

Study on the Resistance of Extended Spectrum β -Lactamases

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Abstract: The multi-drug resistance of bacteria is a serious threat to the treatment of clinical infectious diseases; *Escherichia coli* (*E. coli*) possess plasmid-encoded Extended Spectrum β -Lactamases (ESBLs) that confer multi-drug resistance. In order to overcome this kind of resistance, some experiments were done in this study. In this study for the clinic pathogenic *E. coli*, ESBLs genotype were detected with PCR and for the induced *E. coli*, the plasmid elimination effects of several plasmid-curing agents to ESBLs were investigated using SDS as a control. Results showed that the bacteria producing ESBLs were amplified TEM type ESBLs fully, two strains were amplified both TEM type ESBLs and SHV type ESBLs, only one strain was amplified both TEM type ESBLs and CTX type ESBLs. And to the induced *E. coli* C83845-y, the elimination rate of chlorpromazine, hydrochloride, diazepam, norfloxacin and ciprofloxacin was 14.53, 13.50, 14.59, 8.43 and 8.23%, respectively, the elimination rate of SDS was 11.05%, blank control was 0.05% to induce *E. coli* C83907-y, the elimination rate of chlorpromazine, hydrochloride, diazepam, norfloxacin and ciprofloxacin was 11.25, 12.23, 13.51, 9.91 and 9.03%, respectively, the elimination rate of SDS was 10.72%, blank control was 0.05%. These results indicated that ESBLs-positive *E. coli* in Henan have transmitted resistant gene plasmids among different bacteria, the tricyclic-psychotropic drugs were more effective at eliminating the ESBL-resistant plasmid. This study provides insight into strategies for overcoming the resistance conferred by ESBLs.

Key words: ESBLs, *E. coli*, multi-drug resistance, detection, elimination, China

INTRODUCTION

E. coli infection has become the primary infectious disease of chickens, resulting in 31,700,000 deaths in China annually and causing large profit losses to the poultry industry (Dai *et al.*, 2008). The β -lactam antibiotics as amoxicillin and ceftiofur had healed the diseases well before. But in recent years, the bacteria resistance reduced their effect seriously.

Strains of *E. coli*, producing ESBLs are the most important resistant mechanism to the third-generation cephalosporins and the new β -lactam-antibiotics (Davies *et al.*, 2008). According to a previous report, the gene encoding ESBL is located on a plasmid and can be transmitted from one bacterial strain to another (Pitout and Laupland, 2008) so, bacteria producing ESBLs have multi-drug resistance consequently. When ESBLs-positive bacteria infection has been identified, there is a delay in initiating an appropriate antibiotic treatment (Akujobi *et al.*, 2008). This resistance has

attracted much attention (Bush, 2008; Canton *et al.*, 2008). However, studies on ESBLs in veterinary clinic of Henan is quite rare. In addition to the using of β -lactamases inhibitors another method of overcoming ESBLs-resistance was eliminating the resistant plasmid and reversing the resistance. Bacteria containing plasmids were cultured with normal medium and simultaneously eliminated by physical and chemical methods although, plasmid replication was suppressed but chromosome replication were still continuing so, the plasmid in offspring bacteria were eliminated. The elimination frequency by suppression is more than 10^2 - 10^5 times the spontaneous elimination.

The methods of eliminate resistant plasmids which include physical methods, chemical methods, antibiotics and non-antibiotic methods (Kristiansen and Amaral, 1997). In this study in order to monitor the spread of ESBLs-resist plasmid, the ESBLs genotypes were detected. And in order to screen to the right plasmid-curing agents, the efficacy of several plasmid-curing agents against ESBLs in *E. coli* were evaluated too.

MATERIALS AND METHODS

Bacterial strains and culture conditions: The standard *E. coli* C83907 and C83845 were purchased from the China Institute of Veterinary Drug Control (Beijing, China). The 153 clinic strains of *E. coli* were isolated from distinct regions of Kaifeng, Xinzheng, Luohe, Luoyang, Nanyang and Xinxiang (Henan, China). Some strains were from Swine, the others were from chickens. All strains were cultured in Luria-Bertani (LB) medium at 37°C.

Chemicals and enzymes: Gold view nucleic acid dye and D2000 were purchased from Beijing Solarbio Science and Technology (Beijing, China). Taq DNA polymerase and dNTP were purchased from Takara Biotechnology Co. Ltd. (Dalian, P.R. China). Cefotiofur sodium, formulated as an intramuscular injection was purchased from Henan Pu-Like Co. Ltd (Henan, China).

