum cephalosporins, penicillins+β-lactamase inhibitors, chloramphenicol, cephamycins	0	4
Escherichia coli	Penicillins+β-lactamase inhibitors, chloramphenicol, cephamycins	3	2
	Extended-spectrum cephalosporins; folate pathway inhibitors, penicillins+β-lactamase inhibitors, quinolones	2	1
	Aminoglycosides, extended-spectrum cephalosporins; folate pathway inhibitors, penicillins+β-lactamase inhibitors, Chloramphenicol, Cephamycins	2	1
Proteus mirabilis	Extended-spectrum cephalosporins; folate pathway inhibitors, penicillins+β-lactamase inhibitors	1	2
	Folate pathway inhibitors, penicillins+β-lactamase inhibitors, cephamycins	1	0
Proteus vulgaris	Aminoglycosides, extended-spectrum cephalosporins; folate pathway inhibitors, penicillins+β-lactamase inhibitors, chloramphenicol, cephamycins	1	0
	Aminoglycosides; folate pathway inhibitors, penicillins+β-lactamase inhibitors, chloramphenicol, cephamycins, quinolones	1	1
Serratia marcescens	Aminoglycosides, extended-spectrum cephalosporins; folate pathway inhibitors, penicillins+β-lactamase inhibitors, chloramphenicol, cephamycins	1	0
	Extended-spectrum cephalosporins; folate pathway inhibitors, penicillins+β-lactamase inhibitors, cephamycins, quinolones	1	0
Citrobacter freundii	Aminoglycosides, Extended-spectrum cephalosporins; folate pathway inhibitors, penicillins+β-lactamase inhibitors, cephamycins	1	0
Enterobacter cloaca	Folate pathway inhibitors, penicillins+β-lactamase inhibitors, cephamycins, quinolones, chloramphenicol	1	1
Morganella morgani	Aminoglycosides, Extended-spectrum cephalosporins; folate pathway inhibitors, penicillins+β-lactamase inhibitors, chloramphenicol cephamycins	1	0

**Categories used in this table to identify MDR are those recommended by the European Society of clinical Microbiology and Infectious Diseases (Magiorakos et al., 2012): 1, aminoglycosides: gentamicin, amikacin; 2, carbapenems: imipenem, meropenem; 3, extended-spectrum cephalosporins (third and fourth generation cephalosporins); cefotaxime or ceftriaxone, ceftazidime, cefepime; 4, cephamycins: cefoxitin; 5, quinolones: ciprofloxacin, levofloxacin; 6, folate pathway inhibitors: trimethoprim-sulphamethoxazole; 7, Penicillins + β-lactamase inhibitors: amoxicillin-clavulanic, ampicillin-sulbactam; 8, Phenicols: chloramphenicol.*

gene cassettes were identified relatively more recently than the other cassettes (Jones et al., 1997; Tribuddharat and Fennewald, 1999; Leverstein-van Hall et al., 2002). The *ereA* gene cassette carries its own promoter and is propagated by a class II integron (Biskri and Mazel, 2003). Recently, a cassette carrying a gene and showing about 90% identity with *ereA* was identified in a class I multi-resistant integron (MRI) (Chang et al., 2000; Peters et al., 2001; Thungapathra et al., 2002; Plante et al., 2003). In this study, the occurrence of *ereA* gene carried on class I integron on four isolates (**Table 3**) is reported in Egypt for the first time. Chloramphenicol resistance protein (exporter), (*cmiA5*), were detected in two isolates. This gene is responsible for a less prevalent non-enzymatic mechanism of chloramphenicol resistance observed principally in Gram-negative bacteria (Bissonnette et al., 1991). The extremely low occurrence of chloramphenicol resistance, through a less prevalent mechanism presented by gene cassettes of our isolates, can be explained by the fact that chloramphenicol is no more prescribed in hospitals for the fear of its complications. Cassette (*qac-e*) which encodes for quaternary ammonium compounds resistance was detected in three of our isolates. In *Enterobacteriaceae*, the *qac* genes have been most regularly found together with genes coding for resistance to chloramphenicol, aminoglycosides, beta-lactams, sulphonamides and trimethoprim (Poirel et al., 2000; Riaño et al., 2006; Espedido et al., 2008; Zhao et al., 2012). In our work, *qac-e* has been found together with genes encoding resistance to

TABLE 6 | Relation between integron presence and MDR among *Enterobactericae* isolates.

Integron	Antibiotic		χ^2	*P*-value
	MDR* No. (%)	Other isolates No. (%)		
Integron +ve (*N* = 41)	33 (64.7)	9 (36)	4.491	0.017*
Integron −ve (*N* = 35)	18 (35.3)	16 (64)		
Total	51 (100)	25 (100)		

*χ^2, Chi square test; *level of significans ($p < 0.05$).*

TABLE 7 | Association between risk factors and presence of integron (Chi square test).

Risk factor	No. of cases	Integron +ve		Integron −ve		*P*-value
		No	%	No	%	
Trauma	55	38	69.1	17	30.9	0.029*
Hospitalization more than 7 days	20	17	85	3	15	0.011*
ICU admission	34	29	85.3	5	14.7	0.002*
Ventilator	10	8	80	2	20	0.235
Urinary catheter	28	7	25	21	75	0.357
Central venous catheter	5	4	80	1	20	0.586

**Chi square test.*

TABLE 8 | Logistic regression analysis for significant predictors of the presence of integron among the studied groups.

Variable	*B*	S.E.	Wald	Sig.	OR
Trauma	22.02	7.26	0.02	0.98	3.64 (0.07–5.15)
Hospitalization more than 7 days	20.17	8.27	0.04	0.96	5.73 (1.02–7.45)
ICU admission	42.03	1.10	0.03	0.90	0.01 (0.00–0.06)

B, Regression coefficients; S.E., standard error around the coefficient for the constant; Wald, Wald chi-square test statistic; Sig. P-value for Wald test; OR, odds ratio.

aminoglycosides, and sulphonamides (**Table 3**). The correlation between the *qac* genes and macrolide inactivation genes was reported in *Aeromonas hydrophila* (Poole et al., 2006) and in microflora from a wastewater treatment unit (Szczepanowski et al., 2004). Hence, the use of several cationic biocides may also be accountable for the selection of bacteria resistant to antimicrobials (Russell, 2000).

It is worth mentioning that not only particular genes are shared across species but also some gene combinations. In the current work, *dfrA5* was present across *Klebsiella pneumoniae, Escherichia coli, Proteus mirabilis, Enterobacter cloaca,* and *Proteus vulgaris*. Gene combination, *aadA1-dfrA1*, was identified in *Klebsiella pneumonia* and *Escherichia coli*; the combination comprised of *ereA2-dfrA5* was detected in *Escherichia coli* and *Proteus mirabilis* (**Table 3**). We also observed the presence of more than one isolate of *Klebsiella pneumoniae, Escherichia coli* and *Proteus mirabilis* carrying a particular integron (**Table 3**). Since all these gene cassettes are carried by class I integron which contains integrase 1 (conserved sequence) (White et al., 2001), the transfer of the integrons by both intra- and inter-species is assumed. Integron spread may be achieved through the cross-transmission of integron-carrying clones from one patient to the other, an action known to be facilitated among *Enterobacteriaceae* in hospital settings (Leverstein-van Hall et al., 2002). This is particularly important in the background of poor compliance with infection control in our hospital.

The phenotypic resistance to a specific drug was observed in all isolates carrying the corresponding gene cassette; this coincides with Leverstein-van Hall et al. (2002); Barlow et al. (2004); Kor et al. (2013), and Li et al. (2013). However, it is also evident that integron-carrying organisms had reduced susceptibility not only to antimicrobial agents for which the respective gene cassettes were contained in but also to other classes of agents for which no or very little number of genes are contained within the integrons. In the current work, this applies to the third generation cephalosporins, chloramphenicol, and quinolones. Thus, we support the finding of previous investigators (White et al., 2001; Leverstein-van Hall et al., 2002; Essen-Zandbergen et al., 2007) regarding cephalosporins. Remarkably, not all the resistance profile of the isolates could be explained by the expression of the gene cassettes found within the integrons. Apparently a considerable number of antibiotic resistance genes are located outside the integrons either on chromosomes or plasmids.

More than half of our isolates were MD-resistant (**Tables 5**, **6**) which has also been concluded by other workers (Elsharkawy et al., 2013; Ali et al., 2014). Just as reported by Partridge et al. (2009), class I integrons are significantly associated with MDR.

Logistic regression analysis for risk factors of the presence of integron among the studied group revealed statistically non-significant results (**Table 8**), which correlate with findings of Daikos et al. (2007). It may be due to small sample size. Hence, further investigation on a larger sample size is recommended.

