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

M. J. ALBERT*, M. ANSARUZZAMAN, F. QADRI, A. HOSSAIN, A. K. M. G. KIBRIYA, K. HAIDER, S. NAHAR, S. M. FARUQUE and A. N. ALAM

International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh

Summary. Three strains of *Plesiomonas shigelloides* isolated from patients with diarrhoea were agglutinated with *Shigella flexneri* 6 antiserum in slide and tube tests. All the strains were also agglutinated with a monoclonal antibody to the common group 1 antigen shared between *S. flexneri* serotypes and *S. dysenteriae* type 1. Further studies with one strain also showed sharing of antigenicity in an enzyme-linked immunosorbent assay. The results suggest that the strains share type-specific antigen with *S. flexneri* 6 and the common group 1 antigen with *S. flexneri* serotypes and *S. dysenteriae* 1. The sharing of antigens may have implications for cross-protection. One strain adhered to HEp-2 cell monolayers. None of the strains contained high mol. wt plasmids and there was no sequence homology with the invasiveness plasmid of *Shigella* spp. in DNA probe hybridisation. They were susceptible to the commonly used antibiotics. However, they possessed four other virulence-associated properties of *Shigella* spp. that included Congo-red binding, hydrophobicity, toxicity to HeLa cells and HEp-2 cell invasiveness (although they gave negative results in the Sereny test for invasiveness). These data suggest that the three unique strains might be considered pathogenic. Studies in animal models and human volunteers would be necessary to establish their pathogenic potential.

Introduction

Plesiomonas shigelloides is a member of the family Vibrionaceae, and has been associated with diarrhoeal illness and other diseases.¹ However, its enteropathogenicity is poorly understood as the isolates give generally negative results in the virulence tests for established enteropathogens.^{2–4} Strains of *P. shigelloides* that cross-react with *Shigella sonnei*, *S. dysenteriae* 7 and 8 and *S. boydii* 13 have been reported.⁵ In our clinical laboratory, we have monitored *P. shigelloides* strains that cross-react with shigellae. During 1991, three strains of *P. shigelloides* were isolated from patients with diarrhoea that cross-reacted with *S. flexneri* 6. Further studies suggested that they also shared a common group 1 antigen with *S. flexneri* serotypes and *S. dysenteriae* 1. These strains have been characterised for the basis of their cross-reactions and also studied by standard laboratory methods to determine whether they shared established

virulence properties with diarrhoeagenic bacteria, including shigellae.

Materials and methods

Patients and bacteria

The three strains of *P. shigelloides* (12726, 22074 and 12254) were isolated from the faeces of three patients with diarrhoea. Patients from whom strains 22074 and 12254 were isolated were 4 years old and had bloody, mucoid diarrhoea. The other patient was 6 years old and had watery diarrhoea.

Diarrhoeal stools obtained on admission were cultured for *Salmonella*, *Shigella*, *Vibrio*, *Campylobacter*, *Aeromonas* and *Plesiomonas* spp. by standard methods.^{6,7} Only *P. shigelloides* was isolated from these patients. *P. shigelloides* strains were identified by the API-20E system (API System, Montalieu, Vercieu, France) and reactions in conventional tests as described elsewhere.⁸ The three isolates gave biochemical reactions characteristic of *P. shigelloides*.⁸ Slide agglutination tests were performed with shigella antisera (Wellcome Diagnostics) with live and boiled cells.

Single strains of other bacteria used in the studies

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* Corresponding author: Dr M. John Albert, Laboratory Sciences Division, ICDDR,B, GPO Box 128, Dhaka 1000 Bangladesh.

(table I) were from the culture collection of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B).

MASFB monoclonal antibody

This immunoglobulin M mouse monoclonal antibody (MAb) (ascites fluid) was a gift from N. Carlin, National Bacteriological Laboratory, Stockholm, Sweden. It reacts with the common group 1 antigenic epitope present in the lipopolysaccharide (LPS) structures of all *S. flexneri* serotypes and *S. dysenteriae* 1.⁹

Production of antisera

Antisera were produced against *P. shigelloides* strain 12726 and a local strain of *S. flexneri* 6. Bacteria were grown in trypticase soy broth supplemented with yeast extract 0.6% (TSBY; Gibco, Grand Island, NY, USA) overnight at 37°C as stationary cultures. The growth was pelleted by centrifugation, washed once in physiological saline and reconstituted in physiological saline to 10⁹ bacteria/ml. Adult New Zealand White rabbits were immunised intravenously at 6-day intervals with 0.2, 0.5 and three 1.0-ml doses of bacterial suspension. A booster dose of 2 ml was administered 20 days after the last injection. The rabbits were exsanguinated 7 days after the last dose.

