

Laboratory Observations on *Plesiomonas shigelloides* Strains Isolated from Children with Diarrhea in Peru

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Eleven strains of *Plesiomonas shigelloides* isolated from 10 Peruvian children with diarrhea were examined. All the strains were resistant to two or more antibiotics, most commonly ampicillin, gentamicin, erythromycin, kanamycin, and streptomycin. The strains were all negative in the Sereny and cell culture assays used to test for enteroinvasiveness. One strain showed cytotoxic activity on Vero cells. The strains showed no antigenic relationship with *Shigella* organisms. Both bioassays and enzyme-linked immunosorbent assays used for detection of *Escherichia coli* enterotoxins were negative. Nucleic acid probes for such toxins likewise gave negative results. The strains all possessed a large (approximately 200-megadalton) plasmid in addition to one or more other plasmids. Several different plasmid profiles were observed among these 11 *P. shigelloides* strains, indicating that the isolates were not acquired from a common source or from a single bacterial clone.

Although *Plesiomonas shigelloides* has been implicated in acute gastroenteritis, bacteremia, and meningitis (6, 11, 12, 14, 17, 19, 20, 24, 25), its pathogenesis is not yet understood. Strains are ubiquitous, normally found in the environment, in fresh water, and in fish and birds (1); the potential for transmission to humans is therefore high. In two outbreaks of diarrhea involving more than 1,000 persons, *P. shigelloides* strains were isolated both from the drinking water reservoir and from the feces of the patients (24).

However, one study revealed that the carrier rate of *P. shigelloides* in a group of healthy controls was as high as that in a group with diarrhea (19). *P. shigelloides* strains isolated from people with diarrhea did not induce diarrhea when given orally to adult human volunteers, and it is suggested that the bacterium is more pathogenic to children than to adults (19).

The present report describes a laboratory examination of *P. shigelloides* strains isolated from children with diarrhea, using methods developed for the evaluation of enteropathogenicity in other enteric bacteria such as *Escherichia coli*, *Vibrio cholerae*, *Yersinia enterocolitica*, and *Shigella* spp.

MATERIALS AND METHODS

Strains. A total of 11 strains were isolated from the feces of 10 Peruvian children with diarrhea. The strains were from sporadic cases, and the clinical symptoms are listed in Table 1. The samples were isolated by using different enrichment and growth media (Table 1), and the *P. shigelloides* strains were identified by using the criteria summarized by Pit-arangsi et al. (19). The strains were stored on deep agar slants before further examinations were performed.

Antimicrobial resistance. All strains were tested for antimicrobial susceptibility, using the agar disk method (2). The agents tested are listed in Table 1.

Invasiveness. The strains were tested for invasiveness by the Sereny test (22) and in a cell culture assay with a

monolayer of HeLa cells (15). Invasive *Y. enterocolitica* and *E. coli* strains were used as positive controls. These and all other control strains were from the collection of the Centers for Disease Control.

Cytotoxin. Vero cell cultures were used to test sterile filtered bacterial broth from overnight growth at 37°C in tryptic soy broth (Difco Laboratories, London, England) on a roller drum. Broth samples from known cytotoxic *Shigella* and *E. coli* strains served as positive controls. The test was carried out as described by Konowalchuk et al. (13).

Enterotoxins. The same broths as those used for the cytotoxin testing were used in the infant mouse (7) and the enzyme-linked immunosorbent (28) assays for heat-stable and heat-labile enterotoxins, respectively.

DNA probes for enterotoxins. Purified cloned fragments of DNA encoding parts of the genes for LTI, STIa, STIb, and STII were used as previously described (26). The fragments were labeled with ³²P by nick translation, using a kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) according to the recommendations of the producers, and hybridized on colony blots from the 11 *P. shigelloides* strains and controls, using reagents and conditions as described previously (26). The strains were also tested with two synthetic, alkaline-phosphatase-labeled oligonucleotides encoding parts of the LTI and STI genes (Molecular Biosystem, San Diego, Calif.) as described by Wasteson et al. (27).

Serological examinations. The outer membrane proteins from the *P. shigelloides* strains were isolated and separated by polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane by Western blot (immunoblot), and incubated with convalescent-phase serum from a monkey infected with the major *Shigella* serotypes. Alkaline phosphatase-linked rabbit anti-monkey immunoglobulin G and *p*-nitrophenylphosphate as the substrate were used to develop the signal on the Western blots.

