INFECTION AND IMMUNITY, May 1994, p. 2108–2110
0019-9567/94/$04.00+0

Vol. 62, No. 5

Vibrio cholerae O139 Synonym Bengal Is Closely Related to *Vibrio cholerae* El Tor but Has Important Differences

JUDITH A. JOHNSON,[1,2,3]* CARLOS A. SALLES,[4] PINAKI PANIGRAHI,[1,3,5] M. JOHN ALBERT,[6] ANITA C. WRIGHT,[3] ROBERT J. JOHNSON,[1,2] AND J. GLENN MORRIS, JR.[1,3]

Veterans Administration Medical Center of Baltimore[1] and Department of Pathology,[2] Department of Medicine and Center for Vaccine Development,[3] and Department of Pediatrics,[5] University of Maryland at Baltimore, Baltimore, Maryland 21201; Department of Biochemistry and Molecular Biology, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil[4]; and Department of Laboratory Research, Laboratory Sciences Division, International Centre for Diarrhoeal Diseases Research, Bangladesh, Dhaka, Bangladesh[6]

Received 29 November 1993/Returned for modification 19 January 1994/Accepted 1 February 1994

Although *Vibrio cholerae* O139 synonym Bengal strains, from the current epidemics in India and Bangladesh, are closely related to seventh-pandemic strains, as shown by multilocus enzyme electrophoresis, Bengal strains are encapsulated and portions of the O1 antigen biosynthetic complex genes found in O1 strains are altered or lacking. Encapsulated Bengal strains showed resistance to killing by normal human serum. The presence of the capsule suggests the potential for bloodstream invasion in susceptible hosts and has profound implications for vaccine development.

Vibrio cholerae strains of O group 1 (O1 strains) have traditionally been classified as the etiologic agent for cholera, a well-recognized cause of morbidity and mortality throughout the world: to date there have been seven recorded pandemics of this severe dehydrating diarrheal disease. *V. cholerae* of O groups other than 1 (non-O1, or nonagglutinating, *V. cholerae*) can also cause gastrointestinal disease (6) as well as extraintestinal infections such as wound infections and septicemia (9). Until recently it was believed that only O1 strains had epidemic potential, with isolation of non-O1 strains being restricted to sporadic cases. Late in 1992, large outbreaks of cholera-like disease occurred in southern and eastern India and southern Bangladesh (1), with subsequent spread into other parts of Asia. The causative agent was a toxigenic strain of the previously unidentified serovar O139 (*V. cholerae* O139 synonym Bengal); these strains did not agglutinate with either polyclonal or monoclonal antisera directed against the *V. cholerae* O1 antigen. In the Bangladeshi studies, the incidence of disease was as high in adults as in children, suggesting that prior immunity to *V. cholerae* O1 El Tor was not protective against O139 infection (1).

The appearance of epidemic non-O1 disease raises basic questions about the degree of relatedness between O139 isolates and epidemic O1 strains: do O139 strains represent a simple mutation altering the O antigen of the current pandemic El Tor strain, as has been proposed by Hall et al. (1)?; are they a distantly related non-O1 strain (with non-O1 characteristics, such as encapsulation [5]) that has acquired virulence factors?; or do they lie somewhere between these extremes? An understanding of these relationships is critical as we begin to design vaccines which may be effective against what appears to be the etiologic agent of the eighth cholera pandemic.

To assess the phylogenetic relationship between Bengal strains and other *V. cholerae*, we analyzed three Bengal strains from the Bangladeshi epidemic (strains AI1837, AI1841, and AI1852) by multilocus enzyme electrophoresis. These strains, and control strains from our collection of *V. cholerae*, were confirmed to be *V. cholerae* by API 20E, with the presence of cholera toxin genes confirmed by using CTAP, a 23-base alkaline phosphatase-labeled oligonucleotide probe developed in our laboratory (12). In the enzyme electrophoretic typing system previously described by Salles and colleagues (10), all three O139 strains were classified as being in zymovar 14. *V. cholerae* O1 El Tor isolated during the seventh pandemic in Asia and O1 El Tor strains from the recent South American epidemics are also classified as zymovar 14 in this system. Epidemic *V. cholerae* O1 strains having a classical biotype belong to zymovar 13. The El Tor strain endemic to the U.S. Gulf Coast forms a third zymovar, 71, which differs in one locus from 14. Environmental, nontoxigenic O1 strains are generally not closely related to epidemic strains. Other non-O1 strains occupy over 100 zymovars, with a species diversity which approaches that seen in *Escherichia coli*. Our zymovar data confirm previous reports suggesting that Bengal strains are very closely related to epidemic *V. cholerae* O1 El Tor.

