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Recovery and Characterization of Environmental Variants of *Shigella flexneri* from Surface Water in Bangladesh

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Abstract Little is known about the distribution, survival, and transmission of *Shigella* in environmental surface waters. To gain more insight into the environmental biology of *Shigella* we isolated five bacterial strains serotyped as *Shigella flexneri* 2b from a freshwater lake in Bangladesh using a modified nutrient broth supplemented with nucleic acid bases. The biochemical properties of the isolates, including inability to ferment lactose and a negative lysine decarboxylase test, indicated common physiological characteristics with *Shigella*, but differed significantly from that of standard clinical strains. The isolates possessed the *ipa*H virulence gene and a megaplasmid, but lacked other *Shigella*-related virulence marker genes. Genetic fingerprinting and sequence analysis of housekeeping genes confirmed the strains as *S. flexneri* isolates. An apparent clonal origin of strains recovered with a one-year interval indicates a strong environmental selection pressure on *Shigella* for persistence in the freshwater environment. The lack of a complete set of virulence genes as well as uncommon biochemical properties suggest that these strains might represent a group of non-invasive and atypical environmental *Shigella* variants, with the potential for further elucidation of the survival mechanism, diversity, and emergence of virulent *Shigella* in tropical freshwater environments.

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Introduction

Shigellosis is a major bloody diarrheal disease that occurs as endemic in many developing countries like Bangladesh, causing considerable death and morbidity. Among at least 80 million cases, 700, 000 deaths occur each year due to shigellosis in developing countries. Seventy percent of these cases occur in children less than 5 years of age [23]. Shigellosis is caused by enteric bacteria belonging to the genus *Shigella* and is mainly disseminated through contaminated water [25]. The *Shigella* genus encompasses four species; *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, and *Shigella sonnei*, also known as groups A, B, C, and D, respectively, mainly based on O-antigenic serogrouping. They are further subdivided into more than 47 serotypes [3, 6].

Factors affecting the emergence and decline of *Shigella* epidemics are still not well-defined but normally shigellosis occurs due to drinking of contaminated water from open environmental sources [2, 9]. *Shigella* spp. has been very difficult to recover from environmental waters, possibly due to lack of proper isolation techniques. One previous report on the occurrence of *Shigella* in environmental water bodies is available [5], but the understanding of the diversity, distribution, and survival of these bacteria in aquatic environments is still limited. However, a previous study suggests that these organisms may persist for a

limited period in environmental waters [21]. Prolonged persistence of *Shigella* spp. in the cytoplasm of free-living amoebae has also been demonstrated [17], suggesting that amoebae may serve as an aquatic transmission reservoir.

In this study, we have isolated and described environmental variants of *Shigella* from a freshwater lake in Bangladesh, recovered using a modified medium supplemented with nucleic acid bases in combination with serology-assisted screening. This is the second report describing *Shigella* isolates from the freshwater environment. The results add to our knowledge about the genotypic and phenotypic diversity of *Shigella* in tropical surface waters and confirm the role of environmental water as a reservoir for these microorganisms.

Materials and Methods

Sampling, Isolation, and Serotyping

Water samples were collected from a lake in Narayangonj subdistrict, Bangladesh. Fifty ml of each water sample were filtered through a 0.22 µm membrane filter (Millipore) which was subsequently transferred to 50 ml of a nutrient broth (pH 8.0) containing 0.2% proteose peptone (Difco), 0.1% K_2HPO_4, 0.05% KH_2PO_4, 0.3% NaCl, and 10 µg/ml of each of the nucleotide bases, and incubated for 4–6 h at 37°C. Then, 5 ml of the pre-enrichment was transferred to the same broth supplemented with 50 µg/ml streptomycin and was incubated for 6 h at 37°C. Aliquots were then streaked onto MacConkey agar plates (Oxoid) and xylose lysine desoxycholate agar plates (Oxoid) and were incubated overnight at 37°C. *Shigella*-like colonies were re-streaked on MacConkey agar plates and subjected to slide agglutination testing using commercially available antisera kit (Denka Seiken, Tokyo, Japan) [12] followed by enzyme-linked immunosorbent assay (ELISA) using sonicated whole cell extracts as previously described [10, 16].

