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Resistance of Strains Producing Extended-Spectrum β-lactamases and Genotype Distribution Among *Escherichia coli* in China

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Abstract.- Extended-spectrum β-lactamases (ESBLs) are an increasing cause of resistance to third-generation cephalosporins in Enterobacteriaceae. In order to investigate the resistance of *Escherichia coli* (*E. coli*) strains producing ESBLs and the genotyping of ESBLs from swine in Heilongjiang province in China, 220 *E. coli* strains were isolated consecutively from health swine herds during May to November 2010. Results of antimicrobial susceptibility test showed that most of the isolates were highly susceptible to polymyxin B and aztreonam, but high resistant incidence rates were exhibited to ampicillin. The incidence of ESBL-producing strains was 36.37% among *Escherichia coli* (80/220). The genotypes of ESBLs were analyzed by polymerase chain reaction (PCR), DNA sequencing. PCR and sequence analysis showed that eighty strains produced four type of ESBLs, including CTX-M (27 strains), TEM (36 strains), SHV (2 strains), OXA (2 strains). The resistance of *E. coli* strains producing ESBLs in Heilongjiang Province was a serious issue, and TEM and CTX-M type ESBLs were the most common genotypes. Some ESBLs-producing *Escherichia coli* strains produced more than one type of β-lactamases. These data confirm that ESBL producers are common among nursery strains of *Escherichia coli* in Heilongjiang Province in China. It is important to monitor such strains closely and prevent their spread.

Keywords: β-Lactamases, genotype, *Escherichia coli*, antimicrobial resistance.

INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) represent an important mechanism of resistance in Enterobacteriaceae. ESBLs capable of degrading the extended-spectrum cephalosporins and monobactams are among the most relevant determinants of resistance emerging worldwide in the Enterobacteriaceae. Because ESBL-generating strains often exhibit multidrug resistance, including resistance to aminoglycosides and fluoroquinolones, the therapeutic options associated with these strains are fairly limited. ESBLs are detected most commonly in *Klebsiellae pneumoniae* and *Escherichia coli*. *Escherichia coli* is an important pathogen of humans and animals and is common in their intestinal tracts.

Since the discovery of ESBLs in 1983 (Kliebe *et al.*, 1985), their prevalence has been reported in many countries. In recent years, the prevalence of ESBLs-producing *Escherichia coli* is increasing, and the resistance becomes more and more serious. Until the late 1990s, ESBLs were described mainly as members of the TEM or SHV β-lactamase families in *Klebsiella* and *Enterobacter* spp (Yoo *et al.*, 2010). Since antibiotic prescription pattern varies in different regions, the prevalent genotype of ESBLs is variable (Yu *et al.*, 2007). But there are few such reports from swine in China. With this consideration, we investigated the resistance and probable gene type of ESBLs in 220 *E. coli* isolates collected from swine in Heilongjiang province in China.

MATERIALS AND METHODS

Bacterial isolates

During May and November 2010, 220 *E. coli* strains were isolated consecutively from health swine in Heilongjiang Province of China. The production of ESBLs in the clinical isolates of E.coli was studied by the CLSI diffusion method (CLSI, 2006), as reference test. *E. coli* ATCC25922 was used as reference strains. These strains were stored at -80°C until analysis.

ESBL-producing strains testing

The disk diffusion method was used on

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Mueller-Hinton agar plates inoculated with isolates in a 0.5 McFarland bacterial suspension. ESBL-producing strains were detected by the double-disk synergy test performed as a standardized disk diffusion assay in which an amoxicillin-clavulanic acid disk was placed at the centre of a plate and ceftazidime, cefotaxime,ceftriaxone and aztreonam disks, 25 mm away (centre to centre) from the amoxicillin-clavulanic acid disk.

Their presence was confirmed by the demonstration of synergy between cephalosporin with reduced inhibition zone and clavulanic acid (Synergy was defined when the inhibition zone of the disk of cephalosporin plus clavulanic acid was ≥5 mm greater than that of the disk of cephalosporin alone) by the Clinical and Laboratory Standards Institute (CLSI, 2006). Quality control was performed using *E. coli* ATCC25922.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disk diffusion method on Mueller–Hinton agar. The following antimicrobials were tested: ampicillin, amoxicillin/clavulanic acid, cefalotin, aztreonam, gentamicin, kanamycin, apramycin, neomycin, ciprofloxacin, norfloxacin, enrofloxacinbase, doxycycline, chloramphenicol, florfenicol, polymyxin B, trimesulf, tetracycline.

Molecular characterization of β-lactamase determinants

Detection of genes encoding ESBLs was performed by PCR amplification with the primers listed in Table I. PCR was performed under the following conditions: 94°C for 5 min, 30 cycles of 94°C for 30 s, 56°C for 20s, and 72°C for 40 s followed by 72°C for 7min.

