Characterization of a serologically atypical \textit{Shigella flexneri} Z isolated from diarrheal patients in Bangladesh and a proposed serological scheme for \textit{Shigella flexneri}

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Abstract

**Background**

Atypical \textit{Shigella flexneri} Z variant, that agglutinate with E1037 group factor specific monoclonal antisera against \textit{Shigella flexneri} IV-I but not with other group or type specific antisera, has continuously been isolated in Bangladesh since 1997. Later this serotype has been reported in Indonesia, China and Argentina. Despite being a provisional serotype, continuous isolation of these strains in diverse geographical regions implicated a great necessity to study the overall characteristics of these strains. Therefore, we extensively characterized \textit{S. flexneri} Z strains using various phenotypic and molecular tools.

**Method**

Of 3569 \textit{S. flexneri} isolated between 1997 and 2015, 95 strains were identified as \textit{S. flexneri} Z using a panel of polyvalent absorbed antisera and monoclonal antisera of \textit{S. flexneri} (MASF). Of them, randomly selected 65 strains were molecular O-serotyped using multiplex PCR and characterized using different phenotypic and molecular techniques (i.e. biotyping, plasmid profile, virulence marker and PFGE) to determine relationship with other subserotypes of \textit{S. flexneri}.

**Results**

All these atypical \textit{S. flexneri} Z strains were agglutinated with MASF B and IV-I antisera. Concordantly, these strains were positive to opt-gene, responsible for MASF IV-I sero-positive phenotype. However, molecular O-serotyping of all 65 strains could not differentiate between Z and Yb giving similar amplification products (wzx1-5 and opt). Contrarily, MASF based serotypic scheme distinguished among Z and Yb as well as Ya. All these \textit{S. flexneri} Z showed typical biochemical reaction of \textit{S. flexneri}, harboured a 140 MDa virulence plasmid and virulence markers namely \textit{ipaH}, \textit{ial}, \textit{sen}, \textit{sigA} and \textit{sepA} genes. Along with the virulence plasmid, small plasmids (2.6, 1.8 and 1.6 MDa) were present as core plasmid. Moreover, a
middle ranged plasmid and a 4.0 MDa sized plasmid were observed in 65% and 20% strains, respectively. Analysis of PFGE on XbaI-digested chromosomal DNA of Bangladeshi strains showed that *S. flexneri* Z had a close relatedness with Ya and Yb but completely different than the strains of Xa, Xb, 2a and 2b. This observation was found to be unequivocal while the overall result of biotyping, plasmid profile, and virulence factors was compared. Therefore, we conclude that these atypical serotype Z isolated in Bangladesh had a clonal relationship with Ya and Yb of Bangladesh and the *opt* gene played an important role in serotypic switching among them. Current serotyping scheme of *S. flexneri* strains fails to place many such atypical strains (1c, 1c+6, 1d, type 4, and 4c) including *S. flexneri* Z isolated from different parts of the world. Therefore, an updated serotyping scheme for identification of subserotypes of *S. flexneri* has been proposed to avoid multiple naming of the same subserotype having similar agglutination pattern.

**Introduction**

Shigellosis is one of the major causes of morbidity and mortality in developing countries like Bangladesh, especially among children under five years [1–3]. A shigellosis survey between 2000 and 2004 in six Asian countries including Bangladesh, China, Pakistan, Indonesia, Vietnam and Thailand showed that the overall annual incidence of treated shigellosis was 2.1/1000 per year in all ages and 13.2/1000 per year in children under 5 years old, which was much higher than that in industrialized countries [2]. The Global Burden of Disease Study 2015 estimates that Shigellosis causes 164,410 deaths worldwide in which 98.5% occurred in low and middle income countries and 33% in children under 5 years old [4]. The disease is caused by *Shigella* spp. that colonizes the intestinal mucosa and continues to threaten public health mainly in less developed countries with conditions of poor sanitation. Moreover, clinical infection can be carried out by as little as 10 *Shigella* organisms even without neutralization of gastric acid [5]. Despite a significant global burden of the disease and worldwide spread of antibiotics resistance, no effective vaccine against shigellosis is widely available except for a few candidate vaccines that are under development [6]. The genetic and epidemiological switching leading to chronologic variability between serotypes, and emergence of atypical and new variants strains throughout the world often complicates the development of an effective vaccine [7–11]. As immunity to *Shigella* is serotype specific, vaccine protection will therefore depend on the representation of each serotype in the vaccine or at least the predominant serotypes. Therefore, estimating the disease burden due to *Shigella* infection and understanding it’s epidemiology in countries where the disease is mostly prevalent have utmost importance [12–14].

