A novel serovar of *Shigella dysenteriae* from patients with diarrhoea in Bangladesh

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Every year, around 3% of isolates from patients with diarrhoea at Dhaka Hospital, ICDDR,B, are identified as *Shigella*-like organisms (SLOs) based on their activity in biochemical tests. These isolates do not react with any of the current *Shigella* antisera including all existing and provisional serotypes. Among these SLOs, a unique cluster of seven isolates with an identical plasmid profile was found and these isolates were further characterized by phenotypic and genotypic techniques. All were nonlactose fermenters, with an identical biochemical pattern typical of *Shigella dysenteriae*. They were classified as invasive since they harboured the 140 MDa invasive plasmid, were able to bind Congo red, produced keratoconjunctivitis in the guinea pig eye, and were positive by PCR for the *ipaH* gene and *Shigella* enterotoxin 2 [ShET-2] gene. All isolates were resistant to ampicillin, tetracycline and sulfamethoxazole–trimethoprim but were susceptible to mecillinam, nalidixic acid, ceftriaxone and ciprofloxacin. Six of the isolates were identical in DNA pattern by PFGE with the seventh exhibiting a closely related pattern; both patterns were distinguishable from all other *Shigella* and *Escherichia coli* patterns. An antiserum prepared against one of the isolates reacted with all isolates and did not cross-react with other *Shigella* and *E. coli* serotype reference strains. It is therefore proposed that these isolates represent a new provisional serovar of *S. dysenteriae*, type strain KIVI 162.

**INTRODUCTION**

Shigellosis is one of the major diarrhoeal diseases in Bangladesh and other developing countries. It has been estimated that more than 95,000 children <5 years of age die of shigellosis annually in Bangladesh (Kotloff et al., 1999). *Shigella* is spread by direct faecal–hand–oral contact wherever personal hygiene is compromised (Weissman et al., 1975) and outbreaks are difficult to control because of the low infectious dose of the organisms (DuPont et al., 1989).

The genus *Shigella* comprises four subgroups that historically have been considered species (*Enterobacteriaceae* Subcommittee of the Nomenclature Committee of the International Association of Microbiological Societies, 1958): subgroup A is referred to as *Shigella dysenteriae*, subgroup B as *Shigella flexneri*, subgroup C as *Shigella boydii* and subgroup D as *Shigella sonnei*. Speciation of *Shigella* is not easily achieved based on biochemical properties alone, and definitive identification requires serotyping to be performed (Ewing et al., 1958; Ewing & Lindberg, 1984). Based on somatic O antigens, *S. dysenteriae* is subdivided into 15 serogroups (Bopp et al., 2003) and 2 provisional serogroups (Coimbra et al., 2001; Melito et al., 2005) and *S. flexneri* into 6 serogroups with 14 subserotypes (Bopp et al., 2003). *S. boydii* comprises 19 serogroups. Ewing (1986) acknowledged 17 serogroups, which included two provisional serogroups represented by reference strain 2710-54 as *S. boydii* 16 and 3615-53 as *S. boydii* 17. Gross et al. (1980) reported a new provisional serogroup isolated in Bangladesh and designated serogroup 18 with the reference strain E10163. Recently, provisional *S. boydii* serovar E16553, designated serogroup 19, has been reported by several groups from diverse geographical origins (Gross et al., 1982; Anasaruzzaman et al., 2005); and serogroup 20 was recently proposed and confirmed as novel by Woodward et al. (2005).

In a previous study, we found that a significant number of strains isolated between 1999 and 2002 had biochemical properties typical of *Shigella* but could not be serotyped.
following the current serotyping scheme (Talukder et al., 2003). These strains were designated Shigella-like organisms (SLOs) and approximately 3% of Shigella infections at ICDDR,B hospital are caused by the such organisms (Talukder et al., 2003). The prevalence of SLOs has increased over the years, accentuating the necessity for extensive characterization of these strains. In a retrospective study, we tested all SLOs with all available antisera specific for Shigella including existing, provisional and atypical types. Isolates were also tested for plasmid profiles as most Shigella serotypes contain a wide array of plasmids, some of which are serotype specific (Talukder et al., 2003). This revealed a cluster of seven isolates with identical plasmid patterns and which were not agglutinated by Shigella antisera. We describe here the properties of these isolates with respect to activity in biochemical tests, the presence of pathogenicity-associated genes, chromosomal DNA profiles and reactivity with a serum raised against one of their number.

