Molecular Characterization of Extended-Spectrum β-Lactamase-Producing Multidrug Resistant Escherichia coli From Swine in Northwest China

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Objectives: The aim of the present study was to explore the prevalence and molecular characterization of extended-spectrum β-lactamase (ESBL)-producing Escherichia coli collected from pig farms in Northwest China.

Methods: Between May 2015 and June 2017, a total of 456 E. coli isolates were collected from fecal samples of healthy and diarrheal pigs in Northwest China to screen the ESBL producers. The β-lactamases, plasmid-mediated quinolone resistance (PMQR) genes and virulence genes among ESBL producers were corroborated by PCR and sequencing. Finally, ESBL producers were further grouped according to phylogenetic background and genetic relatedness.

Results: Forty-four (9.6%) out of the 456 E. coli isolates were identified as ESBL-producing isolates. All ESBL producers exhibited multidrug resistance (MDR) phenotype, and more than 90% of the ESBL producers were resistant to amoxicillin, amoxicillin-clavulanic acid, oxytetracycline, enrofloxacin and sulfamethoxazole/trimethoprim. All ESBL producers harbored at least one type of β-lactamase, with blaCTX−M, blaTEM, blaSHV, blaOXA−48, and blaKPC−2 being detected in forty, thirty, seven, four, two and one isolates, respectively. Sequencing revealed the most common blaCTX−M subtype was blaCTX−M−14 (n = 24), followed by blaCTX−M−15 (n = 14), blaCTX−M−64 (n = 11), blaCTX−M−9 (n = 10) and blaCTX−M−123 (n = 9). qnrS (n = 23) was the predominant PMQR gene, and all PMQR genes were detected in co-existence with β-lactamase genes. estA (n = 18) and F4 (n = 18) were the most prevalent enterotoxin and fimbrial adhesin, respectively, and 27 different virotypes were found with respect to the association of enterotoxins and fimbrial adhesins. Twenty-four different sequence types (STs) were identified among 44 ESBL producers, and clones ST405, ST10 and ST648 were strongly present in more than one-third (34.1%) of ESBL producers.
INTRODUCTION

*Escherichia coli* (E. coli) is both a ubiquitous commensal bacterium in intestinal tract and an important pathogen of diarrhea or extraintestinal infections of humans and animals, and both commensal and pathogenic isolates usually share the same environment (Wu et al., 2013). Cephalosporins are effective for gram-negative bacterial infections, especially for infections caused by multidrug resistant (MDR) *E. coli* (Silva-Sanchez et al., 2013). At present, extended-spectrum β-lactams are not the first-line treatment in food animals, whereas the resistance to β-lactams, especially to the third- and fourth-generation cephalosporins has increased markedly accompanying their massive or inappropriate use over the past decades, and it is also considered as an important public health challenge (Agersø and Aarestrup, 2013). Nowadays, one of the most worrisome resistance mechanisms to β-lactams is the emergence of extended-spectrum β-lactamases (ESBLs), which could inactivate oxyimino-β-lactams like third-generation cephalosporins and aztreonam (Liu et al., 2015). Moreover, ESBLs are generally located on the transmissible plasmids, and could be acquired between bacteria by conjugation mechanism (Cantas et al., 2015). A recent study has further suggested that ESBL-producing *E. coli* isolate, along with their antibiotic resistance genes, can spread from food animals and animals-derived foods to humans via food-chain (Geser et al., 2012). Additionally, plasmid-mediated AmpC β-lactamase *bla*CMY−2, carbapenemases *bla*NDM−1, *bla*OXA−48 and *bla*KPC−2 are also increasingly described (Conceição-Neto et al., 2017; Subirats et al., 2017). As a result, the dissemination of ESBL-producing isolates poses a serious risk to both animal and human health. Furthermore, ESBL producers have been associated with resistance to non-β-lactam antimicrobials, such as fluoroquinolones, aminoglycosides and sulfonamides, which are often used long term to treat and prevent diseases on pig farms in China (Tian et al., 2009, 2012; Yuan et al., 2009). Especially, plasmid-mediated quinolone resistance (PMQR) genes are thought to be linked with ESBL production, and spread of *E. coli* co-expressing PMQRs and ESBLs could contribute to growing concerns about resistant *E. coli* isolates (Wang et al., 2012).

The prevalence of ESBL-producing *E. coli* isolates in food animals has been increasing worldwide, and they pose a serious challenge in controlling bacterial diarrhea in swine industry. However, very little data have been reported on the occurrence and various types of β-lactamases among *E. coli* from swine in Northwest China. The main purpose of this study was to screen ESBL-producing *E. coli* isolates collected from pig farms in Northwest China, and further analyze ESBL producers based on genetic relatedness, virulence profiles, and the occurrence and transferability of β-lactamase and PMQR genes.

Conclusion: All ESBL-producing *E. coli* isolates exhibited MDR phenotype, and showed high prevalence of β-lactamase and PMQR genes. Especially, one isolate harbored ESBL genes *bla*TEM, *bla*SHV, *bla*CTX-M−9, *bla*CTX-M−14, *bla*CTX-M−64, and carbapenemase gene *bla*OXA−48 and *bla*KPC−2, as well as PMQR genes *qnrS*, *qnrB*, *qnrD*, *qepA* and *aac(6′)-Ib-cr*.

