Phenotypic and molecular characterizations of multidrug-resistant diarrheagenic E. coli of calf origin

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Abstract

Escherichia coli has become one of the most important causes of calf diarrhea. The aim of this study is to determine the patterns of antimicrobial resistance of E. coli isolates from six cattle farms and to identify prominent resistance genes and virulence genes among the strains isolated from the diarrhea of calves. Antimicrobial susceptibility tests were performed using the disk diffusion method, and PCR was used to detect resistance and virulence genes. The prevalence of multidrug resistant (MDR) E. coli was 77.8% in dairy cattle and 63.6% in beef cattle. There were high resistance rates to penicillin (100%, 100%) and ampicillin (96.3%, 86.4%) in E. coli from dairy cattle and beef cattle. Interestingly, resistance rate to antimicrobials and distribution of resistance genes in E. coli isolated from dairy cattle were higher than those in beef cattle. Further analysis showed that the most prevalent resistance genes were blaTEM and aadA1 in dairy cattle and beef cattle, respectively. Moreover, seven diarrheagenic virulence genes (irp2, fyuA, Stx1, eaeA, F41, K99 and StA) were present in the isolates from dairy cattle, with a prevalence ranging from 3.7% to 22.22%. Six diarrheagenic virulence genes (irp2, fyuA, Stx1, eaeA, hylA and F41) were identified in the isolates from beef cattle, with a prevalence ranging from 2.27% to 63.64%. Our results provide important evidence for better exploring their interaction mechanism. Further studies are also needed to understand the origin and transmission route of E. coli in cattle to reduce its prevalence.

Keywords: Dairy calves, Beef calves, E. coli, Multidrug resistant, Virulence gene

Introduction

Diarrheagenic E. coli (DEC) is a significant cause of gastroenteritis and a major health problem in animals and humans. E. coli infection in calves usually causes a variety of clinical signs, including diarrhea, respiratory infections, and sepsis, and then death due to dehydration and exhaustion because of the difficulties in treatment. Previous studies have shown that diarrhea is the most common problem in young calves, causing more than 52% of deaths in unweaned calves (Diarra et al. 2009). In cattle farms, antimicrobials are the most important therapy for bacterial infection. In dairy cattle farms worldwide, periodic treatment of mastitis after bacterial infection is very common, which not only easily leads to bacterial resistance but also raises concerns about the emergence of multidrug resistant (MDR) bacteria (Yang et al. 2021). The use of antimicrobials to treat infections in beef cattle can increase prevalence of antimicrobial resistance (AMR) in enteric pathogens (Cazer et al. 2017). In addition, antimicrobials are frequently used as growth promoters and preventive agents, which further increases the risk of E. coli resistance (Sivaraman et al.)

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AMR in bacteria of animal origin is considered a major challenge to veterinary medicine and public health (Anes et al. 2020), which not only seriously affects the healthy development of cattle breeding industry but also poses a serious threat to food safety. E. coli has also been used as a sentinel organism for monitoring AMR (de Moyaert et al. 2014). Hence, monitoring AMR in cattle is important to human and animal health.

Some pathogenic E. coli strains use different virulence factors to colonize the hosts’ small intestine, avoiding immune response and stimulating the deleterious inflammatory response to produce diarrhea (Croxen and Brett Finlay 2010). Virulence genes that play significant roles in E. coli pathogenicity are associated with diarrhea in animals, which have been described (Fröhlicher et al. 2008; Huenhn et al. 2010). Among the many virulence genes identified in E. coli isolates from cattle, Shiga toxins (Stx1 and Stx2), Yersinia pathogenicity island (irp2 and fyuA) and intimin (eaeA) were the most significant genes with great public health concerns (Momtaz et al. 2012; Olsson et al. 2003; Momtaz et al. 2013a, b). Cattle are a major reservoir of E. coli, particularly Shiga toxin-producing E. coli (STEC) O157:H7. In addition, E. coli has many serotypes, among which E. coli O157 can cause hemorrhagic colitis and hemolytic uremic syndrome (Iweriebor et al. 2015). Heat-labile enterotoxins (LT) and heat-stable enterotoxins (Sta or STb) are the two most important virulence factors responsible for severe diarrhea in cattle (Nguyen et al. 2011; Kumar et al. 2013). The most important adhesins involved in E. coli host colonization are fimbriae. Well-characterized fimbriae of E. coli isolated from animals include F4 (K88), F5 (K99), F6 (987P), F41 and F18, are associated with E. coli pathotypes (Maciel et al. 2019). Previous studies have shown that the ability of E. coli to acquire many different virulence factors may lead to the emergence of invasive strains, which pose a threat to human and animal health (Mellmann et al. 2011). Therefore, the aim of this study is to characterize AMR and identify different resistance genes and virulence genes in E. coli strains isolated from dairy cattle and beef cattle to provide a reference for clinical practice.

**Results**

**Prevalence of AMR in E. coli isolated from dairy and beef cattle**

A total of 71 E. coli isolates were obtained, including 27 isolates from dairy cattle and 44 isolates from beef cattle diarrheal fecal samples. Subsequently, susceptibility to 15 different antimicrobials was determined for these 71 E. coli isolates. All 27 E. coli isolates from dairy cattle were resistant to penicillin, followed by ampicillin (96.3%), amoxicillin and sulfamethoxydiazine (81.5%), tetracycline and compound sulfamethoxazole (77.8%), with the lowest resistance rate being observed for florfenicol (33.3%) (Fig. 1). Meanwhile, all isolates were sensitive to polymyxin B (100%). Consistent with the results of dairy cattle, the most sensitive antimicrobial was also polymyxin B in the 44 isolates from beef cattle (Fig. 2). The highest resistance rate was also observed for penicillin (100%), which may be related to the widespread use of penicillin for the treatment of E. coli disease. Further analysis showed that the resistance rate of E. coli to antimicrobials (except for florfenicol and polymyxin B) in dairy cattle was higher than that in beef cattle.