Based on biochemical and serological properties, the genus *Shigella* is divided into four species: *S. dysenteriae*, *S. flexneri*, *S. sonnei*, and *S. boydii*. *S. flexneri* causes far more cases of dysentery than other species of *Shigella* in developing countries including Bangladesh [9, 15]. A set of absorbed rabbit antisera against the type specific (I–VI) antigenic factors and group specific (3.4, 6, 7.8) factors are routinely being used for serotyping of *S. flexneri* that subdivide isolates into 13 serotypes (1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b, 5a, 5b, X, Y and 6) [16, 17]. However, this serological scheme of *S. flexneri* is not comprehensive since this cannot type several atypical and novel subserotypes [10, 18]. In addition, the absorbed antisera often show residual cross-reactivity or are of lower titer since there are vast structural similarities between different serotypes of *S. flexneri* [19]. To overcome this constrain, monoclonal antisera of *S. flexneri* (MASF) can be used instead of the commercially available absorbed antisera [20]. Until recent, six type
specific (I, II, IV-2, V, VI and 1c) and four group specific (Y-5, 6, 7.8 and IV-1) monoclonal antibodies are being developed in order to type all the existing subserotypes of *S. flexneri* including nontypeable strains [16, 17].

A Wzy/Wzx dependent pathway, common to all *S. flexneri* except for *S. flexneri* type 6, synthesize the common repeating tetrasaccharide unit (referred to serotype Y), to which type and/or group specific determinants including α-D-glucopyranosyl and/or O-acetyl and/or phosphoethanol amine (PEtN) groups are added exhibiting variation in O antigen [21–23]. These modifications are mostly conferred by prophage-encoded glucosyltransferase and/or acetyltransferase genes [24–26]. Recently a plasmid (6.8 Kb) mediated opt gene conferring the synthesis of a group antigen E1037 has been reported [22], albeit the antigen was first identified in 1984 in a provisional strain E1037 of *S. flexneri* by Berhard Rows [27]. Later, the antigen was detected in some strains of serotypes 4a, X, Y and 6 using E1037 specific antiserum, MASF IV-1 [20]. Immunogenetical studies showed that the optO gene encoding the phosphoethanol amine transferase enzyme that transfers ethanolamine phosphate group predominantly to Rha II and rarely to both Rha II and/or Rha III residues of O antigen [28]. Until recently eight serotype converting genes (gtrI, gtrII, gtrIV, gtrV, oac, gtrX, opt and gtr1c) responsible for the transfer of α-D-glucopyranosyl, O-acetyl and phosphoethanol amine (PEtN) have been identified [24, 29]. Based on the presence of these genes, PCR based O-serotyping technique was developed for the rapid detection of serotypes of *S. flexneri* [29, 30].

Isolation of uncommon serotypes and subserotypes of *Shigella* spp. particularly of *S. flexneri* is not a rare event. Atypical strains or novel subserotypes (i.e. *S. flexneri* 1d, type 4, 7a and 7b etc.) are being isolated in different parts of the world including Bangladesh. Even several atypical serotypes became predominant over the established serotypes in developing countries [9, 10, 18]. For example, in China, the predominant serotype of *S. flexneri* 2a was replaced by atypical strain of *S. flexneri* (-:7.8, E1037) in 2010 [31]. Despite their epidemiological importance, these serotypes are not included in the current serological scheme. Use of monoclonal antisera may provide an extended serological scheme covering these atypical strains which remain in provisional status [18, 32–34]. Among the provisional strains of *S. flexneri*, a group of atypical strains that agglutinate only with MASF IV-1 but with any type or group antigen-specific antiserum tested have been reported earlier (previously reported as 4X) and have continuously being isolated in Bangladesh since 1997 [10]. Later, this serologically atypical strain (as 4X) has also been reported from Indonesia and Argentina in 2002 and in 2010, respectively. Recently, isolates with the same serological characteristics have been reported ( provisionally designated as serotype 4s) form China [35–37]. We renamed this atypical strain as *S. flexneri* Z for the systemic nomenclature of *Shigella flexneri*. This study was aimed to characterize the Bangladeshi strains of *S. flexneri* Z using different phenotypic and molecular techniques. In addition, we proposed an updated MASF based serotyping scheme for the identification of all typical and atypical serotypes and sub-serotypes of *S. flexneri*.

