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Full Length Research Paper

High prevalence of atypical class 1 integrons and class 2 integrons in multi-drug resistance *Shigella flexneri* isolated from China

Xin-mian Wen, Yao-guang Wu, Feng-zhi Bian, Yu-guo Sun, Xiu-feng Zheng, Yan-fang Zhang, Chao Jiang and Guang-ying Yuan*

Department of Laboratory the 4th Hospital of Ji'nan and The second affiliated Hospital of Thaishan Medical College, Ji'nan, 250031, China.

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Shigella flexneri **is an important cause of bacterial dysentery in the developing world. Treatment with most widely used and inexpensive antimicrobial drugs became limited due to globally emerging antimicrobial resistance. Integron-associated antibiotic resistance was observed in *S. flexneri*. This study described the antimicrobial susceptibility and the characteristic of classes 1 and 2 integrons in *S. flexneri* in China. 90.5% (48/53) *S. flexneri* strains accounted for multi-drug resistance and carried integrons of class 1 (90.5%), class 2 (86.8%), or both (86.8%). The gene cassettes of typical class 1 integrons, *dfrV* and *dfrA17-aadA5*, were detected in 6 strains and 2 strains, respectively. Atypical class 1 integrons with gene cassettes bla_{OXA-30}-*aadA1* were detected in 45 (84.9%) strains. The typical and atypical class 1 integrons coexisted in 6 strains; 46 (86.8%) strains carried class 2 integrons with gene cassettes *dfrA1-sat1-aadA1*. No *intI3* was detected. The PFGE profiles showed spread of integrons among different serotypes in *S. flexneri*. The majority of *Shigella* strains are resistant to ampicillin, chloramphenicol, tetracycline, streptomycin and trimethoprim-sulfamethoxazole. Atypical class 1 and class 2 integrons are widely present in these *Shigella* strains. Typical and atypical class 1 integrons coexist among some multi-drug resistant *Shigella* strains.**

Key words: *Shigella flexneri*, integrons, multi-drug resistance, pulsed-field gel electrophoresis.

INTRODUCTION

Shigellosis is a global human health problem (Niyogi, 2005) known to cause diarrheal disease and death in a certain proportion of affected individuals. It was reported that more than 140 million cases of shigellosis occurred worldwide, with 600,000 people dying annually; 60% of the deaths were seen in children under the age of 5 (Kotloff et al., 1999; Sur et al., 2004). Among the four *Shigella* species, *S. flexneri* is the most commonly isolated in the developing world and the most frequent cause of bacterial dysentery. Antimicrobial agents have been recommended for the treatment of shigellosis; however, the increase of multi-drug resistance in the *Shigella* spp. has been reported worldwide (Rosewell et al., 2010; Zhu et al., 2011). Multidrug-resistant *Shigella* strains have emerged by the use and abuse of antibiotics, resulting in inefficient antimicrobial treatment (Kansakar et al., 2007; Watson, 2006). One of the mechanisms of resistance development is frequently borne by the mobile genetic elements, these elements include R-plasmids, transposons, integrons and genetic "islands" on the bacterial genomes, which can transfer resistance determinants among species, even genera (Hrabák et al., 2010; Partridge, 2011).

Integrons have been extensively studied due to their association with other mobile genetic elements and multi-resistance phenotypes. Based on the amino-acid

*Corresponding author: Email: guangyingyuan@126.com.

sequence of the IntI protein, five classes of integrons have been described (Cambray et al., 2010). Classes 1, 2, and 3 are the most commonly detected. Classes 4 and 5 have only been detected once (Hochhut et al., 2001). In gram-negative bacteria, classes 1 and 2 integrons are the most widespread, and the correlation between the presence of integrons and antimicrobial resistance were demonstrated (Köseoğlu, 2004; White et al., 2001). In *Shigella* spp., class 2 integron (Ahmed et al., 2006; Gassama-Sow et al., 2006; Ranjbar et al., 2007) were detected more frequently than class 1 integron (Dubois et al., 2007; Pan et al., 2006). In this study, we aimed to investigate the molecular characteristics of integrons in clinical isolates of *S. flexneri* in Jinan, during 2007 to 2009 and the relationship between gene cassettes of integrons and antibiotic resistances.

