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Characterization of *Vibrio cholerae* Non-O1 Serogroups Obtained from an Outbreak of Diarrhea in Lima, Peru

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In February 1994, an outbreak of diarrhea caused by non-O1 *Vibrio cholerae* occurred among volunteers in a vaccine trial study area in Lima, Peru. Clinically, 95% of the patients presented with liquid diarrhea with either no or mild dehydration. Serogrouping of 58 isolates recovered from diarrheal patients affected in the outbreak revealed seven different serogroups, with serogroups O10 (21%) and O12 (65%) being predominant. Most of these isolates were susceptible to a variety of antimicrobial agents. None of the 58 isolates hybridized with a DNA probe previously used to detect the gene encoding the heat-stable enterotoxin NAG-ST or produced cholera toxin as assessed by GM_1 ganglioside enzyme-linked immunosorbent assay. Ribotyping exhibited 10 different *Bgl*I ribotype patterns among the 58 *V. cholerae* non-O1 strains studied. However, ribotyping showed that all isolates belonging to serogroup O12 exhibited identical ribotypes and that 83% of the serogroup O10 isolates belonged to another identical ribotype, thus showing excellent correlation between ribotypes and serogroups. Among a group of O10 and O12 isolates selected for virulence studies, none produced enterotoxin whereas the majority produced a cytotoxin, as assessed in Y1 and HeLa cells. These isolates were also negative for the gene encoding zonula occludens toxin (Zot) as assessed by a PCR assay. The isolates tested showed strong adherence and some degree of invasion in the HEp-2 cell assay, whereas none of the isolates was positive in the PCR assay for the gene encoding the toxin coregulated pilus subunit A antigen (*tcpA*). In the removable intestinal tie adult rabbit diarrhea model, O10 and O12 serogroup isolates produced severe diarrhea and occasionally death when rabbits were challenged with 10^{10} bacterial cells. Fluid accumulation was shown in the rabbit intestinal loop test when whole cultures were injected. No significant difference in virulence was shown between serogroup O10 and O12 isolates. This study provides further evidence that *V. cholerae* non-O1 non-O139 strains have diarrhegenic potential for humans through a yet-undefined mechanism(s) and that such strains can cause outbreaks.

Vibrio cholerae non-O1 strains have increasingly been recognized as the causative agents of sporadic cases of cholera-like disease (24) and isolated outbreaks (5). To date, the only non-O1 serogroup to cause an epidemic of clinical cholera was O139, which was initially isolated in South Asia (44).

Although cholera toxin (CT) and heat-stable enterotoxin (NAG-ST) have been described as important virulence factors for non-O1 *V. cholerae*, these toxins are relatively rare among clinical isolates (16, 31, 33). A wide range of other determinants, including cytotoxins (21), hemolysins (17, 49), colonization factors (6), and a CT-like enterotoxin (47), have been described as virulence factors for non-O1 *V. cholerae.*

Recently, Shimada and colleagues extended a previously described serogrouping scheme from O84 to O155 (41). Serogrouping has been used to study the epidemiology of non-O1 *V. cholerae* by several workers with varying degrees of success (27, 39). Determination of rRNA gene restriction (rDNA) fragment polymorphisms (ribotyping) was first described as a taxonomic tool by Grimont and Grimont (15). The method has proved to be a useful molecular epidemiologic technique in the study of several pathogens, including *V. cholerae* (10, 11, 32).

The primary objective of the study described herein was to evaluate to what extent ribotyping and serogrouping could be used to study the epidemiology of 58 *V. cholerae* non-O1 isolates recovered from an outbreak of diarrhea in Peru. In addition, putative virulence factors were examined by various assays.

MATERIALS AND METHODS

Bacteria. A surveillance for cholera was initiated on 11 January 1994 to evaluate the efficacy of an oral killed whole-cell recombinant B toxin subunit *V. cholerae* vaccine in a peri-urban community in Lima, Peru, with a population of 38,000 (38). The surveillance program was to be conducted for two cholera seasons, with termination in July 1995. A stool specimen was obtained from each diarrheal patient detected by an active (involving household visits) and/or passive (involving hospital referrals) surveillance program. Specimens were transported in Cary-Blair medium to the Microbiology Laboratory, U.S. Naval Medical Research Institute Detachment, Lima, Peru. All stool specimens were screened for pathogenic *Vibrio* spp. and enterotoxigenic *Escherichia coli*. Ten percent of the stool samples were screened for other enteric pathogens such as *Salmonella* spp. and *Shigella* spp. For the analysis of pathogenic *Vibrio* spp., each specimen was enriched for 6 h in alkaline-peptone-water (pH 8.6) and then plated onto thiosulfate-citrate-bile salt-sucrose (TCBS) agar (Difco, Detroit, Mich.). On the basis of standard biochemical reactions (37), all suspected *V. cholerae* isolates were tested by agglutination tests employing polyvalent O1, monospecific Ogawa and Inaba, and O139 polyclonal goat antisera (Naval Medical Research Institute, Bethesda, Md.). Fifty-eight *V. cholerae* non-O1 isolates were selected for anti-