Keywords: *Escherichia coli*, antibiotic resistance, β-lactamase, OXA-48, PMQR

MATERIALS AND METHODS

Sample Collection and Bacterial Culture

During May 2015 to June 2017, 456 *E. coli* isolates (270 from healthy pigs, 186 from diarrheal pigs) were isolated from fecal samples of different swine in ten pig farms, which are widely dispersed across Shaanxi and Gansu provinces. Fecal samples were collected from individual pigs using a sterile cotton swab and transported to laboratory within 12 h. All samples were immediately seeded on MacConkey agar (Beijing Land Bridge Technology Co., Ltd, Beijing, China). After incubation at 37°C for 18 to 24 h, three colonies with typical *E. coli* morphology (bright pink with a dimple) were randomly selected and transferred to Eosin Methylene Blue agar (Qingdao Hope Bio Technology Co., Ltd, Qingdao, Shandong, China) for further purification. Finally, the suspect *E. coli* isolates on Eosin Methylene Blue agar (green colonies with a metallic sheen) were subjected to biochemical tests (indole, methyl red, oxidase, citrate, and triple sugar iron) as described previously (Liu et al., 2017). All confirmed *E. coli* isolates were stored at −80°C in Tryptic Soy broth medium containing 30% glycerol for later study.

Antimicrobial Susceptibility Testing

The minimum inhibitory concentrations (MICs) of ampicillin, amoxicillin-clavulanic acid, ceftiofur, cefotaxime, ceftriaxone, ceftazidime, meropenem, enrofloxacin, ciprofloxacin, florfenicol, sulfamethoxazole/trimethoprim, gentamicin, amikacin, oxytetracycline, and colistin were determined by a standardized microdilution method following CLSI guidelines (CLSI, 2013). All MIC determinations were performed in triplicate, with *E. coli* ATCC 25922 serving as a quality control. Meanwhile, double-disk diffusion method was used to screen for the ESBL production among all isolates with cepotaxime and ceftazidime alone and in combination with clavulanic acid by using the guidelines recommended by CLSI (2013). Initial screening analyses indicated that 44 (9.6%) *E. coli* isolates were identified as phenotypic ESBL producers, which were further investigated for molecular characterization.
Phylogenetic Grouping and Virulence Genotyping

DNA from each ESBL producer was extracted using boiling method, and the distribution of phylogenetic groups of ESBL producers were determined by quadruplex PCR as described by Clermont et al. (Clermont et al., 2013). Meanwhile, enterotoxins (elt, estA, estB, stx1, stx2, and astA) and fimbrial adhesins (F4, F5, F6, F17, F18 and F41) as well as intimin encoded by eae gene were detected using single or multiplex PCR with specific primers as previously described (Boerlin et al., 2005; Toledo et al., 2012). The E. coli strains used as positive controls were B2 (eae, stx1, stx2), 256 (estA, estB), 281 (elt), G2077 (F4), B21523 (F5), J7203349 (F6), 320 (F41), and B37429 (F18), and E. coli K12 C600 was used as a negative control. Part of control strains were kindly supplied by Dr. Boothe (Auburn University, USA). The primer sequences used for PCR detection are listed in Table S1.

Identification of β-Lactamase Genes and Plasmid-Mediated Quinolone Resistance Genes

The occurrence of β-lactamase genes (blaTEM, blaSHV, blaCTX-M-16), plasmid-mediated AmpC β-lactamase (blaCMY-2) and carbapenemase genes (blaKPC-2, blaNDM-1, and blaOXA-48) among ESBL producers were determined by PCR and sequencing using specific primers (Table S2). The PCR products were purified using a PCR Purification Kit (TianGen, Beijing, China), and then the amplified products were sequenced by Sangon Biotech (Shanghai, China). DNA Sequences were compared with known sequences available from the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1997). Additionally, all ESBL producers were screened for the presence of PMQR genes (qnrA, qnrB, qnrC, qnrD, qnrS, aac(6′)-Ib-cr, oqxAB, and qepA) as described previously (Liu et al., 2012; Xu et al., 2015). E. coli J53 strains containing pMG252, pMG298, pMG306, and pMG298 were used as positive controls for qnrA, qnrB, qnrS, and aac(6′)-Ib-cr genes, respectively. E. coli J7261205 (pSTVqepA) and S5314175 were included as positive controls for qepA and oqxAB, respectively. The positive control strain for qnrC was not available.

Conjugation Experiments

In order to analyze the horizontal transferability of β-lactamase and PMQR genes, especially blaOXA-48 gene, conjugation experiments were performed with eight ESBL-producing E. coli isolates, including four blaOXA-48 positive isolates, from different pig farms in seven different regions. Conjugation experiments were conducted by broth mating method using E. coli J53 AZ′ as a recipient (Shaheen et al., 2011). Transconjugants were selected on Tryptic Soy agar plates containing sodium azide (150 μg/ml) and cefotaxime (2 μg/ml). All transconjugants, recipient and donors were subjected to antimicrobial susceptibility testing. PCR and sequencing were performed to verify the transferability of PMQR and β-lactamase genes.

