Serological cross-reactivity of environmental *Escherichia coli* strains with *Shigella*-specific antisera

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Many bacteria of clinical and environmental origin show evidence of sharing common surface antigens. The present study aimed for isolation of *Escherichia coli* strains that were serologically cross-reactive with *Shigella* species from freshwater ecosystems in Bangladesh by conventional cultural methods. Among twenty eight isolates, two isolates, termed 12(35) and 6(50) showed cross-reactivity with four polyclonal serogroup-specific *Shigella* antisera using slide agglutination assay. The isolates were identified and characterized by cultural and biochemical properties and Western blot analysis. The isolates showed typical *Escherichia coli* cell morphology and cultural and biochemical properties and were identified as *Escherichia coli* by API 20E tests. Western blot analysis confirmed the isolates as cross-reactive with all the four group-specific *Shigella* antisera due to presence of immunogenic proteins and LPS. One of the isolates also showed cross-reactivity with multiple type-specific *Shigella boydii* antisera (monovalent) because of immunogenic proteins. Both the isolates were identified as nonpathogenic due to absence of virulence marker genes of diarrheagenic *E. coli* variants. This study revealed that a number of bacteria present in the environment could share important *Shigella* species surface antigens. Naturally occurring nonpathogenic environmental bacteria expressing surface antigens specific for certain types of *Shigella* could be a good choice for vaccine candidates against shigellosis.

**Keywords:** *E. coli*, *Shigella*, environmental bacteria, serological cross-reactivity, fresh water environment

**Introduction**

Diarrheal diseases cause severe health problems in Bangladesh, where 1.3% of infant deaths occur due to these diseases¹. In developing countries like Bangladesh, children under 5 years of age are the most vulnerable group affected by diarrheal diseases due to fecal contamination of surface water bodies². *Shigella* species are one of the major diarrheagenic pathogens, causing bloody diarrhea (bacillary dysentery or shigellosis). Approximately 125 million shigellosis cases and 14,000 related deaths occur annually in Asia³. The need for shigellosis vaccine is thus evident, particularly because of the global emergence of antibiotic-resistant *shigellae* strains. Protective immunity against shigellosis is serotype specific and directed against the surface lipopolysaccharide antigens. Based on serogrouping, the organism is sub-grouped into four different species: *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnet*⁴. Each species encompasses 2 to 20 serologically distinct serotypes, as based on the O-antigen (somatic) properties, thus making it difficult to design a common shigellosis vaccine. At present, no effective vaccine against *Shigella* infection exists⁵. Laboratory diagnosis of *Shigella* infections mostly relies on serological typing usually with a combination of Kligler’s iron agar or triple sugar iron agar tests⁶. Serotyping remains the ‘gold standard’ for identifying *Shigella* species⁷. However, serological cross-reactivity of different types of clinical bacteria such as *Aeromonas caviae*, *Hafnia alvei*, *Plesiomonas shigelloides*, *Providencia alcalifaciens*, *Yersinia enterocolitica* and *E. coli* with *Shigella*-group specific polyclonal antisera was already known⁸. But recently, many bacteria of the *Enterobacteriaceae* family have been recovered from environmental sources that were found to be cross-reactive with a number of *Shigella* serogroup- and serotype-specific antisera due to sharing of *Shigella* species-specific O-antigens or proteins⁹-¹¹. These types of bacterial genera if identified as nonpathogenic could be a good choice as vaccine candidates for combating shigellosis.

The present study was designed for the isolation of environmental *E. coli* strains sharing common surface antigens with *Shigella* species.

**Materials and Methods**

**Sampling and isolation**

Environmental surface water sample was collected from a pond of Azimpur area, Dhaka, Bangladesh in sterile sampling bottles. For the recovery of both cultivable bacterial populations as well
as environmentally stressed and injured cells, the preliminary step included pre-enrichment of the sample with a 50 ml 0.25% Nutrient broth medium (Oxoid, UK). After 10 h incubation, pre-enriched sample was diluted to proper concentrations depending on the growth in the liquid media, and 50–100 µl inoculum from each dilution was spread-plated onto MacConkey agar. As initially the study focused on isolation of members of Enterobacteriaceae family that are serologically cross-reactive with Shigella spp., both lactose fermenter and nonfermenter colonies were selected after 24 h incubation.