We conclude that integrons carrying gene cassettes encoding antibiotic resistance are significantly present among *Enterobacteriaceae* causing nosocomial infection in our hospital. We also report for the first time in Egypt the detection of *blaOXA129* and the carriage of *ereA* gene on class I integrons. These existences have important implications. Unless the use of antimicrobials in our hospital is rationalized, the emergence and the dissemination of these genes as well as other encoding resistance to more antibiotics will be evident in the future. Our study has renewed interest in the use of chloramphenicol since it is an effective and a cheap antimicrobial. In low-income countries, the use of chloramphenicol should be encouraged. The use of several cationic biocides should also be appropriate, since inappropriate usage may account for the selection of bacteria resistant to antimicrobials. A significant relationship was found

between integron and MD-resistant phenotype. It is to be emphasized that the continuing use of antibiotics will drive the numbers of MDR to swell. The risk factors of integron carriage need to be identified. Our study is providing the base-line information that can be helpful in further monitoring and for evaluation.

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Approaches to treatment of emerging Shiga toxin-producing *Escherichia coli* infections highlighting the O104:H4 serotype

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Shiga toxin-producing *Escherichia coli* (STEC) are a group of diarrheagenic bacteria associated with foodborne outbreaks. Infection with these agents may result in grave sequelae that include fatality. A large number of STEC serotypes has been identified to date. *E. coli* serotype O104:H4 is an emerging pathogen responsible for a 2011 outbreak in Europe that resulted in over 4000 infections and 50 deaths. STEC pathogenicity is highly reliant on the production of one or more Shiga toxins that can inhibit protein synthesis in host cells resulting in a cytotoxicity that may affect various organ systems. Antimicrobials are usually avoided in the treatment of STEC infections since they are believed to induce bacterial cell lysis and the release of stored toxins. Some antimicrobials have also been reported to enhance toxin synthesis and production from these organisms. Various groups have attempted alternative treatment approaches including the administration of toxin-directed antibodies, toxin-adsorbing polymers, probiotic agents and natural remedies. The utility of antibiotics in treating STEC infections has also been reconsidered in recent years with certain modalities showing promise.

Keywords: Shiga toxin-producing *Escherichia coli*, hemorrhagic colitis, hemolytic uremic syndrome, antimicrobial agents, Shiga toxin 1, Shiga toxin 2

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Shiga toxin-producing *Escherichia coli* (STEC) are a group of bacterial organisms that are capable of producing one or more types of Shiga toxin (Stx). STEC are associated with a disease spectrum ranging from diarrhea and hemorrhagic colitis (HC) to the potentially fatal hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). STEC infections are typically food-borne (Dupont, 2007) and the production of Shiga toxins (Stx1, Stx2 or a variant) is believed to be central to the pathogenesis of these organisms. STEC strains are the result of an insertion of one of a group of lysogenic lambdoid bacteriophages that harbor an Stx1/2-encoding gene into the *E. coli* genome. The clinical syndromes, pathogenic characteristics, the pathobiology of these organisms and the toxins they produce are reviewed in Melton-Celsa et al. (2012); Farrokh et al. (2013); Kruger and Lucchesi (2015).

In recent years, novel serotypes have emerged culminating in a major outbreak in 2011 caused by a novel pathotype, *E. coli* O104:H4. The review at hand focuses on potential treatment strategies for STEC infections in light of a consensus contraindication of employing antimicrobials for these bacterial pathogens. The rise of *E. coli* O104:H4 and approaches employed in its treatment are highlighted.

Emerging STEC Serotypes

A large number of STEC serotypes has been documented; these have been isolated from various types of animals including cattle, sheep, and goats (Farrokh et al., 2013). More than 380 STEC serotypes have been associated with human disease; some of the most frequently reported serotypes include O111:H-, O26:H11/H-, O103:H2, O113:H21, O91:H21/H-, O117:H7, O118:H16, O121:H19, O145:H28, O128:H2/H-, and O146:H21. The O157:H7 serotype has been the most commonly isolated one in association with HC and HUS in both outbreaks and sporadic cases. It accounts for more than 30% of estimated STEC illness and mortality cases in the United States (Karmali et al., 2010; Scallan et al., 2011). However, there are some indications that non-O157 STEC are gaining traction in the United States and that they may be even more common than O157 strains in severe illnesses caused by STEC in parts of Europe, Latin America, Australia, and Africa (Blanco et al., 2005; Wang et al., 2013).

The epidemiology and pathogenic characteristics of non-O157 serotypes are not well studied; however, the limited reported data indicates some differences between the two types of infections. Non-O157 strains appear to induce a longer period of diarrhea which is less frequently of the hemorrhagic type (Johnson et al., 2006). Nevertheless, studies demonstrate that these non-O157 serotypes can be as virulent as O157 serotypes depending on the strain involved (Ethelberg et al., 2004).

Perhaps highlighting the relevance of monitoring these non-O157 serotypes was the emergence of the rather notorious *E. coli* O104:H4. This novel pathogen was the cause of a 2011 outbreak that affected 16 European countries with the majority of cases reported in Germany. Few cases were reported in Canada and the United States as well; nevertheless, these were travelers who had been to Europe prior to becoming ill. Reports of this novel pathogen started in May of 2011 and had peaked and then dwindled by July of the same year due to control measures that were implemented. The WHO indicates that 4075 cases and 50 deaths were caused by this STEC outbreak. Therefore, a 1.23% mortality rate was observed. On the other hand, the mortality rate of HUS due to *E. coli* O104:H4 in this outbreak was 3.74% (WHO, 2011). *E. coli* O104:H4 appears to be an enteroaggregative *E. coli* (EAEC) that has acquired the ability to produce Stx2, typically produced by enterohemorrhagic *E. coli* (EHEC) rather than EAEC group members. This may have occurred via horizontal gene transfer resulting in a new *E. coli* virotype dubbed the Enteroaggregative Hemorrhagic *E. coli* or EAHEC (Bloch et al., 2012). The O104:H4 serotype harbors two copies of the Stx2-encoding prophage. Therefore, this emergent bacterium seems to have a rather novel epidemiologic and pathogenic profile (Brzuszkiewicz et al., 2011; Mellmann et al., 2011). While ruminants are the reservoir of most STEC serotypes, no animal reservoir has been identified for *E. coli* O104:H4 and humans are believed to be the major reservoir for this organism (Wieler et al., 2011; Auvray et al., 2012; Karch et al., 2012). Whereas, the clinical profile of *E. coli* O104:H4 was relatively similar to that caused by other STEC infections some pertinent differences existed. For instance, about a quarter of subjects affected developed HUS during the 2011 outbreak, which is 2–5 fold higher than the rate usually observed for an STEC infection (WHO, 2011).

Treatment of an STEC Infection

The lack of an effective treatment strategy for an STEC infection has made these agents a prominent public health threat and a burden to the medical community at large. The currently recommended management of an STEC infection mainly relies on supportive therapy and hydration (Thorpe, 2004). The use of antimicrobial agents in treating these infections has been associated with an increased risk of HUS and is therefore contraindicated (Qadri and Kayali, 1998; Guerrant et al., 2001; Safdar et al., 2002).

Novel and Alternative STEC Treatment Strategies

The debatable use of antimicrobial agents for the treatment of an STEC infection has led to the rise of various alternative treatment approaches (**Table 1**). These have ranged from the use of natural products to the development of novel regimens that re-examine employing antimicrobials.

Shiga Toxin Receptor Analog

Various agents that mimic Stx receptors and bind them thus reducing their availability to cellular receptors have been developed. Carbosilane dendrimers harboring Gb3 at their termini neutralize Shiga toxins *in vitro* and were demonstrated to protect challenged mice when administered intravenously (Nishikawa et al., 2002, 2005). Similarly, multivalent carbohydrate compounds, such as STARFISH and Daisy also neutralize Shiga toxins *in vitro* and in animals (Kitov et al., 2000; Mulvey et al., 2003). Gb3 polymers with highly clustered trisaccharides bind Shiga toxins with high affinity and protect challenged mice when administered orally (Watanabe et al., 2004). Recombinant bacterial strains that express toxin receptor mimics have also demonstrated a potential efficacy *in vitro* and upon testing in animals (Paton et al., 2000, 2001; Hostetter et al., 2014). SYNSORB Pk, a synthetic Stx receptor analog consisting of a Pk trisaccharide bound to Chromosorb® P, a multipurpose sorbent medium, was shown to have an abrogative effect on Shiga toxins *in vitro*. This agent, however, was not effective in clinical trials (Trachtman et al., 2003).

Intracellular Interference with Shiga Toxins

Cell permeable agents that can bind Stx2 and potentially interfere with its intracellular trafficking have been reported. These include Ac-PPP-tet (Watanabe-Takahashi et al., 2010) and TVP (Stearns-Kurosawa et al., 2011); both agents have been tested in animal models and have displayed Stx2 neutralization abilities. Manganese has also been reported to interfere with intracellular trafficking of the B subunits of Stx and to protect against Stx1 in mice (Mukhopadhyay and Linstedt, 2012). However, it did not protect against Stx1-S or Stx2a and hence it may be of limited use (Gaston et al., 2013). The small molecule inhibitors Retro-1

TABLE 1 | Experimental approaches to the treatment of Shiga toxin-producing *Escherichia coli* infections.

