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Vibrio cholerae Non-O1 Serogroup Associated with Cholera Gravis Genetically and Physiologically Resembles O1 El Tor Cholera Strains

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Until recently, only *Vibrio cholerae* strains of the O1 serogroup have been associated with epidemic cholera. In December 1992, an outbreak of cholera gravis in Vellore, India, was attributed to a new serogroup of *V. cholerae* recently designated O139. Serogroup O139 cholera has since spread to 13 countries and has reached pandemic proportions. Serogroup O139 cholera evades immunity to O1 cholera and is not detected by the standard O1 antigen test. Understanding the origins of O139 cholera and determining the relatedness of O139 to O1 cholera are necessary to devise strategies for detecting, reporting, and controlling this new pandemic. In order to determine the origins of this novel cholera serogroup, O139 was analyzed for virulence genes, for virulence proteins and their regulation, and for its genomic background. We found that O139 and O1 *V. cholerae* strains of the El Tor biotype possess highly homologous virulence genes encoding cholera toxin and toxin-coregulated pili and that the regulation of virulence protein expression likewise was indistinguishable between O139 and O1. Pulsed-field gel electrophoresis (PFGE) revealed the restriction digest pattern of O139 strains to be closely related to that of O1 serogroup El Tor biotype cholera strains from the Indian subcontinent. However, PFGE showed minor differences among individual O139 cholera isolates, suggesting that O139 *V. cholerae* is evolving.

The emergence of *Vibrio cholerae* non-O1 as an agent of cholera gravis in India and Bangladesh has caused much concern (1, 2, 16). Serologic testing against all known *V. cholerae* typing sera has resulted in the strain being placed in a new, non-O1 serogroup, O139 (2). The current outbreak is atypical of non-O1 disease because non-O1 *V. cholerae* cases are usually of minor severity, are not usually transmitted from person to person, and have not been associated with epidemic spread (15). The emergence of *V. cholerae* O139 as an agent of epidemic cholera gravis is particularly disturbing because individuals with infection- or vaccine-derived immunity to O1 *V. cholerae* may have little or no protective immunity to O139 strains. The Indian subcontinent has a major presence in international traffic and trade, so the emergence of O139 cholera raises the specter of rapid dissemination to other countries, resulting in an eighth cholera pandemic.

The virulence factors of cholera strains from earlier outbreaks have been well characterized at the genetic and physiological levels: to cause cholera, strains require the cholera enterotoxin (CT), the colonization factor toxin-coregulated pilus (TCP), and the global regulatory element ToxR (8, 11, 14, 21). The genes encoding the structural and regulatory proteins of these virulence factors are not all closely linked. Classical and El Tor biotype strains differ in their growth condition requirements for optimal CT and TCP expression (3, 7), and biotype-specific differences in the DNA sequences of their *tcpA* genes have been described (20).

Pulsed-field gel electrophoresis (PFGE), which reveals genomic restriction digest patterns, is valuable for studying the relatedness of strains (13, 19). This approach has been useful in epidemiological studies of *V. cholerae* O1, since the fingerprints of isolates diverged slowly as the seventh pandemic progressed (13). To investigate the origin of this novel pathogen, we compared O139 cholera strains with the well-characterized strains of the seventh cholera pandemic as well as with several other non-O1 strains of clinical and environmental origin. Comparative studies were done at the level of virulence genes and genomic organization and at the level of virulence protein regulation and expression.

Our findings indicate that O139 and El Tor biotype O1 strains possess and express virulence genes in an indistinguishable manner. Furthermore, there is a high degree of genomic conservation seen between O139 strains and El Tor biotype O1 strains. Serogroup O139 strains are distinctly different from other non-O1 strains. We conclude that O139 strains are likely to be derived from a resident O1 El Tor strain by a mutation(s) affecting its antigenic profile rather than derived from a nontoxigenic non-O1 strain that acquired virulence genes.

MATERIALS AND METHODS

Bacterial strains. *V. cholerae* strains studied by one or more of the methods listed below were as follows. (i) Non-serogroup O1 strains included 1837 (O139 from Bangladesh), 330 (O139 from India), T92-0032 (shrimp isolate from Bangladesh), 409-2c1i (shrimp isolate from Ecuador), 366 (from Saudi Arabia), 566-454 (ship isolate from Seattle), and ATCC 25873 (patient isolate from the Mediterranean area). (ii) Serogroup O1 El Tor biotype strains included P5 (Ogawa from Bangladesh), P17 (Ogawa from Bangladesh), P21 (Ogawa from Bangladesh), N16961 (Inaba from Vietnam), C6706 (Inaba from Peru), 406-1 (Ogawa from Thailand), 1410-1 (Inaba from Korea), and 91703 (Ogawa from Indonesia). (iii) Serogroup

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O1 classical biotype strains included 395 (Ogawa from Bangladesh) and 569B (Inaba from India).

