Research Article

Different Effects of Six Antibiotics and Ten Traditional Chinese Medicines on Shiga Toxin Expression by Escherichia coli O157:H7

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This study compared the effects of ten types of traditional Chinese medicines (TCMs) and six different antibiotics on E. coli O157:H7 Shiga toxin gene (stx2) mRNA expression level based on real-time PCR and the expression level of Stx toxin using an ELISA quantitative assay. We also compared their effects on the induction of the SOS response. The results clearly indicated that all ten TCMs had negative results in the SOS response induction test, while most TCMs did not increase the levels of stx2 mRNA and the Stx toxin. Some TCMs did increase the mRNA levels of the stx2 gene and the Stx toxin level, but their increases were much lower than those caused by antibiotics. With the exception of cefotaxime, the six antibiotics increased the Stx toxin level and increased the stx2 gene mRNA level. With the exceptions of cefotaxime and tetracycline, the antibiotics increased the SOS induction response. These results suggest that TCMs may have advantages compared with antibiotics, when treating E. coli O157:H7; TCMs did not greatly increase Stx toxin production and release.

1. Introduction

The major pathogen, enterohemorrhagic Escherichia coli (EHEC), has caused several outbreaks in different areas throughout the world, such as E. coli O157:H7 epidemics in many countries [1–3] and highly pathogenic E. coli O104:H4 epidemics in Germany and other European countries during 2011 [4, 5]. EHEC possesses multiple virulence factors and the most toxic is Shiga toxin (Stx), especially Stx2 [6–8]. EHEC can cause the life-threatening hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) [6, 9, 10].

EHEC patients are treated mainly with supportive therapy. The use of antibiotics is not recommended, because many reports have shown that antibiotics can stimulate E. coli O157:H7 or E. coli O104:H4 to generate or release Stx, which increases the risks of HC patients becoming HUS patients [11–15]. By contrast, it also has been reported that antibiotics do not increase the expression [16–22] of the Shiga toxin-coding gene (stx2) so they can be used for the treatment of EHEC patients.

For a long time, traditional Chinese medicine (TCM) has been used to treat infectious diarrhea, although people at that time did not differentiate between bacterial, nonbacterial, toxic bacterial, or nontoxic bacterial diarrhea [23, 24]. TCMs are still in use today, and they play important roles in treating infectious diseases other than enteric diseases because they are chemically complex, widely applied, not easily resisted by bacteria [25–29], and have lower toxicity [30–36]. In recent years, a hot research topic has been the extraction of effective compounds or compound complexes from TCMs to treat EHEC infections without inducing Shiga toxin overexpression. We selected ten TCMs from about fifty TCMs which are widely used in clinical Chinese medicines in China, because these TCMs had higher E. coli O157:H7 biofilm forming inhibition activity (data not shown in this paper). This study compared six antibiotics and these ten TCMs to assess their different effects on stx2 expression and the SOS response induction.

2. Materials and Methods

2.1. Bacterial Strain. Enterohemorrhagic E. coli O157:H7 EDL933 was kindly provided by China Disease Prevention and Control Center.
2.2. Antibiotics. Streptomycin was purchased from Sangon Biotech Co. Ltd, Shanghai, China (CAS no. 3810-74-0); tetracycline was purchased from Xin Jing Ke Biotecnology Co., Ltd., Beijing, China (CAS no. 3963-45-9); chloramphenicol was purchased from Guo Chang Sheng Biotecnology Co., Ltd., Beijing, China (CAS no.56-75-7); erythromycin was purchased from Bio Basic Inc., Canada (CAS no. 114-07-8); cefotaxime sodium was purchased from Qilu Pharmaceutical Co., Ltd., Jinan, China (CAS no. 64485-93-4); and hydrogenic acid levofloxacin (injection) was purchased from Yangtze River Pharmaceutical Group Co., Ltd., Yangzhou, China (CAS no. 82419-36-1). All antibiotics were stored as 50 mg/mL stock solutions at –20°C.