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TABLE 1. Serogroups and ribotypes of 58 *V. cholerae* non-O1 isolates recovered from patients during an outbreak of diarrhea in Lima, Peru, in February 1994

Serogroup	No. of isolates ribotyped	*Bgl*I ribotype pattern
O12	38	1
O10	12	2, 7, 9[a]
O53	1	3
O2	1	5
O28	1	6
O34	1	10
O34	1	8
NT[b]	1	4
NT	1	ND[c]
Rough[d]	1	8

[a] Of the 12 isolates, 10 showed pattern 7, 1 showed pattern 9, and 1 showed pattern 2.
[b] NT, nontypeable.
[c] ND, not done.
[d] The rough strain isolate could not be serotyped.

biotic susceptibility testing, serotyping, ribotyping, colony hybridization with NAG-ST probes, and hemolysin production. Antibiotic susceptibilities to 13 antibacterial drugs were tested by disc diffusion (29) on Mueller-Hinton II agar (Difco). Isolates that did not agglutinate O1 or O139 antiserum were tested for agglutination of other O serogroup antisera at the National Institute of Health, Tokyo, Japan, according to the scheme established by Shimada et al. (40).

Ribotyping. Fifty-eight *V. cholerae* non-O1 strains were ribotyped. Total bacterial DNA was extracted by the method of Murray and Thompson (26). On the basis of previous studies (10, 32) and preliminary chromosomal digestion experiments with *Hin*dIII, *Eco*RI, and *Bgl*I (Promega, Madison, Wis.), *Bgl*I provided the best discrimination among *V. cholerae* non-O1 strains and was therefore used to digest chromosomal DNA. Ribotyping was performed by the procedure described by Dalsgaard et al. (10) with digoxigenin-labelled 16S and 23S rRNA probes. A 1-kb molecular weight standard (GIBCO BRL, Gaithersburg, Md.) was used as a weight marker. Ribotype patterns were considered to be different when there was a difference of one or more bands between isolates, and each ribotype was given an arbitrary number.

Colony hybridization with a NAG-ST probe. The *V. cholerae* non-O1 isolates listed in Table 1 were examined by the colony hybridization technique for DNA sequences encoding NAG-ST (3) with an alkaline phosphatase-labelled oligonucleotide probe as previously described (20, 45).

Enterotoxin and cytotoxin production. Four serogroup O10 isolates (VIG 1597, VIG 1644, VIG 1975, and VIG 1980) and four serogroup O12 isolates (VIG 1581, VIG 1587, VIG 1603, and VIG 1613) were selected. The following control bacteria were included: a patient isolate of *Shigella flexneri* 2a, a patient isolate of *Shigella dysenteriae* type 1, localized adherence-positive strain of enteropathogenic *E. coli* E2348/69 (serotype O127:H6), a nonpathogenic laboratory strain of *E. coli* K-12, and *V. cholerae* 569B (a CT-producing O1 biotype El Tor serotype Inaba strain). Bacteria were grown in Casamino Acids-yeast extract (CAYE) broth (Difco) (12) and brain heart infusion broth (BHIB; Difco) for 20 h at 37°C in a shaking water bath. The bacteria were pelleted, and the supernatant was passed through a membrane filter with 0.2-μm-diameter pores (Sartorius, GmbH, Göttingen, Germany). The filtrate (neat and diluted 1:10 in phosphate-buffered saline, pH 7.2) was used to test for toxins with adrenal tumor Y1 cells (35) and HeLa cells (14). A filtrate prepared from *V. cholerae* 569B and a filtrate prepared from *S. dysenteriae* type 1 were used as enterotoxin and cytotoxin controls, respectively. Furthermore, the isolates listed in Table 1 were tested for CT production by GM_1–enzyme-linked immunosorbent assay (ELISA) (36), a method that uses the purified GM_1 ganglioside receptor as a capture molecule.

Cell-associated hemagglutinating activity. Cell-associated hemagglutinating activities were determined for eight selected strains—VIG 1597, VIG 1644, VIG 1975, and VIG 1980 (all serogroup O10) and VIG 1581, VIG 1587, VIG 1603, and VIG 1613 (all serogroup O12)—by the methods described previously (33). Briefly, bacteria grown on colonization factor antigen agar were tested on slides for agglutination of human group O erythrocytes. Inhibition of agglutination was checked against 1% (wt/vol) D-mannose.

PCR assays for *zot* and *tcpA*. The eight selected isolates were tested for the presence of genes encoding the zonula occludens toxin (Zot, a minor enterotoxin) and the toxin coregulated pilus subunit A antigen (TcpA [Tcp is the major colonization antigen]) of *V. cholerae* O1 with sets of primers described previously (13, 18).

El Tor-like hemolysin. Isolates were streaked on 10% sheep blood agar and examined for hemolysin production after incubation at 37°C for 20 h.

