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# MINIREVIEW

# *Vibrio cholerae* O139 Bengal

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## INTRODUCTION

There have been seven pandemics of cholera in recorded history. Even though the etiological agents of the first four pandemics are not known since they occurred in the time before such agents could be recognized, the last three pandemics are known to be due to *Vibrio cholerae* serogroup O1. The seventh pandemic of cholera caused by the El Tor vibrio originated in Celebes, Indonesia, in 1961 and has spread far and wide over the last 30 years, reaching the South American continent in 1991 (9, 12). *V. cholerae* non-O1 serogroups were not known to cause epidemics of diarrhea; they were known, however, to cause sporadic cases and small outbreaks of diarrheas and extraintestinal infections (34). A departure from this pattern occurred in October 1992, when an epidemic of cholera-like disease due to a *V. cholerae* non-O1 serogroup broke out in the southern Indian port city of Madras. Over the next few months, it spread to other southern Indian cities and reached the northeastern Indian city of Calcutta (50). In December of that year, there was an outbreak of cholera-like illness in southern coastal Bangladesh, which over the subsequent several months spread to the entire country (1, 6, 8). The strain also caused epidemics of diarrhea in other parts of India at the same time (23). The disease affected thousands of individuals, mainly adults, and caused many deaths in the Indian subcontinent, indicating that the population was virgin to the organism (6).

**Nomenclature.** The epidemic strain was not related to the 138 known serogroups of *V. cholerae* (serogroup O1 and 137 non-O1 serogroups); therefore, a new serogroup, O139 was assigned to the strain with the synonym Bengal to indicate its first isolation from the coastal areas of the Bay of Bengal (57).

At last count, *V. cholerae* O139 infection had been reported from India, Bangladesh, Nepal, Burma, Thailand, Malaysia, Saudi Arabia, China, and Pakistan (1, 15, 22). There were also imported cases in the United Kingdom (14) and the United States (13). Therefore, it is believed that *V. cholerae* O139 might be the agent of eighth pandemic of cholera (38, 58). Since the strain causes a disease which is virtually indistinguishable from cholera due to *V. cholerae* O1, it should be considered the second etiological agent of cholera (10, 62). It also shares several properties with *V. cholerae* O1 biotype El Tor: morphology, culture, fimbrial antigens, cholera toxin (CT), genes for zonula occludens toxin (*zot*), and accessory cholera enterotoxin (*ace*), in vitro invasiveness for HEp-2 cells, possession and location of CTX element in the core region of the chromosome, possession of toxin coregulated pilus structural gene (*tcpA*) and iron-regulated genes (*IrgA*, *ViuA*, and *fur*) and their locations on similar sites in the chromosomes, outer membrane protein profile, sequence structures for *ctxAB* and 16S rRNA genes, regulation of virulence genes by *toxR*, and patterns in multilocus enzyme electrophoresis, ribotyping, *ctxA* genotyping, and pulsed-field gel electrophoresis. In this report, I will review the laboratory aspects of the organism.

**Morphological, cultural, and biochemical characteristics.** The morphological, cultural, and biochemical characteristics of *V. cholerae* O139 are similar to those of *V. cholerae* O1. It is a gram-negative, facultative, anaerobic, curved bacillus, measuring 2 to 3 by 0.5 μm and having a single polar flagellum (Fig. 1), and shows the typical darting motility of *V. cholerae*. It grows in media containing 0 to 3%, but not 8%, salt. It grows on a variety of nonselective media such as nutrient agar and sheep blood agar and on selective media for *V. cholerae* such as thiosulfate-citrate-bile salt-sucrose agar and taurocholate-tellurite-gelatin agar (TTGA). On thiosulfate-citrate-bile salt-sucrose agar, it produces typical yellow colonies, and on TTGA, it produces grayish colonies with dark centers surrounded most often by a zone of opacity (due to gelatinase production) (6). On many media including Luria agar, gelatin agar, and TTGA, like certain other *V. cholerae* non-O1 organisms, two colony variants can be seen, translucent and opaque. Some isolates produce either form, and others produce a mixture of both. Opaque variants on gelatin-containing media such as gelatin agar and TTGA produce, in addition, a zone of opacity around the colonies as a result of gelatinase activity, whereas this characteristic is absent or minimal with translucent variants. Upon incubation for longer periods (beyond 24 h), this distinction becomes less apparent, and translucent forms become opaque. Like some *V. cholerae* non-O1 serogroups, opaque colonies of *V. cholerae* O139 possess a capsule, whereas this capsular layer seems to be negligible or absent in translucent colonies (see below) (7, 37, 61).

