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Virulence of Environmental *Stenotrophomonas maltophilia* Serologically Cross-reacting with *Shigella*-specific Antisera

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Abstract: This research involved an environmental strain of *Stenotrophomonas maltophilia* which has been reported to produce serological cross-reactivity with *Shigella dysenteriae* type 8 specific antisera. Since clinical diagnosis of shigellosis is largely based on culture and serology, the investigation was aimed at *in vivo* and *in vitro* virulence comparison between the culturally similar environmental *S. maltophilia* isolate and the reference *S. dysenteriae* strains. The findings of this study revealed the absence of virulent genes of *Shigella* sp. like *ipa*H, *vir*A and *stx*1 and characteristic invasive large plasmid in the test isolate. The Western blot analysis revealed that serological cross-reactivity of *Stenotrophomonas maltophilia* was due to certain protein component(s) in its outer membrane. The isolate was capable of producing extracellular protease, exhibited alpha hemolysis and was negative for hemagglutinating assay. The isolate gave negative reaction with rabbit ileal loop and Sereny tests. The *S. maltophilia* isolate did not possess any enterotoxic or invasive property as that of virulent *S. dysenteriae* strains. Further characterizations and adequate genetic manipulations of this environmental isolate may contribute to the development of a potential vaccine candidate for shigellosis.

Key words: Serological cross-reactivity, *Shigella*-specific antisera, *Stenotrophomonas maltophilia*, virulence

INTRODUCTION

Shigellosis or bacillary dysentery caused by *Shigella* sp. is endemic in many developing countries including Bangladesh. 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 (WHO, 2005). By the age of 2, about 37% of the children in Bangladesh have acquired the infection (Seidlein *et al.*, 2006). The poor sanitary conditions in developing countries like Bangladesh contribute to the spread of the disease and increasing antibiotic resistance further complicates the treatment. A safe and effective *Shigella* vaccine offers great potential as a means of preventing shigellosis.

Laboratory diagnosis of *Shigella* is often based on the isolation of the organism from feces of the patients by means of cultural and biochemical characteristics (Kelly *et al.*, 1985). The slide agglutination test using a number of commercially available antisera specific to different groups and types, is a common and indispensable test for serological typing of *Shigella* sp. (Ewing and Lindberg, 1984; Evins *et al.*, 1988).

Bacterial strains carrying identical or similar antigenic components on their surface structure might also react with the antibodies produced against other strains (Grubor *et al.*, 2006; Houpikian and Raoult, 2003; Khan *et al.*, 2003). It was found that various types of microorganisms such as *Hafnia alvei*, *Plesiomonas shigelloides*, *Providencia alcalifaciens* and *Yersinia enterocolitica* serotype 03 and *E. coli* (O114:H32, O157:H7, O157:H19, etc.) gave serological cross-reactivity with polyclonal group-specific *Shigella* antisera (Lefebvre *et al.*, 1995). A number of environmental isolates were also found to cross-react with different types of *Shigella*-specific monovalent antisera. But the major cellular components giving rise to such cross-reactivity remains to be identified. Rahman *et al.* (2007) demonstrated that various environmental isolates cross-reacted with six different serotypes of *Shigella* and were, in each case, highly type-specific. Amongst those isolates, two environmental *S. maltophilia* exhibited significant serological cross-reactivity with *S. dysenteriae* type 8 specific monovalent antisera. These environmental isolates producing 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 (Rahman *et al.*, 2007).

The present investigation involved the comparison of virulent properties of the previously reported environmental *Stenotrophomonas maltophilia* isolate (Rahman *et al.*, 2007) with *Shigella dysenteriae* strains and to identify the putative cellular components giving rise to this type of serological cross-reactivity.

MATERIALS AND METHODS

Bacterial strains: The test organism *Stenotrophomonas maltophilia* (RBSD4), was an environmental isolate and three clinical *Shigella dysenteriae* strains (*S. dysenteriae* type 2 (602), type 3 (C253) and type 4 (613)) were used as reference. All the organisms were obtained from the Environmental Microbiology Laboratory, Department of Microbiology, University of Dhaka, Bangladesh in 2009.

