Association of Serotype With Antimicrobial Resistance Patterns Among Shigella flexneri Isolates From Pakistan

Nisa, Iqbal; Haroon, Mohammad; Qasim, Muhammad; Driessen, Arnold; Nijland, Jeroen; Yasin, Nusrat; Jalal, Abdullah; Ali, Nawab; Ullah, Waheed; Khan, Taj Ali

Published in:
Pediatric infectious disease journal

DOI:
10.1097/INF.0000000000002791

Association of Serotype With Antimicrobial Resistance Patterns Among *Shigella flexneri* Isolates From Pakistan: The Importance of Serotype 2b

Iqbal Nisa, MS, Mohammad Haroon, MBBS, FCPS,† Muhammad Qasim, PhD,* Arnold Driessen, PhD,‡ Jeroen Nijland, PhD,‡ Nusrat Yasin, MS,* Abdullah Jalal, PhD,§ Nawab Ali, PhD,¶ Waheed Ullah, PhD,* Taj Ali Khan, PhD,* Asia Begum, MS,* and Hazir Rahman, PhD‖*

Background: *Shigella flexneri* is an emerging threat in low socioeconomic countries including Pakistan. No previous data is available on the association between *S. flexneri* serotypes and antimicrobial resistance in Pakistan.

Objectives: The objective of the present study was to assess the association between serotypes and antimicrobial resistance patterns among *S. flexneri* isolated from clinical and nonclinical samples.

Methods: A total of 199 *S. flexneri* isolates were subjected to molecular serotyping and antibiotic resistance.

Results: The most prevalent *S. flexneri* serotype was 2b (38%) followed by 1b (24%), 7a (20%), 2a (11%), 1d (5%) and Y (2%). The phylogenetic reconstruction showed 12 clades among which the clades II, III, V, VIII, IX and XI have consisted of serotypes that were found both in human population and environment samples. A high level of multidrug resistance (MDR) was observed in serotype 2b (37.68%) followed by 1b (19.5%) and 7a (19.5%), 2a (11.5%), 1d (5%) and Y (2%). All isolates of serotype 2b showed high level of resistance to amoxicillin/clavulanic acid (100%) followed by quinolone (74.6%) and trimethoprim-sulfamethoxazole (54.6%). Interestingly, none of the serotype was resistant to piperacillin-tazobactam, imipenem and amikacin. The most frequently detected resistance genes among serotype 2b were bla_{OXA} (100%) followed by *qnrS* (88%), *cat* (81%) and *sul2* (63%).

Conclusion: The most frequent *S. flexneri* serotype was 2b while 1d and Y was first time reported in Pakistan. High frequency of MDR serotypes of *S. flexneri* is a serious threat in diarrhea endemic regions and thus require urgent strategies for its continuous monitoring and prevention.

Key Words: *Shigella flexneri*, Serotype 2b, antibiotic resistance, Pakistan

(*Pediatr Infect Dis J* 2020;39:e352–e358)

Shigella flexneri belonging to the Enterobacteriaceae family that causes bacterial dysentery particularly in children and places a great burden on the medical system worldwide, mostly in developing countries with no hygienic water resources and poor sanitation.[1,2] It has been likely to expect that annually up to 160 million *S. flexneri* cases occur with up to 2.6 million deaths.[3] Among *Shigella* species, *S. flexneri* is the most prominent cause of shigellosis in developing countries. Based on O-antigen, *S. flexneri* has 19 different serotypes and in a region where *S. flexneri* is endemic, the serotype distribution may vary over time.[4–6] Among these 7a, 7b, 1d, 4av, Yv, 2 variant and Xv have been recently reported.[7] In 1989, serotype 7a first reported from Bangladesh and later become prevalent in Egypt, Vietnam and Bangladesh.[8] Serotype Xv, 4av, Y and 1d were first reported in China.[7,9] In developing countries, *S. flexneri* serotype 1b is more prevalent followed by *S. flexneri* serotype 2a. There is continuous change in the distribution of *S. flexneri* serotypes and their antibiotic resistance profile in developing countries due to the evolution of diverse circulatory clone of *S. flexneri*.[10,11] Therefore, local antimicrobial resistance profiling is important for effective treatment and prevention of disease dissemination.[10–13] This study describes the first report on the molecular serotyping of *S. flexneri* and its association with antimicrobial resistance patterns in Pakistan. The findings of the present study will be helpful in monitoring and vaccine development for shigellosis in Pakistan.

METHODS

Bacterial Isolates

A total of 199 *S. flexneri* isolates isolated from stool samples of pediatric diarrheal patients up to age of 17 years at tertiary care hospitals of Khyber Pakhtunkhwa, Pakistan, (Nisa et al, 2020)[14] and nonclinical samples (drinking water and raw milk, fruits/vegetables from markets [unpublished work] of Pakistan) from January 2016 to May 2017 were included in the analysis.

Molecular Serotyping of *S. flexneri* Using Multiplex PCR

A molecular serotyping of these *S. flexneri* isolates isolated from clinical and nonclinical (drinking water, retail raw milk, fruits and vegetables) samples was performed by using a multiplex polymerase chain reaction (PCR) assay following a procedure described previously[6] with minor modification. Primers used to amplify *S. flexneri*-specific serotype genes are listed in Table (Supplemental Digital Content 1, http://links.lww.com/INF/E45). The multiplex PCR assay was done by using the 2× *phire* green hot start II PCR master mix (Thermo Fisher Scientific) according to guidelines of the manufacturer's instructions. Thermal cycling was conducted by an initial denaturation step for 90 seconds at 98°C, followed by 32 cycles of 98°C for 10 seconds, 52°C for 90 seconds and 72°C for 45 seconds, with a final extension of 72°C for 55 seconds in a Thermocycler (Bio-Rad, CA). A loading dye was mixed with a 5 µL of the amplified product, subjected to electrophoresis by using 1.5% agarose gel and then visualized in a UV transilluminator.

