

## Serological Cross-Reactivity of Environmental Isolates of *Enterobacter*, *Escherichia*, *Stenotrophomonas*, and *Aerococcus* with *Shigella* spp.-Specific Antisera

Mohammed Ziaur Rahman,<sup>1,2</sup> Munawar Sultana,<sup>1</sup> Sirajul Islam Khan,<sup>1</sup> Nils-Kåre Birkeland<sup>2</sup>

<sup>1</sup>Department of Microbiology, University of Dhaka, Dhaka 1000, Bangladesh

<sup>2</sup>Department of Biology, University of Bergen, P.O. Box 7800, N-5020, Bergen, Norway

Received: 1 August 2006 / Accepted: 17 October 2006

**Abstract.** Using protocols designed for the isolation of *Shigella* from environmental freshwater samples from different regions of Bangladesh, 11 bacterial strains giving rise to *Shigella*-like colonies on selective agar plates and showing serological cross-reaction with *Shigella*-specific antisera were isolated. Phylogenetic analyses revealed that three of the isolates were most closely related to *Escherichia coli*, four to *Enterobacter* sp., two to *Stenotrophomonas*, and two isolates belonged to the Gram-positive genus *Aerococcus*. The isolates cross-reacted with six different serotypes of *Shigella* and were, in each case, highly type-specific. Two of the isolates belonging to the *Enterobacter* and *Escherichia* genera gave extremely strong cross-reactivity with *Shigella dysenteriae* and *Shigella boydii* antisera, respectively. The *Aerococcus* isolates gave relatively weak but significant cross-reactions with *S. dysenteriae*. Western blot analysis revealed that a number of antigens from the isolates cross-react with *Shigella* spp. The results indicate that important *Shigella* spp. surface antigens are shared by a number of environmental bacteria, which have implications for the use of serological methods in attempts for the detection and recovery of *Shigella* from aquatic environments.

In developing countries like Bangladesh, bacillary dysentery is one of the major causes of death, especially among children. The annual number of *Shigella* episodes throughout the world is estimated to be 160 million, and 69% of all deaths attributable to shigellosis involve children less than 5 years of age [9]. Shigellosis occurs as an endemic disease in Bangladesh, and at least three large epidemics caused by *Shigella dysenteriae* type 1 have occurred between 1972 and 1994, causing high morbidity and mortality [3].

Laboratory diagnosis of *Shigella* is often based on the isolation of the organism from feces and its identification by means of cultural and biochemical characteristics [8]. Serological identification is also indispensable because of the common characteristics of *Shigella* species with

members of the genus *Escherichia*, from which they cannot even be differentiated by DNA:DNA hybridization [12]. The slide agglutination test using a number of commercially available antisera specific to different groups as well as different types is a common and indispensable test for serological typing of *Shigella* spp. However, the specificity of commercially produced antisera varies to a significant extent [5, 6], but some of them are useful for routine serological identification purposes [12]. In a previous study, it was found that various types of microorganism such as *Hafnia alvei*, *Plesiomonas shigelloides*, *Providencia alcalifaciens*, and *Yersinia enterocolitica* serotype O3 and of *E. coli* (O114:H32, O157:H7, O157:H19, etc.) gave serological cross-reactivity with polyclonal group-specific *Shigella* antisera [12].

The presence of *Shigella* in environmental water has already been reported and it can be expected that environmental water plays an important role in spread-

ing the disease throughout the community [7]. There are a number of bacteria present in the environment that have very similar biochemical characteristics to *Shigella*; thus, it is crucial to understand the serological cross-reactivity of environmental bacteria with *Shigella* antisera. Previous studies were more or less confined to the serological cross-reactivity of *Shigella* with clinical isolates using polyclonal group-specific antisera. In the present study, attempts have been taken to investigate the cross-reactivity of *Shigella* spp. with environmental micro-organisms isolated using standard procedures for isolation of *Shigella* spp.

