Correlation between the sulfamethoxazole-trimethoprim resistance of *Shigella flexneri* and the sul genes

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Abstract

The aim of this study was to discuss the correlation between the sulfamethoxazole-trimethoprim resistance of *Shigella flexneri* (*S.* *flexneri*) and the antibiotic resistance genes sul1, sul2, and sul3 and SXT element.

From May 2013 to October 2018, 102 isolates of *S. flexneri* were collected from the clinical samples in Jinan. The Kirby–Bauer (K-B) test was employed to determine the antibiotic susceptibility of the *S. flexneri* isolates. The antibiotic resistance rate was analyzed with the WHONETS.4 software. The isolates were subject to the PCR amplification of the sul genes (sul1, sul2, and sul3) and the SXT element. On the basis of the sequencing results, the correlation between the sulfamethoxazole-trimethoprim resistance of the *S. flexneri* isolates and the sul genes was analyzed.

The antibiotic resistance rates of the 102 *S. flexneri* isolates to ampicillin, streptomycin, chloramphenicol, tetracycline, and sulfamethoxazole-trimethoprim were 90.2%, 90.2%, 88.2%, 88.2%, and 62.7%, respectively. The antibiotic resistance rates of these isolates to cefotaxime, ceftazidime, and ciprofloxacin varied between 20% and 35%. However, these isolates were 100% susceptible to cefoxitin. Positive fragments were amplified from 59.8% (61/102) of the 102 *S. flexneri* isolates, the sizes of the sul1 and sul2 genes being 338bp and 286bp, respectively. The sequence alignment revealed the presence of the sul1 and sul2 genes encoding for dihydrofolate synthase. The carrying rate of the sul1 gene was 13.7% (14/102), and that of the sul2 gene was 48.0% (49/102). No target gene fragments were amplified from the 3 isolates resistant to sulfamethoxazole-trimethoprim. The sul3 gene and SXT element were not amplified from any of the isolates. The testing and statistical analysis showed that the resistance of the *S. flexneri* isolates to sulfamethoxazole-trimethoprim correlated to the sul1 and sul2 genes.

The acquired antibiotic resistance genes sul1 and sul2 were closely associated with the resistance of the 102 *S. flexneri* isolates to sulfamethoxazole-trimethoprim.

Abbreviations: CLSI = Clinical and Laboratory Standards Institute, DHPS = dihydropteroate synthetase, *E. coli* = *Escherichia coli*, EB = Ethidium bromide, K-B = Kirby–Bauer, M-H = Mueller–Hinton, *S. flexneri* = *Shigella flexneri*, *S. sonnei* = *Shigella sonnei*, WHO = World Health Organization.

Keywords: *Shigella flexneri*, sul genes, sulfanilamide, SXT element

1. Introduction

*Shigella* infections can lead to acute, chronic, and toxic dysentery. Some isolates may even cause hemolytic-uremic syndrome and Reiter syndrome. In 2015, diarrhea caused more than 1.3 million deaths globally and was the fourth leading cause of death among children younger than 5 years. The *Shigella* bacteria are recognized by the World Health Organization (WHO) as the bacteria with growing antibiotic resistance and bringing a huge threat to human health. *S. flexneri* is the most common *Shigella* species in developing countries, while *Shigella sonnei* (*S. sonnei*) is more prevalent in developed countries. The 2017 Clinical and Laboratory Standards Institute (CLSI) guidelines recommend the use of ampicillin, fluoroquinolones, and sulfamethoxazole-trimethoprim for the treatment of bacterial dysentery. Since the emergence of sulfonamides in the 1930s, they have been widely used in clinical and veterinary medicine to treat bacterial infections. By combining the catalytic enzyme in the folic acid synthesis pathway – dihydropteroate synthetase (DHPS), sulfonamides cause dihydrofolic acid synthesis disorder and inhibit bacterial growth. Sulfonamide resistance is primarily mediated by the sul1, sul2, and sul3 genes encoding DHPS with a low affinity for sulfonamides. The SXT element, first discovered in the *Vibrio* cholerae of the O139 serogroup, was a gene encoding...
sulfamethoxazole-trimethoprim resistance. In this study, the antibiotic resistance of 102 S. flexneri isolates collected in Jinan was detected. The correlation between the resistance to sulfamethoxazole-trimethoprim and the sul1, sul2, and sul3 genes and the SXT element was discussed.