Accepted for publication May 14, 2020.

From the *Department of Microbiology, Kohat University of Science and Technology, Kohat, Pakistan; †Department of Medicine, Khyber Teaching Hospital, Peshawar, Pakistan; ‡Department of Molecular Microbiology, Groningen Biomolecular Sciences, and Biotechnology Institute, Rijksuniversiteit Groningen, Faculty of Science and Engineering, the Netherlands; §Institute of Biotechnology and Genetic Engineering (IBGE), KPAUP Peshawar; Pakistan ¶ Department of Biotechnology and Genetic Engineering, Kohat University of Science and Technology, Kohat, Pakistan; ‖Department of Microbiology, Abdul Wali Khan University, Mardan, Pakistan.

This work was supported by grants from the Higher Education Commission of Pakistan (FTO number 0341-3970-2018).

The authors have no conflicts of interest to disclose.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website (www.pidj.com).

Address for correspondence: Muhammad Qasim, PhD, Department of Microbiology, Kohat University of Science and Technology, 26000 Kohat, Pakistan. E-mail: qasim89@gmail.com.

ISSN: 0891-3668/20/3911-e352

DOI: 10.1097/INF.0000000000002791

Antibiotic Susceptibility Testing and Detection of Antibiotic Resistance Gene

Antibiotic susceptibility testing for all serotypes of *S. flexneri* was performed by Kirby–Bauer disc-diffusion method following the instructions of clinical and laboratory standards institute (CLSI).[15] The discs (OXOID) used in the study were amoxicillin/clavulanic acid (AMC, 30 µg), ofloxacin (OFX, 5 µg), ceftazidime (CAZ, 30 µg), cefpodoxime (CPD, 10 µg), cefixime (CFM, 5 µg), ceftriaxone (CRO, 30 µg), trimethoprim–sulfamethoxazole (SXT, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), imipenem (IPM, 10 µg), nalidixic acid (NA, 30 µg), piperacillin-tazobactam (TZP, 110 µg) and amikacin (AK, 30 µg). In the current study, MDR refers to *S. flexneri* resistance to 3 or more unrelated antibiotic classes. Furthermore for detection of antibiotic resistance genes, a PCR-based assay that targeted bla_{OXA}, $bla_{TEM,}$ bla_{SHV} (genes confer resistance to beta-lactam antibiotics); *sul1 and sul2 (*genes confer resistance to sulfonamides); *cat* (genes confer resistance to chloramphenicol) and *qnr*A, *qnr*B, *qnr*S, *aac* (genes confer resistance to quinolone) was performed as described earlier by Zhu et al. (2017).[16]

Phylogenetic Analysis

A phylogenetic analysis was carried out on nucleotide sequences of all serotypes of *S. flexneri*. *16S rRNA* gene sequences were first aligned using MUSCLE program in MEGA ×10.[17] The multiple aligned sequences were then inferred for evolutionary history by using the maximum likelihood and Kimura 2-parameter model.[18]

Statistical Analysis

Pearson's χ^2 test and odds ratio (OR, binary logistic regression) with 95% confidence interval (CI) were used to evaluate the association of (1) serotype and antibiotic resistance, (2) the drug-resistant serotypes among clinical and nonclinical samples, (3) *S. flexneri* serotypes and multidrug resistance (MDR) patterns and (4) *S. flexneri* serotypes and drug resistance genes. A P value of <0.05 was considered to be statistically significant.

RESULTS

Multiplex PCR Serotypes

The multiplex PCR yielded the expected fragment belonging to specific serotypes. The amplified products were sorted to extract the serotypes belonging to specific fragments patterns (Fig. 1). The distributions of serotypes of *S. flexneri* isolated from different sources (clinical and nonclinical samples) are shown in Figure 2. The *S. flexneri* isolates (n = 199) were grouped into 6 different serotypes (1b, 2b, 1d, 2a, 7a and Y). The most prevalent serotypes was 2b (38%) followed by 1b (24%), 7a (20%), 2a (11%), 1d (5%) and Y (2%). Furthermore, the most prevalent serotype was 2b in both clinical (34.18%) and nonclinical (49.89%) samples (Table 1). Serotype 7a was mostly detected in clinical samples (24.61%) compared with nonclinical samples (2.27%) as shown in table 1. All serotypes except 1b and Y have a significant association ($P < 0.05$) with clinical and nonclinical isolates as shown in Table 1. The study also showed that serotype 2b, 1b and 2a was isolated from all the sources as shown in Table 2. The only significant association was observed between the isolates of clinical and drinking water samples belong to serotypes 2b and 7a (Table 2).

Association Between Serotype and Phenotypic Antibiotic Susceptibility

We studied whether any association was evident between *S. flexneri* serotypes and antimicrobial resistance pattern as documented previously.[10–12,19]

The drug resistance varied among the various serotypes (1b, 2b, 2a, 7a, 1d and Y) as shown in Table (Supplemental Digital Content 2, http://links.lww.com/INF/E46). With the exception of serotype 2a, all serotypes demonstrated high level of resistance to amoxicillin-clavulanic acid. Only serotype 7a showed sensitivity to third-generation cephalosporin compared with other serotypes. Chloramphenicol resistance was observed in all serotypes. High-level quinolone resistance was observed in serotypes 2b (74.6%) and 2a (78.3%) compared with other predominant serotypes (see Table, Supplemental Digital Content 2, http://links.lww.com/INF/E46).

