

Spread of Cholera with Newer Clones of *Vibrio cholerae* O1 El Tor, Serotype Inaba, in India

B. Dutta,¹ R. Ghosh,¹ N. C. Sharma,² G. P. Pazhani,¹ N. Taneja,³ A. Raychowdhuri,¹ B. L. Sarkar,¹
 S. K. Mondal,¹ A. K. Mukhopadhyay,¹ R. K. Nandy,¹ M. K. Bhattacharya,¹
 S. K. Bhattacharya,¹ and T. Ramamurthy^{1*}

National Institute of Cholera and Enteric Diseases, Kolkata,¹ Maharishi Valmiki Infectious Diseases Hospital, Delhi,²
 and Postgraduate Institute of Medical Education and Research, Chandigarh,³ India

Received 24 March 2006/Returned for modification 3 May 2006/Accepted 22 June 2006

During 2004 and 2005, cholera was recorded in 15 states of India, with 7 outbreaks. The newly emerged *Vibrio cholerae* O1 Inaba had a different antibiogram and ribotype, different pulsotypes, and different mutations in the *wbeT* gene. Due to the absence of serogroup O139, the Inaba serotype may have acquired the potential to affect the population at large.

Cholera continues to be a growing concern in most developing countries. Since the emergence of *Vibrio cholerae* O139, the incidence patterns of serogroup O1 have been constantly changing in the Indian subcontinent (12, 14). During 2004 and 2005, we investigated outbreaks of cholera in Delhi, Madhya Pradesh, and West Bengal. *V. cholerae* O1 isolates from outbreaks and those isolates received from four other states of India were included in this study (Table 1). From May 2004 to July 2005, sporadic cases of cholera were reported from 10 other areas (Table 1). To our knowledge, the emergence of the Inaba serotype and its spread in many parts of India, mostly in the form of outbreaks, were first detected during this time.

Stool specimens collected from diarrheal patients were processed for common enteric pathogens following standard methods. *V. cholerae* isolates were grown on thiosulfate citrate bile salts sucrose agar (Eiken Chemical Co. Ltd., Tokyo, Japan) at 37°C for 16 to 18 h and confirmed using polyvalent and monospecific antisera (Denka Seiken, Tokyo, Japan). The Clinical and Laboratory Standards Institute (formerly NCCLS) antimicrobial susceptibility test was adapted for *V. cholerae* O1 (3) with commercially available disks (Becton Dickinson Co., Sparks, MD). All of the *V. cholerae* O1 isolates were typed with El Tor phages (2).

The presence of the cholera toxin gene (*ctxA*) and *tcpA* variants (the major structural subunit gene of the toxin-coregulated pilus) of both biotypes were determined by a multiplex PCR assay (4). Uniplex PCR was performed for the amplification of *wbeT*, encoding the somatic antigen synthesis region (15), and *rstR* gene alleles, encoding regulation of the lysogeny of the CTX phage (12), in a standard PCR mixture. For ribotyping, BglI-digested chromosomal DNAs from the representative isolates were transferred to Hybond N⁺ membranes (Amersham International PLC, Buckinghamshire, England), and hybridization was done with the 7.5-kb BamHI fragment as a probe from plasmid pKK3535 (16) (ECL Nucleic Acid De-

tection System; Amersham). Pulsed-field gel electrophoresis (PFGE) was performed as described previously (20).

For *wbeT* mutation analysis, the 902-bp PCR products were purified (QIAGEN [Hilden, Germany] PCR purification kit), and sequencing was done with a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) using an ABI PRISM 3100 DNA sequencer (Applied Biosystems). The nucleotide and deduced protein sequences were analyzed with DNASIS (Hitachi, Yokohama, Japan), DNASTAR (DNA Star Inc., Madison, WI), and GenBank via the BLAST network.

We analyzed 402 *V. cholerae* O1 isolates from 15 states collected during 2004 and 2005. Among these, 43.3 and 56.7% of the isolates were identified as Ogawa and Inaba serotypes, respectively (Table 1). Serotype Inaba was exclusively identified in five cholera outbreaks (Table 1). Even though *V. cholerae* O1 Inaba was found to coexist with the Ogawa serotype (10, 19), the prevalence of the latter was consistent in many regions where cholera is endemic (11, 17). Outbreaks of cholera exclusively caused by Inaba were reported in a few countries (7, 18). Cycles of serotype switching lasting between 2 and 8 years have been reported in Bangladesh (10). Periodic shifting between *V. cholerae* O1 Ogawa and O139 was observed in India from 1994 to 2000 (14). We assume that, due to the absence of O139 from 2000 to 2004, the O1 Inaba serotype perhaps reemerged in India in a high proportion.