METHODS

Isolates. Seven isolates from 153 SLOs isolated from patients with diarrhoea attending the Dhaka Treatment Centre operated by ICDDR,B in Bangladesh were selected for this study. These isolates were non-reactive with available Shigella antisera and were isolated in the clinical microbiology laboratory with standard biochemical and microbiological methods (World Health Organization, 1987). All isolates were tested further in API 20E kits (bioMérieux). Strain E112707-96, provisional S. dysenteriae serotype, was received from Dr Thomas Cheasty, ESYS Reference Laboratory (Laboratory of Enteric Pathogens, Health Protection Agency, Centre for Infections, London, UK). YSH6000, S. flexneri 2a (Talukder et al., 2002), and an Escherichia coli strain (ATCC 25922) lacking the 140 MDa invasive plasmid and susceptible to all antibiotics were used as positive and strain (ATCC 25922) lacking the 140 MDa invasive plasmid patterns and which were not agglutinated by Shigella antisera. We describe here the properties of these isolates with respect to activity in biochemical tests, the presence of pathogenicity-associated genes, chromosomal DNA profiles and reactivity with a serum raised against one of their number.

Biochemical characterization. Isolates were subjected to a range of biochemical tests by conventional methods as described by Ewing (1986).

Serotyping. Isolates were tested in the local laboratory with commercially available antisera (Denka Seiken) specific for all recognized Shigella serovars and with antisera raised against serovars S. dysenteriae 13, 14 and 15. They were subcultured on MacConkey agar (Difco, Becton Dickinson) and incubated for 18–20 h at 37 °C. Slide agglutination tests were performed as described previously (Talukder et al., 2002). Isolates were also tested in the ESYS reference laboratory with all available serogroups of Shigella and E. coli. An antisera was raised in rabbits against a heat-killed suspension of a representative isolate (KIVI 162) according to the protocol of Ewing & Lindberg (1984). This antisera was tested against other SLOs, S. dysenteriae serotypes 13, 14 and 15, S. boydii serotypes 19 and 20, and enteroinvasive E. coli serotypes O28ac, O29, O124, O136, O143, O144 and O164.

Agglutinin-absorption tests. When cross-reactions were found, reciprocal absorptions were performed to determine the extent of the antigenic relationship between the strains (Wathen-Grady et al., 1985). Serological cross-reactions were considered significant if the heterologous titre was >1/16 of the homologous titre.

Antimicrobial susceptibility. Susceptibility to antimicrobial agents was determined on Mueller–Hinton agar by the disk diffusion method following the guidelines of the Clinical and Laboratory Standards Institute (2004). The antimicrobial discs used were ampicillin (10 μg), tetracycline (30 μg), mecillinam (25 μg), nalidixic acid (30 μg), sulfamethoxazole–trimethoprim (25 μg), ciprofloxacin (5 μg) and ceftixime (30 μg). E. coli ATCC 25922 and Staphylococcus aureus ATCC 25923 were used as control strains.

KERATOCONJUNCTIVITIS ASSAY. This test was performed according to the procedure of Sereny (1957). Briefly, an overnight culture of bacteria, of approximately 10^10 viable cells in 20 μl PBS, was introduced into the conjunctival sac of the guinea pig. The other eye served as the control. The guinea pigs were observed daily for 72 h and inflammatory responses were graded. The experimental procedures were approved by the animal experimentation ethics committee of the ICDDR,B.

Congo red binding ability. Trypsin Case broth containing 0.3% yeast extract with 1.5% agar supplemented with 0.01% Congo red (Sigma) was used to study the pigment binding ability of isolates according to the procedures of Sasakawa et al. (1986).

Detection of virulence genes by PCR. DNA was prepared from the seven isolates and examined by PCR for the presence of the invasion-associated locus (ial) and the invasion plasmid antigen H (iapH) of Shigella (Vargas et al., 1999). PCR to demonstrate the presence of Shiga toxin 1 (stx1) and Shigella enterotoxins (set1 and set2) was performed according to published procedures (Bonnet et al., 1998; Vargas et al., 1999). Primer sequences are listed in Table 1; reactions were performed in a thermal cycler (PTC-200; MJ Research) and amplification products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide.

Plasmid DNA. Plasmid DNA was prepared according to the alkaline lysis method of Kado & Liu (1981) with some modifications (Talukder et al., 2002). The molecular mass of the plasmid DNA bands was assessed by comparison with the mobility of known molecular mass plasmids in E. coli PDK-9, R1, RP4, Sa and V517 in agarose gels (Talukder et al., 2002).

PFGE. Intact agarose-embedded chromosomal DNA was prepared, digested with XbaI (Gibco-BRL), and restriction fragments were resolved in a contour-clamped homogeneous electric field (CHEF-DRII) apparatus (Bio-Rad) following the procedure of Talukder et al. (2002). Pulse times were 1–6 s for 9 h, 3–40 s for 12 h, and 5–70 s for 13 h. The gel was stained, destained and photographed on a gel documentation system and bands were sized against a 48.5–1000 kb molecular mass ladder (Bio-Rad).

