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Original Article

Transfer patterns of integron-associated and antibiotic resistance genes in *S. flexneri* during different time intervals in Tianjin, China

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Abstract

Background: Shigella is one of the common genera of pathogens responsible for bacterial diarrhoea in humans. According to World Health Organisation (WHO), 800,000–1,700,000 patients in China were infected with *Shigella* spp. in 2000, and *Shigella flexneri* is the most common serotype (86%). **Objectives:** We investigated the transfer patterns of integron-associated and antibiotic resistance genes in *S. flexneri* during different time intervals in the city of Tianjin in the People's Republic of China. **Materials and Methods:** The integrase-encoding and variable regions of the integrons of the bacterial strains were amplified by polymerase chain reaction (PCR), followed by gene sequencing. Fifty-six *S. flexneri* strains, 32 of which were stored in our laboratory and the other 24 were isolated from tertiary hospitals in Tianjin during different time intervals, were tested for their sensitivity to 12 antibiotics by using the Kirby–Bauer antibiotic testing method (K-B method). **Results and Conclusion:** Of the 32 strains of *S. flexneri* isolated from 1981 to 1983 and stored in our laboratory, class 1 integron was detected in 28 strains (87.50%), while 27 strains (84.37%) harboured an aminoglycoside resistance gene, aadA, in the variable region of their integrons. Class 1 integron was identified in 22 (91.67%) of the 24 *S. flexneri* strains isolated from 2009 to 2010, whereas the variable region and 3′-end amplification were not present in any of the strains. Class 2 integron was not found in the 1981–1983 group (group A) of strains; although 19 (79.17%) of the 24 strains in the 2009–2010 group (group B) possessed class 2 integron, and the variable region of the integron harboured dfrA1 + sat1 + aadA1 genes, which, respectively, mediate antibiotic resistance to trimethoprim, streptothricin and streptomycin. Seventeen strains of the total 56 possessed both class 1 and 2 integrons. Strains belonging to group A were highly resistant to tetracycline, chloramphenicol and a combination of trimethoprim-sulfamethoxazole; 65.63% of the strains were multi-resistant to three or more antibiotics. In group B, the strains showed high resistance to ampicillin, trimethoprim-sulfamethoxazole, piperacillin and tetracycline; 83.33% of the strains were multi-resistant to three or more antibiotics. Class 1 and 2 integrons exist extensively in *S. flexneri*, and the 3′-conserved segments of class 1 integron may have deletion or other types of mutations. Comparing the antibiotic and multi-drug resistance of group A with that of group B, it is apparent that the antibiotic resistance and the incidence of genes that confer multi-drug resistance have increased over the years in *S. flexneri*.

Key words: *Drug resistance, Integron, S. flexneri, Tianjin*

Introduction

Shigella is one of the common genera of pathogens responsible for bacterial diarrhoea in humans. According to World Health Organisation (WHO),[1] 800,000–1,700,000 patients in China were infected with *Shigella* spp. in 2000, and *Shigella flexneri* is the most common serotype (86%). Since the 1980s, Shigella infection has gradually increased despite the widespread application of antibiotics. In the past, studies on the mechanism of bacterial antibiotic resistance mainly focused on gene mutation. Subsequent research has found that bacteria acquire external antibiotic resistance genes through horizontal gene transfer, which accelerate the emergence of clinical antibiotic-resistant strains. The hereditary structures, which are closely related to horizontal transfer of bacterial antibiotic resistance genes, include plasmids, transposons, integrated phages and a recently discovered gene capture and dissemination system called integron. Previous studies[2-4] have recently reported that class 1 and 2 integrons are linked to drug resistance in Shigella. In this study, we analysed the antibiotic resistance of 56 *S. flexneri* strains carrying class 1 and class 2 integrons, which were isolated during different time intervals. We aimed to understand the horizontal gene transfer of class 1 and class 2 integrons and the changes in antibiotic resistance in the *S. flexneri* strains during different time intervals in Tianjin.

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Materials and Methods

Strains

Our study included 56 heterologous *S. flexneri* strains, 32 of which were stored in our laboratory from 1981 to 1983 (group A). The other 24 strains were isolated from stools of patients (ages ranging from 2 to 70 years) who presented with bacillary dysentery at the gastrointestinal clinics of some tertiary hospitals in Tianjin from 2009 to 2010 (group B).