Like all vibrios, *V. cholerae* O139 is positive for indophenol oxidase and ferments a variety of sugars without gas production. Especially important is that, like O1 vibrios, it ferments D-(+)-mannose and sucrose but not L-(+)-arabinose and thus belongs to Heiberg group 1 vibrios (26). Like El Tor biotype vibrios, it is positive for the Vogues-Proskauer reaction, shows variable hemolysis of sheep erythrocytes in conventional tube tests, agglutinates chicken erythrocytes, produces kappa type phage, and is resistant to polymyxin B. However, it is not attacked by Mukherjee's phages specific for El Tor or classical vibrios. Like most current strains of *V. cholerae* O1 in Bangladesh, *V. cholerae* O139 is resistant to the vibriostatic compound 0/129 (2,4-diamino-6,7-diisopropyl pteridine). All isolates tested to date are susceptible to tetracycline (unlike most of the currently prevalent strains of *V. cholerae* O1 in Bangladesh), ampicillin, chloramphenicol, erythromycin, ciprofloxacin, furazolidone, doxycycline, and nalidixic acid but resistant to trimethoprim-sulfamethoxazole and streptomycin. However, no data are available comparing the MICs of these antimicrobial agents for *V. cholerae* O139 and O1. Like *V.*

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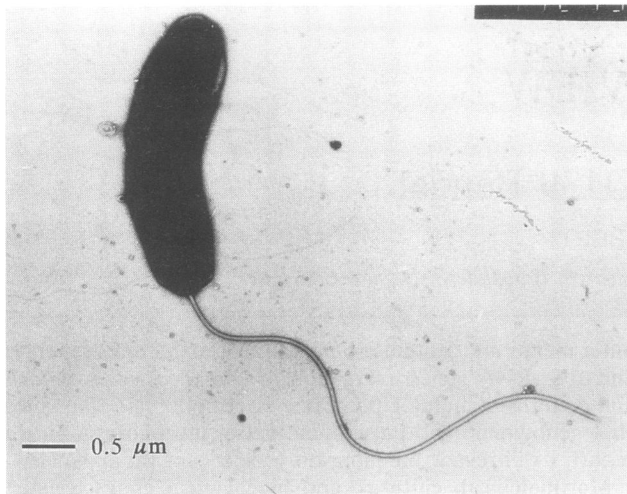


FIG. 1. Transmission electron microscopy of negatively stained *V. cholerae* O139 Bengal which shows a curved organism with a single polar flagellum. (Photo courtesy of M. Ehara, Institute of Tropical Medicine, University of Nagasaki, Nagasaki, Japan.)

*cholerae* O1, *V. cholerae* O139 thrives in an alkaline pH but is susceptible to a pH below 5.0 (6, 27, 41, 46).

**Capsular antigen.** Like a majority of *V. cholerae* non-O1 isolates, but unlike *V. cholerae* O1, *V. cholerae* O139 isolates possess a capsule (37, 61). Preliminary analysis of the capsular layer suggests that it is distinct from the lipopolysaccharide (LPS) antigen (see below) and has the following sugars: 3,6-dideoxyhexose (abequose or colitose), quinovosamine and glucosamine, and traces of tetradecanoic and hexadecanoic fatty acids (37, 61). It is thought that, as in other capsulated *V. cholerae* non-O1 serogroups, the presence of a capsule on *V. cholerae* O139 may confer increased virulence to the organism, such as resistance to serum killing and capacity to produce bacteremia. Like other encapsulated *V. cholerae* non-O1 serogroups, *V. cholerae* O139 produces bacteremia and death in mice upon intradermal inoculation (37). It is also interesting that *V. cholerae* O139 caused bacteremia in an adult patient with chronic underlying liver disease (35). There is difficulty in developing a vibriocidal assay for *V. cholerae* O139 because the organism cannot be easily killed in sera even though the specific antibody content to the organism, as measured by other immunoassays, is high (47). It is hypothesized that this problem may be attributable to the presence of the capsule. What role this capsule plays in antigen recognition is not known. 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 immune response (37). However, rabbits immunized with heat-killed, whole bacterial cells of *V. cholerae* O139 produced antibodies to both capsular and LPS antigens when tested by enzyme-linked immunosorbent assay (61).