2.3. TCMs. Ten TCMS, Coptidis Rhizoma (CR), Fraxini Cortex (FC), Scutellariae Radix (SR), Aucklandiae Radix (AR), Rehmanniae Radix (RR), Radix et Rhizome Rhei (RRR), Achyranthis Bidentatae Radix (ABR), Corni Fructus (CF), Rhizoma seu Radix Notopterygii (RSRN), were all purchased from Beijing Tongrentang Co. Ltd., Jinan branch (Jinan, China). Their decoctions were prepared using the traditional boiling method [25, 36].

2.4. Measuring the MICs of Antibiotics and TCMs. The minimum inhibitory concentrations (MICs) of six antibiotics to E. coli O157:H7 EDL933 were determined with broth double dilution method [37, 38]. The MICs of ten TCMS to E. coli O157:H7 EDL933 were determined with agar double dilution method, because the decoctions of TCMs were somewhat turbid.

2.5. Extracting RNA and Reverse Transcription E. coli. O157:H7 EDL933 was cultured overnight in Luria-Bertani (LB) broth. About 5 x 10^7 colony forming units (CFUs) were mixed with serial dilutions of the antibiotics or TCMS in LB broth, followed by culture at 37°C for about 6 h with rotary shaking at 160 rpm. The bacteria cells were collected, and the total RNA was extracted strictly using a kit (Promega SV Total RNA Isolation System, Z3100), according to the manufacturer's instruction. The purity and concentration of RNA were assessed by denature agarose electrophoresis and Nano Drop. The extracted RNA samples were stored at −70°C until use. The real-time PCR primers were designed according to published sequences of the EDL933 genome [39] using primer 5.0 and were synthesized by Takara Biotechnology Co., Ltd (Dalian, China). The length of the amplified Stx2 fragment was 150 bp. The probe was 5’-(FAM) CACCGAT-GTGTCCTGGGAG (Eclipse)-3’, the forward primer was 5’-CTTGGATTCTCTATCC-3’, and the reverse primer was 5’-GGGGTGATGTTAATACAC-3’. rpoB was used as an internal control, and the length of the amplified rpoB fragment was 79 bp, using the probe 5’-(FAM) AACTGC-CTGCGACCATTCTTCTT (Eclipse)-3’, the forward primer 5’-CAACCTGGCTGTAAGCTAATC-3’, and the reverse primer 5’-CTCTGTTGTTGATGTTAC-3’. The 20 μL PCR reaction mixture contained 10.0 μL of 2x Premix Ex Taq (Probe qPCR), 0.4 μL of PCR forward primer (20 μM), 0.4 μL of PCR reverse primer (20 μM), 0.8 μL of fluorescent probe solution, 2.0 μL of cDNA, and 6.4 μL of ddH2O. The PCR reaction conditions were: 95°C for 15 min, 40 cycles of 95°C for 5 s, 55°C for 30 s, and 72°C for 30 s. The Ct value of each sample was the average of the real-time PCR data for triplicate samples.

2.6. Real-Time PCR Primer Design and Reaction. The real-time PCR primers were designed according to published sequences of the EDL933 genome [39] using primer 5.0 and were synthesized by Takara Biotechnology Co., Ltd

2.7. Quantitative Determination of Stx. The amount of Stx toxin was determined using a double antibody (sandwich) ELISA with a shiga-like toxin (SLT) ELISA kit (Shanghai Jianglai Biotechnology Co., Ltd., China). Absorbance measurements were performed bichromatically at 450/600 nm with an ELISA reader. To determine the specific Stx concentration, the absolute absorbance values were divided by the number of bacteria (OD600 per mL) present in the suspensions.

2.8. The Inductive Effect of Antibiotics and TCMS on SOS Response. Using the methods recommended by ISO [40, 41], we determined the inductive effects of the antibiotics and TCMS on the SOS response at 1/8, 1/4, and 1/2 MIC concentrations, respectively.

2.9. Data Processing and Analysis. The 2^−ΔΔCt method was used for relative quantification of the real-time PCR data [42]. The statistical analyses were carried out using SPSS 13.0.

