

Full Length Research Paper

Chinese pulsatilla extracts eliminate resistance of *Escherichia coli* to streptomycin

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Drug resistance is widely distributed in *Escherichia coli coli*, and related technology is urgently required to control its transmission. The Chinese pulsatilla extracts containing crude drug of 12.5 mg/ml was used to eliminate resistance plasmid against streptomycin in chicken *E. coli*. After screening and identification, plasmids were extracted from resistance-eliminated strain and electrophoresed in order to compare plasmid profiles before and after elimination. After the drug resistance was eliminated, the diameter of inhibition zone became larger and the minimum inhibitory concentration declined. These results indicated that Chinese pulsatilla extracts makes *E. coli* strain that was sensitive to streptomycin become resistant to this antibiotic. The alimantation rate was 15.8% (79/500). Compared with the control bacteria, the resistance-eliminated strain lost an approximately 20 kbp plasmid band, which showed that the Chinese pulsatilla extracts may convert drug resistance by eliminating plasmid against streptomycin.

Key words: Chinese pulsatilla, *Escherichia coli*, streptomycin, resistance plasmid.

INTRODUCTION

Escherichia coli (*E. coli*) is a representative species of *Escherichia* of the family Enterobacteriaceae (Cai et al., 2001). It mainly causes intestinal diseases in mammals and secondary local or systemic infection in poultry, which lead to serious economic losses in poultry industry. Moreover, drug resistance is generated and spread rapidly in *E. coli*; thus, it is more difficult to control colibacillosis (Zhang et al., 2010). In recent years, super- or multi-drug resistant bacteria have appeared in humans and animals, but almost no drug are available for treatment. Therefore, animal-derived resistant bacteria pose great harm to humans, and it is very urgent to study how to control bacterial resistance. Resistance plasmid is small, double-stranded, covalently closed and circular DNA molecule that is separate from chromosomal DNA, and it can replicate independently and is non-essential for bacterial growth (Wang et al., 2011). In addition, it carries genetic information that provides resistance to antibiotic

drugs. Some resistance plasmids are able to transfer themselves between distantly related bacteria, and hence wide resistance (Mazodier and Davies, 1991). Some researchers have confirmed that drug resistance *aadA* gene cassettes, which encode adenylyltransferase that inactivates streptomycin, are commonly found in the resistance plasmids of Gram-positive bacteria (Shaw et al., 1993). Hence, in-depth study of resistance plasmid is in favor of effective prevention and control of bacterial resistance. In this study, we used Chinese pulsatilla extracts to eliminate drug resistance in chicken *E. coli*, and we also explored its elimination mechanism at cellular and molecular level by analyzing plasmid patterns.

MATERIALS AND METHODS

Materials

The Chinese pulsatilla was purchased from Anguo traditional Chinese medicine market (Anguo, China). *E. coli* O₁ strain CVCC-249 was purchased from the China veterinary culture collection center of China institute of veterinary drug control (Beijing, China).

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Drug sensitive paper discs were purchased from Hangzhou Tianhe microorganism reagent company limited. (Hangzhou, China). Streptomycin was purchased from North China pharmaceutical group corporation (Shijiazhuang, China). Plasmid extraction mini kits were purchased from biodev-technology company limited. (Beijing, China). Phage lambda DNA was purchased from TaKaRa Biotech (Dalian, China).

Preparation of Chinese pulsatilla extracts

One hundred grams of Chinese pulsatilla was soaked in 100 ml of distilled water for 30 min, and then distilled water was added to a level 2 to 3 cm above the herbs. The herbs were boiled in water over strong heat. When the water boiled, gentle heat was used to stew the herbs for another 30 min. The decoctions were strained through four-layer gauze. The water was re-added to herb surface, and the herbs were decocted again. All decoctions were mixed and concentrated to 20 ml. After cooling, the concentrate was incubated in 100 ml of alcohol overnight and strained. The filtrates were distilled to remove alcohol, and the yielded non-alcoholic liquid was adjusted to pH 7.2–7.4 and diluted to 100 ml. Following autoclaving and frozen centrifugation (desktop high-speed refrigerated centrifuge CR14RD that was purchased from Shanghai techcomp instrument limited), the supernatant was preserved at 4°C for use. The concentration of crude drug in the Chinese pulsatilla extracts was 1 g/ml.

