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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 WHONET5.4 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 338 bp and 286 bp, 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*

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None of the authors have conflicts of interest to disclose.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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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.[1] 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.[2] *S. flexneri* is the most common *Shigella* species in developing countries, while *Shigella sonnei (S. sonnei)* is more prevalent in developed countries.[3] The 2017 Clinical and Laboratory Standards Institute (CLSI) guidelines[4] 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.[5] Sulfonamide resistance is primarily mediated by the *sul1*, *sul2*, and *sul3* genes encoding DHPS with a low affinity for sulfonamides.[5,6] The *SXT element*, first discovered in the *Vibrio* cholerae of the O139 serogroup, was a gene encoding

sulfamethoxazole-trimethoprim resistance.[7] 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 summarized in Table 1.

2.3. Antibiotic susceptibility test

The Kirby–Bauer (K-B) test was performed to detect the antibiotic susceptibility. The testing procedures were carried out and the results were interpreted in strict accordance with the 2019 CLSI guidelines.[8] The quality-control strain *E. coli* ATCC 25922 was used. The diameter of the zone of inhibition was input into the WHONET 5.4 software.

2.4. PCR amplification of the sul1, sul2, and sul3 genes and SXT element

The primers for the *sul1*, *sul2*, and *sul3* genes and *SXT element* were synthesized according to literature references.[9,10] The primer sequences are presented in Table 1. A sterile inoculation loop was used to pick a single colony and inoculate to the M-H plate. After incubation at 35°C for 16 to 18 hours, an appropriate amount of bacterial lawn was picked with a sterile cotton swab and dissolved in 50 μL 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.[11] The DNA-containing supernatant was collected and stored in a −20°C fridge. PCR conditions for the *sul1*, *sul2*, and *sul3* genes and *SXT element* were as follows: Predenaturation at 94°C for 3 minutes; 94°C 30 seconds, annealing for 30 seconds (the annealing temperature is shown in Table 1), 72°C 40 seconds, 35 cycles; extension at 72°C for 5 minutes. After the PCR reaction was completed, 10 μL PCR product was taken and subject to 1% agarose gel electrophoresis at 120 V for 20 minutes. Ethidium bromide (EB) staining was carried out, and the DNA bands were visualized under the ultraviolet analyzer. The results were recorded, and the pictures were taken. The PCR products were submitted to TaKaRa Biotechnology (Dalian) Co., Ltd. for the sequencing. The genotypes were finally determined by using the NCBI/BLAST tool.

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

Table 1

Sequences of the PCR primers.

Primer name	Primer sequence (5′→3′)	Fragment length, bp	Annealing temperature	Reference
sul1	FP:CTTCGATGAGAGCCGGCGGC RP:GCAAGGCGGAAACCCGCGCC	338	55	[5]
sul2	FP:GCGCTCAAGGCAGATGGCATT RP:GCGTTTGATACCGGCACCCGT	286	55	[5]
sul3	FP: GAGCAAGATTTTTGGAATCG RP: CATCTGCAGCTAACCTAGGGCTTTGGA	799	55	[5]
SXT	FP:ATGGCGTTATCAGTTAGCTGGC RP:GCGAAGATCATGCATAGACC	1035	56	[6]

Table 2

Results of the susceptibility of 102 *S. flexneri* isolates to 9 antibiotics (%).

Antibiotics	R+ I	S
Ampicillin	90.2	9.8
Cefotaxime	30.4	69.6
Ceftazidime	22.5	77.5
Cefoxitin	0.0	100.0
Ciprofloxacin	34.3	65.7
Streptomycin	90.2	9.8
Chloramphenicol	90.2	9.8
Tetracycline	90.2	9.8
Sulfamethoxazole-trimethoprim	62.7	37.3

I = Intermediate, R = Resistance, S = Susceptible.

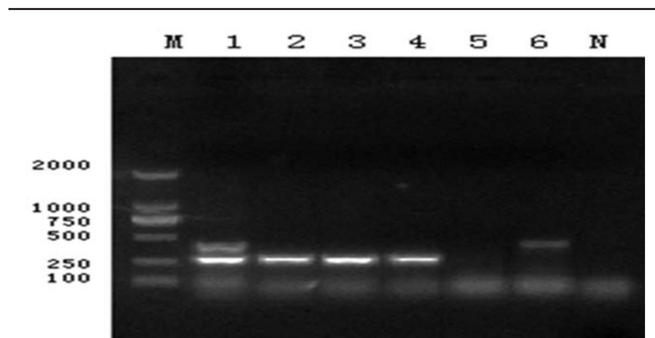

Figure 1. Electrophoretograms of amplified PCR products from the *sul1* and *sul2* genes. The lane M is DL2000. Lanes 1, 2, 3, 4, 5, and 6 are the amplification results of any 6 isolates. Lane N is the *E. coli* ATCC 25922 as the negative control. The size of the *sul1* and *sul2* genes was 338 and 286 bp, respectively.

sequencing. Of all these positive isolates, 11.8% (12/102) only carried the *sul1* gene, 46.1% (47/102) only carried the *sul2* gene, and 2.0% (2/102) carried both. The overall carrying rate of the *sul1* gene was 13.7% (14/102), and that of the *sul2* gene was 48.0% (49/102). No *sul3* gene or *SXT element* was amplified from any isolates. The electrophoretograms and the sequencing diagrams of the amplified PCR products of the *sul1* and *sul2* genes are presented in Figures 1 to 3, respectively.