**Materials and methods**

**Ethics statement**

This study was exempted from institutional review board (IRB) approval at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) given that no experimental procedures were performed and no patient or subject identifiers were collected.

**Bacterial strains**

A total 3569 of *S. flexneri* strains were isolated and identified from patients with diarrhoea attending the Dhaka treatment center of the International Centre for Diarrhoeal Disease...
Research, Bangladesh (icddr,b) following the standard microbiological and biochemical methods [38], between January 1997 and December 2015. In this study, randomly selected 65 strains of \textit{S. flexneri} Z [-:E1037] variants were included for characterization using different phenotypic and genotypic techniques and compared the same with other subserotypes of \textit{S. flexneri} obtained from our laboratory collection. All strains were grown in trypticase soy broth with 0.3% yeast extract (TSBY) and stored at -70˚C after adding 15% glycerol. YSH6000 \textit{S. flexneri} 2a, having 140MDa invasive plasmid, were used as positive control in the polymerase chain reaction (PCR) for detection of \textit{ipaH} gene, \textit{ial}, \textit{Shigella} enterotoxin gene (\textit{set}) and Serine protease autotransporter of Enterobacteriaceae (SPATE) genes (\textit{sigA, pic, sat and sepA}) while an \textit{E. coli} (ATCC 25922) strain lacking 140 MDa plasmid serves as a negative controls in PCR and also antibiotic susceptibility testing.

**Serotyping**

Serotyping of these isolates was performed using (i) a commercially available antisera kit (Denka Seiken, Tokyo, Japan) specific for all type- and group-factor antigens and (ii) a panel of monoclonal antisera (Reagensia AB, Stockholm, Sweden) reagents specific for all \textit{S. flexneri} type- and group-factor antigens [10]. For the serotyped designation, we followed an extended version of serotyping scheme described in Table 1. PCR based assay targeting the O-antigen synthesis gene \textit{wzx} and the O-antigen modification genes: \textit{gtI}, \textit{gtII}, \textit{gtrI}, \textit{gtrII}, \textit{gtrIV}, \textit{gtrV}, \textit{gtrX}, and \textit{opt} were used to detect the molecular serotype of \textit{S. flexneri} according to procedure described elsewhere [29, 30].

| Serotype | Previously designated name | Type antigen specific antisera (MASF) | MASF | Group antigen specific antisera (MASF) |
|----------|-----------------------------|---------------------------------------|------|--------------------------------------|
|          |                             | I         | II    | IV-2    | V      | VI   | Ic  | B  | Y-5 | 6 | 7,8 | IV-1 |
| \textit{S. flexneri} 1a | 1a                           | +               |       | +      |       | +   |     |     |     |     |     |     |
| \textit{S. flexneri} 1b | 1b                           | +               |       | +      |       | +   |     |     |     |     |     |     |
| \textit{S. flexneri} 1d | 1d[18]                      | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} 2a | 2a                           | +               |       |        |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} 2b | 2b                           | +               |       |        |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} 3a | 3a                           | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} 3b | 3b                           | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} 4a | 4a                           | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} 4b | 4b                           | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} 4c | 4c [17]                      | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} 4d | type4 [33]                   | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} 5a | 5a                           | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} 5b | 5b                           | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} 6a | type 6                       | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} 6b | type 6                       | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} 7a | 1c                           | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} 7b | 1c+6 [30]                    | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} Xa | X                            | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} Xb | Xv [22]                      | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} Ya | Y                            | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} Yb | Yv [22]                      | +               |       | +      |       |     |     |     |     |     |     |     |
| \textit{S. flexneri} Z  | 4X, 4s [10, 35]              | +               |       | +      |       |     |     |     |     |     |     |     |
Biochemical characterization

The biochemical reactions of the strains were performed by standard biochemical methods [38].

Antimicrobial susceptibility testing

Bacterial susceptibility to antimicrobial agents including ampicillin (AMP 10 μg), sulfoo méthoxazole-trimethoprim (SXT 23.75/1.25 μg), nalidixic acid (NAL 30 μg), ciprofloxacin (CIP 5 μg), ceftriaxone (CRO 30 μg), ceftazidime (CAZ 30 μg) and amoxicillin–clavulanic acid (AMC 20/10 μg) available from commercial manufacturer (Oxoid, Basingstoke, United Kingdom) were determined by the disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2017). E. coli ATCC 25922, E. coli ATCC 35218 and Staphylococcus aureus ATCC 25923 were used as control strains for susceptibility testing.