## Materials and Methods

**Sampling, Culturing, and Slide Agglutination Test.** Environmental water samples were collected from various Bangladeshi lakes, rivers, and ponds at different places in Dhaka, Gazipur, Karanigonj, Narayan gonj, Mymensingh, and Jamalpur. Fifty milliliters of each water sample were filtered through a 0.22- $\mu$ m membrane filter and the filter was incubated in 50 mL alkaline peptone water (1% peptone, 1% NaCl, pH 8.0) and nutrient broth for 6 h at 37°C. After 6 h, streptomycin was added to the cell suspension to 120  $\mu$ g/mL final concentration, followed by incubation at 37°C for another 6 h. Aliquots were then streaked onto MacConkey agar plates (OXOID, England) and xylose lysine desoxycholate agar plates (OXOID, England), which were incubated overnight at 37°C. Suspected colonies were subcultured on MacConkey plates and tested by slide agglutination using a commercially available antisera kit (Denka Seiken, Japan) specific for all type- and group-factor antigens of *Shigella* spp. according to the procedure described previously [12].

**PCR and DNA Sequencing.** Phylogenetic identification of the strains was based on polymerase chain reaction (PCR) amplification of the highly variable V3 region of the bacterial 16S rRNA gene [14]. The forward primer PRBA338f (5'-ACTCCTACGGGAGGCAGCAG-3') and the universal reverse primer PRUN518r (5'-ATTACCGCGG CTGCTGG-3') were used for this purpose. DNA extraction from each isolate was carried out using the GenElute Bacterial Genomic DNA kit (SIGMA, Germany). PCR reactions were carried out with an initial denaturation step at 94°C for 3 min; 30 cycles of 94°C, 1 min; 55°C, 30 s; 72°C, 1 min, followed by a final extension of 6 min at 72°C. The PCR products were subjected to 2% agarose gel electrophoresis, stained with ethidium bromide and visualized on an ultraviolet (UV) transilluminator for the presence of about 200-bp PCR products. The PCR products were purified by using the StrataPrep® DNA purification kit (Stratagene, California). PCR products were then sequenced using the ABI Prism BigDye sequencing kit (Applied Biosystem, UK).

**Preparation of Sonicated Whole-Cell Extracts.** Forty milliliters of the cell suspension from overnight shake cultures were centrifuged at 10,000g. The pellets were washed with phosphate-buffered saline (PBS) (8.0 g/L NaCl, 0.2 g/L KCl 1.44 g/L Na<sub>2</sub>HPO<sub>4</sub>, 0.27 g/L KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) and resuspended in 5 mL PBS. The cells were then disrupted by sonication (30 s  $\times$  10 times). Sonicates were then centrifuged at 10,000g, filtered, and stored at -20°C.

**Enzyme-Linked Immunosorbent Assay (ELISA).** The protein concentration of the whole-cell extracts was determined by the Bradford method [2]. One hundred fifty milliliters of each sample

were added to the first lane of a microtiter plate at a concentration of 200  $\mu$ g/125  $\mu$ L. The protein samples were then subjected to 1:5-fold serial dilutions in the microtiter plate, which were incubated overnight at 4°C in the dark for coating. The procedure was performed as described elsewhere [10] with minor modifications. Primary antisera were from the Denka Seiken antisera kit. Two types of secondary antisera were used: AP conjugate for polyvalent primary antisera and HRP conjugate for monovalent primary antisera. For the HRP conjugate, the substrate was Sigma Fast OPD (*O*-phenylenediamine dihydrochloride) prepared in citrate buffer (pH 5.2), and for the AP conjugate, the substrate was *p*-nitrophenyl phosphate prepared in diethylether buffer (pH 9.2).

