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Prevalence and Antibiotic Resistance Profiles of Extended-Spectrum β-Lactamase–Producing *Escherichia coli* Isolated from Healthy Broilers in Shandong Province, China

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ABSTRACT

Food-producing animals carrying extended-spectrum β-lactamase–producing *Escherichia coli* (ESBL-EC) have posed a potential threat to human and animal health. However, information regarding ESBL-EC in the intensive broiler breeding areas of Shandong Province, People's Republic of China, is very limited. The goal of our study was to investigate the prevalence and drug resistance characteristics of ESBL-EC in healthy broilers from Shandong Province. A total of 142 ESBL-EC isolates were collected from four prefectures in Shandong Province from October 2014 to February 2015. ESBL-EC isolates were frequently detected (142 of 160 samples, 88.8%) in healthy broilers. Antibiotic susceptibility testing showed that all 142 ESBL-EC isolates were resistant to ampicillin, piperacillin, and cefazolin but were sensitive to imipenem and meropenem. All ESBL-EC isolates carried one or more of the *bla* genes, in which $bla_{\text{CTX-M}}$, $bla_{\text{TEM-1}}$, and $bla_{\text{SHV-5}}$ genes were identified in 142, 106, and 5 isolates, respectively. The $bla_{\text{CTX-M}}$ gene includes $bla_{\text{CTX-M-15}}$ (56), $bla_{\text{CTX-M-65}}$ (42), $bla_{\text{CTX-M-55}}$ (36), $bla_{\text{CTX-M-14}}$ (21), $bla_{\text{CTX-M-79}}$ (1), $bla_{\text{CTX-M-3}}$ (1), $bla_{\text{CTX-M-123}}$ (1), and $bla_{\text{CTX-M-132}}$ (1). In addition, 17 ESBL-EC isolates cocarried the genes of the CTX-M-1 and CTX-M-9 groups. Our findings indicate that healthy broiler flocks in Shandong Province in China are an important reservoir for ESBL-EC, with $bla_{\text{CTX-M}}$ and $bla_{\text{TEM-1}}$ being the prevalent resistance genes identified.

Key words: Broiler flocks; CTX-M-1; CTX-M-9; Extended-spectrum β-lactamase genes; Extended-spectrum β-lactamase–producing *Escherichia coli*

Extended-spectrum cephalosporins, especially third- and fourth-generation cephalosporins, are very important antimicrobial drugs for human and animals health *(10)*. However, extended-spectrum β-lactamase (ESBL)–producing *Enterobacteriaceae* have posed serious challenges in clinical practices. The main resistance mechanism of ESBL-producing *Enterobacteriaceae* is through the production of ESBL or plasmid-encoded AmpC β-lactamase. However, the diversity of ESBL genes in *Enterobacteriaceae* from food-producing animals is much higher than that of AmpC genes *(4, 28)*. ESBL-producing *Escherichia coli* (ESBL-EC) found in food-producing animals is frequently considered to be a potential source of human ESBL-EC infection. The increasing number of ESBL-EC isolates from food-producing animals has also raised great awareness and concern worldwide *(1, 24, 26)*.

Among the prevalent ESBL-EC from food-producing animals, TEM, SHV, and CTX-M are considered to be the most diverse. The ESBL genes are generally located on plasmids, which could promote the dissemination of ESBL genes in gram-negative bacteria *(5)*. Compared with TEM and SHV, CTX-M variants are more diversified and are the most common ESBL genotype in *E. coli* isolates from food-producing animals in the People's Republic of China *(22)*. At present, the prevalent CTX-M types in *E. coli* found in the poultry of European countries include CTX-M-1, CTX-M-2, and CTX-M-14, whereas those found in food-producing animals in China are mainly CTX-M-15, CTX-M-55, CTX-M-14, and CTX-M-65 *(18, 22, 26, 30)*. The high prevalence of CTX-M–producing *E. coli* in food-producing animals in China may be because of the excessive use of antibiotics in poultry, particularly the substantial selective pressure owing to extensive use of β-lactam antibiotics.