**LPS antigen.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of an LPS preparation from *V. cholerae* O139, followed by silver staining of the gel, suggested that LPS does not show a distinct ladder pattern corresponding to lipid A, core oligosaccharide, and high-molecular-weight O antigen side chain of smooth *V. cholerae* O1. Instead, it showed a doublet pattern with an electrophoretic mobility slightly slower than that of the lipid A plus core oligosaccharide region of *V. cholerae* O1 strains, with a lesser amount of comigrating material (39). Thus, *V. cholerae* O139 appeared to have a modified core structure and no high-molecular-weight O-antigen-specific side chain but probably a low-molecular-weight short chain. This LPS pattern resembled a semi-rough-type LPS (28, 39, 48). One group of workers reported that this structure contains a 3,6-dideoxyhexose (identified as colitose) and claimed that this sugar is found for the first time in members of the family *Vibrionaceae* (28). However, a second group of workers claimed that the dideoxyhexose is part of the capsular polysaccharide. The LPS contains glucose, glucosamine, and heptose as component sugars and tetradecanoic acid, hexadecanoic acid, 3-hydroxy-dodecanoic acid, and 3-hydroxy-tetradecanoic acids as component fatty acids (61).

**Fimbrial antigens.** A number of different fimbrial appendages have been demonstrated in *V. cholerae* O1 organisms, but their role in the colonization of the organism remains to be defined. The fimbriae include toxin-coregulated pilus with a subunit molecular mass of 20.5 kDa (TcpA) (25), mannose-sensitive hemagglutinin (36), a 16-kDa subunit structure pilus (31), a hydrophobic pilus with a subunit molecular mass of 18 kDa (19), and a pilus with a subunit structure of 20 kDa (55). The presence of these fimbrial antigens has also been investigated in *V. cholerae* O139. Under optimal AKI-SW cultural conditions (the medium contains 1.5% peptone, 0.4% yeast extract, 0.5% NaCl, and 0.3% $NaHCO_3$ and is initially incubated for 4 h as a stationary culture and then incubated for 16 h as a shaker culture at 37°C) (32) as those for El Tor vibrios, *V. cholerae* O139 produced TcpA (59). Mutant derivatives with insertional inactivation of the structural gene *tcpA* resulted in decreased colonization ability of the mutant for suckling mouse intestine (60). *V. cholerae* O139 also produces the morphologically and immunologically related fimbrial antigens, other than TcpA, listed above (18, 43, 53, 55). In addition, it produces a curved (wavy) pilus with a subunit molecular mass of 2.8 kDa (minipilus) which is shared by many species of gram-negative bacteria (64). In keeping with the presence of various fimbrial (colonization) antigens, the strain adheres very strongly in vitro to HEp-2 cell monolayers (3). It has also been recovered in high numbers from the upper small intestinal fluids of infected patients (6) and has been demonstrated to be adherent to the upper small intestinal mucosal biopsies (2, 63).

**Toxins and other soluble virulence factors.** *V. cholerae* O139 produces CT which is identical to that produced by *V. cholerae* O1 biotype El Tor (41, 46). As with El Tor vibrios, optimal production of CT occurs under AKI-SW culture conditions (24, 59), and the amount of CT produced is equal to or more than that produced by *V. cholerae* O1 (27, 50). Patients infected with *V. cholerae* O139 also excrete large quantities of CT in their stools (6). When cultured in Casamino Acids-yeast extract broth, it produces a cytotonic enterotoxin (6), but in brain heat infusion broth, it produces an enterotoxin which is cytotoxic for Y1 adrenal tumor cells (2). It produces a hemagglutinin/protease which is identical to that produced by *V. cholerae* O1 and non-O1 organisms (42). Even though the production of soluble hemolysin is inconsistent by the conventional tube test, all of the isolates tested to date have been positive for hemolysin by the CAMP test (2). Although studies have not yet been undertaken to demonstrate the production of other enterotoxins of *V. cholerae* O1, namely, zonula occludens toxin (Zot) and the accessory cholera enterotoxin (Ace), the genes encoding these toxins have been demonstrated in the strain (see below). The strain does not produce the heat-stable toxin (NAG-ST) produced by some strains of *V. cholerae* non-O1 (50).

