INFECTION AND IMMUNITY, Dec. 1992, p. 5017–5024
0019-9567/92/125017-08$02.00/0

Vol. 60, No. 12

Pathogenesis of *Providencia alcalifaciens*-Induced Diarrhea

M. JOHN ALBERT,[1]* K. ALAM,[1] M. ANSARUZZAMAN,[1] M. M. ISLAM,[1] A. S. M. H. RAHMAN,[1] K. HAIDER,[1] N. A. BHUIYAN,[1] S. NAHAR,[1] N. RYAN,[2] J. MONTANARO,[3] AND M. M. MATHAN[4]

International Centre for Diarrhoeal Disease Research, Bangladesh, GPO Box 128, Dhaka 1000, Bangladesh[1]; Infectious Disease Hospital, Fairfield,[2] and Royal Children's Hospital, Parkville,[3] Victoria, Australia; and Christian Medical College and Hospital, Vellore, Tamil Nadu, India[4]

Received 2 March 1992/Accepted 14 September 1992

Providencia alcalifaciens* is a member of the family *Enterobacteriaceae*. There are reports that *P. alcalifaciens* can cause diarrhea, but the mechanism(s) by which it causes diarrhea is not known. We studied *P. alcalifaciens* isolated from a child and two adults with diarrhea for enteropathogenicity. The three isolates did not exhibit any characteristic adherence to cultured HEp-2 cell monolayers, and they did not produce enterotoxins, cytotoxins, or keratoconjunctivitis in the Sereny test. Two isolates invaded cultured HEp-2 cell monolayers, producing localized bacterial clusters and actin condensation. The pattern of actin condensation was different from that produced by enteropathogenic *Escherichia coli* but similar to that produced by *Shigella flexneri*. Invasion and actin condensation were poor for the third isolate. Histology of adult rabbit small intestinal loops inoculated with all three isolates revealed bacterial attachment to, penetration of, and microulcer formation on the surface epithelium and hyperemia, edema, and polymorphonuclear cell infiltration of lamina propria. All the isolates produced diarrhea in rabbits with removable intestinal ties, and some of these rabbits developed hindlimb paralysis. Intestinal histology of the rabbits with removable intestinal ties which developed diarrhea showed changes similar to that in adult rabbits on which ileal loop assays had been performed. Transmission electron microscopy of intestinal tissues also confirmed tissue penetration by the isolates. Nerve tissue histology of two rabbits that developed hindlimb paralysis showed focal mononuclear cell infiltration around peripheral nerve sheaths. It is concluded that some strains of *P. alcalifaciens* are enteropathogenic and that they cause diarrhea by invading the intestinal mucosal epithelium. However, the relevance to human disease of the hindlimb paralysis observed in this animal model is not clear.

Providencia alcalifaciens is a member of the family *Enterobacteriaceae* and a component of the normal flora of the feces (7). Some studies in the past have implicated the Providence group of organisms in the causation of diarrhea (16, 18), and a recent study suggested that *P. alcalifaciens* can cause traveler's diarrhea (10). However, the mechanism by which *P. alcalifaciens* causes diarrhea is not known.

In our clinical laboratories, we have sometimes isolated *P. alcalifaciens* either as the predominant organism or as a pure culture from the diarrheal stools of some patients in the absence of recognized enteric pathogens. This prompted us to study the enteropathogenicity of some *P. alcalifaciens* isolates.

MATERIALS AND METHODS

Bacteria. Three isolates of *P. alcalifaciens* were studied. Strain 2939/90 was isolated in Dhaka, Bangladesh, from a child with diarrhea who was dead on arrival. A rectal swab of the dead child grew a pure culture of *P. alcalifaciens*. Strains F90-2004 and R90-1475 were isolated from adult diarrheal patients in Melbourne, Australia. These strains grew as the predominant organisms on culture of the feces. Recognized enteric bacterial pathogens and parasites were absent in rectal swabs or feces of these patients. The specimens were not tested for viruses.

Identification of *P. alcalifaciens*. The identification of non-lactose-fermenting colonies on MacConkey agar was accomplished biochemically as outlined by Ewing (7) and by using an API-20E biochemical strip for the family *Enterobacteriaceae* (API System, Montalieu, Vericieu, France).

Enterotoxin and cytotoxin production. Bacteria were grown in Trypticase soy broth (TSB) supplemented with 0.6% yeast extract (TSBY; GIBCO Laboratories, Grand Island, N.Y.) at 37°C for 20 h with shaking. Cell-free culture filtrates and polymyxin B extracts of bacterial pellets (9) were tested in mouse adrenal tumor Y1 cells for heat-labile enterotoxin (15), in suckling mice for heat-stable enterotoxin (6), and in HeLa cells for cytotoxins (8). The positive controls included were culture filtrates from an *Escherichia coli* strain positive for heat-labile and heat-stable enterotoxins (for the Y1 cell assay and the suckling mouse assay) and *Shigella dysenteriae* 1 (for the HeLa cell assay). One milligram of protein from crude culture filtrate from the control *E. coli* positive for heat-labile toxin had approximately 5×10^2 50% tissue culture dose units for Y1 cells, and 1 mg of protein from crude culture filtrate from the control *S. dysenteriae* 1 positive for cytotoxin had approximately 2×10^4 50% tissue culture dose units for HeLa cells.

Sereny assay. *Shigella*-like invasiveness was tested by the Sereny assay with guinea pigs (17). Shaker cultures (4 and 20 h) in TSBY incubated at 37°C were tested. A *Shigella flexneri* 2a strain, 611R, was included as a positive control.

Cell culture adherence and penetration. Adherence to HEp-2 cells was tested by the method of Cravioto et al. (5) with some modifications. Stationary and shaker cultures (4 and 20 h) in Luria broth incubated at 37°C were used. Bacteria were incubated with HEp-2 cell monolayers at 37°C for 3 h in the presence and absence of 0.5% D-mannose. After the monolayers were washed to remove nonadherent bacteria, they were fixed in 70% methanol and stained with Giemsa stain. A localized adherence-positive serotype

* Corresponding author.

O127:H6 enteropathogenic *E. coli* (EPEC) strain was included as a positive control in the adherence assays.