**Preliminary screening of bacterial isolates by slide agglutination test**

Suspected colonies were subcultured on a nonselective medium and tested by slide agglutination using a commercially available antisera kit (Denka Seiken, Japan) specific for all group- and type-factor antigens of Shigella spp. according to the procedure described previously.

**Identification of bacterial isolates by morphological, cultural and biochemical properties**

The bacterial isolates that showed positive slide agglutination test with Shigella-specific polyvalent and monovalent antisera were then characterized and identified by microscopic, cultural and biochemical properties. Microscopic study was carried out for the determination of size, shape, arrangement and Gram staining of the desired isolates. Two selective and differential media like Xylose-Lysine-Desoxycholate (XLD) agar (Oxoid, UK) and Eosine Methylene Blue (EMB) agar (Oxoid, UK) were used for detection of cultural properties of the bacterial isolates. Initially, five biochemical tests were performed according to the methods as described in “Manual of Methods for General Bacteriology” (ASM, 1981). These biochemical tests were: utilization of glucose and lactose in Kliger’s Iron Agar, Citrate utilization, Motility–Indole–Urea (MIU), Oxidase and Lysine decarboxylase test. The selected bacterial isolates were then subjected to full biochemical profiling using an API 20E kit following the manufacturer’s (BioMerieux, France) instructions. The numerical profile was used for on-line identification of the isolates using API web (https://apiweb.biomerieux.com). A number of amino acids and sugar utilization tests were also carried out for finally selected isolates.

**Bacterial LPS and protein extraction**

A mixture of LPS and proteins were extracted by using a modified version of the protocol originally established by Hitchcock and Brown. Briefly, a clearly isolated colony of fresh culture of each isolate was inoculated into Brain Heart Infusion Broth (Oxoid, UK) and the organisms were grown at 37°C for 24 h in a shaker. Bacterial cell mass were harvested by centrifugation and was added to SDS-PAGE solubilization buffer [62.5 mM Tris-HCl, 10% (v/v) glycerol, 3%(w/v) SDS, 5%(v/v) mercaptoethanol and 0.01% (w/v) bromophenol blue] in proportion of 30 µl buffer for 1 mg of bacterial cell mass, followed by a 10 min incubation in the boiling water bath with occasional mixing. The mixture was centrifuged and supernatant was transferred to fresh tubes which is the mixture of LPS and protein.

**Western blot analysis**

LPS and protein mixture was fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels following the procedure described elsewhere with a Mini Protein III cell system (Bio-Rad, USA). Ten µl of molecular weight standard (Broad Range SDS-PAGE standards, Bio-Rad, USA) was used as a marker. Western blot was performed as described previously. Eight Shigella serogroup-specific polyvalent antisera and forty one Shigella serotype-specific monovalent antisera were used as primary antisera. Alkaline Phosphatase (AP) conjugated secondary serum (anti pig IgG developed in rabbit, Sigma, USA) was used for polyvalent primary antisera and Horse Reddish Peroxidase (HRP) conjugated secondary serum (anti rabbit IgG, developed in goat, Sigma, USA) was used for monovalent primary antisera. For AP, substrate was 10 mg NBT/BCIP (Sigma, USA) in 10 ml H₂O. For HRP, the substrate was diaminobenzoic acid (DAB) dissolved in citrate buffer (pH 5.2) with H₂O₂. LPS and protein mixture of E. coli K-12 strain was used as negative control.

**Detection of diarrheagenic E. coli - specific virulence genes**

Chromosomal DNA of selected isolates was extracted and purified by ’Sigma GenElute Bacterial Genomic DNA Extraction Kit’ (Sigma, USA) according to the manufacturer’s instruction for Gram-negative bacteria. Presence of virulence genes of five diarrheagenic E. coli variants in the selected isolates were assessed by a multiplex PCR assay according to a previously described protocol. Eight virulence marker genes for diarrheagenic E. coli were used, which included: _aeae_ (structural gene for intimin of EHEC and EPEC), _bfpA_ (structural gene for the bundle-forming pilus of EPEC), _vt1_ and/or _vt2_ (Shiga toxins 1 and 2 of EHEC), _eltB_ and/or _esta_ (enterotoxins of ETEC), _ial_ (invasion-associated locus of the invasion plasmid found in EIEC), and _pCDV_ (the nucleotide sequence of the _EcoRI-PstI_ DNA fragment of _pCVD432_ of EAEC). PCR was performed in a 30 µl reaction mixture containing 1 µl of template DNA, 3 µl of 10× buffers for DyNAzyme, 0.6 µl of mixture of deoxynucleoside triphosphates (25 mM each), 0.3 µl of 2 U/µl of DyNAzyme™ DNA polymerase (Finnzymes), and 1.0 µl of a 25 µm solution of each primer (Sigma, Germany). Thermocycling conditions were followed according to the protocol described previously. PCR products (10 µl) were evaluated by electrophoresis through a 1.5%(wt/vol) agarose gel (SeaKem® LE Agarose, USA) at 80 V for 45 minutes with a molecular marker (1-kb DNA ladder, Fermentus, UK) concurrently. The DNA bands were visualized and photographed under UV illuminator (Bio-Rad, USA) after the gel was stained with ethidium bromide.