Ciprofloxacin was purchased from Zhejiang Guobang Pharmaceutical Co., Ltd. (Zhejiang, China); the hydrochloric chlorpromazine and oxapropzin were purchased from Nantong No. 3 pharmaceutical factory (Jiangsu, China). The Sodium Dodecyl Sulfate (SDS) used as control in elimination experiments was purchased from Shanghai Shengzhong Fine Chemical Co. Ltd (Shanghai, China).

Detection of ESBLs: The 153 clinic bacteria isolates were detected by PCR. As described previously (Tenover *et al.*, 1995) according to Plasmid Extraction Kit manual, the DNA was extracted. Quality of the extracted DNA was analyzed by electrophoresis on a 1.0% agarosegel and observed with UVI system (UVI tec ST John’s Innovation Centre, English). The PCR primers for TEM: 5'-GAGTATTCAACATTTCCGTGTCGC-3' (forward) 5'-TACCAATGCTTAATCAGTGAGGC-3'(reverse), SHV: 5'-ATGCGTTATATTCGCCTGTG-3'(forward), 5'-TTAGCG TTGCCAGTGCTTGATC-3' (reverse) and CTX: 5'-GGGC TGAGATGGTGACAA AGAG-3' (forward), 5'-CGTGC GAGTTCGATTTATTCA AC- 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 2 µL DNA templates, 1 µL of each primer, 0.5 µL

of Taq DNA polymerase, 5 µL of 10 x buffer and 4 µL of dNTP. PCR reaction was carried out in a Gene-Amp PCR system 2000 (Biometra, Gottinger, Germany).

PCR reaction program: The PCR program consisted of an initial denaturation step at 94°C for 10 min followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and primer extension f at 72°C or 1.5 min, 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.

Induction of resistant bacteria producing ESBLs: To avoid the other resistant influence for the plasmid cure agents in the clinical resistant bacteria. In this study, the standard strains was induced by cefotiofur and the induced bacteria producing ESBLs was used the eliminated bacteria.

The standard bacteria was inoculated into medium containing the sub-inhibitory concentration drug and incubated for 16~18 h then frozen for 6~8 h. After it was melted, the bacterium was inoculated into the medium containing higher concentration drug again. The above steps were repeated until bacteria had produced ESBLs.

Elimination of ESBL-resistant plasmid: After the MICs of plasmid-curing agents to the induced *E. coli* were detected, using the 2-fold dilution method as previously described (Nakamura *et al.*, 2009) (Table 1). ESBLs resistant-plasmid elimination was carried out according to previous reports (Imre *et al.*, 2006; Schelz *et al.*, 2006). Firstly, the *E. coli* strains were inoculated onto LB agar plate for resuscitation then inoculated into 5 mL of LB broth and incubated shaking for 24 h at 37°C. Secondly, 60 µL of the germ culture containing 10 µL plasmid-curing agents (1/2 MIC) broth was prepared and incubated, shaking for 24 h at 37°C. The above bacteria culture were then inoculated onto 10-12 LB agar plates containing plasmid-curing agents, respectively and incubated for 24 h at 37°C. Thirdly, 100-500 single colonies were picked and inoculated onto LB agar plates containing ampicillin (0.1 mg mL⁻¹) incubated for 24-48 h at 37°C. Some colonies were inoculated onto agar plates without ampicillin as controls.

Table 1: MIC of plasmid-curing agents to *E. coli*

Drug	C83907-y	C83845-y	C83907	C83845	C83907-x	C83907-x
	-----ug mL ⁻¹ -----					
Chlorpromazine	74	74	74	74	74	74
Hydrochloride	74	74	74	74	74	74
Diazepam	74	74	74	74	74	74
Norfloxacin	16	32	0.1	0.1	0.1	0.1
Ciprofloxacin	8	16	0.05	0.05	0.05	0.05

C83907-y was the induced bacteria using C83907, C83845-y was the induced bacteria using C83845. C83907-x was the eliminated bacteria from C83907-y, C83845-x was the eliminated bacteria from C83845-y

Screening for expunction bacteria: After the 24-48 h incubation, bacteria did not grow on ampicillin-containing plates but grow on common plates were selected and subcultured again on ampicillin-containing plates. Bacteria did not grow after subculturing was classed as possible expunction bacteria.