Isolation of plasmid DNA

Plasmid DNA was prepared according to the alkaline lysis method of Kado and Liu with some modifications described elsewhere [33, 40]. The molecular weight of the unknown plasmid DNA was assessed by comparing with the mobility of the known molecular weight plasmids [41]. Plasmids present in strains E. coli PDK-9 (140, 105, 2.7 and 2.1 MDa), R1 (62 MDa), RP-4 (36 MDa), Sa (23 MDa) and V517 (35.8, 4.8, 3.7, 3.4, 3.1, 2.0, 1.8 and 1.4 MDa) were used as molecular mass standards.

Determination of the role of transferable plasmid factor

Three strains of S. flexneri Z, K-5851 (AMP^R^SXT^R^NAL^R^CAZR^CRO^R^AMC^R^), KD-1170 (AMP^R^SXT^R^NAL^R^CAZR^CRO^R^AMC^R^), and K-7468 (AMP^R^SXT^R^NAL^R^CAZR^CRO^R^AMC^R^) were selected as donor strain for the conjugation experiment. Each of the selected donor strains were mated with a recipient strain E. coli K-12 (lac^+^NAL^R^F^-) according to the method described previously [33]. Transconjugants colonies were selected on the MacConkey agar plates containing ampicillin (100 mg/L) and nalidixic acid (50 mg/L) and the transfer frequency of the resistance plasmid was calculated by a method described earlier [42]. Plasmid profile analysis, plasmid curing and antimicrobial susceptibility test of the transconjugants strains were performed to determine the transfer of plasmids with antibiotic resistance phenotype.

Detection of virulence genes and SPATE genes by PCR

All the strains of 2a, 2b, Xa, Xb, Ya (n = 25), Yb (n = 10) and Z (n = 65) were tested for virulence genes: set1 (ShET-1), sen (ShET-2), ial, ipaH genes, and a set of SPATE genes (sat, pic, sepA and sigA) commonly found in Shigella spp. by PCR using primer sets described previously with mentioned annealing temperature [43, 44].

Pulsed-field gel electrophoresis (PFGE)

To determine the clonal relationship of S. flexneri Z variant and closely related subserotypes, 29 representative strains of S. flexneri Z were compared with 13 strains of S. flexneri Ya, three S. flexneri Yb, three S. flexneri Xa and one strains of Xb using PFGE typing. Intact agarose embedded chromosomal DNA was prepared according to the guideline of pulsenet [45, 46]. Genomic DNA was digested with the XbaI restriction enzyme for 4 h at 37˚C and the restriction fragments were separated by using CHEF-MAPPER system apparatus (Bio-Rad Laboratories) under the following conditions: switching time from 5 s to 35 s at 6 V cm⁻¹ for 18 h at 14˚C. PFGE images were analyzed using the fingerprint analysis software BioNumerics version
4.5 (Applied Maths; Kortrijk, Belgium). The dendrogram constructed using the PFGE patterns was generated by the UPGMA algorithm with the Dice-predicted similarity value of two patterns at 1.0% pattern optimization and 1.5% band position tolerance.

Results

Serological typing

All the isolates of \textit{S. flexneri} were confirmed using the MASF B antisera in slide agglutination reaction suggesting that these strains were subserotypes of \textit{S. flexneri}. Among 3569 \textit{S. flexneri}, 95 isolates (2.7\%) were identified as the atypical strains of \textit{S. flexneri}, designated as \textit{S. flexneri} Z that strongly agglutinated only with MASF IV-I but did not agglutinate with other group or type specific antisera of commercially available both absorbed polyvalent and monoclonal antisera. These 95 strains of \textit{S. flexneri} Z neither agglutinated with type specific antisera nor with group specific antisera available in commercial antisera kit. The isolation rate of \textit{S. flexneri} Z was reduced from 9.7\% in 1997–2000 to 1.1\% in 2013–15 (Table 2).

Molecular serotyping

Of the 95 \textit{S. flexneri} Z isolates, 65 were randomly selected for molecular serotyping using multiplex PCR. All the 65 isolates were positive for \textit{wzx}_{1-5} gene which is commonly found in all \textit{S. flexneri}, except for serotype 6. Additionally, \textit{opt} gene was detected in all the \textit{S. flexneri} Z isolates. We detected both \textit{wzx}_{1-5} and \textit{opt} genes in all strains of \textit{S. flexneri} Z and Yb.

