Antibiotic resistance and genetic diversity of *Shigella sonnei* isolated from patients with diarrhoea between 1999 and 2003 in Bangladesh

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Shigella sonnei is a significant cause of diarrhoeal infection in both developing and industrialized countries. From 1999 to 2003, 445 strains of *Shigella sonnei* were isolated from patients admitted to the diarrhoea treatment centre of the International Center for Diarrhoeal Disease Research, Bangladesh. More than 60 % of the isolates were resistant to nalidixic acid, 89 % to sulfamethoxazole-trimethoprim and 9.5 % to ampicillin. In addition, 4 % of strains were resistant to multiple antibiotics (Amp^R Tet^R Sxt^R Str^R) and 4.2 % of strains were sensitive to all antibiotics tested. None of the strains were positive for the *set1* gene, whereas 46 % were positive for the *sen* gene. Forty-six per cent of the strains (stored at –70 °C) harboured the 120 MDa invasive plasmid and representative strains produced keratoconjunctivitis in the guinea pig eye. In addition, three plasmids of approximately 5, 1.8 and 1.4 MDa were found to be present in more than 90 % of the strains. A self-transmissible, middle-ranged plasmid (35–80 MDa) carrying the multiple antibiotic resistance gene was found in some strains. PFGE analysis of the strains identified five unique types with many subtypes, which were characterized into four unique types by ribotyping analysis. It can be concluded that endemic strains of *Shigella sonnei* isolated from patients in Bangladesh are diverse in their genetic pattern.

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INTRODUCTION

Bacillary dysentery caused by *Shigella* species is a serious public health problem in both developing and industrialized countries (Lee *et al.*, 2000; Pan, 1996) and outbreaks due to *Shigella* infection are difficult to control because of their low infectious dose (Dupont *et al.*, 1989). Increased numbers of cases in a community that appear to be sporadic may in fact be due to unrecognized outbreaks (Litwin *et al.*, 1997). Shigellosis is caused by any of the four species of *Shigella*, namely *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*. Of these, *Shigella sonnei* is the most prevalent (77 %) species in industrialized countries and the second most prevalent in developing countries, followed by *Shigella flexneri* (Kotloff *et al.*, 1999). Shigellosis is endemic in Bangladesh and accounts for 20 % of deaths related to diarrhoea among children (Victoria *et al.*, 1993). Fatality rates due to *Shigella sonnei* infection are highest among children less than 1 year old in Bangladesh (Bennish *et al.*, 1990). Species-wise incidence of shigellosis including that caused by *Shigella sonnei* in Bangladesh was reported in a previous study (Talukder *et al.*, 2003b). Unlike developed and industrialized countries, an extensive study on *Shigella sonnei* in terms of clonal diversity and antimicrobial

resistance pattern has not been undertaken in the developing world and particularly in Bangladesh. In the past, several conventional typing methods were used for epidemiological subtyping of *Shigella sonnei* (Lin & Chang, 1992; Morris & Wells, 1974; Pruneda & Farmer, 1977) based on phenotypic properties, but the usefulness of these techniques is now limited, because of the availability of more discriminatory molecular typing methods, including plasmid profiling, ribotyping and PFGE. The aim of this study was to determine the aetiological significance of *Shigella sonnei* among patients with diarrhoea in Bangladesh and to determine its clonal distribution based on phenotypic and genotypic characteristics.

METHODS

Strains. One hundred and eighty-four clinical strains of *Shigella sonnei* were randomly selected from 445 strains isolated from patients attending the Dhaka treatment centre operated by the International Center for Diarrhoeal Disease Research, Bangladesh (ICDDR, B), in Bangladesh between January 1999 and December 2003. The strains were isolated and identified in the Clinical Microbiology Laboratory, according to standard microbiological and biochemical methods (World Health Organization, 1987). The

strains were grown in trypticase soy broth containing 0.3% yeast extract and stored at -70°C after addition of 15% glycerol. *Shigella flexneri* 2a YSH6000 (Sasakawa *et al.*, 1986) and an *Escherichia coli* (ATCC 25922) strain lacking the 140 MDa invasive plasmid and sensitive to all antibiotics were used as positive and negative controls, respectively, in the Sereny test and for PCR. *E. coli* K-12 (Lac^+ , F^-), resistant to nalidixic acid, was used as the recipient in the conjugation experiments (Haider *et al.*, 1989).

Serotyping. Serotypes were determined by using a commercially available antisera kit (Denka Seiken). Strains were subcultured on MacConkey agar (Difco) plates and serological reactions were performed after about 18 h of incubation by using the slide agglutination test, as described previously (Talukder *et al.*, 2001).

