Molecular characterization of serologically atypical provisional serovars of Shigella isolates from Kolkata, India

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During 2000–2004, 13 Shigella strains that were untypeable by commercially available antisera were isolated from children <5 years of age with acute diarrhoea in Kolkata. These strains were subsequently identified as Shigella dysenteriae provisional serovar 204/96 (n=3), Shigella dysenteriae provisional serovar E23507 (n=1), Shigella dysenteriae provisional serovar 19809-73 (n=1), Shigella dysenteriae provisional serovar 93-119 (n=1), Shigella flexneri provisional serovar 88-893 (n=6) and Shigella boydii provisional serovar E16553 (n=1). In this study, characterization of those provisional serovars of Shigella was performed with respect to their antimicrobial resistance, plasmids, virulence genes and PFGE profiles. The drug resistant strains (n=10) of Shigella identified in this study possessed various antibiotic resistance genetic markers like catA (for chloramphenicol resistance); tetA and tetB (for tetracycline resistance); dfrA1 and sul2 (for co-trimoxazole resistance); aadA1, strA and strB (for streptomycin resistance) and blaOXA-1 (for ampicillin resistance). Class 1 and/or class 2 integrons were present in eight resistant strains. Three study strains were pan-susceptible. A single mutation in the gyrA gene (serine to leucine at codon 83) was present in four quinolone resistant strains. The virulence gene ipaH (invasion plasmid antigen H) was uniformly present in all strains in this study, but the stx (Shiga toxin) and set1 (Shigella enterotoxin 1) genes were absent. Other virulence genes like ial (invasion associated locus) and sen (Shigella enterotoxin 2) were occasionally present. A large plasmid of 212 kb and of incompatibility type IncFIIA was present in the majority of the strains (n=10) and diversity was noticed in the smaller plasmid profiles of these strains even within the same provisional serovars. PFGE profile analysis showed the presence of multiple unrelated clones among the isolates of provisional Shigella serovars. To the best of our knowledge, this is the first report on the phenotypic and molecular characterization of provisional serovars of Shigella isolates from Kolkata, India.

INTRODUCTION

Shigellosis or blood dysentery represents a significant public health problem worldwide especially in developing countries. It is estimated that more than 160 million people suffer from shigellosis annually with more than 1 million deaths, the majority of which occur in children less than 5 years of age (Kotloff et al., 1999). The causative agent Shigella spp. is generally represented by four species or subgroups based on biochemical and serological properties: Shigella dysenteriae, Shigella flexneri, Shigella boydii and Shigella sonnei. Except Shigella sonnei, each subgroup may be further subdivided into several serotypes and subspecies based on reactivity (agglutination) with type specific antisera. Current nomenclature of Shigella spp. includes 15 serotypes of Shigella dysenteriae, 14 serotypes of Shigella flexneri, 20 serotypes of Shigella boydii and two phase variations of Shigella sonnei (van der Ploeg et al., 2010; Perepelov et al., 2012). The commercially available antisera

Abbreviations: Ak, amikacin; Amc, amoxicillin–clavulanic acid; Amp, ampicillin; Atm, aztreonam; Azm, azithromycin; Caz, ceftazidime; Cip, ciprofloxacin; Cm, chloramphenicol; Cro, ceftriaxone; Ctx, cefotaxime; CTZ, co-trimoxazole; Gm, gentamicin; Nal, nalidixic acid; Nor, norfloxacin; Ofx, ofloxacin; PMQR, plasmid-mediated quinolone resistance; QRDR, quinolone resistance determining-regions; Strep, streptomycin; Tet, tetracycline.

One supplementary table is available in the online Supplementary Material.
may not cover all possible epitopes of the LPS O-antigen of Shigella serotypes. Probably a multitude of epitopes were not included by the typing scheme currently in use. New serotypes or subserotypes of Shigella are not uncommon and are reported from different parts of the world (Gross et al., 1982, 1989; Shmilovitz et al., 1985; Matsushita et al., 1992, 1998; Ansaruzzaman et al., 1995, 2005; Coimbra et al., 2001; Ueda et al., 2001; Talukder et al., 2007; Qiu et al., 2011). Isolates biochemically resembling Shigella species, but that do not belong to any of the recognized O-serogroups, are described as provisional Shigella serovars until a sufficient number of isolates have been collected to determine whether a provisional serovar should be assigned to a Shigella nomen(s)pecies. Validation is performed by the World Health Organization International Collaborating Center for Shigella located at the Centers for Disease Control and Prevention (CDC), Atlanta (Coimbra et al., 2001; Ansaruzzaman et al., 2005).