**Western Blot Analysis.** Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% polyacrylamide gels following the procedure described elsewhere [11] with a Mini Protean III Cell system (Bio-Rad, USA). The amount of protein loaded was 5  $\mu$ g per sample. Western blot was performed as described elsewhere [15]. For each strain, the same primary antiserum as indicated in Table 1 was used. HRP-conjugated secondary serum (anti rabbit IgG; Sigma) was used for monovalent primary antisera and AP-conjugated secondary serum (anti-pig IgG; Sigma) was used for polyvalent primary antiserum. For HRP, the substrate was diaminobenzoic acid (DAB) dissolved in citrate buffer (pH 5.2) with H<sub>2</sub>O<sub>2</sub>. For AP, substrates were AS-MX naphthol phosphate and Fast Red TR (Sigma) dissolved in 50 mM Tris-HCl, pH 9.14.

## Results

**Isolation of Cross-Reacting Strains.** The enrichment and isolation procedures were designed in such a way so as to isolate *Shigella* spp. from the environmental samples. Initially, colonies that showed similarities with characteristic *Shigella* colonies on selective MacConkey or xylose lysine desoxycholate agar plates were chosen for further characterization. Eleven isolates were obtained that yielded *Shigella*-like colonies and strong positive results in the slide agglutination test with type-specific *Shigella* antisera (Table 1). Six isolates agglutinated with type-specific monovalent *S. dysenteriae* antiserum, three with type-specific monovalent *S. boydii* monovalent antiserum, and two isolates with polyvalent *S. flexneri* antiserum. No isolate agglutinating with polyvalent or monovalent *S. sonnei* antiserum was obtained.

**Phylogenetic Characterization.** Partial sequencing of the 16S rRNA genes of all isolates showed that none belonged to the *Shigella* genus. BLAST searches identified the strains as belonging to a variety of bacterial groups, including the  $\gamma$ -proteobacteria *Escherichia*, *Enterobacter*, and *Stenotrophomonas*, as well as the Gram-positive genus *Aerococcus* (Table 1). This is the first report of cross-reactivity of type-specific *Shigella* antisera with members of these genera.

**ELISA and Western Blot Analysis.** In order to further investigate the serological cross-reaction with *Shigella* antisera, ELISA and Western blot analyses were

Table 1. 16S rRNA gene sequence similarities and serological cross-reactivity with *Shigella*-specific antisera

Strain designation	Cross-reactive <i>Shigella</i> spp. serotype (ELISA titers in parentheses)	Partial 16S rRNA gene sequence analysis	
		Best match (accession No. in parentheses)	Sequence identity (%)
PLMB I	<i>S. boydii</i> Type 2 (1:5 <sup>6</sup> )	<i>E. coli</i> (AY696669)	≥98
LMB I	<i>S. boydii</i> Type 2 (1:5 <sup>5</sup> )	<i>Enterobacter</i> (AY752940)	≥98
IS10B III	<i>S. boydii</i> Type 15 (1:5 <sup>7</sup> )	<i>E. coli</i> (AY776276)	≥97
IS4SD	<i>S. dysenteriae</i> Type 7 (1:5 <sup>3</sup> )	<i>Enterobacter</i> (AJ564061)	≥98
IS11SD	<i>S. dysenteriae</i> Type 10 (1: 5 <sup>7</sup> )	<i>Enterobacter</i> (AY752936)	≥98
RBSD4	<i>S. dysenteriae</i> Type 8 (1:5 <sup>4</sup> )	<i>Stenotrophomonas</i> (AJ518813)	≥97
RBSD5	<i>S. dysenteriae</i> Type 8(1:5 <sup>4</sup> )	<i>Stenotrophomonas</i> (AJ518813)	≥97
NLSD2	<i>S. dysenteriae</i> Type 3(1:5 <sup>3</sup> )	<i>Aerococcus</i> (AF076639)	≥97
IS13SF	<i>S. flexneri</i> poly B (1:5 <sup>4</sup> )	<i>Enterobacter</i> (AY752940)	≥98
I31	<i>S. flexneri</i> poly B (1:5 <sup>5</sup> )	<i>E. coli</i> (AY776275)	≥98
L1SD	<i>S.a dysenteriae</i> Type 3 (1:5 <sup>3</sup> )	<i>Aerococcus</i> (AF076639)	≥97