The high prevalence of ESBL-EC in commensal *E. coli* isolates from healthy animals suggests that commensal *E. coli* may play a significant role in serving as a resistance gene reservoir. Therefore, it is necessary to know the prevalence of ESBL-EC in healthy food animals. Shandong Province is one of the largest broiler breeding provinces in China, but information on the occurrence of ESBL-EC is scarce. Hence, the goal of our study was to investigate the prevalence and drug resistance characteristics of ESBL-EC in healthy broiler chickens from Shandong Province, People's Republic of China.

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FIGURE 1. *Location of sampled sites in Shandong Province, China.*

MATERIALS AND METHODS

Sample collection. Between October 2014 and February 2015, a total of 160 fresh fecal swabs were obtained from four prefectures (Linyi, Weifang, Taian, and Zaozhuang) in Shandong Province, People's Republic of China (Fig. 1 and Table 1). The fecal swabs were kept at 4°C in an ice chest and immediately transported to our lab. Within 12 h of collecting, the swabs were processed for bacterial culture.

Isolation and identification of ESBL-EC. The fecal swabs were plated onto MacConkey plates containing cefotaxime (4 μg/ml) and cultured at 37°C for 24 h. A pure colony per sample with typical *E. coli* morphology was picked up for further characterization by Vitek MS system (bioMérieux, Inc., Marcy l'Étoile, France) and by PCR for the *uidA* gene *(13)* with ExTaq DNA polymerase (Takara, Dalian, People's Republic of China) and primers, as described previously (Table 1). Amplification conditions of the PCR were as follows: initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 in, and a final extension at 72°C for 7 min.

Confirmation of ESBL-EC. Confirmation of ESBL-EC was based on the disk diffusion method *(9)*. Briefly, two pairs of susceptibility disks containing ceftazidime (30 μg) and ceftazidime–clavulanic acid (30 and 10 μg) or cefotaxime (30 μg) and cefotaxime–clavulanic acid (30 and 10 μg) were used in the confirmation test. An *E. coli* isolate was determined to be ESBL producing when the diameter of the inhibition zone following the addition of clavulanic acid to either ceftazidime or cefotaxime was increased ≥5 mm compared with the addition of clavulanic acid alone. *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as quality control strains.

Antibiotic susceptibility testing. Susceptibility testing of ESBL-EC to 17 antimicrobial agents (Tianhe, Hangzhou, People's Republic of China) commonly used in Shandong Province was performed by the disk diffusion method *(9),* including ampicillin (10 μg), piperacillin (30 μg), cefazolin (30 μg), cefotaxime (30 μg), cefepime (30 μg), aztreonam (30 μg), ampicillin-sulbactam (10 and 10 μg), ceftazidime (30 μg), amoxicillin-clavulanate (20 and 10 μg), piperacillin-tazobactam (100 and 10 μg), tetracycline (30 μg), trimethoprim-sulfamethoxazole (1.25 and 23.75 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), gentamicin (10 μg), amikacin (30 μg), imipenem (10 μg), and meropenem (10 μg). Results of the antibiotic susceptibility testing were interpreted according to the guidelines of Clinical and Laboratory Standards Institute *(9)*. Isolates were considered multidrug resistant when they were resistant to three or more classes of antibiotics.

TABLE 1. *Primer pairs used for PCR amplification in this study*

Primer pair	Target gene	Sequence (5′–3′)	Amplicon size	Annealing temp (°C)
CTX-M group 1 F	CTX-M group 1	TTA GGA ARY GTG CCG CTG YA	415	57
CTX-M group 1 R		CGA TAT CGT TGG TGG TRC CAT		
CTX-M group 2 F	CTX-M group 2	CGT TAA CGG CAC GAT GAC	552	57
CTX-M group 2 R		CGA TAT CGT TGG TGG TRC CAT		
CTX-M group 9 F	CTX-M group 9	TCA AGC CTG CCG ATC TGG T	205	57
CTX-M group 9 R		TGA TTC TCG CCG CTG AAG		
TEM F	TEM	CAT TTC CGT GTC GCC CTT ATT C	840	56
TEM R		CGT TCA TCC ATA GTT GCC TGA C		
SHV F	SHV	AGC CGC TTG AGC AAA TTA AAC	1,051	56
SHV R		ATC CCG CAG ATA AAT CAC CAC		
uidA F	*uidA*	ATCACCGTGGTGACGCATGTCGC	147	57
uidA R		CACCACGATGCCATGTTCATCTGC		