Reagents

Twelve types of antibiotic disks produced by the Beijing Tiantan Institute of Biological Products Co., Ltd were used in our study. Each disk was impregnated with antibiotics such as trimethoprim-sulfamethoxazole (SMZ-TMP), norfloxacin, levofloxacin, ampicillin, piperacillin, ceftriaxone, cefotaxime, ceftazidime, cefepime, imipenem, tetracycline and chloramphenicol. Primer synthesis was performed by Sangon Biotech Co., Ltd (Shanghai, China), and gene sequencing was performed by BGI Sequencing (Beijing, China).

Methods

Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed by the Kirby–Bauer antibiotic testing method (K-B method) and the bacteriostatic diameter of the zones of inhibition was used as a standard criterion. The CLSI 2010 criterion was used as a standard for the criteria and interpretation of our experiment's results. *Escherichia coli* (ATCC25922) was adopted as a control strain for antibiotic susceptibility.

Detection and analysis of the integrons

Class 1 integron integrase, 3′-conserved segment and class 2 integron integrase were, respectively, amplified using the primers intI1L/intI1R,[5] qacEΔ1-F/sul1-B[6] and intI 2L/intI 2R.[5] The variable regions of class 1 and class 2 integrons were amplified using the primers hep58/hep59[7] and hep74/hep51[8] [Table 1]. We designed downstream primers dfrA12R, dfrA17R, aadAR and aadA5R based on the GenBank genetic sequences, and amplified corresponding resistance gene cassettes with hep58 as an upstream primer. PCR-amplified integron sequences were analysed by using agarose gel electrophoresis on a 1% gel at 100 V for 30 min, while amplified sequences of the variable region were analysed by electrophoresis on a 1.5% agarose gel at 100 V for 45 min. A gel imaging system (Gel Doc 2000; Bio-Rad) was subsequently used for photographing the gels. Results of direct sequencing of PCR products were compared with standard strains of GenBank's databank using NCBI's BLAST.

Restriction endonuclease reaction

Ten micro litre PCR amplification products, 1.5 μl restriction enzyme buffer and 1 μl HinfI endonucleases were incubated together at 37°C for 2 h. After adding 8 μl loading buffer to the endonuclease products, we performed electrophoresis on a 2% agarose gel at 110 V for 90 min followed by photography of the gels by using a gel imaging system (Gel Doc 2000; Bio-Rad).

Statistical methods

Experimental data were analysed using SPSS 17.0. Statistical analysis was performed using Fisher's exact test. A $P < 0.05$ was considered statistically significant.

Results

Results of the drug sensitivity test

With regard to the *Shigella* sp. in group A, the strains showed highest resistance to tetracycline (93.75%), followed by the resistance rates to chloramphenicol (78.13%), and trimethoprim-sulfamethoxazole (31.25%). The drug resistance rates of the strains to ampicillin and other antibiotics were lower than 20%, discounting the drug resistance to third and fourth generation cephalosporin, fluoroquinolonese and imipenem. Twenty-one strains of the 32 were multi-resistant to three or more antibiotics, which accounted for 65.63%. In group B, the resistance of the strains to ampicillin was the highest (95.83%), followed by the resistance rates to tetracycline (91.67%), trimethoprim-sulfamethoxazole (91.67%), chloramphenicol (58.33%) and piperacillin (54.17%). The resistance rates of the strains to cefotaxime and ceftriaxone were higher than 30%. The strains' rate of resistance to ceftazidime, cefepime, levofloxacin and norfloxacin was lower, discounting the resistance to imipenem [Table 2]. Twenty of the 24 strains (88.33%) were multi-resistant to three or more antibiotics.

Detection of class 1 integron

With respect to the 32 strains in group A, class 1 integron was detected in 28 strains (87.50%), and 27 of those 28 integron-positive strains also possessed the variable region sequences [Table 3, Figure 1]. The PCR-amplified products of the sequences were identical in size, and confirmed to be copies of a 1000 bp fragment through restriction enzyme analysis. The gene sequencing analysis showed that the sequence harboured an aminoglycoside drug resistance gene cassette *aadA*. Among the 24 strains of the group B, 22 strains (91.67%) contained class 1 integron, while both the variable region and the 3′-end amplification were absent [Table 3]. PCR amplification of the gene cassettes showed that five strains were positive for *blaoxa-30* and *aadA*, while four strains were positive for *dfrA12* [Table 4, Figure 2], which had a coincidence