Cell culture adherence and penetration. Adherence to HEp-2 cells was tested by the method of Cravioto et al. (8). Bacteria were grown for 4 h at 37°C in Luria broth (Difco) and incubated with HEp-2 cell monolayers at 37°C for 3 h. After the monolayers were washed to remove nonadherent bacteria, the monolayers were fixed in 70% methanol, stained with Giemsa stain, and examined for adherent bacteria under a light microscope. The localized adherence-positive enteropathogenic *E. coli* strain E2348/69 was used as a positive control in the adherence assays. The HEp-2 cell invasion assay was carried out as described previously (2), with some modifications. In the modified assay, the organisms were grown in BHIB (instead of Luria broth) as shaker cultures at 37°C for 4 h and 10^7 CFU (instead of 10^6 CFU) of bacteria was added to the cells. After infection of the cells, during the invasion period, the tissue culture medium contained 100 μg of gentamicin per ml to kill extracellular bacteria. Experiments were run in duplicate and repeated at least three times per isolate. Bacterial isolates were also exposed to gentamicin-containing tissue culture medium (without HEp-2 cells) to ensure that the bacteria did not survive the antibiotic treatment. The strains *S. flexneri* 2a and *E. coli* K-12 were used as positive and negative controls, respectively. The cell monolayer was lysed with 1% Triton X-100 to release intracellular bacteria, which were enumerated by plating different dilutions on gelatin agar and MacConkey agar (Difco).

Rabbit ileal loop test. One milliliter of a culture containing 10^9 CFU of bacteria (VIG 1644, VIG 1597, VIG 1587, or VIG 1581) grown in CAYE broth as a shaker culture (150 oscillations per min) at 37°C or 1 ml of bacterium-free filtrate (prepared with a Sartorius membrane with 0.2-μm-diameter pores) was injected into an ~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 by parenteral administration of sodium pentobarbitol (50 mg/kg of body weight) and the loop was examined for fluid accumulation and other gross pathological changes (23). Each of the four isolates was tested in two rabbits, and *V. cholerae* 509B and *E. coli* K-12 were tested as positive and negative controls, respectively. The result of fluid accumulation, expressed as a ratio of volume (in milliliters) of fluid accumulated to the length (in centimeters) of the loop, is the average of the results for two rabbits.

RITARD assay. The abilities of bacteria to cause diarrhea were tested with 17 New Zealand White rabbits (13 for *V. cholerae* and 4 for control strain *E. coli* K-12) with removable intestinal ties (removable intestinal tie adult rabbit diarrhea [RITARD] model; rabbits used for this assay are referred to henceforth as RITARD rabbits) (42). Abdominal surgery was performed on rabbits under anesthesia with parenteral administration of sodium pentobarbitol (30 mg/kg body weight) after they had fasted for 24 h, and a permanent tie was introduced in each rabbit in the cecum, close to the ileocecal junction. A temporary tie was introduced in the terminal part of the small intestine above the mesoappendix.

Samples (10 ml, containing either 10^6 or 10^{10} bacteria) of overnight shaker cultures grown in CAYE broth (7) at 37°C were injected into the anterior part of the jejunum. The temporary tie was removed 2 h later. Control experiments were performed with four rabbits with *E. coli* K-12 culture. Unless stated otherwise, the animals were observed for 7 days for diarrhea and death. Rectal swabs were taken daily and plated onto taurocholate-tellurite-gelatin agar (TTGA) (22) to monitor shedding of *V. cholerae*. The animals were sacrificed by parenteral administration of sodium pentobarbitol when they developed diarrhea at various periods after challenge. All animals, including control animals, that did not develop any symptoms (see Results) 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, terminal ileum, cecum, proximal and distal colon, rectum, appendix, and mesenteric lymph nodes. Sections were fixed in 10% formalin for histology (2). Mucosal scrapings of the midjejunum and lower ileum were weighed and cultured quantitatively. Serial 10-fold dilutions of homogenized tissue were prepared in sterile physiological saline, plated on TTGA and MacConkey agar (Difco), and incubated at 37°C for 24 h.

RESULTS

Specimens. The isolation of *V. cholerae* O1 and non-O1 strains from 1,385 cases of diarrhea in the first 4 weeks of surveillance is shown in Fig. 1. In the first week of February 1994, the cases increased markedly, as indicated by the isolation of 54 *V. cholerae* non-O1 strains from patients with diarrhea. After the first week, the isolation rates of both *V. cholerae* O1 and non-O1 strains declined. On the basis of the appearance of the disease outbreak, 58 non-O1 isolates, including 48 strains isolated on 8 February 1994 and 10 strains isolated from 9 to 18 February 1994, were selected for further studies. *V. cholerae* non-O1 were more frequently isolated from females (55%) ranging in age from 0 to 57 years (mean, 14 years). Clinically, 95% of the patients presented with liquid diarrhea either with or without mild dehydration (only 18% had dehydration). None of the patients had fever or vomiting; no case required hospitalization. Cases were scattered all over the

