

Epidemic of Diarrhea Caused by *Vibrio cholerae* Non-O1 That Produced Heat-Stable Toxin among Khmers in a Camp in Thailand

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An epidemic of a cholera-like disease occurred among Khmers in a camp in Aranyaprathet, Thailand, in May 1990. Of 215 patients with diarrhea, *Vibrio cholerae* O1 was isolated from 25 (12%) and *V. cholerae* non-O1 was isolated from 15 (7%). Five of 15 (33%) non-O1 *V. cholerae* isolates hybridized with two different oligonucleotide probes previously used to detect *V. cholerae* non-O1 that produces a heat-stable toxin. This is the first description of an epidemic of diarrhea caused by *V. cholerae* non-O1 that produces heat-stable toxin.

Vibrio cholerae serovar non-O1 is frequently isolated from sewage, estuarine waters, seafood (e.g., oysters and crabs), and animals (4, 7, 16, 25) in cholera-endemic and non-cholera-endemic countries (3); this organism has been associated with sporadic episodes of diarrhea worldwide (1, 5, 11, 14, 18, 21, 23, 26) but does not cause pandemics. The clinical spectrum of diarrhea associated with *V. cholerae* non-O1 includes mild watery diarrhea of 1 or 2 days duration, a severe dehydrating disease resembling cholera, and dysentery (26, 27). A number of different *V. cholerae* non-O1 putative virulence determinants have been identified, including the El Tor and Kanagawa hemolysins (10, 12), shiga-like toxin (20), cholera toxin (CT) (28), and cell-associated hemagglutinins (29). Differences in the presence of virulence factors between *V. cholerae* non-O1 of clinical origins and those of environmental origins have been sought, but no definite association of these virulence determinants with clinical isolates has been found (6, 7, 13, 15). Morris et al. (19) demonstrated in human volunteers that the virulence of *V. cholerae* non-O1 that produced a heat-stable toxin (ST) depended on its ability to colonize the intestine as well as to produce toxin. Two oligonucleotides, one derived from the amino acid sequence of *V. cholerae* non-O1 ST (9) and the other from the cloned and sequenced gene coding for *V. cholerae* non-O1 ST (21), have been described. These two oligonucleotides, as well as a polynucleotide probe to detect genes coding for CT (22), were used to identify genes coding for *V. cholerae* non-O1 ST and CT in *V. cholerae* strains isolated from patients with a cholera-like disease that occurred among Khmers in a camp on the Thai-Cambodian border.

MATERIALS AND METHODS

Patients and specimens. Between 1 May and 11 July 1990, an epidemic of severe watery diarrhea occurred among Khmers in a camp in Aranyaprathet, Thailand. Two hundred and fifteen patients with severe cholera-like diarrhea who were seen at one of three treatment facilities in the camp were referred to a cholera treatment center (this was to facilitate treatment and to collect specimens and epidemio-

logical information). Fecal specimens were collected from patients with a cholera-like disease and their family contacts. Patients were given either a World Health Organization formulation of oral rehydration solution or intravenous fluids. Patients ≥ 10 years of age received tetracycline, 500 mg orally four times a day for 3 days, or doxycycline, 300 mg as a single dose. Patients < 10 years of age and pregnant women received trimethoprim-sulfamethoxazole (8 mg of trimethoprim and 40 mg of sulfamethoxazole per kg per day) in two divided doses for 3 days.

Conventional bacteriology. Rectal or stool swabs were inoculated into Cary Blair transport medium, held at 4°C, and cultured within 48 h of collection on thiosulfate-citrate-bile salts medium (Difco, Detroit, Mich.) at 37°C before and after enrichment in alkaline peptone water. Vibrios were serotyped and biotyped by the method of Sakazaki (24). This includes a positive oxidase reaction; the ability to grow at a NaCl concentration of 0% but not at 7% in 10% tryptone broth; the ability to ferment glucose, saccharose, and mannose but not arabinose; no motility; a positive indole reaction; and production of lysine and ornithine decarboxylase but not arginine dehydroxylase or hydrogen sulfide (detectable in triple sugar iron agar or Kligler iron agar medium).

V. cholerae isolates were tested for hemolysis of sheep erythrocytes, polymyxin B susceptibility, and agglutination in *V. cholerae* O1 Ogawa and Inaba antisera (Difco). Antibiotic susceptibilities were determined by the Bauer-Kirby method (2). Specimens were not examined for diarrheogenic *Escherichia coli*, *Shigella* spp., *Campylobacter* spp., or rotavirus.