Extraction and Analysis of Genomic DNA and Plasmids

Genomic DNA was extracted according to the procedure described by Murray and Thompson [14] with some modifications [7–9, 19]. In brief, cells were treated with 10% SDS and freshly prepared proteinase K at 50°C for 1 h, followed by addition of about 1:7 (vol/vol) of CTAB/NaCl mixture (10% cetyltrimethyl ammonium bromide in 0.7 M NaCl) and incubation at 65°C for about 15 min. The mixture was then extracted with TE-buffered (10 mM Tris–HCL; 1 mM EDTA; pH 8.0) phenol: chloroform: isoamylalcohol (25:24:1) and DNA precipitated by ethanol. The DNA pellet was resuspended in TE, treated with RNAse at 37°C for 1 h and stored at −20°C. Plasmid DNA was prepared according to the alkaline lysis method [22] and analyzed by horizontal electrophoresis in a 0.8% agarose slab gel using Tris–borate–EDTA (TBE) buffer at 80 V for 3 h [19].

PCR Analysis of Virulence Genes and Phylogenetic Analyses

The occurrence of virulence genes, e.g., *ipa*H, *ipa*BCD, and *ial* amongst the test isolates were detected by PCR amplification using the primer sets as described earlier [5, 20]. Sequence analysis of the 16S rRNA V3 gene region was also carried out as described earlier using primer set, PRBA338f and PRUN518r [15, 16]. To identify the closest relatives, the sequences were used in Blastn searches against the NCBI nucleotide collection (nr/nt) database. The multi-locus sequence typing (MLST)-based phylogenetic analysis using seven housekeeping genes, *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA* was done [26]. A concatenated sequence was constructed by merging of the seven housekeeping gene sequences, aligned, and phylogenetically analyzed by the Clustal X 2.0 software [11] using the neighbor-joining algorithm together with the corresponding concatemers from genome-sequenced reference *E. coli* and *Shigella* strains from the Genomes OnLine Database (http://www.genomesonline.org/).

Biochemical and Antibiotic Susceptibility Tests

Biochemical profiling was performed according to standard protocols [24] and by using an API 20E kit following the manufacturer's (BioMerieux Inc.) instructions. Antimicrobial resistance was tested using the agar-disk diffusion method [1] with standard antibiotic disks (Oxoid) at the following concentrations (mg/disk); ampicillin, 10; chloramphenicol, 30; streptomycin, 30; tetracycline, 30; trimethoprim-sulfamethoxazole, 25; erythromycin, 15; ciprofloxacin, 5; norfloxacin, 10; nalidixic acid, 30, and rifampin, 30.

Restriction Fragment Length Polymorphism (RFLP)-Based Genotyping

For RFLP-based genotyping, 16S rRNA (ribotyping) and *ipa*H gene probes (*ipa*H typing) were generated by PCR using primer pairs previously described [5, 13] and genomic DNA from *Shigella boydii* type 15 (ATTC 12034) as template. The experiments were essentially done as described previously [5, 22]. In brief, approximately 5 µg of genomic DNA was digested with 30 U of *Hin*dIII. Southern blots were prepared using a nylon membrane (Hybond-N; Amersham) followed by prehybridization and

hybridization with freshly denatured digoxygenin (Roche Applied Science, Germany) (DIG)-labeled probes. Detection of hybridized fragments was carried out with nitroblue tetrazolium and alkaline phosphatase according to instructions from the manufacturer of the DIG-labeling kit (Roche Applied Science, Germany).

Results

Shigella-like colonies on MacConkey and xylose lysine desoxycholate (XLD) agar plates were picked and serotyped using slide agglutination tests. From several hundred colonies, five isolates, which were given strain designations SNZ5, SNZ6, SNZ9, SF3/S, and SF3/L, were obtained that showed strong reaction with group- and type-specific antiserum for *S. flexneri* 2b. The isolates were recovered from samples obtained in October 2004 (SNZ5, SNZ6, and SNZ9) and October/November 2005 (SF3/S and SF3/L). No other serotypes were recovered. The results were confirmed using ELISA, which yielded high titers ($1{:}5^7$), equal to the titers obtained for the homologous *Shigella* reference strains. Partial sequence analysis of the 16S rRNA genes identified all of the isolates as belonging to the *Escherichia/Shigella* group with similarity values of $\geq$97.

