Molecular characterization of extended-spectrum β -lactamase-producing *Escherichia coli* isolates from chickens in Henan Province, China

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Extended-spectrum β -lactamase (ESBL)-producing *Escherichia coli* has spread rapidly worldwide and poses a serious threat to human and animal health. This study collected 51 non-replicate *E. coli* isolates from 14 different chicken farms in Henan Province in China from December 2007 to August 2008. The prevalence of ESBL-producing *E. coli*, molecular characterization of the ESBL-related *bla* genes, including *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M}, and the susceptibilities of these bacteria to various antimicrobial agents were determined. Thirty-one of the 51 isolates were positive for an ESBL phenotype and 29 of these isolates carried one or more *bla* genes. Twenty-two isolates harboured *bla*_{TEM} genes and 15 isolates carried *bla*_{CTX-M} genes (one CTX-M-14, three CTX-M-24 and 11 CTX-M-65). One isolate carried *bla*_{TEM-57}; the remaining *bla*_{TEM} isolates carried *bla*_{TEM-1} with one silent nucleotide base variation (T18C). We believe that this is the first study to report TEM-57 in *E. coli* isolates. All isolates harbouring *bla*_{CTX-M-24} and *bla*_{CTX-M-14} and five of the *bla*_{CTX-M-65} isolates also harboured the *bla*_{TEM-1} gene. To our knowledge, this study is the first to describe detection of CTX-M-65-producing *E. coli* isolated from chickens. None of the isolates contained the *bla*_{SHV} gene. Conjugation experiments demonstrated that *bla*_{CTX-M} and *bla*_{TEM} genes could be transferred to *E. coli* DH5 α . The results indicate that ESBL frequency has reached an alarming level in chicken isolates in China, with TEM-1 and CTX-M-65 enzymes being the two predominant β -lactamases detected.

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INTRODUCTION

Extended-spectrum β -lactamases (ESBLs) are rapidly spreading worldwide (Tenover *et al.*, 1999) and presently comprise over 500 variants (<http://www.lahey.org/Studies>), which are frequently encountered among human and animal clinical *Enterobacteriaceae* isolates (Bradford, 2001). A typical characteristic of ESBLs is their ability to hydrolyse oxyimino-cephalosporins, which can be inhibited by β -lactamase inhibitors (Paterson & Bonomo, 2005). ESBLs, particularly TEM, SHV and CTX-M enzymes, exhibit a high degree of diversity (Bonnet, 2004; Livermore *et al.*, 2007). ESBL genes are usually carried by plasmids, facilitating their spread among Gram-negative bacilli. Several surveillance studies have revealed a relatively high

prevalence of ESBL-producing organisms in the Asia-Pacific area (Hirakata *et al.*, 2005; Wang *et al.*, 2005). In particular, the CTX-M family is believed to be dominant in Asia, as it has appeared or caused outbreaks in many countries (Bonnet, 2004; Munday *et al.*, 2004; Ryoo *et al.*, 2005; Kiratisin *et al.*, 2008; Xiao *et al.*, 2008).

Since 1994, ESBL-producing bacteria have become widely disseminated in the People's Republic of China and their molecular characterization has focused mainly on human clinical isolates (Cheng & Chen, 1994; Chanawong *et al.*, 2002; Wang *et al.*, 2005; Xiao *et al.*, 2008; Liu *et al.*, 2009). Several studies worldwide have studied the epidemiology and molecular characterization of ESBL-producing animal clinical isolates (Batchelor *et al.*, 2005; Briñas *et al.*, 2005; Riaño *et al.*, 2006; Smet *et al.*, 2008), whereas similar reports in China have been rare (Duan *et al.*, 2006; Liu *et al.*, 2007; Hu *et al.*, 2008). Here, we report the results of the first extensive phenotypic and molecular studies of ESBL-producing *Escherichia coli* isolated from 14 different chicken farms in Henan Province, China. The aim of the

Abbreviations: ESBL, extended-spectrum β -lactamase; MDR, multidrug resistant.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the majority of the β -lactamases from the 31 isolates in this study are FJ405191–FJ405214 and FJ812104–FJ812110.

study was to determine the ESBL frequency in clinical *E. coli* isolates from chickens and to reveal basic aspects of their molecular epidemiology.