3. Results

3.1. MICs of Antibiotics and TCMS in E. coli O157:H7 EDL933. Table 1 shows the results obtained using the broth and agar double-dilution method to measure the MICs of antibiotics and TCMS in E. coli EDL933. The six antibiotics had different antibacterial mechanisms and/or different active targets, and they had much higher bacteriostatic activities than TCMS. Of the ten TCMS Coptidis Rhizoma, Fraxini Cortex, and Schisandraceae Chinensis Fructus had high bacteriostatic activity; Scutellariae Radix, Aucklandiae Radix, and Rehmanniae Radix had medium bacteriostatic activity; while the other four TCMS had weak bacteriostatic activity.

3.2. Reproducibility and Stability of the stx2 Gene Expression Quantitative Measurements. Real-time RT-PCR was used to quantitatively compare the effects of antibiotics and TCMS on stx2 gene expression. We optimized the steps of real-time RT-PCR so that it had good reproducibility and stability. The
The relative quantitative analysis methods (similar, with a relative deviation of less than 5%). Thus, these correlated with greater of the housekeeping gene. A higher antibiotic concentration maximum expression was a thousand times higher than that of the housekeeping gene.

Tetracyclines and erythromycin only showed weak induction. The previous results also indicated that, compared with the antibiotics, six of the TCMs (CF, FC, RsRN, ABR, RR, and CR) had no significant inductive effects on stx2 expression, while four TCMs (AR, RRR, SCF, and SR) had weak inductive effects on stx2 expression at high concentrations, which were similar to tetracycline and erythromycin. Their stx2 expression levels were up to six times that of the control group, which were hundreds or thousands of times below that of chloramphenicol, levofloxacin, and streptomycin. CR, ABR, and RR weakly suppressed the reverse transcription and expression of stx2.

The results showed in Figure 3 indicated that three antibiotics (CHL, STR, and LEV) significantly increased the release of Stx toxin by over ten-fold, while three antibiotics (ERY, CEF, and TET) only weakly increased the release of Stx toxin by about 2.8–5.5 times. However, only three TCMs (AR, RRR, and CR) weakly increased the release of Stx toxin by about 2.0–2.8 times, whereas the other seven TCMs (SR, SCF, CF, FC, RsRN, ABR, and RR) did not increase the release of Stx toxin, that is, Stx toxin release increased less than two-fold.

3.5. Inductive Effects of Antibiotics and TCMs on SOS Response Induction. Stx toxin expression in E. coli O157:H7 is believed to be related to SOS response induction [43]. We also

| Antibiotics       | MIC (µg/mL) |
|-------------------|-------------|
| Levofloxacin (LEV)| 0.01        |
| Streptomycin (STR)| 8.00        |
| Chloramphenicol (CHL)| 1.00     |
| Erythromycin (ERY)| 3.13        |
| Tetracyclines (TET)| 6.25       |
| Cefotaxime sodium (CEF)| 0.25  |

| TCMs               | MIC (mg/mL) |
|--------------------|-------------|
| Captidis Rhizoma (CR)| 3.9       |
| Fraxini Cortex (FC)| 3.9        |
| Schisandrae Chinensis Fructus (SCF)| 7.8     |
| Scutellariae Radix (SR)| 31.3     |
| Aucklandiae Radix (AR)| 62.5     |
| Rehmanniae Radix (RR)| 62.5      |
| Radix et Rhizome Rhei (RRR)| 125.0  |
| Achnarthis Bidentatae Radix (ABR)| 125.0  |
| Corni Fructus (CF)| 125.0      |
| Rhizoma seu Radix Notoperygii (RsRN)| 125.0  |

Optimization results indicate that the extracted RNA was of good quality and the total RNA extracted was of high purity (see Figure 1), that is, A260/A280 = 2.006 ± 0.012 (n = 12), which indicated that there was no contamination with DNA or protein. The brightness ratio of the 23S rRNA band relative to the 16S rRNA band was about 2:1, so the extracted RNA was mostly complete. The real-time PCR expansion curve was generated automatically using a Roche 480 system. Fluorescent signals were not measured in the negative control group, which showed that the reaction system was free from contaminations. The same templates had similar expansion curves, which indicated that this determination method had good reproducibility, where the deviation was small and the data were credible. The expansion efficiencies of the curves, which indicated that this determination method had a good linear relationship, while four TCMs (AR, RRR, SCF, and SR) had weak inductive effects on stx2 expression at high concentrations, which were similar to tetracycline and erythromycin. Their stx2 expression levels were up to six times that of the control group, which were hundreds or thousands of times below that of chloramphenicol, levofloxacin, and streptomycin. CR, ABR, and RR weakly suppressed the reverse transcription and expression of stx2.