MATERIALS AND METHODS

Bacterial isolates

A total of 53 *S. flexneri* isolates including 34 of serotype 2a, 13 of 4a, and 6 of 1a were collected from the 4th hospital of Jinan, Jinan center for Disease Control and Prevention, Shandong province of People's Republic of China during 2007 to 2009. All the strains were isolated from stool samples of clinical sporadic diarrheic patients, 23 females and 30 males, age from six months to seventy years, 38 under five years. All strains were identified biochemically and confirmed by slide agglutination using group- and type-specific Shigella antisera (Lanzhou Institute of Biological Products, Lanzhou, China).

Antimicrobial susceptibility

The MICs of antimicrobial agents were determined by the agar dilution method, according to the guidelines established by the CLSI (Clinical Laboratories Standards Institute). The following antimicrobial agents were tested: ampicillin (AMP); tetracycline (TET); streptomycin (STR); chloramphenicol (CHL); ciprofloxacin (CIP); levofloxacin (LEV); gentamicin (GEN); sulfamethoxazole-trimethoprim (SXT) and cefazolin (CFZ) (National Institute for Food and Drug Control, Beijing, China). *Escherichia coli* ATCC 25922 was used for quality control strain.

DNA template

Total genomic DNA was extracted by boiling methods. Five bacterial colony from Luria-Bertani (LB) plate was suspended in 1.0 ml of phosphate-buffered saline (PBS), centrifuged, and the cell pellet resuspended in 100 µl of TE buffer (10 mM Tris, 1 mM EDTA buffer, pH 8.0) and boiled at 99°C for 5 min. Plasmid DNA were extracted from strains with TIANprep Mini Plasmid kit (Tiangen Biotech, Beijing, China).

PCR and DNA sequencing

Classes 1, 2 and 3 integrase and gene cassettes were detected among the 53 *S. flexneri* strains. The primer pairs used for detecting the integrase and the variable regions of the integrons were shown in Table 1. The 50 µl PCR reaction mix consisted of 2.5 mM $MgCl_2$, 25 μM primers, 200 μM (each) dNTP, 1.25 U *Taq* DNA polymerase (TaKaRa Biotech, Dalian, China) and about 30 ng templates DNA. After predenaturation at 94°C for 3 min, there were 12 cycles of 94°C for 60 s, 68 ~ 55°C for 60 s with degradation of 1°C per cycle and 72°C for 3 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 3 min, with a final extension at 72°C for 5 min. PCR products for gene cassettes of class 2 integrons and atypical class 1 integrons with similar length were examined by restriction fragment length polymorphism (RFLP) analysis with *Hinf*I and *Hind*III (TaKaRa Biotech, Dalian, China). The identical restriction profiles were regarded as the same array of gene cassettes. Purified PCR products were sequenced on an ABI Prism automatic sequencer, as recommended by the manufacturers; the nucleotide sequences were compared online at the National Center for Biotechnology Information (NCBI) website.

Conjugation analysis

Conjugation analyses were carried out to determine whether the integron of *S. flexneri* was on conjugative plasmids and whether the resistance genes could be transferred. Rifampicin-resistant (RifR) and sulfamethoxazole-susceptible (SulS) *E. coli* K12 was used as recipient. All 48 integron-carrying isolates were used as donor strains. Both donor and recipient bacteria were cultured in LB broth until OD660 reached about 0.6. Then 0.1 ml each of the donor and recipient cultures were mixed in 1 ml of fresh LB broth, incubation took place overnight at 37°C. Transconjugants were selected by plating the mating culture on MacConkey agar plates containing both rifampicin (50 µg/ml) and sulfamethoxazole (512 µg/ml). Colonies were selected based on their resistance to both antimicrobials and purified by subculture on MacConkey agar containing antibiotics and then nutrient agar without antibiotics. The transconjugants were tested as described earlier for the presence of integrons.

Pulsed field gel electrophoresis

PFGE was carried out according to a previous protocol (Ribot et al., 2006). DNA was digested with the restriction enzyme *Not*I (TaKaRa Biotech, Dalian, China) at 37°C. The restriction fragments were separated by electrophoresis in 0.5× TBE buffer, for 18.5 h at 14°C in a CHEF Mapper system (Bio-Rad, U.S.A.) using pulsed times of 5 to 35 s. *Xba*I-digested *Salmonella braenderup* H9812 was used as the DNA marker. PFGE data were analyzed using BioNumerics software (version 6.0, Applied Maths).