**Prevalence of multidrug resistant (MDR) E. coli**

Multidrug resistance was defined as resistance by an isolate to at least three antimicrobials of the panel belonging to different classes. Resistance of E. coli to seven different types of antimicrobials were analyzed. The results showed that multidrug resistance rates were 77.8% (21/27) in dairy cattle and 63.6% (28/44) in beef cattle. Most isolates from dairy cattle and beef cattle were resistant to five or six different types of antimicrobials. The prevalence of resistance to five different types of antimicrobials was 37% (10/27) in dairy cattle and 18.2% (8/44) in beef cattle. Compared with the isolates from dairy cattle, isolates from beef cattle had a higher prevalence of resistance to six different types of antimicrobials [dairy cattle 29.6% (8/27) vs. beef cattle 31.8% (14/44)] (Table 1). One isolate from beef cattle was resistant to all antimicrobials.

**Prevalence of resistance genes in E. coli**

Prevalence of 12 different resistance genes was analyzed in E. coli isolates from dairy cattle and beef cattle origins. The results showed that seven different resistance genes were detected in over 50% isolates from dairy cattle (Table 2). Resistance genes that had the highest positive rate were blαTEM (100%), followed by floR, tet (A), aac (3’)-Ila and sul2. Resistance gene with the lowest positive rate was aadB (0%). However, detection rate of seven drug resistance genes in 44 isolates from beef cattle was over 56%, with 100% positive rate of aadA1, followed by blαTEM, tet (A), and tet (B) (Table 2). Overall, the positive rates for blαTEM, aadA1, tet (A), tet (B), floR and sul2 were relatively high in the E. coli isolates of both dairy and beef cattle. Consistent with the AMR results, detection rate of resistance genes in dairy cattle was higher than that in beef cattle.

**Correlation between the resistance phenotype and resistance genes**

Consistency analysis of resistance phenotypes and resistance genes to 11 antibiotics showed that β-lactam (penicillin) resistance phenotype had the highest consistency with β-lactam resistance genes (beef cattle K = 1),
followed by compound sulfamethoxazole (beef cattle \( K = 0.59 \)), gentamicin (beef cattle \( K = 0.56 \)) and florfenicol (beef cattle \( K = 0.41 \)). In dairy and beef cattle, tetracycline resistance phenotype had the lowest consistency (\( K = -0.55, \ K = -0.77 \)) with tetracycline resistance gene \( \text{tet} (C) \). Some isolates presenting drug resistance carried resistance genes, whereas some isolates carried resistance genes without manifesting a resistance phenotype (Table 3).

**Prevalence of virulence genes in \( E. coli \)**

A total of 14 virulence genes were present in \( E. coli \) isolates from dairy cattle and beef cattle. Seven diarrheagenesis-associated virulence genes (\( \text{irp2}, \ \text{fyuA}, \ \text{Stx1}, \ \text{eaeA}, \ \text{hylA} \) and \( F41 \)) were present in isolates from dairy cattle, with a prevalence ranging from 3.7% to 22.22%. In the isolates from beef cattle, six diarrheagenesis-associated virulence genes (\( \text{irp2}, \ \text{fyuA}, \ \text{Stx1}, \ \text{eaeA}, \ \text{hylA} \) and \( F41 \)) were identified, with a prevalence ranging from 2.27% to 63.64%. In addition, 5 (18.52%) isolates from dairy cattle and 19 (43.18%) isolates from beef cattle carried both \( \text{irp2} \) and \( \text{fyuA} \). One (3.7%) isolate from dairy cattle carried \( \text{eaeA}/\text{Stx1}/\text{F41} \) and \( \text{F41}/\text{K99}/\text{STa} \) combination, but such a combination was not detected in isolates from beef cattle. In contrast, 8 (18.18%) isolates from beef cattle carried \( \text{irp2}/\text{fyuA}/\text{Stx1} \) combination, which were not detected in isolates from dairy cattle. \( \text{hylA}/\text{eaeA}/\text{Stx1}, \ \text{irp2}/\text{fyuA}/\text{F41} \) and \( \text{irp2}/\text{F41} \) combinations were detected in 1 (2.27%), 2 (4.54%) and 5 (11.36%) isolates from beef cattle, respectively. These combinations were not observed in isolates from dairy cattle (Table 4).
Further study showed that 49 E. coli isolates carried at least one virulence gene, including 38 isolates from beef cattle and 11 isolates from dairy cattle. Subsequently, the coexistence of virulence genes and AMR genes in these 49 E. coli isolates were analyzed. The results showed that there were at least 4 AMR genes in the isolates containing virulence genes and up to 10 AMR genes (Table 5) in other isolates. Interestingly, all 49 E. coli isolates contained blaTEM and tet(A) genes. In addition, most of 38 isolates from beef cattle contained blaTEM, tet(A), tet(B) and floR

### Table 1 Various antimicrobial resistance patterns in 71 E. coli isolates from dairy cattle (n = 27) and beef cattle (n = 44)