### Table 1: MICs of antibiotics TCMs in E. coli EDL933.

| Antibiotics       | MIC (µg/mL) |
|-------------------|-------------|
| Levofloxacin (LEV)| 0.01        |
| Streptomycin (STR)| 8.00        |
| Chloramphenicol (CHL)| 1.00     |
| Erythromycin (ERY)| 3.13        |
| Tetracyclines (TET)| 6.25       |
| Cefotaxime sodium (CEF)| 0.25  |

| TCMs               | MIC (mg/mL) |
|--------------------|-------------|
| Captidis Rhizoma (CR)| 3.9       |
| Fraxini Cortex (FC)| 3.9        |
| Schisandrae Chinensis Fructus (SCF)| 7.8     |
| Scutellariae Radix (SR)| 31.3     |
| Aucklandiae Radix (AR)| 62.5     |
| Rehmanniae Radix (RR)| 62.5      |
| Radix et Rhizome Rhei (RRR)| 125.0  |
| Achnarthis Bidentatae Radix (ABR)| 125.0  |
| Corni Fructus (CF)| 125.0      |
| Rhizoma seu Radix Notoperygii (RsRN)| 125.0  |
compared the different effects of antibiotics and TCMs on the SOS response induction, and the results are shown in Figure 4. The results indicated that only four antibiotics (LEV, CHL, STR, and ERY) induced a clear SOS response, and their SOS induction factors were >2.0. The other two antibiotics and all ten TCMs did not induce the SOS response, and their SOS induction factors were <2.0.

4. Discussion

4.1. The Different Inductive Effects of Six Antibiotics on \textit{stx2} Gene Expression in \textit{E. coli} O157:H7. The results in Figure 2 showed that, compared with the expression of the housekeeping gene \textit{rpoB}: chloramphenicol treatment caused a sharp increase in \textit{stx2} gene expression, which was thousands of times greater than the control; levofloxacin and streptomycin treatment caused increases that were hundreds of times greater than the control; erythromycin and tetracycline treatment responses were only several times greater than the control; whereas cefotaxime did not increase \textit{stx2} gene expression. The fold increases of \textit{stx2} gene expression varied greatly among the antibiotics, but were these data distorted? First, according to the study by Ichinohe et al. [21], norfloxacin can increase the expression of \textit{E. coli} O157:H7 \textit{stx2} by thousands of times compared with the control, which indicates that our data is credible. Second, our data on the
effects of the six antibiotics on stx2 gene expression were mostly consistent with other reports, although some were contradictory. According to McGannon et al. [16], various antibiotics had different effects on the expression of stx gene. Antibiotics such as ciprofloxacin and sulfamethoxazole, which target the DNA, can increase stx gene expression greatly. However, antibiotics that target the cell wall, transcription, and translation do not increase stx gene expression. Interestingly, azithromycin reduces the stx gene expression. This hypothesis may shed some light on the data in Figure 2, such as why cefotaxime had no inductive effect, whereas tetracycline and erythromycin had relatively weak inductive effects, and levofloxacin had a strong inductive effect. The data in Figure 2 also showed that chloramphenicol and streptomycin, which affect translation, had very strong inductive effects, which contradicts McGannon et al.

There are two contrasting views of antibiotics that affect bacterial cell wall biosynthesis. A previous study [16] suggested that they do not induce stx gene expression, whereas another study [20] showed that cefazidime did not affect stx gene expression whereas panipenem (PAPM) greatly suppressed stx gene expression. Other studies [17, 19] have shown that cefotaxime and meropenem [44] do not affect stx gene expression. By contrast, it was reported [17, 18] that ampicillin increased stx gene expression. However, the present study showed that cefotaxime had little inductive effect on stx2 gene expression.