The HEp-2 cell invasion assay described by Small and Falkow (19), with some modifications, was performed. HEp-2 cells were seeded on glass coverslips (Bellco Glass Inc., Vineland, N.J.) placed in the bottom of 1-dram (3.7-ml) glass shell vials (American Scientific Products, McGaw Park, Ill.) and grown in minimum essential medium with Earle's salts and glutamine (MEM) containing 10% fetal bovine serum and antibiotics (GIBCO). Before infection, the original growth medium was replaced with MEM lacking antibiotics (the monolayers contained approximately 2.0×10^5 HEp-2 cells). After the antibiotic-free medium was removed, the vial was inoculated with bacterial suspension. For each strain, a 4- and 20-h-old stationary Luria broth cultures grown at 37°C were tested. Each 4- or 20-h-old culture was inoculated into three vials. For this, 10 μl of appropriately diluted bacterial culture mixed with 1.0 ml of MEM (approximately 1.0×10^6 CFU) was inoculated into each vial. This resulted in a ratio of approximately 10 bacteria per HEp-2 cell. The bacterial inoculum was centrifuged onto the HEp-2 cell monolayer at $800 \times g$ for 10 min and then incubated at 37°C in 5% CO_2 for 2 h. After the monolayer was washed to remove nonadherent bacteria, it was incubated in MEM containing gentamicin (100 μg/ml) at 37°C for 1 h to kill extracellular bacteria. Two of the three monolayers per bacterial inoculum were lysed with 1.0% Triton X-100 for 5 min to release intracellular bacteria (this treatment did not affect the survival of bacteria). Different dilutions of the lysed monolayer were plated on MacConkey agar (GIBCO) to determine the number of CFU per milliliter. The other infected monolayer was fixed in methanol, stained with Giemsa stain, and observed under a light microscope for integrity of the monolayer and evidence of bacterial invasion. Experiments were run in duplicate and repeated at least three times per isolate. Bacterial isolates were also exposed to gentamicin-containing MEM (without HEp-2 cells) to ensure that the strains did not survive the antibiotic treatment. The positive control used was the invasive *S. flexneri* 2a strain 611R, and the negative control was strain EC101, a local derivative of *E. coli* K-12 (2), hereafter referred to as *E. coli* K-12 EC101.

Fluorescent actin staining test for attachment-effacement lesions. The proxy test for attachment-effacement lesions in the intestine was performed with HEp-2 cell monolayers in 3- and 6-h assays as described previously (11). The localized adherence-positive EPEC serotype O127:H6 strain and the invasive *S. flexneri* 2a 611R strain were included as controls.

Rabbit ileal loop test. One milliliter of a 20-h TSBY shaker culture containing 10^9 bacteria grown at 37°C or bacterium-free filtrate was injected into an approximately 10-cm-long, small intestinal loop of an adult New Zealand White rabbit that had previously fasted for 24 h. After 20 h, the rabbit was sacrificed and the loop was examined for fluid accumulation and other gross pathological changes (12). A portion of the mucosa of the loop was fixed in buffered formal saline, and paraffin sections were stained with hematoxylin and eosin. Another portion was fixed in 3% glutaraldehyde and embedded in araldite, and 1-μm-thick sections were stained with toluidine blue for light microscopy. Each isolate was tested in two rabbits, and nonpathogenic *E. coli* K-12 EC101 was included as a negative control.

RITARD assay. The ability of bacteria to cause diarrhea was tested in 30 rabbits with removable intestinal ties (removable intestinal tie adult rabbit diarrhea [RITARD] model; rabbits used for this assay are referred to herein as RITARD rabbits) (21). Abdominal surgery was performed after rabbits had fasted for 24 h, and a permanent tie was introduced in the cecum close to the ileocecal junction. A temporary tie was introduced in the terminal part of the small intestine above the mesoappendix.

Aliquots (10 ml) of overnight shaker cultures of bacteria grown in TSBY at 37°C (each aliquot contained 10^{10} bacteria) or bacterium-free culture filtrates were injected into the anterior part of the jejunum. The temporary tie was removed 2 h later. Control experiments were performed on four rabbits with *E. coli* K-12 EC101 whole culture. Unless stated otherwise, the animals were observed for 7 days for diarrhea, other symptoms, and death. Rectal swabs were taken daily and plated onto MacConkey agar to monitor shedding of the challenge bacteria. The animals were sacrificed at various periods after challenge when they developed diarrhea or hindlimb paralysis. All animals, including control animals, that did not develop any symptom (see results below) were sacrificed at the end of day 7. At autopsy, the intestines were examined for fluid accumulation and other gross pathological changes. Intestinal sections were taken from the midjejunum, upper and lower ileum, cecum, proximal and distal colon, appendix, rectum, and mesenteric lymph nodes. Sections were fixed in buffered formal saline for histology (1). Mucosal scrapings of the midjejunum and upper and lower ileum were cultured quantitatively. Serial 10-fold dilutions of homogenized tissue were prepared in sterile physiological saline, plated on MacConkey agar, and incubated at 37°C for 24 h.

Electron microscopy. Ileal loop tissues from adult rabbits and intestinal tissues from RITARD rabbits were processed for electron microscopy. The tissues were rapidly fixed in 3% glutaraldehyde and processed and embedded in araldite. After 1-μm-thick survey sections were examined by light microscopy, ultrathin sections of selected areas were cut on an LKB ultratome IV with a diamond knife, stained with uranyl acetate, and examined under a Philips EM201C transmission electron microscope.

Blood culture. Blood was aseptically drawn by syringe from the ear veins of rabbits at 24 h postchallenge. A portion of the blood specimen (0.1 ml) was directly plated onto blood agar and MacConkey agar and incubated for 24 h at 37°C; 1 ml was inoculated into TSB for 24 h of incubation at 37°C and then subcultured onto blood agar and MacConkey agar. Rectal swab culture, quantitative bacteriology of intestine, histology, and blood culture were not done for rabbits inoculated with culture filtrates.