Preparation of bacterial LPS

Bacteria were grown overnight at 37°C in TSBY with shaking. The bacterial cells were pelleted at 6000 *g* for 30 min and used for the preparation of LPS by the hot phenol-water extraction procedure¹⁰ and purified by ultracentrifugation at 100 000 *g* for 4 h.

Tube agglutination test

The antigens used consisted of bacterial growth obtained after overnight still culture in TSBY at 37°C, washed in physiological saline, boiled for 1 h and adjusted to a turbidity corresponding to that of McFarland standard no. 3. Doubling dilutions of antisera, starting at 1 in 50, were tested. Results were read after incubating the test tubes containing antigen-antibody mixtures in a water-bath at 42°C for 1 h and then at 4°C overnight.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed¹¹ with LPS (10 µg/ml) as the coating antigen for the determination of antibody titre. Doubling dilutions of antisera starting with 1 in 1000 dilutions were added to the LPS-coated wells. The rabbit antibody was detected by horseradish peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins, and mouse antibody by horseradish peroxidase-conjugated rabbit immunoglobulins to mouse immunoglobulins (Dakopatts,

Denmark), both diluted (1 in 1000) in PBS containing Tween-20 0.1%. Bound peroxidase was detected with *O*-phenylenediamine dihydrochloride as substrate. The reaction was stopped after 15 min with 4 N sulphuric acid, and optical density (OD) was measured at 492 nm in a Titertek multiscan spectrophotometer. The antibody titre was the highest dilution that gave an OD of 0.2 above control with pre-immune rabbit sera or a non-reactive mouse ascites fluid.

Electrophoretic analysis of LPS

Components of the purified LPS were separated by SDS-PAGE with gels 13.5%.¹² The separated components were visualised by silver staining.¹³

Cell-culture adherence assay

Adherence to HEp-2 cell monolayer was tested according to the method of Cravioto *et al.*¹⁴ Briefly, HEp-2 cell monolayers were inoculated with overnight still cultures grown in Luria broth at 37°C and were then incubated for 3 h in the presence and absence of D-mannose 1%. After washing to remove non-adherent bacteria, cells on coverslips were fixed in methanol 70% and stained with Giemsa. A localised adherence (LA)-positive strain of enteropathogenic *Escherichia coli* (EPEC) serotype O127:H6 and a non-adherent strain of *E. coli* (a derivative of *E. coli* K-12, EC101,¹⁵ hereafter referred to as *E. coli* K-12 EC101) were used as positive and negative controls respectively.

Cell-culture invasion assay

HEp-2 cell invasion assay was performed as described by Small and Falkow¹⁶ with some modifications. Bacterial cells were grown with shaking for 4 h in TSBY at 37°C. Approximately 1 × 10⁶ cfu were added to a HEp-2 cell monolayer (1 × 10⁵ cells in a 6-ml vial, Kimble, Toledo, OH, USA, containing Eagle's minimum essential medium, [MEM]), centrifuged at 800 *g* for 10 min, and then incubated at 37°C for 2 h in an atmosphere of CO₂ 5%, air 95%. After washing to remove non-adherent bacteria, the monolayer was incubated for 1 h in MEM containing gentamicin 100 µg/ml to kill extracellular bacteria. After washing the monolayer, internalised bacteria were released by lysis of the cells with Triton X100 and quantified by plate count. The positive and negative controls included were a strain of *S. flexneri* 2a and *E. coli* K-12 EC101 respectively. Each strain was tested three times in duplicate, and the values were averaged. The isolates were also exposed to MEM containing gentamicin (without HEp-2 cells) to ensure that they did not survive the antibiotic treatment.

Sereny test

Shigella-like invasiveness was investigated by the Sereny test in guinea-pig eyes;¹⁷ 4- and 20-h shaker cultures in TSBY incubated at 37°C were tested. The *S. flexneri* 2a strain was included as a positive control.

