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Atypical Class 1 Integron Coexists with Class 1 and Class 2 Integrons in Multi-Drug Resistant *Shigella flexneri* Isolates from China

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Abstract The antimicrobial resistance and the character of integrons were determined in 58 *Shigella flexneri* strains isolated from China. All isolates were multi-drug resistant and found to carry integrons of class 1 (94.8%), class 2 (100%), or both (94.8%). No *intI*3 was detected. The typical class 1 integrons were found in conjugative plasmids and could be transferred to the recipient *E. coli* DH5α. The gene cassettes of typical class 1 integrons *dfrA17-aadA5* and *dfrA12-orfF-aadA2* were detected in 54 strains (93.1%) and 1 strain, respectively. Atypical class 1 integrons located on the chromosome with gene cassettes $bla_{oxa\text{-}30}$-*aadA1* were detected in 55 isolates (94.8%). All the *intI*2 positive isolates carried gene cassettes *dfrA1-sat1-aadA1*. To our knowledge, this is the first report that atypical and typical class 1 integrons coexisted with class 2 integron in multi-drug resistant *S. flexneri* strains.

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Introduction

Shigella spp. are the important enteric pathogen of the infectious diarrhea. The incidence of shigellosis is high among children under 5 years of age universally. *Shigella flexneri* was the major species (60%) in developing countries and *Shigella sonnei* was the main species (77%) in industrialized countries [5]. In China, it was estimated that 0.8–1.7 million episodes of shigellosis occurred in 2000 and *S. flexneri* was the most frequent serogroup (86%) [19].

Due to the wide use of antibiotics, the emergence of resistant strains of *Shigella* spp. is of great interest in both developing and industrialized countries [12, 17]. The resistance of *Shigella* spp. is commonly associated with the mobile genetic elements (including plasmids, transposons, and gene cassettes in integrons), gene mutations or efflux pumps [7, 21, 23]. Integrons and resistant gene cassettes found in plasmids, transposons, and chromosomes have generated great concern. Class 2 integrons [3, 13, 15] were detected more frequently than class 1 integrons [2, 11] in *Shigella* spp., especially in strains of *S. sonnei*. The present study described the coexistence of integrons in 58 strains of multi-drug resistant *S. flexneri* from China.

Materials and Methods

Bacterial Strains

A total of 58 isolates of *S. flexneri* were investigated in this study. All strains were isolated from stool samples of sporadic diarrheic patients from different hospitals in Sui county, Henan province of People's Republic of China in 2005 and 2006. All strains were identified biochemically by standard procedures [4] and confirmed by slide

agglutination using group- and type-specific *Shigella* antisera (Lanzhou Institute of Biological Products, Lanzhou, China).

Antimicrobial Susceptibility Testing

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

PCR, RFLP, and Sequencing

Total DNA and plasmid DNA were extracted from strains by the Axyprep Bacterial Genomic DNA Miniprep Kit (Axygen Biotechnology, Hangzhou, China) and Tiangen Plasmid Purification Mini kit (Tiangen Biotech, Beijing, China), respectively. The primer pairs used for detecting the integrases and the various regions of the integrons were shown in Table 1. The 50 μl of PCR reaction mixture contained 1× PCR buffer, 10 pmol of each primer, 200 mM (each) dNTP, 1 U of *Taq* DNA polymerase (MBI Fermentas), and about 30 ng template DNA. After predenaturation at 94°C for 5 min, there were 12 cycles of 94°C for 50 s, 68–57°C for 50 s with degradation of 1°C per cycle and 72°C for 4 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 4 min, with a final extension at 72°C for 5 min. The PCR products of various regions with similar length were examined by restriction fragment length polymorphism (RFLP) analysis with PvuI, HindIII, or HinfI (MBI Fermentas). The identical restriction profiles were regarded as the same array of gene cassettes. The interest 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.

Conjugation Analysis

Conjugation analyses were carried out in all isolates of *S. flexneri* by using *E. coli* DH5α as recipient. The over night culture of purified donor and recipient cells were diluted 100-fold in 2 ml of fresh LB broth. The growth continued at 37°C until OD600 reached about 0.6. Then 0.1 ml each of the donor and recipient cultures was mixed in 1 ml of fresh LB broth. After incubation at 37°C without shaking for 16–18 h, the mixture was plated on MacConkey agar plate and SS agar plate. The morphological differences between *E. coli* and *Shigella* on MacConkey agar plate and SS agar plate were used to select the recipients. The extraction of plasmids and PCR tests for the various regions of integrons were applied to confirm the transconjugants that acquire plasmids and integrons. Changes of the susceptibility to antimicrobial agents were measured by the disk diffusion method as described above.