RESULTS

The organisms were negative for enterotoxins, cytotoxins, adherence to HEp-2 cell monolayers (even when grown and assayed under different conditions), and keratoconjunctivitis in the Sereny test, although the controls behaved as expected in these tests. In the gentamicin–HEp-2 cell assay, however, both 4- and 20-h-old cultures of *P. alcalifaciens* were clearly invasive (Table 1). Strain R90-1475 was less invasive than the other two strains were. These differences were evident even in the Giemsa-stained monolayer slides at the end of invasion assay. Strains F90-2004 and 2939/90 produced intracellular bacterial clusters (Fig. 1), which were absent with strain R90-1475. The positive control, *S. flexneri* 2a 611R, was more invasive than *P. alcalifaciens* strains, and the negative control, *E. coli* K-12 EC101, was not invasive. The integrities of the monolayers were not significantly altered at the end of the invasion assays.

TABLE 1. Invasion of HEp-2 cells by *P. alcalifaciens* and control bacteria

Species	Age of culture[a] (h)	CFU of bacteria/ml		% of intracellular bacteria relative to inoculum
		Inoculum[b]	Intracellular[c]	
P. alcalifaciens				
2939/90	4	3.1×10^6	1.7×10^4	0.55
	20	3.0×10^6	1.2×10^4	0.40
R90-1475	4	2.8×10^6	3.3×10^1	0.001
	20	3.3×10^6	6.5×10^1	0.002
F90-2004	4	2.5×10^6	1.3×10^4	0.52
	20	3.0×10^6	2.0×10^4	0.67
S. flexneri 2a 611R	4	1.4×10^6	4.0×10^5	28.6
	20	1.5×10^6	1.6×10^5	10.7
E. coli K-12 EC101	4	7.0×10^6	0	0
	20	2.8×10^6	0	0

[a] Luria broth culture.
[b] Bacteria inoculated into HEp-2 cell monolayers.
[c] Bacteria recovered after gentamicin treatment.

In the 3-h fluorescent actin staining test for attachment-effacement lesions, actin fluorescence was poor (data not shown). But in the 6-h test, localized clusters of fluorescence (which seemed to correspond to intracellular bacterial microcolonies) could be seen with strains F90-2004 and 2939/90. In the fluorescent spots the outlines of some bacteria were evident. This pattern of fluorescence was different from the pattern exhibited by the EPEC serotype O127:H6 strain, in which whole forms of individual bacteria could be seen, but similar to that produced by *S. flexneri* 2a 611R (Fig. 2). Strain R90-1475 showed poor actin staining corresponding to an occasional bacterium (data not shown).

Rabbit ileal loop assay. None of the isolates induced fluid accumulation in the loops of the two rabbits; however, the loops were hemorrhagic. No abnormality was seen in the loops inoculated with bacterium-free culture filtrates. The loops inoculated with *E. coli* K-12 EC101 also remained normal.

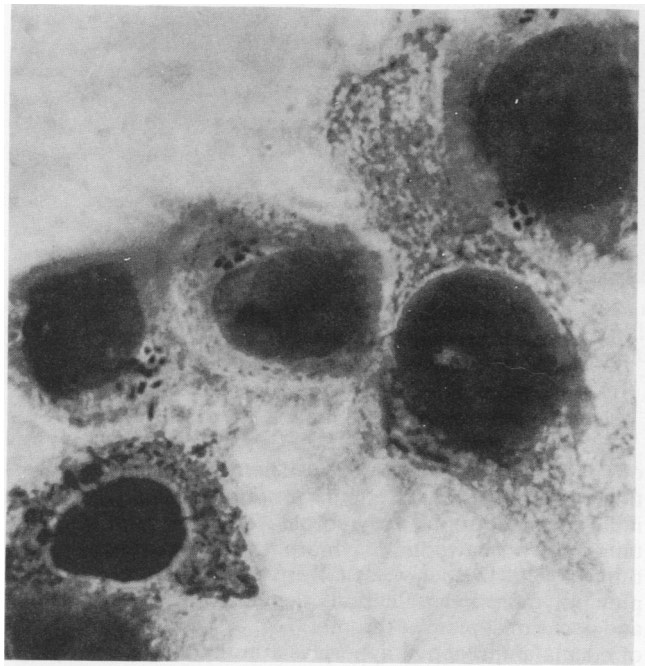


FIG. 1. Intracellular localized bacterial clusters of *P. alcalifaciens* F90-2004 in HEp-2 cell monolayers in the gentamicin-HEp-2 cell invasion assay. Magnification, ×430; Giemsa stain.

RITARD assay. The responses of the rabbits in the RITARD assay are summarized in Table 2. The rabbits inoculated with bacterium-free filtrates of *P. alcalifaciens* and those inoculated with *E. coli* K-12 EC101 remained normal. However, the majority of the animals inoculated with whole cultures of all three *P. alcalifaciens* isolates developed diarrhea and some hindlimb paralysis. One rabbit inoculated with F90-2004 and one inoculated with 2939/90 developed hindlimb paralysis; these rabbits died on days 3 and 2 postsurgery, respectively. Another rabbit that was inoculated with R90-1475 developed hindlimb paralysis on day 3 postsurgery and was then sacrificed.

All rabbits that developed diarrhea shed the challenge organisms in the feces either alone or mixed with *E. coli*. Diarrhea developed between 1 and 4 days after inoculation; the diarrhea lasted in all animals until they were sacrificed (Table 3; data presented are for 9 of the 16 RITARD rabbits that developed diarrhea). One diarrheic rabbit inoculated with F90-2004 passed blood-stained feces; in other diarrheic rabbits, no blood was visible but feces were soft and soiled the perianal region.

At autopsy, rabbits with diarrhea had moderate amounts of greenish yellow frothy fluid in the small intestine and substantial amounts of liquid stool in the portion of the cecum that remained patent and in the colon.

Complete bacteriology and histology tests were performed on only 9 of 16 RITARD animals with diarrhea (Table 3). The mucosal scraping from the small intestine showed pure growth of *P. alcalifaciens* in 5 rabbits (rabbits R63, R65, R81, R99, and R100). In other rabbits the growth was mixed with *E. coli*. The numbers of *P. alcalifaciens* in the jejunum ranged between 1×10^3 and 1.5×10^8 CFU/g of mucosal scraping; in the ileum, the numbers were much higher (between 8×10^3 and 9×10^8 CFU/g of mucosal scraping in the upper ileum and between 6×10^4 and 3.5×10^8 CFU/g of mucosal scraping in lower ileum).