METHODS

Bacterial strains. A total of 51 non-replicate isolates were collected between December 2007 and August 2008 from 14 chicken farms in Henan Province. All samples were obtained aseptically from liver swabs as soon as the sick chickens died, and the organisms were seeded immediately on MacConkey agar. Each isolate was collected from a single chicken. The isolates were identified as *E. coli* using the Vitek-32 system and showed resistance to ampicillin according to the Clinical and Laboratory Standards Institute (CLSI) broth microdiffusion method (CLSI, 2007). The azide-resistant *E. coli* DH5 α was used as a recipient strain for conjugative transfer. *E. coli* ATCC 25922 (Beijing Ordinary Microbiology Strain Store Center), *E. coli* CF1124 (producing TEM-24; Peking University Health Science Center), *E. coli* ZM-7 (CTX-M-type, GenBank accession no. DQ849332, identified by our laboratory) and *Klebsiella pneumoniae* ATCC 700603 (producing SHV-18; Peking University Health Science Center) were used as reference strains.

Identification of ESBL producers. ESBL-producing isolates were screened by a phenotypic confirmatory test using cefotaxime, ceftazidime, cefotaxime/clavulanate (2:1) and ceftazidime/clavulanate (2:1) according to CLSI recommendations (CLSI, 2007). All ESBL-producing isolates were collected for further investigation.

Antimicrobial susceptibility test. The antimicrobial susceptibilities of all ESBL-producing clinical isolates were determined at the same time using the CLSI broth microdilution method (CLSI, 2007). The antimicrobial agents tested were: ceftiofur, ceftiofur/sulbactam (2:1), amoxicillin/clavulanate (2:1), cefoperazone, cefoperazone/tazobactam (2:1), ceftriaxone, cefepime, amikacin, gentamicin, florfenicol, doxycycline, sulfamethoxazole/trimethoprim (5:1) and enrofloxacin. Isolates shown to be resistant to at least three different classes of agent were classified as multidrug resistant (MDR) (Kiratisin *et al.*, 2008).

Characterization of genes encoding β -lactamases. As most ESBL genes have been shown to be plasmid-borne, plasmid DNA was extracted and purified using a TaKaRa MiniBEST Plasmid Purification kit. PCR amplification of *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} was performed with TaKaRa *Ex Taq* DNA polymerase using the primers listed in Table 1, as described previously (Hu *et al.*, 2006). All PCR amplicons were verified by gel electrophoresis on a 1.0% (w/v) agarose gel and stained with ethidium bromide (0.5 μ g ml⁻¹). PCR amplicons were purified, subsequently ligated into the pGEM-T Easy vector (Promega) and then sequenced. Sequencing analyses were performed online using BLAST (www.ncbi.nlm.nih.gov/BLAST/).

Conjugation. Conjugation experiments were carried out using *E. coli* DH5 α . Transconjugants were selected on tryptic soy agar containing

200 μ g sodium azide ml⁻¹ and 50 μ g cefazolin ml⁻¹ and their β -lactamase genes were confirmed by PCR as described above.

RESULTS AND DISCUSSION

E. coli isolates harbouring ESBLs

With the extensive use in poultry of β -lactam antibiotics such as amoxicillin and cephalosporins, especially extended-spectrum cephalosporins, ESBL-mediated resistance in Gram-negative bacilli has become increasingly critical, and therapeutic options for such infections are becoming limited (Hu *et al.*, 2005; Pfaller & Segreti, 2006). It is important to detect ESBL producers in order to know the ESBL prevalence in animal-associated bacteria and to limit the spread of these MDR organisms in veterinary settings (Ramphal & Ambrose, 2006; Liu *et al.*, 2007). In the present study, 60.8% (31/51) of the isolates tested were ESBL-producing *E. coli*. We showed previously that only 12.9% (4/31) of clinical *Enterobacteriaceae* isolates were ESBL producers (Hu *et al.*, 2006). Another survey (Liu *et al.*, 2007), in 2007, showed that the prevalence of ESBL-producing animal-associated bacteria in China was 30% (15/50). These results indicate that the incidence of ESBLs in bacteria isolated from animals in China is increasing.

