Emergence of *Vibrio cholerae* O1 Biotype El Tor Serotype Inaba from the Prevailing O1 Ogawa Serotype Strains in India

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The toxigenic Inaba serotype of *Vibrio cholerae* O1 biotype El Tor reappeared in India in 1998 and 1999, almost 10 years after its last dominance in Calcutta in 1989. Extensive molecular characterization by ribotyping, restriction fragment length polymorphism, and pulsed-field gel electrophoresis indicated that recent Inaba strains are remarkably different from the earlier Inaba strains but are very similar to the prevailing *V. cholerae* O1 Ogawa El Tor biotype strains. The antibiograms of the Inaba strains were also similar to those of the recent *V. cholerae* Ogawa strains. These *V. cholerae* O1 Inaba strains appear to have evolved from the currently prevailing Ogawa strains and are likely to dominate in the coming years.

The disease cholera, caused by toxigenic strains of *Vibrio cholerae* belonging to the O1 or O139 serogroup, is characterized by the passing of voluminous watery stools, which rapidly lead to dehydration and, if left untreated, to death. *V. cholerae* O1 is further classified into two biotypes, classical and El Tor, and into two major serotypes, Inaba and Ogawa. The genes responsible for O1 antigen biosynthesis have been designated *wbe* (previously known as *rfb*) (18) and are localized on a 21.6-kb *Sac* fragment of DNA (7, 24). This region is highly conserved; the only changes observed between the Ogawa and Inaba serotypes are related to a mutation in the *wbeF* region (24, 27). *V. cholerae* O1 strains can undergo serotype conversion or switching between the Inaba and Ogawa serotypes (5, 8, 24). Observations of the epidemic that broke out in Latin America in 1991 have supported this notion. Extensive biochemical analyses and rRNA restriction fragment length polymorphism (RFLP) analysis have shown that the El Tor Inaba epidemic strains were unique to Latin America (13, 21). However, Ogawa isolates that were identical to the epidemic strain in all other respects began to appear in about the seventh month of the epidemic, suggesting that the epidemic strains had undergone a serotype conversion, possibly because of immune pressure in the population (13).

With the advent of the O139 serogroup in 1992 (17), the Inaba serotype of *V. cholerae* O1 was displaced in Calcutta and other parts of India (15) by the O139 serogroup, and the last Inaba predominance in Calcutta was observed in 1989 (17). The isolation of *V. cholerae* O1 belonging to the Inaba serotype became rare; the only isolation reported was from a cholera outbreak in Warangal, which was due to nontoxigenic *V. cholerae* O1 Inaba El Tor biotype strains (20). We began receiving some representative strains belonging to the Inaba serotype from Delhi in December 1998 and from Sewagram in November 1999. This study is an extensive molecular characterization of the recently isolated Inaba strains to determine their clonality and to evaluate their similarity to Inaba strains that were isolated in Calcutta in 1989.

The present study is part of the continuing nationwide surveillance program on cholera of the National Institute of Cholera and Enteric Diseases (NICED), Calcutta, India. In December 1998, we received a representative set of strains from Delhi, two of which were identified as *V. cholerae* O1 Inaba. In November 1999 we received a set of seven strains from Sewagram, six of which were identified as *V. cholerae* O1 Inaba and one of which was *V. cholerae* O1 Ogawa. The purity and identity of the strains were then confirmed by previously published procedures (15).

The strains were examined for resistance to ampicillin (10 μg), chloramphenicol (30 μg), cotrimoxazole (25 μg), ciprofloxacin (5 μg), furazolidone (100 μg), gentamicin (10 μg), neomycin (30 μg), nalidixic acid (30 μg), norfloxacin (10 μg), streptomycin (10 μg), and tetracycline (30 μg) with commercial disks (HiMedia, Mumbai, India). Antimicrobial susceptibility analysis was carried out as described previously (15).