O139 isolates do not react with polyclonal Inaba- or Ogawa-specific sera or monoclonal antibodies specific for A, B, and C antigens, suggesting that the O1-antigen may be missing or altered. The change is not simply loss of the O antigen, as O139 strains are typeable and virulent and do not produce rough colonies. However, small changes in the structure of the O-specific carbohydrate might be sufficient to change its antigenicity. To further examine this question, eight strains (AI1837, AI1838, AI1841, AI1852, AI1854, AI1855, AI4260, and AI4450) from the Bangladeshi epidemic, *E. coli* DH5α, and a collection of O1 and non-O1 *V. cholerae* strains were probed for *rfbR* and *rfbS*, the two penultimate genes in the O1 antigen synthesis operon. A region encoding O1 antigen biosynthesis has been cloned and sequenced for El Tor and classical biotype strains (11). Probes O1SAP (GGATTGGT CACTTGATACCGC) and O1RAP (GGTGAACGCTCTT GCTACAGC), specific for *rfbS* and *rfbR*, respectively, were derived from this sequence information, and alkaline phosphatase was attached to a 5′ amino nucleotide. Strains were grown overnight on L agar, and colony blots were prepared on Whatman 541 filters and hybridized with the probes. Both O1 antigen probes hybridized with 40 strains of O1 *V. cholerae*,

* Corresponding author. Mailing address: Molecular Diagnostics Laboratory, VAMC Baltimore, Room 4D-148, 10 N. Greene St., Baltimore, MD 21201. Phone: (410) 605-7000, ext. 5338. Fax: (410) 605-7911.

