IncA/C Plasmid-Mediated Spread of CMY-2 in Multidrug-Resistant Escherichia coli from Food Animals in China

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Abstract

Objectives: To obtain a broad molecular epidemiological characterization of plasmid-mediated AmpC β-lactamase CMY-2 in Escherichia coli isolates from food animals in China.

Methods: A total of 1083 E. coli isolates from feces, viscera, blood, drinking water, and sub-surface soil were examined for the presence of CMY-2 β-lactamases. CMY-2-producing isolates were characterized as follows: the blaCMY-2 genotype was determined using PCR and sequencing, characterization of the blaCMY-2 genetic environment, plasmid sizing using S1 nuclease pulsed-field gel electrophoresis (PFGE), PCR-based replicon typing, phylogenetic grouping, XbaI-PFGE, and multilocus sequence typing (MLST).

Results: All 31 CMY-2 producers were only detected in feces, and presented with multidrug resistant phenotypes. All CMY-2 strains also co-harbored genes conferring resistance to other antimicrobials, including extended spectrum β-lactamases genes (blaCTX-M-14 or blaCTX-M-53), plasmid-mediated quinolone resistance determinants (qnr, oqxA, and aac(6’)-Ib-cr), floR and rmtB. The co-transferring of blaCMY-2 with qnrS1 and floR (alone and together) was mainly driven by the Inc A/C type plasmid, with sizes of 160 or 200 kb. Gene cassette arrays inserted in the class 1 or class 2 integron were amplified among 12 CMY-2 producers. CMY-2 producers belonged to avirulent groups B1 (n = 12) and A (n = 11), and virulent group D (n = 8). There was a good correlation between phylogenetic groups and sequence types (ST). Twenty-four STs were identified, of which the ST complexes (STC) 101/B1 (n = 6), STC10/A (n = 5), and STC155/B1 (n = 3) were dominant.

Conclusions: CMY-2 is the dominant AmpC β-lactamase in food animals and is associated with a transferable replicon IncA/C plasmid in the STC101, STC10, and STC155 strains.

Introduction

The prevalence of plasmid-encoded AmpC (pAmpC) β-lactamases, which confer resistance to extended-spectrum cephalosporins in Gram-negative bacilli, has increased in both humans and livestock isolates worldwide, and is a global problem [1]. CMY-2 is the most common pAmpC in E. coli from different geographical areas including Asia, North America, and Europe [2–5], and has now been reported in geographical areas including Asia, North America, and Europe [2–5], and has now been reported in China [6]. Some studies reported that the increased incidence of infections in humans with S. enterica serovar Newport possessing CMY-2 in North America was associated with exposure to dairy cattle as well as the consumption of raw milk, raw or improperly cooked beef mince, and the cross-contamination of raw meat with other foods [7,8]. CMY-2-bearing plasmids, predominantly the A/C, I1, or K/B replicon types [9,10], were readily transferable between Salmonella and E. coli from food animals and humans [10,11]. Therefore, livestock-associated plasmid-encoded CMY-2 has posed increasing concerns to public health worldwide [6].

E. coli is one of most common pathogens of nosocomial, healthcare-associated, and community infections [12]. According to CHINET (antimicrobial resistance surveillance networks in China), bacteria isolated from various samples from 14 hospitals in 10 regions or Provinces were predominantly E. coli, comprising 61.8% of the total isolates [13]. CMY-2 AmpC in Chinese pediatric patients was detected first in E.coli between 2003 and 2005; the occurrence of AmpC β-lactamase in E. coli and K. pneumoniae had the highest prevalence [13]. The resistance to multiple drugs, including third-generation cephalosporins, and the carriage of both pAmpC and extended spectrum β-lactamase...
(ESBL) genes in E. coli isolates from food animals has increased rapidly [14–17]. The detection rate of \( \text{bla}_{CMY-2} \) in isolates from chickens, which emerged between 2000 and 2003, increased rapidly from 2004–2007 [16]. However, limited information is available regarding the characteristics of the CMY-2 plasmid type or the clonal dissemination of CMY-2 in E. coli of food animal origin in China. The aim of this study was to assess the molecular epidemiology and characteristics of CMY-2-bearing plasmid-producing E. coli in food animals including pigs, chickens, ducks, and geese.