Furthermore, in the current study, serotype 2b showed high level of resistances to AMC (100%) followed by quinolone [OFX, CIP and NA (74.6%)], SXT (54.6%), cephalosporin [CAZ, CPD and CFM (44%) and CRO (36%)] and C (27%) as shown in Table (Supplemental Digital Content 2, http://links.lww.com/INF/E46). None of the serotype was resistance to IMP, AK and TZPs.

Association Between Drug-Resistant Serotypes Among Clinical and Nonclinical Samples

We also studied whether any association was evident between the drug-resistant isolates belong to different serotypes isolated from clinical with resistant isolates isolated from nonclinical samples. We observed 80.5% of serotype 1b clinical isolates

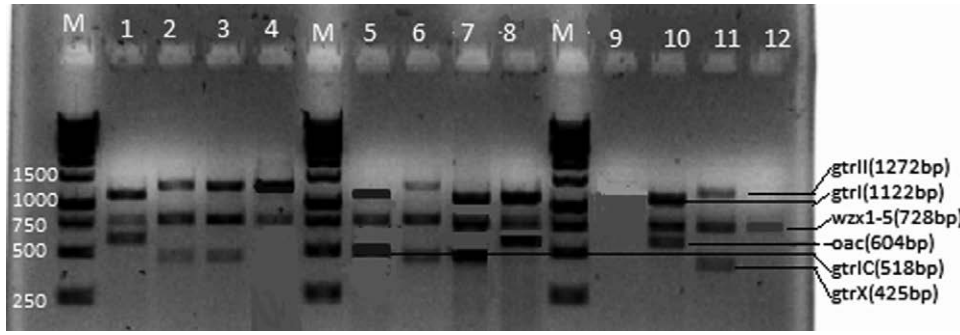

FIGURE 1. Detection of serotypes of *Shigella flexneri* on gel. Multiplex polymerase chain reaction (PCR) products were run on agarose gel of 1.5% and visualized under a ultra-violet transilluminator. Lane M, 1 Kbp DNA ladder; Lane 1, 1b; Lane 2, 2b; Lane 3, 2b; Lane 4, 2a; Lane 5, 7a; Lane 6, 2b; Lane 7, 1d; Lane 8, 1b; Lane 9, negative control; Lane 10, 1b; Lane 11, 1d; Lane 12, Y. *Note:* 1b (wzx 1-5+ gtr I+ oac), 1d (wzx 1-5+ gtr I+ gtrX), 2a (wzx 1-5+ gtrII), 2b(wzx 1-5+ gtrX+ gtrII), 7a (wzx 1-5+ gtr I+ gtrIC) and Y (wzx 1-5).

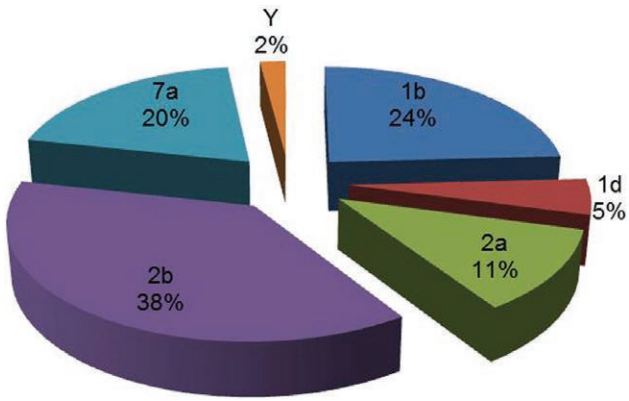

FIGURE 2. Chart showing the distribution of serotypes of *Shigella flexneri* in Pakistan detected by multiplex polymerase chain reaction (PCR).

were resistant to AMC and 83% of nonclinical isolates were resistant whereas both clinical and nonclinical isolates of serotype 2b were 100% resistant to AMC. Similarly, we also noticed 88.6% of serotype 2b clinical isolates were resistant to quinolones but only 45% of nonclinical isolates were resistant, whereas 70.7 % of serotypes 2a clinical isolates were resistant and 55.3% of nonclinical isolates were resistant as shown in Table (Supplemental Digital Content 2, http://links.lww.com/INF/E46). We also noticed a significant association between most of the resistant isolates belongs to serotype 2b and 7a isolated from clinical samples with resistant isolates from nonclinical samples to quinolones ($P < 0.005$) as described in Table (Supplemental Digital Content 2, http://links.lww.com/INF/E46).

Association Between MDR Patterns and *S. flexneri* Serotypes

In the current study, we noticed that the high prevalence of MDR was shown in serotype 2b (37.68%) followed by 1b (19.5%) and 7a (19.5%), 2a (11.5%), 1d (5%) and Y (2%). Furthermore, 13 different phenotypic patterns were observed for all serotypes of *S. flexneri*. A statistically significant association was observed between the most prevalent MDR patterns AMC, CAZ, CPD, CFM, CRO, SXT (52%, $P < 0.0001$); OFX, SXT, NA, CIP (61%, $P < 0.0001$); OFX, AMC, NA, CIP (34.6%, $P < 0.0001$); AMC, SXT, C (97%, $P < 0.0001$); OFX, AMC, CAZ, CPD, CIP (75%, $P < 0.0001$) and AMC, SXT, C (80%, $P = 0.0004$) with a serotypes 1b, 2a, 2b,7a, Y and 1d, respectively (Table 3).