TABLE 2. *Isolation of ESBL-EC from healthy broilers of four prefectures in Shandong Province of China*

Source of samples[a]	No. of samples	No. of ESBL-EC
LY	35	30
WF	45	43
TA	40	36
ZZ	40	33
Total	160	142

[a] LY, Linyi; WF, Weifang; TA, Taian; and ZZ, Zaozhuang.

Detection of β-lactamase genes. The DNAs of ESBL-EC isolates were extracted by the boiling lysis method *(11)*. ESBL-EC isolates were analyzed for the presence of genes encoding TEM, SHV and CTX-M by PCR *(19, 21, 27)* with ExTaq DNA polymerase (Takara) and primers, as described previously (Table 1). Amplification conditions were initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, 56 to 57°C (depending on the primers) for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min.

DNA sequencing analysis. PCR products were sequenced by using an ABI3730 Automated Sequencer (BaseClear, Leiden, The Netherlands). The reagent kit used in this process was purchased from the TianGen Limited Company, Beijing, People's Republic of China. Forward and reverse sequences were aligned with reference sequences in the BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm β-lactamase type (http://www.lahey.org/Studies).

Phylogenetic grouping of ESBL-EC. ESBL-EC was discriminated in phylogenetic groups (A, B1, B2, C, D, E, or F), as previously described by Clermont et al. *(7, 8)*.

RESULTS

Isolation and identification of ESBL-EC. A total of 142 *E. coli* isolates were collected from 160 fresh fecal samples (142 of 160, 88.8%) in four prefectures of Shandong Province in China, and all 142 *E. coli* showed the ESBL phenotype (Table 2).

Antimicrobial susceptibility of ESBL-EC. Antimicrobial susceptibility testing showed that 142 ESBL-EC isolates from four different prefectures had a more or less similar resistance trend, and these isolates were all resistant to ampicillin, piperacillin, and cefazolin. One hundred forty (98.6%), 135 (95.1%), 110 (77.5%), 82 (57.7%), 46 (32.4%), 18 (12.7%), and 12 (8.5%) of 142 ESBL-EC isolates were resistant to cefotaxime, cefepime, aztreonam, ampicillin-sulbactam, ceftazidime, amoxicillin-clavulanate, and piperacillin-tazobactam, respectively.

Of note, some of these isolates were also resistant to other classes of antibiotics: tetracycline (139 of 142, 97.9%), trimethoprim-sulfamethoxazole (130 of 142, 91.5%), ciprofloxacin (121 of 142, 85.2%), levofloxacin (104 of 142, 73.2%), gentamicin (103 of 142, 72.5%), and amikacin (56 of 142, 39.4%). However, 142 ESBL-EC isolates were all susceptible to imipenem and meropenem. In addition, 95.8% (136 of 142) of these ESBL-EC isolates were multidrug resistant.

Characterization of β-lactamase genes. All ESBL-EC isolates carried one or more of the genes from $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} families. Of these 142 ESBL-EC, $bla_{\text{CTX-M}}$ was the dominant genotype, and the most frequently detected $bla_{\text{CTX-M}}$ type was $bla_{\text{CTX-M-15}}$ (56), followed by $bla_{\text{CTX-M-65}}$ (42), $bla_{\text{CTX-M-55}}$ (36), $bla_{\text{CTX-M-14}}$ (21), $bla_{\text{CTX-M-79}}$ (1), $bla_{\text{CTX-M-3}}$ (1), $bla_{\text{CTX-M-123}}$ (1), and $bla_{\text{CTX-M-132}}$ (1). One hundred six (of 142, 74.6%) isolates were found to be positive for $bla_{\text{TEM-1}}$, and these isolates also carried CTX-M-genes. In addition, 5 ESBL-EC isolates carried $bla_{\text{SHV-5}}$, and two $bla_{\text{CTX-M}}$-type genes (CTX-M-1 and CTX-M-9) were confirmed to coexist in 17 ESBL-EC isolates. Of note, two kinds of chimeric genes were found in this study, including $bla_{\text{CTX-M-123}}$ and $bla_{\text{CTX-M-132}}$ (Table 3).