Susceptibility of ESBL-producing *E. coli* to antimicrobial agents

The MIC₅₀/MIC₉₀ values of ceftiofur, ceftriaxone, cefoperazone, ceftiofur/sulbactam, amoxicillin/clavulanate, cefoperazone/tazobactam and cefepime against the 31 ESBL-producing *E. coli* isolates were 40/160, 32/128, 64/128, 5/10, 10/10, 4/8 and 2/4 μ g ml⁻¹, respectively (Table 2). All isolates demonstrated high MICs for extended-spectrum cephalosporins whilst remaining susceptible to β -lactam/ β -lactamase inhibitor combinations and cefepime. It is worth noting that the majority of isolates were also resistant to non- β -lactam agents, especially gentamicin (87.1%), amikacin (80.6%), enrofloxacin (80.6%) and sulfamethoxazole/trimethoprim (90.3%). In addition, 80.6% (25/31) of the ESBL-producing strains expressed an MDR phenotype, suggesting that ESBL-producing isolates have probably acquired additional resistance genes. In order to gain a better understanding of their genetic relationships, further

Table 1. Primers used for PCR amplification of *bla* genes

Target	Primer name	Primer sequence (5'→3')	Product size (bp)	GenBank accession no.
<i>bla</i> _{TEM}	TEM-F	GGGGATGAGTATTCAACATTTCC	861	AF332513
	TEM-R	GGGCAGTTACCAATGCTTAATCA		
<i>bla</i> _{SHV}	SHV-F	GGTTATGCGTTATATTCGCGCTGTG	861	AY036620
	SHV-R	TTAGCGTTGCCAGTGCTCGATCA		
<i>bla</i> _{CTX-M}	CTX-F	GGGCTGAGATGGTGACAAAGAG	905	AF252622
	CTX-R	CGTGCGAGTTCGATTATTCAAC		

Table 2. Susceptibilities of ESBL-producing *E. coli* ($n=31$) to various antimicrobial agents

Antimicrobial agent(s)	MIC ($\mu\text{g ml}^{-1}$)			Resistance (%)
	Range	MIC ₅₀	MIC ₉₀	
Ceftiofur	0.63–160	40	160	87.1
Ceftiofur/sulbactam	0.63/0.32–20/10	5	10	0
Amoxicillin/clavulanate	5/2.5–20/10	10	10	0
Cefoperazone	1–128	64	128	74.2
Cefoperazone/tazobactam	0.5/0.25–16/8	4	8	0
Ceftriaxone	0.5–256	32	128	58.1
Cefepime	0.5–4	2	4	0
Gentamicin	0.63–>640	160	640	87.1
Amikacin	0.63–>640	640	>640	80.6
Florfenicol	0.63–160	5	10	19.4
Doxycycline	0.63–80	5	40	32.3
Sulfamethoxazole/trimethoprim	16/3.2–640/128	>640	>640	90.3
Enrofloxacin	0.63–160	5	10	80.6

studies are needed to characterize the plasmids on which *bla* genes and other resistance genes are located.

Molecular characterization of ESBL genes

Twenty-nine of the 31 ESBL phenotype-positive *E. coli* isolates (93.5 %) carried *bla* genes (Table 3). Two isolates did not contain *bla*_{TEM}, *bla*_{SHV} or *bla*_{CTX-M}; further studies will be needed to look for other ESBL genes in these isolates. Among the 29 isolates, *bla*_{CTX-M} was detected in 15 (51.7 %), and 23 (79.3 %) harboured *bla*_{TEM} genes. Nine of the 29 isolates (31.0 %) carried two *bla* genes (*bla*_{TEM} and *bla*_{CTX-M}). None of the isolates contained the *bla*_{SHV} gene. These findings demonstrate that *bla*_{TEM} and *bla*_{CTX-M} genes are the two dominant types in ESBL-producing chicken isolates and that the CTX-M-type β -lactamase is playing an increasing role in antibiotic resistance in China. Based on a conjugation assay for selected isolates, all *bla* genes detected were successfully transferred to transconjugants, suggesting that these genes were plasmid-borne.

One isolate carried *bla*_{TEM-57}; the remaining *bla*_{TEM} isolates carried *bla*_{TEM-1} with one silent nucleotide base variation (T18C) compared with GenBank accession no. AY293072 (TEM-1 β -lactamase). This indicated that TEM-1 is the