Multidrug resistance has been occasionally reported in provisional serovars of Shigella isolates (Gross et al., 1982; Shmilovitz et al., 1985; Ansaruzzaman et al., 1995; Ueda et al., 2001; Talukder et al., 2007; Qiu et al., 2011). Shigella spp. possesses a multitude of virulence markers associated with colonization of intestinal cells and intracellular invasion (Vargas et al., 1999). Genetic loci like ipaH (invasion plasmid antigen H) and ial (invasion associated locus), located on the chromosomal DNA and/or heavy plasmid DNA (of 120–140 MDa), are important because probes and primers developed for amplifying these loci have been used for rapid identification of culture negative shigellosis cases (Dutta et al., 2001). Apart from Shiga toxin, present only in Shigella dysenteriae type 1 serotype, at least two more enterotoxins like ShET-1 (Shigella enterotoxin 1) and ShET-2 (Shigella enterotoxin 2) have been reported in Shigella spp., which are thought to be responsible for initial watery diarrhoea in shigellosis patients (Vargas et al., 1999). Molecular typing of strains by PFGE is the most popular method to determine the relatedness among the isolates (ECDC, 2013).

In earlier studies, we reported the isolation of seven strains biochemically resembling Shigella spp. but non-agglutinable with commercially available Shigella antisera (Denka Seiken) (Dutta et al., 2003). Subsequently, six more strains that were agglutinable with Shigella flexneri polyclera but untypable with monoserum and isolated from hospital attending children <5 years of age in Kolkata during 2000–2004 were reported (Nandy et al., 2010). These isolates were sent to the Metropolitan Research Laboratory of Public Health, Tokyo, Japan for further identification and serotyping. However, the phenotypic and genetic characterization of the Kolkata isolates was not carried out earlier. Hence, the aim of this study was to characterize those serologically atypical strains of Shigella isolated from Kolkata with respect to their antimicrobial resistance profiles, plasmid profiles, virulence genes profiles and PFGE profiles.

**METHODS**

**Study strains.** Altogether 13 aforesaid atypical strains of Shigella non-agglutinable with either poly- or mono-valent Shigella antisera were included in this study for characterization. Those strains were identified at Metropolitan Research Laboratory of Public Health, Tokyo, Japan as follows: Shigella dysenteriae provisional serovar 204/96 (n = 3), Shigella dysenteriae provisional serovar E23507 (n = 1), Shigella dysenteriae provisional serovar 19809-73 (n = 1), Shigella dysenteriae provisional serovar 93-119 (n = 1), Shigella flexneri provisional serovar 88-893 (n = 6) and Shigella boydii provisional serovar E16553 (n = 1). Contemporary sporadic isolates of Shigella flexneri serotype 2a (n = 4) from Kolkata were included as standards along with the study strains for comparison and interpretation.

**Determination of antimicrobial susceptibility and MIC.** The study strains were tested for their antimicrobial susceptibility against a panel of 17 antibiotics discs (Becton Dickinson) on Mueller–Hinton agar (Difco) following the Kirby–Bauer disc diffusion method. The antimicrobials tested were ampicillin (Amp, 10 μg), chloramphenicol (Cm, 30 μg), tetracycline (Tet, 30 μg), co-trimoxazole (CTZ, 25 μg), nalidixic acid (Nal, 30 μg), ciprofloxacin (Cip, 5 μg), norfloxacin (Nor, 10 μg), ofloxacin (Ofx, 5 μg), gentamicin (Gm, 10 μg), amikacin (Ak, 30 μg), streptomycin (Strep, 10 μg), cefotaxime (Ctx, 30 μg), cefazidime (Caz, 30 μg), ceftriaxone (Ciro, 30 μg), aztreonam (Atm, 30 μg), azithromycin (Azm, 15 μg) and amoxicillin–clavulanic acid (Amc, 30 μg).