Biochemical characterization. The biochemical reactions of the strains were determined by using standard methods (World Health Organization, 1987).

Antimicrobial susceptibility. Bacterial susceptibility to antimicrobial agents was determined by using the disc diffusion method, as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2004) with commercial antimicrobial discs (Oxoid). The antibiotic discs used in this study were ampicillin (Amp; 10 μg), tetracycline (Tet; 30 μg), mecillinam (25 μg), nalidixic acid (Nal; 30 μg), sulfamethoxazole-trimethoprim (Sxt; 25 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), ofloxacin (5 μg), azithromycin (15 μg) and ceftriaxone (30 μg). *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as control strains for the susceptibility tests.

Test for invasiveness and detection of *Shigella* enterotoxin genes. Nine representative strains were subjected to the keratoconjunctivitis assay in the guinea pig eye, according to the procedures described by Mackel *et al.* (1961) and Sereny (1957). The experimental procedures were approved by the animal experimentation ethics committee of ICDDR, B and were in accordance with ethical guidelines for the care of animals. The same bacterial colony was used for the preparation of template DNA for PCR for *set1* and *sen* genes. The primer sets and PCR conditions used were those described by Vargas *et al.* (1999).

Plasmid profile analysis. Plasmid DNA was prepared according to the alkaline lysis method of Kado & Liu (1981), with some modifications as described previously (Talukder *et al.*, 2002). The molecular mass of the unknown plasmid DNA was assessed by comparing the mobility with that of plasmids of known molecular mass (Haider *et al.*, 1989). Plasmids present in *E. coli* PDK-9, R1, RP₄, Sa and V517 described previously (Talukder *et al.*, 2002) were used as molecular mass standards.

Determination of resistance factors. Conjugation experiments between multi-drug resistant (Amp^{R} Sxt^{R} Tet^{R} Str^{R}) *Shigella sonnei* (K-656 and K-386) strains and recipient *E. coli* K-12 (Nal^{R} , Lac^+ , F^-) were carried out according to the method described previously (Munshi *et al.*, 1987). Transconjugant colonies were selected on MacConkey agar plates containing nalidixic acid (30 $\mu\text{g ml}^{-1}$) and ampicillin (50 $\mu\text{g ml}^{-1}$). Plasmid analysis and antimicrobial susceptibility tests of the transconjugants were carried out. Determination of transfer frequency and curing of the resistance plasmid were performed as described previously (Munshi *et al.*, 1987).

Ribotyping. Total cellular DNA was extracted and purified according to the procedures described previously (Talukder *et al.*, 2002). DNA was digested with *Hind*III overnight at 37°C according to the instructions of the manufacturer (Gibco-BRL) and separated by gel electrophoresis in 0.9% agarose in Tris-borate EDTA (TBE) buffer for 18 h at a constant 35 V. Southern blotting to a positively charged nylon membrane (Amersham Pharmacia Biotech) was

performed with a vacuum pump unit (Bio-Rad) and the DNA fragments were fixed to the membrane by exposure to UV light for 3 min. A digoxigenin (DIG)-labelled cDNA probe specific for the 16S rDNA (Kariyama *et al.*, 2000) was constructed, according to the procedure described previously (Talukder *et al.*, 2002). Hybridization of the membrane with the DIG-labelled probe for 18 h at 42°C and development of the membrane with anti-DIG-alkaline phosphatase were performed according to the instructions provided in the DIG DNA labelling and detection kit (Roche Diagnostics). Results were documented by photographing the membrane.

PFGE. Intact, agarose-embedded chromosomal DNA from clinical isolates of *Shigella sonnei* was prepared and PFGE was performed using a contour-clamped homogeneous electric field (CHEF-DRII) apparatus (Bio-Rad), according to procedures described previously (Albert *et al.*, 1997; Okada *et al.*, 1991; Talukder *et al.*, 1999, 2003a) and by Brian *et al.* (1993), but with different pulse times, 1–10 s for 10 h, 3–28 s for 10 h, 3–35 s for 5 h and 5–70 s for 15 h. Genomic DNA was digested with *Xba*I (Gibco-BRL). The restriction fragments were separated by using a CHEF-DRII system apparatus in 1% pulsed-field certified agarose in $0.5\times$ TBE buffer. The gel was stained, destained and photographed using a gel documentation system, according to procedures described previously (Talukder *et al.*, 2002). The DNA size standards used were a bacteriophage lambda ladder ranging from 48.5 to 1000 kb (Bio-Rad) and *Salmonella* Branderup chromosomal DNA ranging from 15.8 to 917 kb. Band patterns were established by the criteria described by Tenover *et al.* (1995).