Biotyping of the test isolate *Stenotrophomonas maltophilia*: Biotyping of the test isolate *S. maltophilia* along with the reference *Shigella dysenteriae* strains were performed to reveal similarities or dissimilarities in their biochemical behavior.

Detection of *Shigella*-specific virulence genes (*ipa*H, *vir*A and *stx*1) by PCR
Extraction of chromosomal DNA: Chromosomal DNA of the test isolate and the reference strains were extracted and purified according to the method described by Sambrook *et al.* (1989). Following this method, pellets from overnight fresh culture were collected by centrifugation at 10,000 rpm for 5 min and were resuspended in 100 µL of solution I containing 50 mM Tris-HCl buffer (pH: 7.5), 50 mM EDTA and 20% (w/v) sucrose. This was followed by addition of 300 µL of solution II containing 1% (w/v) SDS and 50 mM NaCl and 5 µL of proteinase K (20 mg mL^{-1}) to each eppendorf. After gentle shaking, the pellet suspension was incubated at 55°C for 60 min in a water bath. DNA was extracted by using 400 µL phenol: chloroform: isoamylalcohol mixture (25:24:1). The and the top phase was recovered carefully into fresh

tubes were mixed well, centrifuged for 10 min at 10,000 rpm eppendorf tubes. Double volume of ice-cold 100% ethanol followed by 1/10th volume of the 3 M sodium acetate (pH: 5.2) were added to the DNA solution, mixed well and then kept at -20°C overnight. DNA pellet collected by centrifugation at 10,000 rpm for 10 min was washed again with 70% ethanol and was finally dried in a desiccator under vacuum and resuspended in 50 µL TE buffer.

PCR: The PCR was performed in a 25 µL volume consisting of 0.5 µg of genomic DNA, 0.5 mM of each of the oligonucleotide primers (Table 1) for *ipa*H, *vir*A and *stx*1, 2.5 µL of a 10×PCR reaction buffer (500 mM Tris-Cl, pH 8.9, 500 mM KCl and 25 mM MgCl$_2$), 0.5 µL 10 mM dNTPs, 1.25 units of AmpliTaq DNA polymerase (Invitrogen, Life Technologies, USA) and an appropriate volume of sterile MilliQ water. Thermal cycling conditions were set for denaturation at 94°C for 1 min, annealing of primers at 55°C for 1 min 30 sec and primer extension at 72°C for 1 min 30 sec. Amplification was performed for 35 cycles, The expected sizes of the amplicons were ascertained by electrophoresis in 1.0% agarose gels (10 µL PCR product loaded) with an appropriate molecular size marker (1-kb DNA ladder; Sigma, USA).

Plasmid profile analysis: Plasmid extraction from the test isolates was carried out according to the procedure described by Kado and Liu (1981). Overnight fresh culture was centrifuged at 12,000 rpm for 7 min and pellets were resuspended in 100 µL autoclaved solution I (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA at 4°C and pH: 8.0) and kept at room temperature for 5 min. Freshly prepared 200 µL of solution II (0.2 N NaOH, 1% SDS in deionized water) was then added, mixed gently and kept in ice for 10 min. This was followed by addition of 150 µL of solution III (3 M potassium acetate, 5 M glacial acetic acid, pH: 4.8), mixed gently and kept in ice for 5 min. The suspension was centrifuged at 12,000 rpm for 5 min and 300 µL of supernatant was collected in fresh eppendorf tubes. Double volume of ice cold ethanol (95%) was added to the supernatant, mixed well and kept at room temperature for 10 min. Following centrifugation at 12,000 rpm for 5 min, the pellet was taken out and mixed

Table 1: Primers used for the detection of *Shigella*-specific genes (*ipa*H, *vir*A and *stx*1) by polymerase chain reaction (PCR)

