Study on the association between drug-resistance and gene mutations of the active efflux pump *acrAB-tolC* gene and its regulatory genes

QUAN-PING MA[1*], LIANG SU[2,3*], JING-WEN LIU[2,3], MING-XIAO YAO[4] and GUANG-YING YUAN[2,3]

[1]Department of Clinical Laboratory, Jinan Fourth People's Hospital, Jinan, Shandong 250031; [2]Department of Clinical Laboratory, Jinan Infectious Disease Hospital Affiliated to Shandong University, Jinan, Shandong 250021; [3]Collaborative Innovation Center for The Origin and Control of Emerging Infectious Diseases, Taishan Medical University, Taian, Shandong 271016; [4]Bacteria Laboratory, Shandong Center for Disease Control and Prevention, Shandong 250014, P.R. China

Received July 20, 2016; Accepted December 22, 2017

DOI: 10.3892/mmr.2018.8916

Abstract. The aim of the present study was to investigate the correlation between the multi-drug resistance of *Shigella flexneri* and the drug-resistant gene cassette carried by integrons; in the meanwhile, to detect the associations between drug-resistance and gene mutations of the active efflux pump *acrAB-tolC* gene and its regulatory genes, including *marOR*, *acrR* and *soxS*. A total of 158 isolates were isolated from the stool samples of 1,026 children with diarrhoea aged 14 years old between May 2012 and October 2015 in Henan. The K-B method was applied for the determination of drug resistance of *Shigella flexneri*, and polymerase chain reaction amplification was used for class 1, 2 and 3 integrase genes. Enzyme digestion and sequence analysis were performed for the variable regions of positive strains. Based on the drug sensitivity assessment, multi-drug resistant strains that were resistant to five or more antibiotics, and sensitive strains were selected for amplification. Their active efflux pump genes, *acrA* and *acrB*, and regulatory genes, *marOR*, *acrR* and *soxS*, were selected for sequencing. The results revealed that 91.1% of the 158 strains were multi-resistant to ampicillin, chloramphenicol, tetracycline and streptomycin, and 69.6% of the strains were multi-resistant to sulfamethoxazole/trimethoprim. The resistance to ceftazidime, ciprofloxacin and levofloxacin was <32.9%. All strains (100%) were sensitive to cefoxitin, cefoperazone/sulbactam and imipenem. The rate of the class 1 integron positivity was 91.9% (144/158). Among these class 1 integron-positive strains, 18 strains exhibited the resistance gene cassette *dfrV* in the variable region of the strain, four strains exhibited *dfrA17-aadA5* in the variable region and 140 strains exhibited *bla*OXA-30-*aadA1* in the variable region. Four strains showed no resistance gene in the variable regions. The rate of class 2 integron positivity was 86.1% (136/158), and all positive strains harboured the *dfrA1-sat1-aadA* resistance gene cassette in the variable region. The class 3 integrase gene was not detected in these strains. The gene sequencing showed the deletion of base CATT in the 36, 37, 38, 39 site in the *marOR* gene, which is a regulatory gene of the active efflux pump, *AcrAB-TolC*. Taken together, the multi-drug resistance of *Shigella flexneri* was closely associated with gene mutations of class 1 and 2 integrons and the *marOR* gene.

Introduction

Shigella is a genus of pathogens responsible for acute bacterial diarrhea, chronic bacteritic dysentery and toxic dysentery. Certain *Shigella* strains can cause hemolytic uremic syndrome and Reiter's chronic arthritis syndrome, and with the extensive use of antibacterial agents, bacterial drug-resistance is becoming a serious concern. Mobile genetic elements, including plasmids, transposons and integrons are important in the spread of antibiotic resistance (1). Among these elements, class 1, 2 and 3 integrons are associated with drug resistance. A previous study showed that the involvement of the active efflux pump is significant in bacterial multi-drug resistance (2). Previous studies have mainly focused on the active efflux pump of *Escherichia coli,* AcrAB-TolC, which is also expressed in *Shigella* striains (3). The regulation of gene mutation may be important in the multi-drug resistance mediated by the gene (4). In the present study, the characteristics of integron genes were analyzed in clinical *Shigella* isolates from children, mutations of the active effiux pump gene (*acrAB-tolC*) and regulatory genes were examined, and the association between these genes and the drug-resistance of *Shigella* was investigated. This may provide evidence for the

Correspondence to: Dr Guang-Ying Yuan, Department of Clinical Laboratory, Jinan Infectious Disease Hospital Affiliated to Shandong University, 22029 Jingshi Road, Jinan, Shandong 250021, P.R. China
E-mail: yuanguangyin_06@163.com

*Contributed equally

Key words: *Shigella*, drug resistance, integrons, active efflux pump acrAB-tolC, marOR genes

clinical treatment of dysentery and the control of multi-drug resistance of *Shigella*.

Materials and methods

Location of isolated strains. A total of 158 isolates of *Shigella flexneri* were isolated from the stool samples of 94 male cases and 64 female cases aged from 6 months to 14 years old between May 2012 and October 2015, in which there were 106 cases aged less than 5 years old. The isolates were identified using ID32E identificational strips and an ATB expression instrument, and were serotyped using diagnostic-serum of *Shigella*. *Escherichia coli* (cat. no. 25922; American Type Culture Collection) was preserved in the Clinical Microbiology Laboratory of The Fourth Hospital of Jinan City (Jinan, China) and it was the quality control strain for antimicrobial susceptibility assessment. The present study was approved by the Ethics Committee of Jinan Infectious Disease Hospital Affiliated To Shandong University (Shandong, China). Written informed consent was obtained from all patients or patient guardians for the present study.