Hybridization procedures. *V. cholerae* O1 and non-O1 isolates were tested for hybridization with a ³²P-labeled gene probe coding for CT (22) as previously described (8). Two oligonucleotide probes, one described by Hoge et al. (9) and another described by Ogawa et al. (21), were used to detect genes coding for *V. cholerae* non-O1 ST. Colony blots with *V. cholerae* O1 and non-O1 isolates were performed on nitrocellulose filters. ST-producing *V. cholerae* non-O1 strains, NRT 36S and A5, and CT-producing *V. cholerae* 569B were used as positive controls on all filters. An *E. coli* K-12 strain was used as the negative control. Oligonucleotides were end-labeled with [γ -³²P]ATP by a forward kinase procedure (17). Hybridization buffer consisted of 3 \times SSC

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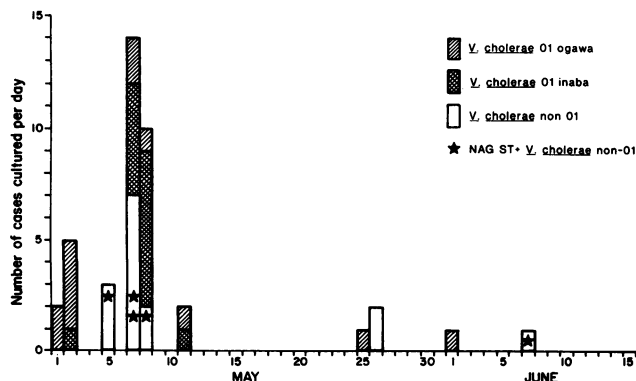


FIG. 1. Isolation of *V. cholerae* from Khmers with cholera-like diarrhea in a camp in Aranyaprathet, Thailand, in May and June 1990. NAG, nonagglutinating.

(1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% bovine serum albumin, 0.5% polyvinylpyrrolidone, and 1% sodium dodecyl sulfate. Hybridization was performed for 16 h at 50°C, followed by a 30-min wash in 3× SSC at room temperature and a final wash in 2× SSC for 1 h at room temperature. Hybridized filters were exposed to X-Omat X-ray film (Kodak, Rochester, N.Y.) for 16 h at -70°C. X-ray film was developed according to the manufacturer's instructions.

RESULTS

The epidemic started at the end of April 1990. *V. cholerae* O1 was isolated from seven patients (six of serotype Ogawa and one serotype Inaba) on 1 and 2 May. Surveillance was maintained from 1 May to 11 June (42 days), during which time *V. cholerae* O1 was isolated from 25 of 215 (12%) patients with diarrhea and 2 of 42 (5%) family contacts. All of the *V. cholerae* O1 isolates were Heiberg group I, biotype El Tor, and were intermediately resistant to streptomycin. Six of the seven *V. cholerae* O1 isolated on 1 and 2 May were of serotype Ogawa. After 2 May, 14 of 18 (78%) *V. cholerae* O1 isolated from patients with diarrhea were determined to be of serotype Inaba (Fig. 1). A total of 27 *V. cholerae* O1 strains (25 patients with cholera and 2 family contacts) were isolated from Khmers during the surveillance period. All O1 isolates hybridized with the CT probe; none hybridized with the two *V. cholerae* non-O1 ST probes.

V. cholerae non-O1 was isolated from 15 of 215 (7%) patients with cholera-like diarrhea. All of these isolates were of Heiberg group II and did not hybridize with the CT probe. Five of 15 (33%) *V. cholerae* non-O1 isolates hybridized with both *V. cholerae* non-O1 ST oligonucleotide probes. None of the 10 other isolates hybridized with either *V. cholerae* non-O1 ST probe. *V. cholerae* non-O1 was isolated from 12 patients with cholera-like diarrhea on May 5, 7, and 8. In contrast to *V. cholerae* O1, 14 of 15 non-O1 isolates from unrelated patients were resistant to three or more antibiotics.

Epidemiologically, it was not possible to identify a source of either O1 or non-O1 *V. cholerae* in this epidemic, and so no environmental cultures were collected. The cases came from different sections of the camp.

DISCUSSION

Seven percent of 103 *V. cholerae* non-O1 isolates isolated in Thailand were reported to produce ST, as determined with an oligonucleotide probe designed from the amino acid sequence of *V. cholerae* non-O1 ST (9). In this small epidemic, 33% of *V. cholerae* non-O1 isolates hybridized with the ST probes. This epidemic was probably caused by a single clone because all isolates were of Heiberg group II. None of these isolates hybridized with the CT probe. The disease associated with these isolates was clinically indistinguishable from the disease caused by *V. cholerae* O1. Because of the setting, it was not possible to compare the clinical outcome of patients infected with *V. cholerae* O1 with that of patients infected with *V. cholerae* non-O1.

