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New Variants of *Vibrio cholerae* O1 Biotype El Tor with Attributes of the Classical Biotype from Hospitalized Patients with Acute Diarrhea in Bangladesh

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The sixth pandemic of cholera and, presumably, the earlier pandemics were caused by the classical biotype of *Vibrio cholerae* O1, which was progressively replaced by the El Tor biotype representing the seventh cholera pandemic. Although the classical biotype of *V. cholerae* O1 is extinct, even in southern Bangladesh, the last of the niches where this biotype prevailed, we have identified new varieties of *V. cholerae* O1, of the El Tor biotype with attributes of the classical biotype, from hospitalized patients with acute diarrhea in Bangladesh. Twenty-four strains of *V. cholerae* O1 isolated between 1991 and 1994 from hospitalized patients with acute diarrhea in Matlab, a rural area of Bangladesh, were examined for the phenotypic and genotypic traits that distinguish the two biotypes of *V. cholerae* O1. Standard reference strains of *V. cholerae* O1 belonging to the classical and El Tor biotypes were used as controls in all of the tests. The phenotypic traits commonly used to distinguish between the El Tor and classical biotypes, including polymyxin B sensitivity, chicken cell agglutination, type of *tcpA* and *rstR* genes, and restriction patterns of conserved rRNA genes (ribotypes), differentiated the 24 strains of toxigenic *V. cholerae* O1 into three types designated the Matlab types. Although all of the strains belonged to ribotypes that have been previously found among El Tor vibrios, type I strains had more traits of the classical biotype while type II and III strains appeared to be more like the El Tor biotype but had some classical biotype properties. These results suggest that, although the classical and El Tor biotypes have different lineages, there are possible naturally occurring genetic hybrids between the classical and El Tor biotypes that can cause cholera and thus provide new insight into the epidemiology of cholera in Bangladesh. Furthermore, the existence of such novel strains may have implications for the development of a cholera vaccine.

New epidemic strains of toxigenic *Vibrio cholerae* have appeared at least twice in recent human history (10). Strains of the classical biotype, which had probably been responsible for most of the epidemic disease in the 19th century and much of the 20th century, were largely replaced as the predominant cause of epidemic cholera by strains of the El Tor biotype in most of the regions where cholera is endemic, beginning in 1961. However, the classical biotype strains reemerged as a predominant epidemic strain in parts of Bangladesh in 1982 (8, 25) and coexisted with the El Tor strains, causing disease until 1993. A second new epidemic strain, carrying the O139 rather than the O1 antigen, emerged in southern Asia in 1992 (7, 24). The O139 and El Tor O1 strains continue to cause epidemics of cholera, and there are indications that the incidence of cholera due to the O139 serogroup is on the rise in parts of India and Bangladesh.

The classical and El Tor biotypes of *V. cholerae* are closely related in their O-antigen biosynthetic genes (21, 31), although these two biotypes differ in many other regions of their genomes (2, 16, 17, 29, 30). Thus, O1 El Tor strains might have arisen following transfer of O1 antigen biosynthetic genes into a previously unknown environmental strain. Conversely, O139 and O1 El Tor strains are closely related in most parts of their genomes but carry different O-antigen genes, suggesting the transfer of O139-specific genes from an unknown donor into a recipient El Tor strain (3, 28). Similar conclusions about gene transfer have emerged from comparisons of serogroups and sequences of diagnostic housekeeping genes of nonepidemic isolates (2).

In this study, we have identified a new variety of *V. cholerae* O1 that appears to be a hybrid of the classical and El Tor biotypes from hospitalized patients with acute diarrhea. The phenotypic traits that distinguish the classical and El Tor biotypes of *V. cholerae* O1 and important discriminating genotypic characteristics of these strains are reported here, and the implications of the existence of such novel strains, especially in relation to cholera vaccine development, are described.

MATERIALS AND METHODS

Twenty-four strains of *V. cholerae* isolated between 1991 and 1994 from hospitalized patients with acute diarrhea in the Matlab hospital, 45 km south of Dhaka, Bangladesh, were included in this study. The basis of a retrospective reexamination of these strains was their unusual response to polymyxin B (50 U), chicken cell agglutination (CCA), Voges-Proskauer (VP) reaction, and sensitivity to group IV and V phages, all of which are phenotypic traits commonly used to differentiate between the classical and El Tor biotypes. The 24 strains were reexamined for the above phenotypic characteristics by standard procedures.