Reagents and equipment. The following were obtained for use in the present study: *Shigella* diagnostic serum (Lanzhou Institute of Biological Products Co. Ltd., Lanzhou, China); antibotic discs of ampicillin, chloramphenicol, tetracycline, streptomycin, trimethoprim, sulfamethoxazole, ciprofloxacin, cefotaxime, ceftazidime, cefoperazone/sulbactam and imipenem, and M-H agar (Oxoid; Thermo Fisher Scientific, Inc., Waltham, MA, USA); 100 bp DNA Ladder marker and D15000+2000 DNA marker (Tiangen Biotech Co., Ltd., Beijing, China); Takara *Taq* kit, DL2000TMDNA marker and restriction enzyme (Takara Biotechnology Co., Ltd., Dalian, China), agarose (Invitrogen; Thermo Fisher Scientific, Inc.); bacterial identification instrument (ATB Expression; BioMérieux, Marcy l'Etoile, France); DNA amplifier (Biometra GmbH, Göttingen, Germany); electrophoresis apparatus (10C type; Beijing Liuyi Biotechnology Co., Ltd., Beijing, China); biosafety cabinets (1200IIA2; Shanghai Lishen Biotechnology Co., Ltd.). Casein hydrolysate acid (M-H) agar (Shanghai Lishen Biological Technology Co., Ltd.).

Strain identification and antimicrobial susceptibility assessment. The preserved strains were removed from the -86˚C ultra-low temperature freezer, thawed at room temprature and re-identified. Subsequently, the strains underwent an agglutination test with *Shigella* diagnostic serum for the serotype identification. In total, 3-4 bacterial colonies of *S. flexneri* were detected following culture of the stains for 16-18 h at 35˚C, followed by adjustment of the turbidity instrument to 0.5 McF with normal saline. Sterile cotton swabs were dipped into the bacteria solution and excess liquid was squeezed out against the tube wall, followed by application of the bacteria onto the M-H plate. The bacteria were applied ontp the M-H plate three times with a 60˚ rotation; the bacteria were also applied along the edge for entire 360˚ rotation. Following 5 min, the antimicrobial susceptibility disks were placed on the colonies and cultured for 16-18 h at 35˚C. Following these procedures, the inhibition zone diameter was detected with vernier calipers and the data were input into WHONET 5.4 software (http://www.whonet.org/). According to the 2010 version of Performance Standards for Antimicrobial Susceptibility Testing of the National Committee for Clinical Standards Laboratory Institute) (5), the quality control strains was *Escherichia coli* (cat. no. 25922).

Assessment and sequencing for resistance gene cassettes of integrons and variable regions. The integron and plasmid DNA templates were prepared using a boiling method for 5 min, and they were preserved in a -20˚C refrigerator. The primers and primer sequences were synthesized in the accordance with relative reports (6-9), as in Table I. The class 1, 2 and 3 integrons, integrase primers and variable region primers were synthesized by Takara Biotechnology Co., Ltd. The reaction system was as follows: 10X buffer containing Mg^{2+} (5 μl), dNTP (4 μl; 2.5 mmol/l), downstream and upstream primers (2.5 μl; 10 μmol/l of each), Taq eznzyme (0.25 μl; 5 U/μl) and DNA templates (5 μl). Deionized water was added to the reaction system to 50 μl. The amplification parameters for integrase were as follows: Pre-denaturation at 94˚C for 5 min, followed by 94˚C for 30 sec, annealing for 30 sec (annealing temperature shown in Table I) and 72˚C for 40 sec, for 35 cycles, with final extension at 72˚C for 5 min. The amplification parameters for variable regions were as follows: Pre-denaturation at 94˚C for 5 min; touchdown polyermase chain reaction (PCR) for 10 cycles, annealing temperature reduction from 60 to 50˚C (temperature decrease by 1.4˚C each cycle), followed by 94˚C for 30 sec, 52˚C for 30 sec and 72˚C for 180 sec, for 25 cycles with final extension at 72˚C for 8 min. For product analysis, the amplification product (10 μl) was used for 1% agarose electrophoresis under 120 V for 20 min. Following EB staining, the products were observed under the UV absorption spectra analyzer. Images were captured and the results recorded. The amplification products were sent to Takara Biotechnology Co., Ltd. for gene sequencing and the sequences were compared with NCBI/BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

BamHI restriction enzyme digestion. Enzyme digestion was performed in the variable regions of the atypical class 1,2 integrons with the restriction enzyme *Hind*III and *Hinf*1. The specific steps were as follows: 10 μl enzyme buffer, 40 μl PCR products and 5 μl restriction enzyme were added to deionized water to 100 μl. The solution was placed at 37˚C for the isothermal reaction for ~1 h. Subsequently, 10X loading buffer was added to terminate the reaction. The 10-μl samples were used for 2.5% agarose electrophoresis under 120 V for 20 min. Following EB staining, the products were observed under the UV absorption spectra analyzer. Images were captured and the results recorded.

Analysis of acrAB-tolC and its associated regulatory genes. The multi-drug resistant *Shigella* strains (resistant to five or more antibiotics) and sensitive strains were used for assessment. The PCR amplication and sequencing methods were applied. The specific steps were in accordance with the above methods. According to previous reports (10,11), the experimental primers were synthesized by Takara Biotechnology Co., Ltd. (Table II). The sequences were compared with NCBI/BLAST to investigate the numbers of mutations and mutational sites.

Table I. Primers of genes associated with class 1 integron.

