

The emerging strains of *Shigella dysenteriae* type 2 in Bangladesh are clonal

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SUMMARY

A total of 113 strains of *Shigella dysenteriae* type 2 isolated from patients attending the Dhaka diarrhoea treatment centre of ICDDR,B: Centre for Health and Population Research during the period 1999–2004 were studied. Serotype of the isolates was confirmed using commercially available antisera. Except for arabinose fermentation, all the strains had similar biochemical reactions. More than 60% of the strains were sensitive to commonly used antibiotics; only 6% ($n=7$) of the strains were resistant to nalidixic acid, and none of the strains were resistant to mecillinam and ciprofloxacin. All strains were invasive as demonstrated by the presence of a 140 MDa plasmid, *ial*, *sen* and *ipaH* genes, Congo Red absorption ability and by the Sereny test performed on representative strains. Plasmid patterns were heterogeneous but more than 50% of strains were confined to a single pattern. All strains possessed a 1.6 MDa plasmid and 87% of the strains contained a 4 MDa plasmid. Middle-range plasmids (90 MDa to 30 MDa) present in 36% of the strains were not associated with antibiotic resistance. All the strains were clustered within a single type with four subtypes by pulsed-field gel electrophoresis while ribotyping patterns of all the strains were identical.

INTRODUCTION

Diarrhoeal disease is one of the leading causes of morbidity and mortality worldwide, and is ranked fourth as a cause of death [1] and second as a cause of years of productive life lost due to premature mortality and disability [2]. Shigellosis is one of the most important diarrhoeal diseases and is caused by members of the genus *Shigella*. Infection and outbreaks associated with this organism are prominent in developing countries and are strongly associated with overcrowding and poor hygienic conditions [3].

It has been estimated that more than 95 000 children <5 years of age die of shigellosis annually in Bangladesh [4]. One of the major problems in combating shigellosis is the increasing frequency of antibiotic resistance in *Shigella* spp. [5]. At present, 50% of *Shigella* are resistant to nalidixic acid in Bangladesh (surveillance data of ICDDR,B, 2002) and 29% in India [6]. Recent studies showed that third-generation cephalosporin- and fluoroquinolone-resistant strains of *Shigella* spp. pose an important threat in the treatment of dysentery especially in case of children [7, 8]. Rarely does susceptibility reappear once resistant strains have become endemic in a region. In order to ensure appropriate treatment, continual surveillance is required to determine which antibiotics are still active [9]. Therefore, the World Health Organization (WHO) has targeted *Shigella*

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as one of the enteric infections for which new vaccines are needed.

Immunity to *Shigella* is serotype specific and vaccine protection will, therefore, depend on the representation of each serotype in the vaccine [10, 11]. Hence, it is crucial to monitor the trends of the prevalence of serotypes, and within each predominant serotype the clonal distribution should be evaluated. In previous studies, we investigated the trends in the prevalence of *S. flexneri* and *S. dysenteriae* infections in Dhaka, Bangladesh [12, 13]. Dominant serotypes changed over time, as was also observed in India [6]. As an endemic zone, there is a huge burden of *Shigella* in Bangladesh with a variety of serotypes prevailing [12–15]. We attempted to characterize all the predominant serotypes of *Shigella* irrespective of the species. A large number of *S. dysenteriae* type 2 strains were isolated during the period 1999–2004 and the prevalence gradually increased over time; it was the most prevalent serotype among *S. dysenteriae* in 2002 (42%) [13]. In the present study, we have conducted a detailed characterization of *S. dysenteriae* type 2 isolates both at the phenotypic and genotypic level to determine the virulence factors and the clonal diversity among the strains.

MATERIALS AND METHODS

Bacterial strains

From January 1999 to December 2004, 113 strains of *S. dysenteriae* type 2 isolated from patients attending the Dhaka treatment centre operated by ICDDR,B were identified in the Clinical Microbiology Laboratory by using standard microbiological and biochemical methods [16]. The strains were grown in trypticase soy broth containing 0.3% yeast extract (TSBY) and stored at -70°C after addition of 15% glycerol. Identification at the serotype level was done by the slide agglutination test [12] using commercially available antisera kit (Denka Seiken, Tokyo, Japan). YSH6000, *S. flexneri* 2a [17] and an *E. coli* (ATCC 25922) strain that lacked the 140 MDa invasive plasmid and was sensitive to all antibiotics were used as positive and negative controls respectively, in the Sereny test and the test for Congo Red binding ability.

