

Multi-drug Resistance and Characteristic of Integrons in *Shigella* spp. Isolated from China*

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Abstract

Objective To investigate the characteristic of integrons and the relationship between integrons and antimicrobial resistance in *Shigella* spp.

Methods Ninety *Shigella* strains (83 *S. flexneri* and 7 *S. sonnei*) were isolated from the stools of patients in China. Susceptibility to 8 antimicrobials was tested for all isolated strains. PCR, RFLP and sequencing analysis of integrons were applied to all of them.

Results High prevalence of multi-drug resistance (95.6%) was identified. Of the isolates 79 (87.8%) carried integrase genes of class 1 integron (3.3%), class 2 integron (10.0%) or both (74.4%). No *intB* was detected in the tested isolates. The prevalence of *int2* was significantly higher in isolates with multi-drug resistance to at least 3 antibiotics than that in isolates with resistance to 2 and less antibiotics ($P < 0.05$). Gene cassettes *dfrA17-aadA5*, *dfrA12-orfF-aadA2* of class 1 integron and *dfrA1-sat1-aadA1* of class 2 integron were identified.

Conclusion The class 2 integron may play a role in the emergence of multi-drug resistance in *Shigella* spp.

Key words: Resistance; *Shigella flexneri*; Integron; Gene cassettes

Biomed Environ Sci, 2011; 24(1):56-61

doi:10.3967/0895-3988.2011.01.007

ISSN:0895-3988

[www.besjournal.com/full text](http://www.besjournal.com/fulltext)

CN11-2816/Q

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INTRODUCTION

As an enteric infectious disease, shigellosis is estimated to affect 164.7 million patients and cause 1.1 million deaths each year globally, of which about 60% are children under 5 years. The occurrence is frequent in developing countries (163.2 million per year) with the major species being *Shigella flexneri* (60%), and rare in industrialized countries (1.5 million per year) with *Shigella sonnei* as the main pathogen (77%)^[1]. In China, 0.8-1.7 million episodes of shigellosis were

reported in 2000 and *S. flexneri* was the most common serogroup (86%)^[2].

Antimicrobial therapy has been effective not only in allaying the dysenteric syndrome of shigellosis, but also in reducing the duration of illness and the fecal excretion of the bacterium to prevent further spread. Since the early 1960s, *Shigella* spp. has been known for acquiring multidrug resistance to commonly used antimicrobials such as trimethoprim/sulfamethoxazole, tetracycline and ampicillin^[3-5]. Efflux pump, plasmids, transposons, and integron have been involved in several

*This work was supported by the Research Fund from Ministry of Health of the People's Republic of China, WKJ2007-2-024.

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Received: May 2, 2010;

Accepted: October 22, 2010

mechanisms investigated^[6-8]. It is a great concern that integrons with resistance gene cassettes have been identified in plasmids, transposons, and chromosome. There are two types of class 1 integron found in *Shigella* spp., classic and atypical class 1 integron, associated with the gene cassettes *dfrA1*, *aadA1*, *estX* and *bla_{oxa-30}*^[9-11]. The common gene cassettes of class 2 integron in *Shigella* spp. were *dfrA1*, *sat1* and *aadA1*^[11-17]. However, these reports either described strains mainly isolated from developed countries where *S. sonnei* was the prevalent species or little referred to strains of *S. flexneri*^[13-17]. Surveys involving relatively large amount of *S. flexneri* were lacking.

The present study described the multidrug resistance and the content of integrons in 83 strains of *S. flexneri*, and 7 strains of *S. sonnei* isolated from China. High frequency of multiresistance and integrons, as well as the relationship between class 2 integron and multiresistance, were revealed.