Primer	Sequence (5'-3')	Primer sequence site
intI1 F	ACATGTGATGGCGACGCACGA	*intI1*
intI1 R	ATTTCTGTCCTGGCTGGCGA	*intI1*
In F	GGCATCCAAGCAGCAAGC	5'-conserved segment in the class 1 integrons
In R	AAGCAGACTTGACCTGAT	3'-conserved segment in the class 1 integrons
qacEΔ1	ATCGCAATAGTTGGCGAAGT	*qacEΔ1*
sul1	GCAAGGCGGAAACCCGCGCC	*sul1*
intI1ca	CGTAGAAGAACAGCAAGG	*intI*1
IS1ca	AGTGAGAGCAGAGATAGC	IS1
intI2 F	GTAGCAAACGAGTGACGAAATG	*intI2*
intI2 R	CACGGATATGCGACAAAAAGGT	*intI2*
intI2ca F	CGGGATCCCGGACGGCATGCACGATTTGTA	*intI2*
intI2ca R	GATGCCATCGCAAGTACGAG	3'-conserved segment in the class 2 integrons
intI3 F	GCCTCCGGCAGCGACTTTCAG	*intI3*
intI3 R	ACGGATCTGCCAAACCTGACT	*intI3*

F, forward; R, reverse.

Table II. Active efflux pump *acrAB-tolC* and its regulatory genes.

Primer	Sequence (5'-3')	Length (bp)	Annealing temperature (˚C)
gyrA	TACACCGGTCAACATTGACG TTAATGTTGCCGCCGTCGG	648	50
parC	GCGTTGCCGTTTATTGGTGAT TGGACATCGTCATACCTCT	469	52
acrA	TGCGGCTTGCTGGTTATT GCGGTCGTTCTGATGCTC	1,131	52
acrB	GATTCCGACCATTGCCGTAC GCCAGAATACCGCCTACGC	510	52
marOR	CACTCTTTAGCTAGCCTTG TGGACATCGTCATACCTCT	604	51
acrR	AAACCCATTGCTGCGTTTAT AAACCGCAAGAATATCACGA	800	55

Results

Results of drug sensitivity tests. A total of 91.1% of the 158 strains were multi-resistant to ampicillin, chloramphenicol, tetracycline and streptomycin, and 69.6% of the strains were multi-resistant to sulfamethoxazole/trimethoprim. The resistance rate to ceftazidime, ciprofloxacin and levofloxacin was <32.9%. All (100%) of the strains were sensitive to cefoxitin, cefoperazone/sulbactam and imipenem. The frequent resistant pattern (ampicillin-tetracycline-chloromycetin-streptomycin) accounted for 91.1% of strains (144/158), and 77.2% (122/158) of the strains were resistant to more than five antibiotics. Multi-drug resistance was a serious problem. Only 14 strains were sensitive strains, which were not drug-resistant (Table III).

Integration assessment results. Among the 158 *S. flexneri* strains, 144 strains were *intI1*-positive and 136 strains were intI 2-positive. The positive rates were 91.1% (144/158) and 86.1% (136/158), respectively (Figs. 1 and 2). All strains were *intI3*-positive. In total, 10 strains were single *ntI1*-positive, accounting for 6.3% (10/158), and 134 strains were *intI1-intI2* positive, accounting for 84.8% (134/158). Among the *intI1*-positive strains, the 3'-conserved segment of 16.5% of the strains (26/156) were positive. They were typical class 1 integrons [*intI1*, (conservative primer of class 1 integrons) *in* and *qacEΔ1-sul1*-positive; Fig. 3]. The 3'-conserved segment of 84.8% (134/158) of the strains were negative. They were atypical class 1 integrons (*intI1* and *intI1-IS1*-positive; Fig. 4. In addition, 10.1% (16/158) strains (*S. flexneri* 2A) possessed typical and atypical class 1 integron class 1 integrons. The integron assessment results are shown in Table III.

Assessment and sequencing for resistance gene cassettes of integrons and variable regions. Among the 26 strains

Table III. Results of class 1 and class 2 integron detection in 79 strains of *Shigella flexneri*.

Serotype	Typical class 1 integron (*intI1*, *in*, *qacEΔ1-sul1*-positive)	Atypical class 1 integron (*intI1*, *intI1-IS1*-positive)	Class 2 integron (*intI2*, *intI2ca*-positive)
S. flexneri 1a	0	16	14
S. flexneri 2a	20	70	74
S. flexneri 2b	2	10	10
S. flexneri 4a	4	34	34
S. flexneri x variant	0	4	4
Total	26	134	136

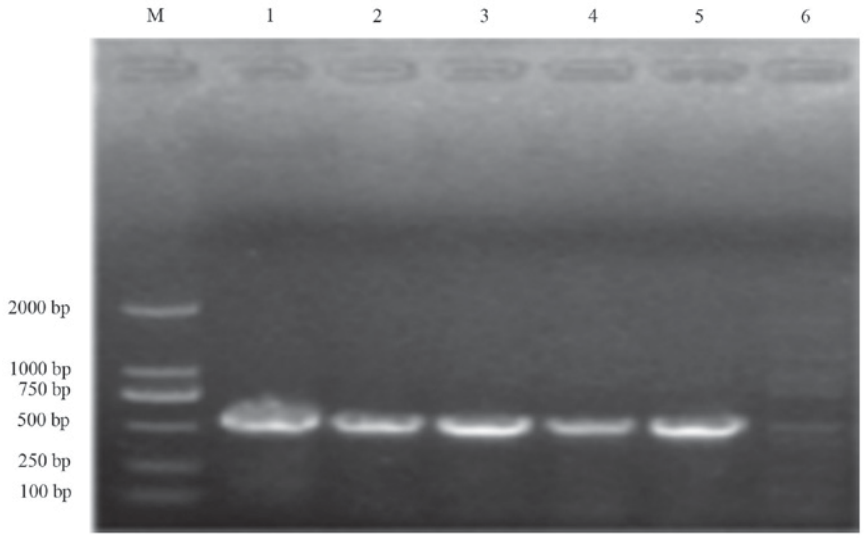

Figure 1. Region of the class 1 integrase polymerase chain reaction electropherogram. M, DNA marker; 1-6 are the specimen numbers.