Elimination of drug resistance of *E. coli* with Chinese pulsatilla extracts

The *E. coli* O₁ strain CVCC-249 was inoculated in LB medium and incubated at 37°C for 12 to 14 h with shaking. Ten microliter culture was added to each tube containing 5 ml of herbal eliminating agent diluted with LB medium (100, 50, 25... 0.39 mg/ml) and then cultured at 37°C for 48 h. After incubation, the culture was inoculated on agar plates by streak culture, three duplicates per tube, and cultured at 37°C for 18 to 24 h. Bacterial growth was observed. The concentration of herbal eliminating agent equal to 50% of its minimum inhibitory concentration (MIC) was taken as its sub-inhibitory concentration on *E. coli* O₁ strain CVCC-249, which was also its working concentration.

Screening of resistance-eliminated bacteria

Single colonies on the plates which derived from the culture that had been incubated in the herbal eliminating agent at the sub-inhibitory concentration were respectively inoculated on nutrient agar plates and agar plates containing 10 µg/ml streptomycin, followed by culture at 37°C for 18 to 24 h. The colonies that could grow on the nutrient agar plates but not on the drug-containing plates were re-cultured on the nutrient agar plates and then re-identified by inoculation on the drug-containing plates. The resistance-eliminated strain was obtained. Bacteria not treated with the herbal eliminating agent were used as control.

Drug susceptibility test

The drug susceptibility was detected by the Kirby–Bauer disc diffusion method as recommended by the World Health Organization (WHO). According to the disc diffusion standard for *E. coli* drug resistance against 24 antibiotics recommended by the national committee for clinical laboratory standards (NCCLS), drug resistance based on size of inhibition zone is at three levels: sensitivity (S), 0–11 mm; intermediate (I), 12–14 mm; and

resistance (R), ≥ 15 mm.

Determination of MIC

Microdilution method was used to determine the MIC. A series of streptomycin solutions at the concentration of 50, 25, 12.5... 0.195 mg/ml was added to UV-disinfected 96-well polystyrene U-bottom microplates, 50 µl per well (from well 1 to well 9). Bacteria control, blank control and drug control were set by adding 50 µl of LB medium to the 10th well, 100 µl of LB medium to the 11th well, and 100 µl of 100 µg/ml streptomycin solution to the 12th well, respectively. Then, LB culture of the resistance-eliminated bacteria was added to the first 10 wells, 50 µl per well. After the plate was covered with a glass slide and mixed through oscillation, it was incubated at 37°C for 18 to 24 h in a square tray padded with wet gauze. The microplate was observed on a black ground. Turbidity and/or a round-dot pellet at the bottom indicated bacterial growth, while no turbidity indicated bacterial growth was inhibited. The lowest drug concentration that inhibited visible bacterial growth was defined as its MIC. Bacteria not treated with the herbal eliminating agent were used as control.

Determination of elimination rate of drug resistance

The *E. coli* culture which had been incubated in the Chinese pulsatilla extracts were inoculated on the nutrient agar plate by streak culture and then cultured at 37°C for 18 to 24 h. When single colonies appeared, five hundred were selected and replica imprinted on the agar plates containing or not containing streptomycin, respectively. After incubation at 37°C for 18 to 24 h, bacterial growth was observed. The percentage of the resistance-eliminated colonies in the detected colonies was taken as elimination rate of *E. coli* drug resistance (%). Bacteria not treated with the herbal eliminating agent were used as control.

Electrophoresis of plasmids extracted from resistance-eliminated *E. coli*

Using alkaline lysis method, plasmids were isolated from the resistance-eliminated strain and from those not treated with the Chinese pulsatilla extracts in accordance with instructions provided by the plasmid extraction mini kits. Using DYY-III 2 constant-voltage constant-current electrophoresis system produced by Beijing Liuyi instrument factory (Beijing, China), the plasmids were electrophoresed in submerged, horizontal agarose gel containing bromophenol blue tracking dye and then visualized by ethidium bromide (EB) staining. Gel imaging system produced by Beijing Liuyi instrument factory (Beijing, China) was used to analyze electrophoregram. The lambda DNA/*Hind* III fragments were used as nucleotide molecular weight standards. Regression equation was obtained with relative mobility as independent variables and number of base pairs as dependent variables. With the regression equation, the molecular weight of plasmid fragments was predicted.