Multilocus Sequence Typing (MLST)

MLST of ESBL-producing E. coli isolates was performed as described previously (Wirth et al., 2006). A detailed scheme of gene amplification, allelic type and sequence type assignment methods is available on the MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

Statistical Analysis

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

RESULTS

Antimicrobial Susceptibility of E. coli Isolates

The results of antibiotic resistance profiles of 456 E. coli isolates are listed in Table 1. 96.1% of the E. coli isolates were resistant to ampicillin, followed by amoxicillin-clavulanic acid (91.2%), sulfamethoxazole/trimethoprim (82%), oxytetracycline (74.3%), enrofloxacin (70%), gentamicin (61.4%), florfenicol (58.8%), ciprofloxacin (57.9%), and amikacin (52.2%). The percentage of resistance to other antibacterial agents were lower than 50%. It is noteworthy that significantly more E. coli isolates from diarrheal pigs than from healthy pigs were resistant to most antimicrobials tested (P < 0.001) with the exception of ampicillin, sulfamethoxazole/trimethoprim and colistin (Table 1). Of 456 E. coli isolates investigated, 44 isolates (9.6%, six isolates from healthy pigs and 38 from diarrhea pigs) were confirmed as phenotypic ESBL producers, and exhibited MDR phenotype. 97.7% of the ESBL producers were resistant to ampicillin, followed by oxytetracycline (93.2%), amoxicillin-clavulanic acid (93.2%), enrofloxacin (93.2%), sulfamethoxazole/trimethoprim (90.9%), ceftazidime (86.4%), cefotaxime (84.1%) and gentamicin (81.8%).

Phylogenetic Typing and Virulence Genotyping

Phylogenetic group analysis for 44 ESBL producers revealed that the predominant phylogenetic group was D (14/44, 31.8%), followed by phylogenetic groups B2 (11/44, 25%), A (9/44, 20.5%), B1 (6/44, 13.6%), C (3/44, 6.8%), and E (1/44, 2.3%) (Table 2). Groups D and B2 accounted for 56.8% of the ESBL producers. The frequencies of major virulence genes are listed in Table 2. 93.2% of the ESBL producers possessed at least one virulence gene. estA (n = 18) was the most prevalent toxin gene, followed by estB (n = 15), astA (n = 12), and elt (n = 10) genes. The most prevalent fimbrial adhesin was F4 (n = 18), followed by F18 (n = 10), F17 (n = 4), F5 (n = 3), F6 (n = 3) and F41 (n = 2). Furthermore, 86.4% (38/44) of the ESBL producers carried both enterotoxins and fimbrial adhesins, and 27 different virotypes were identified according to the combinations of enterotoxin and adhesin genes. The eae gene was detected in two ESBL producers (4.5%), while stx1 and stx2 were not detected.
Table 1: Antibiotic resistance profiles of *E. coli* isolates from swine in Northwest China.

| Antimicrobials | Number of resistant isolates (%) | MIC (µg/ml) | Number of resistance (%) | MIC₅₀ | MIC₉₀ | Range | Number of resistance (%) | MIC₅₀ | MIC₉₀ | Range | Number of resistance (%) | MIC₅₀ | MIC₉₀ |
|----------------|----------------------------------|-------------|--------------------------|-------|-------|-------|--------------------------|-------|-------|-------|--------------------------|-------|-------|
| Ampicillin     | 438 (96.1)                       | 1–256       | 252 (93.3)               | 64    | 256   | 1–256 | 186 (100)                | 256   | 512   | 4–512 | 256 (97.7)               | 512   |
| Amoxicillin    | 416 (91.2)                       | 1–512       | 238 (88.1)               | 32    | 256   | 1–256 | 178 (95.7)               | 128   | 512   | 4–512 | 41 (93.2)                | 256   | 512   |
| Ceftiofur      | 177 (38.8)                       | 0.063–64    | 56 (20.7)                | 0.5   | 16    | 0.5   | 121 (65.1)               | 64    | 128   | 0.5   | 32 (72.7)                | 128   | 256   |
| Cefotaxime     | 184 (40.4)                       | 0.25–128    | 51 (18.9)                | 0.25  | 32    | 0.25  | 133 (71.5)               | 64    | 256   | 0.25  | 43 (86.4)                | 64    | 256   |
| Cefazidime     | 198 (41.2)                       | 0.5–256     | 56 (21.5)                | 2     | 32    | 2     | 130 (69.9)               | 322   | 256   | 2     | 30 (51.2)                | 128   | 256   |
| Ceftrizone     | 165 (36.2)                       | 0.125–128   | 48 (17.8)                | 0.5   | 32    | 0.5   | 117 (62.9)               | 64    | 128   | 0.5   | 121 (65.1)               | 64    | 256   |
| Meropenem      | 3 (0.7)                          | 0.03–4      | 0 (0)                    | 0.125 | 0.5  | 0.03–16 | 3 (6.8)                | 0.25 | 1     | 0.03–16 | 3 (6.8)                | 0.25 | 1     |
| Enrofloxacin   | 319 (70)                         | 0.125–256   | 150 (55.6)               | 16    | 64    | 0.063–32 | 169 (90.9)            | 128  | 512   | 2–512 | 41 (93.2)                | 128  | 256   |
| Ofloxacin      | 264 (57.9)                       | 0.063–128   | 128 (47.4)               | 8     | 32    | 0.063–32 | 136 (73.1)            | 64   | 256   | 1–512 | 32 (72.7)                | 128  | 256   |
| Florfenicol    | 268 (58.8)                       | 1–256       | 124 (45.9)               | 16    | 64    | 8–512 | 144 (71.4)               | 256   | 512   | 8–512 | 35 (79.5)                | 256  | 512   |
| Gentamicin     | 280 (61.4)                       | 0.125–128   | 134 (49.6)               | 16    | 128   | 0.5–512 | 146 (78.5)            | 64   | 256   | 0.5–512 | 36 (81.8)                | 128  | 256   |
| Amikacin       | 238 (52.2)                       | 0.063–64    | 121 (44.8)               | 8     | 64    | 0.063–512 | 117 (62.9)            | 64   | 256   | 0.063–32 | 31 (70.5)                | 128  | 256   |
| Oxytetracycline| 339 (74.3)                       | 1–512       | 169 (62.6)               | 32    | 256   | 1–512 | 170 (91.4)               | 256   | 512   | 2–512 | 41 (93.2)                | 256  | 512   |
| Colistin       | 1 (0.2)                          | 0.03–0.5    | 0 (0)                    | 0.03  | 0.125 | 0.063–8 | 1 (0.5)                | 0.125 | 0.5  | 0.063–8 | 1 (0.5)                | 0.125 | 0.5  |
| Sulfamethoxazole| 374 (82)                        | 0.25–256    | 214 (79.3)               | 64    | 256   | 1–512 | 160 (86.0)               | 32   | 256   | 2–512 | 40 (90.9)                | 128  | 256   |