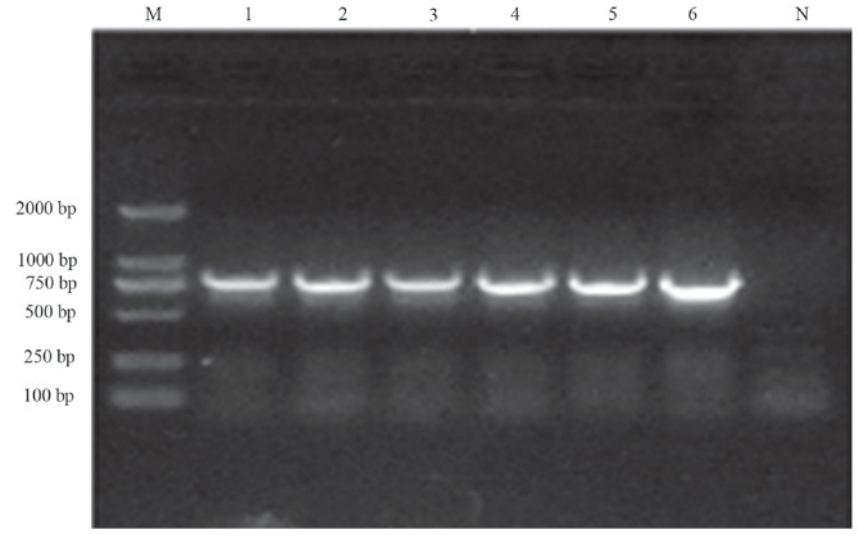

Figure 2. Region of the class 2 integrase polymerase chain reaction electropherogram. M, DNA marker; N, Negative control; 1-6 are the specimen numbers.

with typical class 1 integrons, PCR amplification of the variable regions in 22 strains yielded two types of fragment. The sizes were ~1.6 kb and 0.7 KB, respectively (Fig. 3). Confirmed by the gene sequencing, four *S. flexneri* 4a strains carried the *dfrA17-aadA5* (1,624 bp) drug-resistant gene cassette, which was the dihydrofolate reductase gene, and aminoglycoside acyltransferase gene. A total of 18 *S. flexneri* 2a strains carried the *dfrV* (729 bp) drug-resistant gene cassette, which was the dihydrofolate reductase gene and aminoglycoside acyltransferase gene. The PCR amplification

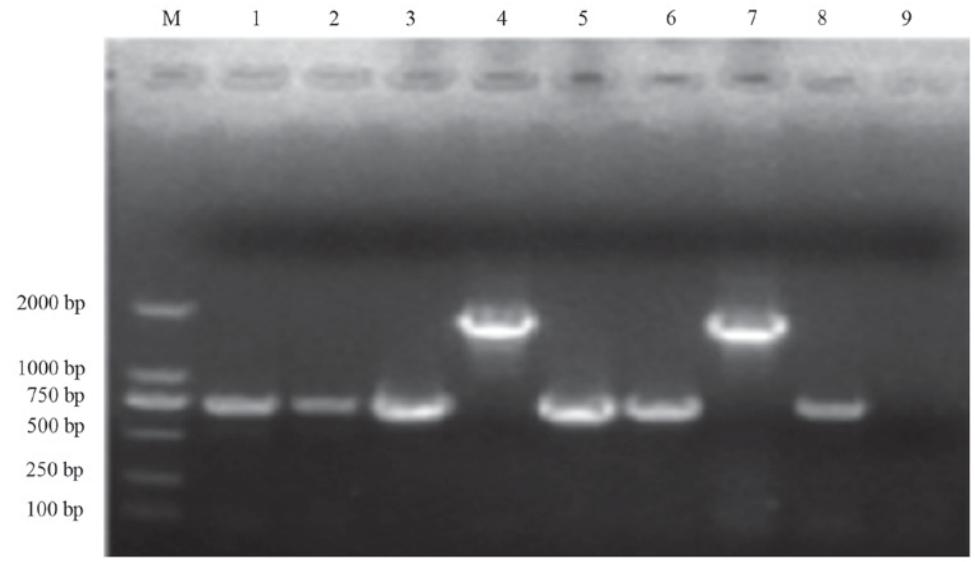

Figure 3. Region of the polymerase chain reaction amplification electrophoretogram of typical class 1 integron variable region. M, DNA marker; 1-8 are specimen numbers.

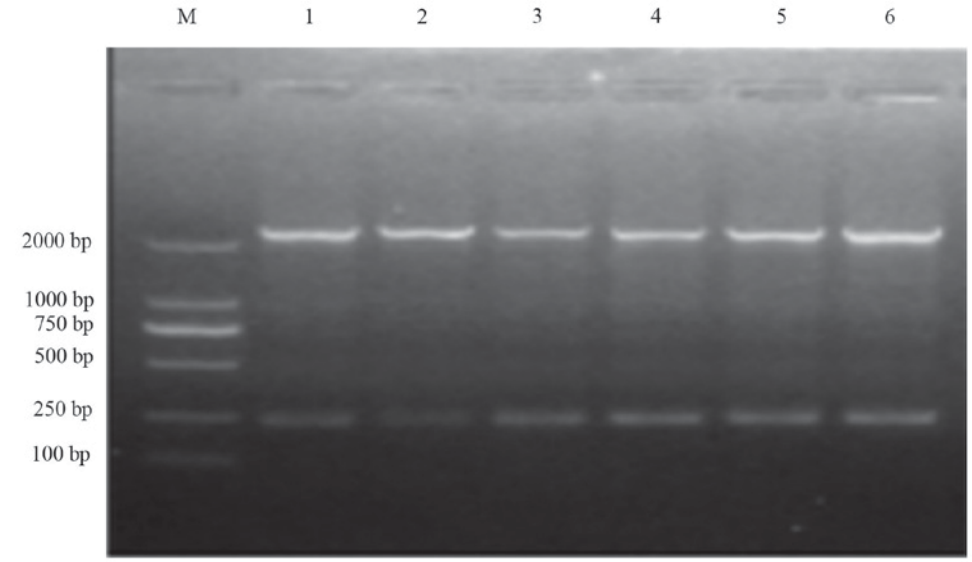

Figure 4. Region of polymerase chain reaction amplification electrophoretogram of atypical class 1 integron variable region. M, DNA marker. 1-6 are the specimen numbers.