TABLE 1. Distribution of Serotypes of *Shigella flexneri* Isolated From Clinical and Nonclinical Samples

Serotypes	Total isolates	Strains (n = 155) isolated from clinical samples, n (%)	Strains (n = 44) isolated from nonclinical samples, n (%)	*P*
1b	48	36 (23.2)	12 (27.24)	0.20
1d	10	9 (5.8)	1 (2.27)	0.004
2a	23	16 (10.32)	7 (15.89)	0.008
2b	75	53 (34.18)	22 (49.89)	0.01
7a	39	38 (24.61)	1 (2.27)	<0.0001
Y	4	3 (1.93)	1 (2.27)	0.20

P value <0.05 was considered statistically significant. Clinical samples includes human diarrheal stool samples. Nonclinical samples includes drinking water, retail raw milk, fruits and vegetables.

TABLE 2. Comparative Distribution of Serotypes Among *Shigella flexneri* Strains Isolated From Clinical Samples, Drinking Water and Retail Raw Foods (Milk, Fruits and Vegetables)

Variables	Total isolates	Positive, n (%)	Negative, n (%)	*P*
1b				
Clinical samples	155	36 (23.2)	119 (76.8)	0.57
Drinking water	22	5 (22.7)	17 (77.3)	0.87
Raw milk	12	3 (25)	9 (75)	0.94
Raw fruits/vegetables	10	4 (40)	6 (60)	0.228
1d				
Clinical samples	155	9 (5.8)	146 (94.2)	0.34
Drinking water	22	0	22 (100)	0.25
Raw milk	12	1 (8.3)	11 (91.7)	0.59
Raw fruits/vegetables	10	0	10 (100)	0.45
2a				
Clinical samples	155	16 (10.3)	139 (89.7)	0.30
Drinking water	22	3 (13.6)	19 (86.4)	0.74
Raw milk	12	1 (8.3)	11 (91.7)	0.71
Raw fruits/vegetables	10	3 (30)	7 (70)	0.06
2b				
Clinical samples	155	53 (34.2)	102 (65.8)	0.05
Drinking water	22	13 (59)	9 (41)	0.02
Raw milk	12	6 (50)	6 (50)	0.36
Raw fruits/vegetables	10	3 (30)	7 (70)	0.60
7a				
Clinical samples	155	38 (24.5)	117 (75.5)	0.001
Drinking water	22	1 (4.5)	21 (95.5)	0.05
Raw milk	12	0	12 (100)	0.07
Raw fruits/vegetables	10	0	10 (100)	0.10
Y				
Clinical samples	155	3 (2)	152 (98)	0.88
Drinking water	22	0	22 (100)	0.47
Raw milk	12	1 (8.3)	11 (91.7)	0.10
Raw fruits/vegetables	10	0	10 (100)	0.64

P value <0.05 was considered statistically significant.

In the current study, we further noticed 8 different phenotypic resistance patterns among *S. flexneri* serotype 2b. The most prevalent phenotypic resistance patterns among *S. flexneri* serotype 2b were OFX, AMC, NA, CIP (34.6%, $P < 0.0001$) followed by OFX, AMC, CAZ, CPD, CFM, CRO, SXT, NA, CIP (28%, p<0.0001); OFX, AMC, NA, CIP, C (10.6%, $P = 0.06$) and AMC, SXT, C (9.3%, $P < 0.0001$) as described in Table 3.

Association Between Genotypic Resistance Patterns and *S. flexneri* Serotypes

All phenotypic resistant isolates were further investigated for the harboring of 10 different antibiotic resistance genes. We observed heterogeneity in the genotypic resistance markers among the different serotypes of *S. flexneri* isolated from different samples as shown in Table (Supplemental Digital Content 3, http://links.lww.com/INF/E47). No expression of resistance genes of bla_{SHV}, *sul1*, *qnrA*, *qnrB* and *aac* genes was detected in the tested isolates. Drug resistance gene bla_{OXA}, bla_{TEM}, *qnrS*, *sul2* and *cat* were detected in all predominant serotypes of *S. flexneri* except for serotype 2a in which only bla_{OXA}, *sul2* and *qnrS* were detected.

In the current study, the most frequently detected resistance gene among *S. flexneri* serotype 2b were bla_{OXA} (100%, $P < 0.0001$), followed by *qnrS* (88%, $P = 0.003$), *cat* (81%, $P < 0.0001$), *sul2* (63%, $P < 0.0001$) and bla_{TEM} (35%, $P < 0.0001$) as shown in Table (Supplemental Digital Content 3, http://links.lww.com/INF/E47).

Furthermore, there were also notable differences in MDR genotypes between the different serotypes as shown in Table 4. For all serotypes of *S. flexneri*, 8 different genotypic resistance patterns

TABLE 3. Multidrug Resistance (MDR) Pattern Among Various Serotypes of *Shigella flexneri*