| Phenotypic resistance | Drug resistance spectrum | Dairy cattle (27) | Beef cattle (44) |
|-----------------------|--------------------------|-------------------|------------------|
|                       |                          | Isolates | Rate | Isolates | Rate |
| 1 PEN                 |                          | 0        | 0%   | 3        | 6.81% |
| PEN-AMP               |                          | 2        | 7.41% | 7        | 15.91% |
| PEN-AMC-AMP           |                          | 1        | 3.7%  | 1        | 2.27% |
| PEN-AMC-AMP-CFZ       |                          |          |      |          |      |
| 2 PEN-SULF            | AMP-SULF                 | 1        | 3.7%  | 1        | 2.27% |
| PEN-TET               | PEN-AMP-COM              | 1        | 3.7%  | 2        | 4.55% |
| PEN-AMM-TET-SULF      | PEN-AMC-AMP-TET-FCC      | 1        | 3.7%  | 1        | 2.27% |
| PEN-AMC-GEN-TET-SULF  | PEN-AMC-AMP-COM-SULF     | 1        | 3.7%  | 2        | 4.55% |
| 3 PEN-AMC-AMP-TET-SULF | PEN-AMC-AMP-TET-COM-SULF | 1        | 3.7%  | 1        | 2.27% |
| PEN-AMC-AMP-CFZ-TET-COM-SULF | PEN-AMC-AMP-CFZ-SULF-CIP-OFX-ENR | 1       | 3.7%  | 1       | 2.27% |
| PEN-AMC-AMP-CFZ-SULF-CIP-OFX-ENR | PEN-AMC-AMP-CFZ-SULF-CIP-OFX-FCC | 1       | 2.27% |
| 4 PEN-AMC-AMP-CFZ-STR-GEN-TET-COM-SULF-CIP-ENR-OFX | PEN-AMC-AMP-CFZ-STR-GEN-TET-COM-SULF-CIP-ENR-OFX-FFC | 1 | 2.27%  | 1 | 2.27% |
| PEN-AMC-AMP-CFZ-STR-GEN-TET-COM-SULF-CIP-ENR-OFX-FFC | PEN-AMC-AMP-CFZ-STR-GEN-TET-COM-SULF-CIP-ENR-OFX-FFC-FC | 1 | 2.27%  | 1 | 2.27% |
| PEN-AMC-AMP-CFZ-STR-GEN-TET-COM-SULF-CIP-ENR-OFX-FFC-FC | PEN-AMC-AMP-CFZ-STR-GEN-TET-COM-SULF-CIP-ENR-OFX-FFC-FC-FC | 1 | 2.27%  | 1 | 2.27% |
| PEN-AMC-AMP-CFZ-STR-GEN-TET-COM-SULF-CIP-ENR-OFX-FFC-FC-FC | PEN-AMC-AMP-CFZ-STR-GEN-TET-COM-SULF-CIP-ENR-OFX-FFC-FC-FC-FC | 1 | 2.27%  | 1 | 2.27% |

*Note: β-lactams: penicillin (PEN), amoxicillin (AMC), ampicillin (AMP), and cefazolin (CFZ); aminoglycosides: streptomycin (STR), gentamicin (GEN), and kanamycin (KAN); tetracyclines: tetracycline (TET); sulfonamides: compound sulfamethoxydiazine (SULF); fluoroquinolones: ciprofloxacin (CIP), enrofloxacin (ENR), and ofloxacin (OFX); chloramphenicol: florfenico (FFC); and polypeptides: polymyxin B (PB)*
| Classification | Gene name | Dairy cattle carry number | Positive detection rate | Beef cattle carry number | Positive detection rate |
|----------------|-----------|----------------------------|-------------------------|--------------------------|-------------------------|
| β-lactams      | βaTEM     | 27                         | 100% (27/27)            | 43                       | 97.7% (43/44)           |
|                | βaSHV     | 5                          | 18.5% (5/27)            | 4                        | 9.1% (4/44)             |
|                | βaOXA     | 4                          | 14.8% (4/27)            | 3                        | 6.8% (3/44)             |
| Aminoglycosides| aadA1     | 19                         | 70.4% (19/27)           | 44                       | 100% (44/44)            |
|                | aac (3′)-Ila | 26             | 96.3% (26/27)           | 25                       | 56.8% (25/44)           |
|                | aadB      | 0                          | 0% (0/27)               | 4                        | 9.1% (4/44)             |
| Chloramphenicol| floR      | 26                         | 96.3% (26/27)           | 26                       | 59.1% (26/44)           |
| Tetracyclines  | tet (A)   | 26                         | 96.3% (26/27)           | 43                       | 97.7% (43/44)           |
|                | tet (B)   | 19                         | 70.4% (19/27)           | 42                       | 95.5% (42/44)           |
|                | tet (C)   | 2                          | 7.4% (2/27)             | 0                        | 0% (0/44)               |
| Sulfonamides   | sul1      | 11                         | 40.7% (11/27)           | 21                       | 47.7% (21/44)           |
|                | sul2      | 26                         | 96.3% (26/27)           | 34                       | 77.3% (34/44)           |

| Antibiotic (resistance gene) | Dairy cattle E. coli isolates (n = 27) | Beef cattle E. coli isolates (n = 44) |
|------------------------------|----------------------------------------|---------------------------------------|
|                              | Genotype | Phenotype | Kappa | Genotype | Phenotype | Kappa |
|------------------------------|----------|-----------|-------|----------|-----------|-------|
| Penicillin                   | S        | 0         | 0     | S        | 1         | 1     |
| (βaTEM)                      | R        | 0         | 27    | R        | 0         | 43    |
| Amoxicillin                  | S        | 0         | 0     | S        | 1         | 0     | 0.06 |
| (βaSHV)                      | R        | 5         | 22    | R        | 19        | 24    |
| Ampicillin                   | S        | 1         | 21    | S        | 6         | 34    | 0.03 |
| (βaOXA)                      | R        | 0         | 5     | R        | 0         | 4     |
| Cefazolin                    | S        | 8         | 15    | S        | 23        | 18    | 0.15 |
| (βaA1)                       | R        | 5         | 14    | R        | 17        | 27    |
| Gentamicin                   | S        | 1         | 0     | S        | 18        | 1     | 0.56 |
| (aac (3′)-Ila)               | R        | 9         | 17    | R        | 9         | 16    |
| Kanamycin                    | S        | 15        | 12    | S        | 24        | 16    | 0.02 |
| (aadA1)                      | R        | 0         | 0     | R        | 1         | 3     |
| Tetracycline                 | S        | 1         | 0     | S        | 0         | 1     | −0.04|
| (tet (A))                    | R        | 6         | 20    | R        | 13        | 30    |
| Tetracycline                 | S        | 4         | 4     | S        | 2         | 0     | 0.19 |
| (tet (B))                    | R        | 4         | 15    | R        | 12        | 30    |
| Tetracycline                 | S        | 5         | 20    | S        | 0         | 30    | −0.77|
| (tet (C))                    | R        | 1         | 1     | R        | 14        | 0     |
| Compound Sulfamethoxazole    | S        | 4         | 12    | S        | 18        | 5     | 0.59 |
| (sul1)                       | R        | 2         | 9     | R        | 4         | 17    |
| Sulfamethoxydiazine          | S        | 1         | 0     | S        | 6         | 4     | 0.26 |
| (sul2)                       | R        | 4         | 22    | R        | 10        | 24    |
| Florfenico                   | S        | 1         | 0     | S        | 18        | 0     | 0.41 |
| (floR)                       | R        | 12        | 14    | R        | 14        | 12    |

Note: Susceptible (S and I) or Resistant (R)
genes, while 11 strains of isolates from dairy cattle carried aac(3\textsuperscript{'})-IIa and sul2 (Table 6).