Approach	Method	Reference(s)
Shiga toxin receptor analogs	Carbosilane dendrimers with terminal Gb3 moieties	Nishikawa et al., 2002, 2005
	Multivalent carbohydrate compounds	Kitov et al., 2000; Mulvey et al., 2003
	Gb3 polymers with highly clustered trisaccharides	Watanabe et al., 2004
	Toxin receptor mimic-producing bacteria	Paton et al., 2000, 2001; Hostetter et al., 2014
	Pk trisaccharide bound to a sorbent medium	Trachtman et al., 2003
Intracellular interference with Shiga toxins	Ac-PPP-tet	Watanabe-Takahashi et al., 2010
	TVP	Stearns-Kurosawa et al., 2011
	Manganese	Mukhopadhyay and Linstedt, 2012; Gaston et al., 2013
	Retro-1, Retro-2, Retro-2^{cycl}	Stechmann et al., 2010; Noel et al., 2013
Antibodies	Anti-lipopolysaccharide antibodies	Paton et al., 1998
	Monocolonal anti-Stx A subunit antibodies	Islam and Stimson, 1990
	Bovine colostrum anti-Shiga toxin antibodies	Huppertz et al., 1999; Kuribayashi et al., 2006, 2009; Seita et al., 2013
	Humanized monoclonal anti-C5 (Eculizumab)	Lapeyraque et al., 2011; Kielstein et al., 2012; Menne et al., 2012; Delmas et al., 2014
Natural Products	Lactic acid	Pittman et al., 2012
	Fruit juices	Nogueira et al., 2003
	Plant, fruit and root products, teas or extracts	Tomita et al., 1997; Isogai et al., 1998; Okubo et al., 1998; Takahashi et al., 1999; Heredia et al., 2005; Takemasa et al., 2009; Lacombe et al., 2010; Lee and Stein, 2011; Voravuthikunchai et al., 2012; Liu et al., 2013; Pellarin et al., 2013
	Green tea extract with an antimicrobial agent (Levofloxacin)	Isogai et al., 2001
Novel/Alternate approaches using antimicrobial agents	Meropenem, chloramphenicol and fosfomycin	Corogeanu et al., 2012
	Ciprofloxacin	Corogeanu et al., 2012; Geerdes-Fenge et al., 2013
	Azithromycin	Nitschke et al., 2012; Nassar et al., 2013
	Rifampicin and Gentamicin	Kanbar et al., 2003; Matar and Rahal, 2003; Rahal et al., 2011a,b; Nassar et al., 2013; Fadlallah et al., 2015
	Imipenem	Nassar et al., 2013

and Retro-2 have also been identified via high throughput screening as agents that interfere with Stx trafficking (Stechmann et al., 2010) and a derivative of Retro-2, referred to as Retro-2^{cycl}, was shown to protect cells in culture against Stx (Noel et al., 2013).

Antibodies

Preparations of antibodies that can bind Shiga toxins and neutralize their effects have been reported. Anti-lipopolysaccharide antibodies have shown protective abilities upon laboratory assessment (Paton et al., 1998) and monocolonal anti-Stx A subunit antibodies have demonstrated potential utility in both laboratory and animal studies (Islam and Stimson, 1990). Bovine colostrum antibodies against Shiga toxins have also been demonstrated to protect challenged animals (Kuribayashi et al., 2006, 2009; Seita et al., 2013). A bovine colostrum preparation, rich in immunoglobulins and harboring a high titer of anti-Stx1 and anti-Stx2 antibodies, has also been assessed; a colostrum-treated group of 13 patients and 14 placebo-treated controls were compared. The median frequency of stool excretion was decreased in the colostrum-treated patients; however, the presence of the bacterial agent in subject stools was not notably affected. Study subjects were not monitored for the effect of this treatment on the development of HUS or other potential sequelae of infection (Huppertz et al., 1999). Eculizumab, a humanized monoclonal antibody against complement component 5 (C5), was shown in small clinical studies to have beneficial effects on recovery from STEC-associated HUS including cases during the 2011 *E. coli* O104:H4 outbreak (Lapeyraque et al., 2011; Delmas et al., 2014). However, some reports have indicated that inclusion of eculizumab in the treatment of *E. coli* O104:H4-induced HUS results in no additional benefits (Kielstein et al., 2012; Menne et al., 2012).

Natural Products

Various natural products have been considered as potential therapeutic agents for STEC infections. These have included lactic

acid (Pittman et al., 2012), fruit juices (Nogueira et al., 2003) in addition to plant, fruit and root products, teas or extracts (Tomita et al., 1997; Isogai et al., 1998; Okubo et al., 1998; Takahashi et al., 1999; Heredia et al., 2005; Takemasa et al., 2009; Lacombe et al., 2010; Lee and Stein, 2011; Voravuthikunchai et al., 2012; Liu et al., 2013; Pellarin et al., 2013). These products have shown promise *in vitro* or in experimental animal models; however, they have not been evaluated in clinical studies. Worth noting is a study that showed a synergistic effect between green tea extract and an antibiotic, levofloxacin, in the treatment of an STEC-infected mouse model (Isogai et al., 2001) indicating that a potential risk imparted by an antibiotic treatment may be lessened by the inclusion of another agent.

Antimicrobial Agents

The use of antimicrobial agents in treating STEC infections has been controversial and the subject of an ongoing debate. While some studies indicated that the use of particular agents may increase the risk of HUS, others have reported a decrease of this risk upon implementation of antimicrobials. While these observations may be particular to certain agents at some doses, the potential risk of antimicrobial treatment inducing HUS has led to a general contraindication of such agents (Qadri and Kayali, 1998; Guerrant et al., 2001; Safdar et al., 2002). Antimicrobials are thought to augment the risk of HUS by enhancing the release of Shiga toxins from bacterial cells via a number of ways. DNA damage that can be caused by some antimicrobials may trigger the bacterial SOS response in STEC cells. The SOS response, whose function is to cope with genomic damage, results in the expression of a number of proteins that may activate the lytic cycle of the bacteriophage encoding a Stx thus enhancing its production. Other types of physiologic stresses caused by antimicrobial agents may also trigger the lytic cycle and result in increased toxin expression (Kimmitt et al., 2000; Los et al., 2009). On the other hand, Stx1 is known to be stored within the periplasmic space of STEC cells; therefore, cellular lysis induced by an antimicrobial agent may result in an enhanced release of this particular type of Stx (Strockbine et al., 1986; Yoh et al., 1997; Sato et al., 2003; Shimizu et al., 2009).

Several antimicrobial agents have been shown to enhance the release or the production of Shiga toxins from STEC cells *in vitro*; these include the quinolones, trimethoprim, and furazolidone (Kimmitt et al., 2000); however, observations indicate that these effect may be strain and antimicrobial agent-specific (Grif et al., 1998). For example some isolates of *E. coli* O104:H4 from the 2011 outbreak in Europe do not display an increase in toxin production upon treatment with meropenem, ciprofloxacin, chloramphenicol, or fosfomycin, unlike *E. coli* O157:H7 (Corogeanu et al., 2012). Our group assessed the effect of sub-MIC levels of various antimicrobial agents on triggering the SOS response and the production of Shiga toxins in *E. coli* O157:H7 and in *E. coli* O104:H4. A sub-MIC concentration may, after all, be the concentration available locally at the site of infection. We noted that the response is variable depending on the isolate used and the concentration of antimicrobial implemented (Nassar et al., 2013; Fadlallah et al., 2015).

Reconsideration of treating STEC infections with antimicrobial agents has nevertheless gained ground in recent years. Ciprofloxacin was recently reported to decrease the risk of HUS in subjects infected with *E. coli* O104:H4 during the 2011 outbreak (Geerdes-Fenge et al., 2013) and a reduced duration of carriage of the organism in subjects treated with azithromycin was detected during this outbreak as well (Nitschke et al., 2012). Worth noting, however, is that only a small number of treated subjects was included in both studies. Our group assessed the use of rifampicin at a concentration that decreases toxin production, but at which *E. coli* O157:H7 cells remain viable, followed by treatment with gentamicin at a bactericidal concentration. This strategy was effective in decreasing toxin release compared to solely treating the cells with a bactericidal gentamicin concentration (Kanbar et al., 2003; Matar and Rahal, 2003). Applying a similar strategy in an *E. coli* O157:H7 infection mouse model resulted in an improved animal survival rate (Rahal et al., 2011a,b). Utilizing the same strategy to treat *E. coli* O104:H4 infected mice similarly resulted in an improved survival rate compared to untreated control mice that were infected with the organism; however, the highest survival rate observed was with mice treated with gentamicin alone, unlike our observations with *E. coli* O157:H7 (Fadlallah et al., 2015). This again highlights observations indicating that different STEC serotypes and even isolates of the same serotype respond differently to antimicrobial treatments.