3.3. Correlation analysis between the sulfamethoxazole-trimethoprim resistance of the S. flexneri isolates and the antibiotic resistance genes

Of the 102 *S. flexneri* strains, 64 strains were resistant to sulfamethoxazole/trimethoprim, accounting for 62.7% (64/102). According to the sequencing of 102 strains of *S. flexneri*, 61 strains carried *sul1* or *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 *S. flexneri* carrying *sul1* or *sul2* gene was significantly higher than that of strains without *sul1* or *sul2* gene ($\chi^2 = 86.184$, $P < .001$), the rate of drug resistance of strains carrying *sul1* or *sul2* gene was about 13.67 times (4.59~40.62) of strains without *sul1* or *sul2* gene, as summarized in Table 3.

The statistical analysis of 100 strains with only *sul1* gene and strains with only *sul2* gene showed that the drug resistance rate of strains with *sul1* gene was statistically significant compared with that without *sul1* gene ($P = .003$). The drug resistance rate of the strains with *sul2* gene was statistically significant compared with that without *sul2* gene ($\chi^2 = 51.351$, $P < .001$), as summarized in Table 4.

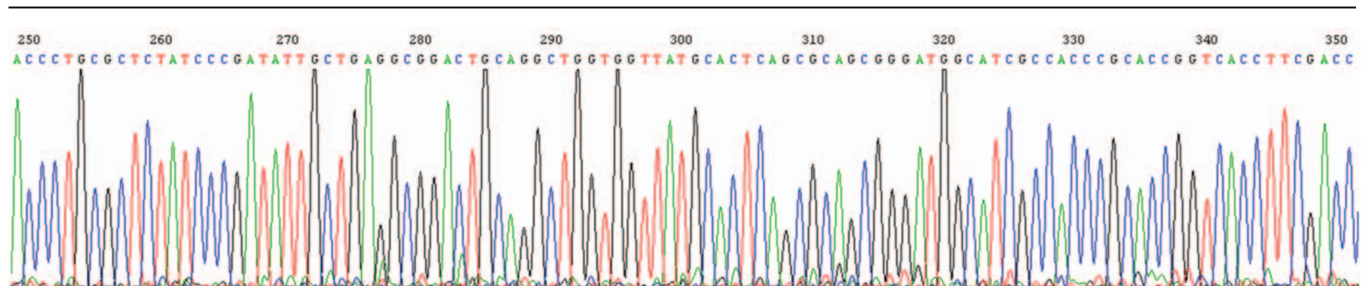

Figure 2. Part of the sequencing diagram of the *sul1* gene.

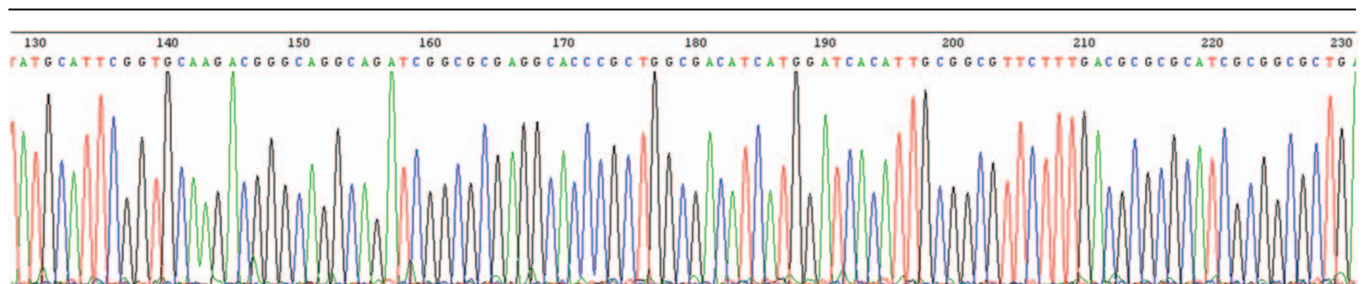

Figure 3. Part of the sequencing diagram of the *sul2* gene.

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	χ2	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	χ2	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. Delfifino 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*.[23,24] 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

Data curation: Quanping Ma, Chengbao Zhu.
Formal analysis: Chengbao Zhu.
Investigation: Quanping Ma, Mingxiao Yao.
Methodology: Chengbao Zhu.
Resources: Quanping Ma, Chengbao Zhu.
Software: Mingxiao Yao.
Supervision: Guangying Yuan, Yuguo Sun.
Writing – original draft: Quanping Ma, Yuguo Sun.
Writing – review & editing: Mingxiao Yao, Guangying Yuan, Yuguo Sun.

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