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.

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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; Ansaruzzaman *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

Abbreviation: SLOs, *Shigella*-like organisms.

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, ESYV 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 negative controls, respectively, in the Sereny test, Congo red binding ability test, and in the PCR assay for detection of *ipaH* and *Shigella* enterotoxin genes (*set*).

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 ESYV reference laboratory with all available serogroups of *Shigella* and *E. coli*. An antiserum was raised in rabbits against a heat-killed suspension of a representative isolate (KIVI 162) according to the protocol of Ewing & Lindberg (1984). This antiserum 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 $\geq 1/16$ of the homologous titre.

Antimicrobial susceptibility. Susceptibility to antimicrobial agents was determined on Mueller–Hinton agar by the disc 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 ceftriaxone (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. Trypticase soy 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 (*ipaH*) of *Shigella* (Vargas *et al.*, 1999). PCR to demonstrate the presence of Shiga toxin 1 (*stx*₁) and *Shigella* enterotoxins (*set1* and *sen*) 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 *Xba*I (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

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

Gene encoding virulence factor	Primer	Oligonucleotide sequence (5'–3')	Location within gene	Size of the amplified product (bp)	Reference
<i>ipaH</i>	Shig-1	TGGAAAACTCAGTGCCTCT	1063–1083	423	Vargas <i>et al.</i> (1999)
	Shig-2	CCAGTCCGTAAATTCATTCT	1466–1485		
<i>ial</i>	<i>ial</i> upper	CTGGATGGTATGGTGAGG	5340–5357	320	Vargas <i>et al.</i> (1999)
	<i>ial</i> lower	GGAGGCCAACAAATTATTTCC	5640–5659		
<i>sen</i>	ShET-2 upper	ATGTGCCTGCTATTATTTAT	380–399	799	Vargas <i>et al.</i> (1999)
	ShET-2 lower	CATAATAATAAGCGGTCAGC	1158–1178		
<i>set1A</i>	ShET-1A upper	TCACGCTACCATCAAAGA	460–477	309	Fasano <i>et al.</i> (1995)
	ShET-1A lower	TATCCCCCTTTGGTGGA	751–768		
<i>set1B</i>	ShET-1B upper	GTGAACCTGCTGCCGATATC	70–89	147	Fasano <i>et al.</i> (1995)
	ShET-1B lower	ATTAGTGGATAAAAATGACG	197–216		
<i>stx₁</i>	<i>stx₁</i> upper	ACCCTGTAACGAAGTTTGCG	–	140	Bonnet <i>et al.</i> (1998)
	<i>stx₁</i> lower	ATCTCATGCGACTACTTGAC			

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 *Xba*I 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.*

Fig. 1. PFGE patterns of *Xba*I-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

–, Negative; +, positive.

Strain no.	Biotyping*	Antibiotic susceptibility†	Presence of virulence/invasive genes					Test for invasiveness			Plasmid typing‡	PFGE typing
			<i>stx</i>	<i>set1</i>	<i>set2</i>	<i>ial</i>	<i>ipaH</i>	140 MDa	Sereny test	CRB§		
KIVI 162	B1	R1	–	–	+	+	+	+	+	+	P1	A
KIVI 165	B1	R1	–	–	+	+	+	+	+	+	P1	A
KIVI 185	B1	R1	–	–	+	+	+	+	+	+	P1	A
KIVI 208	B1	R1	–	–	+	+	+	+	+	+	P1	A
KIVI 216	B1	R1	–	–	+	+	+	+	+	+	P1	A
KIVI 217	B1	R1	–	–	+	+	+	+	+	+	P1	A1
KIVI 219	B1	R1	–	–	+	+	+	+	+	+	P1	A
E112707-96	B2	R2	–	–	–	–	+	–	–	–	P2	B

*B1, Glucose +, arabinose +, maltose +, arginine +; B2, glucose +, arabinose +, maltose +, arginine +, mannose +.

†R1, Amp^r Sxt^r Tet^r Mel^s Nal^s Cip^s; R2, Amp^r Sxt^s Tet^r Mel^s Nal^s Cip^s.

‡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 (Ansaruz-zaman *et al.*, 2005). In this study, we characterized seven SLO isolates recovered over 3 years in hospitalized patients with diarrhoea (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 makes 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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