2. Materials and methods

2.1. Sources of isolates

From May 2013 to October 2018, 102 S. flexneri isolates were isolated from the feces of patients in Jinan. The isolates were analyzed on the ID32E system, and the results were interpreted by ATB expression. Serotyping was performed on the S. flexneri isolates using the Diagnostic Serum for Shigella. The preserved isolates were taken out from the ultra-low temperature freezer at −86°C, thawed at room temperature, and reidentified. The quality-control strain Escherichia coli (E. coli) ATCC 25922 was preserved at the clinical microbiology laboratory of the Fourth People’s Hospital of Jinan. This study had been approved by the medical ethics committee of the Fourth People’s Hospital of Jinan.

2.2. Main reagents and equipment

Ampicillin, chloramphenicol, tetracycline, streptomycin, sulfamethoxazole-trimethoprim, ciprofloxacin, cefotaxime, ceftazidime and cefoxitin drug sensitive slips, and Mueller–Hinton (M–H) agar (OXOID, UK), Diagnostic Serum for Shigella (Lanzhou Institute of Biological Products Co., Ltd.), agarose (Invitrogen); bacterial identification system (Bio mérieux, France, ATB Expression), PCR Instrument (Biometra, Germany), electrophoresis apparatus (10C, Beijing Liuyi Instrument Factory), biosafety cabinet (Shanghai Lishen Scientific Equipment Co., Ltd., 1200IIA2). The synthesis of the PCR primers and the DNA sequencing the amplified PCR products were undertaken by TaKaRa Biotechnology (Dalian) Co., Ltd. Primer sequences are presented in Table 1. A sterile inoculation of the double-distilled water. This was followed by a water bath at 95°C for 5 minutes and centrifugation at 12,000 rpm for 30 seconds.

3. Results

3.1. Results of antibiotic susceptibility test

The antibiotic resistance rates of the 102 S. flexneri isolates to ampicillin, streptomycin, chloramphenicol, and tetracycline were all 90.2% (92/102). The resistance rate to sulfamethoxazole-trimethoprim was 62.7% (64/102). The antibiotic resistance rates of these isolates to cefotaxime, ceftazidime, and ciprofloxacin varied between 20% and 35%. However, these isolates were 100% susceptible to cefoxitin. The antibiotic resistance pattern consisting of ampicillin-streptomycin-chloramphenicol-tetracycline was found in 90.2% of all isolates. The antibiotic resistance pattern consisting of ampicillin-streptomycin-chloramphenicol-tetracycline-sulfamethoxazole-trimethoprim was found in 62.7% of all isolates. See Table 2.

3.2. Results of PCR amplification of the antibiotic resistance genes

Of the 102 S. flexneri isolates, 59.8% (61/102) were positive for the amplification. The presence of the sul1 and sul2 genes encoding for dihydrofolate synthase was then verified by

| Primer name | Primer sequence (5’→3’) | Fragment length, bp | Annealing temperature | Reference |
|-------------|--------------------------|---------------------|-----------------------|-----------|
| sul1        | FP:CTTCGATGAGCCGGGGGC   | 338                 | 55                    | [5]       |
|             | RP:GCAGCGGGGAACGGGCGCC  |                     |                       |           |
| sul2        | FP:GCGCTGAAAGCCGAGTGCATTT | 286                 | 55                    | [5]       |
|             | RP:GGGTGTGATACGGGCGGCGT |                     |                       |           |
| sul3        | FP:GAGGAGGATTTGGGAATCG  | 799                 | 55                    | [5]       |
|             | RP:CATCTGCAGCTAAGGGCTTTGGA |                    |                       |           |
| SXT         | FP:ATGGGCTTATGATCTGCGC | 1035                | 56                    | [6]       |
|             | RP:CGAGATCATGAGTGAACC   |                     |                       |           |
sequencing. Of all these positive isolates, 11.8% (12/102) only carried the \textit{sul1} gene, 46.1% (47/102) only carried the \textit{sul2} gene, and 2.0% (2/102) carried both. The overall carrying rate of the \textit{sul1} gene was 13.7% (14/102), and that of the \textit{sul2} gene was 48.0% (49/102). No \textit{sul3} gene or SXT element was amplified from any isolates. The electrophoretograms and the sequencing diagrams of the amplified PCR products of the \textit{sul1} and \textit{sul2} genes are presented in Figures 1 to 3, respectively.