performed. Some of the environmental isolates (e.g. PLMB I, IS10B III, and IS11 SD) gave very strong results in the ELISA test and yielded even higher titers than the positive control strains (Table 1). As positive controls, *S. flexneri* ATCC12022 and *S. boydii* ATCC12034 were used, both yielding an ELISA titer of 1:5<sup>5</sup> using *S. flexneri* type 2 and *S. boydii* type 15 antisera, respectively. Other *Enterobacter* strains (LMBI, IS4SD, IS13SF) and I31 *E. coli* strains gave equal or lower ELISA values than the positive control, demonstrating a strong serological variety among the environmental isolates. The *Stenotrophomonas* sp. strain RBSD4 and *Aerococcus* sp. strain NLSD2 gave weaker reactions than the positive control, but still the cross-reactivity was significant.

Western blot analysis of nine of the isolates also demonstrated serological cross-reactivity with *Shigella* antisera. Six representative Western blot profiles are shown in Fig. 1, in addition to type strains of *S. boydii* and *S. flexneri*. IS10BIII and IS11SD strains showed a smear on the Western blot, which might be caused by lipopolysaccharides, as well as a number of protein bands. PLMB1 and LMB1 gave a pattern quite different from the other strains, but they were similar to each other. *Stenotrophomonas* strain RBSD4 yielded a unique pattern with at least five protein bands, whereas *Aerococcus* strain NLSD2 only showed two bands (Fig. 1).

## Discussion

Water samples were collected from various ponds, lakes, and rivers to investigate the presence of environmental strains of *Shigella*. It has previously been found that alkaline peptone water (APW) increases the efficiency for isolation of environmental strains of *Enterobacteriaceae* like *Vibrio cholerae*, and even *Shigella* [1]. Use of streptomycin in nutrient broth has also been recom-

mended for isolation of *Shigella* [1]. A combination of these two methods was used in the present work in order to improve the recovery rate. After enrichment, samples were cultured overnight on selective agar plates, and *Shigella*-like colonies were screened serologically using slide agglutination tests with polyvalent *Shigella*-specific antisera. Most of the isolates used in this study gave serological cross-reactivity with monovalent antisera, but two isolates, I31 and IS13SF, which only reacted with polyvalent antisera, have been included, as they exhibited typical *Shigella*-like colonies on agar plates and were also oxidase negative.

16S rRNA sequence analysis revealed that PLMB I, IS10B III and I31 gave closest match to *E. coli*. The V3 region of the 16S rRNA is almost identical for *E. coli* and *Shigella*; thus, it is not possible to distinguish these two closely related bacteria by V3 region sequence analysis alone. One of the phenotypical characteristics most often used to distinguish *E. coli* and *Shigella* is motility. *Shigella* spp. are always nonmotile, whereas *E. coli* is generally motile. IS10B III and I31 were found to be nonmotile, whereas PLMB I was motile. IS10B III demonstrated a very high titer in ELISA (Table 1) and conferred multiple distinguished strong bands in Western blot with the monovalent antisera of *S. boydii* type 15. This isolate might be the first environmental isolate of *S. boydii*, but further molecular analyses (e.g., the presence of *ipaH*, *virA*, *ial*, or plasmid profile analysis) is required for confirmation [16]. I31 was found to cross-react only with polyvalent antisera and is probably an inactive *E. coli* strain.