RESULTS

Biochemical characterization

All the 184 representative strains of *Shigella sonnei* were negative for indole production and positive for arabinose, mannitol, mannose, trehalose, maltose, rhamnose and ornithine fermentation. About 62% ($n=114$) of the strains showed raffinose fermentation, but none of the strains were positive for utilization of sodium acetate, sorbitol, dulcitol, xylose, arginine or lysine. Two different biotypes (BT1 and BT2) were found based on raffinose fermentation (Table 1).

Antimicrobial susceptibility

More than 60% of the strains in the present study were resistant to nalidixic acid, 89% to sulfamethoxazole-trimethoprim and 9.5% to ampicillin. In addition, 4% ($n=18$) of strains were found to be resistant to multiple antibiotics (Amp^{R} Tet^{R} Sxt^{R} Str^{R}) and 4.2% ($n=19$) were sensitive to all antibiotics tested. None of the strains were resistant to ciprofloxacin, norfloxacin, ofloxacin, mecillinam, azithromycin or ceftriaxone.

Test for invasiveness and detection of *Shigella* enterotoxin genes

Five representative strains containing a 120 MDa invasive plasmid were positive for keratoconjunctivitis in the guinea pig eye. In contrast, the representative four strains that did not contain a 120 MDa plasmid were found to be negative in the Sereny test. The *Shigella* enterotoxin 1 (*set1*) gene was not present in the strains of *Shigella sonnei* tested, whereas

Table 1. Characteristics of *Shigella sonnei* strains isolated in Bangladesh

Amp, Ampicillin; Nal, nalidixic acid; Sxt, trimethoprim-sulfamethoxazole.

No. of strains tested	PFGE pattern	Ribotype	Biotype*	Antibiogram (no. of strains)
27	A1	R1	BT1	Sxt ^R Nal ^R (17), Sxt ^R (10)
28	A2	R1	BT1	Sxt ^R Nal ^R (26), Sxt ^R (2)
20	A3	R1	BT1	Sxt ^R (9), Sxt ^R Nal ^R (11)
2	A4	R1	BT1	Sxt ^R Nal ^R (2)
2	A5	R1	BT1	Amp ^R Sxt ^R (2)
4	A6	R1	BT1	Amp ^R Sxt ^R (2), Sxt ^R Nal ^R (2)
4	A7	R1	BT1	Sxt ^R Nal ^R (4)
1	A8	R1	BT1	Sxt ^R (1)
2	B1	R2	BT2	All sensitive (2)
2	B2	R2	BT2	All sensitive (2)
2	C	R3	BT2	All sensitive (2)
2	D	R4	BT2	All sensitive (2)
2	E1	R1	BT2	All sensitive (2)
1	E2	R1	BT2	All sensitive (1)
1	E3	R1	BT2	All sensitive (1)

*BT1: fermentation of ornithine, lactose, arabinose, mannose, mannitol, trehalose, raffinose and maltose;

BT2: fermentation of ornithine, lactose, arabinose, mannose, mannitol, trehalose and maltose.

the *Shigella* enterotoxin 2 gene (*sen*) was present in all strains containing a 120 MDa plasmid.

Plasmid profile analysis

Heterogeneous plasmid profiles ranging in size from approximately 120 to 1.4 MDa were found among the strains. Three plasmids of approximately 5, 1.8 and 1.4 MDa in size were present in more than 90 % of the strains and were considered to be the core plasmids of *Shigella sonnei* (Fig. 1). Interestingly, 54 % of the strains ($n=100$) isolated during the period 1999–2003 were found to lack the 120 MDa invasive plasmid. A middle-ranged plasmid of approximately 35–80 MDa in size was found in 12 % ($n=22$) of the strains.

Determination of resistance factor

Two strains designated K-656 and K-386 with the resistance pattern Amp^R Tet^R Sxt^R Str^R and containing an 80 MDa plasmid were selected for conjugation with *E. coli* K-12 (Lac⁺ and Nal^R). An 80 MDa plasmid was transferred with the multiple antibiotic resistance determinants (Amp^R Tet^R Sxt^R) to the recipient cells via conjugation. The transfer frequency was very high for all the transmissible plasmids (Table 2). The transmissible resistance determinants were confirmed by curing the transconjugants (Table 2).

Ribotyping

Hybridization of *Hind*III-digested chromosomal DNA with the 16S rDNA probe revealed a total of seven to nine fragments ranging in size from 5 to 15 kb in all the

representative strains of *Shigella sonnei* (Fig. 2). Four unique rRNA gene restriction patterns, ribotypes R1, R2, R3 and R4, were obtained among the representative strains.