The isolates yielded positive catalase and methyl red tests, while the urease and oxidase tests, as well as tests for H_2S and indole production were negative. They were also non-motile and non-lactose fermenters, and grew without gas production on trehalose, maltose, and mannose, but not on xylose, dulcitol, arabinose, or raffinose (Table 1). The lysine decarboxylase, arginine dihydrolase, and ornithine decarboxylase tests were all negative. When subjected to biochemical profiling using an API 20E testing kit the isolates were indistinguishable, giving only positive results for glucose and mannose fermentation (profile 0004100), which yielded an identity score of 69.3% against *Shigella* spp. and insignificant scores with other bacterial groups. All the isolates were resistant to erythromycin and streptomycin, but sensitive to ampicillin, chloramphenicol, ciprofloxacin, norfloxacin, and rifampin.

Primers specific for the *Shigella*-related virulence genes *ial*, *ipa*H, and *ipa*BCD, encoding the invasion plasmid locus, the invasion plasmid antigen H and the invasion plasmid antigens B, C, and D, respectively, were used in PCR analysis to evaluate the occurrence of these genes in the isolates. None of the isolates carried *ial* or *ipa*BCD, while they were all *ipa*H positive. To further assess the genetic relatedness between the isolates and *Shigella* spp., ribotyping and genotyping using the *ipa*H probe were carried out. The *ipa*H genotyping revealed a significant similarity to that of a *S. flexneri* reference strain (ATCC 12024), differing only in one band (Fig. 1a). None of the isolates gave patterns with similarity to that of *S. boydii*. Ribotyping yielded identical patterns for the isolates (Fig. 1b), confirming a high genetic relatedness. The patterns were highly similar to that of *S. flexneri* ATCC 12024, differing only in one of seven bands. The results confirm that these five isolates represent true *Shigella* strains belonging to the *S. flexneri* species.

Plasmid analyses identified a megaplasmid in all the environmental isolates (Fig. 1c), thus demonstrating the presence of yet another *Shigella* hallmark [18]. The isolates yielded two slightly different plasmid band patterns, differing only in one plasmid band ($\sim$8 Kb).

An MLST-based phylogenetic analysis using seven housekeeping genes from two representative environmental isolates (SNZ5 and SF3/L) was performed. Concatemers of the seven gene sequences were aligned with concatemers constructed from genome sequences of the four *Shigella* spp. and a variety of *E. coli* reference strains. The resulting phylogenetic tree, based on 3,321 aligned bases, and which included a total of ten reference strains, demonstrated a tight affiliation of the environmental isolates with *S. flexneri*, in accordance with the other correlating characteristics (Fig. 2). In fact, the sequence of the concatemers of the SNZ5 and SF3/L strains were identical with that of the *S. flexneri* type strain, providing an ultimate proof of

Table 1 Biochemical profiles of the environmental isolates

Strain/species	Lac	Tre	Xyl	Mal	Dul	Man	Ara	Raf	Lys_d	Arg_d	Orn_d
Environmental isolates[a]	–	+	–	+	–	+	–	–	–	–	–
Shigella, groups A, B, C[b]	0	80	2	93	2	100	60	50	0	5	1
S. sonnei[b]	2	100	2	99	0	100	95	3	0	2	98
E. coli[b]	95	98	95	98	60	98	99	50	90	17	65

[a] +; Growth without gas production, –; no reaction. The reactions were scored after 2–3 days incubation

[b] *Numbers* in the three last rows indicate percentage of positive isolates [4]

Lac lactose; *Tre* trehalose; *Xyl* xylose; *Mal* maltose; *Dul* dulcitol; *Man* mannose; *Ara* arabinose; *Raf* raffinose; *Lys_d* lysine decarboxylase; *Arg_d* arginine dihydrolase; *Orn_d* ornithine decarboxylase