RESULTS

Antimicrobial susceptibility

All 53 strains except for five completely susceptible to all antimicrobials were resistant to at least four of the following antibiotics: ampicillin, tetracycline, sulfamethoxazole-trimethoprim, streptomycin and chloramphenicol. 3 (5.7%) isolates were resistant to gentamicin, 16 (30.2%) to cefazolin and levofloxacin, 18 (34.0%) to ciprofloxacin, 33 (62.3%) to trimethoprim-sulfamethoxazole, 46 (86.8%) to chloramphenicol, 47 (88.7%) to tetracycline, 48 (90.6%) to ampicillin and streptomycin. The main resistance phenotypes and characteristics of integrons of *S. flexneri* strains are shown in Table 2.

Table 1. The sequence, location and annealing temperature of the PCR primers.

Primer	Sequence (5′→3′)	Location	Product size	Annealing temp (°C)	Reference
intI1F	ACATGTGATGGCGACGCACGA	intI1	569	50	Ploy et al. (2000)
intI1R	ATTTCTGTCCTGGCTGGCGA	intI1			
inF	GGCATCCAAGCAGCAAGC	5′-cs of class 1 integron	varied	52	Dalsgaard et al. (2000)
inR	AAGCAGACTTGACCTGAT	3′-cs of class 1 integron			
qacEΔ1	ATCGCAATAGTTGGCGAAGT	qacEΔ1	798	52	Dalsgaard et al. (2000)
sul1	GCAAGGCGGAAACCCGCGCC	sul1			
intI1ca	CGTAGAAGAACAGCAAGG	intI1	varied	52	Pan et al. (2006)
IS1ca	AGTGAGAGCAGAGATAGC	IS*1*			
intI2F	GTAGCAAACGAGTGACGAAATG	intI2	789	51	Ploy et al. (2000)
intI2R	CACGGATATGCGACAAAAAGGT	intI2			
intI3F	GCCTCCGGCAGCGACTTTCAG	intI3	980	55	Ploy et al. (2000)
intI3R	ACGGATCTGCCAAACCTGACT	intI3			
intI2ca-F	CGGGATCCCGGACGGCATGCACGATTTGTA	intI2	varied	55	White et al. (2001)
intI2ca-R	GATGCCATCGCAAGTACGAG	3′-cs of class 2 integron			

Table 2. Characteristics of *Shigella* strains: Resistance phenotypes, cassette arrays, and PFGE profiles.

Resistant phenotypes	5′-conserved segement (*intI* genes) (No.)	Gene cassettes/PFGE profiles
		Class 1 integron, Atypical class 1 integron, Class 2 integron
AHTR (n = 6)	*intI1* (01)	bla_{OXA-30}-*aadA1* (E)
	intI1, intI2 (05, 06, 07, 08, 36)	*dfrA1-sat1-aadA1*, bla_{OXA-30}-*aadA1*(E)
AHRS (n = 1)	*intI2* (34)	*dfrA1-sat1-aadA1* (E)
ATRS (n = 2)	*intI1, intI2* (02, 04)	*dfrA1-sat1-aadA1*,*dfr V* (E)
AHTRS (n = 11)	*intI1 (50)*	bla_{OXA-30}-aadA1(B)
	intI1, intI2 (03, 09, 35, 37)	*dfrA1-sat1-aadA1*, bla_{OXA-30}-*aadA1*,*dfrV*(E)
	intI1, intI2 (49)	*dfrA1-sat1-aadA1*, bla_{OXA-30}-*aadA1*(A)
	intI1, intI2 (25, 26, 51)	*dfrA1-sat1-aadA1*, bla_{OXA-30}-*aadA1*(B)
	intI1, intI2 (17,24)	*dfrA1-sat1-aadA1*, bla_{OXA-30}-*aadA1*(C)
AHTRSC (n = 9)	*intI1, intI2* (13,39,40,41,42,54,56,58,62)	*dfrA1-sat1-aadA1*, bla_{OXA-30}-*aadA1*(D)
AHTRPL (n = 9)	*intI1, intI2* (14,15,16,18,19,20,21,22,23)	*dfrA1-sat1-aadA1*, bla_{OXA-30}-*aadA1*(C)
AHTRSPL (n = 1)	*intI1, intI2* (48)	*dfrA1-sat1-aadA1*, bla_{OXA-30}-*aadA1*(C)
AHTRSCG (n = 1)	*intI1, intI2* (53)	*dfrA1-sat1-aadA1*, bla_{OXA-30}-*aadA1*(D)
AHTRSPG (n = 2)	*intI1, intI2* (30,32)	*dfrA1-sat1-aadA1*, *dfrA17-aadA5,* bla_{OXA-30}-*aadA1*(A)
AHTRSCPL (n = 6)	*IntI1, intI2* (27,28,29,31,33,38)	*dfrA1-sat1-aadA1*, bla_{OXA-30}-*aadA1*(C)