Probiotics, Phages and Vaccines

Although probiotics may not have a therapeutic benefit in the management of an STEC infection, they may have a relevant preventative utility. Probiotics are probably capable of disrupting host-infectious agent/toxin interactions by occupying cellular receptors themselves, by producing decoy receptors that take up the toxins or by modifying the local milieu, hence making these interactions unfavorable (Corr et al., 2009). Multiple studies have shown *in vitro* beneficial effects of probiotics and that inoculation of animal models with a probiotic prior to an experimental STEC infection has preventative capabilities (Asahara et al., 2004; Reissbrodt et al., 2009; Eaton et al., 2011; Mogna et al., 2012; Chen et al., 2013; Kakisu et al., 2013; Rund et al., 2013; Stanford et al., 2014). The extent of probiotic protective capabilities seen in experimental models is likely dependent on the probiotic strain used and its ability to modify the surrounding medium. For example, the production of acetate by the probiotic agent has been demonstrated to be an important factor (Fukuda et al., 2011, 2012) and the production of butyric acid and lactic acid may be of relevance as well (Ogawa et al., 2001; Takahashi et al., 2004). One *in vitro* study showed that cultivation of STEC organisms in the presence of various *Bifidobacterium*, *Pediococcus,* and *Lactobacillus* strains results in a decreased production of Stx2. This was attributed to a decrease in pH due to the acids produced by these agents (Carey et al., 2008). Worth noting is the recombinant probiotic agent that can produce toxin receptor mimics described in section 3.a. (Paton et al., 2000, 2001; Hostetter et al., 2014). Also of relevance are the various studies indicating that the administration of probiotic agents to cattle may reduce their carriage of STEC organisms (systematically reviewed in Sargeant et al.,

2007), hence effectively reducing the risk of transmitting these toxigenic agents.

Another preventative measure proposed as a means of controlling STEC is the application of lytic phages. Lytic phages have been shown to reduce STEC numbers *in vitro* (Niu et al., 2009; Rivas et al., 2010), in phage-treated food products (Abuladze et al., 2008; Anany et al., 2011), on hard surfaces (Abuladze et al., 2008), in mice and in some ruminants (Raya et al., 2006; Sheng et al., 2006). Phage-containing products that can be sprayed on animal hides or on meat products for the control of STEC organisms are available on the market and are Food and Drug Administration (FDA) approved (Sillankorva et al., 2012). The efficacy of orally treating cattle with lytic phages, however, was reported to be limited and requires the development of an enhanced approach or delivery mode (Stanford et al., 2010). Bacteriophages used to eradicate STEC agents may also have a therapeutic utility should the safety and efficacy of such an application be demonstrated in humans.

Various vaccine approaches have also been attempted including the development of preparations that contain bacterial peptides and virulence factors (Wen et al., 2006; Tiels et al., 2008; Gu et al., 2009; McNeilly et al., 2010; Asper et al., 2011; Cai et al., 2011; Gupta et al., 2011; Wan et al., 2011; Zhang et al., 2012; Rossi et al., 2013; Sato et al., 2013; Cernicchiaro et al., 2014; Garcia-Angulo et al., 2014; Lu et al., 2014; Mejias et al., 2014; Paddock et al., 2014), attenuated bacterial cells (Rojas et al., 2010; Gu et al., 2011; Fujii et al., 2012), bacterial envelope/membrane derivatives (Cai et al., 2010; Choi et al., 2014) in addition to DNA vaccines (Bentancor et al., 2009; Ren et al., 2013). These vaccine preparations have been assessed in animal models with some showing promising results (reviewed in Garcia-Angulo et al., 2013).

In conclusion, despite the passage of more than three decades since STEC organisms were first associated with human clinical illness (CDC, 1982), a generally-accepted successful therapeutic method for these organisms remains undocumented. Various approaches have nevertheless been attempted including ones that reconsider the implementation of antimicrobial agents; beneficial effects have been reported for some agents with outcomes appearing dependent on the antimicrobials used, their dose and the STEC isolate itself. Further studies examining antimicrobial agents in the therapy of STEC infections should be conducted in animals to select the safest and most efficacious regimen that would then be assessed in clinical trials.

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Antimicrobial activities of chicken β-defensin (4 and 10) peptides against pathogenic bacteria and fungi

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Host Defense Peptides (HDPs) are small cationic peptides found in several organisms. They play a vital role in innate immunity response and immunomodulatory stimulation. This investigation was designed to study the antimicrobial activities of β-defensin peptide-4 (sAvBD-4) and 10 (sAvBD-4) derived from chickens against pathogenic organisms including bacteria and fungi. Ten bacterial strains and three fungal species were used in investigation. The results showed that the sAvBD-10 displayed a higher bactericidal potency against all the tested bacterial strains than that of sAvBD-4. The exhibited bactericidal activity was significant against almost the different bacterial strains at different peptide concentrations except for that of *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Streptococcus bovis* (*Str. bovis*) strains where a moderate effect was noted. Both peptides were effective in the inactivation of fungal species tested yielding a killing rate of up to 95%. The results revealed that the synthetic peptides were resistant to salt at a concentration of 50 mM NaCl. However, they lost antimicrobial potency when applied in the presence of high salt concentrations. Based on blood hemolysis studies, a little hemolytic effect was showed in the case of both peptides even when applied at high concentrations. The data obtained from this study indicated that synthetic avian peptides exhibit strong antibacterial and antifungal activity. In conclusion, future work and research should be tailored to a better understanding of the mechanisms of action of those peptides and their potential use in the pharmaceutical industry to help reduce the incidence and impact of infectious agent and be marketed as a naturally occurring antibiotic.

Keywords: antimicrobial activity, synthetic AvBD-4 and 10, bacteria, fungi, natural antibiotic

Introduction

Host Defense Peptides (HDPs) are a diverse group of small and cationic peptides that are present in several organisms (Cuperus et al., 2013). Originally, they were called Antimicrobial Peptides (AMPs) due to their ability to inactivate and control bacteria *in vitro*. Several studies showed that these peptides have additional functions, mainly immunomodulatory ones (Cuperus et al., 2013). Therefore, they were named as "Host Defense Peptides (HDPs)." Recently, some reports indicated that HDPs are responsible for differentiation, activation and chemotaxis of leukocytes. They inhibited Lipopolysaccharide (LPS), enhance phagocytosis, DNA uptake and wound healing (Zanetti, 2005; Semple and Dorin, 2012).

HDPs were first discovered in the 1970's by extraction from tissues of avian species (Evans et al., 1994). Based on new advances in the field of bioinformatics and available sequence data, a number of new HDPs have been identified. The first avian HDPs were confirmed in the mid 1990's, and included five defensins isolated from the leukocytes of some avian species like chickens and turkeys (Evans et al., 1994). Recently, the complete defensin and cathelicidin genes clusters have been sequenced for chickens. Due to the interest in their role as potential therapeutically agents, there is a remarkable amount of information based on various studies using other avian species (Cuperus et al., 2013). Currently, there is a lot of research on the role of these peptides as templates for possible novel anti-microbial agents (Cuperus et al., 2013). Accordingly, they have been sought as possible alternatives to antibiotics which lost their shine due to the increasing resistance that is exhibited against a wide range of bacteria which is a major public health issue especially during the last decade (Cuperus et al., 2013). Defensins peptides are cysteine-rich, cationic in nature and composed of three conserved disulfide bridges, a β-sheet and both hydrophobic and cationic amino acids (Ganz, 2003; Selsted and Ouellette, 2005). The defensin peptides are classified into three main groups: α−, β−, and θ−defensins (Yang et al., 2004; Selsted and Ouellette, 2005). Alpha-defensins are found in mammals and form disulfide bridges between Cys1–Cys6, Cys2–Cys4, and Cys3–Cys5 (Lehrer and Ganz, 2002; Yang et al., 2004). Theta-defensins are present in all vertebrates and form disulfide bonds between Cys1–Cys5, Cys2–Cys4, and Cys3–Cys6 (Lehrer and Ganz, 2002; Klotman and Chang, 2006). Based on extensive search and based on chicken genome analysis, β-defensins were the first to be confirmed which derived from avians species. Using the chicken genome, they were first to be confirmed in avian species (Lehrer and Ganz, 2002; Klotman and Chang, 2006). The underlying mechanism by which AMPs exert their effect is through their interaction with the negatively charged phosolipid bilayer found in the cell membrane under hydrophobic conditions, thus resulting in disruption and killing of the pathogen (Higgs et al., 2005; Lynn et al., 2007). It has been reported that avian β-defensin are more efficient against Gram-positive bacteria than Gram-negative bacteria due to their structural conformation (Cuperus et al., 2013). The mode of action of theses peptides in bacterial killing was reported in some studies due to its net charge. Some studies revealed that the duck β−defensin-12 has the lowest net charge and lower antibacterial activity as compared to duck β−defensin 4 and 7 peptides (Powers and Hancock, 2003; Ganz, 2004; Brogden, 2005).

This study was designed to evaluate the antimicrobial activities of synthetic chicken β-defensin peptides (sAvBD-4 and 10) against both pathogenic Gram-negative and Gram-positive bacteria and fungi. The kinetics were also investigated and the effect of salinity on their activity was delineated.

