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Characterization of Phenotypic, Serological, and Toxigenic Traits of *Vibrio cholerae* O139 Bengal

G. BALAKRISH NAIR,[1] TOSHIO SHIMADA,[2] HISAO KURAZONO,[3] JUN OKUDA,[3] AMIT PAL,[3] TADAHIRO KARASAWA,[3] TATSUYA MIHARA,[3] YOSHIHIKO UESAKA,[3] HIROMASA SHIRAI,[3] SURABHI GARG,[1] PRADIP KUMAR SAHA,[1] A. K. MUKHOPADHYAY,[1] TETSUO OHASHI,[4] JUN TADA,[4] TOMOKO NAKAYAMA,[4] SHIGEHARU FUKUSHIMA,[4] TAE TAKEDA,[5] AND YOSHIFUMI TAKEDA[3,6]*

National Institute of Cholera and Enteric Diseases, Beliaghata, Calcutta, India,[1] and National Institute of Health[2] and Research Institute, International Medical Center of Japan,[6] Shinjuku-ku, Tokyo 162, Department of Microbiology, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-01,[3] Central Research Laboratory, Shimadzu Corporation, Nakagyo-ku, Kyoto 604,[4] and National Children's Medical Research Center, Setagaya-ku, Tokyo 154,[5] Japan

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Biochemical and physiological traits of a collection of strains of *Vibrio cholerae* O139 Bengal isolated from India, Bangladesh, and Thailand showed that these strains formed a phenotypically homogeneous group with identical characteristics that were essentially similar to those of the O1 serogroup. Resistance to 150 μg of the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine) and Mukherjee's El Tor phage 5 and classical phage IV and the nonagglutinability of the strains with O1 antiserum were the only discernible differences between the O139 and O1 serogroups. Extensive serological characterization further revealed the O139 serogroup to be distinct from the existing 138 serogroups of *V. cholerae*. Antiserum raised against the O139 serogroup required absorption with the R reference strain CA385 and with the reference strain representing serogroup O22 to remove cross-reacting agglutinins. All of the 223 representative strains of *V. cholerae* O139 examined hybridized with DNA probes specific for the cholera toxin (CT) gene, zonula occludens toxin gene, and El Tor hemolysin gene but not with the probe specific for the heat-stable enterotoxin gene. The amount of CT present in stool samples of patients infected with the O139 serogroup was higher than that found in stools of patients infected with O1 El Tor, and this echoed findings that the amount of CT produced by O139 strains in vitro was higher than that produced by the O1 El Tor strains. The nucleotide sequences of the genes encoding the A and B subunits of CT of the O139 serogroup were identical to the sequences reported for the CT gene of O1 El Tor. The CT gene of O139 strains could be amplified by using primers developed for detection of the CT gene of the O1 serogroup by a PCR assay, which could also be used to detect the CT gene in stool samples of patients infected with strains of the O139 serogroup.

Until recently, only strains belonging to the O1 serogroup of *Vibrio cholerae* caused epidemic and pandemic cholera (4) while those belonging to the other serogroups (collectively known as *V. cholerae* non-O1) were not associated with large epidemics but were the causative agents of sporadic diarrhea and occasionally caused extraintestinal infections (24). On the basis of variations in the antigenic form and variations in certain traits, the O1 serogroup is further differentiated into two serotypes (Inaba and Ogawa) and two biotypes (El Tor and classical), respectively (5). Spatial and temporal variations in the prevalence of serotypes of *V. cholerae* O1 are usual features and tend to follow a rhythmic pattern, with either of the serotypes dominating at any given time in a given area (4). For instance, the Inaba serotype of *V. cholerae* biotype El Tor dominated in South and Central America in 1991, while most strains isolated in Africa, where a major epidemic affected 21 countries in 1991, belonged to the Ogawa serotype (47). The causative agent of the seventh and ongoing pandemic of cholera, which began in 1961 in Celebes island of Indonesia, is *V. cholerae* O1 belonging to the El Tor biotype. In subsequent years, the El Tor biotype replaced the classical biotype in India and other parts of the world (4), except in southern Bangladesh, where the classical biotype reappeared in 1983 (37).