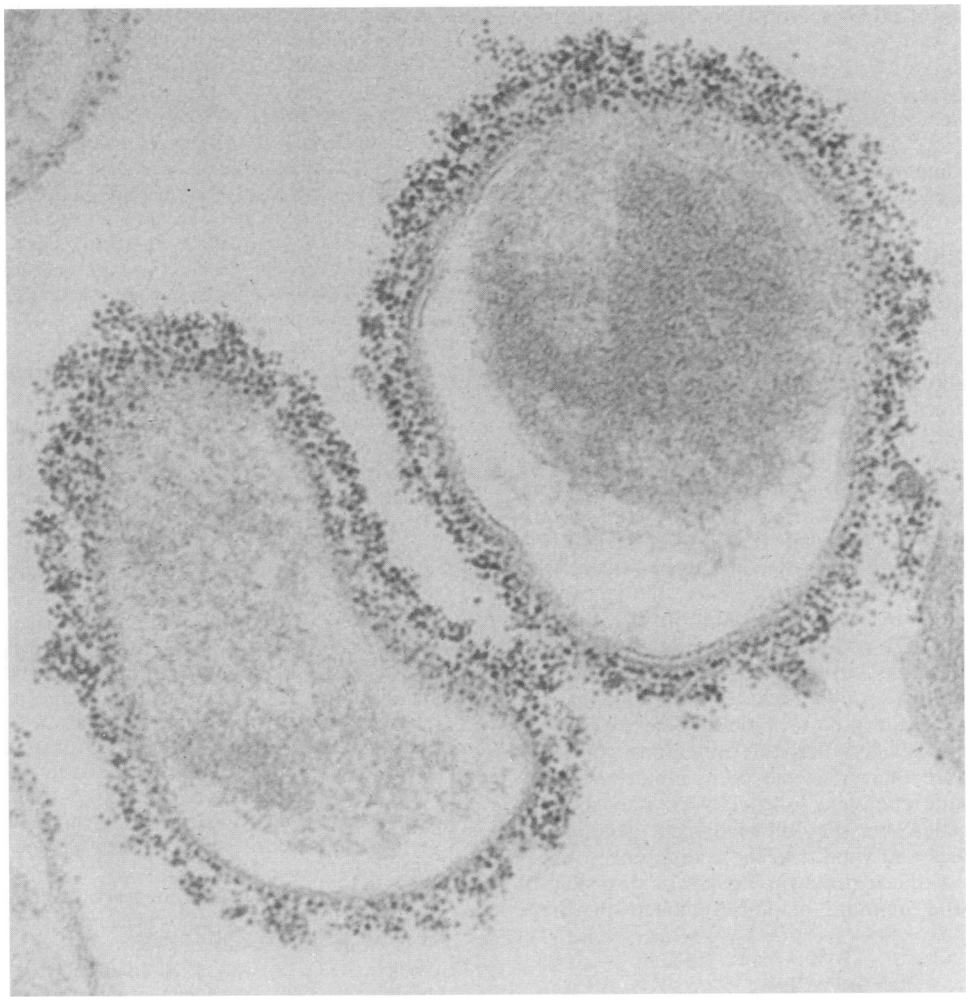

FIG. 1. Polycationic ferritin-stained thin sections of AI1855. Magnification ×38,000.

including El Tor and classical biotypes of both Inaba and Ogawa serotypes. In contrast, O1RAP and O1SAP failed to hybridize with the eight *V. cholerae* Bengal strains or 40 other non-O1 strains, including three cholera toxin-producing strains, suggesting that at least two genes in the biosynthetic pathway for the O1 antigen are missing or altered. Further studies are clearly needed to define the genetic relationship between the O1 and O139 antigen biosynthetic pathways, particularly in light of the apparent close genetic relationship between these two serovars.

As we have previously reported, a majority of non-O1 *V. cholerae* strains are able to produce a polysaccharide capsule (4, 5). Strains are able to shift between an encapsulated form with an opaque colonial morphology and an unencapsulated or minimally encapsulated form with a translucent colonial morphology; the degree of opacity correlates with the amount of capsular material which can be extracted from the cells (4). Encapsulation is associated with resistance to the bactericidal activity of normal human serum and with increased virulence in animal models (5). It has been hypothesized that the ability of non-O1 strains to cause invasive disease is related to capsule expression. *V. cholerae* O1 is not similarly encapsulated and, with very rare exceptions, is not invasive.

To determine whether Bengal strains shared the ability of other non-O1 strains to express a capsule, overnight cultures on L agar were evaluated visually for opaque versus translucent colony morphology and phase shifting. All eight O139 strains had a moderately opaque colony morphology on initial streaks; translucent sectors and colonies appeared after subculturing. Similar changes in colony morphology were not seen when more than 100 O1 strains from clinical and environmental sources were examined. Two Bengal strains (AI1855 and AI1841) were prepared by standard methods, stained with polycationic ferritin, thin sectioned and examined by electron microscopy (5). Both were surrounded by a relatively thin electron-dense capsule (the photomicrograph for strain AI1855 is shown in Fig. 1). Capsular material, extracted and purified as previously described (8), was identified as an animo sugar-containing polysaccharide by high-performance anion-exchange chromatography and magnetic nuclear resonance analysis (1a).

The 50% lethal dose (LD_{50}) after intradermal injection in mice was only marginally lower for Bengal strains than for *V. cholerae* O1 El Tor Ogawa N16961, a highly virulent clinical strain used extensively in prior volunteer studies (LD_{50}s, 1.5×10^8 [geometric mean of the LD_{50}s of three O139 strains] versus 5×10^8 [strain N16961]). However, in contrast to findings with both classical and El Tor O1 strains, which were not isolated from blood, *V. cholerae* could be isolated from the blood of

mice given high doses of O139 or control encapsulated non-O1 strains.

The improved ability of O139 isolates to survive in the blood may be due to increased resistance to complement-mediated killing. Like other encapsulated non-O1 strains (5), Bengal isolates showed a drop in viable counts of less than 1.5 $\log_{10}$ following a 30-min incubation in 65% normal human serum, compared with a circa 5-log drop for classical or El Tor O1 strains.