The 7.5-kb *Bam*HI fragment of plasmid pKK 3535 containing the 16S and 23S rRNA genes of *Escherichia coli* was used as the rRNA probe (4). The *ctxA* probe consisted of a 540-bp *Xba*I-*Cla*I fragment of *ctxA* cloned in pKTN901 using *Eco*RI linkers (11). The DNA probe for the RS element was a 2.7-kb *Nco*I fragment from plasmid pCTSA11 (10). Genomic DNA for ribotyping and for studying the structure and organization of the CTX prophage was extracted as described previously (1). The transfer of digested DNA from a gel to a Hybond N+ membrane (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England) and hybridizations with the rRNA probe for ribotyping and with the *ctxA* and RS1 probes for CTX genotyping were performed as described previously (1) using the ECL Nucleic Acid Detection System (Amersham Pharmacia Biotech). The membranes were then washed, exposed to Kodak Biomax film (Eastman Kodak Co., Rochester, N.Y.), and developed according to the manufacturer’s instructions. Autoradiograms were digitally processed for documentation using the Gel Doc 2000 gel documentation system (BioRad, Richmond, Calif.).

Pulsed-field gel electrophoresis (PFGE) was performed with the genomic DNA of the *V. cholerae* strains by preparing ago-
rose plugs as described previously (12, 28). PFGE of the NorI (Takara, Shuzo Co., Ltd., Shiga, Japan)-digested inserts was performed by using the contour-clamped homogeneous electric field (CHEF) method on the CHEF Mapper system (Bio-Rad) with 1% PFGE grade agarose in 0.5× TBE (44.5 mM Tris-HCl, 44.5 mM boric acid, 1.0 mM EDTA [pH 8.0]) for 40 h, 24 min. Run conditions were generated by the autoalgorithm mode of the CHEF Mapper PFGE system using a size range of 20 to 300 kb. The gels were stained for 30 min in Elix MilliQ water (Millipore Corporation, Bedford, Mass.) containing 1.0 μg of ethidium bromide per ml, then destained in Elix water for 15 min and photographed under UV light using the Gel Doc 2000 gel documentation system (Bio-Rad).

For molecular characterization we included two Inaba strains, V2 and V13, isolated in 1989 in Calcutta, two Inaba strains from Delhi isolated in 1998, six Inaba strains and one Ogawa strain isolated in 1999 in Sewagram and four Ogawa strains from Delhi isolated in 1998, six Inaba strains and one Ogawa strain isolated in 1999 in Sewagram, and four Ogawa strains, V2 and V13, isolated in 1989 in Calcutta, two Inaba strains isolated in Sewagram in 1999 or whether they belonged to a new clone.

The antibiotic susceptibility patterns of these strains revealed that V2 and V13, representing the Inaba strains isolated in 1989, were sensitive to nalidixic acid and streptomycin (16, 17), while the Inaba strains recently isolated in Sewagram and Delhi were resistant to these antibiotics (Table 1). Overall, the antibiotic patterns of the Inaba strains isolated in 1989, V2 and V13 (Fig. 1, lanes 4 and 5), were different from the ribotype patterns shown by the V. cholerae reference strains SG24 (O139) (Fig. 1, lane 1), 569B (O1 classical Inaba) (Fig. 1, lane 2), and CO840 (O1 Ogawa El Tor) (Fig. 1, lane 3). On the other hand, the recent Inaba strains DO182 and DO183 isolated in Delhi in 1998 (Fig. 1, lanes 6 and 7) and the Inaba strains isolated in Sewagram in 1999, SO86 and SO90 (Fig. 1, lanes 8 and 9), showed ribotypes similar to that of the prevailing V. cholerae O1 Ogawa strain CO840 (the slight difference in the mobility of the bands is due to a gel anomaly), which is the RIII type, first described by Sharma et al. (22). The ribotypes of the recent V. cholerae Inaba strains indicate that these strains have molecular traits identical to those of the prevailing V. cholerae O1 Ogawa