Results

Antimicrobial Resistance and Distribution of Integrons in the *Shigella* Strains

All isolates were resistant to three different antibiotics TET, NAL, and SXT and the incidence of multi-drug resistance was 100%. The incidence of resistance to AMP and CHL were 96.5% (56/58) and 79.3% (46/58), to CIP and CFZ were 22.4% (13/58) and 10.3% (6/58). The lowest resistance incidence was to GEN (3.4%, 2/58). Except 3

Table 1 Primers used in this study

Primer	DNA sequence	Location	Product size	Reference
*intI*1F	5′-CCTCCCGCACGATGATC-3′	*intI*1	280 bp	[22]
*intI*1R	5′-TCCACGCATCGTCAGGC-3′	*intI*1		
hep58	5′-GGCATCCAAGCAGCAAGC-3′	5′-CS of class 1 integron	Variable	[1]
hep59	5′-AAGCAGACTTGACCTGAT-3′	3′-CS of class 1 integron		
ISVR	5′-AACACCGACAGGGATGGA-3′	3′-CS of atypical class 1 integron	Variable	[11]
*intI*2F	5′-CACGGATATGCGACAAAAAGGT-3′	*intI*2	789 bp	[14]
*intI*2R	5′-GTAGCAAACGAGTGACGAAATG-3′	*intI*2		
hep74	5′-CGGGATCCCGGAGGCATGCACGATTTG TA-3′	5′-CS of class 2 integron	Variable	[20]
hep51	5′-GATGCCATCGCAAGTACGAG-3′	3′-CS of class 2 integron		
*intI*3F	5′-CGAATGCCCCAACAACTC-3′	*intI*3	922 bp	[16]
*intI*3R	5′-ATCTGCCAAACCTGACTG-3′	*intI*3		

Table 2 Gene cassettes of integrons and resistant types of 58 *Shigella flexneri*

Gene cassettes			No. of *S. flexneri* strains, resistant type (No.)
Of typical class 1 integron with primer pair hep58-hep59	Of atypical class 1 integron with primer pair hep58-ISVR	Of class 2 integron with primer pair hep74-hep51	
dfrA12-orfF-aadA2	bla_{oxa-30}-*aadA1*	*dfrA1-sat1-aadA1*	1, ATSCN I (1)
dfrA17-aadA5	bla_{oxa-30}-*aadA1*	*dfrA1-sat1-aadA1*	54, ATSCN (30); ATSCNI (9); ATSN (8); ATSCNGF (2); ATSCNF (2); ATSCNIF (1); ATSNI (1); TSN (1)
–	–	*dfrA1-sat1-aadA1*	3, ATSN (1); ATSNI (1); TSN (1)

A AMP, *T* TET, *S* SXT, *C* CHL, *N* NAL, *I* CIP, *G* GEN, *F* CFZ

isolates only harboring *intI*2 gene, 55 isolates of *S. flexneri* were found to possess both *intI*1 gene and *intI*2 gene (94.8%). No *intI*3 gene was found. The main resistance types and characteristic of integrons of all strains were shown in Table 2.

Location and the Resistance Gene Cassettes of Integrons

Fifty-five isolates were positive for the PCR detection of the variable region of typical class 1 integron with the primer pair hep58-hep59. Except one isolate exhibiting 1913-bp fragment revealed as *dfrA12-orfF-aadA2* by DNA sequencing, the rest 54 isolates (93.1%) were all found carrying 1665-bp fragments. RFLP analysis with restriction enzyme PvuI showed that all 1665-bp fragments were uniform and DNA sequencing recognized the sequence as *dfrA17-aadA5*. Both of the two gene cassette arrays could be detected by PCR in the transconjugants after the conjugation test. The conjugatable plasmids transferred the resistance to SXT, AMP, and streptomycin(S). Therefore, the typical class 1 integrons were located on conjugatable plasmids. The two sequences of the gene cassette arrays were submitted to the GenBank database (accession numbers FJ895301 and FJ895302, respectively).

The primer pair hep58-ISVR was designed to identify the variable region of atypical class 1 integron. The amplicons of 2140 bp in size were found in 55 isolates. All the amplicons shared the same HindIII profile and were recognized as gene cassettes bla_{oxa-30}-*aadA1* by DNA sequencing. The negative amplification from the plasmids DNA and the negative results of conjugation analysis indicated that atypical class 1 integrons were present on genomic DNA. The sequence of bla_{oxa-30}-*aadA1* was submitted to the GenBank database (accession number GQ214137).