There are also two contradictory views of antibiotics that affect biosynthesis during DNA replication. Studies have shown [14, 22, 44] that ciprofloxacin can increase stx gene expression and that [21] norfloxacin can increase stx2 expression by thousands of times compared with the control. However, another study [17] reported that ciprofloxacin did not increase stx gene expression, while in [12] enrofloxacin reduced stx gene expression. It was reported in [20] that oral intake of quinolones by E. coli O157:H7-infected patients did not increase the possibility of progression to HUS. The present study showed that levofloxacin strongly increased the stx2 gene expression, which agrees with most previous studies.

There are two contradictory views of antibiotics that affect the biosynthesis of proteins, such as aminoglycosides. One study [16] suggested that their restricted translation would not affect stx gene expression. Similarly, another study [44] reported that gentamicin and kanamycin did not affect stx gene expression, which was consistent with a previous study [26]. Another study [18] reported that gentamicin at the concentration of MIC increased the stx2 expression, whereas a sub-MIC concentration reduced the expression of stx2. However, the present study showed that streptomycin at a sub-MIC concentration (1/2, 1/4, and 1/8 MIC) markedly increased stx2 expression.

Antibiotics that affect the biosynthesis of proteins, such as polycyclics, have been shown [16] to have no effects on stx2 gene expression. However, it was reported [44] that tigecycline reduced stx2 expression, while another study [19] reported that bicozamycin reduced stx2 expression, which disagreed with other work [16]. The data in Figure 2 shows that tetracycline had only a weak inductive effect on stx2 gene expression, which agreed with a previous report [16].

For macrolide antibiotics, such as erythromycin and its derivatives, it was reported [21] that azithromycin did not affect stx2 expression, whereas other studies [16, 17, 44] reported that azithromycin reduced stx expression. The present study found that erythromycin had only a weak inductive effect on stx2 gene expression, which agreed with some previous results.

A previous study [44] reported that chloramphenicol reduced stx2 gene expression. By contrast, we found that chloramphenicol strongly induced stx2 gene expression.

4.2. TCMs Had No or Weak Inductive Effects on E. coli O157:H7 stx2 Gene Expression. The inductive effects of antibiotics on E. coli O157:H7 stx2 expression have been reported in many studies, whereas the effects of TCMs have been reported rarely [45, 46]. In the same test conditions used for antibiotics, we found that some TCMs induced the expression of stx2 only weakly, such as Aucklandiae Radix (AR), Radix et Rhizoma Rhei (RRR), Scutellariae Chinensis Fructus (SCF), and Schisandrae Chinensis Fructus (SCF), whereas some TCMs had no inductive effects on stx2 expression, such as Rhizoma seu Radix Notopterygii (RsRN), Corni Fructus (CF), and Fraxini Cortex (FC). Some TCMs actually suppressed stx2 expression, such as Coptidis Rhizoma (CR), Rehmanniae Radix (RR), and Achyranthis Bidentatae Radix (ABR). In general, a preliminary conclusion based on the above analyses of the mRNA levels of the stx2 gene, was that the inductive effects of TCMs were far less than those of the antibiotics. This may be worth exploring in greater depth.

4.3. Differences in the SOS Induction Response to TCMs and Antibiotics. According to the criteria of SOS/umu test system [40, 47], the induction factor (IF) ≥2.0 indicates that the tested compound can induce the SOS response. Results in Figure 4 showed that the ten TCMs did not induce the SOS response because their IF values were <2.0. For antibiotics, however, the results were complex: levofloxacin induced a very strong SOS response; chloramphenicol and erythromycin also induced high SOS responses, whereas cefotaxime, tetracycline, and kanamycin did not induce the SOS response.

TCMs did not induce the SOS response whereas some antibiotics induced strong SOS responses. Identifying the causes of these differences requires further study.