Plasmid profiles. A modified Birnboim procedure was followed to isolate plasmid DNA (4). The plasmids were electrophoresed, stained, and photographed as previously

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TABLE 1. Clinical information on children with diarrhea and characteristics of the 11 strains of *P. shigelloides* isolated

Strain no.	Clinical symptoms				Strain characteristics					
	Fever	Vomiting	Diarrhea with:		Isolation media ^a	Antibiotic resistance ^b	Invasive by:		Cytotoxic (Vero cells)	
			Blood	Mucus			Sereny test	HeLa cell assay		
CG 544	+	-	-	+	GN/H	Tc Amp Gm Km Sm Nm	-	+/- ^c	-	
CG 586	+	-	+	+	H, SS, XLD	Tc Amp Gm Km Sm Nm SSS E	-	-	-	
CG 769	NI ^d	-	-	-	SS, GN/H	Amp Gm Sm E	-	-	-	
CG 778	+	+	-	+	SS, GN/H	Amp Gm Km Sm E	-	+/- ^c	-	
CG 1140	+	-	-	+	H	Amp Gm Km Sm Nm E	-	-	-	
CG 1275	+	NI	-	+	SS	Amp Km Sm E	-	-	-	
O 47A	+	-	+	+	SS	Amp Gm Km Sm Nm SSS E	-	-	-	
G 005	NI	NI	NI	NI	H, SS, XLD	Dox Gm Km Sm Nm SSS E	-	-	-	
G 024	NI	NI	NI	NI	H, SS, XLD	Amp Sm E	-	-	-	
G 031	NI	NI	NI	NI	M, SS, GN/H	Amp Gm Km Sm	-	+/- ^c	+	
G 060	NI	NI	-	-	H, SS, XLD	Amp Sm	-	-	-	

^a H, Hektoen enteric agar; GN/H, gram-negative broth, Hajna enriched and plated on Hektoen enteric agar; SS, salmonella-shigella agar; XLD, xylose-lysine-deoxycholate agar; M, MacConkey agar.

^b Tc, Tetracycline; Amp, ampicillin; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Nm, neomycin; SSS, triple sulfa; E, erythromycin; Dox, doxycycline.

^c The results were not fully reproducible when tests were conducted on different occasions.

^d NI, No information.

described (16). *E. coli* strains containing plasmids with known molecular weights were used as the controls and standards.

RESULTS

The *P. shigelloides* strains were isolated from diarrheal feces, using different growth media as shown in Table 1. All six children with information concerning fever were noted to have fever, but only one child was noted to be vomiting. Six of eight children had mucous diarrhea, and only two had blood in their stools. The antimicrobial susceptibility patterns for the 11 clinical isolates are also presented, indicating several multiply resistant strains. Resistance was found most frequently to ampicillin and streptomycin (10 of 11 strains), gentamicin, kanamycin (7 of 11 strains), and gentamicin, kanamycin, and erythromycin (5 of 11 strains). However, tetracycline resistance was found in only 3 of 11 strains. The monkey serum used did not react with the outer membrane proteins from the *P. shigelloides* strains tested in the Western blot procedure.

None of the 11 clinical isolates of *P. shigelloides* were positive in the Sereny test for enteroinvasiveness. Three strains initially demonstrated invasiveness for HeLa cells, but these results could not subsequently be confirmed. Only one strain, G 031, produced an extracellular product which was cytotoxic to Vero cells.

Examination for *E. coli* ST- and LT-like enterotoxins, using the infant mouse test and enzyme-linked immunosorbent assay, respectively, proved to be negative. Genetic probing with the different DNA probes for these enterotoxins likewise gave negative results.

The plasmid analysis showed the presence of a large (approximately 200-megadalton [MDa]) plasmid in all 11 strains examined in this study. The strains also possessed other plasmids of different sizes (Fig. 1), giving the strains heterogeneous plasmid profiles.

DISCUSSION

The pathogenicity of a bacterial strain depends on both inherent bacterial and host factors. It is therefore difficult to

draw firm conclusions from animal experiments about the potential pathogenicity of a strain to humans. We have, however, used some animal models (7, 22) and laboratory experiments (13, 26, 28) to test for enteropathogenicity in the 11 *P. shigelloides* strains investigated in this study.