Material and Methods

Bacterial Species

Bacterial species used included both Gram-negative and Gram-positive strains. The five Gram-positive bacterial strains used were: *Micrococcus luteus* ATCC 49732 (*M. Luteus*), *Enterococcus faecalis* ATCC 29212 (*Ent. faecalis*), *Streptococcus bovis* ATCC 49147 (*Str. bovis*), *Staphylococcus epidermidis* ATCC 12228 (*Staph. epidermidis*) and Methicillin-Resistance *Staphylococcus aureus* ATCC 43330 (MRSA). Five strains of Gram-negative bacteria included: *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*), *Escherichia coli* ATCC 25922 (*E. coli*), *Salmonella typhimurium* ATCC 14028 (*Salm. typhimurium*), *Klebsiella pneumonia* ATCC 700603(*Kleb. pneumonia*), *Shigella sonnei* ATCC 25931(*Sh. sonnei*). The bacterial strains were cultured on tryptone soy agar and incubated for 24 h at 37°C and stored in slants at 4°C.

Fungal Species

Three types of fungal species were used; *Candida albicans* ATCC 10231 (*C. albicans*), *Aspergillus flavus* (*Asp. flavus*), and *Aspergillus niger* (*Asp. niger*) isolates (obtained from the Department of Biological Sciences, King Abdulaziz University, Jeddah, Saudi Arabia. Filamentous fungi and Candida were cultured in Sabouraud dextrose agar and incubated at 25–28°C for 72 h for multicellular fungi and h at 30°C for 24 in the case of Candida and stored in slants at 4°C.

Chicken β-Defensins Peptides Synthesis

The mature peptide of chicken β-defensin (AvBD-4 and 10) were custom synthesized and purified to 80% level using high-performance liquid chromatography (HPLC) by GL Biochem Ltd. (Shanghai, China) (http://www.glschina.com/en/profile.htm). The full sequence of amino acids of chicken β-defensin (AvBD-4 and 10) are listed in **Table 1**.

TABLE 1 | Amino acid sequence of synthetic AvBDs.

	Amino acid sequence	AA
AvBD-4	AC-MKILCFFIVLLFVAVHGAVGFSRSPRYHM QCGYRGTFCTPGKCPHGNAYLGLCRPKYSCCRWL	64
AvBD-10	AC-MKILCLLFAVLLFLFQAAPGSADPLFPDTVACRT QGNFCRAGACPPTFTISGQCHGGLLNCCAKIPAQ	69

Antimicrobial Activity Assay

MIC assays for the peptides were performed by two-fold broth dilution method with Mueller Hinton II broth according to the procedures as suggested by the CLSI (Clinical and Laboratory Standards Institute) (Wayne, 2009, 2012). In summary, Bacteria and Candida were grew to reach out the exponential phase. Cells were then centrifuged at 2000 × g for 15 min. The resulting pellets were washed and resuspended in 10 mM sodium phosphate buffer saline solution (pH 7.0). Two-fold serial dilutions of the sAvBD-4 and 10 were prepared in appropriate culture medium in 96-microwell plates. 100 μL inoculum from the culture with a bacterial density of 106 CFU/ml were added to each individual well containing 100 μL of either M-H alone, or M-H containing two-fold diluted peptide to give a final concentration of the peptide ranging from 0 to 200 μM. MIC values were recorded as the minimum concentration that showed no visible growth after overnight incubation at 37°C. The cell survival percentage was determined by measuring the absorbance at $\lambda = 570$ nm with a microplate reader. Cell survival % = [(mean optical density of the sample − blank)/(mean optical density of the control ((no treatment) − blank)] × 100.

The minimum bactericidal concentration (MBC) or the minimum fungicidal concentration (MFC) were evaluated by subculture of the contents of the first two clear wells obtained in the MIC assay onto minimal M-H agar plates. The lowest peptide concentration yielding more than 99% of either bacterial or fungal growth inhibition was noted as MBC or MFC.

Two multicellular fungi (*Asp. niger* and *Asp. flavus*) were used to evaluate the anti-fungal activity of those two peptides. Overnight cultures of the fungi were prepared by inoculating 100 ml of Sabouraud dextrose broth with a 105 spores/ml concentration. Tetracycline used as a reference in the case of both Gram-negative and positive bacteria. On the other hand, Ketoconazole was use as a reference in the case of the fungus *C. albicans*, *Asp. Niger*, and *Asp. flavus*. The both reference antibiotics were used at concentration of (30 μg/ml).

Kinetics of Inactivation

In order to study the kinetics of bacterial inactivation for both of the test peptides, three organisms were used: MRSA, and *E. coli* (1×10^8 CFU/ml) and *C. albicans* (1×10^8 CFU/ml). The concentration used was two times of that of the MIC. Overnight bacterial cultures were prepared. The cultures were spun down and resuspended in fresh M-H medium at a concentration of 1×10^8 CFU/ml. sAvBD-4 and 10 were then added to the bacterial suspension, at a concentration equivalent to two times that of the MIC. The mixture was incubated under 35°C. Ten-microliter aliquots were removed with a sterile calibrated loop at (0, 15, 30, 60, 120, and 180 min) and uniformly seeded on M-H medium (Ma et al., 2011). The plates were incubated at 35°C for 24–48 h. After the incubation period, the number of viable cells was counted and expressed in CFU/ml. The results were analyzed and represented graphically, a microbial death curve as a function of time.

Salinity Test

E. coli was used as a test model to evaluate the effects of ionic strength on the antibacterial activity of the two peptides. *E. coli* was subcultured at 37°C to the mid-log phase, and suspended to 10^6 CFU/ml in MH. A suspension of *E. coli* (1 ml) was incubated with different concentrations of peptides (0–200 μg/mL), with different concentrations of NaCl (0, 20, 50, 150 mM) in 10 mM sodium phosphate buffer, pH 7.4. The tested bacteria was cultured at 37°C for 2 h before 1000 times of dilution followed by plating. Survived bacteria were counted (Ma et al., 2011; Wang et al., 2011; Baricelli et al., 2015).

Hemolysis Test

The hemolytic activities of the synthetic defensin were investigated according to what has been reported in the literature (Shin et al., 2001; Yu et al., 2001). Briefly, fresh chicken blood was collected from King Abdulaziz University farm, Jeddah, KSA. The blood was spun down for erythrocytes harvesting by centrifugation (3000 rpm, 10 min, at 20°C). The resulting erythrocytes were then washed twice with sterile PBS at a concentration of 0.5% vol/vol and were used for the assay, by dispensing 90 μl into each well of the 96-well plates. Ten microliters of different peptides concentrations were added to the cells and incubated at 37°C for 2 h. After incubation, the microtiter was spun down at 800 × g for 10 min. The supernatants were withdrawn and transferred to a new 96-well plate and checked for released hemoglobin as measured spectrophotometerically at 405 nm. For (0 hemolysis) as well as (100% hemolysis) controls, cells were resuspended in PBS only and in 1% Triton X-100, respectively (Ma et al., 2013).

Statistical Analysis

Data were entered using IBM SPSS Statistics 20, and was analyzed by Kaplan–Meier analysis. A P level of < 0.05 was considered to significant.

Results

The chicken β-defensin (sAvBD-4 and 10) used in this study were analogs of the natural peptides. These were custom made having the linear N-terminal acetylated as is the case with naturally occurring mature chicken β-defensin peptides. These custom-made peptides were evaluated for their anti-bacterial and anti-fungal activities against 10 bacterial strains and three fungal species. There was a variation in the response of the bacteria to the tested peptides with sAvBD10 showing a better efficacy on average against all the bacteria tested. Statistical analyses showed that the difference was significant at the 95% as shown in (**Table 2**, **Figures 1A,B**). The results showed that sAvBD-4 inhibited the growth of both Gram-negative and positive bacteria with MIC concentrations as follows: 25 μg/ml [(*Staph. epidermidis, Kleb. pneumonia, Sh. sonnei, C. albicans*), 50 μg /ml (MRSA, *M. luteus, Salm. typhimurium, E. coli, Asp. flavus*) and 100 μg/ml for (*Str. bovis, Ent. faecalis, Asp. niger*)]. However, sAvBD-10 was more efficient in achieving bacterial inactivation at the following MIC concentrations: 25 μg/ml (*M. luteus, Kleb. pneumonia, C. albicans*, and *Asp. flavus*) and 50 μg/ml (*Str. bovis, Ent. faecalis*, MRSA, *Salm. typhimurium, E. coli, Staph. epidermidis, Sh. sonnei, Asp. niger*, and *P. aeruginosa*). The MBC levels were also determined and found to be two-fold higher than those of the corresponding MIC values (MBC range, 50–200 μg/ml)

TABLE 2 | The antimicrobial activities of synthetic chicken β-defensin-4 and 10-derived peptide.