**Results**

The study was designed to isolate E. coli strains from fresh water environments which share common surface antigens with Shigella species. After isolation using conventional cultural techniques, preliminary serological screening was done through...
slide agglutination test. Among twenty eight environmental isolates, two isolates, termed strain 12(35) and 6(50), that showed positive agglutination results with all *Shigella* group-specific polyvalent antisera (Denka Seiken, Japan) were selected. Microscopic observation revealed these isolates as Gram-negative, short rods. Both the isolates showed typical *E. coli* colony morphologies in different selective and differential media, i.e., lactose fermenting pink colonies on MacConkey agar, yellow colonies on XLD agar and colonies with green metallic sheen on EMB agar media (Table 1). These isolates also showed typical *E. coli* characteristics in conventional biochemical tests (Table 2). Finally, biochemical identification by API 20E profiling revealed the numerical profile, 5144572, suggesting these isolates to be *E. coli* with very high probability (99.5%).

Primarily, both the isolates 12(35) and 6(50) showed cross-reactivity with group specific *S. dysenteriae, S. flexneri, S. boydii* and *S. sonnei* polyvalent antisera in slide agglutination test. To investigate the reason behind this cross-reactivity, Western blot analyses of protein/LPS extracts of these isolates using *Shigella*-group specific polyvalent antisera was done. Both the isolates showed diverse antigenic properties in Western blot analyses. In most cases, multiple bands representing proteins and/or LPS that reacted with the antisera were detected (Figure 1), and confirmed the slide agglutination results. Further determination of the cross-reaction of these two isolates with type-specific *Shigella* antisera were done by Western blot analyses of the protein-LPS extracts with *Shigella* type-specific monovalent antisera. The isolate 6(50) showed cross-reactivity with a number of *S. boydii* monovalent antisera (type 2-5, 7-8 and 12-18) due to the presence of 1-2 immunogenic proteins (Figure 2). However, the isolate 12(35) didn’t show any cross-reaction with any of the *Shigella*-type specific monovalent antisera.

Finally, both the isolates were subjected to genomic DNA extraction followed by Polymerase Chain Reaction test to determine the presence of virulence marker genes of five diarrheagenic variants of *E. coli*. Presence of eight virulence genes, i.e., *eaeA* (EHEC and EPEC), *bfpA* (EPEC), *vt1* and/or *vt2* (EHEC), *eltB* and/or *estA* (ETEC), *ial* (EIEC), and pCVD (EAEC) were tested. Neither of the strains was positive for any of the virulence marker genes tested and thus identified as nonpathogenic.

### Table 1: Colony morphologies of environmental isolates on selective and differential media

<table>
<thead>
<tr>
<th>Isolates</th>
<th>MacConkey agar</th>
<th>XLD agar</th>
<th>EMB agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>12(35)</td>
<td>Medium, entire, flat, pink, opaque</td>
<td>Small, entire, raised, yellow, opaque</td>
<td>Medium, entire, flat, metallic sheen, opaque</td>
</tr>
<tr>
<td>6(50)</td>
<td>Large, circular entire, flat, pink, opaque</td>
<td>Small, entire, raised, yellow, opaque</td>
<td>Medium, entire, flat, metallic sheen, opaque</td>
</tr>
</tbody>
</table>

### Table 2: Biochemical properties of environmental isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>KIA</th>
<th>MIU</th>
<th>Citrate</th>
<th>Oxidase</th>
<th>Amino acids</th>
<th>Carbohydrates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slant/Bul</td>
<td>Gas</td>
<td>H2S</td>
<td>Motility</td>
<td>Urease</td>
<td>Lysine</td>
</tr>
<tr>
<td>12(35)</td>
<td>A/A</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6(50)</td>
<td>A/A</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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**Note:** A/A= Acid/Acid, ‘+’= Utilization and ‘-’= No utilization, ‘A’= Acid and ‘G’= Gas
Discussions

