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Original Article

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 polyvalent serogroup-specific *Shigella* antisera using slide agglutination assay. The isolates were identified and charcterized 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 diseases[1]. 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 bodies[2]. *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 Asia[3]. 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 sonnei*[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 exists[5].

Laboratory diagnosis of *Shigella* infections mostly relies on serological typing usually with a combination of Kligler's iron agar or triple sugar iron agar tests[6]. Serotyping remains the 'gold standard' for identifying *Shigella* species[7]. 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 polyvalent antisera was already known[8]. 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 proteins[9-11]. 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

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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[8].

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 proterties. 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 Kligler's Iron Agar, Citrate utilization, Motility–Indole–Urea (MIU), Oxidase and Lysine decaroboxylase 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[12]. 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[13] with a Mini Protean 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[14]. 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 antiserum and Horse Reddish Peroxidase (HRP) conjugated secondary serum (anti rabbit IgG, developed in goat, Sigma, USA) was used for monovalent primary antiserum. For AP, substrate was 10 mg NBT/BCIP (Sigma, USA) in 10 ml H_2O. For HRP, the substrate was diaminobenzoic acid (DAB) dissolved in citrate buffer (pH 5.2) with H_2O_2. 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[15]. Eight virulence marker genes for diarrheagenic *E. coli* were used, which included: *eaeA* (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 pCVD (the nucleotide sequence of the *Eco*RI-*Pst*I 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[15]. PCR products (10 µl) were evaluated by electrophoresis through a 1.5% (wt/vol) agarose gel (SeaKem® LE Agarose, USA) at 80 mV for 45 min 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*

Isolates	Culture media		
	MacConkey agar	**XLD agar**	**EMB agar**
12(35)	Medium, entire, flat, pink, opaque	Small , entire, raised, yellow, opaque	Medium, entire, flat, metallic sheen, opaque
6(50)	Large, circular entire, flat, pink, opaque	Small, entire, raised, yellow, opaque	Medium, entire, flat, metallic sheen, opaque

Table 2: *Biochemical properties of environmental isolates*

Isolates	KIA			MIU		Citrate	Oxidase	Amino acids			Carbohydrates							
	Slant/Butt	Gas	H_2S	Motility	Urease			Lysine	Arginine	Ornithin	Salicine	Sucrose	Xylose	Dulcitol	Lactose	Mannose	Mannitol	Arabinose
12(35)	A/A	-	-	+	-	-	-	+	+	+	-	A G	A G	A	A G	A	A G	A G
6(50)	A/A	+	-	+	-	-	-	+	+	+	-	A	A G	A G	A G	A	A G	A