of four stains yielded nothing suggest an empty cassette. The assessment of antibiotic susceptibility showed that the drug resistance of pathogens to ampicillin and other drugs was severe, suggesting that the drug resistance was associated with the insertion of a 3' conservative region into the C-terminal conservative region (9). The variable regions of 134 *S. flexneri* strains were amplified with the *intI1ca-IS1ca* primer. A total of 134 strains yielded 2.4 kb fragments, as shown in Fig. 4. The products had the same enzymatic maps following *Hind*III enzyme digestion. Four strains were randomly selected for sequencing. The carried drug resistant gene cassette was *bla0XA-30-aadA1* (2,453 bp), which was the β-lactamase and aminoglycoside adenosine acyltransferase gene. A total of 134 strains with atypical class 1 integron were resistant to ampicillin, streptomycin, tetracycline and chloromycetin. Only 10 of the 26 strains without atypical class 1 integron were resistant to more than four antibiotics. There was a significant difference between two groups (χ^2=35.96, P<0.01). The detailed results of the resistance gene in class 1 integron variable regions are shown in Table III.

Resistance gene cassette assessment and sequencing in variable regions of integrons. The variable region of integrons in the 136 strains was amplified by integron PCR. Following electrophoresis, all the strains yielded 2.2 kb fragments (Fig. 5). Following *hinf*1 digestion, the amplification products manifested the same characteristic bands in electrophoresis. Verified by gene sequencing, the drug-resistant gene carried by bacteria was the *dfrA1-sat1-aadA1* gene (2,224 bp), which was responsible for the drug resistance to trimethoprim, streptomycin and aminoglycoside. The results

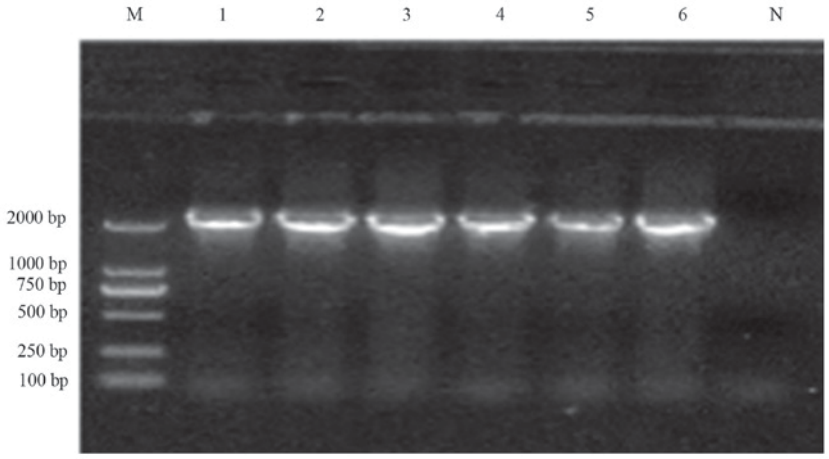

Figure 5. Region of polymerase chain reaction amplification electrophoretogram of class 2 integron variable region. M, DNA marker; N, Negative control; 1-6 are the specimen numbers.

of the resistance gene in class 2 integron variable regions are shown in Table IV.

Analysis of acrAB-tolC and its associated regulatory genes in multi-drug resistant Shigella. There were 12 strains with gene deletions among the 122 multi-drug resistant strains and two strains with gene deletions in 17 sensitive strains. Therefore 110 multi-drug resistant strains and 15 sensitive strains yeilded 131, 510, 604, 1,100 and 800 bp sequences (Fig. 6-D). Verified by gene sequencing, these fragments were the PCR amplification products of *acrA*, *acrB*, *marOR*, *soxS* and *acrB*. There were 110 cases in drug resistant group and four cases in the sensitive group. The gene analysis showed a low mutation rate in the *acrA*, *acrB*, *acrR* and *soxS* genes. In the multi-drug resistant strains, a base CATT deletion in the 36, 37, 38 and 39 sites of *marOR* was detected. However, no *marOR* mutation was found in sensitive strains.

Discussion

Bacillary dysentery caused by *Shigella* is a serious intestinal infectious disease and the incidence ranks third among the national statutory B infectious diseases. In addition to transient immunity following infection, the absence of cross-immunoreactivity between subtypes and problems in the immune system of children, the increased drug-resistance of *Shigella* poses a serious threat to childrens' health. A previous study showed that *Shigella* had the AcrAB-TolC active efflux pump, which caused multi-drug resistance to organic solvents, dyes, detergents and virious antibiotics drugs (12). In another study (13), the high expresion level of the active efflux pump can enhance the resistance of bacteria to organic solvents. It also been reported that the resistance of *Escherichia coli* to organic solvents was affected by certain inherited traits, including the *imp*/*ostA* gene, which coded a protein with a molecular mass of 87,000. The protein was attached to the outer membranes. The low expression of the gene increased the sensitivity to N-hexane (14). Certain cell elements that do not belong to the antivie efflux pump, for example the derivatives of the O-sidechain of lipopolysaccharides or the isomers of fatty acid membranes, may have an effect on the resistance of *Pseudomonas* to organic solvents (15).