The MIC of each antimicrobial for the resistant strains was determined by E-test (AB Biodisk). Interpretation of the result was performed as per the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2013). Escherichia coli ATCC 25922 was used as control.

**Determination of antimicrobial resistance genes and integrons.** The study strains were screened for the presence of the following antimicrobial resistance genes by PCR using genomic DNA as template and published primer sequences (Table S1, available in the online Supplementary Material): _catA_ (for chloramphenicol resistance); _tetA_ and _tetB_ (for tetracycline resistance); _dflA_, _dfvII_, _sul1_, _sul2_ and _sul3_ (for co-trimoxazole resistance); _aadA_, _strA_ and _strB_ (for streptomycin resistance); _blaTEM_, _blaSHV_ and _blaOXA_ (for β-lactamase); _int1_ and _int2_ genes (for presence of class 1 and class 2 integrons, respectively). Presence of the _aacDA1_ gene at the 3’ end of the conserved segment of the class 1 integron was also determined by PCR. Resistance gene alleles and gene cassettes within the integrons were determined by direct sequencing of the PCR product using 3730 DNA Analyser (Applied Biosystems). The obtained sequences were analysed by comparison with the sequences in databases by BLAST (www.ncbi.nlm.nih.gov/blast) and CLUSTAL W (www.ebi.ac.uk/) programs. Suitable positive and negative (reagent) controls were included in each PCR run.

**Determination of chromosomal- and/or plasmid-mediated quinolone resistance (PMQR).** Chromosomal quinolone resistance was determined by the presence of mutations in the quinolone resistance-determining regions (QRDR) of _gryA_, _gryB_, _parC_ and _parE_ genes by PCR followed by sequencing of the PCR products. PMQR was determined by the presence of _qnrA_, _qnrB_, _qnrS_, _aac(6’)-Ib-cr_ and _qepA_ genes by PCR. All PCR was carried out using published primers (Table S1) and bacterial genomic DNA as template. DNA sequences obtained were compared with sequences obtained from databases as described earlier.

**Conjugation experimentation.** Conjugation was performed to determine the potential for transfer of antimicrobial resistance by broth mating procedure in Brain Heart Infusion broth (Difco), using _E. coli_ K-12 MG1655 (all susceptible) as the recipient strain (Gebre-Yohannes & Drasar, 1991) and the study strains as donors. Transconjugants were selected for on MacConkey agar (Difco) containing antimicrobials.
[chloramphenicol (32 μg ml⁻¹) or tetracycline (25 μg ml⁻¹) or streptomycin (50 μg ml⁻¹)] as lactose-fermenting colonies of *E. coli*. The transfer of antimicrobial resistance in transconjugants was checked by determining the antimicrobial resistance profiles by disc diffusion, MIC by E-test, and presence of respective resistance genes by PCR.

**Detection of virulence genes.** The study strains were screened by PCR for the presence of virulence markers like *ipaH*, *ial*, *set1* (*Shigella enterotoxin 1*), *sen* (*Shigella enterotoxin 2*) and *stx* (*Shiga toxin*) using published primer sequences (Table S1). Suitable controls were used in each run.

**Plasmid profile analysis and incompatibility typing.** Plasmid profiling was performed by extracting small and medium sized plasmids using the GenElute Plasmid Miniprep kit (Sigma Aldrich). Large plasmids were extracted following the protocol of Kado & Liu (1981). The plasmids were electrophoresed on 0.8 % (w/v) agarose gels, visualized under UV-transilluminator after staining with ethidium bromide (10 mg ml⁻¹) and photographed by the Gel-Documentation system (Bio-Rad). The approximate plasmid size was determined by Quantity One software version 4.5 (Bio-Rad) using plasmid molecular mass markers as reference. A supercoiled plasmid DNA (2–10 kb) ladder (Bangalore Genei) was used for smaller plasmids; *E. coli* V517 and *Shigella flexneri* YSH6000 were used for heavy plasmids. The gel image was captured by the Gel-Documentation system (Bio-Rad) and the PFGE profiles were analysed using FP Quest software version 4.5 (Bio-Rad). The extent of homology was determined by Dice coefficient and clustering was based on the unweighted pair group method with arithmetic mean (UPGMA) algorithm. The band patterns were interpreted as previously described (Tenover et al., 1995).

