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Full Length Research Paper

Resistance patterns and detection of resistance genes in *Escherichia coli* isolated from diarrheic calves in Northeastern China

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The aim of this study was to investigate the antimicrobial resistance and to characterize the implicated genes in *Escherichia coli* isolated from diarrheic calves during 2008-2011. A total of 104 samples were tested to 15 antimicrobial agents with disk-diffusion method. High resistance to amoxicillin, streptomycin, tetracycline, trimethoprim-sulfamethoxazole and doxycycline was detected. A total of 13 out of the 16 resistance genes were searched in this study. The principal mechanisms of resistance found in the 104 calf isolates were blaTEM (84.6%), aadA1(73.3%), cmlA(46.2%), tet(B)(79.8%), sulI (74.0%) and dhfrV(54.8%). One kind of amino acid change in gyrA (four Ser83→Leu) and sixteen in parC (twelve 80Ser→Arg, four 80Ser→Ile) were identified in the 20 fluoroquinolone-resistant isolates. According to pulsed field gel electrophoresis (PFGE) results, clonal dissemination of multi-drug resistance (MDR) strains played an important role in the severe resistant situation among diarrheic neonatal calves in northeastern China.

Key words: *Escherichia coli*, calf, antimicrobial resistance, resistance genes, clonal dissemination.

INTRODUCTION

Escherichia coli is a common inhabitant of intestinal tract of humans and animals, and can be implicated in many human and animal infectious diseases. Certain pathogenic *E. coli* strains are associated with neonatal or post-weaning domestic animal diseases such as diarrhoea of piglets, calves, lambs and edema of piglets (Vihan et al., 1992; Blanco et al., 1996; Smith et al., 2010). The diarrhea calves associated with certain pathogenic *E. coli* may probably die or be related to primary infection with virus or mycoplasma if not be treated in time and often ended in maldevelopment even if survived (Matsuda et al., 2010). Therefore, for fear of significant economic losses, heavy amounts of antimicrobials are used in calves feed for preventive and curative purposes worldwide (Dheilly et al., 2011). As a result, the inevitable selection of antimicrobial resistance in calf pathogens and commensals may emerge and become a worldwide public health problem, including direct impact on food safety. This situation also led to the prevention and treatment failed because of antimicrobial resistance (Angulo et al., 2004; Okeke et al., 2005). Antimicrobial-resistant bacteria carried by animals can enter the human food chain through the consumption of meat or other animal products, through farm runoff water, and by other pathways (Donnelly, 1999). It has now become clear that antimicrobial resistance poses a threat to human and animal health and should be taken

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seriously (Blondeau and Vaughan, 2000; Mora et al., 2005).

Since *E. coli*, a reservoir of antimicrobial resistance genes, is considered as an excellent indicator of bacterial resistance at a population level investigating the prevalence of antimicrobial resistant isolates can facilitate risk assessment of infection and the choice of effective antimicrobial agents in clinical settings (Bogaard and Stobberingh, 2000).

Few data has been reported on antimicrobial resistant characterization of *E. coli* isolates from diarrheic calves although there are many studies about that of *E. coli* derived from other food animals (Lim et al., 2007; Smith et al., 2010; Li et al., 2010; Jiang et al., 2011), companion animals (Costa et al., 2008; Shaheen et al., 2010) and humans (Zhanel et al., 2006; Hoban et al., 2011). The aim of our present study was to investigate the prevalence of antimicrobial resistances in *E. coli* isolates recovered from diarrheic calves in Northeastern China and the mechanisms of resistance implicated in order to assess whether there is clonal dissemination of MDR strains among different animals in the same farm or among different farms.