The preeminence of the O1 serogroup of *V. cholerae* in causing epidemic and pandemic cholera was rescinded in October 1992, when a large outbreak of choleralike infection caused by *V. cholerae* that did not agglutinate with O1 antiserum occurred in quick succession in Madras, Vellore, and Madurai, three cities in southern India (33). That this was not a local event became evident when an unusual increase in the isolation of *V. cholerae* non-O1 strains compared with that of O1 strains was witnessed in Calcutta, a city in eastern India where cholera is endemic, starting in November 1992 (28). This was quickly followed by a large outbreak of clinical cholera due to the *V. cholerae* non-O1 serogroup in southern Bangladesh (2, 8) and in several other areas in India (14, 28). Serological studies of strains recovered from several cities in India and Bangladesh revealed that the epidemic non-O1 strains were clonal and were assigned to a new serogroup designated O139 synonym Bengal (39). Examination of the restriction fragment length polymorphism of *Sma*I-digested genomic DNA of representative O139 strains from India, Bangladesh, and Thailand by using pulsed-field gel electrophoresis further revealed that the strains were clonal in nature (19). In this report, we describe the phenotypic and serological

* Corresponding author. Mailing address: Research Institute, International Medical Center of Japan, 1-21-1, Toyama, Shinjuku-ku, Tokyo 162, Japan. Phone: 81-3-5273-6844. Fax: 81-3-3202-7364.

characteristics of *V. cholerae* O139 strains isolated from India, Bangladesh, and Thailand and also describe detailed analysis of the cholera toxin (CT) of *V. cholerae* O139 strains and how it compares with the CT produced by El Tor *V. cholerae* O1 strains.

MATERIALS AND METHODS

Bacterial strains. A total of 262 strains of *V. cholerae* O139, which included 223 from India (27, 32), 30 from Bangladesh (2), and 9 from Thailand (9), were characterized in this study. The Indian strains represent isolations from widely separated geographic locales within the country: that is, of the 223 strains, 44, 46, 27, and 86 were isolated from outbreaks or from sporadic cases of choleralike infection from Madras, Vellore, Madurai, and Calcutta, respectively. The remaining 20 strains are from various other locations in India. The Bangladesh strains represent isolates from a single outbreak in southern Bangladesh (2), and the Thailand strains were isolated from sporadic cases of choleralike diarrhea in Bangkok (9).

Stool specimens. Between February and April 1993, when there was a large epidemic of O139 cholera in Calcutta (6), stool specimens from representative patients admitted to the Infectious Diseases Hospital, Calcutta, with acute secretory diarrhea were collected and kept frozen until used.

Phenotypic characterization. Physiological and biochemical characterizations of the *V. cholerae* O139 strains were performed as described by Sakazaki and Shimada (36).

Serology. Polyvalent O1 antiserum and antiserum against each of the 137 serogroups of *V. cholerae* non-O1 strains (35, 38) prepared in our laboratories and commercially available monoclonal antibodies against each of the antigenic factors A, B, and C of the *V. cholerae* O1 serogroup (Denka Seiken, Tokyo, Japan) were used. Antiserum against the reference strain of *V. cholerae* O139 (strain MO45 [ATCC 51394]) was prepared by hyperimmunizing rabbits with heat-killed whole cells (35, 38, 42). In addition, antisera were also prepared against four epidemic strains isolated from Madras, India (strains MO3, MO11, MO23, and MO34), by procedures described in detail by Shimada et al. (38). Agglutination and agglutinin absorption tests were performed as described by Sakazaki and Donovan (35).

CT bead ELISA. Quantification of CT in stool samples of patients yielding a bacteriologically confirmed strain of *V. cholerae* O139 was performed by CT bead enzyme-linked immunosorbent assay (ELISA) as described previously (32, 46). The amount of CT produced by strains of *V. cholerae* O139 and *V. cholerae* O1 El Tor were determined by the bead ELISA. All strains were grown in Casamino Acids-yeast extract medium supplemented with 90 μg of lincomycin (CAYE-L) (11) per ml in stationary culture at 30°C in petri dishes 90 mm in diameter. After overnight incubation, the cultures were centrifuged at 3,000 × *g* at 4°C for 10 min, and the supernatant was used for quantification of the amount of CT. Various dilutions of pure CT (List Biological Laboratories, Campbell, Calif.) served as the positive control, and uninoculated medium or buffer served as the negative control, and these were run concurrently whenever the CT bead ELISA was performed.

DNA probes and colony hybridization. The *Eco*RI fragment (554 bp) of the plasmid pKTN901, which contains the A1 subunit of CT (43), was used to detect the presence of the gene encoding CT. The gene encoding the zonula occludens toxin was detected with the zonula occludens toxin DNA probe constructed by Karasawa et al. (17). A recombinant plasmid, pAO111, containing the coding sequence for the heat-stable enterotoxin of non-O1 *V. cholerae* strains (NAG-ST) was used as the source of the DNA probe for detection of sequences homologous to NAG-ST (30, 44). A 1.4-kb *Eco*RI-*Hin*dIII DNA fragment constructed as described by Alm et al. (3) was used as the El Tor hemolysin probe. Colony hybridization of the test strains was performed under high-stringency conditions as described previously (17, 43, 44).