**Molecular typing of isolates by PFGE.** DNA fingerprinting was performed by PFGE of XbaI digested genomic DNA of the study strains, using CHEF DRIII (Bio-Rad), following the PulseNet one-day standard protocol (CDC, 2013). *Salmonella enterica* serovar Braenderup H9812 was used as the reference strain. The gel image was captured by the Gel-Documentation system (Bio-Rad) and the PFGE profiles were analysed using FP Quest software version 4.5 (Bio-Rad). The extent of homology was determined by Dice coefficient and clustering was based on the unweighted pair group method with arithmetic mean (UPGMA) algorithm. The band patterns were interpreted as previously described (Tenover et al., 1995).

**RESULTS**

From 2000 to 2004, 13 serologically atypical provisional serovars of *Shigella* were isolated in Kolkata. During the same period, a total of 384 shigelae with known serotypes were isolated with *Shigella flexneri* (*n=218*) being the predominant serotype, followed by *Shigella sonnei* (*n=95*), *Shigella dysenteriae* (*n=45*) and *Shigella boydii* (*n=26*). No difference in the clinical signs and symptoms was observed among the patients suffering from shigellosis caused by typical or atypical serovars of *Shigella* isolates.

**Antimicrobial resistance profiles and MICs**

The antimicrobial resistance profiles and MICs of the antimicrobials to which the 13 study strains were resistant are shown in Table 1. Among six profiles observed in the resistant isolates (*n=10*), the predominant profile was *Tet/CTZ/Strep* (*n=3*) followed by *Amp/Cm/Tet/CTZ/Strep* (*n=2*), *Cm/Tet/CTZ/Strep/quinoalone (Nal)* (*n=2*), *Tet/CTZ/Strep/Nal* (*n=1*), *Amp/Cm/Tet/Strep* (*n=1*) and Nal (*n=1*). Three study strains were pan-susceptible. None of the study strains was resistant to fluoroquinolones or third generation cephalosporins.

**Mechanism of antibiotic resistance and transferability**

Presence of antibiotic resistance genes like *catA*, *tetA*, *tetB*, *dfrA1*, *sul2*, *aadA1*, *strA*, *strB* and *blaOXA-1* and integrons among the study strains are shown in Table 1. The antibiotic resistance markers were not transferable to the susceptible recipient strains by conjugation.

Quinolone (Nal) resistance in the study strains (three *Shigella flexneri* and one *Shigella boydii* isolates) was associated with mutation (TCG→TTG, Ser→Leu) at codon 83 position of the gyrA gene (Table 1). No mutations were detected in gyrB, parC or parE genes. Genes encoding PMQR like *qnrA*, *qnrB*, *qnrS*, *aac(6’)-Ib-cr* and *qepA* were absent in the study strains.

Eight MDR study strains harboured integrons of classes 1 and/or 2 (Table 1). More than one class 1 integron, having gene cassettes of different sizes (500 bp and ≥2.5 kb), were found in three study strains by PCR (Table 1). The *qacEΔ1* and *sul1* genes were absent at the 3' end of the conserved segment of the class 1 integron. A class 2 integron with a gene cassette (of approximately 1.3 kb) was detected in five study strains. On sequencing, *dfrA1-sat* resistance genes were found within the gene cassette of class 2 integrons. On sequencing, the 500 bp PCR amplicon of the class 1 integron did not show the presence of any resistance gene cassette. Sequencing of larger PCR products (2.5 kb and 3.5 kb) was not possible in this study.

**Presence of virulence genes**

The virulence gene profiles of the study strains are shown in Table 1. The *ipaH* gene was present in all strains. None of the study strains possessed the *set1* or the *stx* gene. The *ial* and *sen* genes were occasionally present in *Shigella dysenteriae* and *Shigella flexneri* study strains.