MATERIALS AND METHODS

Bacterial strains

A total of 104 *E. coli* isolates were recovered from 357 rectal swabs of 2–10-week-old diseased calves as well as from the intestinal contents of dead calves from cases of post-weaning diarrhoea (PWD) from five different regions of Northeastern China (2008-2011). Each isolate was obtained from an individual calf which had no antibiotics application history. All bacterium samples were stored on ice and dispatched within 12 h to the Veterinary Medicine Academy of Northeast Agricultural University, and isolated and purified on MacConkey agar (TIAN HE Microorganism Reagent Co., Hangzhou, China) and identified using the API 20E kit (API System, Hangzhou, China). All isolates were stored at -80°C until analysis. All confirmed *E. coli* isolates were stored at -80°C in Luria–Bertani broth medium containing 10% glycerol.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed on all 104 *E. coli* isolates using the Disk-diffusion (Kirby–Bauer) method on Mueller–Hinton agar medium (TIAN HE Microorganism Reagent Co.) according to the standards recommended by Clinical and Laboratory Standards Institute (CLSI) (2008). All *E. coli* isolates were examined for the resistance to seven chemical classes including amoxicillin (10 µg), ceftiofur(10 µg), streptomycin (10 µg), gentamicin (10 µg), amikacin sulphate (30 µg), neomycin (30 µg), norfloxacin (10 µg), ciprofloxacin (5 µg), enrofloxacin base (10 µg), tetracycline (30 µg), doxycycline (30 µg), chloramphenicol (30 µg), florfenicol (30 µg), trimethoprim-sulfamethoxazole (23.75 µg/1.25 µg) and polymyxin b (300 u). The antibiotic discs were obtained commercially (TIAN HE Microorganism Reagent Co.). *E. coli* ATCC 25922 (American Type Culture Collection) was used as a sensitive control according to the method described by the CLSI (2008). Results were expressed as susceptible or resistant according to the criteria recommended by the CLSI and the manufacturer (TIAN HE Microorganism Reagent Co.).

PCR screening for antimicrobial resistance genes and DNA sequence analysis

All the 104 *E. coli* isolates were examined for the presence of 16 antimicrobial resistance genes. The presence of genes associated with ß-lactams (blaTEM, blaCTX-M-2, blaSHV and blaOXA), aminoglycosides [aadA1, aac(3)-II], tetracycline (tetA, tetB and tetC), amphenicols (cmlA and floR), sulphonamides(sulI and sulII) and trimethoprim (dhfrI, dhfrV and dhfrXIII) were detected by PCR assay. Oligonucleotide sequences and predicted sizes for PCR amplification of different antimicrobial resistance genes from *E. coli* are in Table 1. The primers were synthesized commercially (finished by Sangon Biological Engineering Technology Inc., Shanghai, China). Plasmid DNA for PCR was extracted by E.Z.N.A^{TM} Plasmid Miniprep Kit (Omega Bio-Tek). The PCR reaction mixture contained 2 µL of template DNA, 2.5 µL of 10X Ex Taq Buffer (Takara, Kyoto, Japan), 0.5 µL of each primer, and 2.5 µL of dNTP mixture (2.5 mM of each dNTP) in a final volume of 25µL.The PCR was done using an ABI 2720 thermal cycler (Applied biosystems, USA). Annealing temperature for each antimicrobial resistant gene is in Table 1. The amplified products were analyzed in 1.0% agarose gels by electrophoresis, and recorded with a gel documentation system. The reactions were run in duplicate to confirm results and representative amplification products were sequenced to verify PCR products. Where positive controls were not available, PCRs were carried out using described parameters, suspected positive amplicons were purified and sequenced. E. coli ATCC 25922 strains was used as negative controls.

Amplification and sequencing of gyrA, gyrB and parC genes

The quinolone resistance-determining region (QRDR) of the gyrase subunit A (gyrA), as well as the analogous region of the gyrase subunit B (gyrB) and topoisomerase IV subunit C (parC), present in all 104 *E. coli* isolates were amplified by PCR. The primers and their annealing temperature are listed in Table 1. The genomic DNA of the resistant *E. coli* isolates extracted by E.Z.N.A® Bacterial DNA Kit (Omega Bio-Tek) was used as the template of PCR. The DNA Sequences obtained were compared with those previously reported for gyrA (GenBank accession number X06373), gyrB (NC_000913) and parC genes (M58408 with the modification included in L22025).