*A, ampicillin; C, cefazolin; H, chloramphenicol; T, tetracycline; S, sulfamethoxazole- trimethoprim; R, streptomycin; G, gentamicin; L, levofloxacin; P, ciprofloxacin.

Integrases genes and gene cassette arrays

Forty-eight (90.5%) strains contained at least one integron. The *intI1* and *intI2* genes were detected in 48 and 46 strains respectively, they were found together in 46 strains, no *intI3* gene was detected. Among 53 *S. flexneri* isolates, 8 (15.1%) strains were positive with three primer pairs (intI1F-intI1R, inF-inR, qacE⊿1-sul1), the integron with 5′-conserved segment and 3′-conserved segment was nominated as typical class 1 integron. Inversely, with 5′-conserved segment but no 3′-conserved segment called as atypical class 1 integron (Pan et al., 2006). Two gene cassette arrays of typical class 1 integron, *dfrA17-aadA5* and *dfrV*, were identified in eight *S. flexneri* strains. The gene cassettes were also detected in the corresponding transconjugants.

In addition, we found the other 40 *intI1* positive strains were negative for the 3′-conserved segment. The variable regions of atypical class 1 integron were detected with the primer pair intI1ca-IS1ca in 48 *intI1* gene-positive strains. The amplicons of the expected size (2.4 kb) were observed for 45 isolates, six of these isolates carried both typical class 1 integrons and atypical class 1 integrons, 3 *intI1* positive isolates carried no any gene cassette. The gene cassettes of atypical class 1 integrons shared the same RFLP profile with *Hind*III were identified as bla_{OXA-30}-*aadA1* by sequencing.

The results indicated that all the atypical class 1 integrons carried the same gene cassettes bla_{OXA-30}-*aadA1*. The positive amplification result of total genomic DNA and negative for plasmids and transconjugants confirmed that the atypical class 1 integrons was located on the chromosome.

The class 2 integrons of all strains were consistent. All 46 *intI2* positive strains carrying *dfrA1-sat1-aadA1* gene cassettes were identified by the same RFLP profile with the restriction enzyme *Hinf*I and DNA sequencing. *Intl2* gene of total genomic DNA and plasmid were amplified with specific primers by PCR. *Intl2*–positive results only in total genome DNA revealed that class 2 integrons were localized on chromosomes.

Distribution of integrons in PFGE types

All the 53 integron carrying strains subtyped into 27 pulsotypes by PFGE of *Not* I-digested chromosomal DNA. Five major clusters (A, B, C, D and E) were typed by Cluster analysis based on 85% similarity cut off point (Blanco et al., 2009) (Figure 1). Cluster A consisted of three strains, two of them habouring gene cassettes *dfrA17-aadA5*. Cluster E consisted of 13 strains, including six strains habouring gene cassettes *dfrV*. Cluster C was the predominate clone; all the strains carried the gene cassettes bla_{OXA-30}-*aadA1* and *dfrA1-sat1-aadA1*. The characteristic of integrons and PFGE clusters were shown in Table 2.