Histology. The histological sections of rabbit ileal loops and of jejunum, ileum, cecum, and colon from the RITARD

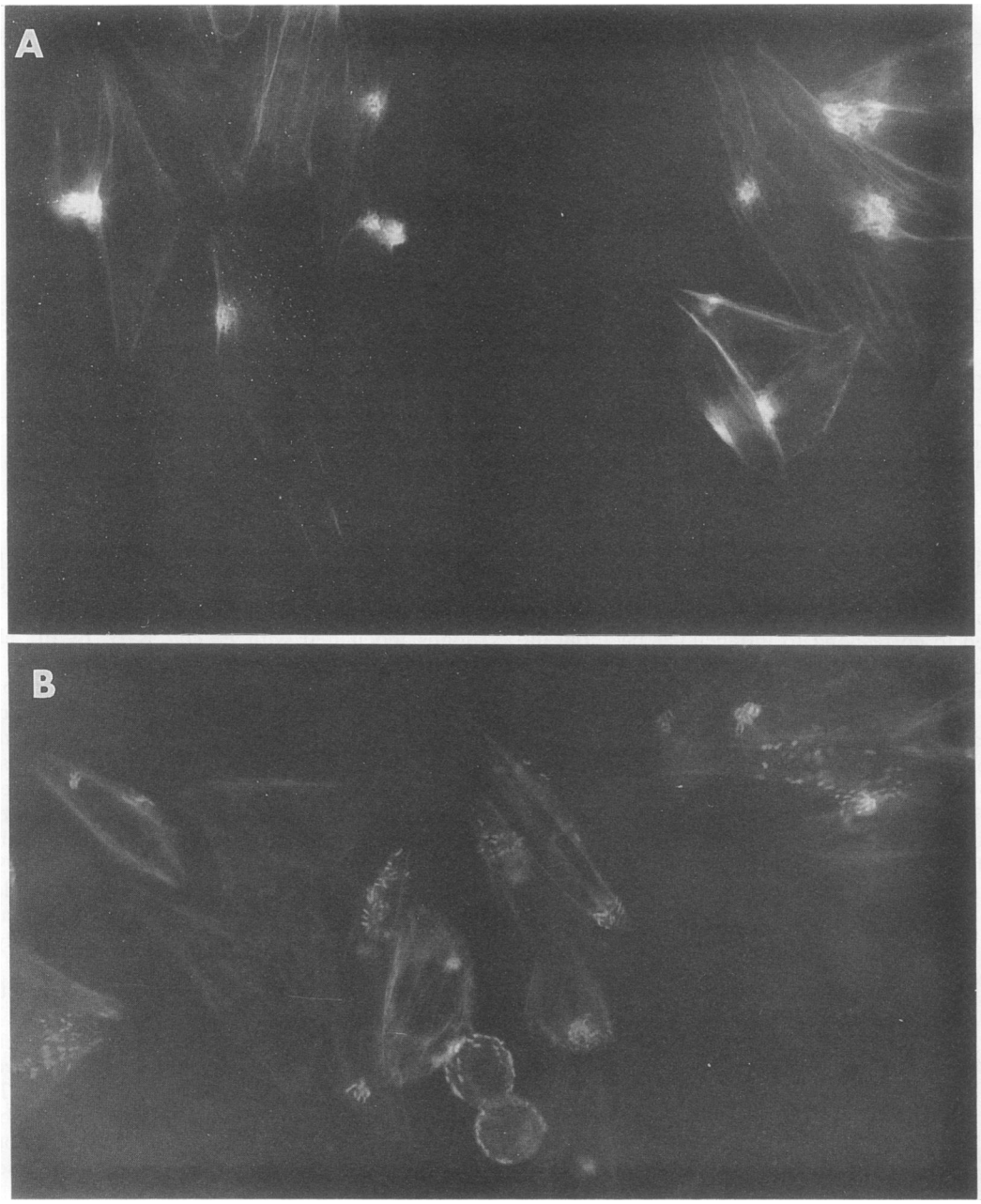
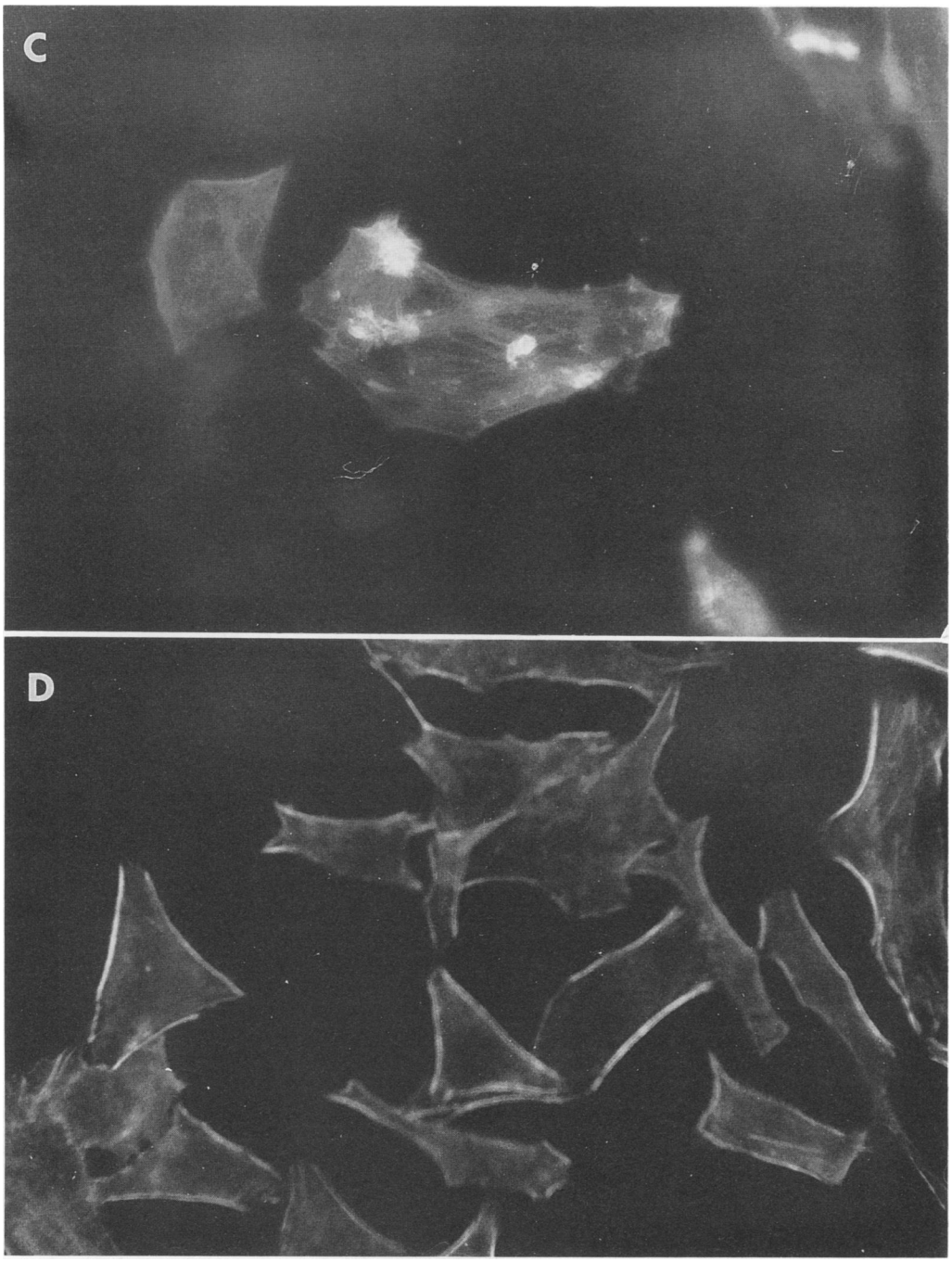