Biochemical characterization

The biochemical reactions of the strains were determined by standard methods [16].

Antimicrobial susceptibility

The susceptibilities of strains to antimicrobial agents were determined by the disc diffusion method, as recommended by the Clinical and Laboratory Standards Institute [18] using commercially available antimicrobial discs (Oxoid, Basingstoke, UK). The antibiotic discs used in this study were ampicillin (10 μg), mecillinam (25 μg), nalidixic acid (30 μg), sulphamethoxazole–trimethoprim (25 μg), and ciprofloxacin (5 μg). *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used as control strains for susceptibility tests.

Tests for invasiveness

To determine the invasive property, the strains were subjected to keratoconjunctivitis assay (Sereny test) and Congo Red absorption ability according to the procedures described previously [13, 15].

PCR assay

Detection of *set1* gene (ShET-1), *sen* gene (ShET-2), *ial* gene, *ipaH* gene and *stx* genes was performed by amplifying *set1A*, *set1B*, *sen*, *ial*, *ipaH* and *stx* primers by PCR according to the procedures described previously [19]. All these primers were synthesized using Oligo 1000 DNA Synthesizer (Beckman, Fullerton, CA, USA) available in our laboratory at ICDDR,B.

Plasmid profile analysis

Plasmid DNA was prepared by the modified alkaline lysis method of Talukder *et al.* [14]. The molecular weight of the unknown plasmid DNA was assessed by comparing the mobilities of the plasmids of known molecular weights [20]. The plasmids present in the strains of *E. coli* PDK-9, R1, RP₄, Sa and V517 as described previously [13] were used as molecular weight standards.

Determination of resistance factors

Conjugation experiments between multidrug resistant (Amp^R, Sxt^R) strains, *S. dysenteriae* type 2 (K-727, K-1075) and the recipient *E. coli* K-12 (Nal^R, Lac⁺, F⁻) were performed by previously described method [21]. Transconjugant colonies were selected on MacConkey agar plates containing nalidixic acid (30 $\mu\text{g}/\text{ml}$) and ampicillin (50 $\mu\text{g}/\text{ml}$). Plasmid analysis

and antimicrobial susceptibility testing of the trans-conjugants were performed to determine the transfer of plasmids with antibiotic resistance. Determinations of transfer frequency and curing of the resistance plasmid were done according to the method described earlier [21].

Pulsed-field gel electrophoresis (PFGE)

Intact agarose-embedded chromosomal DNA from *S. dysenteriae* type 2 strains were prepared and PFGE was performed using the contour-clamped homogeneous electric field apparatus (CHEF-DRII; Bio-Rad Laboratories, Richmond, CA, USA) according to the procedures described earlier [14, 22–24], but with different pulse times, 3–35 s for 8 h, 5–50 s for 10 h, 20–80 s for 10 h, and 60–120 s for 10 h. Genomic DNA was digested with *NotI* restriction enzyme (Gibco-BRL, Gaithersburg, MD, USA). The restriction fragments were resolved by using the CHEF-DRII system apparatus in 1% pulsed-field certified agarose in 0.5×TBE buffer; the gel was stained, de-stained, and photographed on a gel documentation system according to procedures described previously [14]. The DNA size standards used were the bacteriophage lambda ladder ranging from 48.5 kb to 1000 kb (Bio-Rad) and *Saccharomyces cerevisiae* chromosomal DNA ranging from 225 kb to 2200 kb (Bio-Rad). Band patterns were established by the criteria described previously [25].