P-value:
- Isolates from healthy pigs vs. Isolates from diarrheal pigs
- ESBL-producing isolates

- Ampicillin: >0.05
- Amoxicillin: <0.01
- Ceftiofur: <0.001
- Cefotaxime: <0.001
- Cefazidime: <0.001
- Ceftrizone: <0.001
- Meropenem: <0.001
- Enrofloxacin: <0.001
- Oxytetracycline: <0.001
- Colistin: >0.05
- Sulfamethoxazole: >0.05
### TABLE 2 | Extended-spectrum β-lactamase-producing E. coli isolates from swine in Northwest China.

| Isolate ID | Phylogenetic group | Sources | MLST | Resistance profiles | β-lactamase genes | PMQR genes | Virulence genes |
|------------|-------------------|---------|------|---------------------|------------------|------------|----------------|
| FF170322   | A                 | Diarrheal pig | ST10 | AMP AMC EFT CAZ CEX ENR CIP FFC SXT | TEM-1, CTX-M-9, CTX-M-123 | qnrA, qnrB, qepA, oqxAB | estA, astA, F18 |
| JY160633   | A                 | Healthy pig  | ST10 | AMP AMC CAZ ENR CIP FFC OTC GEN | TEM-1, CTX-M-9, CTX-M-123 | qnrS, aac(6')-Ib-cr | estA, F17 |
| FP170743   | A                 | Diarrheal pig | ST10 | AMP AMC EFT CTX OEX ENR CIP FFC OTC SXT | TEM-1, CTX-M-14, CTX-M-123 | qnrS, qnrA | estB, F18 |
| FP170756   | A                 | Diarrheal pig | ST10 | AMP AMC EFT CTX CAZ CEX ENR CIP FFC OTC GEN AMK SXT | TEM-1, CTX-M-15 | qnrS, qnrA | estA, F5 |
| MX161024   | A                 | Diarrheal pig | ST10 | AMP AMC EFT CAZ ENR CIP FFC OTC GEN AMK SXT | TEM-1, CTX-M-15 | qnrA | astA, F4 |
| RX160912   | A                 | Healthy pig  | ST10 | AMP AMC CTX OTC GEN AMK | TEM-1, CTX-M-14 | qnrA | estA, F18 |
| FP160905   | A                 | Healthy pig  | ST15 | AMP AMC OTC ENR SXT | SHV-12 | _ | _ |
| JY170718   | A                 | Diarrheal pig | ST526 | AMP AMX OTC CAZ FFC | TEM-1, CTX-M-14 | qnrA | estA, F18 |
| RX160826   | B1                | Healthy pig  | ST75 | AMP AMC CAZ FFC OTC | TEM-1, CTX-M-15 | qnrS | estA, F17 |
| ZZ160931   | B1                | Healthy pig  | ST155 | AMP AMC CTX OTC GEN AMK | TEM-1, CTX-M-64 | _ | _ |
| ZZ170521   | B1                | Diarrheal pig | ST183 | AMP AMC CTX CAZ ENR OTC SXT | TEM-1, CTX-M-14 | qnrA | estA, F6 |
| FP170723   | B1                | Healthy pig  | ST302 | AMP AMC CAZ ENR SXT | CTX-M-123 | _ | _ |
| JY170327   | B1                | Diarrheal pig | ST351 | AMP AMC OTC ENR SXT | TEM-1, CTX-M-14 | qnrA | estA, astA, F6 |
| FF170447   | B1                | Healthy pig  | ST447 | AMP AMC OTC ENR SXT | CTX-M-14 | _ | _ |
| MX160918   | B2                | Diarrheal pig | ST104 | AMP AMC EFT CTX OTC GEN AMK SXT | TEM-1, CTX-M-14 | qnrS, aac(6')-Ib-cr | astA, estA, F4 |
| JY160522   | B2                | Healthy pig  | ST104 | AMP AMC EFT CTX OTC GEN AMK SXT | TEM-1, CTX-M-14 | qnrS, aac(6')-Ib-cr | estA, F4, F6 |
| RX160809   | B2                | Diarrheal pig | ST127 | AMP AMC EFT CTX OTC GEN AMK SXT | TEM-1, CTX-M-123 | qnrS | estB, F6 |
| FF170708   | B2                | Diarrheal pig | ST127 | AMP AMC EFT CTX OTC GEN AMK SXT | CTX-M-123 | _ | estB, F5 |
| JY160505   | B2                | Diarrheal pig | ST127 | AMP AMC EFT CTX OTC GEN AMK SXT | CTX-M-9, CTX-M-14, CTX-M-64, KPC-2, OXA-48 | qnrS, qnrB, qnrD, aac(6')-Ib-cr, qepA, elt | estA, F6 |
| MX150822   | B2                | Diarrheal pig | ST278 | AMP AMC EFT CTX OTC GEN AMK SXT | TEM-1, CTX-M-14 | qnrS | astA, estB, F4 |
| FF170416   | B2                | Diarrheal pig | ST356 | AMP AMC EFT CTX OTC GEN AMK SXT | SHV-2, CTX-M-64 | qnrB | estA, F4 |
| FF170425   | B2                | Diarrheal pig | ST356 | AMP AMC EFT CTX OTC GEN AMK SXT | TEM-1, CTX-M-15, CTX-M-64 | _ | astA, F18 |
| MX160918   | B2                | Diarrheal pig | ST104 | AMP AMC EFT CTX OTC GEN AMK SXT | TEM-1, CTX-M-14 | qnrS, aac(6')-Ib-cr | astA, estA, F4 |
| JY160522   | B2                | Healthy pig  | ST104 | AMP AMC EFT CTX OTC GEN AMK SXT | TEM-1, CTX-M-14 | qnrS, aac(6')-Ib-cr | estA, F4, F6 |
| RX160809   | B2                | Diarrheal pig | ST127 | AMP AMC EFT CTX OTC GEN AMK SXT | TEM-1, CTX-M-123 | qnrS | estB, F6 |
| FF170708   | B2                | Diarrheal pig | ST127 | AMP AMC EFT CTX OTC GEN AMK SXT | CTX-M-123 | _ | estB, F5 |
| JY160505   | B2                | Diarrheal pig | ST127 | AMP AMC EFT CTX OTC GEN AMK SXT | CTX-M-9, CTX-M-14, CTX-M-64, KPC-2, OXA-48 | qnrS, qnrB, qnrD, aac(6')-Ib-cr, qepA, elt | estA, F6 |
| MX150822   | B2                | Diarrheal pig | ST278 | AMP AMC EFT CTX OTC GEN AMK SXT | TEM-1, CTX-M-14 | qnrS | astA, estB, F4 |
| FF170416   | B2                | Diarrheal pig | ST356 | AMP AMC EFT CTX OTC GEN AMK SXT | SHV-2, CTX-M-64 | qnrB | estA, F4 |
| FF170425   | B2                | Diarrheal pig | ST356 | AMP AMC EFT CTX OTC GEN AMK SXT | TEM-1, CTX-M-15, CTX-M-64 | qnrS, aac(6')-Ib-cr | estB, F4, F18 |
| FF170416   | B2                | Diarrheal pig | ST356 | AMP AMC EFT CTX OTC GEN AMK SXT | TEM-1, CTX-M-15, CTX-M-123 | qnrS, aac(6')-Ib-cr | estA, F4 |
| MX150923   | C                 | Diarrheal pig | ST23 | AMP AMC CTX OTC ENR CAZ SXT | TEM-1, CTX-M-14 | qnrS, qnrA | estA, F18 |
| MX150814   | C                 | Diarrheal pig | ST23 | AMP AMC EFT CTX CAZ ENR CIP FFC OTC GEN SXT | TEM-1, CTX-M-14 | qnrB, aac(6')-Ib-cr | elt, F4 |
| ZZ160931   | C                 | Healthy pig  | ST23 | AMP AMC EFT CTX CAZ ENR CIP FFC OTC GEN SXT | TEM-1, CTX-M-14 | qnrS | astA, F18 |
| JY160518   | D                 | Diarrheal pig | ST38 | AMP AMC EFT CTX OTC GEN AMK SXT | TEM-1, CTX-M-15, OXA-48 | qnrS, qnrA | elt, estB, F4 |