**Note: A/A= Acid/Acid, '+'= Utilization and '-'= No utilization, 'A'= Acid and 'G'= Gas

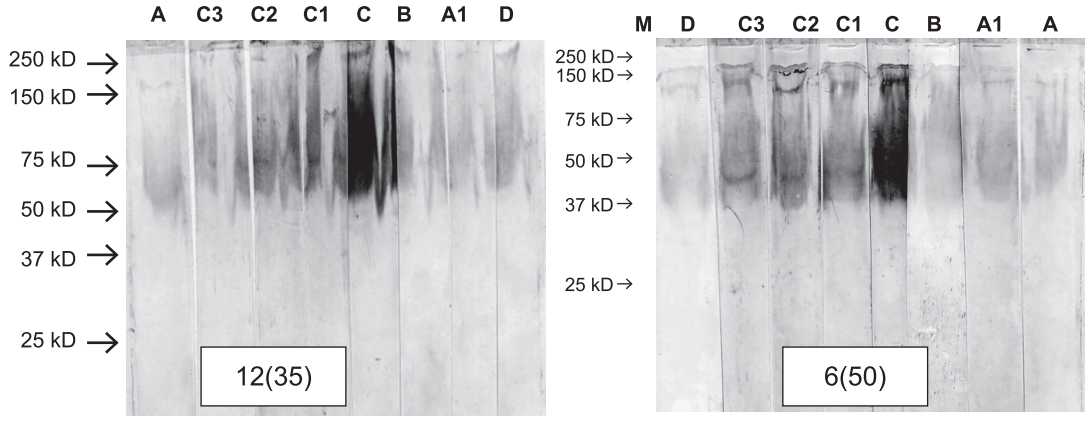

Figure 1. *Western blot showing cross-reacting antigenic components of environmental isolates 12(35) and 6(50) with different Shigella group-specific polyvalent antisera like S. dysenteriae polyvalent A & A1, S. flexneri polyvalent B, S. boydii polyvalent C, C1, C2 & C3 and S. sonnei polyvalent dantisera. The type of antisera used are indicated by letters above the lanes and the isolates' designations are mentioned at the bottom of the panels. 'M' indicates molecular weight standard*

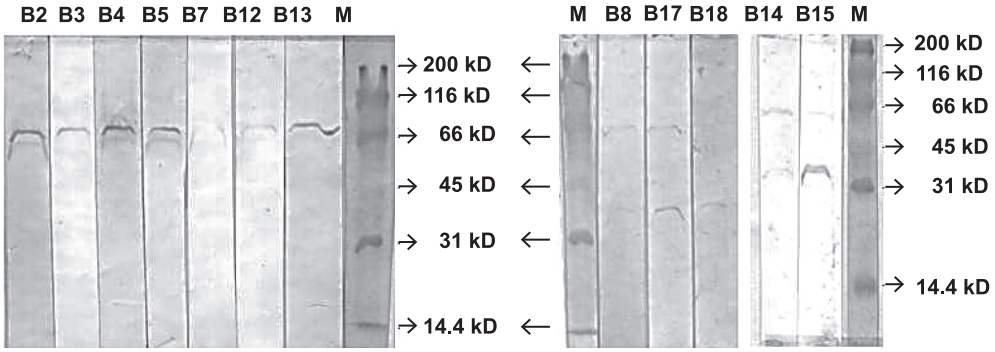

Figure 2. *Western blot showing cross-reacting antigenic components of environmental isolate 6(50) with Shigella type-specific monovalent antisera like S. boydii monovalent antisera B2, B3, B4, B5, B7, B8, B12, B13, B14, B15, B17 and B18 (as indicated above the lanes). 'M' indicates molecular weight standard*

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 chracterization 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[16]. It was observed that various types of bacteria of clinical origin such as *Hafnia 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*[17]. Among the current 34 distinct O antigens in *Shigella* species, 21 are either identical or closely related to an *E. coli* O antigen[18] . Cross-reactivity of many other bacteria with different *Shigella*-type specific antisera has also been reported[19-22]. 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[9-11]. 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