Frequency of virulence gene occurrence in isolated E. coli strains exhibiting antimicrobial resistance

The frequencies of virulence gene occurrence in isolated E. coli strains exhibiting antimicrobial resistance were detailed in Table 7. The majority of β-lactam-, aminoglycoside-, tetracycline-, sulfonamide-, fluoroquinolone- and chloramphenicol-resistant beef cattle E. coli isolates (more than 50%) were positive for irp2 and fyuA genes with a significant association. Significant associations between the rest of virulence genes and antibiotic resistance were not observed.

Discussion

The emergence and spread of AMR bacteria have become a growing problem and a threat to global public health (WHO 2017). In veterinary practice, penicillin, ampicillin, florfenicol, sulfadiazine, streptomycin, gentamicin and tetracycline are all commonly used antimicrobials for treating E. coli-associated infections. Previous studies showed that all 100 E. coli isolates from Irish cattle farms were resistant to streptomycin, with a resistance rate of 100%, followed by resistance rates of 99% for tetracycline, 98% for sulfonamides, and 82% for ampicillin (Karczmarczyk et al. 2011). Aasmäe Birgit et al. also reported that the highest proportion of E. coli isolates from diseased cattle (clinical submissions) was resistant to streptomycin (Aasmäe et al. 2019). However, in this study, we showed that E. coli isolates from dairy cattle and beef cattle with diarrhea were highly resistant to penicillin. Similar to our results, Barigye Robert et al. reported that 23 of 23 (100%) virulent isolates from diarrheic neonatal calves were resistant to penicillin (Barigye et al. 2012). In contrast, we found that E. coli isolated from beef and dairy cattle were both susceptible to polymyxin B. These results indicated that E. coli with different origins may have undergone different evolutionary processes and thereby acquired different resistance genes. Interestingly, this research showed that the resistance rate of E. coli to antimicrobials (except for florfenicol and polymyxin B) from dairy cattle was higher than that of beef cattle. Multidrug resistance analysis showed that most isolates from dairy cattle and beef cattle were resistant to five or six types of antimicrobials. Similarly, multidrug resistance rate in E. coli isolated from dairy cattle is higher than that isolated from beef cattle. In dairy cattle, periodic treatment of mastitis after bacterial infection is very common, and antimicrobials are the most important therapies for bovine mastitis, which may be one potential reason for the high resistance rate of E. coli from dairy cattle (Call et al. 2008; Mazurek et al. 2013). Meanwhile, these results suggested that more rational use of antimicrobials in cattle farms was needed to prevent the development of AMR in E. coli.

E. coli resistance genes bla\textsubscript{TEM} and bla\textsubscript{SHV} were the first described extended spectrum β-lactamase (ESBL) genes in the 1980s, and they were predominant until 2000 (Poirel et al. 2018). Currently, the production of ESBL, especially bla\textsubscript{TEM}, is one of the most important mechanisms of AMR from the clinical and epidemiological point of view (Poirel et al. 2018). Indeed, previous studies reported that bla\textsubscript{TEM} was detected in 78.94% isolates from dairy cattle farms in the Nile Delta in Egypt, whereas bla\textsubscript{SHV} and bla\textsubscript{OXA} were detected only in 0.87% isolates (Braun et al. 2016). In China, previous studies

Table 4 Distribution pattern of virulence genes in isolates from dairy cattle and beef cattle

| Virulence Gene | Isolates from dairy cattle n (%) Total = 27 | Isolates from beef cattle n (%) Total = 44 | P value |
|---------------|------------------------------------------|------------------------------------------|--------|
| irp2          | 22.22% (6/27)                            | 63.64% (28/44)                           | P < 0.01 |
| fyuA          | 22.22% (6/27)                            | 61.36% (27/44)                           | P < 0.05 |
| Stx1          | 3.70% (1/27)                             | 22.73% (10/44)                           | P < 0.05 |
| eaeA          | 3.70% (1/27)                             | 2.27% (1/44)                             | –      |
| hylA          | 0% (0/27)                                | 2.27% (1/44)                             | –      |
| F41           | 14.81% (4/27)                            | 15.91% (7/44)                            | P < 0.05 |
| K99           | 3.70% (1/27)                             | 0% (0/44)                                | –      |
| STa           | 3.70% (1/27)                             | 0% (0/44)                                | –      |
| irp2, fyuA    | 18.52% (5/27)                            | 43.18% (19/44)                           | P < 0.05 |
| eaeA, Stx1, F41 | 3.70% (1/27)                           | 0% (0/44)                                | –      |
| F41, K99, STa | 3.70% (1/27)                             | 0% (0/44)                                | –      |
| irp2, fyuA, Stx1 | 0% (0/27)                              | 18.18% (8/44)                            | –      |
| hylA, eaeA, Stx1 | 0% (0/27)                             | 2.27% (1/44)                             | –      |
| irp2, fyuA, F41 | 0% (0/27)                             | 4.54% (2/44)                             | –      |
| irp2, F41    | 0% (0/27)                                | 11.36% (5/44)                            | –      |