4.4. Explanation of the Different SOS Induction Responses of the Six Antibiotics. The present study showed that levofloxacin had the strongest SOS response inductive effect of the six antibiotics, chloramphenicol had the second strongest induction, cefotaxime had the weakest induction, while erythromycin, streptomycin, and tetracycline had the intermediate inductive effects (see Figure 4). The different SOS responses of these six antibiotics may probably be due to their antimicrobial mechanisms [48]. First, the antimicrobial mechanism of levofloxacin correlated with high SOS induction. Levofloxacin produces a bacteriostatic
effect by interfering with the DNA helicase activity, which interrupts or hinders DNA replication, thereby producing DNA fragments or terminals with single strands. These DNA molecules are inducers of the SOS response [49–52]. Second, the antimicrobial mechanism of cefotaxime is correlated with low SOS induction. Cefotaxime achieves its bacteriostatic effect by inhibiting the biosynthesis of bacterial cell walls, rather than interfering with DNA replication or DNA damage repair, so cefotaxime did not induce the SOS response. Third, chloramphenicol, tetracycline, streptomycin, and erythromycin achieve their bacteriostatic effects by interfering with protein biosynthesis, although they have different specific targets [48]. However, it was difficult to understand why chloramphenicol had the highest SOS response inductive effects, whereas streptomycin and erythromycin had similar SOS response inductive effects, and tetracycline had no SOS response inductive effects. Chloramphenicol contains a chlorine atom, which dissociates from the chloramphenicol molecule to produce a chloride ion after the chloramphenicol is absorbed by bacteria cells. The chloride ion may combine with hydrogen peroxide and peroxidase in bacterial cells to form ternary complexes, which may produce reactive oxygen species (ROS) that have very strong oxidative activities [53–57]. The high oxidative effects of ROS may damage DNA molecules and produce single- and double-stranded DNA breaks, thereby inducing the SOS response [54, 55, 57].

4.5. Effects of Antibiotics and TCMs on the Stx Toxin of E. coli O157:H7. The effects of antibiotics and TCMs on the Stx toxin of E. coli O157:H7 were shown in Figure 3. Their effects on Stx toxin were similar to their effects on the stx2 gene mRNA levels, except in the cases of cefotaxime and Coptidis Rhizoma (CR). Chloramphenicol, levofloxacin, and streptomycin had the strongest inductive effects on the Stx toxin of E. coli O157:H7, and these three antibiotics had the strongest inductive effects on the stx2 mRNA levels. Erythromycin and tetracycline had inductive effects on Stx toxin and the stx2 mRNA levels. By contrast, cefotaxime had inductive effects on Stx toxin but not on the stx2 mRNA levels. Of the TCMs, Aucklandiae Radix (AR) and Radix et Rhizoma Rhei (RRR) had weak inductive effects on the Stx toxin of E. coli O157:H7, and they also had weak inductive effects on the stx2 mRNA levels. Coptidis Rhizoma (CR) had weak inductive effects on Stx toxin but no inductive effects on the stx2 mRNA levels. All of the other seven TCMs had no inductive effects on Stx toxin, that is, Scutellariae Radix (SR), Schisanthae Chinensis Fructus (SCF) had weak inductive effects on the stx2 mRNA levels, whereas the other five TCMs had no inductive effects on the stx2 mRNA levels. Cefotaxime and Coptidis Rhizoma (CR) had the biggest differences in their Stx toxin and stx2 mRNA level effects, which may be because both of them increased the permeability of bacterial cells [25, 48].

5. Conclusions

Given the results of this study and our discussions, all ten of the TCMs had negative effects on SOS response induction, most of them did not increase the stx2 gene mRNA or Stx toxin levels, only a few TCMs increased the stx2 gene mRNA levels and Stx toxin levels, but their increases were many times lower than those with antibiotics. However, six antibiotics increased the Stx toxin level, increased the stx2 gene mRNA levels (except cefotaxime), and increased SOS response induction (except cefotaxime and tetracycline). Thus, TCMs may have advantages compared with antibiotics in the treatment of infections caused by O157:H7, that is, the TCMs treatment used to control E. coli O157:H7 infections might not increase Stx toxin production and release. TCMs have been used for a long time to treat infectious diseases of the digestive tract in China, this paper gives a case of experimental evidence and a possible explanation.

Conflict of Interests

All authors have no personal, professional, or financial conflict of interests to declare.

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