Almost all of the Ogawa, as well as Inaba, isolates belonged to phage type T4 and type 27 with a new set of phages (2), and these types prevailed in India for many years (17). Compared to Ogawa, the Inaba isolates were resistant to chloramphenicol and streptomycin. The pattern of susceptibility of *V. cholerae* isolates to these two antimicrobials has changed over time (6). A majority (91%) of the Inaba isolates showed reduced susceptibility to ciprofloxacin, whereas 32% of the Ogawa isolates remained resistant to the drug. Increase in resistance to ciprofloxacin is a cause for concern, as this drug is extensively used in India for the treatment of diarrhea (5).

The *rstR* region is classified into *rstR*^{class}, *rstR*^{ET}, and *rstR*^{Calc} for classical, El Tor, and O139 alleles, respectively (9). Interestingly, genetic hybrids of the El Tor and classical biotypes, which lack common phenotypic traits, have been detected in

* Corresponding author. Mailing address: National Institute of Cholera and Enteric Diseases, P-33, C. I. T. Road, Scheme XM, Beliaghata, Kolkata-700 010, India. Phone: 91-33-2370-1176. Fax: 91-33-2350-5066. E-mail: tramu@vsnl.net.

TABLE 1. *Vibrio cholerae* isolates

Place/state of isolation	Mo and yr of isolation	No. of <i>V. cholerae</i> isolates		
		Ogawa	Inaba	Total
Delhi ^a /Delhi	May 2004	36	69	105
Ludhiana/Punjab	May 2004	23	25	48
Tripura ^a /Assam	May 2004		6	6
Bhind ^a /Madhya Pradesh	May 2004		2	2
Chandigarh	July 2004	1	1	2
Madurai/Tamil Nadu	July 2004	25	1	26
Uttar Pradesh	September 2004		6	6
Haryana	September 2004		5	5
Trivandrum/Kerala	October 2004		2	2
Ahmedabad/Gujarat	October 2004, May 2005	29	27	56
Goa	February 2005	7	9	16
Kolkata ^a /West Bengal	April 2005		4	4
Berhampur ^a /Orissa	April 2005		4	4
Chennai/Tamil Nadu	July 2005	44	3	47
Hyderabad/Andhra Pradesh	July 2005		34	34
Manipal ^a /Karnataka	July 2005	9	18	27
Alleppy ^a /Kerala	November 2005		12	12
Total		174	228	402

^a Cholera outbreak-affected area.

Mozambique and Bangladesh (1, 12). When they were screened for *rstR* alleles by PCR, we found that all the recent isolates were of the *rstR*^{ET} type. In addition, phage typing and polymyxin B susceptibility results confirmed our isolates as El Tor biotype.

Up to 1993, three ribotypes (RI through RIII) were identified in the *V. cholerae* O1 serogroup (16). The RII and RIII ribotypes were identified after the emergence of *V. cholerae*

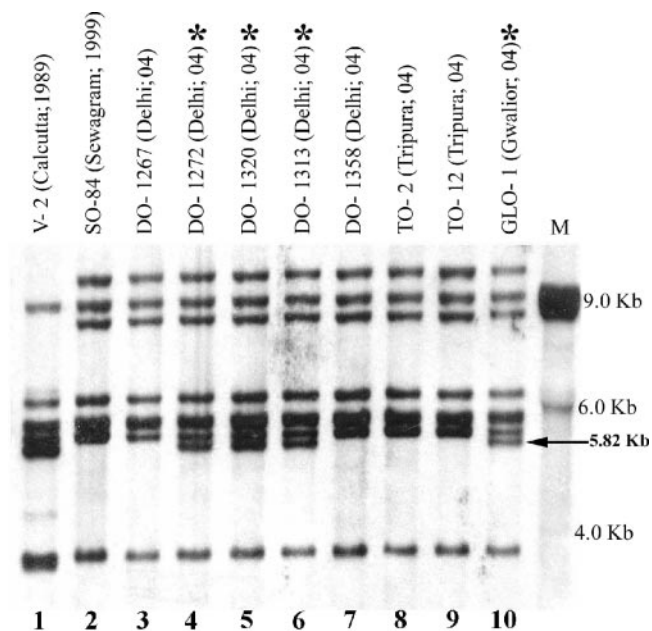


FIG. 1. Ribotypes of the representative *V. cholerae* O1 Inaba isolates using BglI. The place and year of isolation are given in parentheses. The isolates indicated with asterisks belong to ribotype RIV. M, molecular size marker.

