# Detection of ESBLs and Antimicrobial Susceptibility of *Escherichia coli* isolated in Henan, China

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**Abstract:** A total of 51 pathogenic *Escherichia coli* isolates recovered from fowls and pigs were tested for production of Extended Spectrum β-Lactamases (ESBLs) by the Double Disc Synergy Test (DDST). For nine Enterobacteriaceaes tested for ESBLs by double-disk method we designed and synthetized two primer: TEM-1and SHV-1 studied ESBLs gene type with amplified, sequenced and analyzed by PCR; the Minimal Inhibitory Concentrations (MICs) of Cetiofur, Ceftriaxone, Amoxicillin, Ampicillin to the nine Enterobacteriaceae producing ESBLs were carried out with two fold dilution method. The ESBLs producing bacteria amplified TEM type ESBLs fully but no SHV type ESBLs, the Minimal Inhibitory Concentrations (MICs) of Cetiofur, Ceftriaxone, Amoxicillin and Ampicillin were 10-80 μg mL$^{-1}$, 40-80 μg mL$^{-1}$, >128 μg mL$^{-1}$, >128 μg mL$^{-1}$, respectively. This study reveals that *E. coli* isolated from chickens and swines in henan are resistant to the 3rd Cephalosporin Seriously and produce ESBL in large proportions.

**Key words:** ESBLs, resistance, detection, *Escherichia coli*, minimal inhibitory concentrations, antimicrobial susceptibility

## INTRODUCTION

Enterobacteriaceae are major pathogens in animals as well as in humans. In recent years, the bacterial resistance to β-lactam antibiotics has risen dramatically (Katayama *et al.*, 2004). Contributing to this has been the spread of extended spectrum β-Lactamases (ESBLs), enzymes that hydrolyze the expanded-spectrum Cephalosporins like cefotaxime and Ceftiofurand/or the monobactam (Davies *et al.*, 2008).

ESBLs, a kind of β-lactamase were classified in group 2b and subgroup 2be of the Bush-Jacoby-Medeiros classification schemeand were classified kind A in Ambler class (Bush *et al.*, 1995).

The first β-lactamase originating from Enterobacteria was discovered and purified in 1964 it was marked as TEM-1 β-lactamase (Katsanis *et al.*, 1994; Vinodkumar and Neelagund, 2006). The first ESBL which can hydrolyze the 3rd Cephalosporin detected in Klebsiella in German in 1985 (Kliebe *et al.*, 1985). With the widespread use of 3rd generation cephalosporins, new ESBL has emerged continuously, there are more 300 kinds ESBLs now.

When bacteria were caused by ESBL producers, there is a delay in initiating an appropriate antibiotic treatment and high mortality rates (Rodriguez-Bano *et al.*, 2006; Melzer and Perersen, 2007). β-Lactam antibiotics as Amoxicillin, Ceftiofur are frequently used to treat infections in veterinary clinic in China. In this study, the prevalence of ESBL production was evaluated and antimicrobial resistance in *Enterobacteriaceae* species in order to provide a guideline for the treatment about resistant bacterial infections in veterinary practices.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions:** From January 2006-September 2009, 51 non-duplicateg *E. coli* isolates were consecutively collected from different poultry and swine farms in Henan in China. The clinical isolates were identified as *E. coli* by using GNI cards on the VITEK-32 automatic system (bioMerieux, France).

*Escherichia coli* ATCC 25922, C83907 and C83845 were used as the control strain were purchased from the China Institute of Veterinary Drug Control (Beijing, China). The strains were stored at -4°C in nutritional broth until used.

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**Chemicals and enzymes:** Taq DNA polymerase and dNTP were purchased from Takara Biotechnology Co. Ltd., (Dalian, P.R. China). GoldView nucleic acid dye and D2000 was purchased from Beijing Solarbio Science and Technology (Beijing. China). Cetiofur sodium and Ceftriaxone sodium-formulated as an intramuscular injection; Tazobactam sodium, Amoxicillin powder and Ampicillin powder were purchased from Qilu Pharmaceutical Co., Ltd (Shandong, China).

**Detection of ESBLs by Double Disc Synergy Test (DDST):** As described by Livermore and Brown (2001), bacteria were suspended equivalent to McFarland 0.5 turbidity standard in nutritional broth~with Mueller-Hinton broth as test medium. After inoculating a Mueller-Hinton Agar plate, disks containing cefotaxime (CTX, 30 µg each), ceftriaxone (CRO, 30 µg each), ceftazidime (CAZ, 30 µg each) were placed 25 mm (centre to centre) from an Amoxicillin-clavulanic acid disk ( 30 and 10 µg, respectively), incubated at 35°C over night. If there is synergy (combination of paper near the edge of the side appears to expand or enhance), determine the strains producing ESBLs. *Escherichia coli* ATCC 25922 was used as the quality control strain.