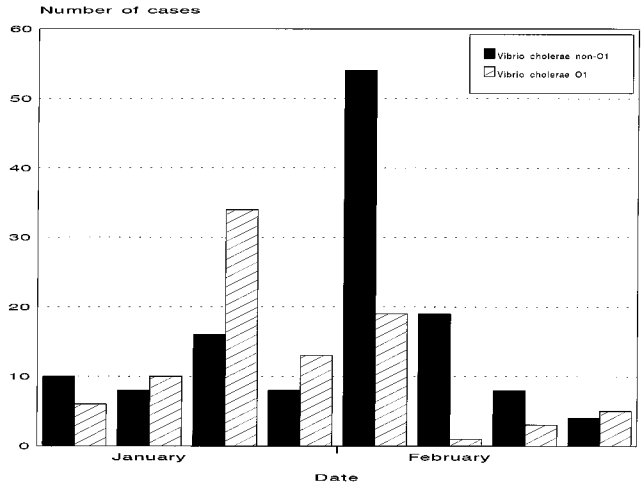

FIG. 1. Weekly isolation of *V. cholerae* O1 and non-O1 strains from patients with diarrhea in a peri-urban community in Lima, Peru, in 1994.

community. No evidence of a common source of infection was found.

Biochemical reactions, hemolysis, and antibiotic susceptibility patterns. The *V. cholerae* non-O1 strains produced yellow colonies on TCBS agar and grew in NaCl concentrations of 0% and 3% but not of 8%. Of the 48 isolates studied from cases seen on 8 February, all were oxidase and indole positive; fermented glucose, saccharose, and mannose; were motile in motility indole-ornithine medium; produced lysine and ornithine decarboxylases but not arginine dehydroxylase; and were negative for hydrogen sulfide production. All isolates produced strong β-type hemolysis on sheep blood agar. The results of the antibiotic susceptibility testing showed that the non-O1 strains were susceptible to tetracycline, doxycycline, and fluorquinolones. Ten percent of the non-O1 strains were resistant to ampicillin. Thirty percent were resistant to trimethoprim-sulfamethoxazole or trimethoprim alone.

Serogrouping. The results of the serogrouping are shown in Table 1. None of the *V. cholerae* isolates agglutinated with antiserum to O1 or O139. Of the 58 *V. cholerae* non-O1 isolates studied, 95% (55 of 58) could be serogrouped, with serogroups O10 (22%) and O12 (69%) being predominant. Serogroup O12 strains were all isolated on 8 February 1994, whereas strains belonging to serogroup O10 were isolated on 8 February (six strains), 14 February (five strains), and 16 February (one strain).

Ribotyping. The results of the ribotyping are shown in Fig. 2 and Table 1. Of the 38 isolates examined belonging to serogroup O12, all isolates showed identical ribotypes (type 1). Of the 12 isolates of serogroup 10, 10 isolates showed ribotype 7, 1 showed ribotype 2 (which differed from type 7 by a single band), and 1 isolate showed a unique pattern, ribotype 9. Thus, among the 58 *V. cholerae* non-O1 isolates studied, 10 different *Bgl*I ribotype patterns were present (Table 1). Five isolates, including one isolate of unknown serogroup, showed unique ribotypes. Ribotype pattern 8 was shared by one rough isolate, VIG 1994, and a serogroup O34 isolate. Ribotypes did not correlate with antibiograms.

Colony hybridization. None of the 58 non-O1 isolates (Table 1) hybridized with the NAG-ST probe.

GM_1-ELISA. None of the 58 non-O1 isolates produced cholera toxin as assessed by GM_1-ELISA. One *V. cholerae* 569B O1 was strongly positive for the toxin.

Enterotoxin and cytotoxin assays in tissue culture cells. None of the eight selected isolates produced an enterotoxin (rounding of Y1 cells was 0 to 2%; with filtrate from *V. cholerae* O1 569B, rounding was almost 100%). However, all except three isolates (VIG 1644, VIG 1587, and VIG 1603) produced a cytotoxin. Evidence of this consisted of rounding and granulation of cells and ultimately sloughing of cells from the plastic surface (>50% of the cells were affected). The cytotoxins could be detected in both Y1 and HeLa cells and were expressed when the organisms were grown in BHIB only. For four isolates (VIG 1597, VIG 1975, VIG 1980, and VIG 1581), the titers of the cytotoxins were <1:10; for the fifth isolate (VIG 1613), the titer was 1:10. The control filtrates from *V. cholerae* 569B and *S. dysenteriae* type 1 were strongly positive for enterotoxin and cytotoxin, respectively.

Cell-associated hemagglutinating activity. All eight isolates agglutinated human group O erythrocytes. This activity was inhibited by 1% D-mannose.

PCR assays for *zot* and *tcpA*. The eight isolates did not generate PCR products of 840 bp (corresponding to the internal segment of the *zot* gene of *V. cholerae* O1), 617 bp (corresponding to classic *tcp*A), or 471 bp (corresponding to El Tor *tcp*A). Therefore, the isolates were deemed negative for *zot* and *tcpA*.

HEp-2 cell adherence. All eight isolates tested showed strong adherence. Carpet-like aggregates of bacteria adhered to cells and glass surfaces (Fig. 3). *E. coli* E2348/69 showed localized adherence and *E. coli* K-12 showed no adherence, as was expected (data not shown).