Despite the clinical similarity and temporal clustering of cases, *V. cholerae* non-O1 isolates were distinctly different from O1 strains. *V. cholerae* non-O1 strains did not contain genes coding for CT and were of Heiberg group II; 93% were resistant to multiple antibiotics, and 33% contained genes coding for ST.

Both case series (18) and a volunteer challenge study (19) have suggested that *V. cholerae* non-O1 is an enteric pathogen. If ST is the essential virulence determinant for *V. cholerae* non-O1, then only a proportion (33% in this epidemic) caused diarrhea. Other virulence determinants may have been present in the other 10 isolates. This epidemic provided additional evidence that *V. cholerae* non-O1 isolates that produce ST cause cholera-like diarrhea.

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REFERENCES

- Aldova, E., K. Laznickova, E. S. Kova, and J. Lietava. 1968. Isolation of non-agglutinable vibrios from an enteritis outbreak in Czechoslovakia. *J. Infect. Dis.* **118**:25-31.
- Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck. 1966. Antibiotic susceptibility testing by a standardized single disc method. *Am. J. Clin. Pathol.* **45**:493-496.
- Blake, P. A., R. E. Weaver, and D. G. Hollis. 1980. Diseases of humans (other than cholera) caused by vibrios. *Annu. Rev. Microbiol.* **34**:341-367.
- Colwell, R. R., J. Kaper, and S. W. Joseph. 1977. *Vibrio cholerae*, *Vibrio parahaemolyticus* and other vibrios: occurrence and distribution in Chesapeake Bay. *Science* **198**:394-396.
- Dakin, W. P. H., D. J. Howell, R. G. A. Sutton, M. F. O'Keefe, and P. Thomas. 1974. Gastroenteritis due to non-agglutinable (non-cholera) vibrios. *Med. J. Aust.* **2**:487-490.
- Datta-Roy, K., A. C. Banerjee, S. P. De, and A. C. Ghose. 1986. Comparative study of expression of hemagglutinins, hemolysins, and enterotoxin by clinical and environmental isolates of non-O1 *Vibrio cholerae* in relation to their enteropathogenicity. *Appl. Environ. Microbiol.* **52**:875-879.
- DePaola, A. 1981. *Vibrio cholerae* in marine foods and environmental waters, a literature review. *J. Food Sci.* **46**:66-70.
- Hanchalay, S., J. Seriwatana, P. Echeverria, J. Holmgren, C. Tirapat, S. L. Moseley, and D. N. Taylor. 1985. Non-O1 *Vibrio cholerae* in Thailand: homology with cloned cholera toxin genes. *J. Clin. Microbiol.* **21**:288-289.
- Hoge, C. W., O. Sethabutr, L. Bodhidatta, P. Echeverria, D. C. Robertson, and J. G. Morris, Jr. 1990. Use of a synthetic oligonucleotide probe to detect strains of non-serovar O1 *Vibrio cholerae* carrying the gene for heat-stable enterotoxin (NAG-ST). *J. Clin. Microbiol.* **28**:1473-1476.