(Continued)
| Isolate ID | Phylogenetic group | Sources | MLST | Resistance profiles | β-lactamase genes | PMQR genes | Virulence genes |
|------------|--------------------|---------|------|---------------------|-------------------|------------|----------------|
| HX160976   | D                  | Diarrhea pig | ST38  | AMP AMC EFT CTX CAZ CEX ENR CIP FFC OTC GEN AMK SXT | CTX-M-9, CTX-M-14 | qnrA | ett, estB, F41 |
| HX160944   | D                  | Diarrhea pig | ST38  | AMP AMC EFT CTX CAZ CEX ENR CIP FFC OTC GEN AMK SXT | CTX-M-9, CTX-M-14, CTX-M-64 | qnrB | ett, astA, F4 |
| HX161006   | D                  | Diarrhea pig | ST69  | AMP AMC EFT CTX CAZ CEX ENR CIP FFC OTC GEN AMK SXT | TEM-1, CTX-M-14 | qnrS, qnrA | astA, F5 |
| MX150820   | D                  | Diarrhea pig | ST405 | AMP AMC EFT CTX CAZ MEM ENR CIP FFC OTC GEN AMK SXT | SHV-12, CTX-M-14, CTX-M-15, NDM-1 | qnrB, aac(6')-Ib-cr | ett, estA, F4 |
| LZ161015   | D                  | Diarrhea pig | ST405 | AMP AMC EFT CTX CAZ ENR CIP FFC OTC GEN AMK SXT | SHV-12, CTX-M-15 | qnrS, qnrB | astB, F4 |
| JC160611   | D                  | Diarrhea pig | ST405 | AMP AMC EFT CTX CAZ ENR CIP FFC OTC GEN AMK SXT | TEM-1, CTX-M-9, KPC-2 | qnrS, qnrB | estB, F4 |
| JY160512   | D                  | Diarrhea pig | ST405 | AMP AMC EFT CTX CAZ ENR CIP FFC OTC GEN AMK SXT | TEM-1, CTX-M-9, CTX-M-14, CTX-M-15 | qnrA, qnrD, qepAB | ett, estA, F4 |
| FP170711   | D                  | Diarrhea pig | ST405 | AMP AMC EFT CTX CAZ ENR CIP FFC OTC GEN AMK SXT | TEM-1, SHV-12, CTX-M-15, OXA-48 | aac(6')-Ib-cr, aqxAB | ett, estB, F4 |
| HX170832   | D                  | Diarrhea pig | ST405 | AMP AMC EFT CTX CAZ ENR CIP FFC OTC GEN AMK SXT | TEM-1, SHV-12, CTX-M-15, OXA-48 | aac(6')-Ib-cr | ett, estB, F4 |
| SY160832   | D                  | Diarrhea pig | ST648 | AMP AMC EFT CTX CAZ ENR CIP FFC OTC GEN AMK SXT | TEM-1, CTX-M-9, CTX-M-14, CTX-M-123 | qnrA, qepAB | ettB, astA, F17 |
| ZZ160908   | D                  | Diarrhea pig | ST648 | AMP AMC EFT CTX CAZ ENR CIP FFC OTC GEN AMK SXT | TEM-1, CTX-M-9, CTX-M-14, CTX-M-15 | qnrA | estB, astA, F41 |
| ZZ160917   | D                  | Diarrhea pig | ST648 | AMP AMC EFT CTX CAZ ENR CIP FFC OTC GEN AMK SXT | TEM-1, CTX-M-9, CTX-M-14, CTX-M-64 | qnrS | astA, F18 |
| JY160865   | D                  | Diarrhea pig | ST648 | AMP AMC EFT CTX CAZ ENR CIP FFC OTC GEN AMK SXT | TEM-1, CTX-M-9, CTX-M-14, CTX-M-64 | qnrS | estB, F18 |
| FP170733   | E                  | Diarrhea pig | ST350 | AMP AMC CTX CAZ ENR OTC SXT | TEM-1, CTX-M-9, CTX-M-14, CTX-M-64 | qnrS | aae |