The results of the present study showed that 91.1% *S. flexneri* strains were multi-drug resistant. According to the *shigella* treatment recommendations of the American Clinical and Laboratory Standards Institute, ampicillin, sulfamethoxazole/trimethoprim were not suitable for the empirical clinical medication. Although the drug resistance rate of ciprofloxacin and levofloxacin was 32.9%, the fluroquinolones were not suitable for pediatric patients due to the potential damage to cartilage in infants and children. The drug-resistance mechanism was associated with multiple-site mutations of gene *gyrA* and/or *parC*. The drug-resistance rate to cefotaxime and cefotaxime was <30.4%, which meant that these drugs were suitable for use in the treatment of bacillary dysentery in children. It is noteworthy that the drug resistance was significantly higher than that reported previously In China and elsewhere (16,17), sugesting that, in case of treatment failure with third generation cephalosporins or in case of pediatric dental patents, cefoxitin, cefoperazone/sulbactam and imipenem may be applied in the treatment.

The integron-mediated muhidrug-resistance has been verified previously. Class 1 integron was detected in 91.1% of 158 *S. flexneri* strains. Class 2 integron was detected in 86.1% of 158 *S. flexneri* strains. The propotions were significantly higher, compared with those reported in South Korea and Iran (18,19). The islolating regions and epidemic strains may accout for this. The typical class 1 integron gene was present in 15.2% of the isolates screened. The drug resistance gene cassette was *frV* and *dfrA17-aadA*5. The atypical class 1 integron accounted for 86.1% strains. The drug resistance gene cassette was *bla*OXA30-*aadA*1. The typical and atypical class 1 integrons were present in 10.1% of isolates screened, similar with the relative reports (20,21). The typical class 1 integron was present in 11.4% of the *S. flexneri* isolates screened. This result has not been reported previously, to the best of our knowledge. In individual studies, the *dfrv* gene was present in *Escherichia coli*, *Salmonella* bacteria and Gram-negative bacilli in waste water (20-22). Whether the resistance genes were transfered from these bacteria requires further investigation.

Table IV. Drug-resistant phenotype integrons and gene cassette of 56 *Shigella flexneri* strains.

	Class 1 integron and gene cassettes		Class 2 integron and gene cassettes	
Drug-resistant phenotype (number of strains)	Class 1 integrase gene	Gene cassettes	Class 2 integrase gene	Gene cassettes
ATCR (2)	*intI1* (n=2)	*bla*OXA-30-*aadA1*	*intI2* (n=0)	-
	intI1 (n=4)	-	*intI2* (n=2)	*dfrA1-sat1-aadA1*
	intI1 (n=16)	*bla*OXA-30-*aadA1*	*intI2* (n=16)	*dfrA1-sat1-aadA1*
ATCRS (40)	*intI1* (n=12)	*bla*OXA-30-*aadA1* *dfr V*	*intI2* (n=12)	*dfrA1-sat1-aadA1*
	intI1 (n=6)	*dfr V*	*intI2* (n=6)	*dfrA1-sat1-aadA1*
	intI1 (n=4)	*bla*OXA-30-*aadA1*	*intI2* (n=0)	-
	intI1 (n=18)	*bla*OXA-30-*aadA1*	*intI2* (n=18)	*dfrA1-sat1-aadA1*
ATCRP (2)	*intI1* (n=2)	*bla*OXA-30-*aadA1*	*intI2* (n=2)	*dfrA1-sat1-aadA1*
ATCRPL (10)	*intI1* (n=10)	*bla*OXA-30-*aadA1*	*intI2* (n=10)	*dfrA1-sat1-aadA1*
ATCRSPL (22)	*intI1* (n=18)	*bla*OXA-30-*aadA1*	*intI2* (n=18)	*dfrA1-sat1-aadA1*
	intI1 (n=4)	*bla*OXA-30-*aadA1 dfrA17-aadA5*	*intI2* (n=4)	*dfrA1-sat1-aadA1*
ATCRSPLX (12)	*intI1* (n=12)	*bla*OXA-30-*aadA1*	*intI2* (n=12)	*dfrA1-sat1-aadA1*
ATCRSXZ (30)	*intI1* (n=30)	*bla*OXA-30-*aadA1*	*intI2* (n=30)	*dfrA1-sat1-aadA1*
ATCRSPLXZ (6)	*intI1* (n=6)	*bla*OXA-30-*aadA1*	*intI2* (n=6)	*dfrA1-sat1-aadA1*

A, ampicillin; T, tetracycline; C, chloramphenicol; R, streptomycin; S, sulfamethoxazole/trimethoprim; P, ciprofloxacin; L, levofloxacin; X, cefotaxime; Z, trimethoprim.