DNA hybridisation

The DNA probe used was constructed from the invasiveness plasmid of *S. flexneri* 5 (M9OT) and consisted of a 17-kb *EcoRI* digestion fragment of pRM17.¹⁸ The appropriate restriction fragment was purified as described by Moseley *et al.*¹⁹ The probe was labelled by nick-translation²⁰ with [α^{32} -P] dCTP (Amersham International plc, Aylesbury, Bucks) and a nick-translation kit (Bethesda Research Laboratory, Bethesda, MD, USA). Colony blots were prepared, processed and hybridised under stringent conditions as described by Echeverria *et al.*²¹

Plasmid analysis

Bacterial plasmids were extracted by the methods of Birnboim and Doly²² and Kado and Liu,²³ separated by agarose gel electrophoresis and stained by ethidium bromide as described previously.²⁴

Enterotoxin and cytotoxin production

Bacteria were grown as shaken cultures in TSBY at 37°C for 20 h. Supernates sterilised by membrane filtration (0.45 μ m pore size; Millipore Corp, Bedford, MA, USA) were used for enterotoxin and cytotoxin assays. Heat-labile enterotoxin was tested in Y1 adrenal tumour cells,²⁵ heat-stable enterotoxin in suckling mice²⁶ and cytotoxin in HeLa cells.²⁷ The positive controls included were filter-sterilised supernates from an *E. coli* strain that produced heat-labile and heat-stable enterotoxins (for Y1 cell assay and suckling mouse assay) and *S. dysenteriae* 1 (for HeLa cell assay). Neutralisation of cytotoxicity for HeLa cells was done by mixing equal volumes of a 1 in 10 dilution of rabbit antiserum to Shiga toxin with ten 50% cytotoxic doses of toxin contained in filtered supernates and then incubating the mixtures for 1 h at 37°C, followed by overnight incubation at 4°C. Rabbit antiserum to Shiga toxin was elicited against purified Shiga toxoid²⁸ by an immunisation protocol described previously.²⁸ Toxin-antitoxin mixture was applied to HeLa cell monolayers and observed for neutralisation of cytotoxicity.

In the Y1 cell assay, rounding of $\geq 50\%$ cells was considered to be a positive result for heat-labile toxin, in the HeLa cell assay, detachment of $\geq 50\%$ cells was considered to be a positive result for cytotoxin, and in the suckling mouse assay, the gut weight to remaining body weight ratio of ≥ 0.085 was considered a positive result for heat-stable toxin.

Congo-red binding

Congo-red binding of bacteria was tested by streaking the organisms on Congo-red agar and observing for the development of pigmented colonies as described previously.²⁹

Salt aggregation test

Bacteria grown on trypticase soy agar for 18 h at

37°C were used to determine cell surface hydrophobicities by checking their agglutination in different concentrations of ammonium sulphate in a slide test.²⁹

Antibiogram

The antibiotic susceptibilities of bacteria were tested by the disk diffusion method³⁰ against tetracycline, ampicillin, chloramphenicol, streptomycin, kanamycin, gentamicin, carbenicillin, pivmecillinam, ceftriaxone, nalidixic acid and trimethoprim-sulphamethoxazole.

Results

Antigenic characteristics of isolates

All three isolates of *P. shigelloides* (12726, 22074 and 12254) gave strong and rapid slide agglutination reactions with *S. flexneri* polyvalent antiserum (Wellcome Diagnostics, Dartford), *S. flexneri* 6 monospecific polyclonal antiserum (Wellcome Diagnostics) and the MASFB MAb. They were not agglutinated with other shigella antisera. The reaction patterns of shigellae against *P. shigelloides* antiserum are shown in table I. The *S. dysenteriae* 1 isolate and all *S. flexneri* serotypes were agglutinated by the antiserum.

In the tube agglutination test (table II) with *P. shigelloides* antiserum, there was one dilution difference between homologous and heterologous titres,

Table I. Slide agglutination of live and boiled cells of shigellae with antiserum to *P. shigelloides* strain 12726

Organism	Agglutination*
<i>S. dysenteriae</i> 1	+
<i>S. dysenteriae</i> 2	—
<i>S. dysenteriae</i> 10	—
<i>S. flexneri</i> 1b	+
<i>S. flexneri</i> 2a	+
<i>S. flexneri</i> 3a	+
<i>S. flexneri</i> 6	+
<i>S. flexneri</i> Y	+
<i>S. boydii</i> (1–6)†	—
<i>S. boydii</i> (7–11)†	—
<i>S. boydii</i> (12–15)†	—
<i>S. sonnei</i>	—

* Both live and boiled cells gave identical agglutination reactions.

† Bacteria agglutinated with indicated polyvalent antisera; typing not attempted with individual antisera of the pool.