All 58 positive isolates of *intI*2 gene were found carrying gene cassettes *dfrA1-sat1-aadA1* that were identified by the same RFLP profile with the restriction enzyme HinfI and DNA sequencing. The negative results of PCR with plasmids DNA and conjugation analyses revealed that class 2 integrons were localized on chromosomes. The sequence was submitted to the GenBank database (accession number EF634237).

Discussion

Most of *S. flexneri* strains in this study had acquired the resistance to TET, SXT, CHL, and AMP. The resistance frequencies were similarly high in other reports from both developing and developed countries [12, 17]. These inexpensive and ready available antibiotics are hardly effective against most of *S. flexneri* strains now. The high frequencies of resistance to NAL and other quinolones in isolates of *Shigella* spp. were revealed in both this study and earlier other studies from Asia [12, 18]. Considering the resistance frequency to CFZ (10.3%), which is the second-line drug for the treatment of shigellosis, the high incidence of antimicrobial resistant *S. flexneri* should be of general concern.

Class 2 integrons have been reported mainly in *S. sonnei* strains in earlier reports [3, 15]. In this study, however, a large percentage (94.8%, 55/58) of isolates harbored both class 1 and class 2 integrons. High distribution of class 1 and class 2 integrons have also been evaluated in strains of *S. flexneri* recently [2, 11], but the amount of isolates involved were limited (about 30 isolates). Our results about 58 isolates of *S. flexneri* indicated that the high incidence of both classes of integrons correlated with the high prevalence of multi-drug resistance in *S. flexneri* strains.

The gene cassettes *dfrA12-orfF-aadA2* and *dfrA17-aadA5* of typical class 1 integrons have been reported rarely or only in outbreak-related isolates of *Shigella* spp. previously [9–11]. But in isolates of *Klebsiella pneumoniae*, they have also been detected [8]. The variation of the gene cassettes in typical class 1 integron may reflect the

horizontal transfer of integrons within or out of the family of *Enterobacteriaceae*.

The atypical class 1 integron was first reported on the chromosome of *S. flexneri* 2a strain YSH6000 from Japan [6]. The sequence of the gene cassettes bla_{oxa-30}-*aadA1* in this study was 99% identical to the corresponding sequence of strain YSH6000. The atypical class 1 integron was adjacent to two resistance determinants of chloramphenicol and tetracycline in YSH6000 and the whole fragment was defined as STL PAL (the *Shigella* resistance locus, pathogenicity island). This may explain the finding in this study that most of the positive isolates (45/55, 81.8%) with gene cassettes bla_{oxa-30}-*aadA1* were resistant to three antibiotics (AMP, TET, and CHL) while all the three negative isolates lacked the triple resistance phenotype (Table 2). This indicated that the presence of the atypical class 1 integron containing the gene cassettes bla_{oxa-30}-*aadA1* is likely associated with the resistance to AMP, TET, and CHL.

The atypical class 1 integrons were also found in *Shigella* spp. isolated from Asia [11], Africa, Europe, and Central America [2]. However, the atypical class 1 integron and typical class 1 integron have not been found to coexist in *Shigella* spp. It's notable that 55 isolates of *S. flexneri* in this study that harbored atypical class 1 integron also carried the typical class 1 integron. The coexistence of the typical and atypical class 1 integron in so many individual isolates of *Shigella* spp. has not been previously detected. The present results gave an expanded view on the linkage between the resistance and integrons in *Shigella* spp. The atypical class 1 integron has been found to be followed by part of IS*1* in previous study [11]. It's known that two copies of the IS element flanking the gene can make it mobile. Strains containing an atypical class 1 integron related with IS element and a typical class 1 integron located on conjugatable plasmid may have more potential to acquire and transfer resistance under antibiotic selection pressure. Moreover, the coexistence of atypical and typical class 1 integrons may indicate the genetic affinity or the trace of removal of the two integrons. The reason or the advantage of this phenomenon is unclear and the interaction of the integrons needs further exploration.

The gene cassettes of class 2 integron were invariable in this study and other reports as *dfrA1-sat1-aadA1* [3, 15], and all isolates harbored the same class 2 integron.

Despite the limited classes of integrons and the high stability of gene cassettes, the atypical class 1 integron, typical class 1 integron and class 2 integron coexisted in each of the 55 multi-drug resistant isolates of *S. flexneri*. Further studies are needed to explore the interaction of the integrons and the possible gene linkages between integron-mediated resistance and other resistant genes.

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