There has already been one case report of septicemia due to an O139 strain (3); in keeping with observations with other non-O1 isolates (9), sepsis occurred in a patient with underlying liver disease. The O139 strains which we have examined do not have the thick capsules usually associated with non-O1 *V. cholerae* blood isolates (4), suggesting that the risk of disseminated disease in infected persons is relatively low. Nonetheless, these observations indicate that disseminated disease can occur; if O139 strains follow the pattern of other non-O1 strains, the risk of dissemination will be greatest in persons with chronic underlying illnesses (9). This risk raises theoretical questions about the advisability of administering oral attenuated vaccines that still carry the capsule to persons who may have underlying illnesses. The presence of the capsule may also have an effect on antigen recognition: in volunteer studies with other non-O1 strains, the presence of a capsule appeared to mask certain critical surface antigens, with a resulting decrease in host immunologic response (7).

The presence of a capsule and the apparent absence of some O1 biosynthetic genes suggest that the appearance of O139 strains is due to more than a simple point mutation in the O-antigen biosynthetic complex. Further work is needed to determine whether these are the only significant genetic and phenotypic differences and what role these differences, especially the presence of the capsule on Bengal strains, play in pathogenesis and the immune response elicited by these isolates.

REFERENCES

1. **Albert, M. J., M. Ansaruzzaman, P. K. Bardhan, A. S. G. Faruque, S. M. Faruque, M. S. Islam, D. Mahalanabis, R. B. Sack, M. A. Salam, A. K. Siddique, M. D. Yunus, and K. Zaman.** 1993. Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. Lancet **342**:387–390. (Letter.)

1a. **Bush, C. A., and C. P. Reddy.** Unpublished data.

2. **Hall, R. H., F. M. Khambaty, M. Kothary, and S. P. Keasler.** 1993. Non-O1 *Vibrio cholerae*. Lancet **342**:430. (Letter.)

3. **Jesudason, M. V., A. M. Cherian, and T. J. John.** 1993. Blood stream invasion by *Vibrio cholerae* O139. Lancet **342**:431. (Letter.)

4. **Johnson, J. A., A. Joseph, P. Panigrahi, and J. G. Morris, Jr.** 1992. Frequency of encapsulated versus unencapsulated strains of non-O1 *Vibrio cholerae* from patients with septicemia or diarrhea, or from environmental sources, abstr. B-277. Abstr. 92nd Annu. Meet. Am. Soc. Microbiol. 1992.

5. **Johnson, J. A., P. Panigrahi, and J. G. Morris, Jr.** 1992. Non-O1 *Vibrio cholerae* NRT36S produces a polysaccharide capsule that determines colony morphology, serum resistance, and virulence in mice. Infect. Immun. **60**:864–869.

6. **Morris, J. G., Jr., T. Takeda, B. D. Tall, G. A. Losonsky, S. K. Bhattacharya, 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.

7. **Panigrahi, P., S. Srinivas, J. A. Johnson, and L. J. DeTolla.** 1992. Modulation of immunity in non-O1 *Vibrio cholerae*, abstr. B-316. Abstr. 92nd Annu. Meet. Am. Soc. Microbiol. 1992.

8. **Reddy, G. P., U. Hayat, C. Abeygunawardana, C. Fox, A. C. Wright, D. E. Maneval, C. A. Bush, and J. G. Morris, Jr.** 1992. Purification and structure determination of *Vibrio vulnificus* capsular polysaccharide. J. Bacteriol. **174**:2620–2630.

9. **Safrin, S., J. G. Morris, M. Adams, V. Pons, R. Jacobs, and J. E. Conte.** 1987. Non-O1 *Vibrio cholerae* bacteremia: a case report and review. Rev. Infect. Dis. **10**:1012–1017.

10. **Salles, C. A., and H. Momen.** 1991. Identification of *Vibrio cholerae* by enzyme electrophoresis. Trans. R. Soc. Trop. Med. Hyg. **85**:544–547.

11. **Stroeher, U. W., L. E. Karageorgos, R. Morona, and P. A. Manning.** 1992. Serotype conversion in *Vibrio cholerae* O1. Proc. Nat. Acad. Sci. USA **89**:2566–2570.

12. **Wright, A. C., Y. Guo, J. A. Johnson, J. P. Nataro, and J. G. Morris, Jr.** 1992. Development and testing of a nonradioactive DNA oligonucleotide probe that is specific for *Vibrio cholerae* cholera toxin. J. Clin. Microbiol. **30**:2302–2306.