Detection of expunction bacteria: Possible expunction bacteria were inoculated into 10 mL broth culture and incubated, shaking for 48 h at 37°C. Plasmid DNA was extracted using a plasmid extraction kit (Takara Biotechnology Co. Ltd., Dalian, China), separated on 1.0% agarose gels (Takara Biotechnology Co. Ltd.) and observed with a UVI system (UVI tec, ST John's Innovation Centre, UK) according to the manufacturer's instructions.

Calculation of the plasmid elimination rate: Bacteria which grew in ordinary plates but did not grow in plates containing antibiotic were chosen and confirmed in plates containing AP were recorded as bacteria eliminated ESBLs plasmid. Percentages of elimination were calculated.

The control experiment: The conventional plasmid eliminator agent SDS (0.5%) was used as the control. Bacteria producing ESBLs were incubated in water at 45°C, other steps are as same as the previous. Bacteria that had naturally eliminated the plasmid were used as a blank control.

RESULTS AND DISCUSSION

In 153 clinic strains, plasmids from ESBLs were extracted in 35 *E. coli*. And 35 strains were amplified TEM type ESBLs fully, two strains were amplified TEM type ESBLs and SHV type ESBLs, only one strain was amplified TEM type ESBLs and CTX type ESBLs (Fig. 1-3) were pictures of these results.

PCR amplifications of extraction of ESBLs-resistant elimination before and after were showed in Fig. 4. It can be seen from the figure; ESBLs resistant-plasmid was clearly visible in the induced *E. coli* but not amplified in the eliminated bacteria. Elimination rate of agents on ESBL-resistant plasmid *in vitro*. All elimination results were showed in Table 2. For the induced C83845-y strain, the elimination rate of chlorpromazine, hydrochloride, diazepam, norfloxacin, ciprofloxacin was 14.53, 13.50, 14.59, 8.43 and 8.23%, respectively, for the other induced bacteria (C83907-y), the elimination rate of chlorpromazine, hydrochloride, diazepam, norfloxacin, ciprofloxacin was 11.25, 12.23, 13.51, 9.91 and 9.03%, respectively. And the elimination rates of SDS to C83845-y and C83907-y were 11.05 and 10.72%, blank control was 0.05 and 0.06%.

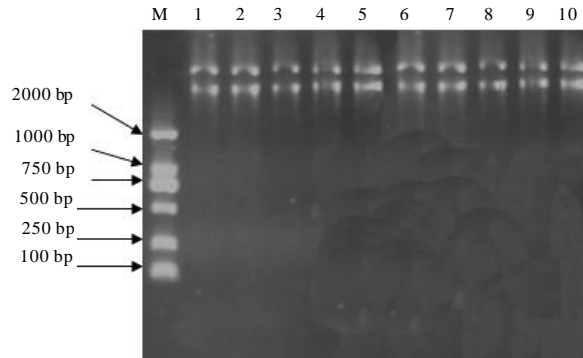


Fig. 1: Results of plasmid of some *E. coli* extracted of ESBLs M. Marker; 1~10 were *E. coli* in chickens from Kaifeng, Xinzheng, Luohu, Luoyang, Xinyang, Zhongmou and *E. coli* in Swine from Xinzheng, Xinyang, Anyang, Nanyang

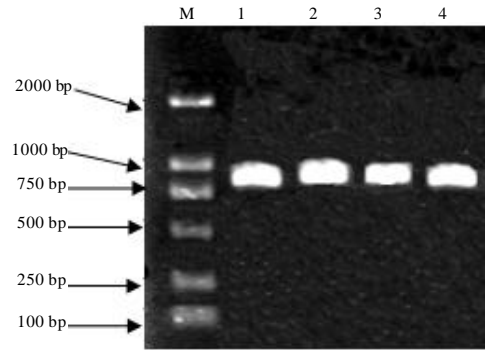


Fig. 2: PCR amplification of plasmid extracted from ESBLs M. Marker; 1, 2 PCR amplification of TEM and SHV primer of ESBLs for *E. coli* from Swine in Anyang. 3, 4. PCR amplification of TEM and SHV primer of ESBLs for *E. coli* from Chicken in Xinyang