FIG. 2. Fluorescent actin staining of HEp-2 monolayers infected with *P. alcalifaciens* F90-2004 (A), a strain of EPEC serotype O127:H6 (B), and *S. flexneri* 2a 611R (C) and an uninoculated monolayer (D). Outlines of some individual bacteria in fluorescent spots can be seen in panel A. Note that actin fluorescence associated with *P. alcalifaciens* is different from that associated with EPEC but similar to that associated with *S. flexneri*. Magnification, ×400 for all panels.

rabbits inoculated with whole bacterial cultures of *P. alcalifaciens* showed marked disorganization, degenerative changes, and denudation of villus epithelium (Fig. 3A). The degenerated epithelial cells were either swollen and pale or stained dark with pyknotic nuclei and condensed cytoplasm. Bacteria were present in the mucus layer and adhered to the enterocytes; they penetrated the cells but seldom deep to the basement membrane, except in tips of villi, where microulcers were present. On the sides of villi, there were focal losses of epithelial cells leading to microulceration and formation of bacterial microcolonies, especially at the base of epithelial infolding (Fig. 3B). There was marked congestion and edema of the lamina propria with an increase in infiltrating neutrophil polymorphs. The blood vessels contained many neutrophil polymorphs, especially in the upper third of villi. Deeper vessels were also dilated with neutrophil polymorph margination, denudation of endothelium, and occasional platelet thrombi (Fig. 4). There was evidence of edema and reactive hyperplasia in the mesenteric lymph nodes. Ileal loops of rabbits inoculated with *E. coli* K-12 EC101 culture and bacterium-free culture filtrates of *P. alcalifaciens* or RITARD rabbits challenged with *E. coli*



K-12 EC101 showed no histological abnormalities. The histology of RITARD rabbits challenged with bacterium-free culture filtrates of *P. alcalifaciens* was not determined.

The histology of nerve tissue in two rabbits that developed hindlimb paralysis and one rabbit that developed diarrhea but no paralysis was determined. Focal mononuclear cell infiltration around the peripheral nerve sheath was found in the two rabbits with paralysis but not in the rabbit with diarrhea only.

Electron microscopy. The ileal loops of rabbits and intestines of RITARD rabbits that developed diarrhea showed evidence of penetration of intestinal epithelial cells by bacteria (Fig. 5); this penetration was absent in animals inoculated with nonpathogenic *E. coli* K-12 EC101.

Blood culture. None of the blood cultures from any of the rabbits grew *P. alcalifaciens*.

DISCUSSION

P. alcalifaciens was suspected as a cause of diarrhea in the three patients because it was either the only organism cultured or present in abundance in the stools in the absence

TABLE 2. Responses of RITARD rabbits inoculated with whole bacterial cultures (BC) of *P. alcalifaciens* and *E. coli* K-12 EC101 and bacterium-free culture filtrates (CF) of *P. alcalifaciens*

Challenge bacterium	Inoculum	No. of animals studied	No. of rabbits with the following response:		
			Diarrhea	Hindlimb paralysis	No change
P. alcalifaciens					
F90-2004	BC	11	10	1	0
	CF	3	0	0	3
2939/90	BC	5	4	1	0
	CF	3	0	0	3
R90-1475	BC	5	2	1	2
	CF	3	0	0	3
E. coli K-12 EC101	BC	4	0	0	4

of recognized enteric pathogens. None of the isolates produced any toxin, but all of them invaded the HEp-2 cell monolayer. Two isolates (F90-2004 and 2939/90) were more invasive than the third isolate (R90-1475) in this assay system. The former two isolates also showed intracellular bacterial clusters on light microscopic examination of Giemsa-stained cell monolayers and produced striking actin condensation in the in vitro fluorescent actin staining test. The actin condensation seemed to correspond to the bacterial microcolonies within the cells. This pattern of actin condensation is similar to that produced by another invasive organism, *S. plexneri* (4), but different from that due to EPEC (11). The third isolate of *P. alcalifaciens*, R90-1475, showed weaker properties of penetration and actin condensation. The negative control of *E. coli* K-12 EC101 did not show any invasion of HEp-2 cells. This is contrary to observations in other studies (14, 20), in which negative controls showed some degree of invasion. The property of cell invasion might be strain dependent; the strain in the present study was a local derivative of *E. coli* K-12 (2). None of the *Providencia* isolates showed any characteristic adherence to HEp-2 cells, even though bacteria were grown and assayed under different conditions. This situation is perhaps analogous to that of *S. flexneri* (the prototype invasive organism), with which it is difficult to demonstrate adherence to cell monolayers (4).