Table II. Antigenic cross-reactivities between *P. shigelloides* and *S. flexneri* 6 in the tube agglutination test

Antigen	Titre of antisera to	
	<i>P. shigelloides</i> 12726	<i>S. flexneri</i> 6
<i>P. shigelloides</i>		
12726	3200	1600
12254	3200	1600
22074	3200	1600
<i>S. flexneri</i> 6	1600	1600

Table III. Antigenic cross-reactivities between *P. shigelloides* and *S. flexneri* 6 in ELISA

LPS antigen from	Titre of antisera to			Titre of MASFB MAb
	<i>P. shigelloides</i> 12726	<i>S. flexneri</i> 6*	<i>S. flexneri</i> 6†	
<i>P. shigelloides</i> 12726	1000000	10000	10000	100000
<i>S. flexneri</i> 6	10000	1000000	100000	100000

* Unabsorbed rabbit polyclonal antiserum.

† Absorbed Wellcome rabbit polyclonal, monospecific antiserum.

Figure. Adherence of: A, *P. shigelloides* 12254 to HEp-2 cells, compared with B, no adherence to HEp-2 cells inoculated with *P. shigelloides* 22074. Bar, 5 μ m; Giemsa stain.

whereas homologous and heterologous titres were identical with *S. flexneri* 6 antiserum.

Cross-reactivities in ELISA are shown in table III. The homologous titres of polyclonal antisera were higher than heterologous titres and the differences ranged from 10-fold to 100-fold. However, the MASFB MAb gave identical titres against LPS from both bacteria.

The silver-stained gel patterns of LPS from *P. shigelloides* 12726 and *S. flexneri* 6 showed patterns of smooth organisms—ladder-like patterns representing the O-polysaccharide side-chain and the core lipid A (data not shown).

Cell-culture adherence

Only *P. shigelloides* strain 12254 showed good adherence to HEP-2 cells both in the presence and absence of D-mannose. The majority of cells had numerous adherent bacteria (fig. A). However, the other two strains did not show any adherence (fig. B).

Invasion

None of the three isolates caused keratoconjunctivitis in the Sereny test and none gave positive hybridisation results with the *S. flexneri* invasiveness plasmid probe. In the quantitative cell-culture invasion assay, the intracellular survival of bacteria in cfu/ml was 4.0×10^3 each for strains 12254 and for 12726, and 4.1×10^3 for strain 22074. The corresponding values for the positive control *S. flexneri* 2a and the negative control *E. coli* K-12 EC101 strains were 1.0×10^5 cfu and 0 cfu respectively. Thus, it appeared that the *P. shigelloides* strains were invasive for cell cultures.

Enterotoxin and cytotoxin production

None of the three isolates produced either heat-labile or heat-stable enterotoxins (rounding of Y1 cells 0–1%, gut weight to remaining body weight in suckling mouse assay 0.077–0.079) but all of them produced cytotoxins (50–80% HeLa cells affected) that were not neutralised by Shiga antitoxin. In the assays for cell-culture adherence, invasion and toxins, the control organisms behaved as expected.

Hydrophobicity

All three isolates formed smooth suspensions in physiological saline. However, all were highly hydrophobic as they agglutinated in 0.06 M ammonium sulphate.

Congo-red binding

The three isolates bound Congo red, as they produced pigmented colonies on Congo-red agar.

Plasmid profile

Strain 12726 had a single plasmid (2.0 MDa), strain 22074 had two plasmids (2.0 and < 1 MDa) and strain 12254 had two plasmids (2.7 and 1.8 MDa).

Antibiogram

All the isolates were uniformly susceptible to tetracycline, chloramphenicol, kanamycin, gentamicin, nalidixic acid, trimethoprim-sulphamethoxazole and ceftriaxone, but resistant to ampicillin, streptomycin and carbenicillin.

Discussion

The three *P. shigelloides* strains agglutinated with Wellcome monospecific polyvalent *S. flexneri* 6 antiserum, which suggested that they possessed the *S. flexneri* 6 type-specific antigen. However, antiserum to live cells of *P. shigelloides* 12726 agglutinated *S. dysenteriae* 1 and all serotypes of *S. flexneri* tested. This suggested that in the bacterial cell, there was an additional antigen(s) shared with *S. dysenteriae* 1 and all *S. flexneri* serotypes. Such an assumption was confirmed by the reactivity of *P. shigelloides* strains with MASFB MAb, which is directed against the common group antigen 1 of *S. flexneri* spp. and *S. dysenteriae* 1. This common antigenic determinant is α -1, 2 or α -1,3 linked L-rhamnopyranosyl residue in the O-antigenic polysaccharide.⁹ Furthermore, agglutination of boiled cells and the reactivities of purified LPS in ELISA would suggest that the cross-reacting antigens are LPS. Higher titres were obtained in ELISA than in the tube agglutination test, which is attributable to the greater sensitivity of ELISA.