Materials and Methods

Bacterial isolates and antimicrobial susceptibility

A total of 1083 unique E. coli isolates from a range of food animal species (pigs, \( n = 424 \); chickens, \( n = 306 \); ducks, \( n = 175 \); geese, \( n = 178 \)) were recovered between October 2010 and January 2012 from 58 fixed farms described previously [15]. Of the 1083 E. coli isolates, 587 were cultured from feces, 456 from the viscera, 14 from blood samples, and 26 from drinking water and subsurface soil of duck farms. These isolates were collected partially from the Guangdong Province Surveillance Program on Antibiotic Resistance in bacteria isolated from animals. The program was carried out by the Laboratory of Clinical Microbiology, Veterinary Research Institute, Guangdong Academy of Agricultural Sciences. Methods for sample collection and bacterial isolation were described previously [18]. Susceptibility testing was determined for all isolates using the standard agar dilution method on Mueller–Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [19,20]. The 13 antimicrobials tested were ampicillin (Amp), ceftriaxone (Cfx), cefotaxime (Ctx), cefpodoxime (Cdp), cefoxitin (Cxt), cefuroxime (Cfx), cefoperazone (Cpo), cefotaxime (Ct), ciprofloxacin (Cip), levofloxacin (Lfx), and streptomycin (Str).

Genetic environment of \( \text{bla}_{CMY-2} \) gene and detection of integrons

The genetic environment of the \( \text{bla}_{CMY-2} \) genes was investigated using PCR and sequencing. The lsaE1 primer forward primer and CMY-2 reverse primer were used to investigate regions upstream of the \( \text{bla}_{CMY-2} \) genes. The CMY-2 forward primer and reverse primers for \( \text{lsaE1}, \text{lsaF1}, \text{lsaA}, \text{lsaC1}, \) and \( \text{lsaS13} \) were used to characterize regions downstream of the \( \text{bla}_{CMY-2} \) genes. Integrons of class 1 or class 2 as well inserted gene cassettes were detected. Sequences of the above primers are listed in Table S1.

Conjugation and plasmid analysis

Conjugation experiments were performed on \( \text{bla}_{CMY-2} \)-containing strains using streptomycin-resistant E. coli C600 as the recipient [4]. Transconjugants were selected on MacConkey agar plates supplemented with streptomycin (1000 mg/L) and cefoxitin (8 mg/L). Transconjugants were tested for the presence of drug resistance genes and antimicrobial susceptibility, as described above.

Plasmids were typed using PCR-based replicon typing (PBRT) [21]. PFGE with S1 nuclease (TakaRa Biotechnology, Dalian, China) digestion of whole genomic DNA was performed for all 15 donor strains and transconjugants, as described previously [22]. After Southern transfer to a Hybond-N* membrane (GE Healthcare, Little Chalfont, United Kingdom), the plasmids were probed with the \( \text{bla}_{CMY-2} \) gene and respective replicons (DIG High Prime DNA Labeling and Detection Starter Kit I, Roche Applied Science, Mannheim, Germany).

Population structure analysis

All CMY-2-producing E. coli isolates were classified according to E. coli phylogenetic groups A, B1, B2, and D using multiplex PCR [23]. XbaI-pulsed-field gel electrophoresis (PFGE) patterns were typed using a CHEF-MAPPER System (Bio-Rad Laboratories, Hercules, CA) as described previously [15]. For multi-locus sequence typing (MLST) analysis, seven conserved housekeeping genes (\( \text{adhE}, \text{fumC}, \text{gyrB}, \text{idh}, \text{pucA}, \text{mdh}, \) and \( \text{recA} \)) were analyzed by PCR amplification using specific primers (Table S4) and sequencing. Allelic profiles and sequence type (ST) determinations were performed according to the E. coli MLST website (http://mlst.ucc.ie/mlst/dbs/E.coli) scheme.