Organisms species	sAvBD-4 peptide		sAvBD-10 peptide		Tetracycline	
Bacteria	**MIC (μg/ml)**	**MBC (μg/ml)**	**MIC (μg/ml)**	**MBC (μg/ml)**	**MIC (μg/ml)**	**MBC (μg/ml)**
Str. bovis ATCC (49147)	< 100 ± 0.0	200 ± 0.0	50 ± 0.0	100 ± 0.58	10 ± 3.46	20 ± 0.0
Staph. epidermidis ATCC (12228)	25 ± 3.46	100 ± 0.58	50 ± 0.0	100 ± 0.58	10 ± 1.73	10 ± 0.0
Staph. aureus MRSA ATCC (43330)	50 ± 0.0	100 ± 1.0	50 ± 1.3	100 ± 1.0	20 ± 0.0	< 20 ± 1.73
Ent. faecalis ATCC (29212)	100 ± 0.0	200 ± 0.58	50 ± 0.0	100 ± 1.73	10 ± 0.0	20 ± 0.0
M. luteus ATCC (49732)	50 ± 3.0	50 ± 1.58	25 ± 0.0	50 ± 1.73	5 ± 0.0	10 ± 0.0
E. coli ATCC (25922)	50 ± 1.3	100 ± 0.00	50 ± 1.73	100 ± 3.46	10 ± 0.0	20 ± 1.73
P. aeruginosa ATCC (27853)	–	–	50 ± 0.0	200 ± 3.46	10 ± 1.73	20 ± 1.73
Salm. typhimurium ATCC (14028)	50 ± 0.58	100 ± 3.46	50 ± 0.58	100 ± 3.46	10 ± 0.0	20 ± 0.0
Kleb. pneumonia ATCC (700603)	25 ± 0.0	50 ± 0.58	25 ± 0.0	100 ± 1.73	5 ± 0.0	20 ± 0.0
Sh. sonnei ATCC (25931)	25 ± 3.46	100 ± 3.46	50 ± 1.73	100 ± 0.58	5 ± 0.0	10 ± 1.0
Fungi		**MFC**		**MFC**	**Ketoconazole**	
C. albicans ATCC (10231)	25 ± 1.0	100 ± 0.58	25 ± 0.0	50 ± 0.58	3 ± 0.0	3 ± 0.0
Asp. Flavus	50 ± 1.0	100 ± 3.46	25 ± 0.0	50 ± 0.58	10 ± 0.0	25 ± 0.0
Asp. Niger	100 ± 1.73	–	50 ± 3.0	100 ± 1.3	10 ± 0.0	10 ± 0.0

Determination of MICs (μg/ml) and MBCs (μg/ml), or MFCs (μg/ml) for each peptide and bacteria was performed at least three-times in doublets. sAvBD-4, synthetic chicken β-defensin-10; sAvBD-10, synthetic chicken β-defensin-10; MIC, minimal inhibition concentration; MBC, minimal bactericidal concentration; MFC, minimal fungicidal concentration.

(**Table 2**). At those lower concentrations, sAvBD-10 had a significantly better antimicrobial activity as compared to sAvBD-4 against all the bacteria tested ($P < 0.002$). However, at higher concentrations of 100 μg/ml, both peptides showed no significant difference in their bactericidal efficacy (**Table 2**).

There was a dose dependent decline in antifungal inhibition and inactivation by both peptides (**Table 2**). Again, sAvBD-10 showed a significantly better anti-fungal activity as compared to sAvBD-4 as far as both MIC and MFC are concerned. In the case of sAvBD-10 (**Figure 1B**), the MIC varied among the three fungi with 25 μg/ml needed for both *C. albicans* and *Asp. flavus* and 50 μg/ml for *Asp. niger*. There was a significant difference in the MIC reported regarding sAvBD-4 among the three fungal species tested (**Table 2**, **Figure 1B**) with highest MIC observed in the case of *Asp. niger*.

The data showed that the MFC activity of sAvBD-10 was 25 μg/ml in the case of *C. albicans* and *Asp. flavus* and 50 μg/ml for *Asp. niger*. A much higher concentration was needed concerning sAvBD-4 (**Table 2**, **Figure 1B**). A better bactericidal activity was noted in the case of tetracycline as compared to the tested peptides. This, also, applies in the case of ketoconazole.

Kinetics of Inactivation

In the kinetics study, MRSA, *E. coli*, and *C. albicans* were used (**Figure 2**). Both peptides showed similar efficacy against MRSA during the first hour of application. However, faster decline was prominent in the case of sAvBD-4 for the next 2 h. This was not the case in term of *E. coli* where the two peptides started the same for the first hour and sAvBD-10 produced higher bactericidal efficacy for the remaining 2 h. As far as the kinetics of killing of *C. albicans* is concerned, there was a consistent better efficacy of sAvBD-10 over sAvBD-4.

Salinity Test

The effect of salinity on the antibacterial efficacy of both peptides was evaluated using various salt concentrations ranging from 0 to 150 mM (**Figure 3**). The results revealed that the synthetic peptide's efficacy was not reduced in the presence of salt concentrations ranging from 0 to 50 mM. However, such an effect was significantly compromised in a dose dependent manner when higher concentrations of salt were present (**Figure 3**) ($P < 0.001$).

Hemolytic Activity of Synthetic Peptide

The hemolytic activities of the two peptides were evaluated using freshly isolated chicken erythrocytes. The hemolysis was done using spectrophotometric measurements at a wavelength of 405 nm (**Figure 4**). The results showed that the peptides did not produce significant hemolytic effects.

Discussion

Around 1000 HDP has been discovered so far. The β-defensins, cathelicidens and liver antimicrobial peptide-2 are three of those HDPs that originated from chickens. In this investigation, the antimicrobial potential of two synthetic peptides (AvBD-4 and 10) was evaluated against 10 Gram-positive and -negative bacterial strains, as well as unicellular and multicellular fungi. This work was initiated to better understand the efficacy of those novel peptides against bacteria and fungi and their possible application in conjunction either with already existing antimicrobial agents or with possible substitutes for them. The uncontrolled use, misuse, and abuse of commonly administered antimicrobial agents has resulted in the horizontal gene transfer among microorganisms and led to the stimulation of the evolutionary potential of the bacteria to develop some kind of resistance