RESULTS

Biochemical characterization

All isolates showed biochemical reactions typical of Shigella species. They were oxidase- and catalase-negative, and did not ferment D-mannitol, sucrose, lactose, rhamnose, raffinose, xylose, trehalose or dulcitol. All utilized arginine and were lysine- and ornithine-negative. API 20E tests identified all isolates as Shigella species.

Serotyping

All isolates were negative in agglutination tests for Shigella and E. coli serotypes and reacted only with the antiserum
raised against KIVI 162. This was confirmed by the ESYV reference laboratory in London, but the isolates did react with an antiserum against a provisionally recognized *S. dysenteriae* serotype, E112707-96 (unpublished data).

**Antibiotic susceptibility**

All isolates were resistant to trimethoprim–sulfamethoxazole, tetracycline and ampicillin and were susceptible to nalidixic acid, ciprofloxacin, ceftriaxone and mecillinam.

**Test for invasiveness**

Congo red dye was bound by all isolates and they caused keratoconjunctivitis in the guinea pig eye within 48 h.

**Virulence pathogenicity**

All isolates were positive for *ial*, *ipaH* and *sen* genes and were negative for *stx1* and *set1* genes. In contrast, the provisional strain E112707-96 was negative for *ial* and *sen* genes and only positive for *ipaH*.

**Plasmid DNA**

All isolates harboured the 140 MDa invasive plasmid and two other plasmids of approximately 2.7 and 2.0 MDa in size. This plasmid pattern was different from that of other *Shigella* serotypes tested, including the provisional strain E112707-96, which lacked the 140 MDa plasmid but contained another plasmid of approximately 65 MDa.

**PFGE**

All isolates were indistinguishable from each other by their *XbaI* restriction pattern. This pattern differed markedly from that of other serotypes, including the provisional *S. dysenteriae* serotype E112707-96 (Fig. 1).

**DISCUSSION**

SLOs are consistently isolated from the hospitalized diarrhoeal population in Bangladesh. During the period 1999–2002, *S. flexneri* accounted for 60% of the total *Shigella* infections followed by *S. boydii* (17%), *S. flexneri*.

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**Table 1.** Primers and conditions used for identification of virulence genes of *Shigella* spp.

<table>
<thead>
<tr>
<th>Gene encoding virulence factor</th>
<th>Primer</th>
<th>Oligonucleotide sequence (5′–3′)</th>
<th>Location within gene</th>
<th>Size of the amplified product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ipaH</em></td>
<td>Shig-1</td>
<td>TGGAAAAACTCAGTGCCTCT</td>
<td>1063–1083</td>
<td>423</td>
<td>Vargas et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>Shig-2</td>
<td>CCAGTCCGGTAATTTCATTCT</td>
<td>1466–1485</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>ial</em></td>
<td><em>ial</em> upper</td>
<td>CTGGATGGTATATGGTGAGG</td>
<td>5340–5357</td>
<td>320</td>
<td>Vargas et al. (1999)</td>
</tr>
<tr>
<td></td>
<td><em>ial</em> lower</td>
<td>GGAGGCCAACAATTTCATTCC</td>
<td>5640–5659</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>sen</em></td>
<td>ShET-2 upper</td>
<td>ATGTGCTGCTATAATTGAT</td>
<td>380–399</td>
<td>799</td>
<td>Vargas et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>ShET-2 lower</td>
<td>CATATAATAAACCTGCTCACGC</td>
<td>1158–1178</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>set1A</em></td>
<td>ShET-1A upper</td>
<td>TCCGGCTACATCAAAGA</td>
<td>460–477</td>
<td>309</td>
<td>Fasano et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>ShET-1A lower</td>
<td>TATCCCTCCTTGGTATGTA</td>
<td>751–768</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>set1B</em></td>
<td>ShET-1B upper</td>
<td>GTGACCCTCTGCGCATTAC</td>
<td>70–89</td>
<td>147</td>
<td>Fasano et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>ShET-1B lower</td>
<td>ATTAGTGGATAAAAAATGACG</td>
<td>197–216</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>stx1</em></td>
<td><em>stx1</em> upper</td>
<td>ACCCTGTAAAGTTGGCCG</td>
<td>–</td>
<td>140</td>
<td>Bonnet et al. (1998)</td>
</tr>
<tr>
<td></td>
<td><em>stx1</em> lower</td>
<td>ATCTCATGCGACTACTGGAC</td>
<td>–</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** PFGE patterns of *XbaI*-digested chromosomal DNA from seven SLOs and provisional *S. dysenteriae* serotype E112707-96. Lanes: 1 and 10, lambda molecular mass ladders; 2–8, SLO isolates; 9, provisional *S. dysenteriae* serotype E112707-96.
Table 2. Characteristics of the SLOs studied