Ribotyping

Ribotyping of *S. dysenteriae* type 2 strains was performed according to the procedure described previously [14]. In brief, chromosomal DNA was extracted, purified and digested with *HindIII* restriction enzyme for overnight at 37 °C and separated by gel electrophoresis in 0.8% agarose in Tris borate EDTA (TBE) buffer according to the procedures described previously [14]. Southern blotting to a positively charged nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) was performed with a vacuum pump unit (Bio-Rad) and the DNA fragments were fixed on the membrane by exposing to UV light for 3 min. Hybridization was performed with a digoxigenin (DIG)-labelled cDNA probe specific for the 16S ribosomal DNA (rDNA) (25) for 18 h at 42 °C and development of the membrane with anti-DIG-alkaline phosphatase was performed according to the procedures

described previously [15]. Results were documented by taking the photographs of the membrane in which the probe had hybridized with the resolved DNA fragments.

RESULTS

Biochemical characterization

A detailed biochemical study of representative strains of *S. dysenteriae* type 2 ($n=42$) showed that all the strains were positive for indole production and rhamnose fermentation within 48 h of incubation. Although, trehalose was fermented by all the strains, 22% showed a positive reaction within 48 h while the remaining strains (78%) fermented after 48 h. Arabinose was fermented by 29% of the strains of which 7% were positive within 48 h, while the remaining 22% strains fermented after 48 h. None of the strains could utilize sodium-acetate, xylose, raffinose, maltose, mannitol, dulcitol and sorbitol.

Antibiotic susceptibility

There were no consistent changes in antimicrobial sensitivity patterns observed over the time period studied. More than 60% of the strains were sensitive to all antibiotics examined in this study. During 1999, 25% were resistant to ampicillin (Amp), while, 29, 19, 39, 31 and 30% of the strains were resistant to Amp in the years 2000, 2001, 2002, 2003 and 2004 respectively. *S. dysenteriae* type 2 strains resistant to sulphamethoxazole–trimethoprim (Sxt) were 31% in 1999, 32% in 2000, 13% in 2001, 45% in 2002, 52% in 2003 and 30% in 2004. All the strains isolated in 1999 and 2004 were sensitive to nalidixic acid (Nal) while 9, 6, 11 and 5% of the strains were resistant to Nal in the years 2000, 2001, 2002 and 2003 respectively. All the strains of *S. dysenteriae* type 2 were sensitive to mecillinam and ciprofloxacin. Depending on the susceptibility results, the strains were grouped into six patterns (Table 1).

Tests for invasiveness

Plasmid analysis showed that all the *S. dysenteriae* type 2 strains harboured the 140 MDa invasive plasmid (Table 2). All the strains ($n=113$) had the ability to absorb Congo Red dye. Five representative strains containing the 140 MDa plasmid were selected at random and were subjected to Sereny test. All the

Table 1. Antibiotic susceptibility pattern of *S. dysenteriae* type 2

Antibiotype	No. of strains (%) isolated in the year					
	1999	2000	2001	2002	2003	2004
Amp ^S Sxt ^S Nal ^S Mel ^S Cip ^S (S1)	10 (62)	21 (62)	12 (75)	10 (56)	8 (42)	7 (70)
Amp ^R Sxt ^R Nal ^S Mel ^S Cip ^S (S2)	3 (19)	9 (26)	2 (13)	5 (28)	4 (21)	3 (30)
Amp ^S Sxt ^R Nal ^S Mel ^S Cip ^S (S3)	2 (12)	1 (3)	0 (0)	1 (6)	5 (26)	0 (0)
Amp ^S Sxt ^S Nal ^R Mel ^S Cip ^S (S4)	0 (0)	2 (6)	1 (6)	0 (0)	0 (0)	0 (0)
Amp ^R Sxt ^R Nal ^R Mel ^S Cip ^S (S5)	0 (0)	1 (3)	0 (0)	2 (11)	1 (5)	0 (0)
Amp ^R Sxt ^S Nal ^S Mel ^S Cip ^S (S6)	1 (6)	0 (0)	1 (6)	0 (0)	1 (5)	0 (0)

Table 2. Plasmid profile analysis of *S. dysenteriae* serotype 2

Plasmid profile	No. of strains (%)	Molecular weight (MDa)							
		140	90-30	5·6	4	3·2	1·8	1·6	1·2
P1	61 (54)	+			+			+	
P2	32 (29)	+	+		+			+	
P3	8 (7)	+						+	
P4	5 (4)	+	+					+	
P5	2 (2)	+	+		+	+		+	
P6	1 (1)	+			+		+	+	
P7	1 (1)	+		+	+	+		+	
P8	1 (1)	+					+	+	
P9	1 (1)	+	+		+			+	+
P10	1 (1)	+			+			+	+

+, Indicates the presence of plasmid.

representative strains produced keratoconjunctivitis in a guinea pig's eye.