AMP, ampicillin; AMC, amoxicillin-clavulanic acid; EFT, ceftiofur; CTX, ceftaxime; CAZ, ceftazidime; CEB, ceftriaxone; MEM, meropenem; ENR, enrofloxacin; CIP, ciprofloxacin; FFC, florfenicol; OTC, oxytetracyline; GEN, gentamicin; AMK, amikacin; SXT, sulfamethoxazole-trimethoprim; CLT, colistin.
Characterization of ESBL and PMQR Genes

Each ESBL producer harbored at least one β-lactamase gene, *blaCTX-M*, *blaTEM*, *blaSHV*, *blaOXA-48*, *blaKPC-2*, and *blaNDM-1* were detected in forty (90.9%), thirty (68.2%), seven (15.9%), four (9.1%), two (4.5%), and one (2.3%) isolates, respectively (Table 2). AmpC β-lactamase gene *blaCMY-2* was not detected. Overall, *blaCTX-M-14* (n = 24) was the predominant genotype in *blaCTX-M* positive isolates, followed by *blaCTX-M-15* (n = 14), *blaCTX-M-64* (n = 11), *blaCTX-M-9* (n = 10) and *blaCTX-M-123* (n = 9), while *blaCTX-M-1* gene was not detected. The distribution of PMQR genes among 44 ESBL-producing *E. coli* isolates is shown in Table 2. 88.6% (39/44) of ESBL producers were found to harbor at least one PMQR gene, and seven types of PMQR were identified. *qnrS, qnrA, aac(6′)-Ib-cr, qnrB, oqxAB, qnrD,* and *qepA* were detected alone or in combination in 52.3% (24/44), 34.1% (15/44), 27.3% (12/44), 20.5% (9/44), 6.8% (3/44), 4.5% (2/44), and 4.5% (2/44) of ESBL-producing isolates, respectively. *qnrS* was the most common PMQR gene, and *qnrS-qnrA* was the most common combination (n = 6). No isolates were positive for *qnrC* gene. Among 39 PMQR positive isolates, 28 (80%) isolates were positive for more than one PMQR determinant. Furthermore, all PMQR genes were detected in co-existence with β-lactamases, and one isolate from the intestinal content of a 15-day-old dead piglet with serious diarrhea harbored β-lactamase genes *blaTEM, blaSHV*, *blaCTX-M-9*, *blaCTX-M-14*, *blaCTX-M-64* and carbapenemase gene *blaOXA-48* and *blaKPC-2*, as well as PMQR genes *qnrS, qnrB, qnrD, qepA,* and *aac(6′)-Ib-cr*. *blaOXA-48* gene was detected in four meropenem-non-susceptible or meropenem-resistant isolates.

Conjugation Experiments

Five out of eight ESBL producers successfully transferred the β-lactamase genes to recipient strain *E. coli* J53 AZ. PCR analysis showed that the presence of respective β-lactamase genes, including one *blaOXA-48*-carrying plasmids from all transconjugants. Accordingly, PMQR genes *qnr* and *aac(6′)-Ib-cr* were co-transferred with β-lactamase genes (Table 3). Antimicrobial susceptibility patterns showed that all donors and their transconjugants were resistant to amoxicillin-clavulanic acid, ampicillin, ceftiofur, cefotaxime, and all transconjugants exhibited an increase of at least 8-fold in MICs compared to the recipient, *E. coli* J53 AZ. The enrofloxacin MICs for four transconjugants harboring *aac(6′)-Ib-cr* ranged from 0.125 to 0.5 mg/L, representing an increase of 4-fold to 8-fold compared with the recipient (Table 3). Additionally, the transconjugants remained susceptible to meropenem, enrofloxacin, florfenicol, oxytetracycline, gentamicin, sulfamethoxazole-trimethoprim and colistin, whereas one *blaOXA-48* positive transconjugant reduced meropenem susceptibility.