Serotype	Resistance pattern	Total isolates	No. of drug-resistant isolates, n (%)	*P*
1b	AMC, SXT, C	48	7 (14.5)	0.006
1b	AMC, CAZ, CPD, CFM, CRO, SXT	48	25 (52)	<0.0001
1b	SXT	48	9 (18.7)	<0.0001
1b	AMC, CAZ, CPD, CFM, CRO, SXT, C	48	4 (8.3)	0.44
1b	AMC, CPD, CFM, SXT, NA, C	48	1 (2)	0.7
1b	OFX, AMC, NA, CIP, C	48	2 (4)	0.44
2b	AMC, SXT, C	75	7 (9.3)	<0.0001
2b	AMC, CAZ, CPD, CFM, CRO, SXT	75	1 (1)	0.0001
2b	AMC, CAZ, CPD, CFM, CRO, SXT, C	75	5 (6.6)	0.25
2b	OFX, AMC, NA, CIP, C	75	8 (10.6)	0.06
2b	AMC, CAZ, CPD, CFM, SXT	75	6 (8)	0.13
2b	OFX, AMC, NA, CIP	75	26 (34.6)	<0.0001
2b	OFX, AMC, SXT, NA, CIP	75	1 (1)	0.19
2b	OFX, AMC, CAZ, CPD, CFM, CRO, SXT, NA, CIP	75	21 (28)	<0.0001
2a	OFX, AMC, NA, CIP, C	23	3 (13)	0.17
2a	AMC, CAZ, CPD, CFM, SXT	23	3 (13)	0.06
2a	OFX, SXT, NA, CIP	23	14 (61)	<0.0001
2a	AMC, CPD, CFM, CRO, SXT, NA, C	23	3 (13)	<0.0001
7a	AMC, SXT, C	39	38 (97)	<0.0001
7a	OFX, AMC, NA, CIP	39	1 (2.5)	0.02
1d	AMC, SXT, C	10	8 (80)	0.0004
1d	AMC, CPD, CFM, SXT, NA, C	10	2 (20)	<0.0001
Y	AMC, CAZ, CPD, CFM, SXT	4	1 (25)	0.06
Y	OFX, AMC, CAZ, CPD, CIP	4	3 (75)	<0.0001

P value < 0.05 was considered statistically significant.

TABLE 4. Distribution of Genetic MDR Pattern Among Serotypes of *Shigella flexneri*

Serotype (n)	Drug resistance genes pattern	MDR isolates, n (%)	*P*
1b (48)	bla_{OXA}, bla_{TEM}, *sul2*, *qnrS*, *cat*	2 (4)	<0.0001
1b (48)	bla_{OXA}, *qnrS*, *cat*	5 (10)	0.158
1b (48)	bla_{OXA}, *sul2*, *qnrS*, *cat*	1 (2)	0.901
1b (48)	*sul2*, *qnrS*	4 (8)	0.31
1b (48)	bla_{OXA}, bla_{TEM}, *sul2*	28 (58)	<0.0001
1b (48)	bla_{OXA}, bla_{TEM}, *sul2*,*cat*	5 (10)	0.009
1b (48)	bla_{OXA}, *sul2*, *cat*	3 (6)	0.5
2b (75)	bla_{OXA}, bla_{TEM}, *sul2*, *qnrS*, *cat*	22 (29)	0.14
2b (75)	bla_{OXA}, *qnrS*, *cat*	27 (36)	<0.0001
2b (75)	bla_{OXA}, *sul2*, *qnrS*, *cat*	3 (4)	0.7
2b (75)	bla_{OXA}, bla_{TEM}, *sul2*	1 (1)	<0.0001
2b (75)	bla_{OXA}, bla_{TEM}, *sul2*, *cat*	2 (3)	0.45
2b (75)	bla_{OXA}, *sul2*, *cat*	6 (8)	0.06
2b (75)	bla_{OXA}, *sul2*, *qnrS*	13 (17)	<0.0001
2b (75)	bla_{OXA}, bla_{TEM}, *qnrS*, *cat*	1 (1)	0.19
2a (23)	bla_{OXA}, *qnrS*, *cat*	2 (9)	0.25
2a (23)	*sul2*, *qnrS*	21 (91)	<0.0001
7a (39)	bla_{OXA}, bla_{TEM}, *sul2*, *qnrS*, *cat*	39 (100)	<0.0001
1d (10)	bla_{OXA}, bla_{TEM}, *sul2*, *qnrS*, *cat*	8 (80)	0.002
1d (10)	bla_{OXA}, *sul2*, *qnrS*	1 (10)	0.7
1d (10)	bla_{OXA}, *sul2*, *qnrS*, *cat*	1 (10)	0.25
Y (4)	bla_{OXA}, *sul2*, *qnrS*, *cat*	2 (50)	<0.0001
Y (4)	bla_{OXA}, bla_{TEM}, *sul2*	1 (25)	0.57
Y (4)	bla_{OXA}, bla_{TEM}, *sul2*, *cat*	1 (25)	0.03

P value < 0.05 was considered statistically significant.

were noticed. The most common antibiotic resistance pattern among different predominant serotypes of *S. flexneri* were bla_{OXA}, bla_{TEM}, *sul2* in 1b (58%, $P < 0.0001$); bla_{OXA}, *qnrS*, *cat* in 2b (36%, $P < 0.0001$); *sul2*, *qnrS* in 2a (91%, $P < 0.0001$); bla_{OXA}, bla_{TEM}, *sul2*, *qnrS*, *cat* in 7a (100%, $P < 0.0001$) and 1d (80%, $P = 0.002$) and bla_{OXA}, *sul2*, *qnrS*, *cat* in Y(50%, $P < 0.0001$).

We also further observed 8 different genotypic resistance patterns among *S. flexneri* serotype 2b. The most prevalent genotypic resistance pattern were bla_{OXA}, *qnrS, cat* (36%, $P < 0.0001$) followed by bla_{OXA}, bla_{TEM}, *sul2*, *qnrS*, *cat* (29%, $P = 0.14$), bla_{OXA}, *sul2*, *qnrS* (17%, $P < 0.0001$) and bla_{OXA}, *sul2*, *cat* (8%, $P = 0.06$) as shown in Table 4.