PCR assays

Shigella enterotoxin 2 (*sen*), *ial* and *ipaH* genes were present in all the strains while *Shigella* enterotoxin 1 (*set1*) and *stx* genes were absent in all 113 strains of *S. dysenteriae* type 2.

Plasmid profile analysis

All the strains of *S. dysenteriae* type 2 contained multiple numbers of plasmids ranging from 140 to 1·0 MDa (Fig. 1). All the strains contained 140 MDa and 1·6 MDa plasmid, 89% of strains contained 4 MDa plasmid and 36% of strains harboured middle-range plasmids (90 MDa to 30 MDa). Plasmid patterns were created according to the number and size of the plasmid. Ten different patterns were found, of these, P1 (54%) was the predominant followed

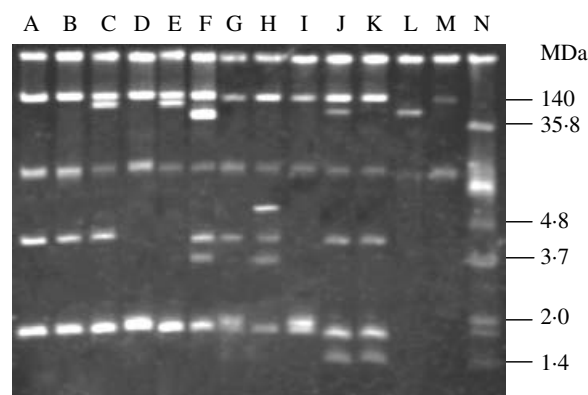


Fig. 1. Agarose gel electrophoresis of plasmid DNA showing the representative patterns among the isolates of *S. dysenteriae* type 2. Lanes A, B, *S. dysenteriae* type 2 (pattern P1); lane C, *S. dysenteriae* type 2 (P2); lane D, *S. dysenteriae* type 2 (P3); lane E, *S. dysenteriae* type 2 (P4); lane F, *S. dysenteriae* type 2 (P5); lane G, *S. dysenteriae* type 2 (P6); lane H, *S. dysenteriae* type 2 (P7); lane I, *S. dysenteriae* type 2 (P8); lane J, *S. dysenteriae* type 2 (P9); lane K, *S. dysenteriae* type 2 (P10); lanes L, M, N, *E. coli* R-1, PDK-9, and V-517 respectively.

by P2 (29%), P3 (7%), P4 (4%), P5 (2%), P6 (1%), P7 (1%), P8 (1%), P9 (1%) and P10 (1%), these are described in detail in Table 2.

Determination of resistance factors

Antibiotic resistance pattern and plasmid profile analyses showed that 23% of the strains were resistant to ampicillin, and Sxt, of which 36% contained a middle-range plasmid having a molecular weight between 30 MDa and 90 MDa. Two strains designated as K-727 and K-1075 containing 62 MDa and 88 MDa respectively, having the antibiotic susceptibility pattern S2 (Table 1), were selected for conjugation experiments with *E. coli* K12. After conjugation, both the plasmids were transferred

Table 3. Transfer of resistance plasmid to *E. coli* K-12 by conjugation

Strain no.	Parent strain		Transconjugant			Cured strain	
	Resistance pattern	Plasmid profile (MDa)	Resistance pattern	Plasmid profile (MDa)	Frequency of R-plasmid transfer	Resistance pattern	Plasmid profile (MDa)
K-727	Amp Sxt	140, 62, 4, 1.6	Amp Sxt Nal	62	2.0×10^{-3}	Nal	No plasmid
K-1075	Amp Sxt	140, 88, 4.0, 1.6	Amp Sxt Nal	88	2.0×10^{-4}	Nal	No plasmid

Amp, Ampicillin; Sxt, Sulphamethoxazole–trimethoprim; Nal, Nalidixic acid.

independently with the complete spectrum of drug resistance (Amp^R Sxt^R). The transfer frequency was very high for all transmissible plasmids. All the transconjugants were cured by Acridine Orange losing the plasmids and became sensitive to all antibiotics (Table 3).