Results

Antimicrobial susceptibility

A total of 233 (21.3%) out of 1083 E. coli isolates were resistant to cefoxitin with MIC values ranging from 0 mg/L to >512 mg/L. Only 33 of the cefoxitin-resistant isolates produced CMY, including 19 from pigs, 10 from chickens, and two each from ducks and geese. Of the 33 \( \text{bla}_{CMY-2} \)-bearing isolates, 31 carried \( \text{bla}_{CMY-2} \) from feces (Table 1), and two from the feces and viscera of geese carried \( \text{bla}_{CMY-41} \) and \( \text{bla}_{CMY-64} \), respectively (data not shown). All 33 CMY-producing isolates exhibited multi-drug resistance profiles, and showed resistance to both \( \beta \)-lactam drugs and more than two non-\( \beta \)-lactam drugs. The most common resistance pattern was \( \text{Amp-CTX-Caz-CiF-Gen-Kan-Tet-Cip-Opx} \) (20/33, 61%). The presence of resistance to olaquindox (a growth promoter used extensively in pig and poultry farms) was detected in the majority of \( \text{bla}_{CMY-2} \)-bearing strains (20/33, 61%). Twenty-three \( \text{bla}_{CMY-2} \)-bearing isolates were resistant to cefotaxime, a newly approved \( \beta \)-lactam for veterinary use in China. Resistance to amikacin was relatively low (8/32, 25%).

Characterization of \( \text{bla}_{CMY-2} \)-harboring isolates

All except one CMY-2-producing isolate harbored more than one resistance gene conferring resistance to different antimicrobial drugs (Table 1). Of the detected ESBLs genes, \( \text{bla}_{CTX-M-14} \) and \( \text{bla}_{CTX-M-55} \) were identified in two strains; no isolate harbored the \( \text{bla}_{SHV} \) gene. The narrow \( \beta \)-lactamase-encoding genes \( \text{bla}_{TEM-1} \) and \( \text{bla}_{OXA-1} \) were found in 22 and three strains, respectively. Among the detected PMQR determinants, \( \text{qepA} \), \( \text{qnrS} \), and \( \text{qnrB} \) were detected in 17, 15, and five strains, respectively (Table 1). Of the 15 \( \text{qnrS} \) genes, 14 were \( \text{qnrS1} \), and one was \( \text{qnrB6} \). No \( \text{qnrA} \), \( \text{qnrD} \), \( \text{qnrE} \), or \( \text{qepA} \) genes were detected. The \( \text{floR} \) and \( \text{mtdB} \) genes were identified in 18 and four strains, respectively. Both ESBL producers also carried the \( \text{qnrS1} \), \( \text{qepA} \), and \( \text{floR} \) genes. Remarkably, one strain (L461) contained five genes conferring resistance to five antimicrobial drug classes.
## Table 1. Overall results of co-resistance, phylogenetic grouping, MLST and plasmid replicon analysis of CMY-2-producing E. coli isolates of food producing animals in China.