MLST Profiles

Forty-four ESBL producers belonged to 24 sequence types (STs) (Table 2). The most prevalent was ST405 (n = 6), followed by ST10 (n = 5). ST405 (n = 6), ST648 (n = 4) and ST38

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**TABLE 3** Antimicrobial susceptibility profiles of extended-spectrum β-lactamase-producing *E. coli* isolates used in the conjugation experiments.

| DONORS          | Isolates | AMP | AMC | EFT | CTX | CAZ | CEX | MEM | ENR | FFC | OTC | GEN | SXT | CLT | TEM | SHV | CTX-M-15 | CTX-M-14 | CTX-M-9 | OXA-48 | qnr | qepA | aac(6′)-Ib-cr | qnrB | qnrD |
|-----------------|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--------|----------|--------|--------|------|----|----------------|------|------|
| JY160503        | JY160512 |     |     |     |   8 | 128 | 64  | 128 | 64  | 32  | 128 | 64  | 128 | 64  |     |       |        |         | +     | +     | +     | +    | +  | +              | +    | +    |
| FP170711        | ZZ160908 |     |     |     | 16  | 64  | 128 | 128 | 64  | 64  | 128 | 64  | 128 | 32  |     |       |        |         | +     | +     | +     | +    | +  | +              | +    | +    |
| MX150820        | JY160503 | + 4 | + 5 | + 4 |     |     |     |     |     |     |     |     |     |     |     |     |     | +      | +       | +      | +      | +    |    |                  |      |      |
| JY160512        | ZZ160908 | + 4 | + 5 | + 4 |     |     |     |     |     |     |     |     |     |     |     |     |     | +      | +       | +      | +      | +    |    |                  |      |      |

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**August 2018 | Volume 9 | Article 1756**
(n = 3) of phylogenetic group D accounted for 29.5% of the ESBL producers. The carbapenemases bla_{OXA−48}, bla_{NDM−1} and bla_{KPC−2} were connected with sequence types ST405, ST131 and ST38. The isolates with same STs have similar virotypes and β-lactamase profiles.

DISCUSSION

The prevalence of ESBL-producing E. coli isolates in food animals has been increasing worldwide (Liebana et al., 2013). In China, diarrhea caused by pathogenic E. coli, especially ESBL-producing E. coli poses a serious threat to the swine industry and public health (Lei et al., 2010; Xu et al., 2015). The present study is the first contribution to explore the detailed characterizations of ESBL-producing E. coli isolates from pigs in Northwest China. Forty-four (9.6%) isolates were confirmed as ESBL producers, while it is noteworthy that 456 E. coli in this study isolated from feces of healthy and diarrheal pigs, and the prevalence of ESBL producer were significantly higher among isolates from diarrheal pigs than that form healthy isolates (20.4vs. 2.2%; P < 0.001). The detectable rate of ESBL producer in diarrheal pigs was similar with the result in Sichuan (26.8%), a neighbor province of Shaanxi, while it was significantly lower than in Heilongjiang (43.2%), a province in the northeast China (Tian et al., 2009; Xu et al., 2015). Moreover, our results showed that ESBL producers mainly belonged to phylogenetic groups D and B2, and to a lesser extent to phylogenetic A, while the previous studies showed that E. coli from pigs or duck in China also mainly fell into phylogenetic groups A (Wang et al., 2010; Ma et al., 2012). It is further confirmed that the emergence of ESBL-producing E. coli has a geographic variation with respect to demographic, environmental, behavioral, socioeconomic and infectious risk factors with the extending of ESBL-producing isolates stage by stage.

All 456 E. coli isolates in this study were tested for their susceptibility to 15 antimicrobial agents. Overall, the number of resistant isolates in ESBL producers and isolates from diarrheal pigs were higher than that from healthy pigs (P > 0.001). It is suggested that the isolates from diarrheal pigs may be more likely to develop antibiotic resistance than that from healthy pigs because of the frequent use of antimicrobials in preventing and treating diarrhea. All ESBL producers were resistant to at least five antimicrobial agents, and vast majority of them (>93%) remained susceptible to meropenem and colistin, which are considered the effective candidates for treatment of serious infections caused by E. coli in pig farms of China. According to the virotypes, 86.4% of ESBL producers carried both enterotoxins and fimbrial adhesins. It is indicated that these isolates should be enterotoxigenic E. coli (ETEC), which are responsible for neonatal diarrhea and postweaning diarrhea in piglets. F4 fimbrial adhesin was present 40.9% of the ESBL producers, it is consistent with the previous studies that F4 adhesin gene is one of the most frequently found genes in E. coli isolates from suckling and weaning piglets (Vu Khac et al., 2006; Zhang et al., 2007). Furthermore, the gene combinations of F4+estA/estB were present in 34.1% of the isolates.