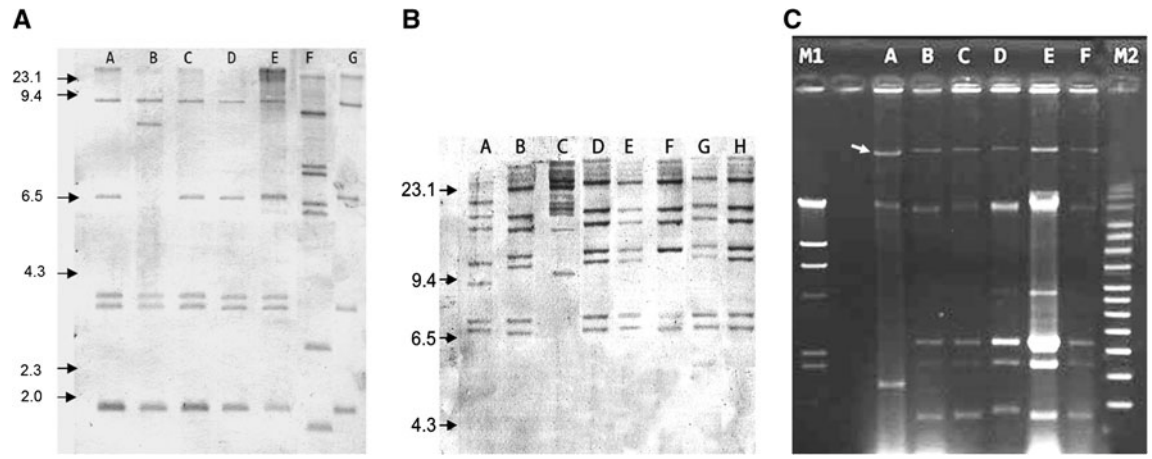

Fig. 1 **a** Southern blot analysis of the *ipa*H gene. Southern blots of *Hin*dIII digested DNA of environmental *Shigella* isolates were hybridized with *ipa*H probe. *Lanes A*, SNZ5; *B*, SNZ6; *C*, SNZ9; *D*, SF3/L; *E*, SF3/S; *F*, *S. boydii* (ATCC 12034); *G*, *S. flexneri* (ATCC 12024). *Arrows* indicate size markers in Kbp. **b** Ribotyping pattern of *Hin*dIII digested DNA of environmental *Shigella* isolates. *Lanes A*, *S. dysenteriae* type 4; *B* SNZ9; *C S. boydii* (ATCC 12034); *D* SNZ 6; *E* SF3/L; *F S. flexneri* (ATCC 12024); *G* SNZ5; *H* SF3/S. **c** Plasmid profile analysis. *Lanes M1 Hin*dIII digested Lamda DNA ladder; *A S. boydii* (ATCC 12034), *B* SF3/S; *C* SF3/L; *D* SNZ5; *E* SNZ6; *F* SNZ9; *M2* Supercoiled DNA ladder. The *arrow* indicates the ~220 Kbp megaplasmid