<table>
<thead>
<tr>
<th>Strain no. (serotype)</th>
<th>Isolation yr. place</th>
<th>Antibiotic*</th>
<th>RPb</th>
<th>Fragment size(s) (kb) with the following probe and restriction enzyme:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BglI</td>
</tr>
<tr>
<td>V2, (Inaba) 1989, Calcutta</td>
<td>A Co Fz N S</td>
<td>PD</td>
<td>18.5</td>
<td>20.0</td>
</tr>
<tr>
<td>V13 (Inaba) 1989, Calcutta</td>
<td>A Co Fz N</td>
<td>PD</td>
<td>19.0</td>
<td>23.0</td>
</tr>
<tr>
<td>DO182 (Inaba) 1998, Delhi</td>
<td>A Co Fz N Na S</td>
<td>RIII</td>
<td>14.0</td>
<td>21.0</td>
</tr>
<tr>
<td>DO183 (Inaba) 1998, Delhi</td>
<td>A Co Fz N Na S</td>
<td>RIII</td>
<td>23.0</td>
<td>23.0</td>
</tr>
<tr>
<td>SO85 (Inaba) 1999, Sewagram</td>
<td>A Co Fz N Na S</td>
<td>RIII</td>
<td>12.0</td>
<td>19.5</td>
</tr>
<tr>
<td>SO86 (Inaba) 1999, Sewagram</td>
<td>A Co Fz N Na S T</td>
<td>RIII</td>
<td>13.0</td>
<td>19.5</td>
</tr>
<tr>
<td>SO87 (Inaba) 1999, Sewagram</td>
<td>A Co Fz N Na S</td>
<td>RIII</td>
<td>13.0</td>
<td>20.0</td>
</tr>
<tr>
<td>SO88 (Ogawa) 1999, Sewagram</td>
<td>A Co Fz N Na S</td>
<td>RIII</td>
<td>14.0</td>
<td>20.5</td>
</tr>
<tr>
<td>SO89 (Inaba) 1999, Sewagram</td>
<td>A Co Fz N S T</td>
<td>RIII</td>
<td>13.0</td>
<td>13.0</td>
</tr>
<tr>
<td>SO90 (Inaba) 1999, Sewagram</td>
<td>A Co Fz N Na S</td>
<td>RIII</td>
<td>14.0</td>
<td>21.0</td>
</tr>
<tr>
<td>SO84 (Inaba) 1999, Sewagram</td>
<td>A Co Fz N Na S T</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PG81 (Ogawa) 1998, Calcutta</td>
<td>A Co Fz N Na</td>
<td>RIII</td>
<td>ND</td>
<td>26.0</td>
</tr>
<tr>
<td>PG117 (Ogawa) 1998, Calcutta</td>
<td>A Co Fz N Na</td>
<td>RIII</td>
<td>ND</td>
<td>26.0</td>
</tr>
<tr>
<td>CO840 (Inaba) 1995, Calcutta</td>
<td>A Co Fz N Na S</td>
<td>RIII</td>
<td>ND</td>
<td>23.0</td>
</tr>
</tbody>
</table>

* A, ampicillin; Cf, ciprofloxacin; Co, cotrimoxazole; Fz, furazolidone; N, neomycin; Na, nalidixic acid; S, streptomycin; T, tetracycline.

b RP, ribotype pattern; PD, previously described by Dalsgaard et al. (6); ND, not done.
strains. This result also points out that the recent Inaba strains are quite different from the Inaba strains isolated in 1989, when Inaba was the dominant serotype (16, 17).