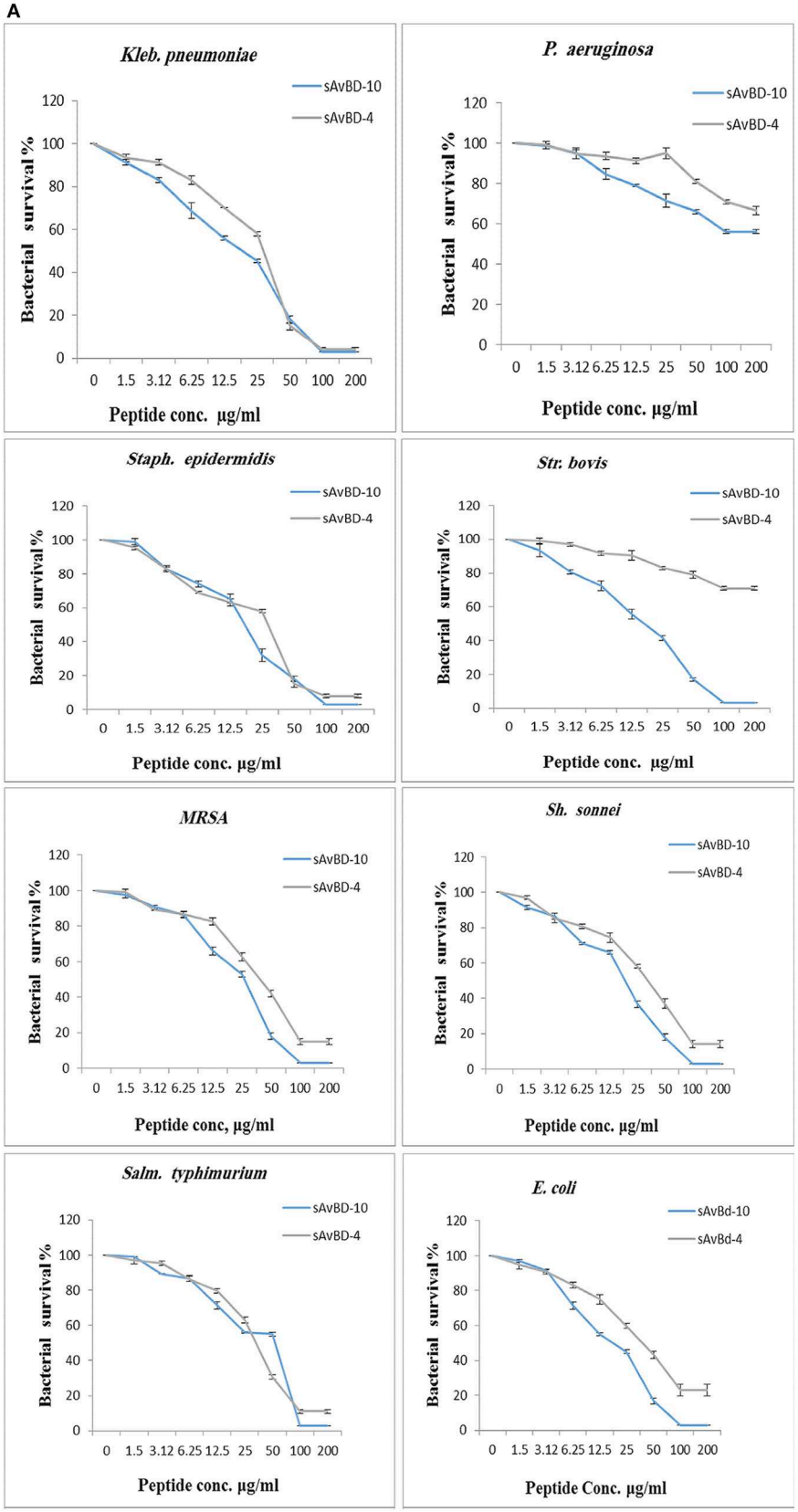

FIGURE 1 | Continued

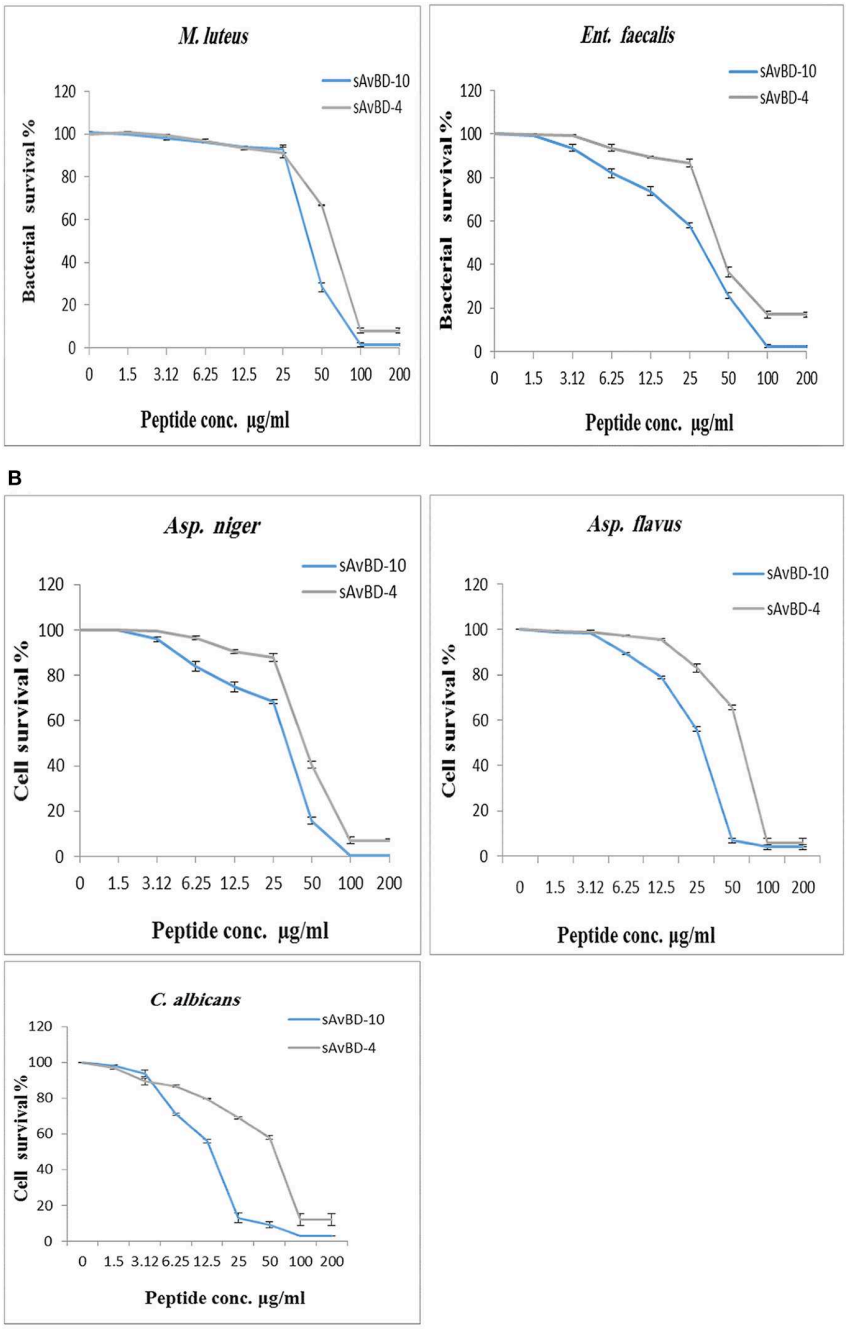

FIGURE 1 | Antimicrobial activities of synthetic chicken β-defensin-4 and 10-derived peptide (sAvBD) against (A) bacteria and (B) Fungal species. All assays were performed in three independent experiments and each point is the mean ± SE, ($P < 0.002$).

against the antimicrobial agents and consequently resulted in the emergence of multi-drug resistant bacteria. Such a resistance to antimicrobials is a worldwide problem in the treatment of infectious diseases especially in patients who are immunocompromised patients with hospital-acquired infections. For this reason, alternative agents have been sought to deal with the issue of multi-drug resistance. The AMPs are naturally occurring products, which have undergone a long-term evolution in nature. These peptides may have a promising potential as possible substitutes for antimicrobial agents or to potentiate the efficacy of already existing drugs used for the treatment of infectious agents.

The findings of this study revealed that the synthetic AvBD-4 and 10 displayed a potent efficacy against a broad-spectrum of bacteria. The current results were compatible with those of other studies on the antimicrobial activity of other known AvBDs (Evans et al., 1994, 1995; Harwig et al., 1994; Thouzeau et al.,

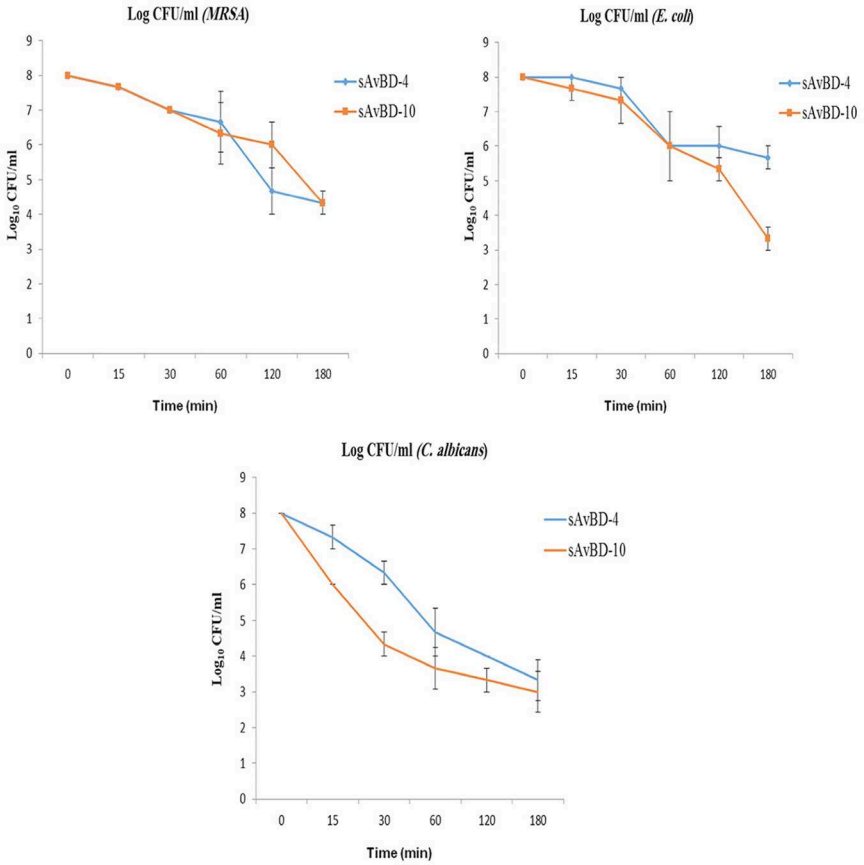

FIGURE 2 | The kinetic inactivation of synthetic chicken β-defensin-4 and 10-derived peptide (sAvBD) against MRSA, *E. coli* and *C. albicans* species. All assays were performed in three independent experiments and each point is the mean ± SE.

2003; Lynn et al., 2004; Higgs et al., 2005; van Dijk et al., 2008; Ma et al., 2009a,b, 2011, 2012a,b,c; Soman et al., 2010; Wang et al., 2010; Zhang et al., 2011, 2012). For instance, geese derived synthetic peptides showed an effect against both Gram-positive and -negative bacteria, like *S. aureus* and *E. coli*, thus consistent with the findings on synthetic β-defensins potency in their natural forms as reported elsewhere (Sass et al., 2010; Ma et al., 2013). The results of a study on recombinant AvBD2 derived from ducks exhibited a strong bactericidal potential against *B. cereus*, *S. aureus*, and *P. multocid*, and weak bactericidal activity against *E. coli* and *S. choleraesuis* like the results obtained using defensins (Evans et al., 1994; Harwig et al., 1994; Yu et al., 2001; Thouzeau et al., 2003; Sugiarto and Yu, 2006), were chemically synthesized (Higgs et al., 2005; van Dijk et al., 2007) or were produced by recombinant expression (Milona et al., 2007; van Dijk et al., 2007; Ma et al., 2008). In another study using Recombinant HBD2 showed that it inhibited three Gram-negative bacteria known for their resistant to antimicrobial agents (*S. marcescen, P. aeruginosa, A. baumannii*) that are opportunistic nosocomial pathogens present among immunocompromised individuals (Baricelli et al., 2015).