DNA sequencing and data analysis

PCR products were excised from 1.0% agarose gels and purified using AxyPrep™ DNA Gel Extraction Kit (Axygen Scientific, inc., USA) and then cloned into a T-tailed vector, pMD18-T and transformed using JM109 competent cells (TaKaRa, Dalian, China). Three recombinant DNA clones of each product were sequenced by Beijing Genomics Institute (China). The nucleotide sequences were edited, analyzed with EditSeq software (version 7.1.0, DNASTAR Inc., USA) and the program NCBI-BLAST (www.ncbi.nlm.nih.gov).

Table I.- Sequences of primers for PCR used in this study

Primers	Primer sequence (5′→ 3′)	Size (bp)	Ref.
TEM-F	GTATCCGCTCATGAGACAATA	717	This study
TEM-R	AGAAGTGGTCCTGCAACTTT		
SHV-F	TCTCCCTGTTAGCCACCCTG	593	This study
SHV-R	CCACTGCAGCAGCTGCCGTT		
CTX-M-F	GGGCTGAGATGGTGACAAAGAG	905	This study
CTX-M-R	CGTGCGAGTTCGATTTATTCAAC		
OXA-F	GCAGCGCCAGTGCATCAAC	198	Thi Thu Hao *et al.* (2008)
OXA-R	CCGCATCAAATGCCATAAGTG		

Nucleotide sequence accession numbers

The nucleotide sequence data in this paper have been submitted to the GenBank nucleotide sequence database. The accession numbers are as follows. CTX-M-14: HZ17 (HQ162125), HZ22 (HQ162126), JZ9 (HQ162127), JZ28 (HQ162128). TEM-2: MZ28 (HQ162129), HZ54 (HQ162130), JZ37 (HQ162131). SHV-7: JZ14 (HQ162132), JZ16 (HQ162133). OXA-1: JZ13 (HQ162134), HZ29 (HQ162135).

RESULTS

Antimicrobial resistance rates

Antimicrobial susceptibility results of 80 ESBL-producing *E. coli* strains isolated from swine to 17 antimicrobial agents are shown in Table II and Table III. Most of the isolates were highly susceptible to polymyxin B (91.25%), apramycin (88.25%), aztreonam (86.25%). But high resistant incidence rates were exhibited to ampicillin (90.5%). Such higher resistance rates in the ESBL-producing isolates were also observed in each species.

Genotyping of ESBLs

In this study, we identified 4 different types of ESBL genes detected: 36 TEMs, 27 CTX-Ms, 2 SHVs and 2OXAs.TEM was the main type of β-lactamase among ESBL producing *E. coli* followed by CTX-M of ESBLs. Some ESBL-producing *E. coli* produced more than one type of β-lactamase (Such as TEM+CTX-M type) in Table IV.

Table II.- *In vitro* susceptibilities of 80 *E. coli* strains isolated from health swine herds to 17 antimicrobial agents

Antimicrobial agents	No. of resistant strains (%)*		
	R	I	S
Ampicillin	90.50	2.50	7.00
Amoxicillin/clavulanic acid	70.50	7.00	22.50
Cefalotin	51.25	7.50	41.25
Aztreonam	12.50	1.25	86.25
Gentamicin	32.50	2.50	65.00
Kanamycin	20.00	2.50	77.50
Apramycin	8.25	3.50	88.25
Neomycin	38.75	3.75	57.50
Norfloxacin	32.50	6.25	61.25
Ciprofloxacin	38.75	5.25	56.00
Enrofloxacin base	36.25	8.75	55.00
Tetracycline	56.25	3.75	40.00
Doxycycline	68.75	1.25	30.00
Chloramphenicol	18.75	5.00	76.25
Florfenicol	22.25	7.50	70.25
Trimesulf	78.75	5.00	16.25
Polymyxin B	3.75	5.00	91.25

* R, resistant; I, intermediate; S, susceptible.

Table III.- Antibiotic resistance patterns of 80 *E. coli* strains.

Resistant patterns*	No. of drug resistant strains
AMC-AMP-DOX	3
AMC-AMP-TET-DOX	4
AMP-DOX-CEF-FFC-PB	5
AMP-TET-CEF-SXT-APR-KAN	6
AMC-AMP-DOX-TET-CEF-SXT	6
AMC-ATM-DOX-TET-KAN-SXT-NE	7
AMP-NOR-DOX-CIP-TET-KAN-SXT-EN	8
AMC-AMP-NOR-CH-TET-KAN-SXT-FFC-PB	9
AMP-AMC-DOX-CIP-TET-KAN-SXT-CEF-NOR	9
AMC-AMP-DOX-CIP-CEF-KAN-SXT-ATM-CH-CN-NOR	11
AMC-AMP-DOX-CIP-TET-CEF-KAN-SXT-EN-CN-NE-NOR	12
AMP-AMC-DOX-TET-KAN-SXT-EN-NE-NOR-CN-CH-FFC-ATM	13

*Abbreviation for antimicrobial agents: AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CEF, cefalotin; ATM, aztr-eonam;CN, gentamicin; KAN, kanamycin; APR, apramycin; NE, neomycin; CIP, cip-rofloxacin; NOR, norfloxacin; EN, enrofloxacinbase; DOX, doxycycline; CH, chloramphenicol; FFC, Fl-orfenicol; B,PB, Polymyxin SXT, Trimesulf; TET, tetracycline.