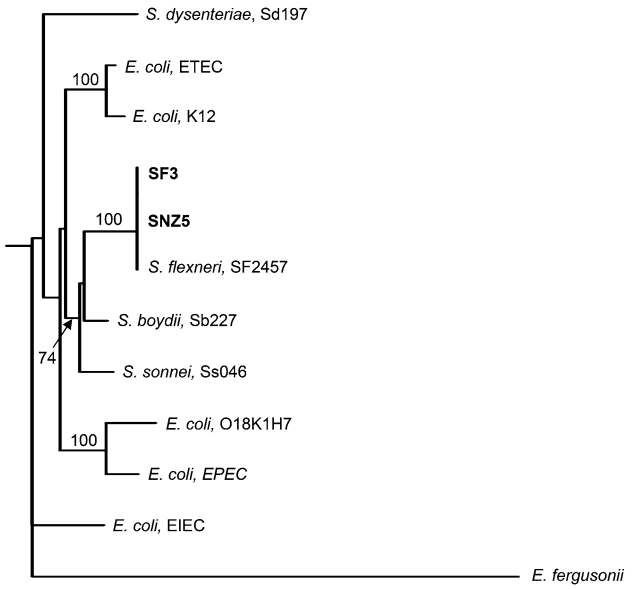

Fig. 2 Phylogenetic analyses using housekeeping genes. The phylogenetic relationships of the environmental isolates SF3/L and SNZ5 (in *bold*) and reference strains of *E. coli* and *Shigella* spp. were assessed based on concatenated sequences of seven housekeeping genes. The reference sequences were retrieved from the following database sequence entries: *E. coli* K12, NC000913; *E. coli* ETEC, NC009801; *E. coli* O18:K1:H7, CP001969; *E. coli* EPEC, NC011601; *S. sonnei*, NC007384; *S. boydii*, NC007613; *S. dysenteriae*, CP000034; *S. flexneri*, NC004741; *E. fergusonii*, NC011740. The *E. fergusonii* concatemer was used as outgroup. Bootstrap values ≥74% are indicated at *nodes*

the phylogenetic affiliation with this species. The isolates form a cluster including *S. boydii* and *S. sonnei*, while *S. dysenteriae* forms a separate lineage (Fig. 2).

Discussion

In this study, a nutrient broth supplemented with nucleic acid bases was successfully used for isolation of five *Shigella* strains from a Bangladeshi lake. The isolates were shown to belong to the *Escherichia/Shigella* group according to the 16S rRNA gene sequence analysis and were serotyped specifically as *S. flexneri* 2b. PCR analysis targeting virulence marker genes demonstrated the presence of *ipa*H in the isolates, but the additional invasion genes, *ipa*BCD and *ial*, were not detected. A previous study also revealed a similar occurrence of *S. flexneri* in surface water in Bangladesh [5], and most of those isolates (six out of seven) also lacked the *ipa*BCD invasion genes. The lack of *ipa*BCD and *ial* indicates that the strains constitute a non-invasive variant of *S. flexneri*. These genes might have been lost during survival/persistence in the aquatic environment. The emergence of virulent *Shigella* strains is still not understood. Sequencing of the megaplasmids found in all these environmental isolates might reveal valuable information about the genetic processes involved in possible gene loss or gene gain events. Antibiotic resistance screening of the environmental *Shigella* isolates revealed resistance to streptomycin and erythromycin, but sensitivity to all the other antibiotics tested. This contrasts with the previous study reporting that seven out of seven environmental *S. flexneri* isolates were resistant to rifampin but were erythromycin sensitive [5]. Our isolates thus differ significantly from the previously reported isolates in antibiotics resistance patterns.

The negative results for the lactose utilization and lysine decarboxylase tests strongly conform to the biochemical

properties of *Shigella* (Table 1). However, the biochemical profile as revealed by the API 20E testing did not match perfectly with *Shigella* (69.3% identity score) except for the positive glucose and mannose tests, demonstrating a distinct biochemical difference from clinical *Shigella* strains, which might be related to long-term persistence in the aquatic environment. Ribotyping indicated a clonal origin of the five strains with patterns slightly different from the *S. flexneri* type strain, suggesting a strong clonal selection pressure for persistence in the freshwater environment. Clonal relationships between environmental *Vibrio cholerae* isolates has also been observed before [7], supporting the notion about a strong selection pressure. MLST-based analysis of two of the strains confirmed the clonal relationship as well as the affiliation with *S. flexneri*.

This study suggests that *Shigella* has a reservoir in the tropical aquatic environment and is able to survive or persist for a certain period of time in freshwater. The environmental isolates were, however, non-invasive as determined by PCR. Recovery of *Shigella* from the freshwater environment using the protocol for enrichment and isolation described in this report may provide more information about the epidemics and seasonality of shigellosis as well as the fate of *Shigella* following discharge to the aquatic environment. The isolation of avirulent *Shigella* strains might also open up a new avenue for searching of potential candidates for live *Shigella* vaccine development. Further analysis of the strains may provide important clues for the environmental selection pressure and survival strategy involved in the persistence of this pathogen in the environment.

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