The presence of the *ctxA* gene and the variants of the classical and El Tor *tcpA* genes was determined by a multiplex PCR assay (18). The expected size of the PCR amplicons was ascertained by electrophoresis in agarose gels. The identities of all PCR products were further verified with specific oligonucleotide probes. The probes for El Tor and classical biotype-specific CTX prophage repressor *rstR*

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TABLE 1. Phenotypic traits of Matlab types I, II, and III of toxigenic *V. cholerae* O1 isolated from patients hospitalized with acute secretory diarrhea in Bangladesh

Type	No. of strains	No. of strains of serotype:		VP test[a]	Sensitivity to polymyxin B (50 U)[b]	CAA[a]	Phage sensitivity[a]	
		Inaba	Ogawa				Group IV (El Tor biotype specific)	Group V (classical biotype specific)
Matlab I	2	2	0	−	R	−	R	R
Matlab II	1	0	1	−	S	−	S	R
Matlab III	21	0	21	−	R	−	S	R
El Tor MAK757	1	0	1	+	R	+	S	R
Classical 154	1	0	1	−	S	−	R	S

[a] +, positive; −, negative.
[b] Abbreviations: R, resistant; S, sensitive.

were *Sac*I-*Xba*I fragments of pHK1 and pHK2, respectively (19). The *acfB* gene probe was prepared from the PCR amplicon with previously reported *acfB*-specific primers (13). The rRNA gene probe consisted of a 7.5-kb *Bam*HI fragment of *Escherichia coli* rRNA clone pKK3535 (5). Colony blots or Southern blots were prepared with nylon filters (Hybond; Amersham International plc., Aylesbury, United Kingdom) by standard methods (27). The probes were labeled by random priming (14) with a random-primer DNA labeling kit (Bethesda Research Laboratories, Gaithersburg, Md.) and [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham). Colony blots and Southern blots were hybridized with the probes and autoradiographed as described previously (11–13).

RESULTS

The commonly used phenotypic traits used to distinguish between the El Tor and classical biotypes of *V. cholerae* differentiated the 24 strains into three types (Table 1), which we classified as Matlab types I, II, and III (named after the place where these strains were first isolated). Matlab type I included two strains belonging to the Inaba serotype that were resistant to both the El Tor-specific group IV and the classical biotype-specific group V phages, negative by the CCA and VP tests (both are classical traits), and resistant to polymyxin B (an El Tor trait). Matlab type II included one strain belonging to the Ogawa serotype that was sensitive to the group IV phage but showed negative responses in the CCA and VP tests and was sensitive to polymyxin B, all of which are classical biotype characteristics. Matlab type III included 21 Ogawa strains that showed the sensitivity to phages and polymyxin B typical of the El Tor biotype but were negative by the CCA and VP tests (both classical biotype traits).

Genotypically, all of the strains carried the *ctxA* gene, a constituent gene of the CTX prophage that encodes cholera toxin, and *acfB* and *tcpA*, which are located in different gene clusters (*acf* and *tcp* gene clusters) on the *V. cholerae* pathogenicity island. The type I strains appeared to belong more to the classical biotype because they carried the *tcpA* gene and the CTX prophage repressor gene (*rstR*) of the classical type (Table 2). The *tcpA* gene of the single type II strain was of the classical type, while the *rstR* gene was of the El Tor type. The six representative strains of *V. cholerae* representing Matlab III also carried the *tcpA* gene of the classical type. Five of the strains had the El Tor-type *rstR* gene, while one carried both the El Tor and classical *rstR* types.

The ribotypes of the *V. cholerae* strains examined in this study, compared to those of selected reference strains of the El Tor and classical biotypes, are shown in Fig. 1. The ribotypes of different strains representing the three Matlab types of *V. cholerae* were similar to the ribotypes of El Tor biotype strains and different from that of typical classical biotype strains described previously (11, 12). The ribotypes of two type I strains (lanes 9 and 10) were similar to that of toxigenic El Tor strains 1849 (lane 11), isolated in 2001, and G-3669 (lane 1), isolated in 1969 in Bangladesh. The Matlab type III strains belonged to three different ribotypes (Fig. 1, lanes 2 through 7), and the single type II strain had the same ribotype as a type III strain.