PCR. PCR was performed with the primers and procedure developed for amplification of a 302-bp DNA fragment of the *V. cholerae* O1 CT gene (43). Additionally, another pair of primers recently developed in this laboratory that amplify a 307-bp DNA fragment of the CT gene were used concurrently under the same amplification conditions described in reference 43. The additional pair of primers were ACAGAGTGAG TACTTTGACC and ATACCATCCATATATTTGGGAG.

Nucleotide sequence analysis of the CT gene of *V. cholerae* O139 strains. Chromosomal DNA from *V. cholerae* O139 strain MO35 was digested to completion with *Bgl*II and *Pst*I, and the fragments were separated by agarose gel electrophoresis. A 4.4-kb fragment containing the CT gene was identified by Southern hybridization with a polynucleotide DNA probe specific for the CT gene (43), extracted, and cloned into the *Bam*HI and *Pst*I sites of pUC119. After subcloning, the nucleotide sequence of the CT gene was determined by the dideoxy chain termination method (23).

RESULTS

Phenotypic characterization. The biochemical and physiological characteristics of the collection of *V. cholerae* O139 strains isolated from India, Bangladesh, and Thailand were essentially similar, and the O139 strains formed a phenotypically homogeneous group with identical characteristics. Resistance to 150 μg of the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine) and to Mukherjee's El Tor phage 5 and classical phage IV and the nonagglutinability of the strains with O1 antiserum were the main differences between strains belonging to the O139 serogroup and those belonging to the O1 serogroup. Inadvertently, strain MO45 (ATCC 51394), chosen as the reference strain for producing antiserum against the O139 serogroup, did not reduce nitrate but otherwise exhibited characteristics typical of the other O139 strains. All other tests evoked universally positive or negative responses, and the O139 serogroup showed responses identical to those of the O1 serogroup.

Serological characterization. Serological studies revealed that the reference strain of O139 (MO45, ATCC 31594) and four other epidemic strains of *V. cholerae* from Madras (MO3, MO11, MO23, and MO34) were not agglutinated by polyvalent O1 antiserum or by monoclonal antibodies against factors A, B, and C of the *V. cholerae* O1 serogroup. Moreover, they were not agglutinated by antisera prepared against the 137 non-O1 serogroups.

Antiserum prepared against reference strain MO45 (ATCC 31594) of the O139 serogroup and absorbed with the R strain of *V. cholerae* (strain CA385) did not agglutinate *V. cholerae* O1 strains of either serotype (Ogawa or Inaba) belonging to the classical (NIH 41, NIH 35A3) or El Tor (P1418, P6973) biotype. However, when examined with the panel of 137 reference strains representing serogroups O2 to O138, it was observed that the O139 antiserum cross-agglutinated with the reference strain representing serogroup O22. Therefore, the antiserum was absorbed with the O22 reference strain to remove cross-reacting agglutinins. Similar results were obtained with the other four epidemic strains.

Production of CT. All of the representative 223 strains of the

TABLE 1. Comparison of the amount of CT produced in CAYE-L medium by *V. cholerae* O139 and O1 strains

Amt of CT/ml of culture supernatant	No. of strains	
	O139	O1
>25 pg–<500 pg		2
>500 pg–<1 ng		7
>1 ng–<10 ng	17	17
>10 ng–<100 ng	12	2
>100 ng	1	
Total	30	28

V. cholerae O139 serogroup examined by the bead ELISA produced CT. The amount of CT produced by 30 representative O139 strains was quantified. It was observed that all of the strains produced more than 1 ng of CT per ml, with 13 strains producing more than 10 ng/ml (Table 1). On the other hand, there was a tendency for the amount of CT produced by 28 randomly selected representative O1 El Tor strains to be smaller than that produced by the O139 strains. Furthermore, 100 representative O139 strains examined hybridized with DNA probes specific for CT, zonula occludens toxin, and El Tor hemolysin genes but did not hybridize with the DNA probe specific for the NAG-ST gene.