**Plasmid profiles and incompatibility typing**

We observed major diversity in the plasmid patterns of all the 13 study strains including strains within the same provisional serovars (Fig. 1a, b). Interestingly, all the four *Shigella flexneri* 2a contemporary strains with identical antimicrobial resistance profiles exhibited identical plasmid profiles with plasmid sizes 3.7, 4.8 and 7.2 kb (Fig. 1b, lanes 7–10). A large plasmid of 212 kb (140 MDa) was present in most (*n=10*) of the study strains (Table 1; not shown in the figures). Plasmid of incompatibility type IncFIIA was found in all the study strains harbouring a large plasmid of 212 kb, while strains harbouring only small plasmids remained untypable (Table 1).

**Molecular typing by PFGE**

The PFGE analysis showed non-clonality of the strains belonging to the provisional serovars of *Shigella* whereas 93.2 % relatedness was observed among four contemporary *Shigella flexneri* 2a strains (Fig. 2). A total of 13 provisional strains of *Shigella* generated 12 different pulsotypes.
<table>
<thead>
<tr>
<th>Sl. no.</th>
<th>Shigella provisional serovars (current designation)</th>
<th>No. of isolates</th>
<th>Sample no.</th>
<th>Year of isolation</th>
<th>R-profile (MIC in ( \mu g , ml^{-1} ))</th>
<th>Genes mediating antimicrobial resistance</th>
<th>Mutations in the QRDR of gyrA</th>
<th>Presence of integron (approximate size of gene cassette in kb)</th>
<th>Presence of heavy plasmid (212 kb)</th>
<th>Plasmid type by PCR</th>
<th>Virulence genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Shigella boydii</em> E16553 (Shigella boydii serotype 19)</td>
<td>1</td>
<td>BCH-6347</td>
<td>2000</td>
<td>Tet (96), CTZ (&gt;32), Strep (96), Nal (&gt;256)</td>
<td>tetA, dfra1, sul2, strA-B, aadA1</td>
<td>S83L</td>
<td>Class 2 (1.3)</td>
<td>Present</td>
<td>IncFIIA</td>
<td>ipaH, ial, sen</td>
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<td>2</td>
<td><em>Shigella dysenteriae</em> 204/96</td>
<td>3</td>
<td>BCH-2588</td>
<td>2000</td>
<td>Tet (96), CTZ (&gt;32), Strep (96)</td>
<td>tetB, dfra1, sul2, strA-B</td>
<td>NA</td>
<td>Class 2 (1.3)</td>
<td>Present</td>
<td>IncFIIA</td>
<td>ipaH, ial, sen</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BCH-3824</td>
<td>2001</td>
<td>Amp (&gt;256), Cm (&gt;256), Tet (96), CTZ (&gt;32), Strep (48)</td>
<td>blaOXA-1, catA, tetB, dfra1, aadA1</td>
<td>NA</td>
<td>Class 1 (0.5 and 2.5), class 2 (1.3)</td>
<td>Present</td>
<td>IncFIIA</td>
<td>ipaH, ial, sen</td>
</tr>
<tr>
<td>3</td>
<td><em>Shigella dysenteriae</em> 93-119</td>
<td>1</td>
<td>BCH-2834</td>
<td>2000</td>
<td>Tet (96), CTZ (&gt;32), Strep (128)</td>
<td>tetA, sul2, strA-B</td>
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<td>NA</td>
<td>Present</td>
<td>IncFIIA</td>
<td>ipaH, ial, sen</td>
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<td>4</td>
<td><em>Shigella dysenteriae</em> E23507 (Shigella dysenteriae serotype 15)</td>
<td>1</td>
<td>BCH-3853</td>
<td>2001</td>
<td>Pan-susceptible</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Present</td>
<td>IncFIIA</td>
<td>ipaH, ial, sen</td>
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<td>5</td>
<td><em>Shigella dysenteriae</em> I9809-73 (Shigella dysenteriae serotype 13)</td>
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<td>BCH-4139</td>
<td>2001</td>
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<td>blaOXA-1, catA, tetB, dfra1, aadA1</td>
<td>NA</td>
<td>Class 1 (0.5 and 2.5), class 2 (1.3)</td>
<td>Present</td>
<td>IncFIIA</td>
<td>ipaH, ial</td>
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<td>6</td>
<td><em>Shigella flexneri</em> 88-893 (Shigella flexneri serotype 7b)</td>
<td>6</td>
<td>BCH-4191</td>
<td>2002</td>
<td>Cm (&gt;256), Tet (96), CTZ (&gt;32), Strep (128), Nal (&gt;256)</td>
<td>catA, tetB, dfra1, aadA1</td>
<td>S83L</td>
<td>Class 1 (0.5)</td>
<td>Present</td>
<td>IncFIIA</td>
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<td>BCH-4285</td>
<td>2002</td>
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<td>ipaH</td>
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<td></td>
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<td>BCH-5435</td>
<td>2003</td>
<td>Nal (&gt;256)</td>
<td>NA</td>
<td>S83L</td>
<td>NA</td>
<td>Present Absent untypable</td>
<td>ipaH</td>
<td></td>
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<td></td>
<td></td>
<td>BCH-5449</td>
<td>2003</td>
<td>Amp (&gt;256), Cm (&gt;256), Tet (96), Strep (96)</td>
<td>blaOXA-1, catA, tetB, dfra1, aadA1</td>
<td>NA</td>
<td>Class 1 (0.5 and 3.5)</td>
<td>Present Absent untypable</td>
<td>ipaH</td>
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<td>BCH-5863</td>
<td>2004</td>
<td>Pan-susceptible</td>
<td>NA</td>
<td>S83L</td>
<td>Class 1 (0.5)</td>
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<td>ipaH</td>
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<td>BCH-6141</td>
<td>2004</td>
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<td>catA, tetB, dfra1, sul2, aadA1</td>
<td>NA</td>
<td>NA</td>
<td>Absent untypable</td>
<td>ipaH, ial</td>
<td></td>
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</table>