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have shown that detection rate of bla\textsubscript{TEM} was the highest (58.7%); however, detection rate of bla\textsubscript{SHV} was only 2.7% in dairy cattle farms (Yang et al. 2018). In the present study, 27 E. coli isolates from dairy cattle farms were tested and it was found that detection rate of bla\textsubscript{TEM} was as high as 100%, and detection rates of bla\textsubscript{SHV} and bla\textsubscript{OXA} were also higher than previous studies. Similar to the results in dairy cattle, 44 E. coli isolates from beef cattle also showed the highest detection of bla\textsubscript{TEM} (97.7%). In addition, a previous study reported the resistance rates of bla\textsubscript{SHV} (0%) and bla\textsubscript{OXA} (0%) in Japanese beef cattle (Yamamoto et al. 2014), while they were 9.1%
and 6.8% in this work, respectively. These results indicated that blaTEM was still the most common AMR gene in China and other countries regardless of whether the isolates were from dairy or beef cattle. Furthermore, it is worth noting that detection rates of blaSHV and blaOXA may have a tendency to increase. This research further showed that chloramphenicol and aminoglycoside resistance genes were present in *E. coli* isolates. Detection rates of floR in dairy cattle and beef cattle were 96.3% and 59.1%, respectively, which were similar to previous reports (Belaynehe et al. 2018; Wu et al. 2011). In addition, aminoglycoside (resistance) genes aadA1 and aadB were detected in 70.4% and 0% of 27 *E. coli* isolates from dairy cattle and in 100% and 9.1% of 44 *E. coli* isolates from beef cattle. In Ireland, aadA1 and aadB were identified in 19% and 1% of 100 (MDR) *E. coli* isolates recovered from dairy cattle (Karczmarczyk et al. 2011). In Iran, aadA1 was detected in 26.2% of *E. coli* isolates from dairy cattle (Jamali et al. 2018). In Mexico, aadA1 was detected in 17% of *E. coli* isolates from beef cattle (Martínez-Vázquez et al. 2018). Detection rate of aadA1 in this study is much higher than that reported in other countries. Interestingly, the detection rate of aac(3’)-Iia that has not been reported in previous studies was 56.8% in beef cattle and 96.3% in dairy cattle, which is worth further investigation detection rate of tetracycline resistance gene tet (A) was 97.7%, followed by tet (B) (95.5%) and tet (C) (0%) in 44 *E. coli* isolates from beef cattle. In isolates from dairy cattle, detection rate of tet (A) was 96.33%, followed by tet (B) (70.4%) and tet (C) (7.4%). sul1 gene was detected in 40.7% and 47.7% while sul2 gene was detected in 96.3% and 77.3% of *E. coli* isolates from dairy cattle and beef cattle, respectively. These results are similar to those previously reported data (Karczmarczyk et al. 2011; Belaynehe et al. 2018; Shin et al. 2015; Navajas-Benito et al. 2017). Further analysis found that the overall detection rate of resistance genes in dairy cattle was higher than that of beef cattle, suggesting the widespread resistance of *E. coli* in dairy cattle.

Totally 14 different virulence genes were analyzed in *E. coli* isolates from dairy cattle and beef cattle. However, only 7 diarrheagenesis-associated virulence genes (irp2, fyuA, stx1, eaeA, F41, K99 and STa) were detected in isolates from dairy cattle, and 6 diarrheagenesis-associated virulence genes (irp2, fyuA, Stx1, eaeA, hytA and F41) were detected in isolates from beef cattle. In beef cattle, 28 out of 44 *E. coli* isolates were positive for irp2 (63.64%), and 27 were positive for fyuA (61.36%). Detection rates of irp2 and fyuA in isolates from dairy cattle were also the highest, both at 22.22%. These results suggested that irp2 and fyuA in *E. coli* isolates from dairy cattle and beef cattle were the main virulence genes, which was similar to the results of previous studies (Ewers et al. 2004; de Verdier et al. 2012). The results also indicated that detection rate of the main virulence genes irp2 and fyuA in isolates from beef cattle was higher than that in isolates from dairy cattle. Furthermore, detection rates of F41 and eaeA genes were not significantly different between beef and dairy cattle, which was consistent with the results of previous reports (Andrade et al. 2012; Hornitzky et al. 2005; Fremaux et al. 2006). However, the percentage of stx1-positive isolates was higher in beef cattle (22.73%) than in dairy cattle (3.7%), which was different from the results of a previous study (Bok et al. 2015). Further analysis showed that detection rate of irp2/fyuA combination in *E. coli* isolates from beef cattle was also higher than that in dairy cattle. Interestingly, irp2/fyuA/Stx1, hytA/eaeA/Stx1, irp2/fyuA/F41 and irp2/F41 combinations were not detected in dairy cattle but were detected in beef cattle. These results lay a foundation for further understanding the distribution of virulence genes in *E. coli* isolated from dairy cattle and beef cattle and provide a basis for reducing *E. coli* infections.

### Table 6 Dairy cattle *E. coli* resistance genes and virulence genes

| Dairy cattle strain | Virulence gene | Resistance gene |
|---------------------|----------------|-----------------|
| SH160413            | irp2           | blaTEM sul1, sul2, aac (3’)-Iia, aadA1, tet (A), tet (B), floR |
| SH160417            | irp2, fyuA     | blaTEM blaoxa, blaoxa, sul1, sul2, aac (3’)-Iia, aadA1, tet (A), tet (B), tet (C), floR |
| SH160418            | irp2          | blaoxa sul1, sul2, aac (3’)-Iia, aadA1, tet (A), tet (B), tet (C), floR |
| JS160808            | eaeA, stx1, F41 | blaoxa sul2, aac (3’)-Iia, tet (A), floR |
| JS160809            | F41, K99, STa | blaoxa sul2, aac (3’)-Iia, tet (A) |
| JS160810            | F41            | blaoxa sul2, aac (3’)-Iia, tet (A) |
| JS160811            | F41            | blaoxa sul2, aac (3’)-Iia, tet (A) |
| KD161102            | irp2, fyuA     | blaoxa sul1, sul2, aac (3’)-Iia, aadA1, tet (A), tet (B), floR |
| KD161103            | irp2          | blaoxa sul2, aac (3’)-Iia, aadA1, tet (A) |
| KD161106            | irp2, fyuA     | blaoxa sul1, sul2, aac (3’)-Iia, aadA1, tet (A), tet (B), floR |
| KD161108            | fyuA           | blaoxa sul1, sul2, aac (3’)-Iia, aadA1, tet (A), floR |
Conclusions

The results of this study indicated that MDR diarrheagenic *E. coli* were more common in dairy and beef calves, with frequent MDR, ESBL and the presence of tetracycline resistance gene *tet* (A). The prevalence rate in dairy cattle is higher than that in beef cattle, which may be related to the prevalence of resistance genes and highlights the importance of the rational use of antimicrobials and strict enforcement of preventive measures in cattle farms. Furthermore, detection rate of virulence genes in the isolates from dairy cattle was lower than that in beef cattle. Although the link between resistance and virulence genes has been extensively studied and virulence genes *irp2* and *fyuA* have a high detection rate in MDR strains, it is still not conclusive. Our results provide important evidences for better exploring their interaction mechanism.