RESULTS

Sub-inhibitory concentration of the Chinese pulsatilla extracts against *E. coli*

When 100, 50 and 25 mg/ml herbal eliminating agents were used to eliminate drug resistance, no bacterial growth was observed on the agar plates inoculated with

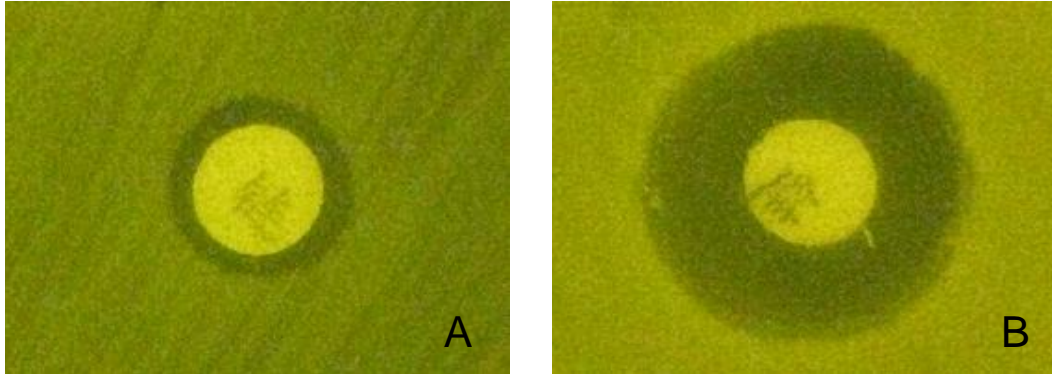


Figure 1. Streptomycin sensitivity testing of *E. coli* before (A) and after (B) resistance elimination.

the *E. coli*. When 12.5 mg/ml herbal eliminating agent was used, a few colonies appeared. Normal bacterial growth was observed in the residual agar plates. Therefore, the sub-inhibitory concentration of the herbal eliminating agent was 12.5 mg/ml for drug resistance elimination.

Obtainment of resistance-eliminated *E. coli*

After the drug resistance was eliminated by the Chinese pulsatilla extracts at its sub-inhibitory concentration, the bacteria were distinguished through inoculation on the nutrient agar plates and drug-containing plates. A bacterial strain not having drug resistance was obtained, as manifested by growth on the nutrient agar plates but no growth on the drug-containing plates. Its performance was always stable during three times repeat selection. Due to no resistance elimination, the control bacteria could grow normally on both the nutrient agar plates and the drug-containing plates.

Identification of resistance-eliminated *E. coli* strain

According to the results of susceptibility test, the resistance-eliminated bacteria and control bacteria had the same or similar resistance to the antibiotics recommended by the national committee for clinical laboratory standards (NCCLS), except that the diameter of inhibition zone of the resistance-eliminated bacteria was 17 mm compared with that of the control bacteria (9 mm). After the elimination of drug resistance, the bacteria resistant to streptomycin became sensitive to this antibiotic (Figure 1).

In addition, the sensitivity of *E. coli* to streptomycin was also enhanced, as supported by the determination results of MIC. Turbidity and/or a round-dot pellet at the well bottom of microplates indicated bacterial growth. The growth of the resistance-eliminated strain first appeared in the 9th well. The result showed its MIC against

streptomycin was 0.39 $\mu\text{g/ml}$, lower than that before the resistance elimination (6.25 $\mu\text{g/ml}$).

Elimination rate of drug resistance

As observed in replica plating test, all 500 inoculated colonies could grow on the nutrient agar plates, but only 421 colonies could grow on the plates containing streptomycin. Thus, the elimination rate of streptomycin resistance was 15.8% (79/500). The number of the control bacteria growing on the nutrient agar plates was the same as that growing on the plates containing streptomycin.

Plasmid profiles of the resistance-eliminated *E. coli* strain

As evidenced by the electrophoresis, five bands appeared in the plasmid pattern of the control bacteria. However, after the resistance elimination, an approximately 20 kbp band disappeared in the resistance-eliminated strain (Figure 2). The results demonstrated that the Chinese pulsatilla extracts had eliminated the resistance plasmid against streptomycin in *E. coli* O₁ strain CVCC-249.

DISCUSSION

The mechanisms of resistance in *E. coli* can be explained at genetic and biochemical levels. The genetic factors include spontaneous mutations combined with drug selection, transfer of drug resistance genes between cells, and DNA transformation. The biochemical factors include production of catabolic enzymes or inactive enzymes which can change antibiotic structure, modification of antibiotic targets, changes of membrane permeability, deletion of porin, as well as metabolic disorder. Drug resistance genes in *E. coli* are generated