most common β -lactamase among *E. coli* isolates from chickens in China. TEM-1, the most prevalent *bla*-encoded enzyme in human clinical isolates worldwide (Ryoo *et al.*, 2005; Carattoli *et al.*, 2008; Kiratisin *et al.*, 2008; Smet *et al.*, 2008), is not classified as an ESBL. However, several TEM-1 derivatives confer ESBL properties (Paterson & Bonomo, 2005). In our study, the 13 isolates carrying *bla*_{TEM-1}, in the absence of other *bla* genes, exhibited resistance to third-generation cephalosporins, including ceftiofur, ceftriaxone and cefoperazone. The MIC₅₀/MIC₉₀ values of these drugs were 40/80, 32/64 and 64/64 $\mu\text{g ml}^{-1}$, respectively. The results suggest that these 13 isolates may carry other ESBL genes that this study did not evaluate, for example OXA-type ESBLs (Paterson & Bonomo, 2005). Compared with TEM-1, TEM-57 contains a Gly→Asp substitution at position 92 (Ambler numbering), which was first described in *Proteus mirabilis* in 1999 (Bonnet *et al.*, 1999). However, we believe that this is the first study worldwide to report TEM-57 in *E. coli* and the first *bla*_{TEM-57} sequence deposited in GenBank (accession no. FJ405211). Studies are needed to characterize this enzyme further.

The CTX-M family, first described in 2002 in China (Chanawong *et al.*, 2002) and known to be the most dominant non-TEM, non-SHV ESBLs among members of the *Enterobacteriaceae*, is recognized as a rapidly growing family of ESBLs in Europe and Asia (Bonnet, 2004; Livermore *et al.*, 2007), with geographical variation in the prevalence of CTX-M cluster groups. In Asia, the CTX-M-9 cluster was found to be the most common (Cantón & Coque, 2006; Ho *et al.*, 2008; Kiratisin *et al.*, 2008; Song *et al.*, 2009), whilst in Europe the CTX-M-1 cluster was found to predominate (Woodford *et al.*, 2004). The current study showed that, among 29 ESBL-producing *E. coli* isolates, *bla*_{CTX-M} genes encoding CTX-M-14, CTX-M-24 and CTX-M-65 were found in 3.5, 10.3 and 37.9 % of the isolates, respectively, suggesting that there might be wide dissemination of different CTX-M-type β -lactamases in chicken isolates in China. Based on amino acid sequence

Table 3. Molecular characterization of *bla* genes among ESBL-producing *E. coli* isolates ($n=31$)

<i>bla</i> gene	No. of isolates
TEM-1	13
TEM-57	1
CTX-M-65	6
TEM-1 and CTX-M-14	1
TEM-1 and CTX-M-24	3
TEM-1 and CTX-M-65	5
Non-TEM, non-SHV and non-CTX-M	2

similarities, CTX-M-14, CTX-M-24 and CTX-M-65 are classified in the CTX-M-9 subgroup. CTX-M-14 was found to be the predominant β -lactamase in human clinical ESBL-containing *E. coli* isolates in China (Munday *et al.*, 2004; Liu *et al.*, 2009) and its sequence was determined from China in 2000 (GenBank accession no. AF252622). The nucleotide change in the protein coding region (A823C) between CTX-M-24 and CTX-M-14 leads to the amino acid alteration Ser275Arg, but the resistant profiles are similar. CTX-M-65, another variant of CTX-M-14, was first detected in *E. coli* isolates from Germany in 2007 (GenBank accession no. EF418608). In 2008, CTX-M-65-producing isolates were identified in China (GenBank accession no. EF394372) and America (Doi *et al.*, 2008), but, to our knowledge, this is the first study to report CTX-M-65 in animal isolates, and the CTX-M-65 prevalence (37.9%) was remarkably high. The amino acid sequence of CTX-M-65 was found to differ from that of CTX-M-14 by the substitutions of Ala80Val and Ser275Arg. Antimicrobial susceptibility tests demonstrated that the CTX-M-65 isolates exhibited a higher level of resistance (from two- to eightfold) to ceftriaxone, ceftiofur, cefoperazone, gentamicin, amikacin and florfenicol compared with CTX-M-14 and CTX-M-24 isolates, i.e. the CTX-M-65 isolates were more prone to exhibit an MDR phenotype. Therefore, these surveys indicated increasing trends not only in the prevalence of CTX-M-65 ESBLs but also in MDR phenotypes in China.

In summary, we have reported the first extensive study of the prevalence and molecular characterization of ESBL-producing *E. coli* isolated from chickens in Henan Province, China. This study clearly indicated that TEM-1 and CTX-M-65 are widespread in this region. Most ESBL producers were resistant to extended-spectrum cephalosporins and exhibited a high rate of the MDR phenotype.

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