Further studies are also needed to understand the origin and transmission route of *E. coli* in cattle to reduce its prevalence.

### Table 7 Frequency of virulence genes among antibiotic-resistant *E. coli* isolates

| Antibiotic resistance (beef and dairy cattle) | Beef cattle *E. coli* carry virulence genes n (%) | Dairy cattle *E. coli* carry virulence genes n (%) |
|---------------------------------------------|-----------------------------------------------|-----------------------------------------------|
|                                             | *irp2* | *fyuA* | Stx1 | F41 | hylA | eaeA | *irp2* | *fyuA* | Stx1 | F41 | hylA | eaeA | K99 | Sta |
| PEN (43) and (27)                           | 28/43  | 27/43  | 10/43 | 7/43 | 1/43 | 1/43 | 6/27   | 6/27  | 4/27 | 1/27 | 1/27 | 1/27 | 1/27 | 1/27 |
| AMC                                         | 18/24  | 13/24  | 4/24  | 7/24 | 0/24 | 0/24 | 6/22   | 5/22  | 4/22 | 1/22 | 1/22 | 1/22 | 1/22 | 1/22 |
| AMP                                         | 25/38  | 22/38  | 7/38  | 7/38 | 1/38 | 1/38 | 6/26   | 5/26  | 4/26 | 1/22 | 1/22 | 1/22 | 1/22 | 1/22 |
| CFZ                                         | 17/21  | 11/21  | 3/21  | 7/21 | 0/21 | 0/21 | 4/19   | 4/19  | 4/19 | 1/22 | 1/22 | 1/22 | 1/22 | 1/22 |
| STR (21) and (19)                           | 11/21  | 8/21   | 3/21  | 7/21 | 0/21 | 0/21 | 4/18   | 4/18  | 4/18 | 1/22 | 1/22 | 1/22 | 1/22 | 1/22 |
| GEN                                         | 16/20  | 11/20  | 4/20  | 6/20 | 0/20 | 0/20 | 3/17   | 2/17  | 0/17 | 0/17 | 0/17 | 0/17 | 0/17 | 0/17 |
| KAN (20) and (17)                           | 80%    | 55%    | 20%   | 30%  | 0%   | 0%   | 17.6%  | 11.8% | 0%   | 0%   | 0%   | 0%   | 0%   | 0%   |
| AMC                                         | 14/19  | 11/19  | 4/19  | 6/19 | 0/19 | 0/19 | 2/12   | 2/12  | 3/12 | 0/12 | 0/12 | 1/22 | 1/22 | 1/22 |
| COM (19) and (12)                           | 73.7%  | 57.9%  | 21.1% | 31.6%| 0%   | 0%   | 16.7%  | 16.7% | 25%  | 0%   | 0%   | 0.05%| 0.05%| 0.05%|
| TET (31) and (21)                           | 22/31  | 17/31  | 7/31  | 7/31 | 1/31 | 1/31 | 6/21   | 5/21  | 4/21 | 1/21 | 1/21 | 1/21 | 1/21 | 1/21 |
| COM                                         | 18/26  | 14/26  | 4/26  | 7/26 | 0/26 | 0/26 | 5/21   | 5/21  | 4/21 | 1/21 | 1/21 | 1/21 | 1/21 | 1/21 |
| SULF                                        | 20/30  | 16/30  | 5/30  | 7/30 | 1/30 | 1/30 | 6/22   | 6/22  | 4/22 | 1/22 | 1/22 | 1/22 | 1/22 | 1/22 |
| CIP (30) and (22)                           | 66.7%  | 53.3%  | 16.7% | 23.3%| 0.03%| 0.03%| 27.3%  | 27.3% | 18.2%| 0.05%| 0.05%| 0.05%| 0.05%| 0.05%|
| ENR                                         | 18/22  | 12/22  | 4/22  | 6/22 | 0/22 | 0/22 | 4/18   | 3/18  | 4/18 | 0/18 | 1/18 | 1/18 | 1/18 | 1/18 |
| OFX                                         | 17/19  | 13/19  | 4/19  | 6/19 | 0/19 | 0/19 | 3/17   | 3/17  | 3/17 | 0/17 | 0/17 | 0/17 | 0/17 | 0/17 |
| FFC (19) and (17)                           | 89.5%  | 68.4%  | 21.1% | 31.6%| 0%   | 0%   | 17.6%  | 17.6% | 17.6%| 0%   | 0%   | 0%   | 0%   | 0%   |
| PB (17) and (9)                             | 70.6%  | 41.2%  | 11.8% | 41.2%| 0%   | 0%   | 22.2%  | 22.2% | 11.1%| 11.1%| 0%   | 0%   | 0%   | 0%   |
| (1) and (0)                                 | 0%     | 100%   | 0%    | 0%   | 0%   | 0%   | 0%     | 0%    | 0%   | 0%   | 0%   | 0%   | 0%   | 0%   |