Table 1: PCR amplification reaction primer

Primer name	Primer sequencing	Fragment length (bp)
intI1L	ACA TGT GAT GGC GAC GCA CGA	569bp
intI1R	ATT TCT GTC CTG GCT GGC GA	
hep58	TCA TGG CTT GTT ATG ACT GT	Variable
hep59	GTA GGG CTT ATT ATG CAC GC	
qacEΔ1F sul1B	ATC GCA ATA GTT GGC GAA GT	798 bp
	GCA AGG CGG AAA CCC GCG CC	
dfrA12R	AGC GTG CGA CAG CGT TGA	Variable
dfrA17R	TTC CGT TCT TTG ACA CTA CTG	Variable
aadAR	CGC CTC GAA TAG ATC CTG	Variable
aadA5R	CGG AGC GAA TCG TTA GGT G	Variable
intI2L intI2R	GTA GCA AAC GAG TGA CGA AAT G	789 bp
	CAC GGA TAT GCG ACA AAA AGG T	
hep74	CGG GAT CCC GGA CGG CAT GCA CGA TTT GTA	Variable
hep51	GAT GCC ATC GCA AGT ACG AG	

Table 2: Drug-sensitivity of the 56 *S. flexneri* strains

Antibiotic drugs	Group A (32 strains)				Group B (24 strains)				*P* value
	R (strains)	I (strains)	S (strains)	Drug resistance rate	R (strains)	I (strains)	S (strains)	Drug resistance rate	
Ampicillin	5	0	27	15.63	23	0	1	95.83	<0.001
Piperacillin	5	0	27	15.63	13	7	4	54.17	<0.001
Cefotaxime	0	0	32	0.00	9	1	14	37.50	<0.001
Ceftazidime	0	0	32	0.00	2	1	21	8.33	0.071
Ceftriaxone	0	0	32	0.00	9	0	15	37.50	<0.001
Cefepime	0	0	32	0.00	3	0	21	12.50	0.073
Imipenem	0	0	32	0.00	0	0	24	0.00	
Tetracycline	30	1	1	93.75	22	0	2	91.67	0.757
chloromycetin	25	1	6	78.13	14	2	8	58.33	0.255
Norfloxacin	0	0	32	0.00	3	2	19	12.5	0.011
Levofloxacin	0	0	32	0.00	2	2	20	8.33	0.030
Trimethoprim/sulfamethoxazole	10	7	15	31.25	22	0	2	91.67	<0.001

R: Resistant; I: Intermediary; S: Sensitive; resistance rate=R/R+I + S; $P<0.05$ was considered significant

Table 3: Results of class 1 and class 2 integron detection

Groups	Strain number	Class 1 integron			Class 2 integron	
		Integrase (+)	variable region (+)	3′-end (+)	Integrase (+)	variable region (+)
A	32	28	27	28	0	0
B	24	22	0	0	19	19

Table 4: Results of gene cassette detection

Antibiotic-resistance gene cassettes	Positive	Negative
aadA	5	17
aadA5	0	22
dfrA12	4	18
dfrA17	0	22

rate of 99% according to homology analysis (BLAST) with the sequence obtained from the GenBank database. The amplification of *DfrA17* and *aadA5* was negative.

Results of class 2 integron detection

Class 2 integron amplification of 32 strains in group A was negative. Class 2 integron in group B was detected in 19 strains (79.17%) [Table 3], the variable region of which harboured dfrA1 + sat1 + aadA1 genes that mediate antibiotic resistance to trimethoprim, streptothricinm and streptomycin. Among those 19, 17 strains were positive for class 1 integron amplification. Nineteen strains with both class 1 and 2 integrons were analysed via HinfI restriction enzyme digestion. The band

pattern of the sequences obtained via restriction enzyme digestion were identical with the sequence of the class 2 integron detected in the strains of our study [Figure 3]. The gene sequencing of a randomly selected sample with positive class 2-integron amplification showed that the class 2 integron sequence was about 2200 bp, which was identified to harbour the dhfl gene responsible for antibiotic resistance to trimethoprim, the satl gene encoding for streptothricin resistance, and the aadA gene conferring streptomycin resistance. These findings were verified by homologous comparison with the corresponding sequences of standard strains from the GenBank databank (No. AY140652) with a coincidence rate of 99%.