**PCR detection of ESBLs genetype:** The screening 9 clinic bacteria isolates further confirmed by Polymerase Chain Reactions (PCRs). As described previously (Tenover *et al.*, 1995), the DNA extracted according to Plasmid Extraction Kit Manual. The DNA extraction was analyzed by electrophoresis on a 1.0% agarosegel and observed with UVI system (UVItec ST John's Innovation Centre, English).

PCRs for ESBL were conducted as the previously described data (Costa *et al.*, 2009). The PCR primers for TEM: 5'-GAGTATTCAACATTTCCGTGTCGC-3' (forward), 5'-TACCAATGCTTAATCAGTGAGGC-3' (reverse) and SHV: 5'-ATGCGTTATATTCGCCTGTG-3' (forward), 5'-TTAGCGTTGCCAGTGCTTGATC-3' (reverse).

The length of the expected amplified fragment was about 860 bp. The PCR amplification reactions were performed in a total volume of 50 µL. Each reaction mixture contained 5 µL of 10×buffer, 4 µL of dNTP, 1 µL of each primer, 2 µL DNA templates and 0.5 µL of *Taq* DNA polymerase. PCR reaction was carried out in a Gene-Amp PCR system 2000 (Biometra, Gottinger, Germany) Programmed to perform. PCR reaction program: initial denaturation for 10 min at 94°C followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and an extension for 1.5 min at 72°C, 35 cycles; the last extension step was extended to 8 min longer. PCR products were evaluated for the expected size and quantified on 1% agarosegels.

**Sequencing:** Both strands of PCR products were sequenced and identified kinds of ESBLs genes. Sequencing of the amplified products was performed with the ABIPRISM 377DNA automated sequencer (Takara Biotechnology Co. Ltd., Dalian, P.R. China). Sequences were analyzed and constructed the phylogenetic tree using the DNASTAR 7.10 (Integrated Sequence Analysis Software), software available on the website of the National Center for Biotechnology Information (accessed 26 Dec 2009).

**Antimicrobial susceptibility testing:** The Minimal Inhibitory Concentrations (MICs) of Amoxicillin, Ampicillin, Ceftriaxoneand Ceftiofur to the *E.coli* strains producing ESBLs were determined with the two-fold dilution method as previously described (Nakamura *et al.*, 2009).

The concentration of the final inoculum was $1-5 \times 10^5$ cfu mL$^{-1}$. The suspension were incubated aerobically at 35°C for 18-24 h. The Minimum Inhibitory Concentrations (MICs) for each isolate was determined as the lowest dilution showing no visible bacteria growth~clarify. The two quality control strains C83907 and C83845 were included in duplicate in each batch tested. Throughout the study, results were interpreted using the National Committee for Clinical Laboratory Standards (Clinical and Laboratory Standards Institute, 2006) for broth dilution and disc diffusion.

## RESULTS AND DISCUSSION

Nine out of the 51 *E. coli* isolates from animal farms in Henan were found to be ESBLs-positive with DDST, (Fig. 1) resulting in an overall prevalence of 17.65%. They were No 1 from chiaken in Kaifeng, No 3 from chicken in Xingyang, No 13 from chicken in Xinzheng, No 24 from chicken in Xinzheng, No. 25 from chicken in Yuanyang,
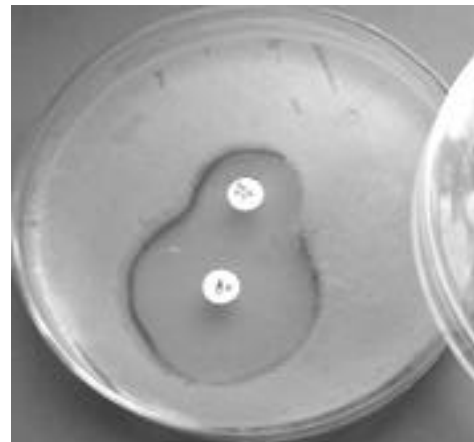