Table IV.- Distribution of genotypes of ESBLs in 80 *E. coli* strains.

Genotype	Total
TEM	36
CTX-M	27
SHV	2
OXA	2
TEM+CTX-M	4
TEM+SHV	1
TEM+OXA	1
CTX-M+SHV	1
Not detect the ESBLs genotype	13

DISCUSSION

The ESBL producers usually carry a multi-resistant plasmid. As shown in Table 2, most of the isolates were high susceptible to polymyxin B, aztreonam, apramycin. On the other hand, the existence of high resistant incidence rates to ampicillin, amoxicillin/clavulanic acid, doxycycline and trimesulf may be derived from the numerous, long-term and widely use of these antimicrobials in the studied farms. These results for amoxicillin/ clavulanic acid were similar to previous report (Spanu *et al.*, 2002).

ESBLs are divided into five types: TEM, SHV, CTX-M, OXA and others, based on the homogeneity of coding genes. Most ESBLs derived from plasmid-mediated penicillinases belonging to TEM or SHV families. Recently, the CTX-M group with a typical ESBL resistance phenotype but that does not originate from TEM or SHV families have been described. The CTX-M group is a new family of plasmid-mediated ESBLs that preferentially hydrolyze Cefotaxime (Xiong *et al.*, 2002).

In recent years, ESBL producing strains have been reported more and more frequently in China (Xiong *et al.*, 2004). The predominant genotype of ESBLs was TEM and CTX-M in chicken breeding farms of Henan province, but SHV was not found (Yuan *et al.*, 2009). There were 17 strains produced TEM type ESBLs , 14 strains produced SHV type ESBLs and 15 strains produced CTX-M type ESBLs from cattle in Ningxia (Zhou *et al.*, 2010). The predominant genotype of ESBLs was TEM (100% detection rate) in chicken breeding farms in

Liaoning Province, and then were CTX-M, but SHV was not found (Zhang *et al.*, 2011). TEM and CTX-M are major genotype in Chongqing (Zhang *et al.*, 2009).

The prevalent genotypes vary in different countries, such as the major genotypes TEM-10, TEM-12 and TEM-26 in U.S., (Jacoby and Medeiros, 1991).TEM-10 and TEM-12 in United Kingdom.SHV-3, SHV-4 and TEM-3 in France. Previously, the most prevalent ESBLs in *E. coli* isolates from Korea were recognized as SHV-12 and CTX-M, as well as a prototype of β-lactams, TEM-1 (Kim and Lin, 2005). TEM- and SHV-type ESBLs remain more common in North American. CTX-M type ESBLs have been mainly detected in South America, Eastern Europe, Japan and more recently, in Spain and Kenya (Yu *et al.*, 2007).

In 2000, a Spanish multicenter study reported a much lower frequency of ESBLs than that detected in the present study (Herna´ndez *et al.*, 2005). In Korea, ESBL rates in *E. coli* were 10.2% and 14.2% in previous studies (Ko *et al.*, 2008; Song *et al.*, 2009). On the basis of several recent reports from Korea, the prevalence of ESBL-producing isolates in *E. coli* was reported as 9.2% to 11.7% (Jeong *et al.*, 2004; Kim and Lin, 2005).

Although the present ESBL production rates in *E. coli* isolates from Korea were lower than those in China and Hong Kong (24.5% and 14.3%, respectively), they were higher than those in other Asia-Pacific countries, including Australia, Japan, Philippines, and Taiwan (0.5–5.9%) (Hirakata *et al.*, 2005). Differences between the provinces come from the differences in the use of antibacterial agents and prevalence of plasmids which harbor ESBLs genes.

In this study, we determined most of 80 *E. coli* isolates were the prevalent TEM–type ESBLs. TEM was the main type of β-lactamase, and CTX-M was the second. SHV was detected in 2 isolates. In addition to OXA was detected in 2 isolates. Most ESBL-producing *E. coli* were resistant. Another interesting point is that some ESBL-producing *E. coli* produced more than one type β-lactamase, which is rare in countries other than China.

In conclusion, genotypes of ESBLs in Heilongjiang Province of China have a very high prevalence of *E. coli*. TEM was main type of β-lactamase, and the CTX-M type of ESBL was common among these isolates. Many hypotheses suggest that resistant bacteria or resistance genes could spread from food animals to humans. Thus, public health efforts are necessary to prevent the spread of ESBL-producing between farms. In addition, studies to assess the risk factors for ESBL carriers in China from swine and regular antimicrobial resistance surveillance for swine are required. It is important to monitor such strains closely to prevent their spread.

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