| Strains | Source   | Other resistance genes | Phylogen. group | MLST | **bla**<sub>CMY-2</sub>, linked-element upstream | Gene cassettes inserted in class1 and 2 integrons | Plasmid transfer | Co-transferred resistant gene | Plasmid replicon types and approx. size (kb) |
|---------|----------|------------------------|----------------|------|--------------------------------------------------|-------------------------------------------------|-----------------|-------------------------------|---------------------------------------------|
| L145    | Pig      | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup>,floR | A              | 10   | STC 10                                          | ISE<sup>cp</sup>1                                 | ND              | ND                            | ND                                          |
| L147    | Pig      | <sup>oqxA</sup>        | A              | 10   | STC 10                                          | ND                                                | ND              | ND                            | ND                                          |
| L393    | Chicken  | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup>,<sup>floR</sup> | A              | 2690 | STC 10                                          | ISE<sup>cp</sup>1                                 | ND              | +                             | qnrS1,floR                                  | A/C,160                                    |
| L699    | Chicken  | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup>,floR | A              | 48   | STC 10                                          | ISE<sup>cp</sup>1                                 | class2: sat1+aadA1 | +                             | qnrS1,floR                                  | A/C,200                                    |
| L78     | Pig      | bla<sub>CTX-M-14</sub>,<sup>oqxA</sup>,<sup>floR</sup> | A              | 3244 | STC 10                                          | ISE<sup>cp</sup>1                                 | ND              | +                             | bla<sub>TEM</sub>-<sup>oqxA</sup>,<sup>floR</sup> | A/C,160                                    |
| L667    | Pig      | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup> | B1             | 3403 | STC 10                                          | ISE<sup>cp</sup>1                                 | class1: dfIA17+aadA5 | -                             | -                                           | -                                           |
| L671    | Pig      | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup>,<sup>oqxA</sup> | B1             | 359  | STC 10                                          | ISE<sup>cp</sup>1                                 | class1: dfIA17+aadA5 | +                             | qnrS1                                      | K200                                       |
| L669    | Pig      | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup> | B1             | 359  | STC 10                                          | ISE<sup>cp</sup>1                                 | class1: dfIA17+aadA5 | -                             | -                                           | -                                           |
| L670    | Pig      | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup>,<sup>oqxA</sup>,<sup>floR</sup> | B1             | 359  | STC 10                                          | ISE<sup>cp</sup>1                                 | class1: dfIA17+aadA5 | -                             | -                                           | -                                           |
| L679    | Pig      | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup>,<sup>floR</sup> | B1             | 101  | STC 10                                          | ISE<sup>cp</sup>1                                 | ND              | -                             | -                                           | -                                           |
| L1119   | Pig      | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup>,<sup>floR</sup>,<sup>oqxA</sup>,<sup>floR</sup> | B1             | 101  | STC 10                                          | ISE<sup>cp</sup>1                                 | ND              | +                             | qnrS1,floR                                  | A/C,160                                    |
| L518    | Pig      | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup>,<sup>oqxA</sup>,<sup>oqxA</sup>,<sup>floR</sup> | D              | 648  | none                                            | ISE<sup>cp</sup>1                                 | ND              | -                             | -                                           | -                                           |
| L461    | Duck     | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup>,<sup>oqxA</sup> | D              | 648  | none                                            | ISE<sup>cp</sup>1                                 | class1: oadA22  | +                             | bla<sub>CTX-M-55</sub>, qnrS1               | FIB,40                                     |
| L351    | Chicken  | floR | B1             | 155  | STC 10                                          | ISE<sup>cp</sup>1                                 | ND              | +                             | floR                                      | A/C,160                                    |
| L391    | Chicken  | floR | B1             | 155  | STC 10                                          | ISE<sup>cp</sup>1                                 | class1: dfIA17+aadA1 | +                             | qnrS1,floR                                  | A/C,160                                    |
| L392    | Chicken  | floR | B1             | 2294 | STC 10                                          | ISE<sup>cp</sup>1                                 | class1: dfIA17+aadA1 | +                             | floR                                      | A/C,160                                    |
| L813    | Pig      | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup>,<sup>oqxA</sup>,<sup>oqxA</sup>,<sup>floR</sup> | B1             | 156  | none                                            | ISE<sup>cp</sup>1                                 | ND              | +                             | qnrS1                                      | FIB,40                                     |
| L361    | Duck     | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup>,<sup>oqxA</sup> | D              | 156  | none                                            | ISE<sup>cp</sup>1                                 | ND              | -                             | -                                           | -                                           |
| T117    | Pig      | bla<sub>TEM</sub>-<sub>1</sub> | A              | 1114 | none                                            | ISE<sup>cp</sup>1                                 | ND              | -                             | -                                           | -                                           |
| A10-2   | Pig      | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup> | A              | 1114 | none                                            | ISE<sup>cp</sup>1                                 | ND              | -                             | -                                           | -                                           |
| L1039   | Pig      | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup>,<sup>oqxA</sup>,<sup>oqxA</sup>,<sup>floR</sup> | D              | 457  | STC 157                                         | ISE<sup>cp</sup>1                                 | ND              | -                             | -                                           | -                                           |
| T43     | Pig      | <sup>oqxA</sup>        | D              | 3376 | STC 157                                         | ND                                                | ND              | -                             | -                                           | -                                           |
| C42     | Pig      | floR | A              | 3402 | none                                            | ISE<sup>cp</sup>1                                 | ND              | -                             | -                                           | -                                           |
| T26     | Pig      | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup>,<sup>floR</sup> | A              | 3404 | none                                            | ISE<sup>cp</sup>1                                 | ND              | +                             | bla<sub>TEM</sub>-<sup>floR</sup>          | A/C,160                                    |
| L1166   | Pig      | floR | A              | 3269 | none                                            | ISE<sup>cp</sup>1                                 | ND              | +                             | floR                                      | A/C,160                                    |
| L653    | Pig      | bla<sub>TEM</sub>-<sub>1</sub>,<sup>oqxA</sup>,<sup>floR</sup> | A              | 3014 | none                                            | ISE<sup>cp</sup>1                                 | ND              | -                             | -                                           | -                                           |
| L215    | Chicken  | bla<sub>TEM</sub>-<sub>1</sub> | D              | 362  | none                                            | ISE<sup>cp</sup>1                                 | class1: dfIA17+aadA5 | -                             | -                                           | -                                           |
| L349    | Chicken  | qnrS1,floR | D              | 354  | none                                            | ISE<sup>cp</sup>1                                 | class1: dfIA17+aadA5 | +                             | qnrS1,floR                                  | A/C,200                                    |
| L394    | Chicken  | qnrS1,bla<sub>TEM</sub>-<sub>1</sub>,floR | B1             | 3245 | none                                            | ISE<sup>cp</sup>1                                 | class1: orf1+aadA2 | +                             | bla<sub>TEM</sub>-<sup>oqxA</sup>,<sup>floR</sup> | H12,220                                   |
| L398    | Chicken  | qnrS1 | B1             | 1431 | none                                            | ND                                                | class1: dfIA17+aadA5 | -                             | -                                           | -                                           |
| L399    | Chicken  | qnrS1 | D              | 69   | none                                            | ISE<sup>cp</sup>1                                 | ND              | +                             | qnrS1                                      | K160                                       |