Primer	Description	Sequence	Amplicon size (bp)	Reference
*ipa*H-f	Forward primer for *ipa*H	5'-GCTGGAAAAACTCAGTGCCT-3'	421	Venkatesan *et al.* (1989)
*ipa*H-b	Backward primer for *ipa*H	5'-CCAGTCCGTAAATTCATTCT-3'		
*vir*A-f	Forward primer for *vir*A	5'-CTGCATTCTTGGCAATCTCTTCACATC-3'	215	Jin *et al.* (2005)
*vir*A -b	Backward primer for *vir*A	5'-TGATGAGCTAACTTCGTAAGCCCTTCC-3'		
*stx*1-f	Forward primer for *stx*1	5'- ATAAATCGCCATTCGTTGACTAC-3'	180	Paton and Paton (1998)
*stx*1 -b	Backward primer for *stx*1	5'- AGAACGCCCACTGAGATCATC-3'		

with 1.0 mL ethanol (70%). This was centrifuged again at 12,000 rpm for 5 min. Finally, the collected pellet was dried and resuspended in 50 μL TE buffer and kept at 4°C.

Plasmid DNA was separated by horizontal electrophoresis in 1.0% agarose gel slab in Tris-acetate EDTA (TAE) buffer at room temperature at 50 volts for 3 h. The gel was stained with ethidium bromide and DNA bands were visualized using UV transilluminator (Gel Doc, Bio-Rad, USA).

Extraction of Outer Membrane Protein and Lipopolysaccharide (LPS): The outer membrane proteins were extracted from the isolates by the water extraction method described by Hale *et al.* (1985). Briefly, an isolated colony from a fresh culture in Nutrient Agar (NA) was inoculated into brain heart infusion broth and incubated at 37°C for 24 h in an orbital shaker. Following incubation, the whole culture media was harvested by centrifugation at 8,000 rpm for 15 min. The pellet was washed in normal saline and resuspended in 5.0 mL of sterilized deionized water and was shaken (100 oscillations per min) for 6 h at room temperature. The suspension was then centrifuged at 10,000 rpm for 20 min and the supernatant was collected and filtered through a Millipore filter having pore size of 0.45 μm. LPS was extracted using extraction kit (iNTRON Biotechnology, http://eng.intronbio.com/product/LPS.htm). Finally, 70 μL of 10 mM Tris-HCl buffer (pH: 8.0) was added to the LPS pellet and was dissolved by boiling for 1 min.

Western blot analysis: Outer Membrane Proteins (OMP) and LPS were fractionated separately by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% polycrylamide gels following the procedure described by Laemmli (1970). Twelve microlitre of OMP and LPS were loaded. These were followed by Western blot analyses (Towbin and Gordon, 1984). LPS and OMP were translocated from the gel to the nitrocellulose membrane by overnight transfer in transfer buffer (1.8% glycine, 0.36% Tris, 25% methanol in deionized water). The membrane was then cut into strips, washed with PBS (Phosphate Buffer Saline), PBS-Tween20 (0.1%) and subjected to blocking solution (3% skim milk in PBS). Commercially available antisera specific to *S. dysenteriae* type 2, 3, 4 and 8 were used at a concentrations of 1:50 dilutions as primary antisera (Denka Seiken, Tokyo, Japan). Horse radish peroxidase (HRP)-conjugated antibody (anti-rabbit IgG; Sigma) at 1:2000 dilutions was used as secondary antibody. Diaminobenzoic acid (DAB) (0.1%) dissolved in 100 mM citrate buffer (2.94% Na-citrate in deionized water, pH 5.2) with 0.15% H_2O_2 was used as the substrate.

Proteolytic, hemolytic and hemagglutinating activity: Proteolytic activity was determined based on the ability to hydrolyze milk protein casein. Fresh cultures were inoculated in skim milk agar plate and incubated overnight at 37°C (Murray *et al.*, 1999).

The hemolytic activities of the test *S. maltophilia* isolate and reference *S. dysenteriae* strains were determined by streaking aseptically on the blood agar plates. After incubation at 37°C for 18-20 h in aerobic and anaerobic jar incubator, the hemolysis zone and type under both the conditions were determined respectively (Atlas, 2006).