Fig. 1. (a) Agarose gel electrophoresis of plasmid DNA showing the representative patterns among isolates of *Shigella sonnei*. Lanes: 1, *E. coli* PDK-9 (marker); 2, *S. sonnei* K-476; 3, *S. sonnei* K-544; 4, *S. sonnei* K-548; 5, *S. sonnei* K-564; 6, *S. sonnei* K-567; 7, *S. sonnei* K-586; 8, *S. sonnei* K-1038; 9, *S. sonnei* K-1051; 10, *S. sonnei* K-1036; 11, *S. sonnei* K-1075; 12, *S. sonnei* KD-446; 13, *S. sonnei* KD-462; 14, *S. sonnei* KD-226; 15, *E. coli* V517 + R1. (b) Agarose gel electrophoresis of plasmid DNA of *Shigella sonnei* strains run for 16 h. Lanes: 1, *E. coli* PDK-9 (marker); 2, *S. sonnei* KD-412; 3, *S. sonnei* K-544; 4, *S. sonnei* K-567; 5, *S. flexneri* YSH6000.

Table 2. Results of conjugation between two strains of *Shigella sonnei* and *E. coli* K-12

Amp, Ampicillin; Nal, nalidixic acid; Str, streptomycin; Sxt, trimethoprim-sulfamethoxazole; Tet, tetracycline.

Strain no.	Parent strain		Transconjugant		Transfer frequency of R-plasmid	Cured strain	
	Resistance pattern	Plasmid profile (MDa)	Resistance pattern	Plasmid profile (MDa)		Resistance pattern	Plasmid profile (MDa)
K-656	Amp Sxt Str Tet	140, 80, 5·0, 1·8, 1·4	Amp Sxt Tet Nal	80	$4·0 \times 10^{-4}$	Nal	No plasmid
K-386	Amp Sxt Str Tet	140, 80, 2·8, 4·0, 2·1, 1·8, 1·0	Amp Sxt Tet Nal	80	$1·2 \times 10^{-4}$	Nal	No plasmid

PFGE

Of 184 strains, 100 were randomly selected for PFGE analysis. *Xba*I-digested chromosomal DNA of these strains yielded 15–24 reproducible DNA fragments, ranging in size from approximately 20 to 485 kb (Fig. 3). These strains yielded five unique PFGE types designated A, B, C, D and E with numerous subtypes (Fig. 3), of which 88 % of the strains belonged to type A, 4 % belonged to types B and E each and 2 % belonged to types C and D each (Fig. 3). Type A was further subdivided into five subtypes (A1–A5), and types B and E into two (B1 and B2) and three (E1–E3) subtypes, respectively.

DISCUSSION

We examined 184 clinical strains of *Shigella sonnei* that were isolated between January 1999 and December 2003, using biochemical characterization, antimicrobial susceptibility testing, plasmid profile analysis, PFGE and ribotyping, in order to define the aetiological significance of endemic shigellosis due to infection by *Shigella sonnei* in Bangladesh. According to our previous study, during the period 1999–2002, each year about 8 % of the total number of cases of shigellosis were caused by infection due to *Shigella*

sonnei (Talukder *et al.*, 2003b). A similar trend was observed in 2003. The first step in the characterization scheme for all strains in the present study was biochemical reaction pattern, in which we did not observe any significant change from the typical reactions of *Shigella sonnei* described by Ewing (1986). Based on the biochemical reaction profiles, strains could not be divided into different clonal groups.

Resistance of *Shigella* species to commonly used antimicrobial agents has been increasing in Bangladesh (Hossain *et al.*, 1998). Ampicillin or trimethoprim combined with sulfamethoxazole has been used to treat shigellosis during the past two decades. However, a high proportion of *Shigella sonnei* strains were resistant to these antibiotics (Kim *et al.*,

Fig. 2. Ribotyping for the representative strains of *Shigella sonnei*. Lanes: 1, K-655 (R1); 2, KD-446 (R1); 3, K-633 (R1); 4, K-656 (R1); 5, KD-412 (R1); 6, KD-501 (R1); 7, KD-632 (R1); 8, K-701 (R2); 9, K-631 (R2); 10, K-366 (R3); 11, K-368 (R4); 12, K-436 (R1); 13, KD-223 (R1); 14, K-436 (R1).

Fig. 3. PFGE banding patterns of *Xba*I-digested chromosomal DNA of representative strains of *Shigella sonnei*. Lanes: 1, K-655 (PFGE type A1); 2, KD-446 (PFGE type A2); 3, K-633 (PFGE type A3); 4, K-656 (PFGE type A4); 5, KD-412 (PFGE type A5); 6, K-564 (PFGE type B1); 7, K-701 (PFGE type B2); 8, K-631 (PFGE type B1); 9, K-366 (PFGE type C); 10, K-368 (PFGE type D); 11, K-436 (PFGE type E); 12, *Salmonella* Branderup (marker); 13, lambda ladder (marker).