1. WHO. 2009. World health statistics.
2. Caldwell BK, Caldwell JC, Mitra SN & Smith W. 2003. Searching for an optimum solution to the Bangladesh arsenic crisis. *Soc Sci Med.* **56(10)**:2089-2096.
3. Bardhan P, Faruque AS, Naheed A & Sack DA. 2010. Decrease in shigellosis-related deaths without *Shigella* spp.-specific interventions, Asia. *Emerg Infect Dis.* **16(11)**:1718-1723.
4. Hale TL. 1991. Genetic basis of virulence in *Shigella* species. *Microbiol Rev.* **55(2)**:206-224.
5. Levine MM, Kotloff KL, Barry EM, Pasetti MF & Sztein MB. 2007. Clinical trials of *Shigella* vaccines: two steps forward and one step back on a long, hard road. *Nat Rev Microbiol.* **5(7)**:540-553.
6. WHO. 2003. Manual for the laboratory identification and antimicrobial susceptibility testing of bacterial pathogens of public health importance in the developing world.WHO/CDS/CSR/EPH/2002. **15**:121-140.
7. Melito PL, Woodward DL, Munro J, Walsh J, Foster R, Tilley P, Paccagnella A, Isaac-Renton J, Ismail J & Ng LK. 2005. A novel *Shigella dysenteriae* serovar isolated in Canada. *J Clin Microbiol.* **43(2)**:740-744.
8. Lefebvre J, Gosselin F, Ismail J, Lorange M, Lior H & Woodward D. 1995. Evaluation of commercial antisera for *Shigella* serogrouping. *J Clin Microbiol.* **33(8)**:1997-2001.
9. Rahman MZ, Sultana M, Khan SI & Birkeland NK. 2007. Serological cross-reactivity of environmental isolates of *Enterobacter*, *Escherichia*, *Stenotrophomonas*, and *Aerococcus* with *Shigella* spp.-specific antisera. *Curr Microbiol.* **54(1)**:63-67.
10. Azmuda N, Rahman MZ, Sultana M, Jenssen EL, Khan SI & Birkeland NK. 2012. Evidence of interspecies O antigen gene cluster transfer between *Shigella boydii* 15 and *Escherichia fergusonii*. *APMIS.* **120(12)**:959-966.
11. Rahman MZ, Akter S, Azmuda N, Sultana M, Weill FX, Khan SI, Grimont PAD & Birkeland NK. 2013. Serological cross-reaction between O-antigens of *Shigella dysenteriae* type 4 and an environmental *Escherichia albertii* isolate. *Curr Microbiol.* **67(5)**: 590-595.
12. Hitchcock PJ & Brown TM. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J Bacteriol.* **154(1)**:269-277.
13. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227(5259)**: 680-685.
14. Towbin H, Staehelin T & Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A*. **76(9)**:4350-4354.
15. Svenungsson B, Lagergren A, Ekwall E, Evengard B, Hedlund KO, Karnell A, Lofdahl S, Svensson L & Weintraub A. 2000. Enteropathogens in adult patients with diarrhea and healthy control subjects: a 1-year prospective study in a Swedish clinic for infectious diseases. *Clin Infect Dis.* **30(5)**:770-778.
16. Orskov I, Orskov F, Jann B & Jann K. 1977. Serology, chemistry, and genetics of O and K antigens of *Escherichia coli*. *Bacteriol Rev.* **41(3)**:667-710.
17. Henrik C, Daniel RMA & Cheasty T. 2009. The expression of lipopolysaccharide by strains of *Shigella dysenteriae*, *Shigella flexneri* and *Shigella boydii* and their crossreacting strains of *Escherichia coli*. *FEMS Microbiol Lett.* **292**:21-26.
18. Liu B, Knirel YA, Feng L, Perepelov AV, Senchenkova SN, Wang Q, Reeves PR & Wang L. 2008. Structure and genetics of *Shigella* O antigens. *FEMS Microbiol Rev.* **32(4)**:627-653.
19. Albert MJ, Ansaruzzaman M, Qadri F, Hossain A, Kibriya AK, Haider K, Nahar S, Faruque SM & Alam AN. 1993. Characterisation of *Plesiomonas shigelloides* strains that share type-specific antigen with *Shigella flexneri* 6 and common group 1 antigen with *Shigella flexneri* spp. and *Shigella dysenteriae* 1. *J Med Microbiol.* **39(3)**: 211-217.
20. Albert MJ, Qadri F, Ansaruzzaman M, Kibriya AK, Haider K, Neogi PK, Alam K & Alam AN. 1992. Characterization of *Aeromonas caviae* antigens which cross-react with *Shigella boydii* 5. *J Clin Microbiol*. **30(5)**:1341-1343.
21. Ansaruzzaman M, Albert MJ, Holme T, Jansson PE, Rahman MM & Widmalm G. 1996. A *Klebsiella pneumoniae* strain that shares a type-specific antigen with *Shigella flexneri* serotype 6. Characterization of the strain and structural studies of the O-antigenic polysaccharide. *Eur J Biochem.* **237(3)**:786-791.
22. Rabbi F, Sultana N, Rahman T, Al-Emran HM, Uddin MN, Hossain M, Anwar KS, Yasmin M, Nessa J & Ahsan CR. 2008. Analysis of immune responses and serological cross reactivities among *Vibrio cholerae* O1, *Shigella flexneri* 2a and *Haemophilus influenzae* b. *Cell Mol Immunol.* **5(5)**:393-396.