HEp-2 cell invasion. A typical result of the HEp-2 cell invasion assay is shown in Table 2. Five isolates showed some degree of invasion. The positive control strain of *S. flexneri* 2a

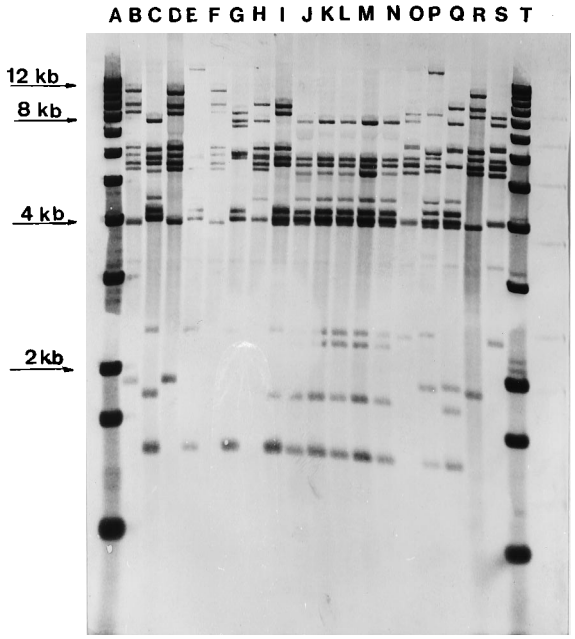

FIG. 2. *Bgl*I ribotypes of *V. cholerae* non-O1 isolates recovered from an outbreak of a cholera-like disease in Peru. Lanes: A, 1-kb molecular weight standard; B, VIG 1659, ribotype 1; C, VIG 1660, type 2; D, VIG 1661, type 1; E, VIG 1789, type 3; F, VIG 1617, type 1; G, VIG 1890, type 4; H, VIG 1956, type 5; I, VIG 1974, type 6; J, VIG 1975, type 7; K, VIG 1976, type 7; L, VIG 1980, type 7; M, VIG 1981, type 7; N, VIG 1982, type 7; O, VIG 1994, type 8; P, VIG 2144, type 9; Q, VIG 2156, type 10; R, VIG 1624, type 1; S, VIG 2309, type 8; T, 1-kb molecular weight standard.