Detection of virulence genes. A PCR-based assay (9) was used to determine the presence of *ctxA* (encoding the enzymatic subunit of CT) and *tcpA* (encoding the major structural subunit of TCP), as described elsewhere. Primer pairs were used to detect the following: a 301-bp sequence of *ctxA* (18), 470-bp fragment of the El Tor variant of the *tcpA* sequence, and a 671-bp sequence of the classical variant of *tcpA* (20). After electrophoresis, PCR amplicons of particular interest were recovered from gels and sequenced by the primer extension method of dideoxy sequencing (17).

Assays for CT expression. Culture supernatants of O139 strain 1837 grown in a variety of media were obtained by centrifugation, filter sterilized, and stored on ice. To determine if O139 strain 1837 excreted a protein immunologically related to CT, these supernatants were tested by reversed passive latex agglutination and Chinese hamster ovary (CHO) cell elongation assays (5). Neutralization activity of specific rabbit anti-CT antibody was determined in the CHO assay by titrating a sample of sterile culture filtrate of strain 1837 grown in AKI conditions alongside pure CT with and without anti-CT antibody. Western blot (immunoblot) analysis of culture filtrates of strain 1837 was conducted with rabbit anti-CT and alkaline phosphatase-conjugated anti-rabbit antiserum by standard techniques.

Analysis of virulence factors: TCP. To determine if the *tcpA* virulence gene identified by PCR was expressed, we cultured non-O1 strain 1837 in several media and assayed for expression of fimbriae by electron microscopy (7). Strain 1837 was cultured in AKI conditions, negative stained with 1% sodium phosphotungstate (pH 7.2), and examined with a Philips 400 transmission electron microscope operating at an accelerating voltage of 80 kV.

Analysis of genomic background. DNA-containing agarose plugs were prepared by described methods (13, 19). The embedded DNA was digested with a restriction endonuclease (*Apa*I or *Not*I), and the restriction fragments generated were resolved by PFGE with the Bio-Rad CHEF DR II apparatus.

RESULTS AND DISCUSSION

Detection of virulence genes. To cause the disease symptoms of cholera, a *V. cholerae* strain must express CT, the colonization factor TCP, and the ToxR regulator (14). We used a multiplex PCR method (9) to detect the essential structural genes *ctxA* and *tcpA* among a range of O1 and non-O1 *V. cholerae* strains of clinical and environmental origin (Fig. 1). While all of the clinical cholera strains yielded the same *ctxA* amplicon irrespective of serogroup or biotype, the *tcpA* amplicon obtained correlated with the biotypes of the strains determined previously by traditional methods. Of the non-serogroup O1 strains tested, only O139 strain 1837 yielded PCR amplicons. The banding pattern of O139-derived amplicons was identical to that of the *ctxA* and *tcpA* amplicons from O1 El Tor cholera strains and distinctive from that of the amplicons from classical biotype strains. Over the 471-bp target sequence of the El Tor *tcpA* fragment, El Tor and classical biotype strains differ at 100 bases. The *tcpA* amplicon from O139 strain 1837 was extracted from agarose gels and sequenced, revealing a 100% homology with the corresponding sequence from El Tor *tcpA*. The identity of O139 *tcpA* with El Tor *tcpA*, but not with classical biotype *tcpA*, was striking. This region of *tcpA* contains many epitopes on the essential colonization factor TCP (20), further supporting the observation that naturally acquired anti-TcpA antibody confers negligible protection in humans (6).

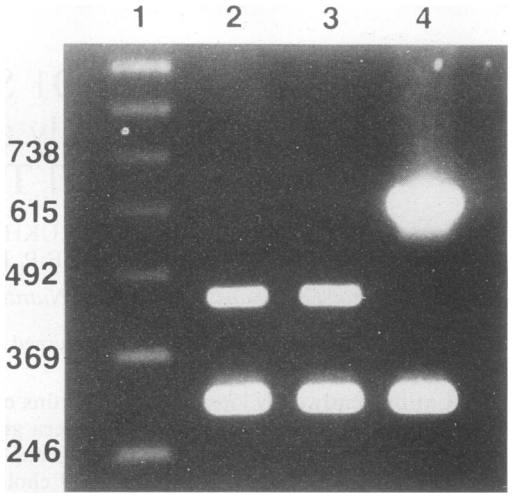

FIG. 1. Detection of virulence genes. A PCR-based assay (9) was used to detect *ctxA*, *tcpA* (classical variant), and *tcpA* (El Tor variant). Lane 1, molecular weight markers; lane 2, amplicons from O139 strain 1837; lane 3, amplicons from O1 El Tor strain E7946; lane 4, amplicons from O1 classical biotype strain 395.