Phylogenetic grouping of ESBL-EC. Phylogenetic grouping revealed, among 142 ESBL-EC isolates, 62 (43.6%) belonged to group A, 46 (32.4%) to group D, 22 (15.5%) to group B1, and 12 (8.5%) to group B2 (Table 4).

DISCUSSION

A total of 142 ESBL-EC isolates were collected in this study, which showed relatively high resistance rates (77.5 to 100%) to β-lactam antibiotics, including ampicillin, piperacillin, cefazolin, cefotaxime, cefepime, and aztreonam. In addition, these isolates demonstrated an alarming resistance to combinations of β-lactams and β-lactamase inhibitors, with high susceptibility to ceftazidime only. One hundred forty-two ESBL-EC isolates were divided into four phylogenetic groups: group A was dominant, followed by group D, group B2, and group B1. The results are consistent with a previous investigation conducted in Northeast China *(26)*.

In this study, 136 (95.8%) of 142 ESBL-EC were multidrug resistant, which is significantly higher than the proportion (80.6%) reported from Henan Province, People's Republic of China by Yuan et al. *(29)*. These results indicate that a multidrug resistant phenomenon in chicken ESBL-EC is becoming increasingly serious. Although we could not obtain detailed information on the antibiotics added to the poultry feed, under normal circumstances, its use in the feed exerts a selective pressure on the colonization of intestinal bacteria in the animals *(10)*.

In recent years, the number of ESBL-producing gram-negative bacteria from food-producing animals has been increasing due to the extensive use of extended-spectrum β-lactam antibiotics in the broiler breeding industry of China *(14, 15, 26)*. PCR results showed that 106 ESBL-EC isolates were positive for the $bla_{\text{TEM-1}}$ gene, with a detection rate of 74.6%. This indicates that TEM-1 is a relatively common β-lactamase in chicken ESBL-EC isolated from Shandong Province in China. Of note, compared with previous studies conducted in other provinces in China *(16, 30)*, this study indicates an increasing carrier rate of TEM-1 in ESBL-EC isolated from food animals.

Bauernfeind et al. *(2)* first discovered the CTX-M–type ESBL in *E. coli*, STX-M-1, so named owing to its hydrolytic activity on cefotaxime. It has been verified that the bla_{CTM} gene originally came from bacteria of the genus *Kluyvera* *(23)*. Currently, CTX-M–type β-lactamase, the most prev-

TABLE 3. *Characteristics of β-lactamase genes among ESBL-EC isolates from healthy broilers of Shandong Province of China*[a]

β-Lactamase genes	No. of isolates (LY, WF, TA, and ZZ)[b]	No./total no. (%)
CTX-M-15	8 (1/3/2/2)	8/142 (5.6)
TEM-1+CTX-M-15	37 (8/9/11/9)	37/142 (27.5)
TEM-1+CTX-M-14+CTX-M-15	6 (1/2/2/1)	6/142 (4.2)
CTX-M-65	5 (1/1/2/1)	5/142 (3.5)
TEM-1+CTX-M-65	32 (8/9/8/7)	32/142 (22.5)
CTX-M-55+CTX-M-65	5 (1/2/0/2)	5/142 (3.5)
CTX-M-55	6 (1/2/1/2)	6/142 (4.2)
TEM-1+CTX-M-55	21 (5/6/6/4)	21/142 (14.8)
CTX-M-55+CTX-M-14	4 (1/2/0/1)	4/142 (2.8)
CTX-M-14	6 (2/2/1/1)	6/142 (4.2)
TEM-1+CTX-M-14	3 (0/1/1/1)	3/142 (2.1)
CTX-M-14+CTX-M-3	1 (0/1/0/0)	1/142 (0.7)
CTX-M-14+CTX-M-79	1 (0/0/1/0)	1/142 (0.7)
TEM-1+CTX-M-123	1 (0/1/0/0)	1/142 (0.7)
TEM-1+CTX-M-132	1 (0/1/0/0)	1/142 (0.7)
TEM-1+CTX-M-15+SHV-5	5 (1/1/1/2)	5/142 (3.5)
Total	142 (30/43/36/33)	142/142 (100.0)