Pulsed field gel electrophoresis (PFGE)

PFGE was used to analyze the genomic relatedness among *E. coli* isolates from diseased calves. We randomly selected 16 typical isolates with the same phenotypes of resistance (AMC- STR-TET-SXT- DOX) from five different regions of northeastern China. PFGE of chromosomal DNA digested with the restriction enzyme XbaI was carried out according to a standard protocol using a CHEF-MAPPER System (Bio-Rad Laboratories, Hercules, CA, USA) as described by Gauton (1997). The gels were run at 6.0V cm^{-1} with an angle of 120 at 14°C for 19 h. The Bio-rad 170-3605 CHEF DNA served as size marker.

RESULTS

Antimicrobial susceptibility of *E. coli* isolates from the diseased calves

The susceptibility to 14 antimicrobial agents for these 104

Table 1. Primer sequences used to amplify antimicrobial resistance genes and quinolone resistance-determining region.

Antimicrobial family	Genes	Primer sequence (5'→3')	Annealing (℃)	Fragment size (bp)	Reference
β-lactams	blaCTX-M-2	GCGACCTGGTTAACTACAATCC CGGTAGTATTGCCCTTAAGCC	55	351	Pitout et al. (2004)
	blaTEM	GAGTATTCAACATTTTCGT ACCAATGCTTAATCAGTGA	50	286	Maynard et al. (2003)
	blaSHV	TCGCCTGTGTATTATCTCCC CGCAGATAAATCACCACAATG	50	698	Maynard et al. (2003)
	blaOXA	GCAGCGCCAGTGCATCAAC CCGCATCAAATGCCATAAGTG	50	888	Maynard et al. (2003)
Aminoglycosides	aadA1	TGATTTGCTGGTTACGGTGAC CGCTATGTTCTCTTGCTTTTG	58	284	Van et al. (2008)
	aac(3)-II	ACTGTGATGGGATACGCGTC CTCCGTCAGCGTTTCAGCTA	60	237	Saenz et al. (2004)
Tetracycline	tetA	GTGAAACCCAACATACCCC GAAGGCAAGCAGGATGTAG	50	887	Van et al. (2008)
	tetB	CCTTATCATGCCAGTCTTGC ACTGCCGTTTTTTCGCC	50	773	Van et al. (2008)
	tetC	ACTTGGAGCCACTATCGAC CTACAATCCATGCCAACCC	50	880	Van et al. (2008)
Sulphonamides	sulI	TTCGGCATTCTGAATCTCAC ATGATCTAACCCTCGGTCTC	50	822	Maynard et al. (2003)
	sulII	CGGCATCGTCAACATAACC GTGTGCGGATGAAGTCAG	50	722	Saenz et al. (2004)
trimethoprim	dhfrI	AAGAATGGAGTTATCGGGAATG GGGTAAAAACTGGCCTAAAATTG	50	391	Maynard et al. (2003)
	dhfrV	CTGCAAAAGCGAAAAACGG AGCAATAGTTAATGTTTGAGCTAAAG	50	432	Maynard et al. (2003)
	dhfrXIII	CAGGTGAGCAGAAGATTTTT CCTCAAAGGTTTGATGTACC	50	294	Maynard et al. (2003)
Amphenicols	floR	CACGTTGAGCCTCTATAT ATGCAGAAGTAGAACGCG	55	868	Sáenz et al. (2004)
	cmlA	TGTCATTTACGGCATACTCG ATCAGGCATCCCATTCCCAT	55	455	Sáenz et al. (2004)
Quinolones	GyrA	TACACCGGTCAACATTGAGG TTAATGATTGCCGCCGTCGG	64	647	Bansal et al. (2011)
	GyrB	CTCCTCCCAGACCAAAGACA TCACGACCGATACCACAGCC	64	447	Bansal et al. (2011)
	ParC	AAACCTGTTCAGCGCCGCATT GTGGTGCCGTTAAGCAAA	64	395	Bansal et al. (2011)

Table 2. Antimicrobial resistance rate of *E. coli* isolated from different regions around Harbin.

Antimicrobials	No. of isolates		
	Resistant	Intermediate	Susceptible
AMC	104	0	0
CTF	20	0	84
STR	104	0	0
GM	0	0	104
AMI	0	0	104
NEO	0	0	104
TET	104	0	0
DOX	100	4	0
CMP	20	58	26
FLR	20	12	72
P(B)	0	0	104
SXT	104	0	0
CIP	8	20	76
NOR	12	22	70
EN	4	14	86

AMC, Amoxicillin-clavulanic acid; CTF, ceftiofur; STR, streptomycin; GM, gentamicin; AN, amikacin; NEO, neomycin; TET, tetracycline; DOX, doxycycline; CMP, chloramphenicol; FLR, florfenicol; PB, polymyxin; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; NOR, ciprofloxacin; EN, enrofloxacin base.