Phylogenetic Relationship of *S. flexneri* Serotype by Partial Genome Sequencing

The phylogenetic reconstruction showed that all 199 isolates formed 3 major clusters as shown in Figure 3. These clusters were further placed into 12 clades according to how strains were closely linked. The clades I, IV, VI, VII, X and XII have strains of *S. flexneri* serotypes that were circulating within a human population while other clades II, III, V, VIII, IX and XI have consisted of *S. flexneri* serotypes that were found both in human population and nonclinical samples. Furthermore, we found that the serotypes Y fall in same clades with 2b and 1b serotypes while serotype 1d showed close sequence identity with 2b, 1b and 7a serotypes isolated from different sources.

DISCUSSION

Shigellosis caused by the *S. flexneri* has been a leading health problem in developing countries. Molecular characterization of *S. flexneri* isolates is important to understand the disease burden caused by new *Shigella* serotypes, vaccine development and to prevent the emergence of MDR bacteria.[7–13,20,21] Several studies reported evidence about association of *S. flexneri* serotype with antibiotic resistance pattern.[4–14] The current study is highlighting the molecular serotyping of *S. flexneri* and their association with antimicrobial resistance patterns in Pakistan. The serotype 2b was observed as most frequent serotype followed by serotype 1b which is in line with previous studies.[12,20] A previous study from Pakistan reported serotype was 2a as a most frequent serotype.[12] However, serotypes 1d and Y had not been previously been reported

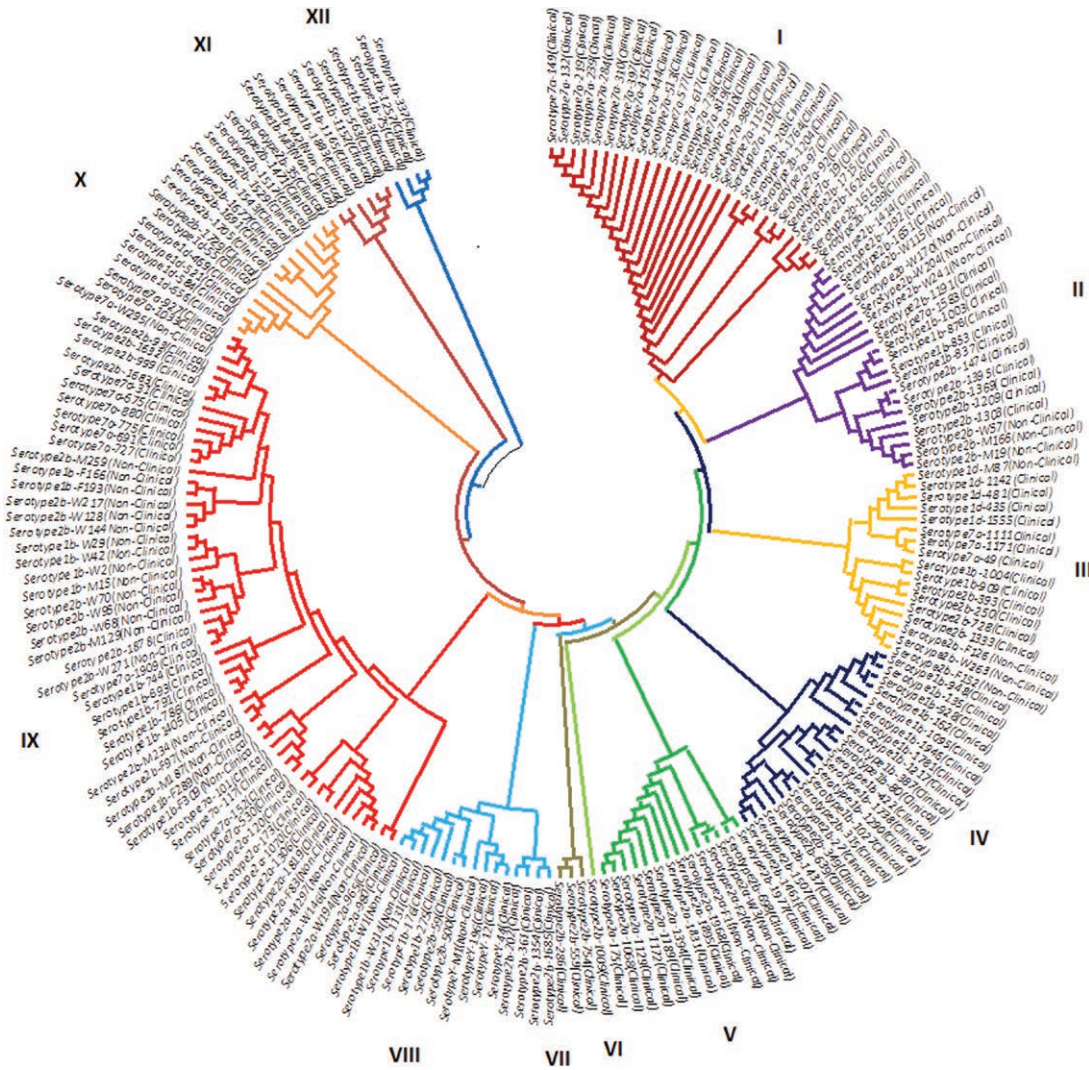

FIGURE 3. Phylogenetic tree displaying similarity of *Shigella flexneri* serotypes between clinical and nonclinical isolates. The evolutionary history was inferred by using the maximum likelihood method and Kimura 2-parameter model. The tree with the highest log likelihood (–2189.40) is shown. Initial tree(s) for the heuristic search was obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.8052)). This analysis involved 199 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 543 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

in Pakistan. This changing trend in *S. flexneri* serotypes have also been reported in Bangladesh and other regions of the world.[12,21,22]