The hemagglutinating study was performed in microtiter plate. The chicken blood cells were washed thrice in 0.01 M cold PBS and finally, suspended to a concentration of 10% (v/v). Fifty microlitre of chicken blood cell suspension was added to each well. Bacterial suspension containing 10^{10} colony forming unit (cfu) mL^{-1} was subjected to two-fold serial dilution in 0.01 M PBS. Fifty microlitres of serially diluted test isolate and *Shigella* strains were then added to the wells and the microtiter plate was incubated at 22°C for 1 h. The highest dilution giving complete hemagglutination was recorded as the end point (Franzon and Manning, 1986).

Test of invasiveness: Sereny (keratoconjunctivitis) test was used as confirmatory test of invasiveness. Bacterial suspension adjusted to a concentration of 3×10^8 cfu mL^{-1} was used as inocula for Sereny test. Thirty microliter of this suspension from each organism was dispensed into the left eye of each guinea pig. Cell suspensions of *Shigella flexneri* 2a was used as positive control (Sereny, 1955).

Rabbit Ileal Loop (RIL) assay for enterotoxicity: Preparation of inocula was done following the method of De and Chatterje (1953). Both live cells and culture filtrates were used as inocula. Prepared inoculum cell suspension and culture filtrate (1.0 mL each) of the test isolate and reference strain together with positive (*Vibrio cholerae* 569B) and negative (PBS) controls were injected separately in the ligated loops of an Albino rabbit. The same method was repeated twice to check for reproducibility of results. The animals were sacrificed after 18 h by intravenous administration of 10% magnesium sulfate. Enterotoxicity was determined according to the fluid accumulation ratio suggested by De and Chatterje (1953).

Antibiotic susceptibility pattern: Susceptibility of the test isolate and the reference strains were determined by using the standardized agar-disc-diffusion method

known as Kirby-Bauer (Barry *et al.*, 1985). A total of seventeen different commercially available (Oxoid Limited, England) antibiotic discs were used in this study. The zone diameter for individual antimicrobial agent was then translated into sensitive, intermediate or resistant categories according to the interpretation table (Oxoid Limited, England).

RESULTS

Biotyping of the test isolate: The test isolate *Stenotrophomonas maltophilia* and the reference *Shigella dysenteriae* strains had many similar biochemical properties. But in contrast to the reference strains, the test isolate was unable to utilize any of the carbohydrates and possessed lysine decarboxylase activity (Table 2).

Detection of *Shigella*-specific virulence genes and plasmid profile analysis: The test isolate *S. maltophilia* did not harbor *ipa*H, *vir*A or *stx*1 genes (Fig. 1, 2), whereas all the reference strains demonstrated *ipa*H and *vir*A positive results and 421 and 215 bp amplified bands were observed in the agarose gel respectively. In addition, the reference strain *S. dysenteriae* type 3 (C253) was also *stx*1 positive and gave 384 bp amplified band.

Analysis of plasmid DNA by agarose gel electrophoresis revealed that the test isolate *S. maltophilia* had no plasmid. Only the reference strain *S. dysenteriae* type 4 (613) had large plasmid and other reference strains contained plasmid bands ranging between approximately 2.9 and 14 kb (Fig. 3).

LPS and OMP profile analysis: Western blot analysis of the LPS of the test isolate *S. maltophilia* did not reveal any cross-reactivity with *S. dysenteriae* type 8 specific

antisera. This suggested that, the cross reactivity of this isolate was not due to its LPS. Any other outer membrane protein or surface protein might contribute to such type of serological cross-reactivity. Therefore, this assumption was confirmed by the OMP profile analysis (Fig. 4) which showed antigenic bands on the nitrocellulose membrane.

Proteolytic, hemolytic and hemagglutinating activity: The test isolate *S. maltophilia* gave clear zone of proteolysis on skim milk agar whereas all the reference *Shigella dysenteriae* strains demonstrated negative result.