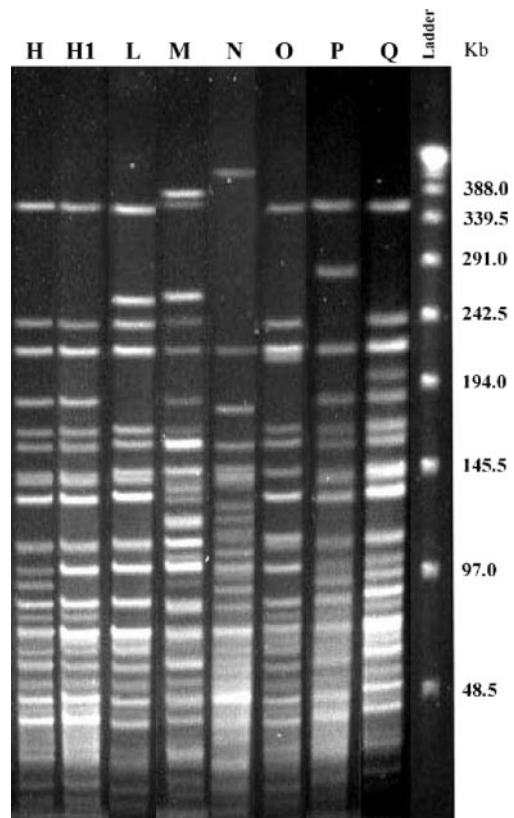


FIG. 2. PFGE profiles generated with NotI-digested genomic DNAs of *V. cholerae* O1 isolates. The new pulsotypes H1, M, and P and L, N, and O were detected among Inaba and Ogawa isolates, respectively.

O139 (16), and these two types were not recorded in the previous scheme (13). Most of the recent Inaba isolates from different states of India were identified as a new ribotype, RIV, and the Ogawa isolates during the same period were identified as RIII (Fig. 1). Prevalence of ribotype RIII was also detected in some of the 2004 Inaba isolates.

Twenty-seven *V. cholerae* isolates (20 Inaba and 7 Ogawa) were tested following the PFGE typing scheme (20), which consisted of 11 pulsotypes (A through K). The majority of the Inaba isolates encountered in this study belonged to the “H” type (12 isolates) or its subtype “H1” (Fig. 2). The H pulsotype was dominant among *V. cholerae* O1 isolates and was identified in India in July 1993. Six new pulsotypes (L through Q) were identified in this study (Fig. 2). Such an assortment of pulsotypes of *V. cholerae* O1 in a span of 2 years has not been reported before.

Mutations in the *wbeT* gene were responsible for serotype conversion in *V. cholerae* O1 (8). The DNA sequence analysis revealed that *wbeT* was homologous in Inaba isolates from Tripura, Madurai, Ludhiyana, Ahmedabad, and Kolkata. In all of these isolates, a novel mutation (substitution of C for T at position 538) was detected, which changed serine to proline. More molecular studies are warranted to confirm our findings in relation to the epidemiology of cholera with recent Inaba serotype isolates.

Nucleotide sequence accession number. The nucleotide sequence of *wbeT* has been deposited in the NCBI database under accession number DQ401028.

We acknowledge Kasturba Medical College, Mannipal; Trivandrum Medical College, Trivandrum; T. D. Medical College, Alleppey; Sheth V. S. General Hospital, Ahmedabad; Sir Ronald Ross Institute of Tropical and Communicable Diseases, Hyderabad; M. K. C. G. Medical College Hospital, Berhampur; Madurai Medical College, Madurai; Christian Medical College, Ludhiana; Communicable Diseases Hospital, Chennai; IGM Hospital, Tripura; and Goa Medical College, Punjim, for sending the *V. cholerae* isolates.

The work was supported in part by the Indian Council of Medical Research, Japan International Cooperation Agency (JICA/NICED Project 054-1061-E-O), and the Ministry of Health, Labor and Family Welfare of Japan (Project H17-Shinkou-3).