### 3.3. Correlation analysis between the sulfamethoxazole-trimethoprim resistance of the \textit{S. flexneri} isolates and the antibiotic resistance genes

Of the 102 \textit{S. flexneri} strains, 64 strains were resistant to sulfamethoxazole/trimethoprim, accounting for 62.7% (64/102). According to the sequencing of 102 strains of \textit{S. flexneri}, 61 strains carried \textit{sul1} or \textit{sul2} genes (2 strains carried 2 genes at the same time), all of which were resistant to antibiotics. Only 3 strains (7.32%) of the remaining 41 strains that did not carry these two genes were resistant. After statistical analysis, the drug resistance rate of \textit{S. flexneri} carrying \textit{sul1} or \textit{sul2} gene was significantly higher than that of strains without \textit{sul1} or \textit{sul2} gene ($\chi^2 = 86.184$, $P < .001$), the rate of drug resistance of strains carrying \textit{sul1} or \textit{sul2} gene was about 13.67 times (4.59~40.62) of strains without \textit{sul1} or \textit{sul2} gene, as summarized in Table 3.

The statistical analysis of 100 strains with only \textit{sul1} gene and strains with only \textit{sul2} gene showed that the drug resistance rate of strains with \textit{sul1} gene was statistically significant compared with that without \textit{sul1} gene ($P = .003$). The drug resistance rate of the strains with \textit{sul2} gene was statistically significant compared with that without \textit{sul2} gene ($\chi^2 = 51.351$, $P < .001$), as summarized in Table 4.
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Table 3
Correlation analysis of drug resistance gene and drug susceptibility test results (N=102).

| Drug resistance gene | Sulfamethoxazole-trimethoprim |
|----------------------|--------------------------------|
|                      | Drug resistance | No drug resistance | \(\chi^2\) | \(P\) |
| sul1 gene or sul2 gene | Carry | 61 | 0 | 86.184 | <.001 |
|                      | Don’t carry | 3 | 38 |        |        |

Table 4
Correlation analysis of drug resistance gene and drug susceptibility test results (N=100).

| Drug resistance gene | Sulfamethoxazole-trimethoprim |
|----------------------|--------------------------------|
|                      | Drug resistance | No drug resistance | \(\chi^2\) | \(P\) |
| sul1 gene            | Carry | 12 | 0 | 6.625 | <.01 |
|                      | Don’t carry | 50 | 38 |        |        |
| sul2 gene            | Carry | 47 | 0 | 51.351 | <.001 |
|                      | Don’t carry | 15 | 38 |        |        |

4. Discussion
In recent years, cross-resistance and multidrug resistance to Shigella bacteria have become a critical concern.\(^{[12,13]}\) In 1996, Shigella bacteria were recognized by WHO as the bacteria causing a huge threat to human society due to the growing antibiotic resistance.\(^{[2]}\) The prevalence of Shigella infection is closely related to economic status, public health, life habits, and prevalent serotypes in different regions. In China, bacterial dysentery is a severe public health problem.\(^{[14,15]}\)