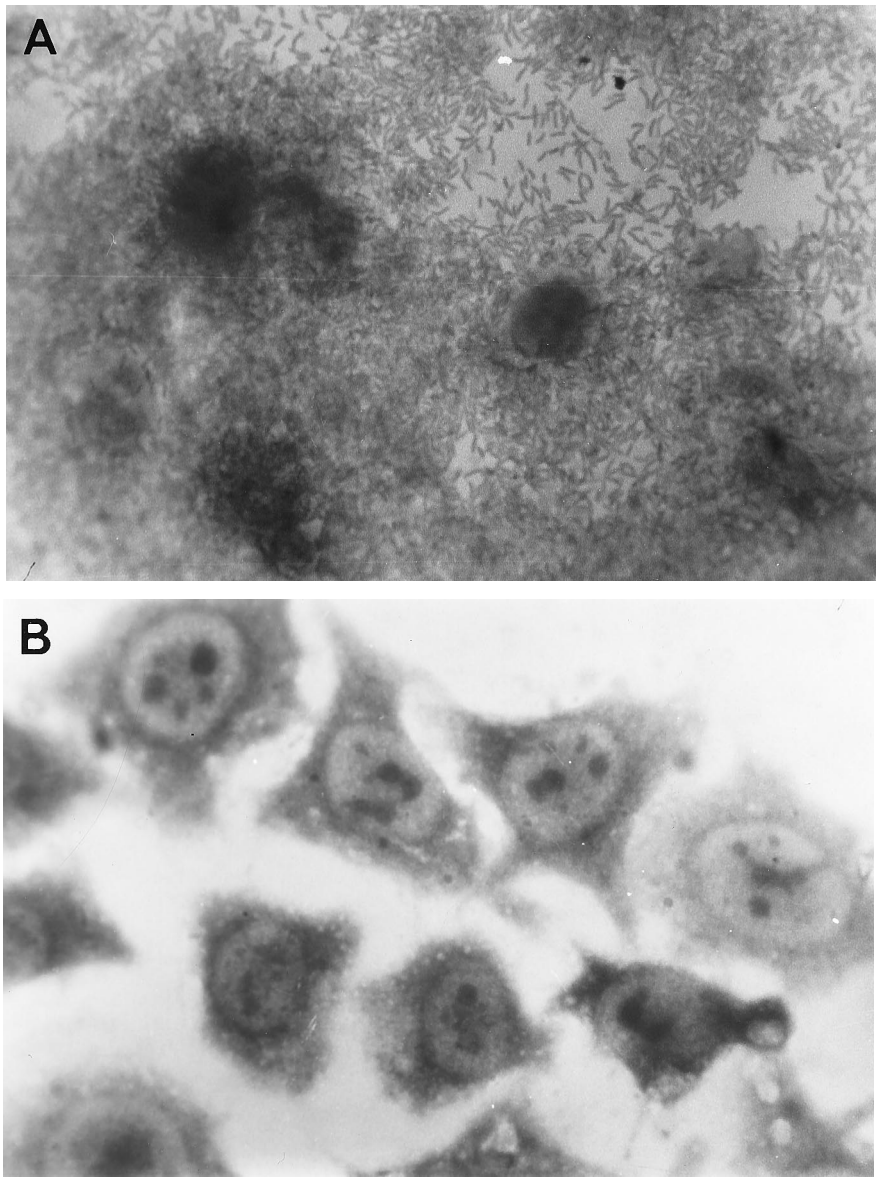

FIG. 3. (A) Carpet-like adherence of *V. cholerae* VIG 1587 (serogroup O12) to HEp-2 cells and a glass surface. (B) No adherent bacteria were seen on the cells or the glass surface when the cells were infected with nonpathogenic *E. coli* K-12 (magnification, ×400; Giemsa stain).

showed strong invasion and the negative control strain of *E. coli* K-12 showed no invasion, as was expected.

Rabbit ileal loop assay. Four isolates (VIG 1644, VIG 1597, VIG 1587, and VIG 1581) were tested in this assay. All four isolates produced a volume-to-length ratio of >1.0 when whole cultures were injected. However, when filtrates were injected the ratios were 0.8 (for both VIG 1581 and VIG 1587), 0.2 (for VIG 1597), and 0.4 (for VIG 1644). Whole culture of 569B and its filtrate produced ratios of >1.0.

RITARD assay. Only two *V. cholerae* strains (VIG 1644 and VIG 1587) and the control *E. coli* K-12 were used in RITARD studies (Table 3). Four rabbits were initially challenged with 10^{10} CFU of strain VIG 1644; three of them died within 24 h, and the fourth died within 48 h because of severe diarrhea and dehydration. The perianal region was stained with liquid stool, and the trays used to collect droppings were found to contain large quantities of liquid stool. Since the deaths of these rab-

TABLE 2. Invasion of HEp-2 cells by *V. cholerae* non-O1 strains and control bacteria

Bacterium and strain	Amt of bacterium (CFU/ml) in:		Intracellular bacteria/inoculum (%)
	Inoculum[a]	Intracellular region[b]	
V. cholerae			
VIG 1597	2.3×10^7	8.0×10^2	0.004
VIG 1644	2.6×10^7	6.0×10^3	0.023
VIG 1975	2.0×10^7	3.3×10^2	0.002
VIG 1980	2.3×10^7	0	0
VIG 1581	1.5×10^7	1.4×10^3	0.009
VIG 1587	2.0×10^7	7.0×10^2	0.004
VIG 1603	1.3×10^7	0	0
VIG 1613	3.0×10^7	0	0
S. flexneri 2a	1.5×10^7	3.0×10^5	2.0
E. coli K-12	2.0×10^7		0

[a] Bacteria were inoculated into HEp-2 cell monolayers.
[b] Bacteria were recovered after gentamicin treatment.

TABLE 3. Clinical presentations of RITARD rabbits inoculated with non-O1 *V. cholerae* or *E. coli* K-12

Rabbit no.[a]	Diarrhea[b] (day after inoculation)	Organisms[c] shed in stool (day)	No. of organisms (species)/g of mucosal scraping of:		Histological evidence in[d]:					
			Midjejunum	Lower ileum	Jejunum	Ileum	Cecum	Colon	Rectum	Appendix
371	+ (2)	VE (2)	1.2×10^5 (V) 3.5×10^4 (E)	2.5×10^7 (V) 3.5×10^5 (E)	A	—	A, I	A	A, I	A
372	+ (3)	VE (3)	1.5×10^6 (V) 8.4×10^5 (E)	1.1×10^8 (V) 6.0×10^6 (E)	—	—	A	A	A	—
373	+ (2)	VE (3)	5.5×10^4 (V)	1.2×10^5 (V)	—	A	I	A	—	—
374	+ (1)	VE (3)	2.5×10^3 (V) 1.0×10^2 (E)	3.0×10^4 (V) 1.2×10^3 (E)	—	—	—	—	—	—
375	+ (1)	VE (2)	ND[e]		—	I	A	—	—	—
376	+ (1)	VE (3)	6.8×10^4 (V)	1.8×10^5 (V)	—	—	—	—	I	—
377	No diarrhea for 4 days	NE[f]	NQ[g]	NQ	—	—	—	—	—	—
388	No diarrhea for 4 days	NE	NQ	NQ	—	—	—	—	—	—

[a] Rabbits 371 and 372 were challenged with strain VIG 1587 (serogroup 12), rabbits 373 to 376 were challenged with strain VIG 1644 (serogroup 10), and rabbits 377 and 388 were challenged with *E. coli* K-12.
[b] +, presence of diarrhea.
[c] V, *V. cholerae*; E, *E. coli*. Shedding of the indicated organisms occurred until the day of sacrifice (in parentheses).
[d] A, evidence of bacterial adhesion; I, evidence of bacterial invasion; —, no evidence of bacterial adhesion or invasion.
[e] ND, bacteriology not done.
[f] NE, not examined.
[g] NQ, not quantified.

bits occurred at night, bacteriological and histological studies were not performed. However, when the abdomens of the rabbits were opened the following morning, the colon contained liquid stool and the distal ileum appeared hemorrhagic in each rabbit.