PFGE

PFGE analysis of *NotI*-digested chromosomal DNA of *S. dysenteriae* type 2 strains yielded 12–14 reproducible DNA fragments ranging in size of ~20 kb to 800 kb (Fig. 2). According to the criteria of interpretation for PFGE [25], all the strains were grouped into a single type (designated as A), which was further subdivided into four subtypes (A1–A4). Of these, A1 was the predominant pattern shared by 74% of the strains followed by A2 (18%), A3 (4%) and A4 (4%) (Fig. 2).

Ribotyping

Hybridization of *HindIII*-digested chromosomal DNA of representative strains of *S. dysenteriae* type 2 with the 16S rDNA probe revealed a total of six fragments ranging in size of approximately 5–15 kb (Fig. 3). DNA fragments were arranged in a similar fashion in all the strains suggesting their identity in ribotyping pattern.

DISCUSSION

As previously noted, the trend in prevalence of *S. dysenteriae* serotype has changed over the period 1999–2002. *S. dysenteriae* type 1, the most prevalent serotype in 1999 was replaced by serotypes of *S. dysenteriae* types 2 and 4 [13]. Recently, we have reported an unusual outbreak of *S. dysenteriae* type 4 that occurred between June and December 2000, in Dhaka,

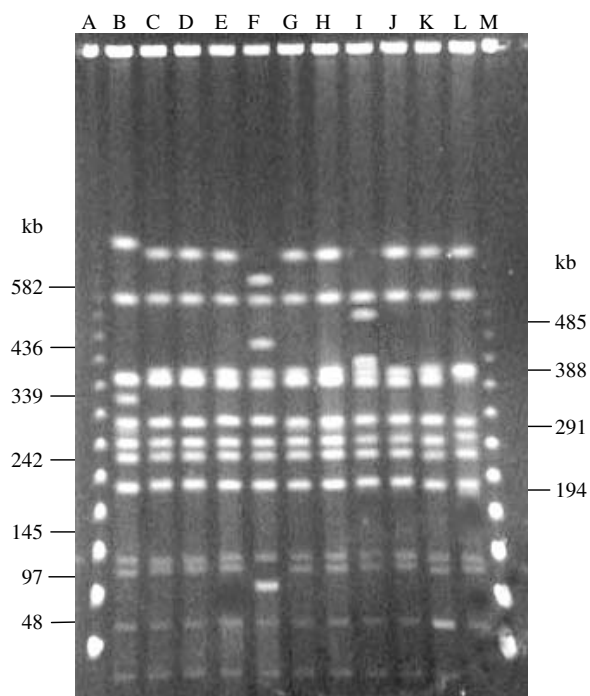


Fig. 2. PFGE banding patterns of *NotI*-digested chromosomal DNA of representative strains of *S. dysenteriae* type 2. Lane A, λ ladder (marker); lanes B–L, *S. dysenteriae* type 2, K-467 (PFGE type A2); K-490 (A1); K-1075 (A1); K-1157 (A1); K-1455 (A3); K-1913 (A1); K-1141 (A1); K-1639 (A4); K-727 (A1); K-1822 (A1); K-1827 (A1) respectively; lane M, λ ladder (marker).

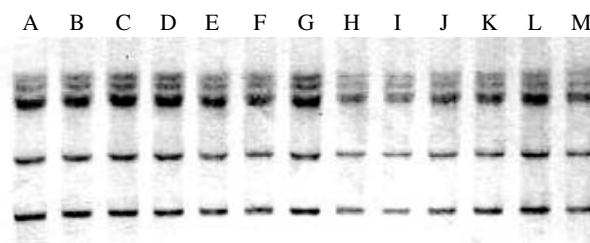


Fig. 3. Ribotyping patterns of representative strains of *S. dysenteriae* type 2. Lanes A–M, *S. dysenteriae* type 2 (K-467, K-490, K-727, K-774, K-1075, K-1083, K-1141, K-1157, K-1158, K-1455, K-1639, K-1827 and KD-11), all strains showed same ribotype (R1).