MATERIALS AND METHODS

Bacterial Strains

Ninety strains of *Shigella* spp. investigated in this study were isolated from stool samples of sporadic diarrheal patients in China. Eighty three strains of *S. flexneri* included 11 strains isolated in Tonggu county, Jiangxi province from 1995 to 1996; 5 strains isolated in Zhengzhou city, Henan province from 1998 to 2001; and 67 strains isolated in Sui county, Henan province from 2004 to 2006. Seven strains of *S. sonnei* included 6 strains isolated in Zhengzhou city, Henan province from 1998 to 2001 and 1 strains isolated in Sui county, Henan province in 2004. All the strains were identified at the serogroup level by standardized procedures.

Antimicrobial Susceptibility Testing

Ninety *Shigella* spp. isolates susceptibility to eight antimicrobial agents were determined by the disk diffusion method on Mueller-Hinton agar according to the recommendations of the Institute of Clinical and Laboratory Standards. The following antimicrobial agents were tested: ampicillin, AMP(10 µg); tetracycline, TET(30 µg); trimethoprim-sulfamethoxazole, SXT(25 µg); chloramphenicol, CHL(30 µg); nalidixic acid, NAL(30 µg); ciprofloxacin, CIP(5 µg); gentamicin, GEN(10 µg) and cefazolin, CFZ (30 µg). *Escherichia coli* ATCC 25922 was used as a quality control strain.

PCR, RFLP and Sequencing

Total DNA and plasmid DNA were extracted from the 90 *Shigella* strains by Axyprep Bacterial Genomic DNA Miniprep Kit (Axygen Biotechnology, Hangzhou, China) and Tiangen Plasmid Purification Mini kit (Tiangen Biotech, Beijing, China) respectively. The 50 µL of PCR reaction mixture contained 1× PCR buffer, 10 pmoles of each primer, 200 mmol/L (each) dNTP, 1 U of *Taq* DNA polymerase (MBI Fermentas) and about 30 ng template DNA. PCR amplification was performed by the following procedure: pre-denaturation at 94 °C for 5 min followed by 30 cycles at 94°C for 50 s, 56-58 °C for 50 s and 72°C for 1-4 min, with a final extension at 72 °C for 10 min. The primer pairs used for detecting the integrase gene of class 1 integron (*int1*) were *int1* F: 5'-CCT CCC GCA CGA TGA TC-3' and *int1* R: 5'-TCC ACG CAT CGT CAG GC-3'^[18]. Amplification fragment contains 280 bp. The primer pairs for *int2* were *int2* F: 5'-CAC GGATATGCGACAAAAGGT-3' and *int2* R: 5'-GTAGCAAAC GAG TGA CGA AAT G-3'^[19]. Amplification fragment contains 789 bp. The primer pairs for *int3* were *int3* F: 5'-CGA ATG CCC CAA CAA CTC-3' and *int3* R: 5'-ATC TGC CAA ACC TGA CTG-3'^[20]. Amplification fragment contains 922 bp. The primer pairs used for detecting the gene cassettes of class 1 and class 2 integron were hep58: 5'-GGC ATC CAA GCA GCA AGC-3' and hep59: 5'-AAG CAG ACT TGA CCT GAT-3'^[21], hep74: 5'-CGG GAT CCC GGA GGC ATG CAC GAT TTG TA-3' and hep51: 5'-GAT GCC ATC GCA AGT ACG AG-3'^[22], respectively. Amplification fragments are various.

PCR products were revealed by horizontal electrophoresis on 1%-1.5% agarose gel stained by GoldView™ DNA dye (SBS Genetech, Beijing, China). The PCR products of gene cassettes with similar length were examined by restriction fragment length polymorphism analysis with *Pvu* I, *Hind* III or *Hinf* I (MBI Fermentas). The identical restriction profiles were regarded as the same array of gene cassettes.

The interested PCR product of representative isolates were cloned by the TaKaRa TA cloning kit pMD-18 (TaKaRa, Dalian, China), transformed into *E. coli* DH5α and sequenced. The nucleotide sequences obtained were analyzed by using software BLAST on the website of the National Center for Biotechnology Information.