Fig. 1: Detection ESBLs by DDST

Table 1: Results for detection of ESBLs by DDST

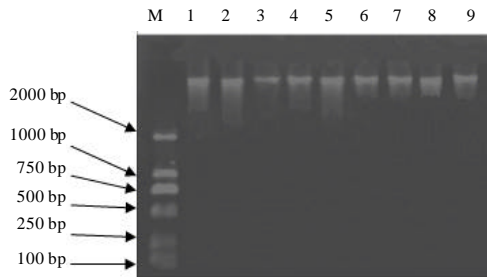| Location of *E. coli* | Bacteria | |
|---|---|---|
| | Strain amount | Positive strain amount |
| Kaifeng | 10 | 2 |
| Luohe | 6 | 1 |
| Yuanyang | 10 | 1 |
| Anyang | 7 | 1 |
| Xingyang | 12 | 2 |
| Xanzheng | 6 | 2 |
| Stand strains | 1 | 0 |



Fig. 2: Result of plasmid of Enterobacteriaceae ertracted of ESBLs, M. Marker, 1-9, T-1, T-2, T-3, T-4, T-5, T-6, T-7, T-8 and T-9
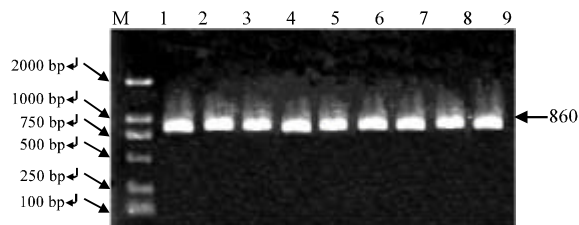


Fig. 3: PCR amplification of plasmid extracted from ESBLsM. DL2000 Marker, 1-9. PCR amplification of TEM primer of ESBLs for T-1, T-2, T-3, T-4, T-5, T-6, T-7, T-8 and T-9

27 from chicken in Luohe, No 32 from chicken in Xingyang, No. 37 from swine in Anyang, No 43 from swine in Kaifeng. They were marked as T-1, T-2, T-3, T-4, T-5, T-6, T-7, T-8, T-9, respectively, The results were shown in Table 1. Among the most relevant determinants of resistance emerging the worldwide in the Enterobacteriaceae. ESBLs are mainly associated with Enterobacteriaceae (Jacoby and Munoz-Price, 2005; Paterson and Bonomo, 2005). The similar proportion of ESBL-producing isolates in *E. coli* (17.65 %) has been reported in previous studies ( Ko *et al.*, 2008).

Figure 2 shows that ESBLs resistant plasmids were extracted in all ESBL-positive bacteria. This results show that results by DDST was in agreement with PCR. For the method DDST was convenient, it was concluded that it fit in with the laboratory. This was in agreement with previous findings (Akujobi *et al.*, 2008). According to
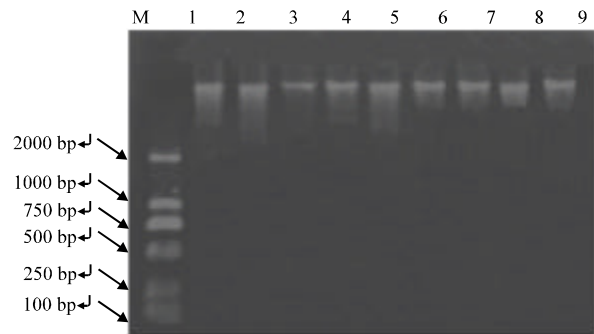


Fig. 4: PCR amplification of plasmid extracted from ESBLs M. DL2000 Marker, 1-9. PCR amplification of SHV primer of ESBLs, for T-1, T-2, T-3, T-4, T-5, T-6, T-7, T-8 and T-9

PCR results (Fig. 3), the ESBLs producing bacteria by DDST, amplified TEM type ESBLs fully but no SHV type ESBLs (Fig. 4). And no bacteria both have TEM and SHV kind ESBL. In sequencing experiments, results show that those homologies among T-3 from Xinzheng, T-4 from Xinzheng, T-5 from Yuanyang were 99.9%, the TEM ESBLs were same as each other in the two bacteria from Yuanyang.

Those homologies between T-1 from chickenin Kaifeng and T-9 from swine in Kaifeng were 100.0% but those homologies among T-6 from Luohe and this above ESBLs were 25.2-26.7% only And those homologies among T-8 from Anyang and this above ESBLs were 23.0-26.5% (Table 2 and Fig. 5).

Sequencing analysis results show that those homologies among ESBLs in the outskirts Zhengzhou were 31.9-00.0%. But those homologies among T-6 bacteria from Luohe (a suburb of Zhengzhou)and this ESBL in the outskirt of Zhengzhou were 25.2-6.7% only those homologies among T-8 from Anyang and ESBLs in outskirt around Zhengzhou were 23.0-26.5% only. These result shows that ESBL producing was possible relation with the frequency of drug using.

