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 from December 2007 to August 2008. The prevalence of ESBL-producing *E. coli*, molecular characterization of the ESBL-related *bla* genes, including *blaTEM*, *blaSHV* and *blaCTX-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 *blaTEM* genes and 15 isolates carried *blaCTX-M* genes (one CTX-M-14, three CTX-M-24 and 11 CTX-M-65). One isolate carried *blaTEM-57*; the remaining *blaTEM* isolates carried *blaTEM-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 *blaCTX-M-24* and *blaCTX-M-14* and five of the *blaCTX-M-65* isolates also harboured the *blaTEM-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 *blaSHV* gene. Conjugation experiments demonstrated that *blaCTX-M* and *blaTEM* genes could be transferred to *E. coli* DH5α. 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.

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 oxymino-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.
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* DH5α 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 microdiffusion method (CLSI, 2007). The antimicrobial agents tested were: ceftiofur, ceftiofur/sulbactam (2:1), amoxicillin/clavulanate (2:1), cefoperazone, cefoperazone/tazobactam (2:1), ceftriaxone, cefepine, amikacin, gentamicin, florfenicol, doxycycline, sulamethoxazole/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 μg 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* DH5α. Transconjugants were selected on tryptic soy agar containing 200 μg sodium azide ml⁻¹ and 50 μg 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; Pfaffer & 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 μg 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 sulamethoxazole/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. |
| *bla*TEM | TEM-F | GGGGATGAGTATTCACATTTCC | 861 | AF332513 |
| *bla*TEM | TEM-R | GGGGCTTTACCAATGCGTTAC |
| *bla*SHV | SHV-F | GGGTATGGCGTTATATTGCCTGTC | 861 | AY036620 |
| *bla*SHV | SHV-R | TTAGCGTTGCCAGTGCTCGATCA |
| *bla*CTX-M | CTX-F | GGCGGCTGAGAGTGCTGCAAAAGAG | 905 | AF252622 |
| *bla*CTX-M | CTX-R | CGTGCGAGTTCGATTTATTCAAC |
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 blaTEM, blaSHV or blaCTX-M; further studies will be needed to look for other ESBL genes in these isolates. Among the 29 isolates, blaCTX-M was detected in 15 (51.7 %), and 23 (79.3 %) harboured blaTEM genes. Nine of the 29 isolates (31.0 %) carried two bla genes (blaTEM and blaCTX-M). None of the isolates contained the blaSHV gene. These findings demonstrate that blaTEM and blaCTX-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 blaTEM-57; the remaining blaTEM isolates carried blaTEM-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 blaTEM-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 μg 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 blaTEM-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, blaCTX-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)

<table>
<thead>
<tr>
<th>bla gene</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-1</td>
<td>13</td>
</tr>
<tr>
<td>TEM-57</td>
<td>1</td>
</tr>
<tr>
<td>CTX-M-65</td>
<td>6</td>
</tr>
<tr>
<td>TEM-1 and CTX-M-14</td>
<td>1</td>
</tr>
<tr>
<td>TEM-1 and CTX-M-24</td>
<td>3</td>
</tr>
<tr>
<td>TEM-1 and CTX-M-65</td>
<td>5</td>
</tr>
<tr>
<td>Non-TEM, non-SHV and non-CTX-M</td>
<td>2</td>
</tr>
</tbody>
</table>
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 $\beta$-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, cefotiofur, 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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REFERENCES