DISCUSSION

A high percentage of the *S. flexneri* isolates were resistant to ampicillin, trimethoprim- sulfamethoxazole, tetracycline, chloramphenicol and streptomycin. These antimicrobials were used as first-line therapy in diarrhea and other infectious diseases, but now it is hardly effective against *Shigella* spp. infection (Rosewell et al., 2010; Zhu et al., 2011). With quinolone and cephalosporin used to treat diarrhea, the resistance to them was revealed (Bhattacharya et al., 2011; Rahman et al., 2007). In present study, the resistance rate of ciprofloxacin was up to 34.0% and resistant to cefazolin to 30.2%, which are considered to be an indication of reduced susceptibility. In order to ensure appropriate treatment for shigellosis, continual surveillance is required to determine which antibiotics are still active.

In enterobacteriaceae, class 2 integron have been reported mainly in *Shigella* with high percentage (Gassama-Sow et al., 2006; Ranjbar et al., 2007), and the gene cassettes of class 2 integron were relatively conservative. Up to now, it was reported as two types of cassette arrays, one as *dfrA1-sat1-aadA1*, and the other without *aadA1* and/or the *orfx* (Jin et al., 2010; Gassama-Sow et al., 2010). In our study, 86.8% (46/53) isolates were found to harbour *dfrA1-sat1-aadA1* gene cassette in class 2 integron, encoding resistance to trimethoprim and streptomycin.

Class 1 integrons were relatively diversity. 15.1% (8/53) strains carried typical class 1 integrons with *dfrV* (6/8) and *dfrA17-aadA5* (2/8) gene cassettes. In enterobacteriaceae, the *dfrV* gene cassette encoding the resistance to trimethoprim has been reported only in China in *S. flexneri* (Pan et al., 2006). The gene cassette *dfrA17-aadA5* encoding the resistance to trimethoprim and streptomycin was a common array in class 1 integron not only in the *Shigella* (Pan et al., 2006; Zhu et al., 2011), but also in *E. coli* (Ben Slama et al., 2011) and *S. enteric* (Kim et al., 2011). But the gene cassette *dfrA17-aadA5* in *Shigella* was found only in China, it might be the popular resistant gene which could be captured by class 1 integron in our country. Since the typical class 1 integrons was confirmed locating on the conjugative plasmid, it was more inclined to transfer the antimicrobial resistant genes, and the transfer can take place among *Shigella* spp. and/or other bacteria.

The atypical class 1 integeron was first reported on the SRL PAI (the *Shigella* resistance locus, pathogenicity island) of the chromosome of *S. flexneri* 2a strain YSH6000 (Luck et al., 2011). It was adjacent to two resistance determinants of chloramphenicol and tetracycline. These findings may explain why most bla_{OXA-30}-*aadA1* positive strains were resistant to three antibiotics (AMP, TET, and CHL) while the others were not resistant to triple antibiotics (Table 2). Furthermore, atypical class 1 integrons always showed carrying the gene cassettes bla_{OXA-30}-*aadA1* when the insertion