Table 3. Phenotypes of resistance detected among the 104 *E. coli* isolates from calves.

Phenotype of resistance	No. of isolates	Percentage of isolates (%)
AMC-STR-TET- SXT	4	3.8
AMC- STR-TET- SXT- DOX	56	53.8
AMC- STR-TET- SXT- DOX-CTF	12	11.5
AMC- STR-TET- SXT- DOX- NOR	8	7.7
AMC- STR-TET- SXT- DOX- NOR-CTF	4	3.8
AMC- STR-TET- SXT- DOX- CMP-FLR	8	7.7
AMC- STR-TET- SXT- DOX- CMP-FLR-CTF	4	3.8
AMC- STR-TET- SXT- DOX- CMP-FLR- CIP	4	3.8
AMC- STR-TET- SXT- DOX- CMP-FLR- CIP-EN	4	3.8

isolates is presented in Table 2. 100% of the isolates showed resistance to amoxicillin, streptomycin, tetracycline and sulfamethoxazole-trimethoprim, and 96.15% exhibited doxycycline resistance. The resistant rate to the other antimicrobial agents was in all cases below 19.23%, and no resistant isolates were detected to gentamicin, amikacin sulphate, neomycin and polymyxin b.

The phenotypes of resistance exhibited by 104 *E. coli* isolates are presented in Table 3. All the 104 isolates showed resistance to more than four antimicrobials and all of them were multidrug-resistant (MDR) isolates. Obviously, the most frequent detected phenotype was AMC- STR-TET- SXT- DOX which was found among 53.8% of these resistant isolates, followed by AMC- STR-TET- SXT- DOX-CTF (11.5%). The phenotypes of these *E. coli* isolates from the five regions showed highly similarity which could be easily found from the resistance to amoxicillin, streptomycin, tetracycline, doxycycline, sulfamethoxazole-trimethoprim and the susceptibility to gentamicin, amikacin sulphate, neomycin, polymyxin b. This reveals the same uses of antimicrobial agents of the nearby areas or there maybe clonal dissemination of identical resistant *E. coli* clones among farms nearby.

Antimicrobial resistance genes and QRDR detected in *E. coli* isolates from diseased calves

All the 104 isolates were screened for the presence of genes coding for 16 resistance determinants. Thirteen (13) out of the 16 resistance genes [bla_{TEM}, $bla_{CTX-M-2}$,

Table 4. Genes of resistance detected among 104 E. coli isolates of calf origins.

Antibiotics	Resistant			Susceptible and Intermediate		
	Number of isolates	Genes detected		Number of isolates	Genes detected	
		Genes	Number of isolates		Genes	Number of isolates
AMC	104	bla_{TEM}	80	0	—	—
		bla_{TEM}+bla_{CTX-M}	8[a]			
		$bla_{CTX-M-2}$	12[a]			
Streptomycin	104	aadA1	76	0	—	—
Gentamicin	0	—	—	104	aac(3)-II	12
Tetracycline	104	tetA	17[b]	0	—	—
		tetB	65[c]			
		tetA +tetB	18[c]			
Chloramphenicol	20	cmlA+floR	20[d]	84	cmlA	28
SXT	104	sulI	5	0	—	—
		sulI+ dhfrV	49			
		sulI+ dhfrXIII	19			
		sulI+ dhfrI	4			
		sulII+ dhfrV	8			
		sulII+ dhfrXIII	17			

[a] These isolates also showed resistance to ceftiofur. [b] Thirteen of these isolates also showed resistance to doxycycline. [c] These isolates also showed resistance to doxycycline. [d] These isolates also showed resistance to florfenicol.

aadA1, aac(3)-II, tetA, tetB, cmlA, floR, sulI, sulII, dhfrI, dhfrV and dhfrXIII)] were detected. All 104 isolates carried one or more antimicrobial resistance genes evaluated. The resistance genotypes for beta-lactams, aminoglycosides, tetracycline, amphenicols, sulphonamides and trimethoprim in all the 104 *E. coli* isolates are presented in Table 4.