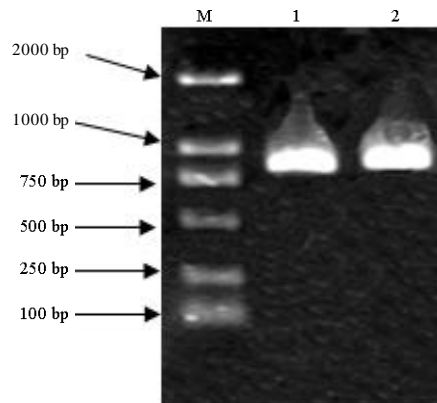


Fig. 3: PCR amplification of plasmid extracted from ESBLs M.Marker; 1, 2 PCR amplification of TEM and CTX primer of ESBLs for *E. coli* from Chicken in Xinzheng

Table 2: Elimination rate of plasmid-curing agents to plasmid of ESBLs *in vitro*

Bacteria	Drug (%)						
	Chlorpromazine	Hydrochloride	Diazepam	Norfloxacin	Ciprofloxacin	SDS	Blank control
C83845-y	14.53	13.50	14.59	8.43	8.23	11.05	0.05
C83907-y	11.25	12.23	13.51	9.91	9.03	10.72	0.06

C83907-y was the induced bacteria using C83907, C83845-y was the induced bacteria using C83845

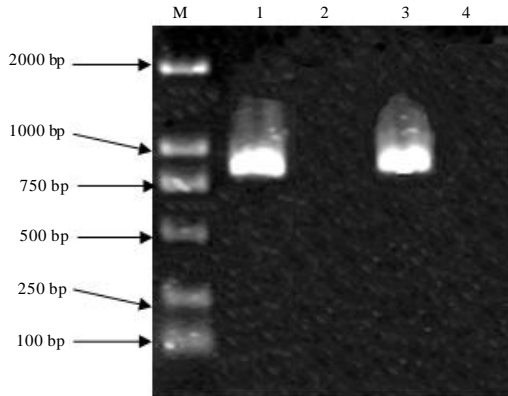


Fig. 4: PCR products of plasmid before/after elimination
M. Marker; 1, 3. Before elimination of ESBLs in C83907-y and C83845-y; 2, 4. After elimination of ESBLs in C83907-y and C83845-y

Bacterial resistance is a public health problem worldwide. In recent years, bacterial resistance to β -lactam antibiotics has risen dramatically (Katayama *et al.*, 2004). Contributing to this increase has been the spread of ESBLs. In this study, the produced bacteria ESBLs were amplified TEM type ESBLs fully. It illustrated that TEM type ESBLs are the most ESBLs in veterinary clinic in Henan province, the *E. coli* from Anyang and Xinyang produced both TEM-type ESBLs and SHV-type ESBLs. *E. coli* from Xinzheng produced both TEM-type ESBLs and CTX-type ESBLs. This result illustrated that the clinic *E. coli* producing ESBLs have transmitted resistant gene through plasmids in Henan.

Under antibiotic selection pressure, resistant plasmid bonded and transferred in the bacteria will increase the clinical therapeutic difficulty. Ceftiofur, a third-generation broad-spectrum cephalosporin has been used to treat diseases in Swine, ruminants and horses (Hornish and Kotarski, 2002). But after the standard strain were induced by it for 18 times in this study, it produced ESBLs and the MIC was increased significantly. This showed that the bacteria resistance was associated with the frequent use of drugs.

In the last 30 years, many researchers in the world have been actively looking for effective ways to eliminate resistant plasmids. In this study, the elimination rates of chlorpromazine, hydrochloride and diazepam were higher than natural elimination rate were similar with SDS. But the

SDS has higher toxicity was not suitable for using *in vitro*. And the MICs of tricyclic-psychoactive drugs to the standard strains C83845 and C83907 were same the MICs of them to the induced producing bacteria, this illustrated that tricyclic-psychoactive drugs have not pharmacology effect to *E. coli*, only played the elimination in the experiment. As reported by Molnar *et al.* (1992) and Kristiansen *et al.* (2007), the tricyclic-psychoactive drugs can eliminate hemolysin-encoded plasmid, increase the permeability of bacterial membrane and inhibit the activity of DNA helicase to format supercoiled plasmid, resulting in the inhabitation of plasmid replication. We can conclude that tricyclic-psychoactive drugs have the potential power to overcome the antibiotic resistance conferred by ESBLs.

CONCLUSION

These result shows that ESBLs-positive *E. coli* in Henan have not transmitted resistant gene through plasmids. The tricyclic-psychoactive drugs were proposed to be used as plasmid-cure agent in veterinary clinics in China.

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