*STC, ST complex.
ND, not detected.

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Genetic environment of blaCMY-2 and detection of integrons

Twenty-eight of 31 blaCMY-2 were linked to an upstream ISEsp1 element; blaCMY-41 was also associated with the upstream ISEsp1 element. No IS elements were detected upstream of blaCMY-64 or downstream of blaCMY. Of the 31 CMY-2 producers, 27 contained class 1 integrines, and one harbored a class 2 integrase. Of the 27 class 1 integrase-positive isolates examined, 11 were found to possess cassettes inserted within the integrons, including the four-gene cassette arrays dfrA17+aadA5, dfrA17+aadA1, aadA2, and aadA22 (Table 1). dfrA17+aadA5 was most common (7/11, 64%), followed by dfrA17+aadA1 (2/11, 18%); aadA2 and aadA22 were found in single isolates. The class 2 integron present in one strain (L699) harbored the 1.0 kb sat1+aadA1 arrays. One CMY-41 producer from geese also contained a dfrA17+aadA5 cassette array.

Population structure analysis

All 31 blaCMY-2 strains were distributed into groups B1 (n = 12) and A (n = 11) of the commensal strains, strains associated with enterotoxigenic and enterohemorrhagic infections, and the potentially virulent phylogenetic group D (n = 7). The strain carrying blaCMY-41 from the liver of a diseased goose belonged to group D, and the strain containing blaCMY-64 from a goose intestine belonged to group A.

A total of 18 different PFGE-types were detected among 22 typeable CMY isolates (20 CMY-2, one CMY-41, and one CMY-64), including 19 single types, and two clusters (>90% similarity) containing two (Cluster 2) and four isolates each (Cluster 1) (Fig. 1). Interestingly, the four isolates in Cluster 1 were obtained from two different locations and further divided into two phylogenetic groups, each containing two isolates. The Cluster 2 isolates belonged to the same phylogenetic group and originated from the same place.

MLST analysis of the 31 CMY-2 producing isolates identified 24 different STs, including seven novel ones (ST3244, ST3245, ST3403, ST3269, ST3402, ST3404, and ST3376) (Table S5). A total of 20 isolates were related to six STs or ST-complexes (STCs) described previously: 10, 101, 155, 156, 648, and 1114. The prevalent ST/STCs were 101 (n = 6, 19%), 10 (n = 5, 16%), 155 (n = 3, 10%), 156 (n = 2, 6%), 457 (n = 2, 6%), 1114 (n = 2, 6%), and 648 (n = 2, 6%). The remaining isolates were each of a single ST type, including four novel ST types (Table S5).