Beta-lactam resistance gene blaTEM was found in 88 (86.4%) isolates out of 104 AMC-resistant isolates. 20 (19.2%) isolates (8 bla_{TEM} positive isolates and 12 bla_{TEM} negative isolates) showed ceftiofur resistance and all of them harboured the resistance gene $bla_{CTX-M-2}$ (Table 4). No β-lactamases genes were identified in the remaining four AMC-resistant isolates. Other beta-lactam resistance genes (bla_{OXA} and bla_{SHV}) were not detected.

Of 104 streptomycin-resistant *E. coli* isolates, 76 (73.3%) isolates harboured aadA1 gene. However, it seems paradoxical that aac(3)-II was found in 12(11.5%) isolates with none of the isolates showed resistance to gentamicin (Table 4). In addition, 20 isolates showed resistance to both chloramphenicol and florfenicol. The floR gene was amplified from these 20 (19.2%) isolates and the cmlA gene was amplified from these 20 isolates and other 28 susceptible isolates (Table 4). Tetracycline resistance genes [tet(A) and/or tet(B) genes] were found in all 104 tetracycline-resistant. The tetB gene was most common, found in 83 (79.8%) isolates, and the tetA gene in 35 (33.7%) isolates. Fourteen (14) isolates carried two (tetA and tetB) genes (Table 4). TetC was not detected in any isolates.

Among 104 SXT-resistant E. coli isolates, the sulfonamide efflux resistance gene sulI was found in 77 (74.0%) isolates and sulII found in only 25 (24.0%). Three common trimethoprim resistance (dhfr) genes were targeted: dhfrI, dhfrV and dhfrXIII. The dhfrV and dhfrXIII genes were observed in 57 (54.8%) and 36 (34.6%) isolates, whereas dhfrI was only found in 4(3.8%) isolates (Table 4). But five SXT-resistant isolates did not carry any of dhfr genes and two isolates did not carry either sulfonamide efflux resistance genes or dhfr genes evaluated. They might carry other or even novel genetic resistance determinants.

The gyrA, gyrB and parC genes were amplified and sequenced in all 104 isolates and the deduced amino acid changes detected in GyrA and ParC proteins are shown in Table 5. One amino acid changes in GyrA

Table 5. Amino acid changes in GyrA and ParC proteins deduced from the sequences of the corresponding genes in our quinolone-resistant *E. coli* isolatesa.

Phenotype of resistance to quinolones[a]	Number of *E. coli* isolates	Amino acid changes in	
		GyrA	ParC
Norfloxacin	2	Ser83Leu	wild
Norfloxacin	10	wild	Ser80Arg
Ciprofloxacin	4	wild	Ser80 Ile
Ciprofloxacin-enrofloxacin base	2	Ser83Leu	wild
Ciprofloxacin-enrofloxacin base	2	wild	Ser80Arg

[a]Sequences were compared with gyrA and parC genes included in the GenBank database with the accession numbers X06373 for gyrA and M58408 with the modification in L22025 for parC.