Therefore, another five rabbits were challenged with lower doses (10^6 CFU) of the strain VIG 1644. Studies were, however, carried out with another four rabbits with 10^{10} CFU of the strain VIG 1587. Three of the four rabbits inoculated with VIG 1587 developed diarrhea within 24 h, and one rabbit did not develop diarrhea until 7 days of observation. All five rabbits inoculated with VIG 1644 (10^6 CFU) developed diarrhea within 48 to 72 h (Table 3; data presented are for six of the eight rabbits that developed diarrhea). None of the four rabbits challenged with *E. coli* K-12 developed diarrhea.

At autopsy, rabbits with diarrhea each had large amounts of frothy liquid stool in the portion of the cecum that remained patent and in the colon. Bacteriology of the mucosal scrapings suggested colonization of the mucosae with the challenge organisms (Table 3).

Histology of the small and large intestines showed marked changes. There was bacterial adhesion, and sometimes there was invasion with necrosis of tips of the villi. In the lamina propria, there were congestion, edema, hyperemia, and acute inflammation. The mesenteric lymph nodes showed reactive changes (an increased number of free macrophages in the sinuses as well as hyperplasia of lymphocytes). The pathological changes were more marked in VIG 1587-infected rabbits than in VIG 1644-infected rabbits. Some histological changes are shown in Fig. 4. Examinations of two of four rabbits inoculated with *E. coli* K-12 did not show any histological abnormalities of the intestinal mucosae.

DISCUSSION

We have studied 58 strains of non-O1 *V. cholerae* isolated on February 1994 because they were clearly associated with an outbreak of diarrhea. Serogrouping suggested that two serogroups (O10 and O12) were involved in the outbreak. Ours is the first report in which *V. cholerae* O10 and O12 are shown to have caused an outbreak of diarrhea. However, epidemiological investigations did not identify a source of infection. Investigations of previous outbreaks of diarrhea due to *V. cholerae* non-O1 strains have implicated food as an important source of infection (9). Ribotyping of the two main serogroup isolates showed that all the serogroup O12 isolates belonged to a single ribotype, ribotype 1, and that 10 of 12 serogroup O10 isolates belonged to another ribotype, ribotype 7. Thus, an excellent correlation between serogrouping and ribotyping results was obtained, emphasizing the usefulness of these typing methods in the investigation of outbreaks caused by non-O1 *V. cholerae* (11). However, serogrouping is not always practical, because it can only be performed by reference laboratories. On the other hand, ribotyping seems to be a useful substitute which is increasingly carried out in epidemiological investigations. Ribotyping is cost-effective, and a person trained in elementary molecular biology techniques can perform the technique. In addition, with the use of digoxigenin-labelled probes radioactivity is avoided.

The enteropathogenicity of non-O1 *V. cholerae* is multifactorial (33). The various virulence factors identified in non-O1 *V. cholerae* include CT-like enterotoxin (7), NAG-ST (4), Zot (19), El Tor-like hemolysin (46), thermostable direct hemolysin similar to that of *Vibrio parahaemolyticus* (48), shiga-like toxin (30), hemagglutinins (12), fimbrial antigens (28), and invasiveness (34). The isolates of serogroups O10 and O12 did not have sequences encoding NAG-ST and did not produce CT-like enterotoxin. Among four serogroup O10 isolates and four serogroup O12 isolates, none contained sequences encoding Zot. However, they were positive for El Tor-like hemolysin, cytotoxin, invasiveness, and mannose-sensitive hemagglutinin. A combination of these factors and perhaps an unknown factor(s) could have produced diarrhea. The bacteria also strongly adhered to HEp-2 cells. Along with the ability to produce hemagglutinin, this is an indicator of the ability to colonize the intestinal mucosa, which is a prerequisite for the causation of diarrhea (25). The organisms did not contain *tcpA* sequences, and to date, *tcpA* sequences have only been shown for *V. cholerae* O1 and O139 strains (18, 43). However, *V. cholerae* non-O1 strains possess other fimbrial antigens (28), and studies are in progress to identify such antigens.