2002). In this study, none of the strains were found to be resistant to ciprofloxacin, norfloxacin, ofloxacin, mecillinam, azithromycin or ceftriaxone.

Plasmid profile analysis has been applied extensively in epidemiological investigations of various enteric pathogens, particularly if there is a wide range of stable plasmids present in the bacterial population (Shahid *et al.*, 1985; Surdeanu *et al.*, 2000; Prado *et al.*, 1987). *Shigella sonnei* usually harbours a heterogeneous population of plasmids, which may range in number from two to as many as 10 (Haider *et al.*, 1989) and from 1.4 to 120 MDa in molecular mass. Of 184 strains, 54 % did not harbour the 120 MDa plasmid, which carries the major determinants for invasiveness. One of the possible explanations for this result might be the loss of the 120 MDa plasmid because of: (i) prolonged storage of the strains at -70°C ; and (ii) repeated subculturing of the strains, which has also been described previously by Vargas *et al.* (1999). In addition to the large plasmid, about 90 % of the strains contained three plasmids of approximately 5, 1.8 and 1.4 MDa in size. In order to validate this cluster of plasmids as core plasmids of *Shigella sonnei*, it was compared with the plasmid profiles of all the serotypes and subserotypes of *Shigella* species (data not shown). The cluster of these three plasmids was found to be unique to *Shigella sonnei* and did not match the plasmid profiles of any of the existing *Shigella* serotypes.

Conjugative plasmids encoding resistance to antibiotics have been detected in numerous studies on *Shigella sonnei* (DeLappe *et al.*, 2003). The experiment in the present study demonstrated that middle-range plasmids were self-transmissible, conferring resistance to ampicillin, tetracycline and trimethoprim-sulfamethoxazole. The co-transfer of streptomycin resistance was not observed, suggesting that the resistance determinants of this antibiotic in *Shigella sonnei* are not associated with the conjugative plasmids.

Invasiveness is an important property of pathogenic *Shigella* species. *Shigella flexneri* produces an enterotoxin (Kinsey *et al.*, 1976; Rout *et al.*, 1975), mainly of two types, *Shigella* enterotoxin 1 (ShET-1) and *Shigella* enterotoxin 2 (ShET-2). In our study, ShET-1 was not found in *Shigella sonnei*, confirming previous results (Nataro *et al.*, 1995). In contrast to the study of Vargas *et al.* (1999), which showed that 100 % of *Shigella sonnei* strains isolated between 1996 and 1998 were positive for ShET-2 (*sen*), we found that only 46 % of *Shigella sonnei* strains in this study were positive for this gene. In addition, a correlation between the presence of the 120 MDa plasmid and the *sen* gene was observed.

PFGE and ribotyping have been used more widely to determine the genetic diversity of various enteropathogens including *Shigella sonnei* (Hinojosa-Ahumada *et al.*, 1991; Korpela *et al.*, 1995; Pace *et al.*, 1986). Applying these two techniques, we identified four ribotypes, R1, R2, R3 and R4, whereas five PFGE types (A, B, C, D and E) were found in the same set of strains of *Shigella sonnei*. Among these four ribotypes, one (R1) was shared by two different PFGE types

(A and E). The present study showed the poor discriminatory power of ribotyping for *Shigella sonnei* isolates, compared with PFGE. This finding is similar to previous reports on strains from Taiwan (Henry, 1991; Liu *et al.*, 1995). However, ribotyping has been used successfully for epidemiological investigation of *Shigella sonnei* in Spain (Pace *et al.*, 1986) and other countries (Hinojosa-Ahumada *et al.*, 1991; Korpela *et al.*, 1995). It is interesting to note here that PFGE type A belonged to biotype BT1 and all the other types (PFGE types B, C, D and E) belonged to biotype BT2 only. PFGE type A was found to be predominant, encompassing approximately 88 % of the strains in the present study. A good correlation was observed between PFGE type, ribotype, antimicrobial resistance pattern and biotyping (Table 1). Strains that were resistant to single or multiple drugs belonged to PFGE type A, whereas strains that were sensitive to all antibiotics tested were more heterogeneous in PFGE type. Although *Shigella sonnei* is a more common problem in the developed and industrialized countries, this study underlines a significant burden of this pathogen in overall *Shigella* infections in Bangladesh, with strains of heterogeneous traits.

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