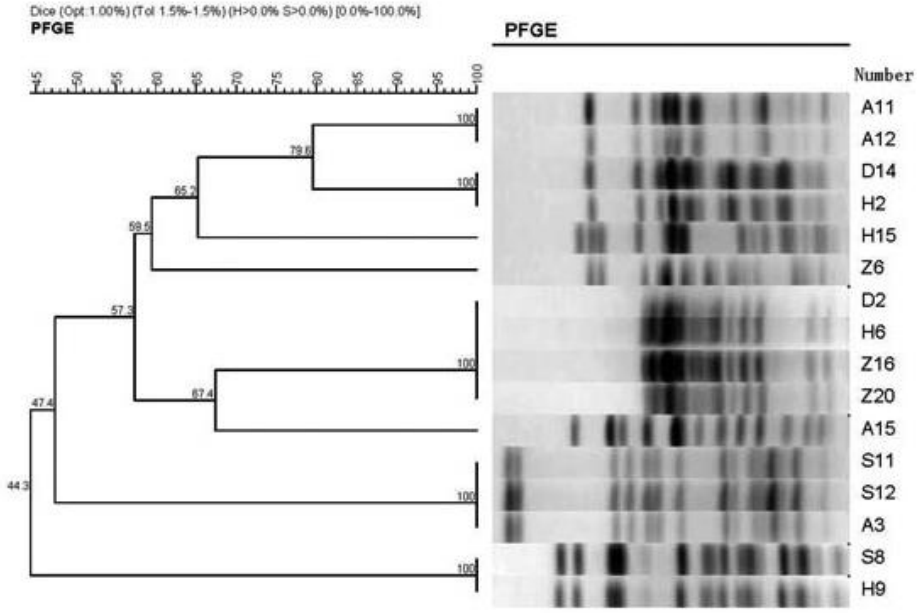

Figure 1. Dendogram showing clustering of XbaI-digested PFGE patterns for isolates; A, D, H, S, represent isolates from different regions.

(Ser83Leu) in two norfloxacin-resistant isolates and one in ParC (Ser80Arg) in other ten norfloxacin-resistant isolates found in this study. Only one amino acid change in ParC (Ser83 Ile) was found in the four ciprofloxacin-resistant isolates. And one amino acid changes in GyrA (Ser83Leu) in two ciprofloxacin-enrofloxacin base-resistant isolates and one in ParC (Ser80 Arg) in the other two ciprofloxacin-enrofloxacin base-resistant isolates. No mutation was detected in gyrB in this study.

Pulsed field gel electrophoresis (PFGE)

To investigate the possibility of clonal spreading, a total of 16 isolates with the same phenotype of resistance from five different geographical regions were analyzed by PFGE. The resulting dendrogram shows diverse group clusters in XbaI-digested PFGE patterns of isolates (Figure 1). Isolates D14 and H2 had closely related banding patterns, and isolates D2, H6, Z16 and Z20 had closely related PFGE profiles. Isolates S11, S12 and A3 had closely related banding patterns, and isolates S8 and H9 had closely related PFGE profiles. Genomic typing demonstrated that at least four closely related M. catarrhalis clones had been transmitted in five regions of northeastern China.

DISCUSSION

It is noteworthy that our study revealed a very high incidence rate of resistance for amoxicillin (100%),

streptomycin (100%), tetracycline (100%), trimethoprim-sulfamethoxazole (100%), and doxycycline (96.2%). The overuse of these antimicrobial agents for a long term might explain the high resistance rates. In contrast to this situation of china, the resistance of *E. coli* isolates from calves to above five antimicrobials was lower than that in other countries (You et al., 2006; Dolejská et al., 2008; Rybaríková et al., 2010). However the percentages of resistance for gentamicin, amikacin sulphate, neomycin and polymyxin b were much lower in this study than any other countries (You et al., 2006; Dolejská et al., 2008; Rybaríková et al., 2010). The enormous difference might reflect the different uses of antimicrobial agents in different regions and different countries. The incidence rate of resistance for ceftiofur, FQNS (ciprofloxacin, norfloxacin and enrofloxacin base) and amphenicols (chloramphenicol and florfenicol) (3.8%-19.2%) were in the average extent (Lim et al., 2007; Dolejská et al., 2008; Rybaríková et al., 2010).

It is interesting to point out that all of the 104 isolates were resistant to streptomycin but were absolutely susceptible to gentamicin, neomycin, and amikacin sulphate. This phenomenon showed that there was no cross resistance among the aminoglycosides in the local farms until now. Chloramphenicol had been forbidden for many years in China, but resistance to chloramphenicol could still be detected. It concur with some studies reporting that resistance to chloramphenicol could still be found from either animal origin isolates or/and human origin isolates in China or other countries (Lim et al., 2007; Jiang et al., 2011). The presence of chloramphenicol resistance could be attributed to the antibiotics resistance genes (ARGs). ARGs which providing resistance to historically used antimicrobial agents will still persist in the local bacterial population, and may increase over time due to co-selection even in the absence of selective pressure (Smith et al., 2010).