Our results indicated that the antibiotic resistance rate of the 102 S. flexneri isolates to ampicillin, streptomycin, chloramphenicol, and tetracycline was 90.2%. The resistance rate to sulfamethoxazole-trimethoprim was 62.7%. These results were consistent with those by Xu et al\(^{[16]}\) but lower than the resistance rates reported in Peru\(^{[17]}\) and Iran,\(^{[18]}\) and higher than the 44.4% reported in Xinjiang.\(^{[15]}\) Moreover, the antibiotic resistance pattern consisting of ampicillin-streptomycin-chloramphenicol-tetracycline was found in 90.2% of the isolates. The antibiotic resistance pattern consisting of ampicillin-streptomycin-chloramphenicol-tetracycline-sulfamethoxazole-trimethoprim was found in 62.7% of the isolates. In India\(^{[19]}\) and Bangladesh,\(^{[20]}\) the antibiotic resistance pattern consisting of ampicillin-nalidixic acid-sulfamethoxazole-trimethoprim prevailed. Delfino et al\(^{[21]}\) reported that as nalidixic acid and ciprofloxacin were rarely used in Mozambique, the antibiotic resistance pattern consisting of ampicillin-chloramphenicol-tetracycline-sulfamethoxazole-trimethoprim prevailed. The variation of the antibiotic resistance pattern has a close connection to the use of different antibiotics and the prevalent antibiotic resistance genotypes across the regions.

PCR results showed the presence of the sul1 and sul2 genes in the S. flexneri isolates resistant to sulfamethoxazole-trimethoprim. However, no fragments of the sul1 and sul2 genes were amplified from the isolates susceptible to sulfamethoxazole-trimethoprim. Statistical analysis suggested a strong correlation between antibiotic resistance and the presence of the sul1 and sul2 genes. It was thus implied that the sul1 and sul2 genes encoding for dihydrofolate synthase were involved in the resistance of the S. flexneri isolates to sulfamethoxazole-trimethoprim. Shuyu et al\(^{[22]}\) also detected the sul1, sul2, and sul3 genes in 45%, 65%, and 12% of the E. coli isolates resistant to sulfonamides in Denmark, respectively. These genes could be transferred via the plasmid of 33 to 160 kb, which was related to the spread of the sulfonamide-resistant E. coli. In the UK, although the prescription rate of sulfonamides dropped dramatically in the 1990s (by 97%), the resistance of the sulfonamide-resistant E. coli isolates from the patients did not weaken. It has been reported that gene transfer was mediated by the sul2-carrying plasmid in E. coli.\(^{[13,24]}\) Byrne-Bailey et al\(^{[25]}\) reported the identification of an S. flexneri isolate from the soil slurry fertilized with the pig manure at a pig farm in the UK, this isolate presented with resistance to multiple drugs, including sulfonamides, and also carried the sul2 and intI1 genes. Lluque et al\(^{[17]}\) detected 36 clinical isolates of S. flexneri resistant to sulfamethoxazole-trimethoprim. Among them, 94% (34/36) of the isolates carried the sul2 gene, and 61% (22/36) carried the dfrA1 gene. Mohd et al\(^{[20]}\) reported the presence of sul2 in all 146 isolates of S. flexneri 2a resistant to sulfamethoxazole-trimethoprim. The transfer of the sul2 gene was mediated by the 4.3 MDa plasmid.

The SXT element, first discovered in the Vibrio cholerae of the O139 serogroup, was a gene encoding sulfamethoxazole-trimethoprim resistance.\(^{[26]}\) In recent years, the SXT element has been successively discovered in other bacteria, and the antibiotic resistance genes carrying the SXT element also varies.\(^{[27,28]}\) To observe whether the SXT element is also present in Shigella, we detected 102 strains of S. flexneri and found no SXT element. Of the 64 isolates resistant to sulfamethoxazole-trimethoprim, 95.3% (61/64) carried the sul1 or sul2 gene or both; the remaining 4.7% (3/64) were negative for the sul1 and sul2 genes. Whether other antibiotic resistance mechanisms were also involved was not yet fully clarified. Fragments of the sul1 and sul2 genes were not amplified from 40 isolates susceptible to sulfamethoxazole-trimethoprim. This result implied that the presence of the sul1 and sul2 genes induced the sulfamethoxazole-trimethoprim resistance in the 102 S. flexneri isolates collected in Jinan. The occurrence mechanism of multidrug resistance is very complex. One possible explanation is that the mobile genetic elements in bacteria are able to move within the same species or across the different species, thereby accelerating the antibiotic resistance to S. flexneri and the generation of the multidrug-resistant isolates. This study has some limitations. Due to our limited sample size, it may have an impact on the
experimental results; in future studies, we will further improve our experiment.

**Author contributions**

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