Nucleotide Sequence Accession Numbers

The sequences of the gene cassettes in class 1 integron and class 2 integron were submitted to the GenBank database and got the accession numbers

FJ895301, FJ895302 and EF634237 respectively.

Statistical Method

In order to analyze the relationship between the prevalence of integrase gene and multi-drug resistance, the software of SPSS 12.0 for Windows (SPSS Inc. 2003) was used to evaluate the *P* value of Fisher's Exact Test.

RESULTS

Antimicrobial Resistance of the *Shigella* Strains

The higher rates of resistance were to TET, NAL, AMP, SXT, and CHL (Table 1). The lowest rate of resistance was to GEN. Multi-drug resistance (resistance to at least 3 different antimicrobial agents) was detected in 86 isolates (95.6%). All isolates of *S. flexneri* were multi-drug resistant. As for 7 isolates of *S. sonnei*, 2 isolates were susceptible to all the antibiotics tested while the other 2 were resistant to TET or TET-AMP. And the remaining 3

isolates of *S. sonnei* had the multiresistant pattern as TET-SXT-NAL (2 isolates) and AMP-CHL-CFZ. Among all the *Shigella* spp., the most common resistant profiles were AMP-TET-SXT-CHL-NAL (31/90, 34.4%) and AMP-TET-SXT-CHL-NAL-CIP (17/90, 18.9%).

Distribution of Integrons in the *Shigella* Strains

The integrase genes were detected by PCR (Figures 1 and 2). No *intB* was identified. There were 87.8% (79/90) isolates found positive for integrons. Table 2 presents isolates positive for both class 1 and class 2 integron (74.4%), class 1 integron only (3.3%) and class 2 integron only (10.0%). There were still 11 isolates without any integrons. All strains harboring a class 2 integron or accompanied with a class 1 integron were resistant to at least three different antibiotics tested.

The prevalence of *int2* was significantly higher in isolates with multi-drug resistance to at least 3 antibiotics than that in isolates with resistance to 2 and less than 2 antibiotics ($P < 0.05$) (Table 3).

Table 1. The Antimicrobial Resistance of *Shigella* spp. Isolated from China

Species	No. of Isolates	No. of Resistant Isolates (%)							
		AMP	TET	SXT	CHL	NAL	CIP	GEN	CFZ
<i>S. flexneri</i>	83	80(96.4)	81(97.6)	73(88.0)	67(80.7)	82(98.8)	30(36.1)	5(6.0)	19(22.9)
<i>S. sonnei</i>	7	2(28.6)	3(42.9)	1(14.3)	1(14.3)	1(14.3)	0	0	1(14.3)
Total	90	82(91.1)	84(93.3)	74(82.2)	68(75.6)	83(92.2)	30(33.3)	5(5.6)	20(22.2)

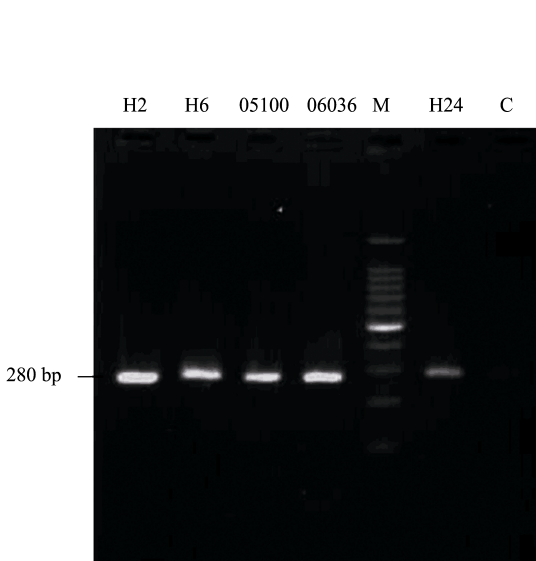


Figure 1. PCR product of *int1*. M: 100 bp DNA ladder. C: blank control.