The RITARD model has previously been used to investigate the diarrheagenic properties of a variety of organisms (1, 13, 21). All three *P. alcalifaciens* isolates produced diarrhea in this model, supporting the suspicion of diarrheagenic potential of these organisms. The isolates colonized the gut, resulting in the excretion of bacteria in feces, sometimes with total displacement of normal flora. Again, as in the in vitro virulence tests, differences were noted in the disease-producing abilities of the three isolates. Isolates F90-2004 and 2939/90 produced diarrhea in 91% (10 of 11 rabbits) and 80% (4 of 5 rabbits) of the animals, respectively, and also colonized the intestines well. However, isolate, R90-1475 produced diarrhea in only 40% (two of five rabbits) of the animals and was not recovered from the small intestine of one rabbit (R108) at the time of sacrifice (Tables 2 and 3). These data suggest that there are differences in the disease-producing abilities of different strains of *P. alcalifaciens*. None of the strains produced bacteremia, which suggests that these bacteria do not invade beyond the intestinal mucosa.

Histology showed mucosal lesions suggestive of invasive diarrhea. The lesions included bacterial attachment to, penetration of, and microulcer formation on the surface epithelium and edema, hyperemia, and polymorphonuclear cell infiltration of lamina propria. Transmission electron microscopy of the intestinal mucosa of affected animals also showed that the organisms are invasive. The finding of

TABLE 3. Fecal shedding, diarrhea, and colonization of the gut in RITARD rabbits inoculated with *P. alcalifaciens*

Rabbit no.[a]	Diarrhea[b] (day after inoculation)	Organism(s)[c] shed in stool	No. of organisms (species)/g of mucosal scraping of:			Histological evidence of bacterial adhesion (A) and/or invasion (I) in:					
			Midjejunum	Upper ileum	Lower ileum	Jejunum	Ileum	Cecum	Colon	Rectum	Appendix
R63	+ (2)	PE (3)	4×10^5 (P)	5×10^6 (P)	2.5×10^7 (P)	—[d]	AI	—	—	—	A
R64	+ (1)	P (5)	2×10^3 (P) 4×10^2 (E)	8×10^3 (P) 4.5×10^2 (E)	6×10^4 (P)	A	A	A	A	—	—
R65	+ (2)	P (5)	1.6×10^5 (P)	4×10^7 (P)	1.4×10^8 (P)	—	I	A	—	—	—
R79	+ (2)	PE (5)	3.5×10^5 (P) 1×10^4 (E)	1×10^6 (P) 2×10^5 (E)	4.2×10^6 (P) 1.5×10^5 (E)	—	—	A	A	A	—
R81	+ (2)	PE (3)	5×10^7 (P)	2.6×10^8 (P)	2.5×10^8 (P)	A	AI	AI	A	—	—
R99	+ (2)	PE (3)	1.5×10^8 (P)	1×10^8 (P)	3.5×10^8 (P)	AI	I	A	AI	AI	—
R100	+ (2)	PE (5)	4×10^6 (P)	1.5×10^7 (P)	2×10^8 (P)	—	AI	AI	AI	—	—
R101	+ (3)	PE (5)	2×10^7 (P)	9×10^8 (P)	1.5×10^7 (P) 2×10^4 (E)	AI	AI	AI	—	—	—
R108	+ (4)	PE (4)	1×10^3 (E)	1.4×10^5 (E)	1.8×10^5 (E)	—	—	A	—	—	—

[a] Rabbits R63, R64, R65, R79, and R81 were challenged with strain F90-2004; rabbits R99, R100, and R101 were challenged with strain 2939/90; and rabbit R108 was challenged with strain R90-1475.

[b] +, presence of diarrhea.

[c] P, *P. alcalifaciens*; E, *E. coli*. Shedding of the indicated organism(s) occurred until the day of sacrifice (in parentheses).

[d] —, no evidence of adhesion or invasion.

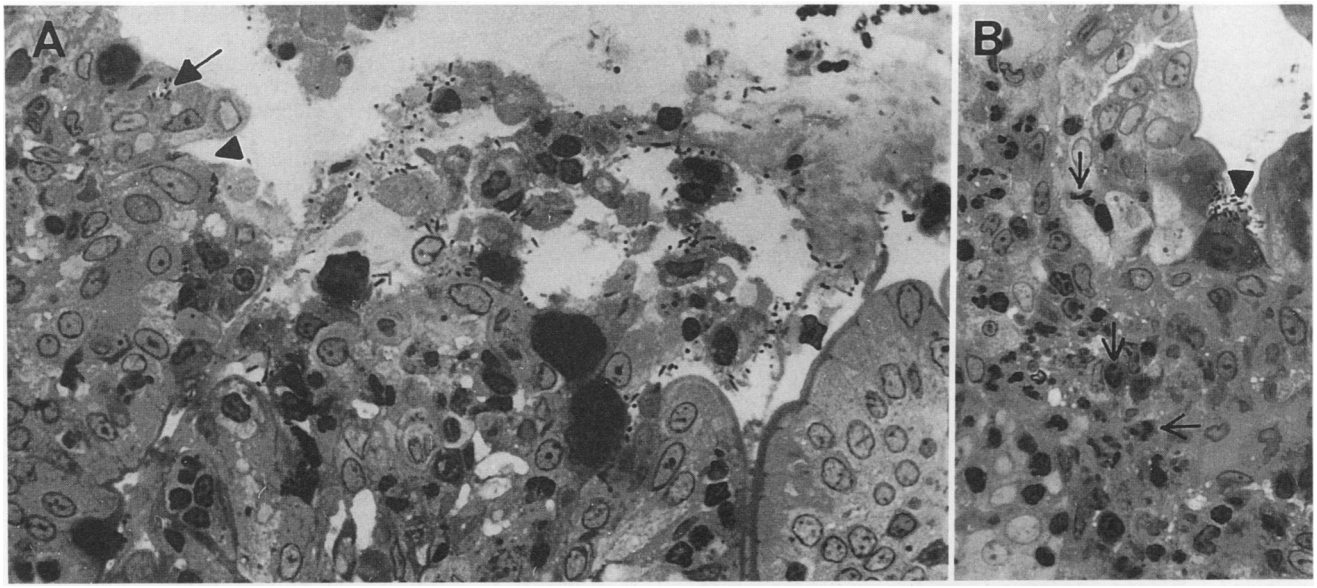


FIG. 3. Luminal aspect of the jejunal mucosa of a RITARD animal inoculated with *P. alcalifaciens* F90-2004 showing bacterial invasion (arrow) with luminal cell degeneration and desquamation forming surface ulceration (arrowhead). Marked congestion of microvasculature is seen in the lamina propria (A). The adjacent mucosa shows many bacteria adherent to a degenerating enterocyte (arrowhead). The lamina propria shows many neutrophil polymorphs (arrows) (B). Magnifications, ×750 for panel A and ×580 for panel B; toluidine blue stain.