10. Honda, T., M. Arita, T. Takeda, M. Yoh, and T. Miwatani. 1985. Non-O1 *Vibrio cholerae* produces two newly identified toxins related to *Vibrio parahaemolyticus* hemolysin and *Escherichia coli* heat-stable enterotoxin. *Lancet* ii:163-164.
11. Hughes, J. M., D. G. Hollis, E. J. Gangarosa, and R. E. Weaver. 1978. Non-cholera infections in the United States. *Ann. Intern. Med.* 88:602-606.
12. Ichinos, Y., K. Yamamoto, N. Nakasome, M. J. Tanabe, T. Takeda, T. Miwatani, and M. Iwanaga. 1987. Enterotoxicity of El Tor-like hemolysin of non-O1 *Vibrio cholerae*. *Infect. Immun.* 55:1090-1093.
13. Kabir, S., and S. Alf. 1983. Characterization of surface properties of *Vibrio cholerae*. *Infect. Immun.* 39:1048-1058.
14. Kamal, A. M. 1971. Outbreak of gastroenteritis by non-agglutinable (NAG) vibrios in the Republic of Sudan. *J. Egypt. Public Health Assoc.* 44:125-129.
15. Kaper, J., H. Lockman, R. R. Colwell, and S. W. Joseph. 1979. Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. *Appl. Environ. Microbiol.* 36:91-103.
16. Kaysner, C. A., C. Abeyta, M. M. Wekell, A. DePaola, R. F. Stott, and J. M. Leitch. 1987. Incidence of *Vibrio cholerae* from estuaries of the United States West Coast. *Appl. Environ. Microbiol.* 53:1344-1348.
17. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual, p. 122-123. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. Morris, J. G., R. Wilson, B. R. Davis, I. K. Wachsmuth, C. F. Riddle, H. G. Wathen, R. A. Polard, and P. A. Blake. 1981. Non-O group 1 *V. cholerae* gastroenteritis in the United States: clinical, epidemiological, and laboratory characteristics of sporadic cases. *Ann. Intern. Med.* 94:656-658.
19. Morris, J. G., Jr., T. Takeda, B. D. Tall, G. A. Losonsky, S. K. Battacharya, B. D. Forrest, B. A. Kay, and M. Nishibuchi. 1990. Experimental non-O group 1 *Vibrio cholerae* gastroenteritis in humans. *J. Clin. Invest.* 85:697-705.
20. O'Brien, A. D., M. E. Chen, R. K. Holmes, J. B. Kaper, and M. M. Levine. 1984. Environmental and human isolates of *Vibrio cholerae* and *Vibrio parahaemolyticus* produce a *Shigella dysenteriae* 1 (Shiga-like) cytotoxin. *Lancet* i:77-78.
21. Ogawa, A., J.-I. Kato, H. Watanabe, B. G. Nair, and T. Takeda. 1990. Cloning and nucleotide sequence of a heat-stable enterotoxin gene from *Vibrio cholerae* non-O1 isolated from a patient with traveler's diarrhea. *Infect. Immun.* 58:3325-3329.
22. Pearson, G. D. N., and J. J. Mekalanos. 1982. Molecular cloning of *Vibrio cholerae* enterotoxin genes in *Escherichia coli* K12. *Proc. Natl. Acad. Sci. USA* 79:2976-2980.
23. Piergentili, P., M. Gastellani-Pastoris, R. D. Fellini, G. Farisano, C. Bonello, E. Rigoni, and A. Zampieri. 1984. Transmission of non-O group 1 *Vibrio cholerae* by raw oyster consumption. *Int. J. Epidemiol.* 13:340-343.
24. Sakazaki, R. 1992. Bacteriology of vibrio and related organisms, p. 37-55. In D. Barua and W. B. Greenough (ed.), *Cholera*. Plenum Publishing Company, New York.
25. Sanyal, S. C., S. J. Singh, I. C. Tiwari, D. C. Sen, M. Marwah, S. M. Hazarika, R. H. Singh, T. Shimada, and R. Sakazaki. 1974. Role of household animals in maintenance of cholera infection in a community. *J. Infect. Dis.* 130:575-579.
26. Spira, W. M., and R. R. Daniel. 1979. Biotype cluster on the basis of virulence characteristics in non-O group 1 *Vibrio cholerae*, p. 440-457. In N. Pierce and K. Takeya (ed.), *Symposium on cholera*: Bethesda 1979. Proceedings of the 15th Joint Conference U.S.-Japan Cooperative Medical Science Program, Cholera Panel. NIH publication no. 80-2003. National Institutes of Health, Bethesda, Md.
27. Spira, W. M., R. R. Daniel, Q. S. Ahmed, A. Huq, A. Yusuf, and D. A. Sack. 1978. Clinical features and pathogenicity of O group 1 non-agglutinating *Vibrio cholerae* and other vibrios isolated from cases of diarrhea in Dacca, Bangladesh, p. 137-153. In K. Takeya and Y. Zinnaka (ed.), *Symposium on cholera*: Karatsu 1978. Proceedings of the 14th Joint Conference U.S.-Japan Cooperative Medical Science Program, Cholera Panel, Toho University, Tokyo, Japan.
28. Yamamoto, T., Y. Takeda, T. Miwatani, and J. P. Craig. 1983. Purification and some properties of non-O1 *Vibrio cholerae* enterotoxin that is identical to cholera enterotoxin. *Infect. Immun.* 39:1128-1135.
29. Yamamoto, T., and T. Yokota. 1988. *Vibrio cholerae* non-O1: production of cell-associated hemagglutinins and *in vitro* adherence to mucus coat and epithelial surfaces of the villi and lymphoid follicles of human small intestines treated with formalin. *J. Clin. Microbiol.* 26:2018-2024.