Table 2. Prevalence of \textit{Shigella flexneri} in Bangladesh between 1997 and 2015.

|             | 1997–00 | 2001–04 | 2005–08 | 2009–12 | 2013–15 | 1997–2015 |
|-------------|---------|---------|---------|---------|---------|-----------|
| \textit{S. flexneri} 1a | 2       | 6       | 5       | 1       | 1       | 15        |
| \textit{S. flexneri} 1b | 69      | 73      | 103     | 22      | 5       | 272       |
| \textit{S. flexneri} 1d | 0       | 0       | 0       | 0       | 0       | 0         |
| \textit{S. flexneri} 2a | 70      | 713     | 234     | 382     | 203     | 1602      |
| \textit{S. flexneri} 2b | 105     | 217     | 59      | 20      | 3       | 404       |
| \textit{S. flexneri} 3a | 70      | 263     | 111     | 100     | 46      | 590       |
| \textit{S. flexneri} 3b | 0       | 1       | 1       | 4       | 1       | 7         |
| \textit{S. flexneri} 4a | 0       | 2       | 0       | 2       | 0       | 4         |
| \textit{S. flexneri} 4b | 0       | 1       | 0       | 1       | 0       | 2         |
| \textit{S. flexneri} 4c | 0       | 0       | 0       | 0       | 0       | 0         |
| \textit{S. flexneri} 4d | 17      | 32      | 16      | 9       | 11      | 85        |
| \textit{S. flexneri} 5a | 0       | 0       | 0       | 1       | 0       | 1         |
| \textit{S. flexneri} 5b | 0       | 0       | 0       | 3       | 1       | 5         |
| \textit{S. flexneri} 6a | 12      | 58      | 38      | 68      | 22      | 198       |
| \textit{S. flexneri} 6b | 10      | 23      | 16      | 35      | 13      | 97        |
| \textit{S. flexneri} 7a | 37      | 80      | 13      | 79      | 37      | 246       |
| \textit{S. flexneri} 7b | 0       | 0       | 0       | 0       | 0       | 0         |
| \textit{S. flexneri} Xa | 1       | 1       | 2       | 1       | 1       | 6         |
| \textit{S. flexneri} Xb | 0       | 0       | 0       | 1       | 0       | 1         |
| \textit{S. flexneri} Ya | 10      | 7       | 1       | 8       | 2       | 28        |
| \textit{S. flexneri} Yb | 9       | 10      | 0       | 4       | 2       | 25        |
| \textit{S. flexneri} Z  | 39      | 30      | 17      | 5       | 4       | 95        |
| Total       | 404     | 1477    | 603     | 734     | 351     | 3569      |

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Biochemical characterization

All *S. flexneri* serotype Z isolates possessed the biochemical characteristics typical of *S. flexneri*, including negative reactions in utilization of sodium acetate, rhamnose, xylose, raffinose, ornithine, arginine, and lysine [47]. Around 58% of the isolates showed positive reaction in arabinose fermentation while 70% showed positive reaction in trehalose fermentation at variable time intervals. Interestingly, all the *S. flexneri* Z, Ya and Yb isolates produced indole after utilizing tryptophan, which was in contrast to the strains of *S. flexneri* 2a, 2b, Xa and Xb. Based on the biochemical reaction patterns *S. flexneri* Z isolates appeared to be more similar to *S. flexneri* Ya and Yb (Table 3).

Antibiotic susceptibility test

Of the 65 *S. flexneri* Z isolates tested for antibiotic susceptibility, 61% were resistant to ampicillin, followed by 57% of SXT, 55% to nalidixic acid and 41% to ciprofloxacin. Around 45% of isolates were resistant to three or more classes of antibiotics and thus identified these as multidrug resistant (MDR). Two isolates (K-7468 and KD-1170) were resistant to third generation cephalosporin including ceftriaxone, ceftazidime and amoxicillin-clavulanic acid. Both isolates were positive for extended spectrum beta lactamase (ESBL) as detected by using double disc synergy method. None of the isolates were resistant to nalidixic acid and ciprofloxacin.

Plasmid profile analysis

Analysis of plasmid DNA revealed that all the strains of *S. flexneri* Z harbored the 140 MDa plasmid along with three or four small sized (<6 MDa) plasmids (Fig 1). Additionally, a middle ranged plasmid (20–80 MDa) was detected in 37 strains (62%) of Z variant. The prevalent plasmid pattern P1a (140, 2.6, 1.8 and 1.6 MDa) was found in 39 (87%) strains while the remaining four strains were belonged to P1b (140, 4.0, 2.6, 1.8 and 1.6 MDa). Both plasmid patterns were observed in serotype Ya and Yb (Table 4). However, serotype Xa and Xb contained plasmid pattern P2 (140, 3.4 2.7 and 2.1 MDa) while serotype 2a and 2b had plasmids of slightly different sizes (140, 2.7 and 2.1 MDa) (P3).