invasive diarrhea in the animal model is supported by the invasive picture in the stool of diarrheal patients excreting organisms of the *Providencia* group (16). Unfortunately, there was no information on what types of diarrheas were present in the three patients from whom the present study isolates originated.

The organisms also produced hindlimb paralysis in some rabbits, and the clinical relevance of this observation is not clear. It is known that *S. dysenteriae* 1 toxin induces hindlimb paralysis when injected into mice (3). It is not clear whether a similar toxin is elaborated by *P. alcalifaciens*, although no evidence of cytotoxicity was found in the in vitro tissue culture systems inoculated with bacterium-free filtrate and bacterial extract or in the rabbit ileal loop challenged with bacterium-free filtrate. Further studies are needed to elucidate the mechanism of paralysis.

Histology of rabbit ileal loops injected with bacterium-free culture filtrates showed no abnormality. Moreover, RI-

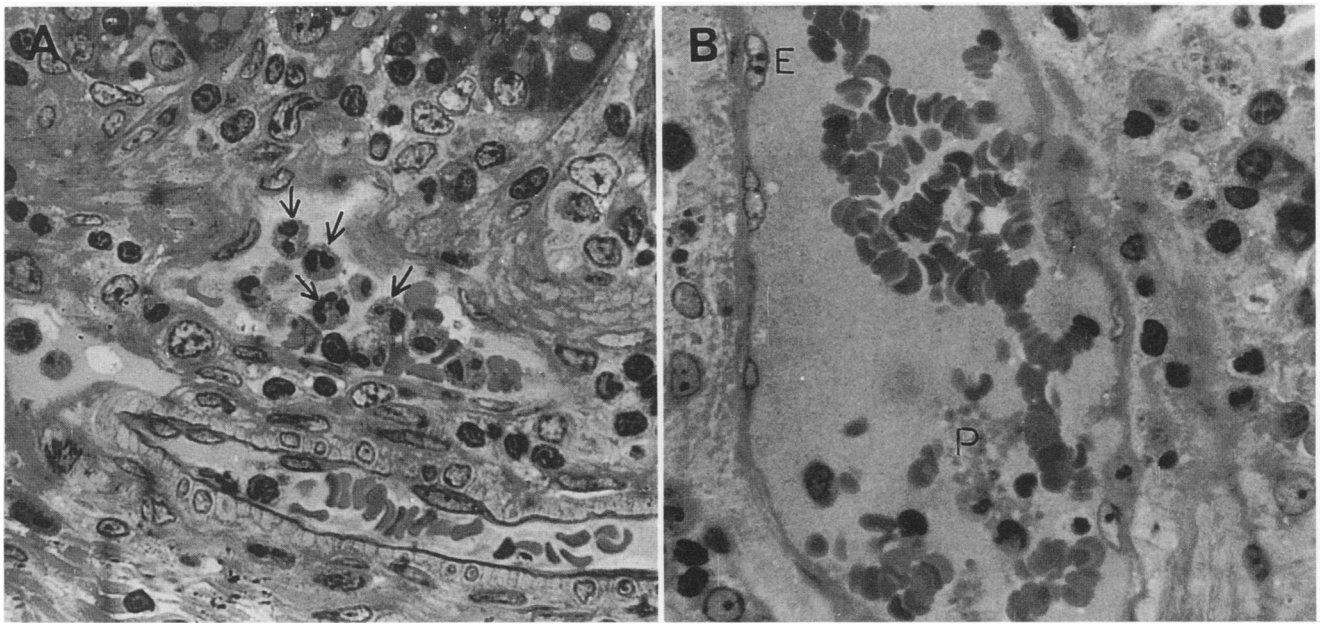


FIG. 4. Same section as that shown in Fig. 3. (A) The capillary near the crypt base shows many neutrophil polymorphs (arrows). (B) Note the markedly congested venule with endothelial swelling (E), degeneration, and endothelial cell loss. The lumen shows platelet thrombi (P). Magnification, ×800 for both panels; toluidine blue stain.

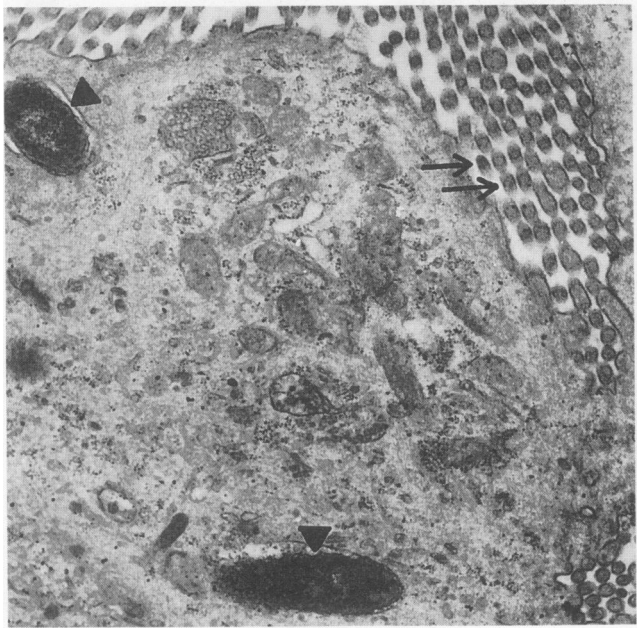


FIG. 5. Transmission electron microscopy of an ileal section from the same RITARD rabbit as that shown in Fig. 3. Note the two intracellular bacteria within vacuoles (arrowheads). The microvilli of the enterocyte are vesiculated (arrows). Magnification, ×17,500.