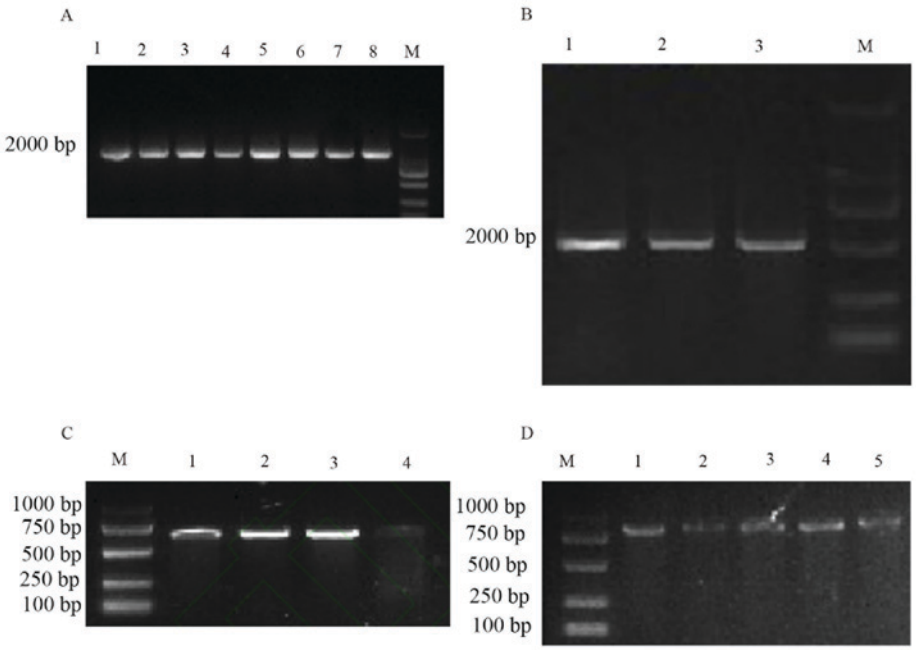

Figure 6. Polymerase chain reaction amplification and electrophoretogram of multi-drug resistance gene *acrAB-tolC* and its regulatory genes (A) multi-drug resistance gene *acrA*, (B) multi-drug resistance gene *acrB*, (C) multi-drug resistance gene *marOR* and (D) multi-drug resistance gene *acrR*. 1, 368 strains; 2, 157 strains; 3, 3,171 strains; 4, 187 strains; 5, 4,536 strains; 6, 1,113 strains; 7, 22 strains; 8, N8; M, DNA marker (DL2000). Upper to lower progressively 2,000, 1,000, 750, 500, 250 and 100 bp. M, DNA marker; 1-8 are the specimen numbers.

A total of 134 strains with atypical class 1 integron were resistant to ampicillin, streptomycin, tetracycline and chloromycetin. Only 10 of the 26 strains without the atypical class 1 integron were resistant to more than four antibiotics. There was a significant difference between them (χ^2=35.96, P<0.01). This difference is associated with a situation that the *bla*OXA-30-*aadA1* gene cassette in the atypical class 1 integron variable region was adjacent to the chloramphenicol- and tetracycline-resistant determinants (23). Those strains with no atypical class 1 integrons, which were resistant to the

four antibiotics may have other drug resistance mechanisms. There were 10.1% strains (*S. flexneri* 2A) possessing typical and atypical class 1 integron class 1 integrons (24), which deteriorate the drug resistance of *S. flexneri*. The typical class 1 integron was found in conjugative plasmids and can mediate the horizontal gene transfer. In the present study, 86.1% *S. flexneri* isolates carried the class 2 integron and the gene cassette was *dfrA1-sat1-aadA1*, which was consistant with other reports (25,26). The gene cassettes reported currently comprise *dfrAl-sat1, dfrAl-sat2-aadA1* and sat2-aadA1 (27-29). As the class 2 integron integrase is inadequate for integrating novel resistance genes from externally or to resect the resistance genes from its own variable region, the drug resistance gene cassettes of class 2 integron variable regions are relatively conservative.

The drug resistance rate to sulfamethoxazole/trimethoprim was 69.6%. The strains resistant to sulfamethoxazole/trimethoprim were compared with the *sull* gene carried in the atypical class 1 integron, the *dfrAl* gene carried in the class 2 integron, and the two together, suggesting that the two genes were not associated with the drug resistance. The two genes may be nonfunctional structural genes. The mechanism undferlying the drug resistance to sulfamethoxazole/trimethoprim requires further investigation.

The single-stranded conformation polymorphism suggested a low mutation rate of *acrA, acrB* and *tolC* in drug-resistant *Shigella*. The expression of AcrA and AcrB are regulated by several regulators. Of all the regulators the *acrR* suppressors and *marOR* operon are important. The results demonstrated a low mutation rate of *marOR* in drug-resistant *Shigella*, suggesting that the mutaion of the reglatory gene contributed more to the drug resistance of *Shigella* than the mutaion of a pump gene. Further investigations aim to examine the association between the mutation of regulatory gene *marOR* and the expression of *acrA, acrB* and *tolC*, which may provide a theoretical basis for the drug-resistant mechanism mediated by the active efflux pump of *Shigella*.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

QPM was responsible for specimen collection, bacterial identification, and experimental procedures. MXY was responsible for experimental operations and data collection. LS and JWL were responsible for data collection and statistical analysis. GYY was responsible for experimental design and data review.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Jinan Infectious Disease Hospital Affiliated To Shandong University (Shandong, China).

Consent for publication

Written informed consent was obtained from all patients or patient guardians for the present study.

Competing interests

The authors declare they have no competing interests.