In case of the test *Stenotrophomonas maltophilia* isolate, zones of α-hemolysis (greenish hue) were detected in blood agar plates incubated under aerobic as
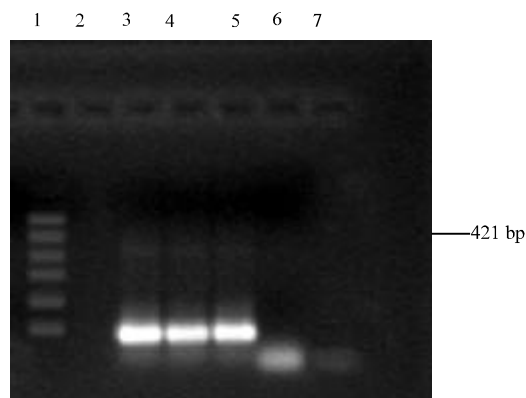

Fig. 1: PCR analysis of the test *S. maltophilia* isolate for *Shigella*-specific virulence gene, *ipa*H. Products of a PCR assay for the *ipa*H gene are shown. Lane 1: 1 kb+ DNA ladder marker; Lane 3: *S. dysenteriae* type 2 (602); Lane 4: *S. dysenteriae* type 5 (613); Lane 5: *S. dysenteriae* type 3 (C253); Lane 6: *S. maltophilia*; Lane 7: Negative control

Table 2: Biochemical characteristics and antibiotic susceptibility pattern of the environmental test isolate *S. maltophilia* along with the reference *S. dysenteriae* strains

Biochemical parameters	Antibiotic tested	*S. maltophilia* (RBSD4)	*S. dysenteriae* Type 2 (602)	*S. dysenteriae* Type 3 (C253)	*S. dysenteriae* Type 4 (613)
KIA	Amoxycillin 30 µg	K/K*	K/A, S	K/A*	A/A, S*
Simmon's Citrate	Cefotaxime 30 µg	- ve	- ve, S	- ve, S	- ve, S
Motility	Ceftriaxone 30 µg	- ve	- ve, S	- ve, S	- ve, S
Indole	Gentamicin 10 µg	- ve, S	- ve, S	- ve, S	- ve, S
Urease	Ciprofloxacin 5 µg	- ve	- ve	- ve	- ve
Oxidase	Sulfamethoxazole 25 µg	- ve, S	- ve	- ve	- ve
Catalase	Tetracycline 30 µg	+ ve	+ ve, S	+ ve	+ ve
H₂S	Nalidixic acid 30 µg	- ve	- ve, S	- ve, S	- ve, S
Lysine decarboxylase	Chloramphenicol 30 µg	+ ve, S	- ve, S	- ve	- ve, S
MR	Methicillin 5 µg	+ ve	+ ve	+ ve	+ ve
VP	Azithromycin 15 µg	- ve, S	- ve	- ve	- ve
Glucose	Rifampicin 30 µg	- ve	+ ve	+ ve	+ ve
Lactose	Polymyxin B 30 µg	- ve	+ ve	- ve	- ve
Maltose	Penicillin G 10 µg	- ve	+ ve	+ ve	+ ve
Sucrose	Kanamycin 30 µg	- ve	+ ve	- ve	+ ve
Arabinose	Erythromycin 15 µg	- ve	+ ve	+ ve	+ ve
Ornithine decaroxylase	Neomycin 30 µg	- ve, S	+ ve, S	- ve, S	+ ve

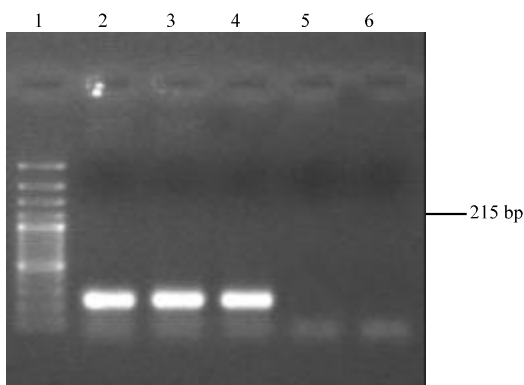*K: Alkaline reaction, A: Acid reaction, S: Sensitive to antibiotic