Bangladesh [26]. The prevalence of type 2 increased steadily from 1999 to 2002 [13], although major outbreaks caused by these type 2 strains have not yet been reported.

Our results suggest that *S. dysenteriae* type 2 strains in Bangladesh may be emerging from a common clone. Among the 22 biochemical tests carried out, only five reactions were positive of which, rhamnose, trehalose and glucose were utilized and indole was produced by all the strains within 48 h, however, extended incubation times were needed for 78% of the strains to ferment trehalose. Although limited responses were observed in biochemical reactions, all the strains exhibited the same reaction pattern, which matched the previous biochemical study [27]. Therefore, this biochemical reaction pattern can be used as a phenotypic marker for the preliminary identification of *S. dysenteriae* type 2 strains.

Antibiotic susceptibility pattern of strains isolated in different years did not show any significant variations. Although resistance to nalidixic acid was noted for strains isolated from 2000 to 2003, none of the strains isolated in 2004 were resistant to nalidixic acid. Additionally, among all the antibiotypes, S1 was predominant (>60%), which represented the strains sensitive to all antibiotics (Table 1). This is consistent with the limited antibiotic selection pressure put on strains that are circulating at a low level in the population [14].

Invasiveness is a key property of pathogenic *Shigella* strains, and is mainly conferred by a 140 MDa plasmid. However, the invasiveness genes on this plasmid are not fully operational without the activity of some chromosomal sequences. According to previous studies [28–32], it was observed that the virulence factor of *Shigella* is mainly conferred by a large plasmid of ~140 MDa in which the key sequence of the invasive plasmid antigen is located, although it is not fully operative without the activity of some chromosomal sequences. All the strains in present study were actively virulent as is evident by the presence of the 140 MDa plasmid, *IpaH*, *sen* and *ial* genes and the ability to bind Congo Red and to produce keratoconjunctivitis in a guinea pig's eye.

In epidemiological studies, clonal distribution may be important to characterize for preventive treatment particularly in the early diagnosis and management of the disease. According to a previous report, plasmid profile analysis is well documented as

meaningful in epidemiological studies of enteric pathogens [33]. It has been shown previously that *S. dysenteriae* type 2 strains possessed multiple numbers of plasmids with a heterogeneous pattern but three plasmids of 140, 4 and 1.6 MDa were commonly present in most of the strains designated as core plasmids [13]. Haider *et al.* [34] suggested that middle-range plasmids in *Shigella* are associated with antibiotic resistance with a self-transmissible property. In this study, 28% of strains were resistant to multiple drugs having a self-transmissible middle-range plasmid but at the same time 8% of strains were sensitive to all antibiotics carrying this plasmid. Therefore, it can be interpreted that all the middle-range plasmids are not associated with antibiotic resistance. Munshi *et al.* [21] reported that a 20 MDa plasmid was associated with nalidixic-acid resistance of *S. dysenteriae* type 1. In the present study, 6% ($n=7$) of *S. dysenteriae* type 2 strains were found to be resistant to nalidixic acid but none of the strains contained 20 MDa plasmid. Moreover, the strains did not harbour any middle-range plasmid, which concludes that nalidixic-acid resistance in *S. dysenteriae* type 2 strains is not transmissible via the plasmid.

PFGE and ribotyping have been considered to be highly discriminatory in subtyping strains of *Shigella* and other enteric pathogens. A single PFGE type was found in all the strains denoting their possible circulation from a single origin. However, little variation (2–6 bands) was observed in the banding patterns of the strains of single PFGE type, which grouped the strains into four subtypes, therefore characterizing them as possibly related clones. Additionally, there was no variation among strains observed by ribotyping.

The data presented here showed a high degree of conformance of the isolates by biochemical measures and ribotyping. Somewhat more variability was seen by PFGE and more was found in cases of antimicrobial sensitivity patterns and plasmid profiles. This strongly suggests a clonal expansion of the strains, with some diversification along the way. However, the variability being seen is occurring in characteristics that are most susceptible to change by the environmental influence or by local molecular events. It would be very interesting to compare the clones of *S. dysenteriae* type 2 in Bangladesh with the strains from other parts of the world in order to explain the epidemiological significance of this serotype in the global burden of shigellosis.

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DECLARATION OF INTEREST

None.

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