Determination of the role of transferable plasmid

Three strains of *S. flexneri* Z with different plasmid patterns were selected for conjugation experiment with *E. coli* strain K-12 (Lac<sup>+</sup> F<sup>-</sup> NAL<sup>R</sup>). After conjugation, a plasmid of 36 MDa from both K-7468 and KD-1170 strains was transferred to the recipient strain of *E. coli* K12 with the full spectrum of resistance to AMP, SXT, CRO, CAZ and AMC. Transfer frequency for both isolates were similar. However, in case of K-5851, a plasmid of 62 MDa was transferred with a frequency of 10-fold higher (6×10<sup>-4</sup>) than the previous two isolates with resistance to AMP and SXT only (Table 5). Transfer of antimicrobial resistance through transmissible plasmids was confirmed by curing the plasmids of transconjugants.

Detection of virulence and SPATE genes by PCR

*ipaH, ial, sen, sigA and sepA* genes were detected in all the tested *S. flexneri* Ya, Yb and Z strains, but none of the isolates were found to be positive for *shet1, sat, pic* and *pet* genes. However, all these nine genes were detected in each of the strains of serotype Xa, Xb, 2a and 2b.

Pulsed-field gel electrophoresis

The dendrogram generated based on *XbaI*-digested PFGE banding patterns demonstrated that all the 29 strains of *S. flexneri* Z isolated from Bangladesh were formed a single cluster (cluster 1) at 80% similarity level which was further divided into four subclusters (1A, 1B, 1C.
Table 3. Biochemical characteristics of representative strains of *S. flexneri* serotype Z and other serotypes.

| Subserotypes of *S. flexneri* | No of tested strains | Indole prod\(^a\) | Mannitol | Arginine decarboxylase | Glucose | Arabinose | Raffinose | Rhamnose | Trehalose | Sodium acetate | Maltose | Xylose | Mannose | Biotype |
|-------------------------------|----------------------|-------------------|----------|------------------------|---------|-----------|-----------|----------|-----------|----------------|---------|--------|---------|---------|
| Z                             | 65                   | 100%              | 100%     | 4.5%                   | 100%    | (58%+)\(^2\) | 0%        | 0%       | 30% (70% +) | 0%     | 100%    | 0%      | 100%    | B1      |
| Yb                            | 10                   | 100%              | 100%     | 0%                     | 100%    | (60%+)\(^2\) | 0%        | 0%       | 20% (80% +) | 0%     | 100%    | 0%      | 100%    | B1      |
| Ya                            | 25                   | 100%              | 100%     | 4%                     | 100%    | (60%)\(^2\) | 0%        | 0%       | 33% (67% +) | 0%     | 100%    | 0%      | 100%    | B1      |
| Xa                            | 4                    | 0%                | 100%     | 0%                     | 100%    | 0%        | 0%        | 0%       | (100%+) | 0%     | 100%    | 0%      | 100%    | B2      |
| Xb                            | 1                    | 0%                | 100%     | 0%                     | 100%    | 0%        | 0%        | 0%       | (100%+) | 0%     | 100%    | 0%      | 100%    | B2      |
| 2a                            | 5                    | 0%                | 100%     | 0%                     | 100%    | 80% (20% +) | 0%        | 0%       | (100%+) | 0%     | 100%    | 0%      | 100%    | B2      |
| 2b                            | 5                    | 0%                | 100%     | 0%                     | 100%    | 100%      | 0%        | 0%       | (100%+) | 0%     | 100%    | 0%      | 100%    | B2      |

\(^a\) in parenthesis, strains required prolong incubation time (≥4 days) for fermentation.

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and 1D at 85%). Strains in each of the subclusters were grouped and intermingled with strains of Ya and Yb serotypes. On the other hand, serotypes 2a, 2b, Xa and Xb grouped in cluster 2 were completely different from the cluster 1 (Fig 2).