Sl., serial; R-profile, antibiotic resistance profile; blaOXA-1, ampicillin resistance; catA, chloramphenicol resistance; tetB, tetracycline resistance; dfra1, co-trimoxazole resistance; sul2, co-trimoxazole resistance; aadA1, streptomycin resistance; NA, not applicable.
Strains belonging to *Shigella dysenteriae* and *Shigella flexneri* (except one BCH-5435) provisional serovars formed distinct clusters A and B, respectively. Cluster A showed two different subclusters (A1 and A2) which were 63.5% similar. Subcluster A1 comprised *Shigella dysenteriae* provisional serovar 204/96 (*n*=3) strains, which showed similarity of 69.6 to 90.3% among them. Subcluster A2 consisted of three different provisional serovars of *Shigella dysenteriae* with three distinct pulsotypes and more than 77.8% relatedness. *Shigella flexneri* provisional serovar 88-893 (*n*=6) generated two separate subclusters (B1 and B2), which were 60.6% similar. *Shigella flexneri* provisional serovar 88-893 (*n*=3) strains of the B1 subcluster were 70.8% related, whereas *Shigella flexneri* provisional serovars 88-893 (*n*=2) of the B2 subcluster were clonal (100% related) and 74.4% related to *Shigella flexneri* 2a strains. The *Shigella flexneri* provisional serovar 88-893 strain (BCH-5435), which did not belong to cluster B, showed 69% similarity to *Shigella boydii* provisional serovar E16533.