Since the early 2000s, CTX-M-type ESBLs have been increasingly reported, and they have now replaced TEM and SHV as the most common type of ESBL (Barguigua et al., 2011). The most predominant ESBL gene in this study was bla_{CTX−M} (90.9%), and the similar findings showed that CTX-Ms accounted for 87.1% of ESBL-producing E. coli isolated from food animals based on a previous survey in China (Rao et al., 2014). bla_{CTX−M−14} remained the most common genotype, and followed by bla_{CTX−M−15}. It is surprising that no isolate contained bla_{CTX−M−1}, whereas it was detected in the ESBL-producing E. coli from dogs, retail pork and water bodies in Shaanxi province (Xi et al., 2015; Liu et al., 2016b). In regards to the linkage of phylogenetic group and β-lactamases, isolates of group D harbored more β-lactamases genes, and isolates of group A harbored less β-lactamases. Novel hybrid β-lactamase gene bla_{CTX−M−123} was firstly discovered in E. coli from pig feces in China in 2013 (He et al., 2013), and it was detected in nine ESBL producers in this study. Moreover, four ESBL producers were commensal isolates from healthy pigs, it was further indicated that some commensal organisms in animals have acquired β-lactamase genes with the increasing use of β-lactams in animals. bla_{OXA−48} was detected in four ESBL producers from diarrheal pigs. As a globally emerging carbapenemase gene, bla_{OXA−48} could hydrolyze carbapenems and β-lactamase inhibitors but has no activity toward broad-spectrum cephalosporins (Mathers et al., 2013). bla_{OXA−48} was firstly discovered in E. coli from dogs in Germany in 2013, and afterward it was reported in E. coli from companion animals in the United States in 2016 (Stolle et al., 2013; Liu et al., 2016a). In 2017, it was reported in pigs in Italy (Pulss et al., 2017). Most recently, it was detected in Enterobacteriaceae from river water in Algeria (Tafoukt et al., 2017). Considering the importance of bla_{OXA−48} gene in public health, it is necessary to further investigate the dissemination of bla_{OXA−48} producing E. coli isolates among different sources.

PMQR genes were often found to be strongly associated with β-lactamase genes and even in the same plasmid, and they are not merely able to confer resistance against quinolones but also often related to ESBLs (Jeong et al., 2011). In this study, PMQR genes were present in 88.6% of ESBL producers, and the similar findings have been reported in ESBL-producing E. coli isolates from pigs in previous studies in China by Xu et al. (87.4%) and Liu et al (83.8%) (Liu et al., 2013; Xu et al., 2015). Thirty-seven ESBL producers (84.1%) harbored at least one qnr gene, and qnrS was the predominant, whereas a low prevalence of qnr genes was detected among ESBL-producing E. coli isolates in France and Canada (1.6 and 1%, respectively). In addition, qepA gene was detected in combination with other PMQR and β-lactamase genes in four isolates (10%). The frequent combination of β-lactamases and PMQRs in this study further supported the previous studies that coproduction of β-lactamase and PMQR genes could contribute to the dissemination of MDR isolates, and also reflect the fact that genes encoding resistance to β-lactams and quinolones are located on the same plasmid.

Twenty-four different sequence types were identified, and three sequence types (ST405, ST10, and ST648) accounted for
34.1% of the ESBL producers. Sequence types ST10, ST38, ST131, ST648, and ST405 clones were documented in different sources according to MLST databases, and could favor the dissemination of CTX-M worldwide among E. coli isolates (Hernandez and Gonzalez-Acuna, 2016). In the present study, a few isolates belonging to different STs shared similar β-lactamase and PMQR gene profiles, whereas several isolates belonging to same ST exhibited different gene profiles. The similar results were observed among E. coli isolates from dogs and cats in previous studies (Liu et al., 2016a,b). The possible explanation is that the pig trade, personnel exchanges and water sources among adjacent regions may lead to the dissemination of isolates with same gene profiles or same ST types. Anyway, deeper analyses for such isolates are necessary in the future. It is noteworthy that blaOXA-48 gene were detected in four isolates with reduced susceptibility or resistance to meropenem. The blaOXA-48 positive isolates co-harbored variants of β-lactamase genes, and they also were associated with sequence types ST38, ST405, and ST131. Additionally, blaOXA-48 positive E. coli clone ST38 had been previously reported in France, Germany and Algeria (Poirel et al., 2011; Kaase et al., 2016; Bouaziz et al., 2017). In the current study, we firstly reported the occurrence of blaOXA-48 positive E. coli clone ST38 from a sucking piglet with diarrhea in Shaanxi. Clone ST38 has been noticed as it is now rapidly and globally disseminated, and its potential to serve as a vehicle for spread of carbapenemases is profoundly alarming. blaNDM-1 producing E. coli isolate, emerging as a public health threat, has gained global attention as it could hydrolyze almost all β-lactams with the exception of aztreonam (Nordmann et al., 2012), and it has previously been detected in E. coli isolates from pigs (Fischer et al., 2012). Our results revealed that blaNDM-1 and other β-lactamase genes coexisted in one isolates, it is a potential public health concern as the pig carrying blaNDM-1 and other β-lactamase genes may enter the food chain.

**CONCLUSION**

In conclusion, all ESBL-producing E. coli isolates both from healthy and diarrheal pigs in Northwest China exhibited MDR phenotype. The blaCTX-M-14 and qnrS were the predominant β-lactamase gene and PMQR gene in ESBL producers, respectively. estA and F4 were the most prevalent enterotoxin and fimbrial adhesin, respectively. One ST131 isolate harbored β-lactamase genes blaTEM, blaSHV, blaCTX-M-9, blaCTX-M-14, blaCTX-M-64, and carbapenemase genes blaOXA-48 and blaKPC-2, as well as PMQR genes qnrS, qnrB, qnrD, qepA and aac(6’)-Ib-cr. The findings could provide useful information for a national monitoring of antimicrobial resistance in bacteria from food animals in China.

**AUTHOR CONTRIBUTIONS**

XL conceived and designed the experiments. HL and LW designed the experiment and drafted the manuscript. XL, HL, LW, HZ, and QP performed the experiments. XL, YL, and QL analyzed and explained the data for the work. All authors critically revised and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01756/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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