**Invasiveness.** Cholera due to *V. cholerae* O1 is the prototype secretory diarrheal disease, although there are occasional

reports of bloodstream invasion by *V. cholerae* O1 in some patients (33, 51). Non-O1, non-O139 vibrios, on the other hand, produce invasive disease in patients especially with chronic underlying conditions (54). As alluded to in a preceding section, there is one report of a bacteremic infection with *V. cholerae* O139 in a patient. This suggests that this bacterium, like other vibrios, has the potential to cause invasion. This ability was assessed in vitro by using the standard HEp-2 cell culture invasion assay. Several *V. cholerae* O1 isolates and opaque and translucent colony variants of *V. cholerae* O139 invaded the cells in low numbers (7). This property of invasion may be another aspect of *V. cholerae* O1 and O139 infection, as in the case of another agent of secretory diarrhea, enterotoxigenic *Escherichia coli* (20).

**Molecular characterization.** Molecular analysis of *V. cholerae* O1 strains has suggested that most of the genes that encode virulence factors are carried on a 4.5-kb virulence cassette or core region of the chromosome. The genes that have been identified in the core region are *cxtAB* (that encodes CT), *zot* (that encodes zonula occludens toxin), *ace* (that encodes accessory cholera enterotoxin), and *cep* (that encodes a pilin antigen which enhances colonization). This core region is flanked by RS1 elements (repetitive sequence 1, an insertion sequence 2.7 kb long normally found at the junction of tandem duplication of *ctxAB* and which is responsible for amplification of *ctxAB*). The core region and the RS1 element constitute the CTX element (45). Normally, *V. cholerae* O1 isolates carry multiple copies of the CTX element. Similarly, *V. cholerae* O139 isolates also carried two or more copies of the CTX element at the same chromosomal site as that of the CTX element in El Tor vibrios (37, 59). Moreover, they carried the *tcpA* gene and three iron-regulated genes, *irgA* (a virulence gene), *viuA* (the gene for the receptor for the siderophore vibriobactin), and *fur* (an iron regulatory gene) previously described for O1 vibrios in the same chromosomal location as in El Tor vibrios (11). Further relatedness with El Tor vibrios was suggested by the identity of sequences for *ctxAB* (41, 46) and 16S rRNA genes (46) in *V. cholerae* O139 and El Tor vibrios. Furthermore, with the construction of *toxR* (a positive regulator gene for *ctxAB*) null mutants, it was found that the expression of CT, TcpA, and outer membrane protein OmpU in O139 vibrios is dependent on ToxR as it is in O1 vibrios (59). Similarity between O1 and O139 vibrios was also found in the iron-regulated outer membrane protein profiles (11).

The *rfb* region encoding the O-antigen synthesis in *V. cholerae* O139 was examined with *V. cholerae* O1-specific *rfb* gene probes. It was found that all of the genes involved in O-antigen synthesis and Ogawa serotype modification in *V. cholerae* O1 are absent in *V. cholerae* O139 (39). These genetic data thus complement the structural data of the LPS antigen.

Molecular epidemiological studies have been carried out by using multilocus enzyme electrophoresis (37, 46), ribotyping (21, 46), *ctxA* genotyping (21, 29), and pulsed-field gel electrophoresis (46). In all of these typing methods, *V. cholerae* O139 isolates were either indistinguishable from, or similar to, the seventh pandemic strain (El Tor biotype) of cholera and were distinctly different from other non-O1 vibrios. However, by some of these molecular typing techniques, outbreak strains appeared to be heterogeneous (29, 46). This may suggest that *V. cholerae* O139 may be more prone to mutation and that it is important to monitor the strains over extended periods of time. With a molecular subtyping scheme, it may be possible to associate specific clones with certain geographical areas as in the case of *V. cholerae* O1 (44).

**Origin of *V. cholerae* O139.** From the foregoing data, it is obvious that there are striking cultural, physiological, and genetic similarities between *V. cholerae* O139 and *V. cholerae* O1, especially the El Tor biotype (summarized above). The most obvious difference between the two seems to be the possession of a capsule by *V. cholerae* O139, which makes it resemble a non-O1 vibrio. Therefore, I believe that there are two possibilities regarding the origin of *V. cholerae* O139: either an El Tor strain mutated and became a new serogroup, or a hitherto-unknown non-O1 *V. cholerae* acquired the necessary virulence genes from an El Tor strain. For the second possibility, it is important to demonstrate the existence of an ancestral strain of *V. cholerae* O139. In this regard, it is interesting that a CT-negative but NAG-ST-positive *V. cholerae* O139 strain was isolated from a patient with diarrhea in Argentina (52). However, for such a precursor strain to become a full-fledged epidemic strain would require the acquisition of a large amount of genetic material, which, although difficult, cannot be ruled out. Even though there are more proponents for the first possibility (i.e., mutant of an El Tor strain), it is equally important to study the second possibility to unequivocally establish the origin of *V. cholerae* O139.