Fig. 2: PCR analysis of the test *S. maltophilia* isolate for *Shigella*-specific virulence gene, *vir*A. Products of a PCR assay for the *vir*A gene are shown. Lane 1: 1 kb+ DNA ladder marker; Lane 2: *S. dysenteriae* type 2 (602); Lane 3: *S. dysenteriae* type 4 (613); Lane 4: *S. dysenteriae* type 3 (C253); Lane 5: *S. maltophilia*, Lane 6: Negative control
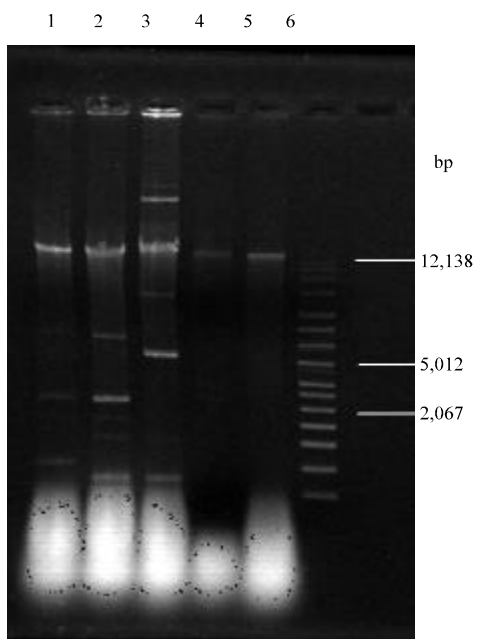

Fig. 3: Plasmid profile analysis. Lane 1: *S. dysenteriae* type 3 (C253); Lane 2: *S. dysenteriae* type 2 (602); Lane 3: *S. dysenteriae* type 4 (613); Lane 5: *S. maltophilia*; Lane 6: Supercoiled DNA ladder

well as anaerobic conditions. But the reference strains (*S. dysenteriae* type 2, 3 and 4) did not show any hemolysis (gamma hemolysis) on blood agar plate incubated either in aerobic or in anaerobic condition.
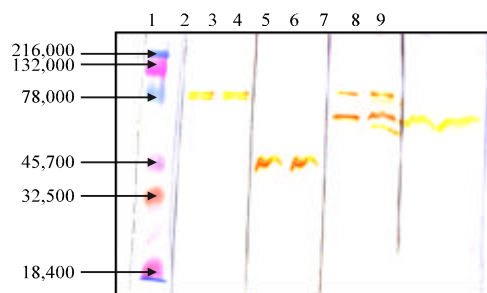

Fig. 4: OMP profile of the test isolate *S. maltophilia* (RBSD4) along with three clinical *S. dysenteriae* strains. Lane 1: protein marker; Lane 2 and 3: test isolate *S. maltophilia*; Lane 4 and 5: *S. dysenteriae* type 2 (602); Lane 6 and 7: *S. dysenteriae* type 4 (613); Lane 8 and 9: *S. dysenteriae* type 3 (C253)
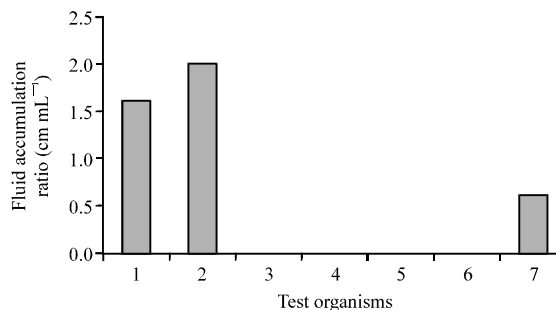

Fig. 5: Bar chart showing the ratio of fluid accumulation (mL cm^{-1}) by the test isolate, reference strains and positive control in RIL assay. Key: 1: *V. cholerae* 569B live cells (positive control); 2: *V. cholerae* 569B culture filtrate (positive control); 3: Test *S. maltophilia* live cells; 4: Test *S. maltophilia* culture filtrate; 5: *S. dysenteriae* type 2 (602); 6: *S. dysenteriae* type 3 (C253); 7: *S. dysenteriae* type 4 (613) live cells

Neither the test isolate *S. maltophilia* (RBSD4) nor the reference strains (*S. dysenteriae* type 2, 3 and 4) exhibited any hemagglutinating activity *in vitro*.