TARD rabbits inoculated with culture filtrates also remained normal. These findings suggest that soluble bacterial products may not be involved in the pathogenesis.

Past studies (16, 18) have looked at the roles of Providence group organisms as a whole in the causation of diarrhea, and there are no reliable data on the frequencies of isolation of *P. alcalifaciens* from patients with diarrhea and from controls without diarrhea. Clearly, case control studies are needed to assess the importance of this organism in diarrheal illness.

ACKNOWLEDGMENTS

This research was supported by the International Centre for Diarrhoeal Disease Research, Bangladesh, which is supported by countries and agencies that share its concern for the health problems of developing countries. Current donors include the aid agencies of the governments of Australia, Bangladesh, Belgium, Canada, Denmark, France, Japan, the Netherlands, Norway, Saudi Arabia, Sweden, Switzerland, the United Kingdom, and the United States; international organizations including the United Nations Development Programme, the United Nations Children's Fund, and the World Health Organization; and private foundations, including the Ford Foundation and the Sasakawa Foundation.

We thank Manzurul Haque of the International Centre for Diarrhoeal Disease Research, Bangladesh, for his secretarial assistance.

REFERENCES

1. **Albert, M. J., K. Alam, M. Islam, J. Montanaro, A. S. M. H. Rahman, K. Haider, M. A. Hossain, A. K. M. G. Kibriya, and S. Tzipori.** 1991. *Hafnia alvei*, a probable cause of diarrhea in humans. Infect. Immun. **59**:1507–1513.
2. **Ashraf, M. H., Z. U. Ahmed, and D. A. Sack.** 1991. Unusual association of a plasmid with nalidixic acid resistance in an epidemic strain of *Shigella dysenteriae* type 1 from Asia. Can. J. Microbiol. **37**:59–63.
3. **Cavanagh, J. B., J. C. Howard, and J. L. Whitby.** 1956. The neurotoxin of *Shigella shigae*. A comparative study of the effects produced in various laboratory animals. Br. J. Exp. Pathol. **37**:272–278.
4. **Clerc, P., and P. J. Sansonetti.** 1987. Entry of *Shigella flexneri* into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation. Infect. Immun. **55**:2681–2688.
5. **Cravioto, A., R. J. Gross, S. M. Scotland, and B. Rowe.** 1979. An adhesive factor found in strains of *Escherichia coli* belonging to the traditional infantile enteropathogenic serotypes. Curr. Microbiol. **3**:95–99.
6. **Dean, A. G., Y. C. Ching, R. G. Williams, and L. B. Harden.** 1972. Test for *Escherichia coli* enterotoxin using infant mice: application in a study of diarrhea in children in Honolulu. J. Infect. Dis. **125**:407–411.
7. **Ewing, W. H.** 1986. Edwards and Ewing's identification of *Enterobactericeae*, 4th ed, p. 443–459. Elsevier Science Publishing Co., Inc., New York.
8. **Gentry, M. K., and J. M. Dalrymple.** 1980. Quantitative microtiter cytotoxicity assay for *Shigella* toxin. J. Clin. Microbiol. **12**:361–366.
9. **Griffin, D. F., and P. Gemski, Jr.** 1983. Release of Shiga toxin from *Shigella dysenteriae* 1 by polymyxin B. Infect. Immun. **40**:425–428.
10. **Haynes, J., and P. M. Hawkey.** 1989. *Providencia alcalifaciens* and travellers' diarrhoea. Br. Med. J. **299**:94–95.
11. **Knutton, S., A. D. Phillips, H. R. Smith, R. J. Gross, R. Shaw, P. Watson, and E. Price.** 1991. Screening for enteropathogenic *Escherichia coli* in infants with diarrhea by the fluorescent actin staining test. Infect. Immun. **59**:365–371.
12. **Moon, H. W., S. C. Whipp, R. A. Argenzio, M. M. Levine, and R. A. Gianella.** 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. Infect. Immun. **41**:1340–1351.
13. **Pazzaglia, G., R. B. Sack, A. L. Bourgeois, J. Froehlich, and J. Eckstein.** 1990. Diarrhea and intestinal invasiveness of *Aeromonas* strains in the removable intestinal tie rabbit model. Infect. Immun. **58**:1924–1931.
14. **Robins-Browne, R., and V. Bennett-Wood.** 1992. Quantitative assessment of the ability of *Escherichia coli* to invade cultured animal cells. Microb. Pathog. **12**:159–164.
15. **Sack, D. A., and R. B. Sack.** 1975. Test for enterotoxigenic *Escherichia coli* using Y1 adrenal cells in miniculture. Infect. Immun. **11**:334–336.
16. **Sen, R.** 1962. Isolation of strains of *Providence* group from cases with diarrhoea in Ibadan, Nigeria, West Africa. Indian J. Med. Res. **50**:622–626.
17. **Sereny, B.** 1957. Experimental keratoconjunctivitis shigellosa. Acta Microbiol. Acad. Sci. Hung. **4**:367–376.
18. **Singer, J., and J. Bar-Chay.** 1954. Biochemical investigation of *Providence* strains and their relationship to the *Proteus* group. J. Hyg. Camb. **52**:1.
19. **Small, P. L. C., and S. Falkow.** 1988. Identification of regions on a 230-kilobase plasmid from enteroinvasive *Escherichia coli* that are required for entry into HEp-2 cells. Infect. Immun. **56**:225–229.
20. **Small, P. L. C., R. R. Isberg, and S. Falkow.** 1987. Comparison of the ability of enteroinvasive *Escherichia coli*, *Salmonella typhimurium*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* to enter and replicate within HEp-2 cells. Infect. Immun. **55**:1674–1679.
21. **Spira, W. M., R. B. Sack, and J. L. Froehlich.** 1981. Simple adult rabbit model for *Vibrio cholerae* and enterotoxigenic *Escherichia coli* diarrhea. Infect. Immun. **32**:739–749.