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**Fig. 1.** Plasmid profiles of sporadic isolates of provisional serovars of *Shigella* from Kolkata, India, 2000–2004 on 0.8% (w/v) agarose gel. (a) Plasmid profiles of provisional *Shigella boydii* and *Shigella dysenteriae* study strains. Lanes: 1, supercoiled plasmid DNA ladder; 2, BCH-6347 (*Shigella boydii* E16553); 3, BCH-2588 (*Shigella dysenteriae* 204/96); 4, BCH-3824 (*Shigella dysenteriae* 204/96); 5, BCH-4318 (*Shigella dysenteriae* 204/96); 6, BCH-3853 (*Shigella dysenteriae* E23507); 7, BCH-2834 (*Shigella dysenteriae* 93-119); 8, BCH-4139 (*Shigella dysenteriae* 9809-73); 9, *E. coli* V517 plasmid molecular mass marker. (b) Plasmid profiles of provisional *Shigella flexneri* 88-893 (lanes 1–6) and *Shigella flexneri* 2a (lanes 7–10) sporadic isolates. Lanes: M, supercoiled plasmid DNA ladder; 1, BCH-4191; 2, BCH-6141; 3, BCH-4285; 4, BCH-5449; 5, BCH-5863; 6, BCH-5435; 7, BCH-5909; 8, BCH-6023; 9, BCH-6037; 10, BCH-6152.

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**Fig. 2.** PFGE profiles of XbaI digested DNA of sporadic isolates of provisional serovars of *Shigella* by cluster analysis and comparison with sporadic *Shigella flexneri* 2a isolates, Kolkata, India, 2000–2004. R-profile, antibiotic resistance profile.
**DISCUSSION**

Shigella flexneri provisional serovar 88-893, having both group 6 and a new type antigen (distinct from known type antigen 1-VI of Shigella flexneri) was first reported from Tokyo during 1986–1989 from stool cultures of domestic as well as imported cases and during 1993–2000 from travellers with diarrhoea at Osaka and Kansai Airport-Quarantine Station (Matsushita et al., 1992; Toshima et al., 1992; Ueda et al., 2001). Recently, the structure of the repeating unit of new O-antigen was elucidated and this provisional serovar has been proposed to be included as Shigella flexneri serotype 7b in the Shigella typing scheme (Foster et al., 2011). Since 1979, Shigella boydii provisional serovar E16553 has been isolated from stools of patients in Britain, Finland, Iceland, Sweden and Japan who had mainly travelled to India (Gross et al., 1982). During 1995–1997, 15 isolates of Shigella boydii provisional serovar E16553 were reported from Bangladesh and this provisional serovar has been given the status of a new serotype Shigella boydii serotype 19 (Ansaruzzaman et al., 2005). During 1972–1980, 17 strains of Shigella dysenteriae provisional serovar 19809-73 were isolated from stool cultures of sporadic acute diarrhoeal cases in Israel. During the same period, Shigella dysenteriae provisional serovar 19809-73 was also isolated from the USA and the UK. Subsequently, in 1984 these strains were also isolated from 14 out of 70 cases involved in an institutional outbreak of diarrhoea in Israel (Shmilovitz et al., 1985). Isolation of another Shigella dysenteriae provisional serovar E23507 was reported from various geographical locations like Sweden, Japan and Bangladesh (Gross et al., 1989; Ansaruzzaman et al., 1995). Based on biochemical, serological, virulence profiles of the atypical Shigella dysenteriae strains and their isolation from various geographical regions, Shigella dysenteriae provisional serovar 19809-73 and Shigella dysenteriae provisional serovar E23507 have been included in the Shigella dysenteriae typing scheme as Shigella dysenteriae serotype 13 and Shigella dysenteriae serotype 15, respectively (Wathen-Grady et al., 1990; Ansaruzzaman et al., 1995). In 1997, a single strain of Shigella dysenteriae provisional serovar 93-119 was first reported from one imported diarrhoeal case in Japan and in 1998, five strains of Shigella dysenteriae provisional serovar 204/96 were isolated from diarrhoeal patients in Japan having history of travel to India and Nepal (Matsushita et al., 1997; 1998). Subsequently, two strains of Shigella dysenteriae provisional serovar 93-119 and four strains of Shigella dysenteriae provisional serovar 204/96 were reported from diarrhoea cases at Osaka and Kansai Airport-Quarantine Station, from patients who had travelled to South West Asia (Ueda et al., 2001). Twenty-two strains of Shigella dysenteriae provisional serovar 204/96 were also reported from dysentery patients in France during 1997–1998 (Coimbra et al., 2001). Shigella dysenteriae provisional serovar 93-119 and Shigella dysenteriae provisional serovar 204/96 are yet to be included in the Shigella typing scheme. Multidrug resistance (resistance to two or more classes of antibiotics) was observed in nine study strains from Kolkata (Table 1). In the past, multidrug resistance (resistance to two or more of the following antimicrobials: Strep, Cm, Tet, CTZ, Amp and Nal) was also reported in Shigella flexneri provisional serovar 88-893, Shigella dysenteriae provisional serovar 204/96 and Shigella dysenteriae provisional serovar 93-119 from Japan; in Shigella boydii provisional serovar E16553 from Britain and Japan; in Shigella dysenteriae provisional serovar 19809-73 from Israel and Japan; and in Shigella dysenteriae provisional serovar E23507 isolates from Sweden and Bangladesh (Gross et al., 1982, 1989; Shmilovitz et al., 1985; Ansaruzzaman et al., 1995; Matsushita et al., 1998; Ueda et al., 2001). None of the study strains was resistant to fluoroquinolones or third generation cephalosporins, but resistance to ciprofloxacin and ceftriaxone has been reported in typical Shigella serovars from India as well as other Asian countries (Kuo et al., 2008; Nandy et al., 2010; Tariq et al., 2012).