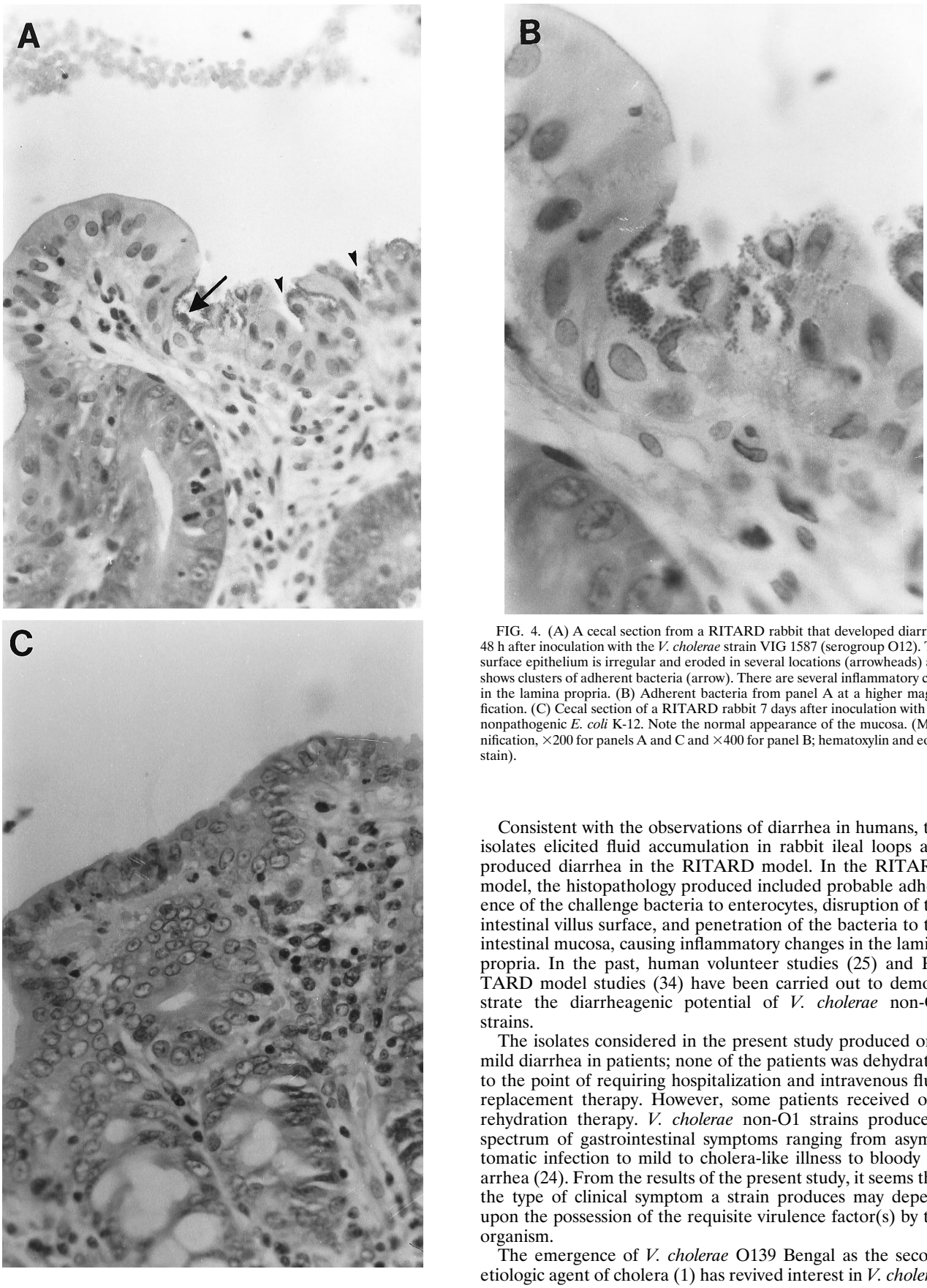

FIG. 4. (A) A cecal section from a RITARD rabbit that developed diarrhea 48 h after inoculation with the *V. cholerae* strain VIG 1587 (serogroup O12). The surface epithelium is irregular and eroded in several locations (arrowheads) and shows clusters of adherent bacteria (arrow). There are several inflammatory cells in the lamina propria. (B) Adherent bacteria from panel A at a higher magnification. (C) Cecal section of a RITARD rabbit 7 days after inoculation with the nonpathogenic *E. coli* K-12. Note the normal appearance of the mucosa. (Magnification, ×200 for panels A and C and ×400 for panel B; hematoxylin and eosin stain).

Consistent with the observations of diarrhea in humans, the isolates elicited fluid accumulation in rabbit ileal loops and produced diarrhea in the RITARD model. In the RITARD model, the histopathology produced included probable adherence of the challenge bacteria to enterocytes, disruption of the intestinal villus surface, and penetration of the bacteria to the intestinal mucosa, causing inflammatory changes in the lamina propria. In the past, human volunteer studies (25) and RITARD model studies (34) have been carried out to demonstrate the diarrheagenic potential of *V. cholerae* non-O1 strains.

The isolates considered in the present study produced only mild diarrhea in patients; none of the patients was dehydrated to the point of requiring hospitalization and intravenous fluid replacement therapy. However, some patients received oral rehydration therapy. *V. cholerae* non-O1 strains produce a spectrum of gastrointestinal symptoms ranging from asymptomatic infection to mild to cholera-like illness to bloody diarrhea (24). From the results of the present study, it seems that the type of clinical symptom a strain produces may depend upon the possession of the requisite virulence factor(s) by the organism.

The emergence of *V. cholerae* O139 Bengal as the second etiologic agent of cholera (1) has revived interest in *V. cholerae*

non-O1 strains. These organisms are ubiquitous in nature. However, only a minority of strains seems to be enteropathogenic. The pathogenic mechanisms by which these strains cause diarrhea remain to be elucidated.

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