Note: β-lactams: penicillin (PEN), amoxicillin (AMC), ampicillin (AMP), and cefazolin (CFZ); aminoglycosides: streptomycin (STR), gentamicin (GEN), and kanamycin (KAN); tetracyclines: tetracycline (TET); sulfonamides: compound sulfamethoxydiazine (SULF); fluoroquinolones: ciprofloxacin (CIP), enrofloxacin (ENR), and ofloxacin (OFX); chloramphenicol: florfenico (FFC); polypeptides: polymyxin B (PB)
| Classification       | Gene       | Primer sequence (5′ → 3′)                      | Annealing temperature | Fragment length | Reference            |
|----------------------|------------|------------------------------------------------|-----------------------|-----------------|----------------------|
| β-lactams            | *bla*OXA   | F:TTTTCTGTTGTTTGGAATTCC R:TTTCTTGGCTTTTATGCTTG | 53 °C                | 447 bp          | This work            |
|                      | *bla*SHV   | F:CTGATTATCTCCCTGTTGAC R:TTAGCGTTGCCCACTGTTG   | 55 °C                | 843 bp          | This work            |
|                      | *bla*TEM   | F:CAAGAAGCCTGTTGAAAG R:TTACCAATGTTAATACCTG     | 54 °C                | 788 bp          | This work            |
| Tetracyclines        | tet (A)    | F:GCTCATCCTGCTTGCTTTC R:CATAGATCGCCGTGAAGAGG  | 59.5 °C              | 210 bp          | Ng et al. 2001       |
|                      | tet (B)    | F:TTGTTAGGGGCAAGTTTTCR:GTATAGGCCCAATAACCCG    | 59.5 °C              | 659 bp          | This work            |
|                      | tet (C)    | F:CTCTGAGACCTTCAACCCG R:ATGCTGTCATCTACCTGGCC  | 59.5 °C              | 418 bp          | This work            |
| Sulfonamides         | sul1       | F:TCGAGACACGGCGCTCTAAG R:GGGTATCGGAGCGTTTGC    | 63 °C                | 925 bp          | This work            |
|                      | sul2       | F:CTGTTTTGCGCCCGACACAG R:GAAGCGCAGCCGCAATTCAT | 60 °C                | 435 bp          | This work            |
| Aminoglycosides      | aadA1      | F:GCAGCGCAATGACATTCTTG R:ATCCTCGGCGGATTTT      | 60 °C                | This work        | Sáenz et al. 2004    |
|                      | aadB       | F:GAGGAGTTGGACTATGGATT R:CTTCATGGCAATAAGAAAA   | 53 °C                | 208 bp          | This work            |
|                      | aac (3′)-IIa| F:GGCCGACTCTCCGTITCT R:GGACGATACCCCTACGAG     | 54 °C                | 412 bp          | This work            |
| Chloramphenicol s    | floR       | F:GAACACAGCGACCCCGCTAT R:TTCCGCGGCTGCTATGAG   | 54 °C                | 601 bp          | This work            |
| Yersinia             | irp2       | F:AAAGATTCCGTGTTACCCGGA R:TCCCGCGTGGATTGATTTGG | 60 °C                | 301 bp          | This work            |
|                      | FyuA       | F:ACACGGCTTATCCTTGCGC R:GGCCATATGGGCATGAAGAAA | 58 °C                | 953 bp          | This work            |
|                      | eaeA       | F:ATTACTGAGATTAAGGCGCTATG R:TTTATTTGCGAGCCCCCAT | 57 °C                | 682 bp          | This work            |
| Fimbriae             | F41        | F:GAGGGACTTTCTCCTTCTTAG R:AGTCTGCTCACTATTAGGCC | 58 °C                | 431 bp          | This work            |
|                      | K88        | F:GGTCGACTGTCGTGCTCTGGT R:CCATTGCTGCTCGATGCC   | 60 °C                | 792 bp          | This work            |
|                      | K99        | F:TATATCTGAGTTGAGTGGG R:RGGTATCCCTTACGCGAGTAGT | 56 °C                | 314 bp          | This work            |
|                      | 987P       | F:TCTGCCTTTAAAGCTACTG R:RAACTCCACCTTTGATCAC   | 55.5 °C              | 333 bp          | This work            |
|                      | F18        | F:GTGAAAAGACTAGTGTGTTTTCC R:CTTCTTGAAGTACCAGCTG | 55 °C                | 510 bp          | This work            |
| Hemolysin            | hylA       | F:GCATCATCAAGCGCTACCTCC R:RAAGTACGCTAACGCTGTAAGT | 60 °C                | 534 bp          | This work            |
| Shiga toxins         | Stx1       | F:TTAGACTTCTCAGCTGCACAG R:RTTGTGACAAATCCCCTCG | 52 °C                | 579 bp          | This work            |
|                      | Stx2       | F:CCATGAAAACCGGCGACAGCAGT R:CCCTGCTAACGACGACCTG | 58 °C                | 779 bp          | This work            |
| Heat-stable enterotoxins | STa      | F:TCCCTCTTTTATGCTAAGCTGC R:GCGACGGGCAATTACACAAAGT | 56 °C                | 163 bp          | This work            |
|                      | STb        | F:GCAATTAGGTGAGTTGCT R:GCGCTGCAAGAAATTTGACG   | 60 °C                | 368 bp          | This work            |
| Heat-labile enterotoxins | LT       | F:GGCGACAGATTATACGCCTGC R:CGGTCTCTATATATTCCCTG | 54 °C                | 450 bp          | This work            |
Materials and methods

Sample collection and identification of E. coli
From April 2016 to November 2018, we collected fecal samples from sick dairy calves with diarrhea in Suihu, Jiusan and Kedong and fecal samples from sick beef calves in Harbin, Zhaodong and Daqing in Heilongjiang Province, China. The aseptically collected intestinal and fecal samples were inoculated onto MacConkey agar and eosin methylene blue agar (Momtaz et al. 2013a, b). After overnight incubation at 37 °C, only pure pink colonies were selected and transferred to nutrient agar. The isolate was identified by 16S rDNA and stored in 50% glycerol at −80 °C.

Antimicrobial susceptibility test
The antimicrobial susceptibility of E. coli isolated from diarrheal dairy cattle and beef cattle was tested using the Kirby-Bauer disk diffusion method according to standards of the Clinical and Laboratory Standards Association (Clinical and Laboratory Standards Institute 2014). Nutrient agar was used to determine the susceptibility of E. coli to 15 different antimicrobials using commercial disks: penicillin (PEN, 10 μg), amoxicillin (AMC, 10 μg), ampicillin (AMP, 10 μg), cefazolin (CFZ, 30 μg), streptomycin (STR, 10 μg), gentamicin (GEN, 10 μg), kanamycin (KAN, 30 μg), polymyxin B (PB, 300 units), tetracycline (TET, 30 μg), compound sulfamethoxazole (COM, 23.75/1.25 μg), sulfamethoxazole (SULF, 5 μg), florfenico (FFC, 30 μg), ciprofloxacin (CIP, 5 μg), enrofloxacin (ENR, 5 μg), and ofloxacin (OFX, 5 μg). Lab-stored E. coli ATCC 25922 was used as a control strain.