Discussion

The common epidemic *Shigella* genotypes in China are mainly *S. flexneri* and *S. sonnei*. In 2007, results from national monitoring sites for bacillary dysentery showed[9] that *S. flexneri* and *S. sonnei* were the dominant causative pathogens of bacillary dysentery in China. Nearly 57.21% cases of bacterial diarrhoea were caused by *S. flexneri* and 42.41% cases of bacterial diarrhoea were caused by *S. sonnei*. The drug sensitivities of *S. flexneri* strains, which were isolated from 2009 to 2010, showed that the resistance of *S. flexneri* to antibiotics such as ampicillin, piperacillin, trimethoprim-sulfamethoxazole, and third and fourth generation cephalosporin and fluoroquinolones had evidently increased than the antibiotic resistance of the *S. flexneri* strains found in group A with significant differences, while the resistance rates of the strains to tetracycline and chloramphenicol remained essentially similar.

The integron system, first proposed by Stokes *et al*.,[10] in 1989, consists of three components: A 5′-conserved segment (5′-CS), a 3′-conserved segment (3′-CS) and a variable region between them. The 5′-CS is the elementary structure of an integron and includes the integrase-encoding *intI*, a recombination site *attI* and a variable region promoter (Pant). The variable region harbours different numbers of gene cassettes with different functions, but it is not an elementary structure of the integron, since some integrons have no gene cassettes between the 5′-CS and 3′-CS. An integron can recognise and capture (or delete) mobile gene cassettes through the action of integrase, and can be expressed under the action of a promoter located upstream of the integron in order to confer antibiotic resistance, and even multi-drug resistance, to the bacteria. Currently, several studies have been investigating the integron system, which has been found in various antibiotic-resistant bacteria, including the members of *Shigella*. Among them, class 1 and class 2 integrons are closely related to multi-drug resistance of *Shigella* spp., but the detection rate may vary between different regions and species.

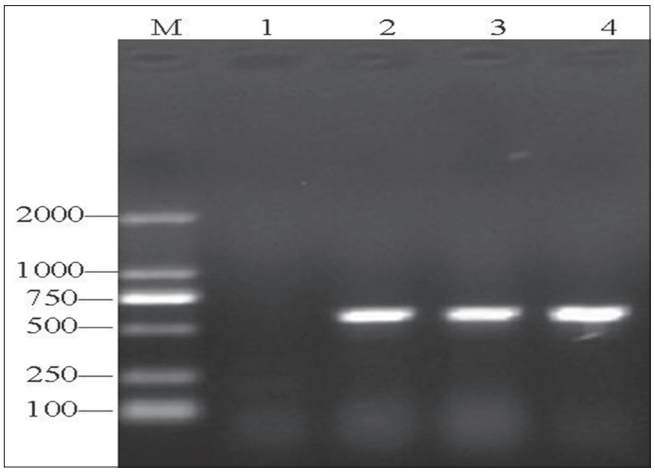

Figure 1: PCR amplification of class 1 integrase M, DL2000 DNA Marker; 1, Negative control; 2-4, class 1 integrase amplification-positive strains

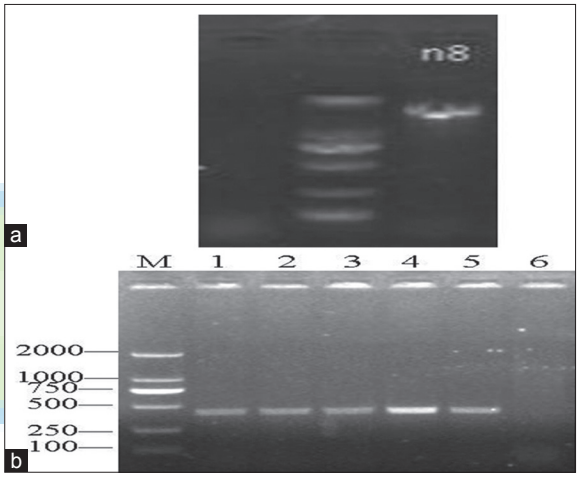

Figure 2: PCR amplification of aadA, dfrA12 gene cassettes (a) M, DL2000 DNA Marker; 1, Negative control; 2, aadA-positive strains N8; (b) M, DL 2000 DNA Marker; 1-5, dfrA12 amplification-positive strains; 6, negative control