Analysis of virulence factors. (i) Growth conditions. The CT and TCP of O1 serogroup El Tor biotype strains are coordinately up-regulated in AKI conditions and coordinately down-regulated in Casamino Acids-yeast extract media. Classical biotype strains give the opposite results, inducing coordinate up-regulation of CT and TCP in Casamino Acids-yeast extract but not in AKI conditions (3, 7, 14, 21, 22). We found that O139 serogroup strain 1837 expressed CT-like toxin activity and bundles of pili resembling the TCP that were up-regulated in AKI conditions and down-regulated in Casamino Acids-yeast extract, matching results with O1 El Tor strains. After growth in AKI conditions, CT activity was purified (Fig. 2), and bundles of pili resembling the TCP were detected by electron microscopy (Fig. 3). In terms of the environmental regulation

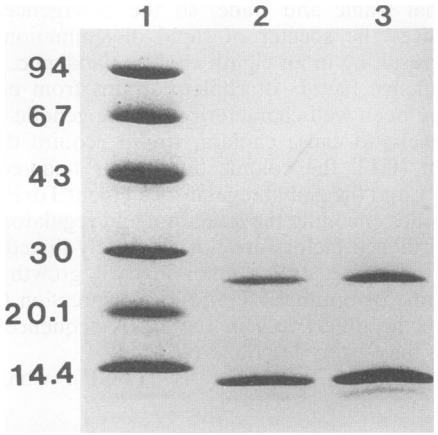

FIG. 2. SDS-PAGE of CT from O139 strain 1837. Lane 1, molecular weight markers; lane 2, CT subunits (Sigma Chemical Co.) from O1 *V. cholerae* ($CtxA_1$, CtxB, and $CtxA_2$, migrating, respectively, with apparent molecular weights of 21,000, 11,500, and 7,000); lane 3, CT subunits from O139 *V. cholerae* strain 1837.