Through the high resistant rate to amoxicillin, streptomycin, tetracycline, doxycycline, and trimethoprim-sulfamethoxazole, it can be easily found that the phenotypes of resistance exhibited by the *E. coli* isolates from different farms showed highly similarity. This reveals that above antimicrobial agents have been widely used as therapeutics or feed supplements in these farms or there maybe dissemination of identical *E. coli* clones between these farms.

A total of 13 out of 16 resistance genes were searched in this study. The principal mechanisms of resistance found in the 104 calf isolates were blaTEM (84.6%), aadA1(73.3%), cmlA(46.2%), tet(B)(79.8%), sulI (74.0%) and dhfrV(54.8%).

In our study, all 104 isolates were resistant to amoxicillin-clavulanic acid, and TEM gene was indentified in 84.6% of these isolates which was more frequently detected than CTX-M-2 gene (9.2%), SHV (0) and OXA (0). It demonstrated that TEM β-lactamase was the main mechanism of amoxicillin - clavulanic acid resistance among our isolates from five farms. These findings were similar with previous studies reporting that TEM β-lactamases were the most frequent mechanism in ampicillin-resistant *E. coli* isolates from food-producing animals (Mora et al., 2005; Lim et al., 2007; Jiang et al., 2011; Ryu et al., 2012). Our findings have provided another confirmation that TEM β-lactamase was the main mechanism of amoxicillin resistance.

In our 104 streptomycin-resistant isolates, aminoglycoside resistant gene aadA1 was found in 76 (73.3%) isolates, which was similar to some previous studies (Chang et al., 2007; Costa et al., 2008; Ryu et al., 2012). In cases where the gene aadA1 was not identified, streptomycin resistance may be due to other resistance gene (strA or strB for instance) that was not screened in the present study. Four classes of aac(3) acetyltransferases have been reported associated with gentamicin resistance in *E. coli* (Costa et al., 2008). In this study, aac(3)-II was found in 12(11.54%) isolates with none of the isolates showed gentamicin resistance. Nevertheless, the similar phenomenon had been previously reported (Chen et al., 2005). From these results, we can see that it is possible that resistance genes may not be expressed or expressed in a low level in actuality (Meacham et al., 2003; Ryu et al., 2012). Therefore, the search for antimicrobial resistance genes should not only be limited to phenotypically resistant isolates (Ryu et al., 2012).

In our study, 20 isolates showed chloramphenicol-florfenicol cross-resistance and chloramphenicol resistance gene cmlA and florfenicol resistance gene floR were both amplified in the 20 isolates. There is a previous report about *E. coli* isolates from calf harbouring both floR and the cmlA genes (Du et al., 2004). Since the cmlA gene shares 50.4% homology with floR gene, it is very possible that at some point they may have evolved from a common ancestor resistance gene (Doublet et al., 2002). And in our study, isolates contained gene floR and gene cmlA showed resistance to chloramphenicol while isolates only contained gene cmlA showed resistance to chloramphenicol. So we could imagine that the main mechanism of chloramphenicol resistance was gene floR which was verified chloramphenicol-florfenicol cross-resistance gene and could developed majority of chloramphenicol resistance in calf isolates (Dolejska´ et al., 2008).

The detection of tet(A) and/or tet(B) genes in all our tetracycline-resistant isolates indicates that the main mechanism of tetracycline resistance in calf *E. coli* isolates is by active efflux. A predominance of tet(B) gene has been observed among tetracycline-doxycycline-resistant *E. coli* isolates of diarrheic calves in this study which was also reported in clinical isolates of calves by other authors from different countries (Dolejska´et al., 2008).

The high percentage of resistance to SXT correlates with the high prevalence of genes of antibiotic resistance

to both antibiotics. Such high levels of resistance to this combined antibiotic in *E. coli* isolates from calf had never been reported previously. One given explanation was their widespread use in the treatment of diseases associated with Gram-negative bacteria, especially with acute infectious diarrhoea. And the majority of sul and dhfr genes are related to mobile elements of antibiotic resistance such as integron, which would be consistent with antibiotic exposure explaining their high prevalence in the study population (Costa et al., 2008; Smith et al., 2010; Soufi et al., 2011).