DNA extraction and amplification of resistance genes and virulence genes
Primers used to amplify resistance genes (blaTEM, blaSHV, blaOXA, tet(A), tet(B), tet(C), sul1, sul2, aadA1, aadB and aac(3′)-Ia, floR) and virulence genes (irp2, fyuA, eaeA, hlyA, K88, K99, F41, 987P, F18, Stx1, Stx2, Sta, Stb and LT) were shown in Table 8. Primers were synthesized by the Shanghai Bioengineering Co., Ltd. E. coli genomic DNA was extracted according to the manufacturer’s instructions of the extraction kit (Beijing Tiange Biotechnology Co., Ltd.). PCR was carried out in a 25 μL volume containing 12.5 μL of 2× Taq MasterMix (ComWin Biotech Co., Ltd., Beijing, China), 1 μL of forward and reverse primer, 1 μL of DNA template and 9.5 μL of ddH2O. The parameters for PCR included an initial annealing at 95 °C for 5 min and 30 cycles of 94 °C for 30 s, 53–63 °C for 45 s (the annealing temperature varied according to the primers), and 72 °C for 60 s, followed by a final extension at 72 °C for 5 min. PCR products were analyzed by electrophoresis in a 1% agarose gel.

Statistical analysis
All statistical analyses were performed using GraphPad Prism® 8.00 software (GraphPad Software, Inc., USA). For all experiments, differences were considered to be statistically significant at P < 0.05 values.

Abbreviations
E. coli: Escherichia coli; MDR: Multidrug resistant; DEC: Diarrheagenic E. coli; AMR: Antimicrobial resistance; STEC: Shiga-toxin producing E. coli; PEN: Penicillin; AMC: Amoxicillin; AMP: Ampicillin; CFZ: Cefazolin; STR: Streptomycin; GEN: Gentamicin; KAN: Kanamycin; TET: Tetracycline; COM: Compound sulfamethoxazole; SULF: Sulfamethoxazole; CIP: Ciprofloxacin; ENR: Enrofloxacin; OFX: Ofloxacin; FFC: Florfenico; PB: Polymyxin B; ESBL: Extended spectrum β-lactamases

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Authors’ contributions
S.Y., Y.Z., and Z.Z. contributed to the conception and design of this work; S.Y., C.W., W.H., and N.C. participated in sample collection, laboratory experiments and data analysis; S.Y and Y.L. drafted the manuscript; and S.Y., Z.Z., Y.L., and Z.Z. revised the manuscript. All authors have read and approved the final version of the manuscript.

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Availability of data and materials
All data can be shared upon reasonable request. The data can be obtained by email.

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflicts of interest.

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References
Aasmåe, B., L. Häkkinen, T. Kaart, and P. Kalmus. 2019. Antimicrobial resistance of Escherichia coli and Enterococcus spp. isolated from Estonian cattle and swine from 2010 to 2015. Acta Veterinaria Scandinavica 61 (1): 5. https://doi.org/10.1186/s13028-019-0441-9.
Andrade, G.I., F.M. Coura, E.L.S. Santos, M.G. Ferreira, G.C.F. Galinari, E.I. Facury Filho, A.U. Carvalho, A.P. Lage, and M.B. Heinemann. 2012. Identification of virulence factors by multiplex PCR in Escherichia coli isolated from calves in Minas Gerais, Brazil. Tropical Animal Health and Production 44 (7): 1783–1790. https://doi.org/10.1007/s11250-012-0139-8.
Shin, S.W., M.K. Shin, M. Jung, K.M. Belaynehe, and H.S. Yoo. 2015. Prevalence of antimicrobial resistance and transfer of tetracycline resistance genes in *Escherichia coli* isolates from beef cattle. *Applied and Environmental Microbiology* 81 (16): 5560–5566. https://doi.org/10.1128/AEM.01511-15.

Sivaraman, G.K., S. Sudha, K.H. Muneeb, B. Shome, M. Holmes, and J. Cole. 2020. Molecular assessment of antimicrobial resistance and virulence in multi drug resistant ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* from food fishes, Assam, India. *Microbial Pathogenesis* 149: 104581. https://doi.org/10.1016/j.micpath.2020.104581.

WHO., 2017. World Health Organization. Critically important antimicrobials for human medicine: ranking of antimicrobial agents for risk management of antimicrobial resistance due to non-human use.

Wu, R.B., T.W. Alexander, J.Q. Li, K. Munns, R. Sharma, and T.A. McAllister. 2011. Prevalence and diversity of class 1 integrons and resistance genes in antimicrobial-resistant *Escherichia coli* originating from beef cattle administered subtherapeutic antimicrobials. *Journal of Applied Microbiology* 111 (2): 511–523. https://doi.org/10.1111/j.1365-2672.2011.05066.x.

Yamamoto, S., M. Nakano, W. Kitagawa, M. Tanaka, T. Sone, K. Hirai, and K. Asano. 2014. Characterization of multi-antibiotic-resistant *Escherichia coli* isolated from beef cattle in Japan. *Microbes and Environments* 29 (2): 136–144. https://doi.org/10.1264/jsme2.me13173.

Yang, F., S.D. Zhang, X.F. Shang, L. Wang, H.S. Li, and X.R. Wang. 2018. Characteristics of quinolone-resistant *Escherichia coli* isolated from bovine mastitis in China. *Journal of Dairy Science* 101 (7): 6244–6252. https://doi.org/10.3168/jds.2017-14156.

Yang, Y., Y.L. Peng, J.Y. Jiang, Z.C. Gong, H. Zhu, K. Wang, Q.N. Zhou, Y. Tian, A.J. Qin, Z.P. Yang, et al. 2021. Isolation and characterization of multidrug-resistant *Klebsiella pneumoniae* from raw cow milk in Jiangsu and Shandong provinces, China. *Transboundary and Emerging Diseases* 68 (3): 1033–1039. https://doi.org/10.1111/tbed.13787.

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