STC 101 included ST101, the single locus variant (SLV; isolate ST359), and one double locus variant (DLV; isolate ST3403). STC 10 included ST10, one SLV isolate (ST48), one DLV isolate (ST2690), and one triple locus variant (TLV) isolate (the novel ST3244). STC155 included two ST155 and one SLV isolate (ST2298).

Transferability of blaCMY-2 and plasmid analysis

The transferability of blaCMY-2 was observed in 15 out of 31 isolates at transfer frequencies ranging from 10⁻³ to 10⁻⁷ transconjugants per recipient. Of the 15 transferable blaCMY-2, 10 were located on the IncA/C type plasmid with sizes of 160 to 200 kb, two 40 kb IncFIB plasmids, two IncK plasmids sized 160 kb and 200 kb, and one 220 kb H12 plasmid. Genes encoding resistance to other antimicrobials also co-transferred in some strains. Remarkably, qnrS1 and floR co-transferred with blaCMY-2 in eight of 15 isolates. The co-transfer of blaCMY-2, blaCTX-M-55, and qnrS1 was observed in one isolate from a duck.

Discussion

In the present study, we performed a broad molecular epidemiological characterization of CMY-2-producing E. coli collected from the Guangdong Province Surveillance Program on antibiotic resistance in bacteria isolated from animals between 2010 and 2012. Compared with earlier surveillance on E. coli antibiotic resistance from fixed food animal farms, resistance to a panel of cephalosporins was increased significantly from <7% to between 20 and 60% (data not shown) for all tested drugs in 2003 to 2005 [14,15]; resistance to cefotaxime, ceftriaxone, cefoxitin, cefditoren, and ceftazidime was 55.8, 59.7, 21.5, 60, and 20.8%, respectively. The rapid increase in resistance to third-generation cephalosporins, particularly for cefditoren, which is newly approved for use in veterinary clinical settings, was consistent with previous reports from other Provinces in China [16,17].

Cefoxitin/cefidotetan resistant isolates from E. coli and Salmonella occur frequently worldwide, which is likely to be associated with the production of CMY enzyme(s) [24]. The use of cefditoren in the veterinary clinical setting has encouraged selection of CMY-2 AmpC in both Salmonella and E. coli [6]. In China, the occurrence of CMY-2 in E. coli originating in chickens increased rapidly [16]; the detection rate of blaCMY-2 in chicken isolates was higher than in those from pigs [25], which is consistent with reports from Japan [26]. In the present study, all CMY-2 producers were isolated from feses, consistent with a previous study [14], suggesting that the gastro-intestinal tract of animals is a reservoir for CMY-2 producers. Only half of the samples were collected from animal feses in the present study, which might explain the lower occurrence of CMY-2. Of note, many CMY-2 producers were isolated from pigs, which was an increased prevalence compared with previous reports [14,25]. The use of cefditoren was approved for pigs in 2005 in China, which may have contributed to the rapid increase.

Salmonella and E. coli isolates carrying blaCMY-2 have been associated with community-acquired infections [1,6,27]. Plasmids carrying AmpC genes often carry other genes that confer resistance to non-β-lactams, but rarely ESBL genes [28]. Previous studies demonstrated that CMY-2-producing E. coli easily acquired other resistance genes, resulting in a multidrug resistant profile. The floR gene was the most common gene acquired, which conferred resistance to florfenicol. Some studies have shown that the increasing occurrence of CMY-2 was associated with the use of florfenicol in the veterinary clinic [29]. In the present study, most CMY-2 producers harbored not only genes encoding β-lactamases (including ESBLs), but also many diverse genes encoding resistance to other antimicrobials. In addition to floR, variants of qnr, a plasmid-mediated quinolone resistant gene, were detected frequently. The presence of qnr or floR, either alone or in combination, was detected frequently in CMY-2 isolates. Trans-conjugation experiments confirmed that the blaCMY-2 gene could co-transfer with multiple antibiotic resistance genes, frequently with qnrS1-floR or qnrS1, driven by 160 and 200 kb-sized IncA/C plasmids. Previous studies demonstrated that plasmids carrying CMY-2 could self-transfer between different strains alone, but rarely co-transferred with multiple genes [4,27,30]. The spread of blaCMY-2 was driven mainly by the IncA/C, IncI1, or IncK plasmids [31].