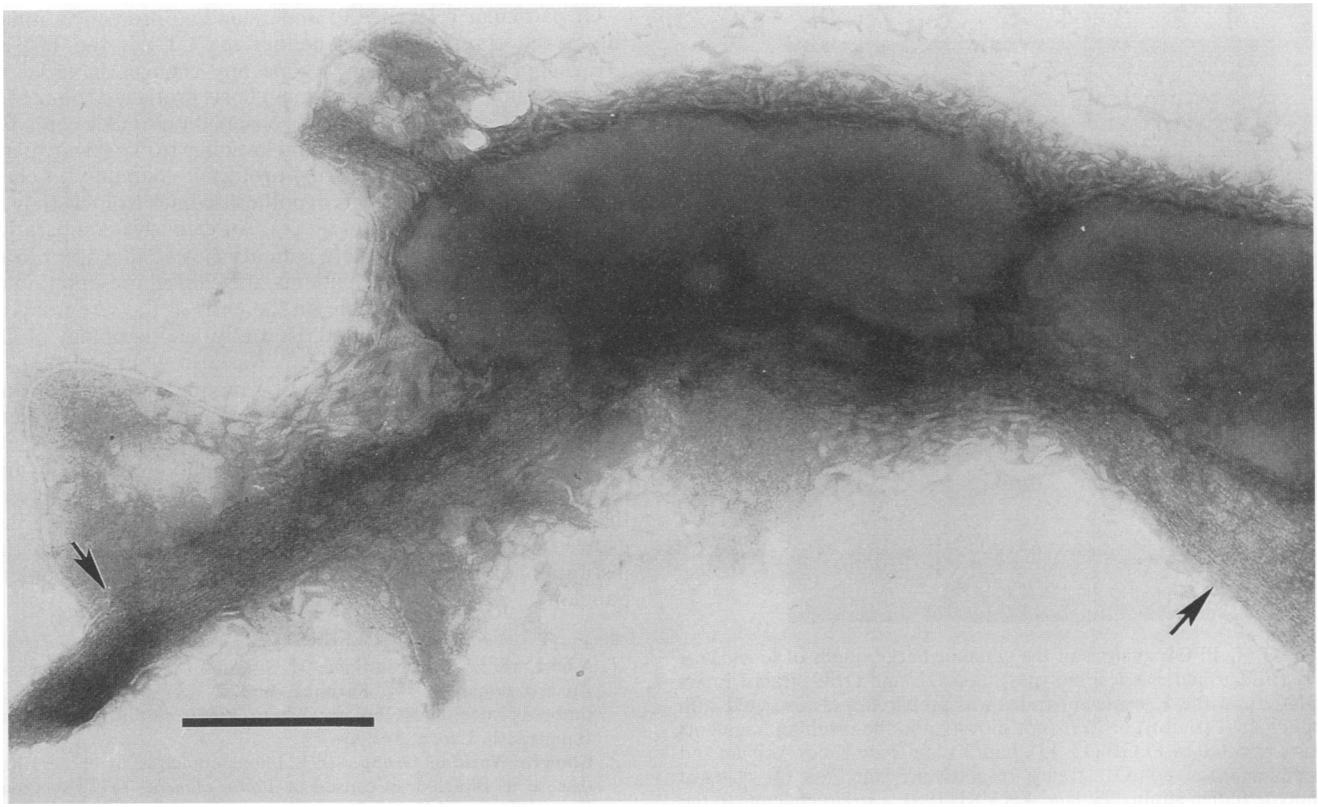

FIG. 3. Transmission electron photomicrograph of O139 *V. cholerae* expressing pili. Bundles of pili resembling the TCP of O1 *V. cholerae* are indicated by the arrows. Bar = 0.5 μm.

of CT and TCP expression, the physiological relatedness of this strain to O1 El Tor cholera isolates was thus remarkable.

(ii) Purification of toxin from O139 strain 1837. Supernatants of O139 strain 1837 grown in AKI conditions tested positive when assayed for CHO cell-elongating activity (5). The toxin activity was purified and compared with CT by a range of biochemical and immunological assays.

(a) Copurification. Proteins were precipitated from filter-sterilized culture supernatants with $(NH_4)_2SO_4$ at 60% saturation, resuspended, dialyzed, concentrated, and applied to an affinity chromatography column of cyanogen bromide-activated Sepharose 4B linked with immunoaffinity-purified rabbit anti-CT antibodies. Proteins were eluted with magnesium chloride, and fractions were assayed for A_{280} and were subjected to a CHO assay.

(b) Biochemical identity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed three bands which migrated with apparent molecular weights of 21,000, 7,000, and 11,500, which corresponded to $CtxA_1$, $CtxA_2$, and CtxB, respectively. After the proteins wre transblotted to polyvinylidene difluoride membranes, the subunits were sequenced by automated Edman degradation (12). The amino-terminal sequences of the first 30 residues of each of the three subunits had a strong homology with the previously reported sequences of the respective CT subunits. Analysis of the preparation by thin-layer isoelectric focusing indicated that the O139 toxin and CT had the same isoelectric point (ca. 6.6).

(c) Immunological cross-reactivity. The identity of O139 CT with O1 CT was further demonstrated by (i) neutralization of CHO cell-elongating activity by specific rabbit anti-CT antibody, (ii) reversed passive latex agglutination activity corresponding to 500 ng of CT per ml of AKI culture supernatant, (iii) immunoaffinity purification with Sepharose 4B linked with immunoaffinity-purified anti-CT, (iv) Western blot analysis with anti-CT, and (v) identity with the absence of an immunoprecipitated spur that was revealed by immunodiffusion of CT (Sigma Chemical Company, St. Louis, Mo.) and CT (O139 strain 1837) against rabbit anti-CT.

These experiments established the genetic, biochemical, and immunological homology of the O139 toxin to CT expressed by cholera strains of the seventh pandemic. Thus, O139 and El Tor biotype O1 strains have a striking resemblance in physiology: they possess, express, and regulate essential cholera virulence genes in an indistinguishable manner.

Analysis of genomic background. PFGE of genomic restriction digests is a powerful method for studying the relatedness of bacterial strains (13, 19). PFGE was used to compare three O139 strains with a wide range of O1 cholera and non-O1 vibrios isolated from a range of sources. The banding patterns of O1 strains were generally conserved, although there were consistent differences between classical biotype and El Tor biotype strains (Fig. 4). Non-O1 strains isolated from the environment or from patients with gastroenteritis have much greater diversity, with the notable exception of non-O1 cholera strains of the new O139 serogroup, which bore a close resemblance to O1 El Tor strains from India and Bangladesh (Fig. 4). The O139 strains all showed highly similar though nonidentical patterns, suggesting that O139 strains evolved from an O1 El Tor strain. Minor differences in banding patterns were seen among O139 strains (data not shown), suggesting that although O139 has a clonal origin, this serogroup is undergoing changes in a manner analogous to that seen among El Tor strains of the