The emergence and dissemination of *S. flexneri* MDR isolates have a great impact in low socioeconomic countries.[7–13,20–22] *S. flexneri* serotypes revealed high resistance to widely used antibiotics. In the present study, we observed high MDR in different *S. flexneri* serotypes, especially serotype 2b compared with the earlier reports from Pakistan[12,23,24] and China.[6,20] We observed high drug resistance of *S. flexneri* serotype toward amoxicillin, chloramphenicol, quinolone, cephalosporin and sulfamethoxazole-trimethoprim and thus care must be taken while prescribing these drugs. As reported in an earlier study, when the effectiveness of first-line generation antibiotics therapy becomes reduced because of the development of the resistant isolates then quinolones and cephalosporin were suggested as frontline antibiotics for the treatment of shigellosis.[25] But, in our study, all the predominant serotypes were resistant to a third-generation cephalosporin (except 7a) and quinolones. Because of the presently used frontline antibiotics, these phenotypic resistances threaten the efficacy of treatment. Thus, preventive policies should be needed to prevent the antimicrobial resistance spreading among *S. flexneri* serotype 2b and other predominant serotypes.[20]

The drug resistance pattern of *S. flexneri* also varied among serotypes isolated from clinical and nonclinical source. In the present study, bla_{OXA} gene was detected in all predominant serotypes including serotype 2b while the bla_{TEM} gene was found in all predominant serotypes of *S. flexneri* except in serotype 2a isolates.

In the current study, only *qnrS* gene was observed in all predominant serotypes of *S. flexneri* including serotype 2b and is in parallel with the previous studies.[26,27] The *qnrS* gene detection may

enable chromosomal selection mutations that produce resistance against quinolone.[9]

We further observed heterogeneity in phenotypic and genotypic resistance patterns among the same or different serotypes of *S. flexneri* pattern, which implies either the choice of antimicrobial therapy may vary between different serotypes, the clonal spread of resistant microorganism or antibiotic cross-resistance between different classes of the antibiotic that leads to therapeutic failures. This dissimilarity may be justifying the need for guideline implementation on antimicrobial agents.[28] A phylogenetic tree was constructed to know the genetic similarity between the first times reported *S. flexneri* serotypes isolates Y and 1d in Pakistan with other predominant serotypes like 2b, 2a, 1b and 7a from clinical and nonclinical samples. The phylogenetic tree showed 12 clades in which clade I, IV, VI, VII, X and XII were *S. flexneri* serotypes strains that were circulating only in the human population. These clades did not indicate any *S. flexneri* serotype movement from human to environment and vice versa. The remaining clades were intermixed with *S. flexneri* serotypes including all Y and 1d serotypes except 4 isolates of 1d serotype isolated from clinical and nonclinical samples. The present finding suggests that these serotypes were closely similar and might be related to the same lineage, recommending that shigellosis caused by *S. flexneri* was possibly transmitted from the environment (drinking water, retail raw milk, fruits and vegetables) to humans. These findings favor the notion that the environment is a statistically significant source of shigellosis.[29] Seeing that *S. flexneri* serotypes Y and 1d strains from clinical and nonclinical samples shared the same clade, it might be assumed that clinical strains most probably originated from nonclinical strains. The results further suggested that serotypes Y shared the same clade with serotype 2b and 1b while serotype 1d shared the same clade with serotype 2b, 1b and 7a suggesting that 1d and Y serotypes display similarity with these serotypes.

The findings of the present study are important because it was the first report to reveal association of molecular serotypes and drug resistance in diarrhea endemic region of Pakistan since there is no such data available in other regions of Pakistan. This study had some limitations such as (1) 6 serotypes were studied though 19 serotypes has been reported in various regions of the world, (2) limited number (n = 4) of Y serotypes has been observed so data would not a representative of general Y serotypes population and (3) we have studied selected drug resistance genes though many other additional targets such as CTX-M or gyrA could had been included in the study.

In conclusion, the current findings provide serotypes 2b was predominant in the region and serotypes 1d and Y had not been previously been reported in Pakistan. Phylogenetic analysis showed that shigellosis from the studied population possibly has been acquired from nonclinical sources. Most of the serotypes especially serotype 2b showed a high level of MDR to the most commonly used antibiotics in Pakistan, which is a serious threat to diarrhea endemic regions of Pakistan. We encourage the government health authorities to develop surveillance system for continuous monitoring of emergence of novel *S. flexneri* serotypes and drug resistance, which could be effectively control shigellosis cases in Pakistan.

ACKNOWLEDGMENTS

The authors would like to acknowledge the staff of the Department of Molecular Microbiology Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, the Netherlands; Veterinary Research Institute Peshawar, Lady Reading Hospital Peshawar, Pakistan; and Kohat university of Science and Technology, Kohat, Pakistan, for their excellent technical assistance. M.Q., I.N., A.D. and J.N. conceived and designed the study. I.N. wrote the main manuscript and performed the experiments. M.Q., I.N., A.D., J.N. and H.R. analyzed the data. M.Q., A.D., J.N., M.H., N.Y., A.J., N.A., W.U., T.A.K. and A.B. review and commented on the manuscript. All authors read and approved the final manuscript.