Isolates LMB I, IS4SD, IS11SD, and IS13SF were identified as *Enterobacter* spp. according to the 16S rRNA gene sequence analysis. Although these isolates were all *Enterobacter* spp, they revealed different types of serological cross-reactivity with monovalent or polyvalent antisera (Table 1). RBSD4 and RBSD5 were

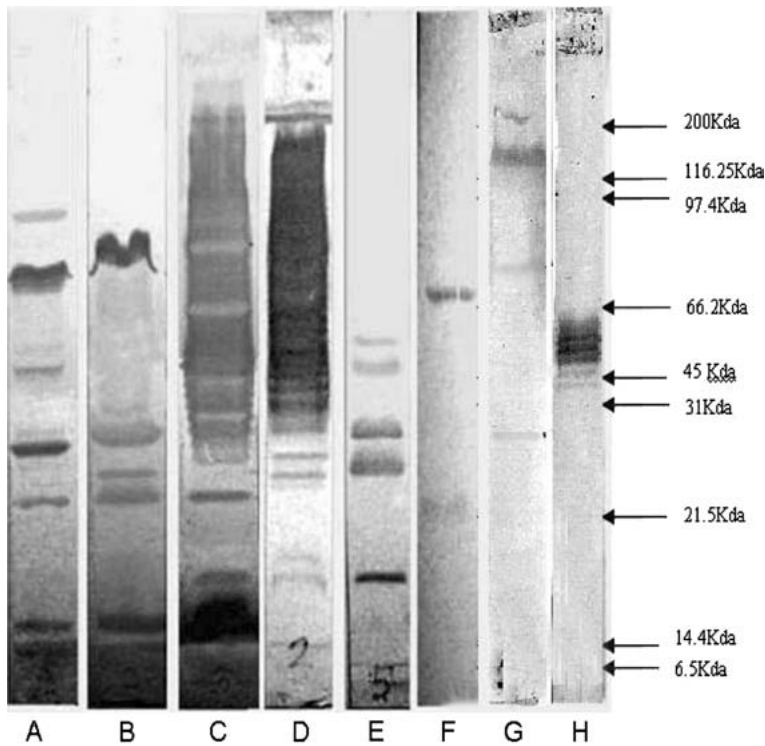


Fig. 1. Western blot membrane showing immunogenic proteins. Lane A = PLMB1; lane B = LMB1; lane C = IS10BIII; lane D = IS11SD; lane E = RBSD4; lane F = NLS2; lane G = *S. flexneri* ATCC 12022, lane H = *S. boydii* ATCC 12034. Arrows indicate molecular-weight markers.

identified as *Stenotrophomonas maltophilia* (previously known as *Pseudomonas maltophilia* or *Xanthomonas maltophilia*), a nonfermentative, Gram-negative bacterium, which plays an increasingly important role as a nosocomial pathogen in compromised patients [4]. Cross-reactivity between *Stenotrophomonas* and *Shigella* spp. has not been reported previously.

Interestingly, NLS2 and L1SD were found to be Gram-positive bacteria but agglutinated with *S. dysenteriae*-specific antisera. These strains were isolated following prolonged incubation of MacConkey agar plates. The 16S rRNA sequence analysis of NLS2 and L1SD revealed that these isolates were most closely related to *Aerococcus*. This is the first report on the serological cross-reactivity of a Gram-positive bacterium with *Shigella*.

Sonicated whole-cell extracts of the isolates were subjected to ELISA for quantification, even more than that of the positive controls. IS4SD and NLS2 exhibited relatively low titer obtained with ELISA. The similarities in the surface structure of NLS2 and IS4SD with those of *Shigella* might be significantly lower than the other isolates.

Representatives of each group of isolates were subjected to Western blot analysis. The banding pattern and the number of bands varied significantly from organism to organism. A smear of bands was found in the case of IS10B III and IS11SD, indicating that these

isolates possess a huge similarity in the surface structures with that of *S. boydii* type 15 or *S. dysenteriae* type 10. Only one band was observed after Western blotting in the case of the NLS2 isolate. Normally, cross-reactivity among Enterobacteriaceae is due to similarities in the outer membrane proteins, but cross-reactivity between *E. coli* O164 and *S. dysenteriae* type 3 has previously been shown to be due to a similar O-antigen structure [13].