[a] Two $bla_{\text{CTX-M}}$ genes (CTX-M-1 and CTX-M-9) coexist in the same ESBL-EC: $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$ (six isolates); $bla_{\text{CTX-M-55}}$ and $bla_{\text{CTX-M-65}}$ (five isolates); $bla_{\text{CTX-M-55}}$ and $bla_{\text{CTX-M-14}}$ (four isolates); $bla_{\text{CTX-M-79}}$ and $bla_{\text{CTX-M-14}}$ (one isolate); and $bla_{\text{CTX-M-3}}$ and $bla_{\text{CTX-M-14}}$ (one isolate).
[b] LY, Linyi; WF, Weifang; TA, Taian; and ZZ, Zaozhuang.

alent non-TEM and non-SHV type ESBL in *Enterobacteriaceae (6),* is the world's most common and widely disseminated ESBL. The CTX-M–type β-lactamase family shows a high degree of plasticity, and the CTX-M cluster is prevalent and is widely distributed geographically *(3)*. The CTX-M–type β-lactamases found in European countries, such as $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, and $bla_{\text{CTX-M-14}}$, are mainly associated with *E. coli* isolated from poultry *(17)*. In China, $bla_{\text{CTX-M-14}}$ is the most prevalent type of ESBL in isolates from animals, and $bla_{\text{CTX-M-55}}$ is slowly replacing $bla_{\text{CTX-M-15}}$ as the second most prevalent $bla_{\text{CTX-M}}$ gene *(22, 30)*.

In this study, all 142 ESBL-EC isolates carried $bla_{\text{CTX-M}}$ genes that were mainly $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$ from the CTX-M-1 and CTX-M-9 enzyme clusters, respectively. These data are consistent with those reported from other areas of China *(22, 26)*. We found two kinds of chimeric genes, including $bla_{\text{CTX-M-123}}$ and $bla_{\text{CTX-M-132}}$. According to He et al. *(12),* these chimeric $bla_{\text{CTX-M}}$ genes are composed of different fragments from the CTX-M-1 and CTX-M-9 gene clusters. These gene cluster fragments may have resulted from homologous recombination of two genes (CTX-M-1 and CTX-M-9 clusters) that were colocalized within the same isolate. Here, we determined that genes from the CTX-M-1 and CTX-M-9 clusters were simultaneously present in 17 ESBL-EC isolates. In fact, it has already been reported that multiple CTX-M types of β-lactamase can be present in the same isolate, which may contribute to the emergence of chimeric enzymes *(26, 29)*.

TABLE 4. *Distribution of phylogenetic groups among ESBL-EC isolates from healthy broilers of Shandong Province of China*

Phylogenetic groups	No. of isolates (LY, WF, TA, and ZZ)[a]	Percentage (LY, WF, TA, and ZZ)[a]
A	62	43.6
B1	46	32.4
B2	22	15.5
D	12	8.5
Total	142	100.0

[a] LY, Linyi; WF, Weifang; TA, Taian; and ZZ, Zaozhuang.

In the past few years, bla_{SHV} has been occasionally detected in *E. coli* isolates from animals in China, with only a few studies reporting the presence of $bla_{\text{SHV-5}}$ in *E. coli* isolates from dogs and chickens *(14, 25, 26)*. In this study, we detected the $bla_{\text{SHV-5}}$ gene in 5 ESBL-EC isolates; however, further studies are required to determine its source of origin.

In summary, 142 *E. coli* isolates from 160 fresh fecal samples (142 of 160, 88.8%) were all ESBL-EC, and their main resistance mechanism to β-lactams was through the production of TEM-1, CTX-M, and SHV-5 β-lactamases. Rational use of antibiotics in veterinary practice is necessary to reduce the prevalence and dissemination of drug resistance bacteria in food animals.

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