REFERENCES

1. Muthuirulandi Sethuvel DP, Ragupathi N, Anandan S, et al. Update on: *Shigella* new serogroups/serotypes and their antimicrobial resistance. *Lett Appl Microbiol*. 2016;64:8–18.
2. Nikfar R, Shamsizadeh A, Darbor M, et al. A study of prevalence of *Shigella* species and antimicrobial resistance patterns in paediatric medical center, Ahvaz, Iran. *Iran J Microbiol*. 2017;9:277–283.
3. Izard T, Tran Van Nhieu G, Bois PR. *Shigella* applies molecular mimicry to subvert vinculin and invade host cells. *J Cell Biol*. 2006;175:465–475.
4. Ahmed SF, Riddle MS, Wierzba TF, et al. Epidemiology and genetic characterization of *Shigella flexneri* strains isolated from three paediatric populations in Egypt (2000-2004). *Epidemiol Infect*. 2006;134:1237–1248.
5. Brengi BSP, Sun Q, Bolanos H, et al. PCR based method for *Shigella flexneri* serotyping: international multicenter validation. *J Clin Microbiol*. 2019;57:e01592–18
6. Sun Q, Lan R, Wang Y, et al. Development of a multiplex PCR assay targeting O-antigen modification genes for molecular serotyping of *Shigella flexneri*. *J Clin Microbiol*. 2011;49:3766–3770.
7. Cui X, Wang J, Yang C, et al. Prevalence and antimicrobial resistance of *Shigella flexneri* serotype 2 variant in China. *Front Microbiol*. 2015;6:435.
8. Stagg RM, Tang SS, Carlin NI, et al. A novel glucosyltransferase involved in O-antigen modification of *Shigella flexneri* serotype 1c. *J Bacteriol*. 2009;191:6612–6617.
9. Luo X, Sun Q, Lan R, et al. Emergence of a novel *Shigella flexneri* serotype 1d in China. *Diagn Microbiol Infect Dis*. 2012;74:316–319.
10. Cui X, Yang C, Wang J, et al. Antimicrobial resistance of *Shigella flexneri* serotype 1b isolates in China. *PLoS One*. 2015;10:e0129009.
11. Yang C, Li P, Zhang X, et al. Molecular characterization and analysis of high-level multidrug-resistance of *Shigella flexneri* serotype 4s strains from China. *Sci Rep*. 2016;6:29124.
12. Zafar A, Hasan R, Nizami SQ, et al. Frequency of isolation of various subtypes and antimicrobial resistance of *Shigella* from urban slums of Karachi, Pakistan. *Int J Infect Dis*. 2009;13:668–672.
13. Niyogi SK. Increasing antimicrobial resistance—an emerging problem in the treatment of shigellosis. *Clin Microbiol Infect*. 2007;13:1141–1143.
14. Nisa I, Qasim M, Driessen A, et al. Molecular epidemiology of *Shigella flexneri* isolated from pediatrics in a diarrhea-endemic area of Khyber Pakhtunkhwa, Pakistan. *Eur J Clin Microbiol Infect Dis*. 2020;39:971–985.
15. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing*. 28th ed. M100-28. Wayne, PA: CLSI; 2018:30–37
16. Zhu Z, Cao M, Zhou X, et al. Epidemic characterization and molecular genotyping of *Shigella flexneri* isolated from calves with diarrhea in Northwest China. *Antimicrob Resist Infect Control*. 2017;6:92.
17. Kumar S, Stecher G, Li M, et al. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol*. 2018;35:1547–1549.
18. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol*. 1980;16:111–120.
19. Jones ME, Peters E, Weersink AM, et al. Widespread occurrence of integrons causing multiple antibiotic resistance in bacteria. *Lancet*. 1997;349:1742–1743.
20. Liu H, Zhu B, Qiu S, et al. Dominant serotype distribution and antimicrobial resistance profile of *Shigella spp*. in Xinjiang, China. *PLoS One*. 2018;13:e0195259.
21. Talukder KA, Dutta DK, Safa A, et al. Altering trends in the dominance of *Shigella flexneri* serotypes and emergence of serologically atypical *S. flexneri* strains in Dhaka, Bangladesh. *J Clin Microbiol*. 2001;39:3757–3759.
22. Chang Z, Zhang J, Ran L, et al. The changing epidemiology of bacillary dysentery and characteristics of antimicrobial resistance of *Shigella* isolated in China from 2004–2014. *BMC Infect Dis*. 2016;16:685.
23. Ahmed K, Shakoori FR, Shakoori AR. Aetiology of shigellosis in northern Pakistan. *J Health Popul Nutr.* 2003;21:32–39.
24. Lindblom GB, Mazhar K, Khalil K, et al. Occurrence and susceptibility to antibiotics of *Shigella* species in stools of hospitalized children with bloody diarrhea in Pakistan. *Am J Trop Med Hyg*. 1998;58:800–803.

25. Guarino A1, Albano F, Ashkenazi S, et al. European society for paediatric gastroenterology, hepatology, and nutrition/European society for paediatric infectious diseases evidence-based guidelines for the management of acute gastroenteritis in children in Europe. *J Pediatr Gastroenterol Nutr*. 2008;46(suppl 2):S81–S122.

26. Pu XY, Pan JC, Wang HQ, et al. Characterization of fluoroquinolone-resistant *Shigella flexneri* in Hangzhou area of China. *J Antimicrob Chemother*. 2009;63:917–920.

27. Hata M, Suzuki M, Matsumoto M, et al. Cloning of a novel gene for quinolone resistance from a transferable plasmid in *Shigella flexneri* 2b. *Antimicrob Agents Chemother*. 2005;49:801–803.

28. Yahiaoui RY, Bootsma HJ, den Heijer CDJ, et al. Distribution of serotypes and patterns of antimicrobial resistance among commensal *Streptococcus pneumoniae* in nine European countries. *BMC Infect Dis*. 2018;18:440.

29. Dekker JP, Frank KM. Salmonella, Shigella, and yersinia. *Clin Lab Med*. 2015;35:225–246.