DISCUSSION

Classical and El Tor strains of *V. cholerae* are closely related but are not directly derived from each other (16, 17). El Tor vibrios appeared in Bangladesh, causing the first significant outbreak in 1968, and by 1973, they completely replaced the classical vibrios (1). In 1982, the classical biotype reappeared as the predominant epidemic strain in Bangladesh (25). In retrospect, it appears that classical cholera did not completely disappear from Bangladesh during the 1970s or late 1980s, but rather, its frequency varied in different regions of the country (26). The classical and El Tor biotypes have temporally overlapped for over a decade and are likely to have interacted and exchanged genetic material either in the human intestinal milieu or in the aquatic environment. The strains isolated in this study probably represent the amalgam of such an exchange. It is well recognized that genetic exchange between divergent bacterial lineages can contribute importantly to the success of

TABLE 2. Genotypic traits of *V. cholerae* O1 strains isolated from hospitalized patients with acute diarrhea in Bangladesh[a]

Strain	Matlab type	Yr of isolation	*tcpA* PCR	*ctxA* PCR	*acfB* (probe)	*rstR* (probe)
MJ-1236	I	1994	C	+	+	C
MJ-1485	I	1994	C	+	+	C
MG-116226	II	1991	C	+	+	E
MG-116025	III	1991	C	+	+	E
MG-116955	III	1991	C	+	+	E
MG-116926	III	1991	C	+	+	E, C
MG-117086	III	1991	C	+	+	E
MG-117159	III	1991	C	+	+	E
MH-08	III	1992	C	+	+	E
MAK 757 (El Tor)	Ref	1937	E	+	+	E
154 (classical)	Ref	UK	C	+	+	C

[a] C, classical type; E, El Tor type; Ref, reference strain; UK, unknown; +, positive.

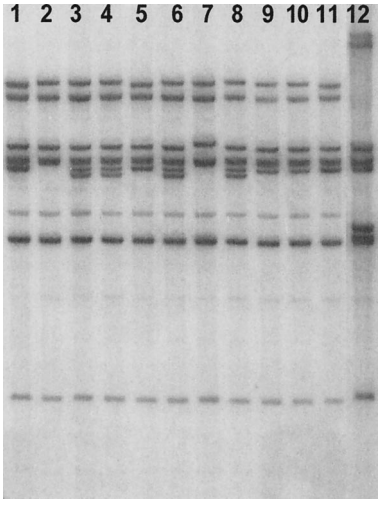

FIG. 1. *Bgl*I restriction patterns of rRNA genes of *V. cholerae* strains compared to those of selected typical strains of the El Tor and classical biotypes of *V. cholerae* O1. A Southern blot of *Bgl*I-digested genomic DNA was hybridized with the 7.5-kb *Bam*HI fragment of *E. coli* rRNA clone pKK3535. Lanes (including strain designations and relevant characteristics): 1, toxigenic El Tor strain G-3669 (isolated in 1969 in Bangladesh); 2 through 10, strains MH-08 (Matlab type III), MG-117159 (Matlab type III), MG-117086 (Matlab type III), MG-116926 (Matlab type III), MG-116955 (Matlab type III), MG-116025 (Matlab type III), MG-116226 (Matlab type II), MJ-1485 (Matlab type I), and MJ-1236 (Matlab type I); 11, toxigenic El Tor strain 1849 (isolated in 2001); 12, toxigenic classical biotype strain (isolated in 1963 in Bangladesh).

a species in complex and inconstant environments, such as those in which *V. cholerae* may reside. Several studies have also pointed to such exchanges as an important factor in *V. cholerae* population genetics and evolution (2, 3, 10).