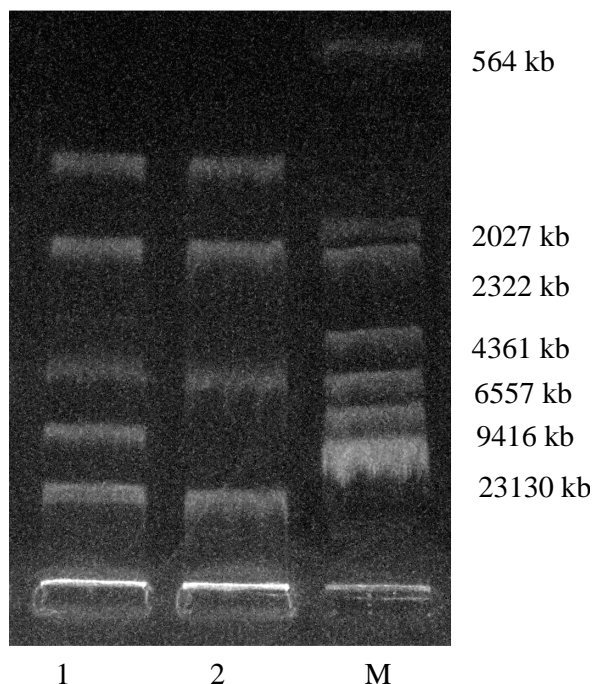


Figure 2. Plasmid profiles of chicken *E. coli* before and after resistance elimination.

After the electrophoresis, five different plasmid bands appear in the bacteria before resistance elimination (Lane 1), but an approximately 20 kbp band disappears after resistance elimination (Lane 2). Lamda DNA / *Hind* III Maker are used to assess plasmid size (Lane M).

due to spontaneous mutation of genetic material in sensitive bacteria. Genetic information of drug resistance in *E. coli* is mainly carried by resistance plasmids and transferred rapidly with the plasmids among bacterial strains. For example, the streptomycin resistance in *E. coli* is produced because *aadA*, *strA* and *strB* genes in resistance plasmid respectively encode adenylase and phosphotransferase (inactive enzymes) which can inactivate streptomycin (Shaw et al., 1993). Resistance genes can transfer from animals to humans directly or indirectly and damage human health. This is a difficult problem of public health.

As chemical eliminating agents have weak antibacterial activity, serious adverse effects and declining sensitivity, researchers have tried to use traditional Chinese medicines for elimination of drug resistance. Its mechanisms are represented as follows. (i) Through eliminating resistance plasmids. Through 48 h incubation in *Coptis chinensis* extracts, the elimination rate of gentamicin resistance in *E. coli* can reach 22.5 % (Chen et al., 1996). In this study, we used the Chinese pulsatilla extracts to eliminate the resistance plasmid in chicken *E. coli*. The reactivity of the obtained resistance-eliminated strain against streptomycin was changed from sensitivity to resistance, and the elimination rate was 15.8%. As demonstrated by the plasmid profiles, the obtained

resistance-eliminated strain lost an approximately 20 kbp band compared with the control bacteria. This result indicated that the Chinese pulsatilla extracts may eliminate the resistance plasmid harboring streptomycin resistance genes and thus change the sensitivity of chicken *E. coli* to streptomycin. (ii) Through inhibiting beta-lactamase activity. Some researches have supported this mechanism. The beta-lactamase activity in penicillin-resistant bacteria can be inhibited by *Adinandra nitida* Merr. ex H. L. Li, *Bulbus allii*, *Flos eriocauli* and *Phellodendron amurense* Rupr (Huang et al., 2000). Production of extended-spectrum beta-lactamases (ESBLs) and AmpC beta-lactamases in bacteria can be inhibited by *Scutellaria baicalensis*, *Coptis chinensis*, *Phellodendron amurense* Rupr., and *Cineraria repanda* (Liu et al., 2006). (iii) Through inhibiting function of bacterial active efflux pump. The external pump-mediated drug resistance of *Staphylococcus aureus* can be converted by *Fritillaria thunbergii* Miq. *Belamcanda sinensis*, *Andrographis paniculata* and *Trapa bispinosa* (Song et al., 2007). Resistance of multidrug resistant cells can be converted by blocking efflux pump with tetrandrine and dauricine (Pan and Tian, 1995). The resistance of *E. coli* to fluoroquinolones, doxycycline and gentamicin can be converted by restoring normal expression of outer membrane channel protein with *Coptis chinensis* extracts (Lei et al., 2004). (iv) Through inhibiting formation of bacterial biofilm. The formation of plaque biofilm can be strongly inhibited by water extracts of *Chinese nutgall* (Xi et al., 2004). The above-mentioned results show Chinese herbal medicines has a good prospect to eliminate bacterial drug resistance in clinic.

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