The present study focused on the isolation and characterization of *E. coli* strains that were serologically cross-reactive with *Shigella* species from natural freshwaters in Bangladesh in order to enhance our knowledge of their distribution, survival and diversity in the environment. Among twenty eight recovered isolates, two environmental isolates were selected as they yielded positive results in the slide agglutination test with *Shigella* group specific polyvalent antisera. The selected isolates – 12(35) and 6(50) showed cross-reaction with all the group specific *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* polyvalent antisera.

The isolates were further subjected to phenotypic characterization which showed that they belonged to the *Enterobacteriaceae* family. The isolates showed typical *E. coli* cell morphology, colony characteristics and metabolic properties. Finally, the strains were identified through the numerical profile index and the APIweb identification tool (https://apiweb.biomerieux.com/servlet/). The numeric profile was 5144572, thus identifying both the isolates as *E. coli* with 99.5% probability.

The serological cross-reactivity of the isolated *E. coli* strains were confirmed by Western blot analysis of the proteins-LPS extract with the group specific *Shigella* antisera (polyvalent). Both the isolates showed cross-reactivity with four *Shigella* groups due to presence of immunogenic proteins/LPS. These proteins-LPS extracts were further analysed with forty one *Shigella*-type specific monovalent antisera (Denka Seiken, Japan) but only the isolate 6(50) showed cross-reaction with multiple type-specific antisera. Although strain 6(50) was found cross-reactive with all the group-specific polyvalent antisera, it showed cross-reactivity with only *S. boydii* type members (type 2-5, 7-8 and 12-18) due to presence of immunogenic proteins. However, the isolate 12(35) did not share any antigens with any of the *Shigella*-type members. The isolates were also found to be nonpathogenic as they lacked all of the virulence marker genes normally found in diarrheagenic variants of *E. coli*.
Several studies have been conducted previously on the serological cross-reaction between *Shigella* and other bacterial genera. Normally, cross-reactivity among *Enterobacteriaceae* is due to similarities in the outer membrane proteins\(^1^6\). It was observed that various types of bacteria of clinical origin such as *Haemalia alvei*, *Plesiomonas shigelloides*, *Providencia alcalifaciens*, *Yersinia enterocolitica* serotype O3 and *E. coli* (O114:H32, O157:H7, O157:H19 etc.) gave serological cross-reactivity with polyclonal group-specific *Shigella* antisera\(^8\). A majority of the *Shigella* spp. strains are also known to cross-react with strains of *E. coli*\(^1^7\). Among the current 34 distinct O antigens in *Shigella* species, 21 are either identical or closely related to an *E. coli* O antigen\(^1^8\). Cross-reactivity of many other bacteria with different *Shigella*-type specific antisera has also been reported\(^1^9-^2^2\). But recently, many environmental bacteria that belong to the *Enterobacteriaceae* family (such as *Enterobacter*, *Escherichia albertii*, *Escherichia coli*, *Escherichia fergusonii*, and *Stenotrophomonas*) cross-reacting specifically with *Shigella* serogroups and -types were reported\(^2^3-^2^6\). These bacteria were found to be cross-reactive with number of *Shigella* serogroup- and serotype-specific antisera due to sharing of *Shigella* species-specific O-antigens or proteins. However, sharing of surface antigens by a single *E. coli* strain that cross-react with several *Shigella* groups and types was not reported earlier.

A nontoxic and nonpathogenic bacterial strain able to colonize the gut epithelial cells as in a natural *Shigella* infection and elicit mucosal and/or humoral immunity could develop protective efficacy against the corresponding wild type *Shigella* strains. Environmental microorganisms are usually less virulent than that of clinical organisms. As development of protective immunity against *Shigella* infection is serotype specific, a single live bacterium that could develop protection against multiple *Shigella* serogroups and/or serotypes could be a good choice as candidate vaccine strain. Therefore, these isolates could be a good choice of bacteria for live *Shigella* vaccine development. Further analysis of strains 12(35) and 6(50) should be carried out to investigate their suitability as candidate vaccine strains against shigellosis.

**References**

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