The presence of antibiotic resistance genes (catA, tetA, tetB, dfrA1, sul2, aadA1, strA, strB and blabOXA-1), (class 1 and class 2) and a mutation (TCG→TTG; Ser→Leu) at codon 83 position of the gyrA gene was not investigated in the earlier studies reporting Shigella provisional serovars (Gross et al., 1982; Ansaruzzaman et al., 1995; Matsushita et al., 1998; Ueda et al., 2001), but these resistance genes were detected in many of the study strains (Table 1). The typical class 2 integron has a gene cassette of 2.2 kb with dfrA1-sat-aadA1 genes conferring resistance to trimethoprim, streptomycin and spectinomycin/streptomycin, respectively (Gassama Sow et al., 2010). However, the study strains (n=5) harboured a truncated class 2 integron with a 1.3 kb gene cassette. A truncated class 2 integron of similar size has been previously reported in typical Shigella serotypes, where the aadA1 gene was absent (Gassama Sow et al., 2010).

With respect to the virulence gene profiles, similar profiles were observed among the provisional Shigella dysenteriae study strains (Table 1). In earlier studies, Shigella dysenteriae provisional serovar 204/96 and Shigella boydii provisional serovar E16553 strains possessing the ipaH and sen genes were reported (Coimbra et al., 2001; Ansaruzzaman et al., 2005).

Profile analysis of smaller plasmids in shigellae has been used as an epidemiological tool for investigating outbreaks and determining strain diversity in a particular serotype (Nandy et al., 2010). The presence of a large plasmid of 212 kb (120–140 MDa) in the study strains was usually associated with invasiveness in Shigella serotypes as reported earlier in Shigella dysenteriae provisional serovar E23507 and Shigella boydii provisional serovar E16533 from Bangladesh (Ansaruzzaman et al., 1995, 2005). Multiple plasmids of different sizes (ranging from 1.4 to 3.4 MDa) were reported in Shigella boydii provisional serovar E16533 from Bangladesh (Ansaruzzaman et al., 2005), as was observed in this study. Plasmids of incompatibility type IncFIIA were found in study strains possessing the large plasmid (212 kb). The plasmid incompatibility type of the provisional serovars of Shigella was not reported earlier.
Molecular typing by PFGE is used to determine the relatedness among the isolated strains and to study the molecular epidemiology of the organism in a specific geographical region. In this study, multiple clones were present among the new serovars of Shigella spp. indicating diverse origin of the study strains.

This is the first report to our knowledge where characterization of the atypical shigellae serovars from Kolkata has been carried out with respect to antimicrobial resistance, virulence, plasmid profiles and molecular subtypes of the isolates. Although the number of atypical shigellae strains in this study was only 13, thorough and stringent monitoring of isolation of such atypical strains would help to understand the actual disease burden caused by the new Shigella serovars and consequently to study the epidemiology of shigellosis as a whole.

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