We found that a variety of environmental isolates cross-reacted with different types of *Shigella*-specific monovalent antisera. Yet, to date, no such findings have been reported. However, based on our results, we cannot identify the major cellular components giving rise to this cross-reactivity. Therefore, further studies should be carried out to confirm this finding. Investigation of proteins and plasmid-encoded antigens of *Shigella* spp. as candidates for vaccine development against shigellosis is currently a major task. The environmental isolates yielding a high cross-reactivity with various types of *Shigella* antisera represent a potential for development of a shigellosis vaccine based on live cells because they probably are not virulent, like clinical organisms. This possibility will be further explored.

#### ACKNOWLEDGMENT

This study was supported by The Norwegian Programme for Development, Research and Higher Education (NUFU) (grant No. PRO 52/03)

## Literature Cited

1. American Public Health Association (1992) Detection of pathogenic bacteria. In: Greenberg AE, Clesceri LS, Eaton AD (eds) Standard methods for the examination of water and wastewater. 18th ed. Washington, DC: American Public Health Association, pp 991–9100
2. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72:248–254
3. Chen LC, Rahman M, Sarder AM (1980) Epidemiology and causes of death among children in a rural area of Bangladesh. *Int J Epidemiol* 9:25–33
4. Oliveira-Garcia D, Dall'Agnol D, Rosales M, et al. (2002) Characterization of flagella produced by clinical strains of *Stenotrophomonas maltophilia*. *Emerg Infect Dis* 8(9):918–923
5. Evins GM, Gheesling LL, Tauxe RV (1988) Quality of commercially produced *Shigella* serogrouping and serotyping antisera. *J Clin Microbiol* 26:438–442
6. Ewing WH, Lindberg AA (1984) Serology of *Shigella*. In: Bergan T. (eds) *Methods in microbiology*. Vol 14. London: Academic Press, pp 113–142
7. Faruque SM, Khan R, Kamruzzaman M, et al. (2002) Isolation of *Shigella dysenteriae* type 1 and *S. flexneri* strains from surface waters in Bangladesh: Comparative molecular analysis of environmental *Shigella* isolates versus clinical strains. *Appl Environ Microbiol* 68:3908–3913
8. Kelly MT, Brenner DJ, Farmer JJ (1985) *Enterobacteriaceae*. In: Lennette EH, Balows A, Hausler WJ Jr, et al. (eds) *Manual of clinical microbiology*. 4th ed. Washington, DC: American Society for Microbiology, pp 263–277
9. Kotloff KL, Winickoff JP, Ivanoff B, et al. (1999) Global burden of *Shigella* infections: Implications for vaccine development and implementation of control strategies. *Bull World Health Org* 77:651–666
10. Krajaejun T, Kunakorn M, Niemhom S, et al. (2002) Development and evaluation of an in-house enzyme-linked immunosorbent assay for early diagnosis and monitoring of human pythiosis. *Clin Diagn Lab Immunol* 9:378–382
11. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
12. Lefebvre J, Gosselin F, Ismail J, et al. (1995) Evaluation of commercial antisera for *Shigella* serogrouping. *J Clin Microbiol* 33:1997–2000
13. Orskov I, Orskov F, Jann B, et al. (1977) Serology, chemistry and genetics of O: An K antigen of *Escherichia coli*. *Bacteriol Rev.* 41:667–710
14. Øvreås L, Forney L, Daae FL, et al. (1997) Distribution of bacterioplankton in meromictic lake Sælenvannet, as determined by denaturing gradient gel electrophoresis of PCR amplified gene fragments coding for 16S rRNA. *Appl Environ Microbiol* 63:3367–3373
15. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedures and some applications. *Proc Natl Acad Sci USA* 76:4350–4354
16. Wei J, Goldberg MB, Burland V, et al. (2003) Complete genome sequence and comparative genomics of *Shigella flexneri* serotype 2a strain 2457T. *Infect Immun* 71(5):2775–2786