Southern hybridization with the ctxA probe using HindIII-digested genomic DNA detected only one band, but of varying size, in the Inaba strains. This result indicates that the CTX prophage is located at a single site in the chromosome, as HindIII does not have any recognition site within the CTX prophage (14). The arrangement and number of copies of the CTX prophage were determined by analysis of the Southern hybridization pattern generated separately with ctxA and RS1 probes using other restriction enzymes which do cut within the CTX prophage but not in ctxA. ctxA RFLP patterns generated with BglI- and PstI-digested genomic DNA showed a single band in all the strains (Table 1), suggesting the presence of a single copy of the CTX prophage. The CTX prophage genome has two regions: a 4.6-kb "core region" that includes ctxAB and a 2.4-kb region termed RS2. The integrated CTX prophage is frequently flanked by an element known as RS1, which is related to RS2 (25). These related elements contain three nearly identical open reading frames (ORFs), while RS1 contains an additional ORF (25). Southern hybridization with the RS1 probe was carried out to determine the organization of the RS sequences upstream and downstream of the core region. The restriction endonuclease PstI, which cuts within the core region (14), was used to digest genomic DNA, which was then hybridized with the RS1 probe. RS1 RFLP patterns generated with PstI-digested genomic DNA exhibited the presence of two bands of 30.0 and 10.0 kb in strains V2 and V13 and of 30.0 and 16.0 kb in strain DO183, while a single band of 30.0 kb appeared in the remaining strains (Table 1). Therefore, strains V2, V13, and DO183 each contain at least one copy of RS1 at both sides of the core region. When the results of hybridization of PstI-digested genomic DNA with the ctxA and RS1 probes were compared, a band common to both was observed for strains V2, V13, and DO183. The size of the common band was 10.0 kb in strains V2 and V13 and 16.0 kb in strain DO183 (Table 1). This result confirms the presence of an RS element downstream of the CTX prophage. The BglII restriction enzyme has a site in the RS region (25). As expected, when BglII-digested genomic DNA was hybridized with the RS1 probe, three bands were observed for V2 and four bands were observed for V13 and DO183; one of these, a 7.0-kb band, is actually the size of the CTX prophage (25). The presence of tandemly arranged RS sequences is not uncommon in toxigenic V. cholerae, and the size of RS1 is reported to be 2.7 kb (25). Since hybridization with the RS1 probe never showed the presence of a 2.7-kb band in V2, the possibility of tandemly arranged RS1 on either side of the core region can be excluded. The presence of a 2.7-kb band in V13 and DO183 indicates the possibility of tandemly arranged RS1 on either side of the core region. When the Inaba strains isolated in Sewagram and strain DO182 were digested with PstI, the RFLP data showed a single band upon hybridization with both the ctxA and RS1 probes (Table 1), indicating the presence of a single copy of the CTX prophage, while BglII digestion and hybridization with the RS1 probe showed a band of 2.7 kb, indicating the presence of two tandemly arranged copies of the RS1 element in these strains. Thus, strain DO183 and the Sewagram strains have a single copy of CTX prophage with one RS1 element upstream of the prophage. This organization is very similar to that of the prevailing Ogawa strains (22). Figure 2 shows schematic representations, based on RFLP analysis, of the CTX prophage of the Inaba strain V2, isolated

![FIG. 2. Schematic representations of the organization of the CTX prophage (not to scale) as deduced by Southern hybridization data for V. cholerae O1 Inaba strains represented by V2 (a) and V. cholerae O1 Inaba strains represented by SO85 (b). Arrows and boxes represent the RS elements and the core region of the CTX prophage, respectively, with the hatched portion of the box representing the ctxAB gene. Restriction site abbreviations: BglI; BglII; PstI; H, HindIII.](http://jcm.asm.org/Downloaded from http://jcm.asm.org/ on October 21, 2017 by guest)
in 1989 (Fig. 2a), and of strain SO85, which is typical of the Inaba strains recently isolated in Delhi and Sewagram (Fig. 2b).

Analysis of PFGE patterns showed that the Inaba strains isolated recently (Fig. 3, lanes 3 to 7) had a profile different from those of strains V2 (Fig. 3, lane 1) and V13 (Fig. 3, lane 2), representing the Inaba strains isolated in 1989. The recent Inaba strains (Fig. 3, lanes 3 to 7) differed from strain CO840 (Fig. 3, lane 8) by the presence of more than one band in the 145-kb region. Interestingly, O1 Ogawa strains isolated during 1998 (Fig. 3, lanes 9 to 12) had a PFGE profile identical to that of the recently isolated Inaba strains, indicating that the Inaba strains that have emerged recently are similar to the prevailing O1 Ogawa strains.

V. cholerae O1 strains are known to interconvert between the Ogawa and Inaba forms (5, 8, 24). The frequency of conversion of Ogawa to Inaba is approximately 10^{-5} (3), whereas conversion from Inaba to Ogawa is rare and may be strain dependent (13). In vivo seroconversion correlates well with the host immune response, and this is supported by observations with germ-free mice (19) and a clinical study carried out by Sheehy et al. (23). The wbtT gene of the highly conserved wbt region is responsible for the serotype conversion, as there is a single-nucleotide change within the gene, resulting in a TGA stop codon and a truncated 32-kDa wbtT protein (24). The product of the wbtT gene of V. cholerae O1 is not essential for O antigen biosynthesis but is required for determining the Ogawa serotype specificity (24). A variety of changes in wbtT could produce an Inaba strain by leading to truncated wbtT proteins of various sizes due to reading frame shifts. Thus, Inaba strains are effectively wbtT mutants and presumably have arisen as a result of selection due to the immune response during cholera infection (24). This study demonstrates that V. cholerae O1 Inaba, after predominating until 1989 (17), suddenly disap-

**REFERENCES**