**Laboratory diagnosis.** Conventional methods of laboratory diagnosis of *V. cholerae* O1 infection are applicable to *V. cholerae* O139. When CT-positive and vibriostatic compound (0/129)-resistant *V. cholerae* organisms that do not agglutinate with *V. cholerae* O1 antisera are encountered and on the basis of the epidemiologic setting, the presence of *V. cholerae* O139 should be suspected (40). However, since *V. cholerae* O139 agglutinates with its specific antiserum, diagnosis can be confirmed by a slide agglutination test. Specific rabbit polyclonal antiserum can be prepared by absorption of the antiserum with a rough strain of *V. cholerae* to remove cross-reacting agglutinins (41, 56, 57). Since *V. cholerae* O139 also shows cross-reaction with *V. cholerae* serogroup O22, further absorption should be carried out with this serogroup (41). (Since antiserum to *V. cholerae* O139 is not yet commercially available, small amounts of antiserum can be obtained from us free of charge, by writing to me at the indicated address).

We have produced specific monoclonal antibodies to *V. cholerae* O139 which can be used for slide agglutination of colonies (48) and for preparation of reasonably sensitive and highly specific coagglutination reagents for direct detection of the organism from stool (49). Other rapid diagnostic tests using these monoclonal antibodies are under evaluation.

**Intervention measures.** Nonvaccine prevention strategies include the improvement of personal and environmental hygiene as in the case of all diarrheal diseases. As with *V. cholerae* O1, surface water seems to be the main vehicle of transmission for *V. cholerae* O139. We have isolated *V. cholerae* O139 from a higher percentage of water samples than *V. cholerae* O1 during the recent epidemic, suggesting that *V. cholerae* O139 may survive better in the environment (30).

*V. cholerae* O1 and O139 share several antigens that include fimbriae and CT. In spite of this, the occurrence of severe diarrhea due to *V. cholerae* O139 in adult populations of cholera-endemic areas of India and Bangladesh suggest that prior infection with *V. cholerae* O1 will not cross-protect against *V. cholerae* O139 infection. It is hypothesized that this may be due either to the poor immune response some common antigens might evoke (25) or the heavy antigenic load (as evidenced by the multiple copies of virulence genes) that might simply overwhelm the host immune response (17, 60). Laboratory studies with adult rabbits also suggested that oral immunization with either live *V. cholerae* O1 or O139 will not cross-protect against diarrhea upon challenge with heterologous organisms. In these studies, the most significant protective antigens seemed to be the LPS antigens (4, 5). It is obvious

that a future cholera vaccine should be bivalent and should incorporate protective antigens from both serogroups.

## CONCLUSIONS

The occurrence of epidemics due to *V. cholerae* O139 marked a turning point in the history of cholera. To date, the possession of O1 antigen alone for vibrios served as a convenient serological marker for recognition of cholera. This situation is now altered by the emergence of *V. cholerae* O139 as an additional etiological agent of cholera. The factor(s) that has contributed to the genesis of *V. cholerae* O139 remains a mystery. The aquatic environment that provides an ecological niche for vibrios (16) may hold the key to unlock the mystery. It is possible that new epidemic strains may emerge in the future. In the meantime, the pandemic potential of *V. cholerae* O139 should not be ignored, and we must be vigilant with good, laboratory-based surveillance to track the movement of the organism. The long-term solutions for containment of outbreaks depend upon provision of safe drinking water to those at risk and improvement of sanitation. In the short term, we have to pin our hopes on the development of suitable vaccines. Certainly, the wealth of experience we have accumulated over the past 100 years studying serogroup O1 vibrios has stood us in good stead; it has helped us to quickly characterize *V. cholerae* O139 by a "consortium of international cholera scientists" [sic] (1). This, in turn, has made it possible to prepare and distribute diagnostic reagents for global surveillance and make prototype vaccines in a relatively short period of time (53, 60).


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## ADDENDUM IN PROOF

Walder and Mekalanos (M. K. Walder and J. J. Mekalanos, Lancet **343:**1366, 1994) reported that *V. cholerae* O139 possesses a unique sequence which may be useful as a diagnostic tool. Lebens and Holmgren (M. Lebens and J. Holmgren, FEMS Microbiol. Lett. **117:**197–202, 1994) reported that the virulence cassette DNA region of *V. cholerae* O139 is identical to that in the Classical rather than in the El Tor biotype of *V. cholerae* O1.

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