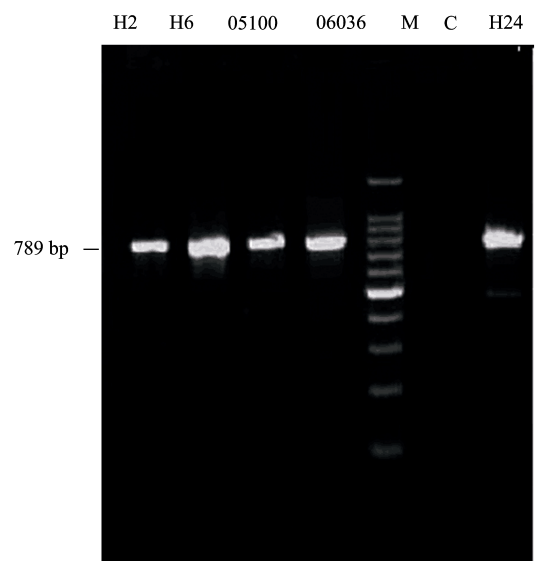


Figure 2. PCR product of *int2*. M: 100 bp DNA ladder. C: blank control.

Table 2. Distribution of Integrons in Different Serotype Isolates of *Shigella*

Species	No. of Isolates	No. of Positive Isolates (%)		
		<i>int1</i> Only	<i>int2</i> Only	both <i>int1</i> and <i>int2</i>
<i>S. flexneri</i>	83	0	8 (9.6)	66(79.5)
<i>S. sonnei</i>	7	3(42.9)	1(14.3)	1(14.3)
Total	90	3(3.3)	9(10.0)	67(74.4)

Table 3. The Positive Integron and Multiresistance in *Shigella* spp.

Group	Number	No. of Isolates (%)		Pvalue*
		Multiresistant to 3 and More than 3 Antibiotics	Susceptible to all or Resistant to 2 and Less than 2 Antibiotics	
Positive of either integron	79	77(97.5)	2 (2.5)	0.072
Negative of all integrons	11	9(81.8)	2(18.2)	
Positive of <i>int1</i> gene	70	68(97.1)	2(2.9)	0.213
Negative of <i>int1</i> gene	20	18(90.0)	2(10.0)	
Positive of <i>int2</i> gene	76	76(100.0)	0(0.0)	0.000
Negative of <i>int2</i> gene	14	10(71.4)	4(28.6)	

Note.* Fisher's Exact Test.

The Resistance Gene Cassettes of Integrons

With the primer pair hep58-hep59 for the gene cassettes of class 1 integron, two different types of gene cassette arrays were found (Figure 3). The first type of 1 913 bp size was detected in only one isolate and confirmed by DNA sequencing as *dfrA12-orfF-aadA2* with the GenBank accession number FJ895301. The second type with size of 1 665bp, sequenced as *dfrA17-aadA5* with the GenBank accession number FJ895302, was identified by RFLP with restriction enzyme *Pvu* I in 55 isolates. The 2 211 bp DNA product of PCR with primer pair hep74-hep51 was present in all 76 isolates positive for *int2* gene and shared the same RFLP profile with the restriction enzyme *Hinf* I (Figure 4). As shown by DNA sequencing analysis, it was comprised of *dfrA1-sat1-aadA1* with the GenBank accession number EF634237.

DISCUSSION

More than 85% of *S. flexneri* isolates in this study were found to possess the resistance to TET and SXT. Similar results had been obtained in other studies from developing countries^[4-5, 23]. Resistance of *S. sonnei* to AMP and CHL was reported in various rates and was relatively rare^[3,5,24], while that of *S. flexneri* were frequent^[25-26]. The high resistant rate to AMP and CHL identified in the present study

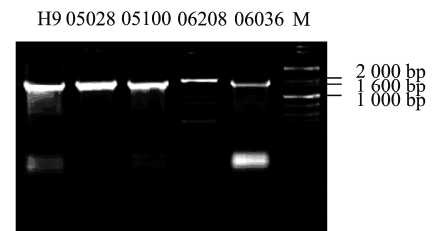


Figure 3. PCR product of class 1 integron gene cassettes. H9, 05028, 05100, and 06036 have the fragment about 1 665 bp while 06208 has the fragment about 1 913 bp. M: 1 kb plus DNA ladder.