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Biotyping*</th>
<th>Antibiotic susceptibility†</th>
<th>Presence of virulence/invasive genes</th>
<th>Test for invasiveness</th>
<th>Plasmid typing‡</th>
<th>PFGE typing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>stx</td>
<td>set1</td>
<td>set2</td>
<td>iai</td>
</tr>
<tr>
<td>KIVI 162</td>
<td>B1</td>
<td>R1</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KIVI 165</td>
<td>B1</td>
<td>R1</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KIVI 185</td>
<td>B1</td>
<td>R1</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KIVI 208</td>
<td>B1</td>
<td>R1</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KIVI 216</td>
<td>B1</td>
<td>R1</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KIVI 217</td>
<td>B1</td>
<td>R1</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KIVI 219</td>
<td>B1</td>
<td>R1</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E112707-96</td>
<td>B2</td>
<td>R2</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* B1, Glucose +, arabinose +, maltose +, arginine +; B2, glucose +, arabinose +, maltose +, arginine +, mannose +.
† R1, Amp′ Sx′ Tet′ Mel′ Nal′ Cip′; R2, Amp′ Sx′ Tet′ Mel′ Nal′ Cip′.
‡ P1, 140, 2.7, 2.0 MDa; P2, 65, 3, 1.8 MDa.
§ CRB, Congo red binding ability.

*dysenteriae* (12%) and *S. sonnei* (8%) then SLOs (3%) (Talukder et al., 2003). Serological classification is insufficient for the identification of novel *Shigella* species and a polyphasic approach utilizing biochemical, serological and molecular characterization is advocated (Anisurzaman et al., 2005). In this study, we characterized seven SLO isolates recovered over 3 years in hospitalized patients with diarrhea (Table 2). They all had biochemical properties compatible with *S. dysenteriae* and had an antimicrobial resistance profile (ampicillin, tetracycline and trimethoprim–sulfamethoxazole) common among *S. flexneri* and *S. dysenteriae* isolates in Bangladesh (Talukder et al., 2002). Among the serotypes of *Shigella*, *S. dysenteriae* type 1 is generally the most resistant to antibiotics and appears to readily acquire resistance to newly introduced antibiotics for *Shigella* infection (Talukder et al., 2004). Indeed, all *S. dysenteriae* type 1 isolates in Bangladesh in 2004 and onwards are resistant to ciprofloxacin (Talukder et al., 2004). However, none of the seven SLO isolates was resistant to ciprofloxacin.

Although pathogenicity tests are not fundamental criteria for the classification of members of the *Enterobacteriaceae*, the findings of a positive Sereny test, the presence of a 140 MDa plasmid and the identification of *ial* and *ipaH* genes provide strong evidence in support of a classification in the genus *Shigella*. Two novel toxins, *Shigella* enterotoxin 1 (ShET-1) encoded by chromosomal gene *set1* and *Shigella* enterotoxin 2 (ShET-2) encoded by *sen* (located in the 140 MDa plasmid), have been associated with the pathogenicity of many *Shigella* species (Nataro et al., 1995; Noriega et al., 1995). To date, *S. dysenteriae* 1 is the only serotype that has been reported to produce Shiga toxin 1 and some *E. coli* strains produce Shiga-like toxin 1 and 2. Strains belonging to the other serotypes of the *Shigella* group have not been shown to produce Shiga toxin.

Serotyping is considered to be essential for the confirmation of an organism as a member of the genus *Shigella*. An antiserum raised against one of the isolates in this study (KIVI 162) reacted only with the seven isolates but not with other *Shigella* or *E. coli* reference strains. Among *E. coli* pathotypes, enteroinvasive *E. coli* are the most similar in biochemical and virulence properties to *Shigella*, which can sometimes make differentiation difficult. However, the SLO isolates did react with antiserum to an existing provisional *S. dysenteriae* serotype, designated E112707-96 (unpublished data), and this strain was strongly agglutinated by serum KIVI 162, supporting their antigenic identity.

A common genetic lineage for the SLO isolates was indicated by their similarity in plasmid and chromosomal DNA profiles. In the latter, one isolate exhibited the loss of a major band and the appearance of two minor bands in comparison with the others, but this difference was insufficient to distinguish between the isolates by the criteria of Tenover et al. (1995).

In summary, the SLO isolates characterized in this report exhibited traits characteristic of the genus *Shigella* in biochemical, serological and molecular properties. No serological cross-reactivity was observed with other *Shigella* and *E. coli* strains, except for an unpublished provisional *S. dysenteriae* serotype, E112707-96, isolated and identified by the *E. coli* reference laboratory in 1996. These isolates possess an antigenic epitope that sets this group apart from all other recognized strains of *Shigella* and we propose that they represent a novel emerging serovar of *S. dysenteriae*, type strain KIVI 162.

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