Interestingly, besides the broad spectrum of antibacterial effect, the synthetic AvBD-4 and 10 exhibited a fungicidal activity against both unicellular and multicellular fungi. More than 90% fungicidal activity was reported in this study. It was interesting to note that avian derived peptides have an anti-fungal potential. The results showed for the first time that those avian peptides had inhibitory and cidal effects against fungi. Also, it was reported that defensin secreted by filamentous fungi displayed strong inhibitory potential against human and plant infectious agents (Lacadena et al., 1995; Meyer, 2002, 2008; Marx, 2004; Galgóczy et al., 2010).

The differences in the inactivation kinetics between the two peptides may be structure related and/or dependent on the type bacteria used. Studies demonstrated that pheasant cathelicidin-1 (Pc-CATH1) inhibited *E. coli* growth during the first hour of application. However, it took 6 h for the bacterial growth to be resumed (Wang et al., 2011). The killing kinetics for synthetic sAvBD-10 was more effective than sAvBD-4 in eliminating fungal species than bacteria as indicated in this investigation.

Salt resistance is a defiance that is highly pertinent for HDPs in order for them to exert their full potential under physiological conditions. The results showed that the NaCl levels as those found under physiological conditions found in the human body did not compromise the efficacy of the tested peptides. Similar to those present in the Similar to other defensins (Sang et al., 2005; Ma et al., 2011). The efficacies of those peptides were compromised in the presence of high salt concentrations. This is

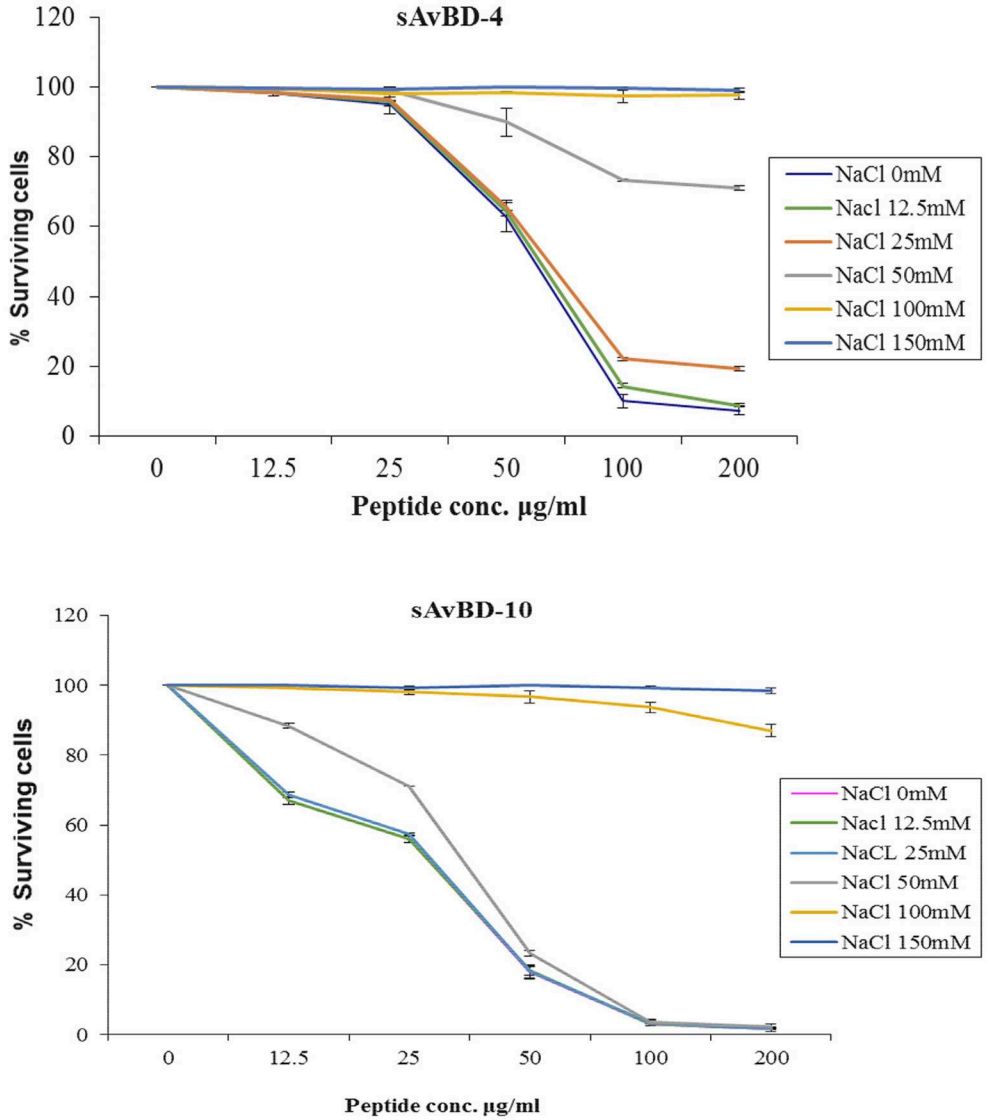

FIGURE 3 | Effects of salinity on the antibacterial activity of synthetic chicken β-defensin-4 and 10-derived peptide (sAvBD) against *E. coli*. All assays were performed in three independent experiments and each point is the mean ± SE, ($P < 0.001$).

consistent with the findings reported by others (Soman et al., 2010) and those of others using ostrich (Ma et al., 2013; Lu et al., 2014). The results revealed that the effect of ionic strength on the antimicrobial impact of investigated peptides were compatible with other reports on most tested defensins and other AMPs (Porter et al., 1997; Bals et al., 1998; Zucht et al., 1998; Veldhuizen et al., 2008).

Furthermore, the antimicrobial potency of synthetic AvBD-4 and 10, these synthetic peptides had low hemolytic effect and low toxicity at higher concentrations. These results are in agreement with other studies on other avian β-defensin and also for mammals defensin (Milona et al., 2007; Veldhuizen et al., 2008; Ma et al., 2011, 2012a,c). Some published studies indicated that the little toxic effect of defensin peptide on animal cells might be due to the partly higher cholesterol levels and a lack of negatively charged phospholipids in the outer leaflet of animal membranes, which inhibit the binding of many AMPs (Matsuzaki et al., 1995; Ishitsuka et al., 2006). This in agreement with the data obtained by other scientists using geese and duck related defensins (Ma et al., 2013; Lu et al., 2014).

The mechanism of action by which those peptides these peptides exert their effects is not well-understood. It is likely to be due to interaction of the peptides which are positively charged with specific structures like the phospholipid membranes found on the negatively charged bacterial membrane. The result of the interaction of the peptides with the phospholipid membranes, will lead to an increase in the pore formation of the membrane which result in increased permeability leading to the demise of the bacterial cell (Zasloff, 2002; van Dijk et al., 2008; Derache et al., 2009; Ma et al., 2013; Lu et al., 2014).

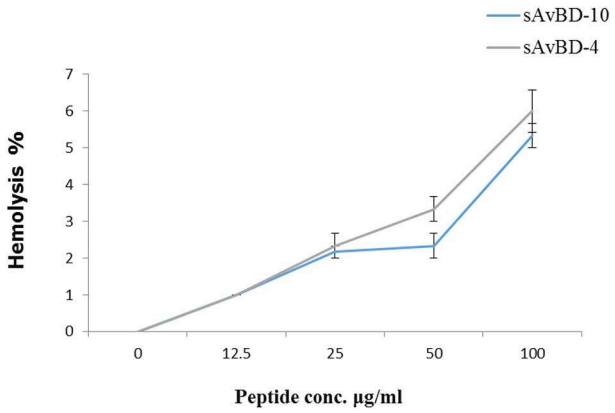

FIGURE 4 | Hemolytic activities of synthetic chicken β-defensin-4 and 10-derived peptides (sAvBD). All assays were performed in three independent experiments and each point is the mean ± SE.

Conclusion

The antimicrobial activity of synthetic chicken β-defensin-4 and 10 were studied against wide spectrum of infectious pathogens, including bacterial and fungal species. These peptides have exhibited significant antimicrobial activities against a broad spectrum of Gram-positive and Gram-negative bacteria of concern to the public health and have been involved in many outbreaks. Interestingly, besides their broad spectrum of antibacterial activities, synthetic AvBD (4 and 10) have shown fungicidal potential against *C. albicans* and *Asp. flavus*. The results showed that the synthetic AvBD (4 and 10) were more efficacious as an anti-fungal agents causing more than 95% reduction in fungi as compared to the control. Both of those synthetic peptides were resistant to salt concentrations of 50 mM NaCl, but lost their antimicrobial potential in a milieu containing salt concentration of 100 and 150 mM NaCl. Based on the data, it would be concluded that synthetic β-defensin peptides have a potent antimicrobial activities against a broad spectrum of pathogens and would merit the need for more studies to investigate their full potential in the treatment of disease in both humans and animals. They are likely to be a possible alternative to antibiotics and serve as natural antimicrobial properties with the hope that they do not produce bacteria that are resistant due to their naturally occurring properties.

Author Contributions

HY and AE collection and assembly of the data, manuscript writing, and data analysis; HY, AE, MM, and SH discussion, manuscript revision; AA, MM, and OA data analysis and discussion; HY and AE concept and design, data analysis, manuscript revision, and final approval of the manuscript.

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