The amount of CT present in stools of patients infected with *V. cholerae* O139 strains examined by the bead ELISA was more than that present in stools of patients infected with *V. cholerae* O1 El Tor strains (Table 2). In at least 15.2% of the O1 El Tor-infected patients (9 of 59), the amount of CT present in stools was <25 pg/ml of stool, if present at all, while CT was detected in the stools of all 36 O139-infected patients examined at levels of >500 pg/ml of stool.

Structure of the CT gene. The CT gene was cloned from O139 strain MO35 and sequenced. The nucleotide sequences of the genes coding for the A and B subunits of MO35 CT were identical to those shown by El Tor strains 62746 (21) and 2125 (12) (data not shown).

Detection of the CT gene by PCR. The CT gene of 39 representative O139 strains examined could be amplified by PCR with both pairs of primers described. Moreover, of the 100 watery stool samples collected from patients with clinically diagnosed cholera, 65 were positive for O139 strains by culture and were positive for the CT gene by PCR. In addition, PCR could also detect the CT gene in 17 stool samples that were negative by culture. The remaining 18 samples were negative both by culture and by the PCR assay.

TABLE 2. Comparison of amounts of CT present in stools of patients infected with *V. cholerae* O139 and O1 strains

Amt of CT/ml of stool	No. of stools of patients infected with:	
	O139	O1
<25 pg		9
>25 pg–<500 pg		21
>500 pg–<1 ng	1	4
>1 ng–<10 ng	20	14
>10 ng–<100 ng	11	10
>100 ng	4	1
Total	36	59

DISCUSSION

This study establishes the phenotypic similarity between serogroups O139 and O1 of *V. cholerae*. Although it is obsolete, according to Heiberg's classification (15), the O139 strains belonged to group I (strains of *V. cholerae* that ferment sucrose and mannose but not arabinose), which has characteristics identical to those exhibited by the O1 serogroup of *V. cholerae*. The production of CT by all O139 strains examined in this study and the complete similarity between the nucleotide sequence of the O139 CT gene and that of the O1 El Tor CT gene further extends the similarity between the O1 and O139 serogroups of *V. cholerae* and demonstrates the difference between the O139 serogroup and the remaining 137 non-O1 serogroups (O2 to O138). Studies of *V. cholerae* non-O1 serogroups have documented that production of CT is an exception to the rule among clinical (29, 31) and environmental (26) strains of *V. cholerae* belonging to the non-O1 serogroups. The only discernible phenotypic difference between the O1 and O139 serogroups was the resistance of the latter to the vibriostatic agent O/129 and Mukherjee's El Tor phage 5 and classical phage IV. However, in recent years, the emergence of O/129-resistant clinical strains of *V. cholerae* O1 (16, 22, 34) and other vibrios (18) has been reported by several workers.

Serologically, however, the O139 serogroup was distinct from the O1 serogroup or from any of the existing non-O1 serogroups of *V. cholerae* and is definitely a new entity. Use of monoclonal antibodies against the various antigenic forms of the O1 strain of *V. cholerae* made it clear that the O139 serogroup did not bear any resemblance to the O1 serogroup. This fact was important in view of the emergence of several bioserogroups of vibrios possessing somatic antigen factors in common with the *V. cholerae* O1 serogroup (40–42).

The appearance of the O139 serogroup, which shares ominous similarities with that of the O1 serogroup, has several implications. For instance, until recently, only O1 *V. cholerae* strains were considered to cause cholera while the non-O1 *V. cholerae* strains and other enteric pathogens caused only a choleralike infection. We have already proposed that the disease caused by O139 strains should be designated as cholera and should be a disease notifiable to the World Health Organization because of the similarity in the clinical profile of the disease and because of the epidemic potential of the O139 serogroup, which is identical to that of the O1 serogroup (6). More importantly, in places like Calcutta, the O139 serogroup of *V. cholerae* is exhibiting the tendency to replace the O1 serogroup, an event that has never before happened in the recorded history of cholera (27, 28). Current trends indicate that O139 will soon become the dominant serogroup, much in the same fashion that El Tor *V. cholerae* entered India in 1964, rapidly outnumbered classical *V. cholerae* serogroups by 1965, and ultimately replaced cases of cholera caused by the classical type (25). The pandemic potential of the O139 serogroup is now obvious with the spread of this serogroup to several countries in the Asian and neighboring subcontinents, including Thailand (9) and Pakistan (13). Furthermore, imported cases of O139 cholera have been reported from the United States (45), United Kingdom (7), Singapore (10), Switzerland (1), Germany (30a), and Japan (20). The progress of the O139 serogroup within countries that it has already invaded and in other areas where cholera is endemic should be carefully monitored.

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