The enterotoxin test using the infant mouse model has successfully detected several heat-stable enterotoxins originating from *Y. enterocolitica*, *Klebsiella pneumoniae*, and *E. coli*. The *P. shigelloides* strains used in the present study were negative when tested in this animal model, although it could be assumed that in vitro production of a possible heat-stable enterotoxin by the *P. shigelloides* strains would have required special cultivation media and growth conditions not provided in these experiments. However, genetic analysis with DNA probes for three different enterotoxins did not reveal any genes encoding enterotoxins. Similar negative results were obtained both in the enzyme-linked immunosorbent assay and with probes for heat-labile enterotoxins. DNA probes for the heat-labile toxin have been

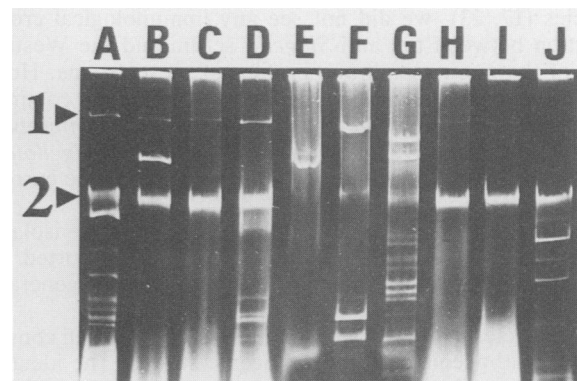


FIG. 1. Plasmid profiles of seven strains of *P. shigelloides* isolated from children with diarrhea. Lanes: A, *P. shigelloides* G 060; B, *P. shigelloides* CG 769; C, *P. shigelloides* G 031; D, *E. coli* strain with control plasmids; E, *E. coli* strain with control plasmids; F, *E. coli* strain with control plasmids; H, *P. shigelloides* CG 778; I, *P. shigelloides* G 005; J, *P. shigelloides* CG 544. 1, Location of the plasmid of approximately 200 MDa; 2, location of the chromosomal band.

used successfully with *V. cholerae*, but attempts to identify similar toxins in *Campylobacter jejuni* were negative (18). Although the presence of enterotoxins in *P. shigelloides* has been reported (9, 21), others have not observed enterotoxins in their strain collections (8, 19).

Cytotoxins are commonly reported in *Aeromonas* spp. (11) and are suspected of contributing to the enteropathogenic properties of these organisms. However, only 1 of the 11 *P. shigelloides* strains was found to produce an extracellular product which was cytotoxic for Vero cells. Holmberg and Farmer (11) and Pitarangsi et al. (19) failed to find any cytotoxic activity in the 31 and 27 *P. shigelloides* strains they tested, respectively. Fisher et al. (8) have recently reported a cytotoxin-producing strain from a patient with diarrhea.

We could not demonstrate invasiveness using the animal test previously employed for *E. coli*, *Shigella*, and *Yersinia* invasiveness in our strains. The cell culture assay gave divergent results and was not regarded as suitable for the study of *P. shigelloides* invasiveness. Similar results have been obtained in other studies (12, 19). However, Binnis et al. (3) reported that 5 of 16 strains tested were invasive in the HeLa cell culture assay. A 140-MDa plasmid has been found to be associated with invasiveness of both *Shigella* spp. and *E. coli* (10). It was therefore of interest to observe that all our *P. shigelloides* strains possessed a plasmid of approximately 200 MDa. The size of the plasmid had to be calculated by extrapolation because of a lack of control plasmids of corresponding size. A large plasmid of similar size has been reported by Holmberg et al. (11, 12), and a plasmid of 280 kilobases (182 MDa) has been reported by Fisher et al. (8). The present data do not exclude the possibility that these plasmids are the same as the one described in this work. A plasmid of more than 100 MDa has been observed in *P. shigelloides* strains by Nolte et al. (17). Because the 11 strains showed generally different plasmid and antimicrobial susceptibility patterns, we concluded that they were not of the same clone. The plasmid of approximately 200 MDa was found in all the *P. shigelloides* strains examined and may be a characteristic of the species or of strains possessing certain phenotypic characters. Only two of eight children in this study had bloody diarrhea, often characteristic of *Shigella*- or invasive *E. coli*-induced diarrhea.

Even though some of the O antigens reported for *P. shigelloides* have been associated with those of *Shigella* species (17, 23), we did not see any immunological cross-reaction between the anti-*Shigella* serum and the Western blot of the outer membrane profiles of these strains. However, a monoclonal antibody presumed to detect a common antigenic epitope in members of the family *Enterobacteriaceae* has been reported to react with all *P. shigelloides* strains tested (5). The antibody was not tested in the current study, however.

The antimicrobial resistance patterns found in the isolates investigated were similar to those previously reported for clinical isolates of this species; the strains were generally resistant to β -lactam antibiotics (8, 16).

The species *P. shigelloides*, like *E. coli*, might well consist of both pathogenic and nonpathogenic strains. The identification of possible pathogenicity factors is therefore of importance. Pathogenicity might also depend on such factors in the host as age and immunological status (17, 19).

Neither our data, originating as they did from a limited number of specimens, nor data presented by others allow any general conclusions concerning the pathogenicity mechanisms of *P. shigelloides* to be drawn. Nevertheless, our study indicates that possible pathogenicity mechanisms in

these strains are dissimilar to those in related enteric species.

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