Sereny (keratoconjunctivitis) assay: Sereny test revealed that with the test *S. maltophilia* did not cause any sign of inflammation in guinea pig eyes. Only those inoculated with the reference strain *S. dysenteriae* type 4 (613) and positive control *S. flexneri* 2a exhibited typical symptoms of keratoconjunctivitis.

Rabbit Ileal Loop (RIL) assay: Level of enterotoxicity was determined according to the ratio of fluid accumulation

(mL) per unit (cm) length of ileal loop. Response is considered positive when the fluid accumulation reflects ≥0.4 mL cm⁻¹. Live cell and culture filtrate of the test *S. maltophilia* (RBSD4) did not cause any fluid accumulation. Enterotoxicity was evident in RIL only with the reference strain *S. dysenteriae* type 4 (613) and the positive control *Vibrio cholerae* 569B (Fig. 5).

Antibiotic susceptibility pattern of the test and reference strains: The test isolate *S. maltophilia* showed resistance to methicillin, erythromycin, amoxycillin, cefotaxime, ceftriaxone and penicillin G. It also showed intermediate resistance to antibiotics like tetracycline, nalidixic acid, rifampicin, polymyxin B and kanamycin. The test isolate was sensitive to gentamicin, ciprofloxacin, chloramphenicol and trimethoprim sulfamethoxazole (Table 2).

DISCUSSION

Shigellosis is one of the dreadful diarrheal diseases due to its invasive character, systemic manifestations, severe nutritional impact and tendency to recur over prolonged periods (Mata *et al.*, 1970). In the present study, the virulent properties of serologically cross-reactive environmental *Stenotrophomonas maltophilia* were compared with reference *Shigella dysenteriae* strains. The serologically cross-reactive *S. maltophilia* isolate revealed many similarities and dissimilarities in their biochemical behavior. In contrast to the reference strains, the *S. maltophilia* isolate was unable to utilize carbohydrates, e.g., glucose, lactose, sucrose, maltose and arabinose. This finding is not in conformity to another report which showed that some clinical *S. maltophilia* strains were capable of utilizing glucose, lactose, sucrose, maltose, xylose and citrate (Travassos *et al.*, 2004). Another distinguishing characteristic of the test isolate was that it possessed lysine decarboxylase activity while all the reference *Shigella* strains were lysine decarboxylase negative. Lysine decarboxylase (LDC) activity is present in ≈90% of *E. coli* strains (Edwards and Ewing, 1972) but all the strains of EIEC and *Shigella* sp. are LDC negative (Silva *et al.*, 1980). When the gene for LDC, *cadA* was introduced into *Shigella flexneri* 2a, virulence became attenuated and enterotoxin activity was inhibited significantly. The enterotoxin inhibitor was identified as cadaverine, a product of the reaction catalyzed by LDC. Comparison between the *S. flexneri* 2a and *E. coli* K-12 genomes in the region of *cadA* revealed a large deletion in *Shigella*. Representative strains of *Shigella* sp. and enteroinvasive *E. coli* displayed similar deletions of *cadA*

(Maurelli *et al.*, 1998). This study is consistent with the present findings with the test *S. maltophilia* isolate which was lysine decarboxylase positive but lacked enterotoxic property.

Presence of *Shigella*-specific virulence genes have been reported in closely related *Escherichia coli* virotypes (Pass *et al.*, 2000). In this study, serologically cross-reactive environmental *S. maltophilia* isolate demonstrated PCR negative results for *Shigella*-specific virulence genes like *ipaH*, *virA* and *stx*1 indicating that this organism did not possess any invasive or enterotoxigenic property. Absence of *Shigella*-specific virulence genes is a prerequisite for a vaccine candidate against shigellosis (DuPont *et al.*, 1972). This serologically cross-reactive environmental *S. maltophilia* isolate fulfils the criterion and may open up the scope for development of shigellosis vaccine based on live cells.