It is unlikely that the three *P. shigelloides* strains are rough and that the cross-reaction is due to an exposed common epitope in rough strains because the strains formed smooth suspensions and reacted with MASFB MAb, which is known to react only with smooth strains, not rough mutants. Moreover, the silver-stained LPS structure showed recognition of antigens corresponding to the ladder-like O-polysaccharide side chain of smooth bacteria, and smooth *P. shigelloides* cross-reacting with other shigellae have been demonstrated previously.⁵

Standard laboratory assays were used to see whether these unique isolates possessed some of the established virulence properties of diarrhoeagenic bacteria. The properties investigated included cell-culture adherence,^{31,32} invasiveness in the Sereny test and cell culture,³³ cytotoxin and enterotoxin production,^{34,35} Congo-red binding,²⁹ possession of high mol. wt plasmid,³³ high hydrophobicity²⁹ and multiresistance to antibiotics.³⁶ Only one strain, 12254, adhered to a HEP-2 cell monolayer, and we know of no previous studies on the cell-culture adherence of *P. shigelloides*. The role of this cell-culture adherence in pathogenicity remains unclear. As found in other studies,^{3,4,37} the strains were not invasive in the Sereny test. However, all strains were invasive in the HEP-2 cell assay although to a lesser extent than the positive *S. flexneri* 2a control strain. A previous report by Binns *et al.*³⁸ also found invasiveness in five of 16 *P. shigelloides*

strains, although other studies failed to identify this property.^{2,4,39} The lack of hybridisation with the 17-kb DNA probe demonstrated that our *P. shigelloides* strains lack the gene sequence common to the large invasiveness plasmids found in *Shigella* spp. and enteroinvasive *E. coli*. Consistent with this observation is the finding that none of the isolates possessed high mol. wt plasmids resembling the invasiveness plasmids of the last two bacteria. Although many strains of *P. shigelloides* were found to harbour high mol. wt plasmids in previous studies,^{2-4,39} sequence homology with the invasiveness plasmids of *Shigella* spp. and enteroinvasive *E. coli* were not observed.⁴ However, our strains had different plasmid profiles, mainly of smaller plasmids, and the functions of these plasmids remain undefined. All strains bound Congo red dye and exhibited high hydrophobicity, as reported previously.² Our strains did not produce enterotoxins, although this property was found to be variable in previous studies.^{2,3,40} However, all three isolates produced toxins (unrelated to Shiga toxin) that were cytotoxic for HeLa cells. It is known that *Shigella* spp. also produce cytotoxins unrelated to Shiga toxin.⁴¹ Previous studies also found similar cytotoxin production by *P. shigelloides* strains^{2,42} and it is possible that such cytotoxic activity could be a potential virulence factor in *P. shigelloides*-associated gastroenteritis. Multiresistance to antibiotics did not seem to be a problem with the isolates, as they were susceptible to most of the antibiotics tested.

Contrary to the previous studies, which found low pathogenic potential for *P. shigelloides*,²⁻⁴ we demonstrated that the three strains that shared antigens with *Shigella* spp. seemed to possess at least four virulence-associated properties of shigellae, namely epithelial cell invasiveness, cytotoxicity, Congo-red binding and high hydrophobicity. One strain (no. 12254) also possessed cell-culture adherence. This would suggest that the three strains might be pathogenic. However, for reasons of economy, studies were confined to pathogens requested by physicians (see *Materials and methods*), and other pathogens such as parasites and viruses were not sought in these patients. Therefore, the role of *P. shigelloides* as the causative agents of diarrhoea in these patients could not be established unequivocally. Further studies with experimental animals and human volunteers would be necessary to establish the diarrhoeagenic potential of these unique isolates.

In a previous study,² some *P. shigelloides* were found to agglutinate with polyvalent *S. flexneri* antiserum, not type-specific antisera. Our study has further demonstrated strains that cross-react with a serotype-specific antiserum. The present and the previous data⁵ suggest that antigenic cross-reactions between *Shigella* spp. and *P. shigelloides* are not uncommon, and this will have implications for cross-protection.

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