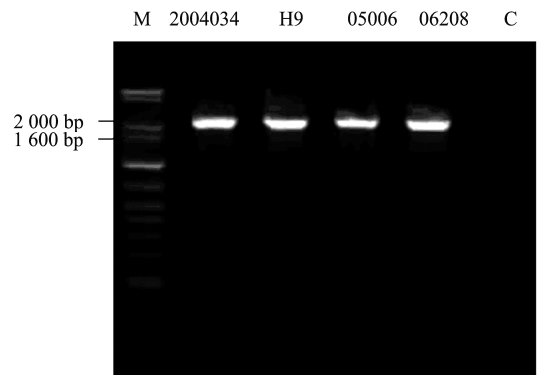


Figure 4. PCR product of class 2 integron gene cassettes. M: 1 kb plus DNA ladder. C: blank control. confirmed that most of *S. flexneri* isolates had

acquired resistance to TET, SXT, CHL, and AMP. These inexpensive and readily available antibiotics are hardly effective against most of *S. flexneri* strains now. Although most of the *Shigella* spp. strains were susceptible to GEN^[2,5], according to the present study, the serious adverse effect was confined to the usage of GEN in non-pediatric practice. Quinolone antibiotics can be used alternatively for those isolates resistant to classical antibiotics. High prevalence of *Shigella* spp. isolated from Asia which is resistant to NAL and other quinolone antibiotics has been reported in previous studies^[4,27]. Indeed, our results proved that majority of *S. flexneri* isolates were resistant to NAL and even more than 30% of them were also resistant to CIP, which provided a clue that *S. flexneri* isolates from certain areas in China had gained the high prevalence of resistance to quinolones. Other than the prevalence of resistance to CFZ (22.9%), which are second-line drugs for the treatment of multiresistant *Shigella* spp. isolates, the great antimicrobial resistance of *S. flexneri* identified in this study should constitute a public concern.

The gene cassettes *dfrA12-orfF-aadA2* of class 1 integron was rarely reported and confined to outbreak-related isolates of *Shigella* spp. in previous studies^[14]. The gene cassettes *dfrA17-aadA5* of class 1 integron was usually found in other species of *Enterobacteriaceae* and seldom detected in *Shigella* spp. previously^[9]. The class 2 integron gene cassettes *dfrA1-sat1-aadA1* in this study was constant as in other studies^[13-17].

Being different from the earlier reports of the sole class 2 integron found in *Shigella* spp, especially in *S. sonnei*^[13-17], a great part of isolates in this study harbored both class 1 and class 2 integrons. As for *S. flexneri*, high distribution of class 1 and class 2 integrons was evaluated recently, but the amount of isolates involved were limited (about 30 isolates)^[9-10]. Although our results revealed that isolates of *Shigella* spp. had high prevalence of multi-drug resistance and high incidence of both classes of integrons at the same time, only the prevalence of *int2* gene was significantly correlated with the multi-drug resistance ($P < 0.05$). Now that the frequency of class 2 integron and multi-drug resistant rate in *Shigella* spp. were uniformly high, the class 2 integron might play a role in the emergence of multi-drug resistance in *Shigella* spp. These results lead to the hypothesis that the gene linkages between class 2 integron and other resistant genes or class 2 integron work together with other genetic resistance factors, which need

further exploratory studies at a molecular level. The linkage of class 2 integron to other antibiotic resistance genes would also make it possible to use class 2 integron as a molecular biomarker to monitor the multi-drug resistance in *Shigella* spp.

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