Co-localization of blaCMY-2 and floR on an IncA/C plasmid was detected commonly. A close relationship between the IncA/C plasmid and the multidrug resistance (MDR) of plasmid bearing isolates from humans, animal, and environmental origins has been reported [9]. Considering the high occurrence of MDR genes (such as floR and qnr) in IncA/C plasmids and the co-transfer of
these genes within the same plasmids in the present study, we suggest that there should be a stable association of these resistance genes with A/C plasmids. In addition, bla\textsubscript{CMY-2} co-transferred with \textit{qnrS1} located on the IncK plasmid, with sizes of 160 and 200 kb. These results highlight the potential risk for co-selection of isolates carrying \textit{bla}\textsubscript{CMY-2} via the use of florfenicol or fluoroquinolones in the raising of food animals [32,33]. Interestingly, the co-transfer of \textit{bla}\textsubscript{CMY-2} with \textit{qnrS1}+\textit{floR} located on the IncHI2 plasmid was first reported in a chicken isolate. The IncHI2 plasmid was most prevalent in \textit{Salmonella} isolates of food animal origin, and could readily capture PMQR [34]. Our results suggest that the dissemination of IncHI2 plasmids carrying CMY-2, PMQR, and \textit{floR} between \textit{E. coli} and \textit{Salmonella} might have occurred. Although the occurrence of \textit{OqxAB} was surprisingly high in China (39%) [35] compared with Denmark (1.8%), and Korea (0.4%) [36,37], the encoded genes rarely co-transferred with \textit{bla}\textsubscript{CMY-2} on the same plasmid. Previous findings demonstrated that \textit{rmtB} was the most prevalent 16S rRNA methylase gene in the \textit{Enterobacteriaceae} isolates that produced ESBLs in China [23,38]. Similarly, only the \textit{mtdB} gene was detected in five strains in this study. However, in contrast with the previously described co-transfer of \textit{mtdB} and ESBL-encoding genes, the co-transfer of \textit{mtdB} with \textit{bla}\textsubscript{CMY-2} was not observed in the current study, as demonstrated by a conjugation experiment.

Plasmids and integrons can contain or capture a variety of resistance genes that are beneficial for survival of the bacterial host and help them adapt to changing environments [39,40]. Integrons are genetic platforms involved in the spread of different previously captured gene cassettes that encode determinants of antimicrobial-resistance and represent a fundamental resource for bacterial evolution [41]. Integrons are divided into five classes based on integrase gene sequence; class 1 integrons are by far the most common in clinical isolates of Gram-negative bacteria [41]. In this study, we found five types of integrons encompassing eight different genes: \textit{aadA1}, \textit{aadA2}, \textit{aadA5}, \textit{aadA22}, \textit{dfrA1}, \textit{dfrA17}, \textit{sat1}, and \textit{orfF}. The most common integron profile (\textit{dfrA17-aadA5}) was found in \textit{Salmonella} and other \textit{Enterobacteriaceae}, and recently in two \textit{Staphylococcus} species isolated in China, suggesting the successful spread of this integron around the world and across bacterial phyla [40]. Different types of integrons contained by CMY-2 producers

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**Figure 1.** Dendrogram of XbaI-PFGE patterns of CMY-producing \textit{E. coli} strains recovered from food-producing animals. All the strains were CMY-2 producers, except for T19 (CMY-41) and T129 (CMY-64). Similarity analysis was performed using the Dice coefficient, and clustering was performed by following the unweighted-pair group method using average linkages (UGPMA). A total of 16 PFGE patterns were identified, and the two clusters with highly similar PFGE patterns were labeled C1 and C2. Abbreviations for the place column: SS, Sanshui; GZ, Guangzhou; KP, Kaiping; ZC, Zengcheng; PY, Panyu; MM, Maoming; QY, Qingyuan; JM, Jiangmen; ZQ, Zhaoqing; SH, Shihui. Abbreviation for PhG: Phylogenetic Group. doi:10.1371/journal.pone.0096738.g001
represented a diverse trend in strain evolution. DNA fingerprints also revealed that most CMY-2 producers were unlikely to be derived from a single *E. coli* clone. However, the clonal dissemination of *bla*<sub>CMY-2</sub> between different farms at the same and different geographical locations was also found.