Four amino acid changes in gyrA (4Ser83→Leu) and sixteen in parC (twelve Ser80→Arg, four Ser80→Ile) were identified in the 20 fluoroquinolone-resistant *E. coli* isolates in this study. No mutation was detected in gyrB. This observation was in agreement with previous reports that mutations in gyrA and parC were common among fluoroquinolone-resistant strains of *E. coli* and *Salmonella*, whereas mutations in gyrB were rare (Bansal et al., 2011; Yang et al., 2012).

Overall, when the *E. coli* obtained the resistance, a corresponding resistance gene may be detected most of the time. In cases where the gene was not identified, resistance may be encoded by other resistance gene that was not screened in the present study or due to other mechanisms of resistance. Another situation should be noted that the resistant genes were detected not only from the resistant isolates but also from the susceptible isolates. The reason was complex and the most credible explanation was that the genes may not be expressed. Therefore, investigating antimicrobial resistance genes should not only be limited to phenotypically resistant isolates, but also to susceptible isolates. So far, some studies reported that antimicrobial resistance genes have been detected in some susceptible *E. coli* isolates (Lanz et al., 2003; Srinivasan et al., 2007; Ryu et al., 2012).

In this study, PFGE was performed for subtyping the isolates to determine the genetic relatedness of isolates with the same resistance phenotype. Although the selected number was small (16/104, 15.4%), we found four closely related clones in five different geographical regions, which indicates that these clones are spreading in Northeastern China. Preventive measures should be adopted to control the clonal dissemination of MDR strains.

According to our knowledge, this is the first report about prevalence of antimicrobial resistance and resistance genes in *E. coli* from diarrheic calves in northeastern China. All the 104 *E. coli* isolates showed multi-drug resistance, and very high percentages of resistance to amoxicillin, streptomycin, tetracycline, doxycycline and sulfamethoxazole-trimethoprim have been detected, which were much higher than those reported in other countries. Observations from this study do not exactly explain the link between antimicrobial usage and an increase of antimicrobial resistance among *E. coli* isolates. However, our observations have suggested that clonal dissemination of MDR strains among different animals in the same farm or among different farms played an important role in the severe antimicrobial resistance in northeastern China and our observations have provided data to display the prevalence of 13 kinds of resistant genes in northeastern China.

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Abbreviations

15 antibiotics

AMC, amoxicillin-clavulanic acid; **CTF**, ceftiofur; **STR**, streptomycin; **GM**, gentamicin; **AMI**, amikacin sulphate; **NEO**, neomycin; **NOR**, norfloxacin; **CIP**, ciprofloxacin; **EN**, enrofloxacin base; **TET**, tetracycline; **DOX**, doxycycline; **CMP**, chloramphenicol; **FLR**, florfenicol; **SXT**, sulfamethoxazole-trimethoprim; **P(B)**, polymyxin b.; **blaTEM**, beta-lactamase TEM; **blaCTX-M-2**, beta-lactamase CTX-M-2; **blaSHV**, beta-lactamase SHV; **blaOXA**, beta-lactamase OXA; **aadA1**, aminoglycoside adenylyltransferase A1; **aac(3)-II**, aminoglycoside N(3')-acetyltransferase III; **floR**, florfenicol resistant gene; **cmlA**, chloramphenicol acetyltransferase 2; **tetA**, tetracycline resistance genes A; **tetB**, tetracycline resistance genes B; **tetC**, tetracycline efflux resistance genes C; **sulI**, sulfonamide resistance gene I; **sulII**, sulfonamide efflux resistance gene II; **dhfrI**, trimethoprim resistance gene I; **dhfrV**, trimethoprim resistance gene V; **dhfrXIII**, trimethoprim resistance gene XIII; **gyrA**, DNA gyrase (type II topoisomerase), subunit A; **gyrB**, DNA gyrase (type II topoisomerase), subunit B; **Ser**, serine parC: DNA topoisomerase IV, subunit A; **Leu**, leucine ; Arg, Arginine ; **MDR**, multi-drug resistance ; **ARGs**, antibiotics resistance genes; **QRDR**, quinolone resistance-determining region; **CLSI**, Clinical and Laboratory Standards Institute; **PFGE**, Pulsed field gel electrophoresis.

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