Invasive organisms like *E. coli* (EIEC) and *Shigella* sp., cause a rapid keratoconjunctivitis when placed on the conjunctiva of the guinea pig eye (known as Sereny test). Manifestations of keratoconjunctivitis include white or yellowish discharge from the eye, crustiness around the eye, redness or swelling in and around the eye and behavior such as pawing at the eyes or sneezing. Sereny test-positive isolates carry large (usually 140-MDa) plasmid responsible for this property. In the present study, the test isolate *S. maltophilia* gave negative response in the Sereny test indicating that it was noninvasive. Only the reference strain of *S. dysenteriae* type 4 (613) produced typical symptoms of keratoconjunctivitis in inoculated guinea pig eyes. This result is consistent with the plasmid profile analysis which showed the presence of a large virulence plasmid only in *S. dysenteriae* type 4 (613) and its absence in all the other reference strains including the test isolate.

The serological cross-reactivity is mostly due to the heat stable LPS antigens or common outer membrane protein antigens (Peterfi *et al.*, 2007). In the present study, Western blot analysis revealed that the cross-reactivity with *S. maltophilia* isolate was not due to its LPS component. As many protein components in the outer membrane are also shared among different Gram negative bacteria, the cross-reaction observed in the test isolate might be due to any outer membrane protein(s). This assumption was eventually confirmed by Western blot analysis involving outer membrane protein (OMP) of the bacterial isolate. The environmental *S. maltophilia* isolate yielding a high cross-reactivity with *Shigella* antisera represents potential for development of a shigellosis vaccine.

The ability of organisms to produce enterotoxins and hence their diarrhoeagenic potential was tested using the

rabbit ileal loop assay. The test isolate *S. maltophilia* live cells and its culture filtrate did not cause any fluid accumulation indicating that it was incapable of producing enterotoxin. Absence of enterotoxic property is consistent with the finding that it did not possess any toxin encoding gene.

Active extracellular protease was secreted by the test isolate. Some clinical *S. maltophilia* isolate also produce alkaline serine protease detected as a major secretion product (Windhorst *et al.*, 2002). *Stenotrophomonas maltophilia* G2 isolated from a soil sample producing extracellular serine protease have been documented in a separate study (Huang *et al.*, 2009). The roles played by protease in this environmental *S. maltophilia* isolate remains to be explored. The test *S. maltophilia* isolate exhibited alpha hemolysis on blood agar plate indicating that it was capable of partially degrading hemoglobin, giving rise to the typical greenish hue around bacterial growth. An earlier study which reported clinical *S. maltophilia* presenting a cell-free hemolytic activity similar to the 'hot-cold' hemolysins (Figueiredo *et al.*, 2006) is in conformity to the present findings. Hemolysins may be channel-forming proteins or phospholipases or lecithinases that destroy red blood cells and other cells by lysis (Figueiredo *et al.*, 2006). No hemagglutinating activity *in vitro* was exhibited by the test isolate and the reference strains. This indicates the absence of specific hemagglutinin on the bacterial cell surface which interacts with the chicken red blood cell membrane component.

Resistance to β-lactam antibiotics was confirmed previously in a study reporting approximately 200 kb plasmid purified from clinical isolates of *S. maltophilia* (Avison *et al.*, 2001). Transformation of a 5.6 kb plasmid, designated pTHB, derived from a *S. maltophilia* isolate into *Escherichia coli* K-12 HB101 resulted in the expression of resistance to the penicillin and to cefazolin (Kelly *et al.*, 1995). However, the occurrence of multiple antibiotic resistance devoid of any plasmid in the test isolate indicates that these resistance genes might be chromosome mediated. This type of antibiotic resistance might be due to the occurrence of certain genes like Smqnr, a new chromosome-borne quinolone resistance gene, in *S. maltophilia* (Shimizu *et al.*, 2008).

The overall findings of this study suggest the *S. maltophilia* isolate to be noninvasive and nonenterotoxic. Multiple antibiotic resistance and production of hemolysin and extracellular protease are some prominent features of the test isolate. Routine procedures to identify *S. dysenteriae* must incorporate virulence tests in addition to culture and serology in order to rule out any possibility of misdiagnosis.

Finally, further investigations are necessary to fully characterize the virulent properties of this environmental isolate and its potential as a vaccine candidate against shigellosis.

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