**Discussion**

Diversity of O antigen in *S. flexneri* evolves due to presence of serotype converting genes on horizontal transmissible elements [24, 29]. These genes encode enzymes which modify the
Characterization of

common tetrasaccharide unit (except serotype 6) of S. flexneri. As a result, atypical strains or newer subserotypes of S. flexneri are being isolated from different parts of the world, including Bangladesh [9, 10, 18]. In 2001, we reported three atypical strains of S. flexneri namely type 4, 1c and 4X which could not be definitively serotyped using commercially available antisera [10]. Therefore, for the identification of subserotypes of S. flexneri, a set of MASF antisera were used for a definitive serotyping of S. flexneri that are not included in the current serotyping scheme of S. flexneri. A group of atypical strains, designated as S. flexneri Z (previously reported as 4X), that only agglutinated with MASF IV-I but not with any other type or group specific monoclonal antibodies, were isolated in Bangladesh [10]. These atypical strains were also reported from Indonesia and Argentina but not characterized in detail [2, 36]. In 2010, this serotype (provisionally designated as 4s) was reported from China having clonal relationship with serotype 2, Xa, Xb, Ya and Yb [35, 37]. Since characterization of the atypical serotype isolated in Bangladesh remained undone, in this study, a comparative study was carried out to determine their relationship with other serotype of S. flexneri using different typing tools based on phenotypic and molecular traits.

S. flexneri Z isolates could not be serotyped using absorbed antisera that are commercially available (Denka Seiken co. Japan), but only agglutinated with MASF IV-I group factor (Table 1). The opt gene, responsible for MASF IV-I seroreactive phenotype was detected in all the S. flexneri Z tested. Although PCR based detection of O-serotype of S. flexneri and serotyping using monoclonal antisera had showed consistent result, but it could not differentiate between the Yb and Z serotypes that gave similar amplification result (wzx1-5, opt). On the other hand, antisera based serotyping could differentiate between these two serotype Yb and Z, since MASF Y-5 specifically agglutinated with Yb strains, but not with Z strains. Since no

Table 4. Plasmid profile of representative strains of S. flexneri serotype Z and other serotypes.

| Serotype | No. of strains | 140 MDa | 80–20 MDa (%) | 4.0 MDa | 3.4 MDa | 2.7 MDa | 2.6 MDa | 2.1 MDa | 1.8 MDa | 1.6 MDa | plasmid pattern |
|----------|----------------|---------|---------------|---------|---------|---------|---------|---------|---------|---------|----------------|
| Z        | 52             | +       | 50%           | +       | +       | +       | p1a     |
| Z        | 13             | +       | 70%           | +       | +       | +       | p1b     |
| Yb       | 10             | +       | 100%          | +       | +       | +       | p1a     |
| Ya       | 20             | +       | 75%           | +       | +       | +       | p1a     |
| Ya       | 5              | +       | 23%           | +       | +       | +       | p1b     |
| Xa       | 4              | +       | 0%            | +       | +       | +       | p2      |
| Xb       | 1              | +       | 0%            | +       | +       | +       | p2      |
| 2b       | 5              | +       | 60%           | +       | +       | +       | p3      |
| 2a       | 5              | +       | 80%           | +       | +       | +       | p3      |

Table 5. Transfer of resistance plasmid to E. coli K-12 in conjugation experiment.

| Donor | Plasmid pattern (MDa) | Acquired plasmid by strain E. coli K-12 | Transferred resistant phenotype | Transfer frequency of R-plasmid |
|-------|-----------------------|------------------------------------------|-------------------------------|--------------------------------|
| K-7468 | 140, 36, 2.6, 1.8, 1.6 | 36                                       | AMP\textsuperscript{R}, SXT\textsuperscript{R}, CRO\textsuperscript{R}, AMC\textsuperscript{R}, CAZ\textsuperscript{R} | 3×10\textsuperscript{−5} |
| KD-1170 | 140, 36, 2.6, 1.8, 1.6 | 36                                       | AMP\textsuperscript{R}, SXT\textsuperscript{R}, CRO\textsuperscript{R}, AMC\textsuperscript{R}, CAZ\textsuperscript{R} | 5×10\textsuperscript{−5} |
| K-5851 | 140, 62, 4.0, 2.6, 1.8, 1.6 | 62                                       | AMP\textsuperscript{R}, SXT\textsuperscript{R} | 6×10\textsuperscript{−4} |

\textsuperscript{R}Resistant to antimicrobial drug
genetic determinants for Y-5 is available, molecular serotyping of these isolates was not possible. Another limitation of the PCR based serotyping is that due to presence of mutation in O-antigen genes, this method may give discrepant results when cross checked with antisera based serotyping [48]. Moreover, for establishing a novel serotypes, producing and verifying a new antisera for the phenotypic schemes are much easier than identifying and validating a new primer pairs of the atypical strain[48]. Therefore, antisera based serotyping scheme is by far the gold standard for serotyping of *S. flexneri* [48].