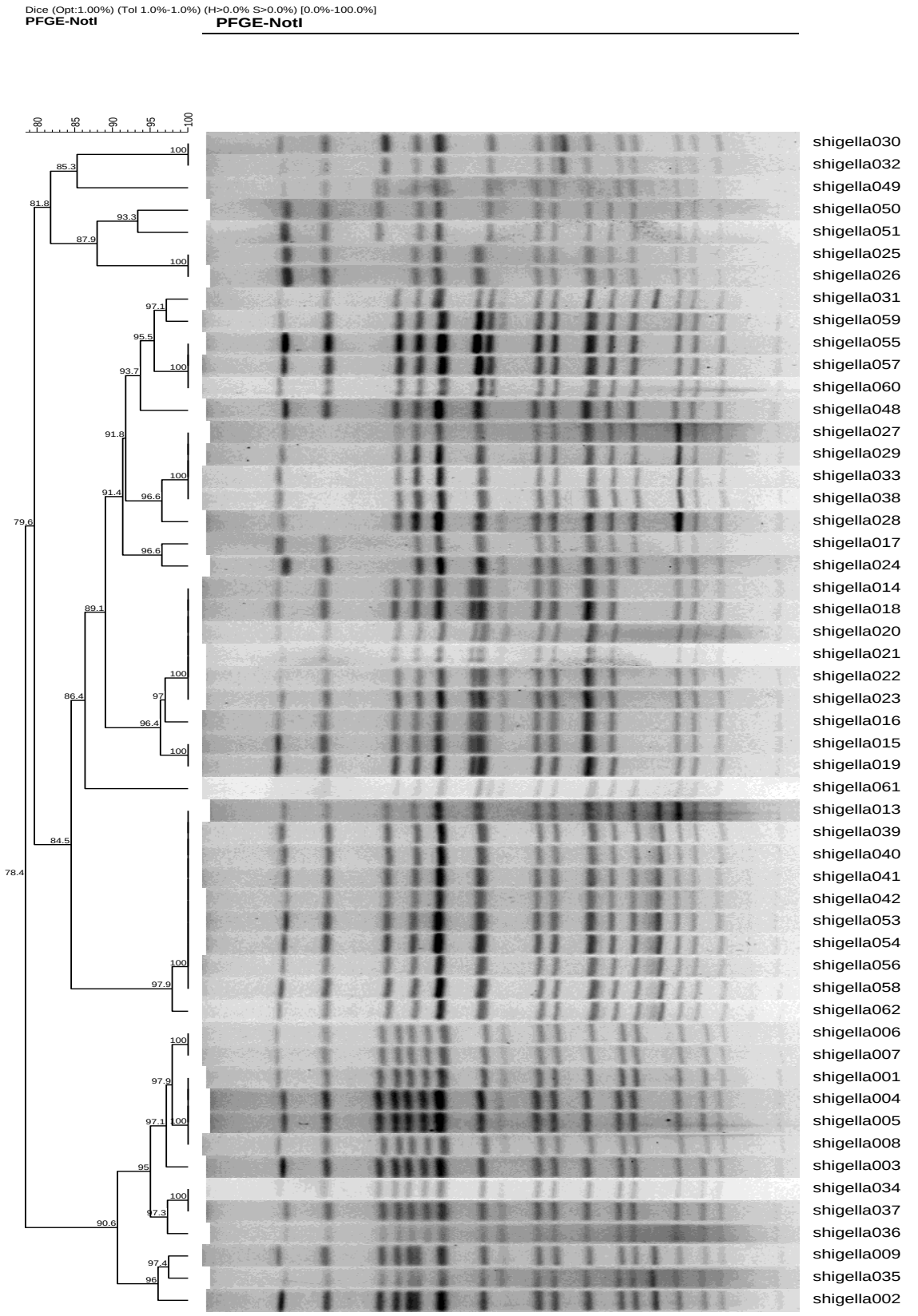

Figure 1. PFGE of *Not*I-digested DNA from 53 *Shigella flexneri* isolates, the scale above the dendrogram indicates percentage similarity using BioNumerics software (Applied Maths). Using a 85% similarity cut-off point, PFGE identified 5 clusters (A, B, C, D, E).

sequence IS*1* at the 3' end instead of the typical 3'-conserved segment (Dubois et al., 2007; Gassama-Sow et al., 2010; Pan et al., 2006; Zhu et al., 2011). Among 45 strains with atypical class 1 integrons, typical and atypical class 1 integrons coexisted in 6 isolates. This is the second report of the coexistence in *Shigella*. While in the first report the coexistence took place in all bacteria used to study strains (Zhu et al., 2011). The difference may be related with the isolates; in our study, the typical class 1 integron was detected with lower percentage than that of previous study (Zhu et al., 2011), so the coexistence probability was relatively lower. The atypical class 1 integron has been found to be followed by part of IS*1* (Pan et al., 2006). It is known that two copies of the IS element flanking the gene can make it mobile. Strains containing atypical class 1 integron related with IS element and typical class 1 integron located on conjugatable plasmid may have more opportunity to acquire and transfer resistance under antibiotic selection pressure. In this study, the coexistence isolates is so limited that from the resistance phenotype, we could not find the significant differences between coexistence and non-coexistence isolates. It remains unclear whether the interaction of typical and atypical class 1 integrons would enhance the resistance and how they obtain the coexistence, and further exploration is needed.

It seemed that no closely relationship was found between the resistance phenotypes, integrons distribution and *Not*I–pulsotypes for all strains (Table 2). But among every cluster, the PFGE profiles were associated with similar resistance phenotypes and the distribution of integrons. These data suggest a clonal relatedness of these *S. flexneri* strains, especially those strains which had the same serotype and isolated from the same locality. The similar result in strains of *Shigella* spp. has been reported previously in Japan (Ahmed et al., 2006).

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