There was strong correlation between phylogenetic groups and STs in this study. One of the most common ST lineages, STC101, was isolated from pigs, and belonged to the avirulent phylogroup B1. Most of these harbored class 1 integron-containing *draF17* and *aadA5* cassette arrays. The global spread of *bla*<sub>CMY-2</sub> and *bla*<sub>NDM-1</sub> was driven by ST101/B1 *E. coli*, and could be explained by the accumulation of a large number of virulence genes [42]. Similarly ST101C/B1 CMY-2, which is prevalent dominantly and consistently at pig farms from different geographical regions, could be explained by its ability for acquiring class 1 integrons with the most common cassettes arrays and antibiotic resistant genes. Capturing and acquiring these genes could help establish the dissemination of STC101/B1 CMY-2 isolates between pig farms. The other most common ST, STC10, belonged to the avirulent phylogroup A, and was distributed widely between pigs, chickens, and geese. The significant contribution of ST10C to the spread of resistance in humans was reported in Europe and Canada [43]. Interestingly, a chicken origin ST48 (belonging to ST10C) contained *qnrS1* and/or *floR*, as well a class 2 integron carrying a *sat1* and/or *aadA1* cassette array. Compared with ST10, its SLV or DLV contained more antibiotic resistant genes, which helps the host bacteria adapt to their surroundings under antibiotic selective pressures. Three strains isolated from a diverse range of animal species corresponded to ST648, which belongs to virulent phylogroup D. It was worthy noting that two of these three strains contained class 1 integrons with different cassette arrays, which might allow the potential persistence between animals. *E. coli* strains of ST648/D clones were reported to cause most cases of ESBL-producing *E. coli* bacteremia in the Netherlands [44]. Additional concerns arise from this ST belonging to phylogroup D, due to its ability to produce New Delhi metallo (NDM-type) carbapenemases in hospitalized patients in Pakistan and the United Kingdom [45,46]. In a recent study, ST648/D clones were the main vectors that allowed the spread of *bla*<sub>CMY-2</sub> between dogs in the Republic of Korea [47]. This study demonstrated the presence of *bla*<sub>CMY-2</sub> in broad host-range conjugative plasmids. To our knowledge, this represents the first comprehensive analysis of CMY-2 plasmids in *E. coli* isolated from food animals in China. *bla*<sub>CMY-2</sub> was co-transferred with *qnrS1* and/or *floR*, linked to diverse lineages of *E. coli* STCs (including 101, 10, and 155), and disseminated among different food producing animals in China. The acquisition of multiple antimicrobial resistant genes and integrons might have allowed CMY-2-positive isolates to persist in the environment and evolve under antibiotic selective pressure. Continued surveillance of CMY-2 in animal reservoirs is necessary to curb the spread of multidrug resistant pathogens from animals to humans.

Supporting Information

Table S1 Primers used for the PCR amplification of antimicrobial resistance genes.

(DOC)

Table S2 Primers used for the PCR amplification of genetic environment of *bla*<sub>CMY-2</sub> gene.

(DOC)

Table S3 Primers used for the PCR amplification of integrons.

(DOC)

Table S4 MLST Primers used for the PCR amplification of *E. coli*.

(DOC)

Table S5 The number of alleles and ST results for thirty-one CMY-2 producing strains.

(DOC)

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Author Contributions

Conceived and designed the experiments: H-XJ Y-HL Z-LZ. Performed the experiments: W-HZ S-QR LY D-HL. Analyzed the data: Y-FG. Contributed reagents/materials/analysis tools: S-QR LY D-HL. Wrote the paper: H-XJ Y-FG.

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