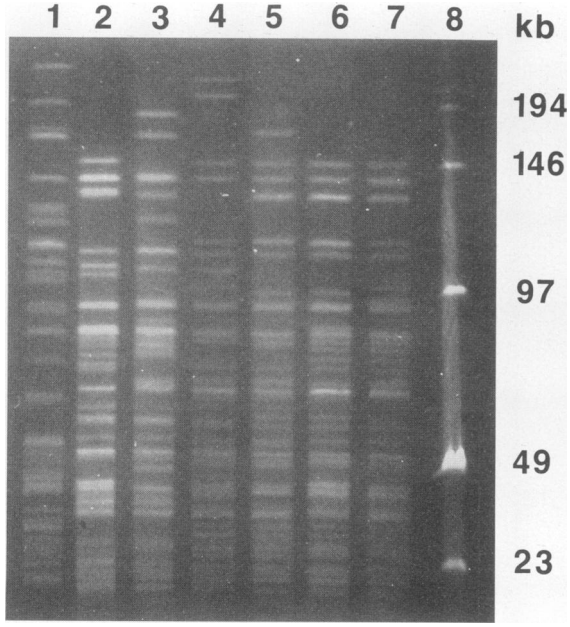

FIG. 4. PFGE analysis of the genomic backgrounds of *V. cholerae* O1 (El Tor and classical biotypes), non-O1, and O139 strains. Intact DNA from the *V. cholerae* strains was prepared and restricted with either *Apa*I (shown) or *Not*I (not shown), and the resulting fragments were resolved by PFGE (13, 19). Lanes 1 and 2 are Saudi Arabian and Mediterranean non-O1 strains, respectively; lane 3 is O1 classical biotype Indian strain 395; lane 4 is VRL 1984, a strain endemic to the U.S. Gulf Coast; lane 5 is O139 Bangladeshi strain 1837; lane 6 is an O1 El Tor strain also from Bangladesh; lane 7 is the O1 El Tor Peruvian strain C6706 involved in the Latin American epidemic; and lane 8 contains size markers.

seventh cholera pandemic (13). These data do not support the hypothesis that O139 cholera arose by the transfer of DNA from a toxigenic El Tor strain to a nontoxigenic non-O1 background.

A clear distinction between the *Apa*I banding patterns of O1 strains and non-O1 strains was seen. While the patterns of the former are highly conserved, the patterns of the non-O1 strains tend to be more diverse. Also, within the O1 serogroup, a pronounced difference between the pattern of the classical biotype strain 395 and those of O1 El Tor strains was observed. The banding pattern of the non-O1 cholera strain 1837 more closely resembled those of the O1 El Tor strains, with very good matches being obtained with O1 El Tor strains from Bangladesh. These findings were reiterated when *Apa*I was replaced with either *Not*I or *Xba*I. These data show the close relationship between the genomic arrangements of non-O1 strain 1837 and O1 antigen El Tor strains and suggest that O139 is derived from one of the latter, possibly by one or more mutations in the O1 synthesis pathway. An alternate mechanism that could explain the origin of strain 1837 is the transfer of DNA from a toxigenic El Tor strain to a nontoxigenic non-O1 background. This, while theoretically feasible, is less likely because it would require the transfer of a very large portion of the donor genome, with the recipient retaining genes responsible for the O139 phenotype. A relatively simple mutation(s) in O1 antigen biosynthesis thus probably accounts for the evasion by O139 of convalescent and infection-derived immunity, conferring on O139 a selective advantage in a human population with a high level of immunity to O1 serogroup cholera.

Of particular relevance to understanding protective immunity is the observation that neither the CT nor the TCP of O139 appears to have undergone any changes in order to evade the immune system of human hosts protected against O1 cholera. These results reiterate observations made with O1 cholera that antitoxin (10, 11) and antipilus (6) immunity plays a negligible role in engendering protective immunity to cholera. Further support for this hypothesis comes from a study in Pakistan by Fisher-Hoch et al. (4). An extensive comparative study of O1 and O139 cholera patients showed that apart from the age distribution of the patients, the clinical presentation of cholera was indistinguishable on the basis of the serogroup of the *V. cholerae* strain isolated. Recently, the homology of O1 regulatory genes to those of O139 was also described (22).

By comparing virulence genes, genomic background, and expressed virulence factors, we found that the non-O1 cholera isolate 1837 was profoundly different from the other non-O1 strains tested while it closely resembled, both genetically and physiologically, O1 serogroup El Tor biotype cholera strains of the seventh pandemic. These results show how cholera may evolve in the future and carry important implications for the institution of public health measures for the eighth cholera pandemic.

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