It has been reported that a plasmid (6.85-10Kb)-borne *opt* gene may transfer to other serotypes via conjugation and eventually can convert their hosts into a MASF IV-I reactive phenotype [37]. Interestingly, we found a similar sized plasmid (~6.4 Kb) in 25% (13/52) strains of *S. flexneri Z*. However, a large proportion (75%) of the isolates was negative for this plasmid indicating that other mechanism might be involved in the acquisition and/or transmission of the gene, which accentuates further genetic investigation. Acquisition and transfer of plasmids carrying antibiotic resistance occur quite frequently in *Shigella* spp We found a plasmid of 62
MDa in size conferring resistance to ampicillin and SXT with a high degree of transfer frequency ($6 \times 10^{-4}$). Interestingly, two strains of *S. flexneri* Z harbored a 36 MDa transmissible plasmid carrying resistance to 3rd generation cephalosporin (ceftriaxone and ceftazidime) along with AMP and SXT, though the transfer frequency was 10-fold lower than that of the 62 MDa plasmid. Emergence of multiple drug resistance genes and their dissemination among these bacteria complicates the treatment strategy. Hence, more emphasis should be given to new/atypical isolates of *Shigella* spp.

In addition to the transferrable plasmids, all strains of *S. flexneri* Z harboured a 140 MDa plasmid along with three small sized stable plasmids (2.6, 1.8 and 1.6 MDa). Although the invasive plasmid of *S. flexneri* (140MDa) is extensively studied, little is known about the function of the small plasmid which constitute as a stable DNA pool. Therefore, these stable plasmids can be used as a molecular typing tool for the characterization of *S. flexneri* [41]. Overall analysis of plasmid DNA indicated that plasmid patterns of *S. flexneri* serotype Z isolates were indistinguishable from that of the serotypes Ya and Yb but distinct from those of serotype X and serotype 2. This observation was also true for biochemical properties, distribution of virulence genes and PFGE banding patterns of the isolates belonged to respective subserotypes and serogroups (Fig 2). The presence of similar plasmid pattern in isolates of different serotypes which are biochemically and genetically related to each other suggests that plasmid profiles may be a useful tool for determining their clonal relatedness.

All strains of serotype Z had biochemical characteristics typical of *S. flexneri* and very similar to that of serotypes Ya and Yb. Unlike serotype 2a, 2b, Xa and Xb, all strains of Z as well as Ya and Yb produced indole from tryptophan (Table 3). Although little is known about the effect of tryptophan utilization and indole production on characteristics of *Shigella* spp. it can be used as a biomarker for detection of *S. flexneri* Z. The dendrogram based on PFGE banding pattern was a better fit with indole production properties in which all strains belonged to cluster 1 (Ya, Yb and Z) produced indole whereas strains of cluster 2 (2a, 2b, Xa and Xb) did not. Furthermore, strains in cluster 1 contained relatively less virulence factors (*ipaH*, *ial*, *sen*, *sigA* and *sepA* genes), compared to the strains in cluster 2 (*ipaH*, *ial*, *set1*, *sen*, *sat*, *pic*, *sepA* and *sigA* genes).

In summary, Bangladeshi isolates of *S. flexneri* Ya, Yb and Z were different from isolates of serotypes 2a, 2b, Xa and Xb both at phenotypic and genotypic levels (Fig 2). Although Chinese isolates of the serotype 4s (= serotype Z) have the similar sero-agglutination pattern like *S. flexneri* Z, Chinese strains showed clonal relationship with serotype 2, Xa, Xb, Ya and Yb [35–37]. This characteristic differences between isolates from two different regions explained that Bangladeshi isolates of *S. flexneri* Z may be emerged from a different clonal population than that of the Chinese isolates. This may be attributed to the insertion of gene carrying MASFIV-1 sero-property into serotype Ya of Bangladeshi isolates through horizontal gene transfer mechanism.

*S. flexneri* Z and a few other atypical *S. flexneri* have been reported from different parts of the world that require further serological classification [9, 10, 17, 18, 22, 30, 35]. In order to meet this growing demand, we proposed an updated serotyping scheme for the identification of subserotypes of *S. flexneri*. Using this MASP based serological scheme it is possible to identify all the reported serotypes and subserotypes of *S. flexneri* including the atypical ones (Table 1).

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