The results for antimicrobial susceptibility are shown in Table 3. The MICs of Ceftiofur, Ceftriaxone, Amoxicillin, Ampicillin to the standard strains was 2, 2, 0.02 and 0.2-0.4 $\mu g \ mL^{-1}$, respectively.

In Table 3, the MIC of Ceftiofur to clinical *E. coli* is higher 80-400 times than the standard strains; the MIC of Ceftriaxone to clinical *E. coli* is higher 800-320 times than the standard strain; the MIC of Amoxicillin to clinical *E. coli* is higher 64 times than the standard strain. The MIC of Ampicillin to clinical *E. coli* is higher 64 times than the standard strain too. As we all known, every antimicrobial agent can select for resistant bacteria and

Table 2: Percent identity about sequences of ESBLs

| Bacteria | T-1 | T-2 | T-3 | T-4 | T-5 | T-6 | T-7 | T-8 | T-9 |
|----------|------|------|------|------|------|------|------|------|-------|
| T-1 | - | 23.2 | 25.8 | 25.6 | 25.7 | 26.5 | 25.6 | 25.2 | 100.0 |
| T-2 | 350.0 | - | 23.1 | 23.1 | 23.0 | 23.0 | 31.9 | 25.2 | 23.2 |
| T-3 | 421.7 | 350.0 | - | 100.0 | 99.9 | 24.9 | 25.3 | 26.7 | 25.8 |
| T-4 | 350.0 | 350.0 | 0.0 | - | 99.9 | 25.1 | 25.1 | 26.6 | 25.6 |
| T-5 | 350.0 | 350.0 | 0.1 | 0.1 | - | 24.8 | 25.5 | 26.7 | 25.7 |
| T-6 | 299.1 | 350.0 | 350.0 | 350.0 | 350.0 | - | 23.8 | 26.7 | 26.5 |
| T-7 | 350.0 | 183.7 | 350.0 | 350.0 | 350.0 | 350.0 | - | 25.2 | 25.6 |
| T-8 | 350.0 | 448.4 | 298.5 | 301.0 | 298.5 | 305.3 | 350.0 | - | 25.2 |
| T-9 | 0.0 | 350.0 | 421.7 | 350.0 | 350.0 | 299.1 | 350.0 | 350.0 | - |

Data above-means were percent identity, data below-means were divergence

Table 3: MIC of β-lactam antibiotics to Enterobacteriaceae producing ESBLs ug mL$^{-1}$

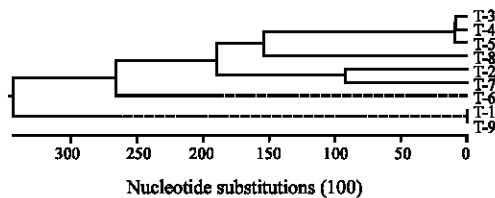| Bacteria | T-1 | T-2 | T-3 | T-4 | T-5 | T-6 | T-7 | T-8 | T-9 | $C_{83907}$ | $C_{83845}$ |
|----------|------|------|------|------|------|------|------|------|------|-------|-------|
| Amoxicillin | >128 | >128 | >128 | >128 | >128 | >128 | >128 | >128 | >128 | 2.00 | 2.00 |
| Ampicillin | >128 | >128 | >128 | >128 | >128 | >128 | >128 | >128 | >128 | 4.00 | 4.00 |
| Ceftriaxone | 128 | 64 | 80 | 64 | 40 | 32 | 64 | 80 | 64 | 0.04 | 0.04 |
| Cetiofur | 32 | 64 | 80 | 80 | 40 | 80 | 64 | 32 | 64 | 0.40 | 0.20 |



Fig. 5: Phylogenetic tree of TEM ESBLs1-9. Sequence of ESBLs for T-1, T-2, T-3, T-4, T-5, T-6, T-7, T-8 and T-9

hence the choice of the most effective antimicrobial agent(s) in the control of bacterial infections. Determination of valid data for the in vitro susceptibility of bacteria is an essential requirement to support the clinician's decision against or for a particular antimicrobial agent.

## CONCLUSION

These result shows that ESBL producing was possible relation with the frequency of drug using. ESBLs-positive *E. coli* in Henan have not transmitted resistant gene through plasmids. The 3rd generation of cephalosporin has been resisted seriously in the veterinary clinic.

## ACKNOWLEDGEMENTS

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