References

1. Partridge SR: Analysis of antibiotic resistance regions in Gram-negative bacteria. FEMS Microbiol Rev 35: 820-855, 2011.
2. Poole K: Efflux-mediated multiresistance in Gram-negative bacteria. Clin Microbiol Infect 10: 12-26, 2004.
3. Yang HY, Duan GC and Xi YL: Distribution and expression of active efflux pump acrAB in clinical isolates of *Shigella*. Chin J Public Health 21: 685-687, 2005.
4. Lv RL, Duan G and Ca and Song CH: Analysis on acrR, marOR multi-drug regulated gene mutations in clinical isolates of *Shigella*. Chin J Public Health 24: 865-965, 2008.
5. Cockerill FR, Cockerill F, Adler J, Dudley MN, Patel JB, Eliopoulos GM and Bradford PA: Performance standards for antimicrobial susceptibility testing: Twenty-first informational supplement. Clin Lab Stand Inst 31: 21-42, 2011.
6. White PA, McIver CJ and Rawlinson WD: Integrons and gene cassettes in the enterobacteiaceae. Antimicrob Agents Chemother 45: 2658-2661, 2001.
7. Ploy MC, Denis F, Courvalin P and Lambert T: Molecular characterization of integrons in Acinetobacter baumannii: Description of a hybrid class 2 integron. Antimicrob Agents Chemother 44: 2684-2688, 2000.
8. Dalsgaard A, Forslund A, Serichantalergs O and Sandvang D: Distribution and content of class 1 integrons in different Vibrio cholerae O-serotype strains isolated in Thailand. Antimicrob Agents Chemother 44: 1315-1321, 2000.
9. Pan JC, Ye R, Meng DM, Zhang W, Wang HQ and Liu KZ: Molecular characteristics of class 1 and class 2 integrons and their relationships to antibiotic resistance in clinical isolates of *Shigella sonnei* and *Shigella flexneri*. J Antimicrob Chemother 58: 288-296, 2006.
10. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, *et al*: Relation of acrAB-tolC efflux pump and marOR regulatory gene mutation with antimicrobial resistance in *Shigella*. spp. J Jilin Univ 36: 45-48, 2010.
11. Rahman M, Mauff G, Levy J, Couturier M, Pulverer G, Glasdorff N and Butzler JP: Detection of 4-quinolone resistance mutation in gyrA gene of *Shigella dysenteriae* type 1 by PCR. Antimicrob Agents Chemother 38: 2488-2491, 1994.
12. Abe S, Okutsu T, Nakajima H, Kakuda N, Ohtsu I and Aono R: n-Hexane sensitivity of *Escherichia coli* due to low expression of imp/ostA encoding an 87 kDa minor protein associated with the outer membrane. Microbiology 149: 1265-1273, 2003.
13. Sikkema J, de Bont JA and Poolman B: Mechanism of membrane toxicity of hydrocarbons. Microbiol Rev 59: 201-222, 1995.
14. Wang XN, Wei DJ, Zhang JL and Li WS: Study on extended-spectrum β-lactamases and their genotypes of *Shigella* isolated from Tianjin. Chin J Infect Dis 30: 71-75, 2012.
15. Varghese SR and Aggarwal A: Extended spectrum beta-lactamase production in *Shigella* isolates-a matter of concern. Indian J Med Microbiol 29: 76-78, 2011.
16. Hong S, Choi YH, Choo YA, Choi Y, Choi SY, Kim DW, Lee BK and Park MS: Genetic characterization of atypical *Shigella flexneri* isolated in Korea. J Microbiol Biotechnol 20: 1457-1462, 2010.

17. Eftekhari N, Bakhshi B, Pourshafie MR, Zarbakhsh B, Rahbar M, Hajia M and Ghazvini K: Genetic diversity of *Shigella* spp. and their integron content. Foodborne Pathog Dis 10: 237-242, 2013.
18. Schaumburg F, Alabi AS, Kaba H, Lell B, Becker K, Grobusch MP, Kremsner PG and Mellmann A: Molecular characterization of *Shigella* spp. from patients in Gabon 2011-2013. Trans R Soc Trop Med Hyg 109: 275-279, 2015.
19. Gassama Sow A, Aïdara-Kane A, Barraud O, Gatet M, Denis F and Ploy MC: High prevalence of trimethoprim-resistance cassettes in class 1 and 2 integrons in Senegalese *Shigella* spp isolates. J Infect Dev Ctries 4: 207-212, 2010.
20. Kim TE, Jeong YW, Cho SH, Kim SJ and Kwon HJ: Chronological study of antibiotic resistances and their relevant genes in Korean avian pathogenic *Escherichia coli* isolates. J Clin Microbiol 45: 3309-3315, 2007.
21. Nógrády N, Gadó I, Tóth A and Pászti J: Antibiotic resistance and class 1 integron patterns of non-typhoidal human Salmonella serotypes isolated in Hungary in 2002 and 2003. Int J Antimicrob Agents 26: 126-132, 2005.
22. Rosser SJ and Young HK: Identification and characterization of class 1 integrons in bacteria from an aquatic environment. J Antimicrob Chemother 44: 11-18, 1999.
23. Luck SN, Turner SA, Rajakumar K, Sakellaris H and Adler B: Ferric dicitrate transport system (Fec) of *Shigella flexneri* 2a YSH6000 is encoded on a novel pathogenicity island carrying multiple antibiotic resistance genes. Infect Immun 69: 6012-6021, 2001.
24. Zhu JY, Duan GC, Yang HY, Fan QT and Xi YL: Atypical class 1 integron coexists with class 1 and class 2 integrons in multi-drug resistant *Shigella flexneri* isolates from China. Curr Microbiol 62: 802-806, 2011.
25. Huang YL, Xu YL, Zhuang L, Qian HM, Gu L, Gu B, Liu GY and Pan SY: Molecular epidemiology and integron analysis of *Shigella flexneri* isolates from Jiangsu province in 2011. Chi J Clin Infect Dis 7: 34-38, 2011.
26. Wang J, Liang F, Wu XM and Qi W: Transfer patterns of integron-associated and antibiotic resistance genes in *S. flexneri* during different time intervals in Tianjin, China. Indian J Med Microbiol 32: 256-260, 2014.
27. Xia R, Ren Y, Guo X and Xu H: Molecular diversity of class 2 integrons in antibiotic-resistant gram-negative bacteria found in wastewater environments in China. Ecotoxicology 22: 402-414, 2013.
28. Chang CY, Lu PL, Lin CC, Lee TM, Tsai MY and Chang LL: Integron types, gene cassettes, antimicrobial resistance genes and plasmids of *Shigella sonnei* isolates from outbreaks and sporadic cases in Taiwan. J Med Microbiol 60: 197-204, 2011.
29. Ud-Din AI, Wahid SU, Latif HA, Shahnaij M, Akter M, Azmi IJ, Hasan TN, Ahmed D, Hossain MA, Faruque AS, *et al*: Changing trends in the prevalence of *Shigella* species: Emergence of multi-drug resistant *Shigella sonnei* biotype g in Bangladesh. PLoS One 8: e82601, 2013.