On the basis of their phenotypic and genotypic traits, Matlab type I strains appeared to be more like the classical biotype while Matlab type II and III strains appeared to be more like the El Tor biotype. Matlab I strains, however, had altered phage receptor sites, since both of the strains were resistant to group IV and V phages. We assessed the similarity of the hybrid strains with classical and El Tor biotype strains on the basis of previously described ribotype patterns of classical and El Tor strains (11, 12). Ribotyping demonstrated that the Matlab I, II, and III strains showed minor differences in fragment patterns shown by the El Tor standard strains, suggesting that the hybrids originated from an El Tor-like clone. Therefore, overall, these strains were of the El Tor biotype displaying traits of the classical biotype. It has been proposed that while El Tor and classical strains are not directly derived from each other but appear to be derived from environmental nontoxigenic strains that are El Tor like (15). Clinical strains might become classical like in some properties simply by loss of function, and this agrees with the finding of the present study. While some genetic exchange has also probably occurred, it appears that the strains have evolved classical biotype properties. With a *V. cholerae* genomic microarray that displayed more than 93% of the predicted genes of the whole genome sequence of El Tor strain N16961, Dziejman et al. (9) showed that only seven genes were absent solely in classical strains but present in other strains, leading them to speculate that classical biotype strains may be derived from a primordial environmental strain that was more El Tor like than previously thought. Mitra et al. have previously reported the involvement of bacteriophage PS166 in the acquisition of some classical biotype-specific properties by El Tor strains (22, 23). Insertion of lysogenic phage genomes in the bacterial chromosome leading to the activation or inactivation of certain genes or expression of new phage-encoded genes is a natural phenomenon in the origination of genetic diversity. However, in the present study, the acquisition of classical properties such as classical type *tcpA* and *rstR* genes by El Tor vibrios by conversion through phage PS166 seems unlikely. It seems more probable that more than one genetic exchanges were involved in the conversion of these strains. Irrespective of the mechanism involved in the generation of the natural hybrid strains, the existence of strains showing a combination of classical and El Tor biotype properties has evolutionary and epidemiological importance.

Interestingly, all of the hybrid strains carried the *tcpA* gene of the classical type. Recently, the dominance of the classical type *tcpA* gene among environmental strains of *V. cholerae* has been reported (6). The primary structure of TcpA is highly conserved among *V. cholerae* serogroups and biotypes shown to be pathogenic to humans, with amino acid identities of nearly 100% between strains of a given biotype and about 80% between classical and El Tor biotype O1 strains (20). We are not certain if El Tor strains with classical *tcpA* are more efficient colonizers, but there is enough evidence showing that classical biotype strains elaborate abundant amounts of toxin-coregulated pilin when grown in vitro, in contrast to El Tor strains (20, 29). The strains analyzed in the present study are not only of academic interest but may well represent precursors of other clones that could lead to a pandemic spread since they have all of the genetic features needed to make a *V. cholerae* strain pandemic. Moreover, these strains were isolated from clinical cases of acute diarrhea. These strains also represent unique natural recombinants that could be judiciously employed in the construction of live-vaccine strains since they have a combination of virulence attributes of both the classical and El Tor biotypes of *V. cholerae* O1.

The classical biotype of *V. cholerae* O1 is believed to be extinct and has not been isolated in the past several years, even in southern Bangladesh, the last of the niches where this biotype prevailed (A. K. Siddique, unpublished data). In this study, we show the existence of El Tor strains that have lost some of the El Tor phenotypes and acquired classical biotype characteristics. Therefore, even though strains that represent the classical biotype in entirety have been completely displaced, a reservoir of the virulence genes of the classical biotype still exists in nature. Previous molecular analyses of classical strains isolated between 1961 and 1992 in Bangladesh support the contention that classical vibrios were never completely replaced in Bangladesh (11) Thus, a vaccine developed against cholera must take this into consideration and must be targeted against both of the biotypes, failing which the global use of a vaccine exclusively against the El Tor biotype might select against El Tor strains and favor strains carrying the classical attributes, such as those isolated in this study.

These hybrid strains of *V. cholerae* may be more common than currently recognized because phenotypic methods are inadequate to precisely distinguish between the two biotypes

and are not routinely used in clinical microbiology laboratories. IS*1004* fingerprinting has determined that an O37 strain of *V. cholerae* that was responsible for a large outbreak of cholera in Sudan in 1968 (32) is closely related to classical O1 strains (4). This indicates that horizontal exchange of genes has occurred not only between O1 biotypes but also between classical biotype and non-O1 strains, and the Sudan strain is a typical example of how a novel genotype can cause a large outbreak. Although the strains characterized in this study were isolated a decade ago, the inadequacy of phenotypic tests did not permit us to place these strains accurately. However, molecular techniques have revealed that these strains carry traits of the two biotypes and that such strains exist in nature and are associated with sporadic diarrhea in Bangladesh and, presumably, other areas of the world where cholera is endemic. The recognition of such strains and tracking of their global prevalence and spread are important because each of these types possesses all of the traits necessary to initiate a pandemic spread.

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