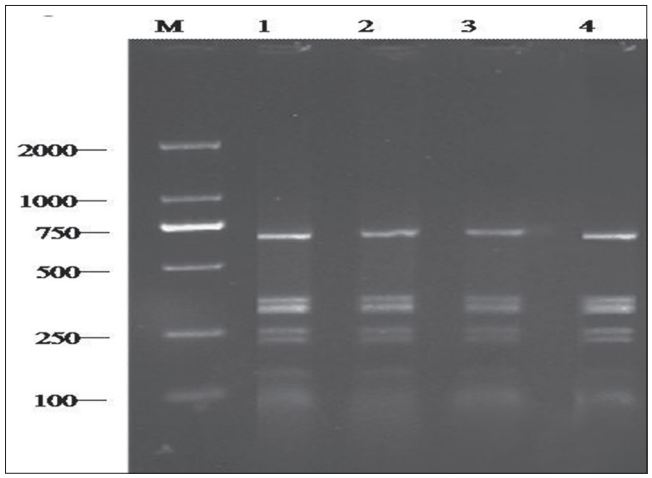

Figure 3: Electrophoretogram of class 2 integron variable region M, DL 2000 DNA Marker; 1, strains A11; 2, strains A6; 3, strains A4; 4, strains N10

It has been reported that the detection rate of class 2 integron has been far higher than that of class 1 integron in *Shigella* spp.[2,3,11]

Our study showed that class 1 integron-positive rate of *Shigella* sp. in Tianjin was 87.50% in group A and 91.67% in group B. There was no significant statistical difference between the class 1 integron-positive rates of the two groups. The amplification of the variable region of class 1 integron showed that group A had 28 strains that contained the integron along with the 3′-CS, of which 27 were positive for the variable region containing the aminoglycoside-resistance gene cassette *aadA*, which was determined through gene sequencing. Group B had 22 integron-positive strains that did not have the variable region or the 3′-CS. To exclude the omission caused by the 3′-CS mutation, we designed downstream primers dfrA12R, dfrA17, aadA and aadA5 based on the drug-resistance gene cassettes *dfrA12*, *dfrA17*, *aadA* and *aadA5*, which are usually carried by the class 1 integron. With hep58 as an upstream primer, corresponding drug-resistance gene cassettes were amplified to confirm whether the variable region was present, which showed that four strains were positive for *dfrA12* amplification, while five strains were positive for *aadA* amplification and the *blaoxa-30* gene located upstream. We speculate that a 3′-CS deletion mutation or mutations in the class 1 integron might exist in the strains belonging to group B. Class 2 integron was absent in the *Shigella* strains of group A, while 79% of the strains in group B possessed the class 2 integron. Mammina *et al*.,[11] speculated that *S. sonnei* have been carrying the class 2 integron since the 1980s, a theory they put forth by comparing the incidence of class 2 integron in *S. sonnei* strains isolated during different time intervals from 1971 to 2003 in Southern Italy, with which our conclusion was similar. Class 2 integron-positive strains possessed the variable region, which was confirmed through restriction endonuclease analysis to be the same fragment, and gene sequencing analysis showed class 2 integron carried the trimethoprim resistance gene *dfrA1*, streptothricin resistance gene *sat1*, and streptomycin resistance gene *aadA1*.

Our results have shown that class 1 integron-positive rate of *S. flexneri* between 2009 and 2010 had slightly increased than that between 1981 and 1983 in Tianjin, but the difference was not statistically significant. Both, variable region of class 1 integron and the 3′-CS of *Shigella* sp. were absent in the strains of group B. We think that the 3′-CS might have deletion or other mutations through the amplification of common antibiotic-resistance gene cassettes. Class 2 integron-amplification positive rate of the *Shigella* strains in group B was 79.17%, of which 17 strains had both class 1 and class 2 integrons. Considering the drug sensitivity results, we believe that the increased resistance of the *Shigella* strains to gentamicin, trimethoprim-sulfamethoxazole and cephalosporin might be linked to mutations in the structure of the class 1 integron, the ability to carry more drug-resistance gene cassettes as well as possessing the class 2 integron. However, this needs to be confirmed through further research on larger quantities of samples.

In conclusion, with the wide use of broad-spectrum antibiotics, the rate of resistance to commonly used antibiotics and the incidence of multiple drug-resistant strains of *Shigella* have increased than earlier. Class 1 and class 2 integrons are widespread in the members of *Shigella*, and 3′-CS deletion or mutations in class 1 integron may exist in some strains.

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