REFERENCES

1. Ansaruzaman, M., N. A. Bhuiyan, G. B. Nair, D. A. Sack, M. Lucas, J. L. Dean, J. Ampuero, C.-L. Chaignat, and The Mozambique Cholera Vaccine Demonstration Project Coordination Group. 2004. Cholera in Mozambique, variant of *Vibrio cholerae*. *Emerg. Infect. Dis.* **10**:2057–2058.
2. Chattopadhyay, D. J., B. L. Sarkar, M. Q. Ansari, B. K. Chakrabarti, M. K. Roy, A. N. Ghosh, and S. C. Pal. 1993. New phage typing scheme for *Vibrio cholerae* O1 biotype El Tor strains. *J. Clin. Microbiol.* **31**:1579–1585.
3. Clinical and Laboratory Standards Institute. 2005. Performance standards for antimicrobial testing. 15th informational supplement M100-S15. Clinical and Laboratory Standards Institute, Wayne, Pa.
4. De, K., T. Ramamurthy, A. C. Ghose, M. S. Islam, Y. Takeda, G. B. Nair, and R. K. Nandy. 2001. Modification of the multiplex PCR for ambiguous difference of the El Tor and classical biotypes of *Vibrio cholerae* O1. *Indian J. Med. Res.* **114**:77–82.
5. Garg, P., S. Sinha, R. Chakraborty, S. K. Bhattacharya, G. B. Nair, T. Ramamurthy, and Y. Takeda. 2001. Emergence of fluoroquinolone-resistance strains of *Vibrio cholerae* O1 biotype El Tor among hospitalized patients with cholera in Calcutta, India. *Antimicrob. Agents Chemother.* **45**:1605–1606.
6. Garg, P., S. Chakraborty, I. Basu, S. Datta, K. Ranjendran, T. Bhattacharya, S. Yamasaki, S. K. Bhattacharya, Y. Takeda, G. B., Nair, and T. Ramamurthy. 2000. Expanding multiple antibiotic resistance among clinical strains of *Vibrio cholerae* isolated from 1992–7 in Calcutta, India. *Epidemiol. Infect.* **124**:393–399.
7. Garg, P., R. K. Nandy, P. Chaudhury, N. R. Chowdhury, K. De, T. Ramamurthy, S. Yamasaki, S. K. Bhattacharya, Y. Takeda, and G. B. Nair. 2000. Emergence of *Vibrio cholerae* O1 biotype El Tor serotype Inaba from the prevailing O1 Ogawa serotype strains in India. *J. Clin. Microbiol.* **38**:4249–4253.
8. Ito, T., K. Hiramatsu, Y. Ohshita, and T. Yokota. 1993. Mutations in the *rfbT* gene are responsible for the Ogawa to Inaba serotype conversion in *Vibrio cholerae* O1. *Microbiol. Immunol.* **37**:281–288.
9. Kimsey, H. H., G. B. Nair, A. Ghose, and M. K. Waldor. 1998. Diverse CTX ϕ and evolution of new pathogenic *Vibrio cholerae*. *Lancet* **352**:457–458.
10. Longini, I. M., Jr., M. Yunus, K. Zaman, A. K. Siddique, R. B. Sack, and A. Nizam. 2002. Epidemic and endemic cholera trends over a 33-year period in Bangladesh. *J. Infect. Dis.* **186**:246–251.
11. Mishra, M., F. Mohammed, S. L. Akulwar, V. J. Katkar, N. S. Tankhiwale, and R. M. Powar. 2004. Re-emergence of El Tor *Vibrio* in outbreak of cholera in and around Nagpur. *Indian J. Med. Res.* **120**:478–480.
12. Nair, G. B., S. M. Faruque, N. A. Bhuiyan, M. Kamruzzaman, A. K. Siddique, and D. A. Sack. 2002. New variants of *Vibrio cholerae* O1 biotype El Tor with attributes of the classical biotype from hospitalized patients with acute diarrhea in Bangladesh. *J. Clin. Microbiol.* **40**:3296–3299.
13. Popovic, T., C. Bopp, O. Olsvik, and K. Wachsmuth. 1993. Epidemiologic application of a standardized ribotyping scheme of *Vibrio cholerae* O1. *J. Clin. Microbiol.* **31**:2474–2482.
14. Ramamurthy, T., S. Yamasaki, Y. Takeda, and G. B. Nair. 2003. *Vibrio cholerae* O139 Bengal: odyssey of a fortuitous variant. *Microbes Infect.* **5**:329–344.
15. Rijpkema, S. G., Z. Durrani, T. Ramamurthy, and G. B. Nair. 2004. Assessing clonality of *Vibrio cholerae* Inaba isolates by characterization of nonsense mutations in *wbeT*. *J. Med. Microbiol.* **53**:1105–1107.
16. Sharma, C., G. B. Nair, A. K. Mukhopadhyay, S. K. Bhattacharya, R. K. Ghosh, and A. Ghosh. 1997. Molecular characterization of *Vibrio cholerae* O1 biotype El Tor strains isolated between 1992 and 1995 in Calcutta, India: evidence for the emergence of a new clone of El Tor biotype. *J. Infect. Dis.* **175**:1134–1141.
17. Sundaram, S. P., J. Revathi, B. L. Sarkar, and S. K. Bhattacharya. 2002. Bacteriological profile of cholera in Tamil Nadu. *Indian J. Med. Res.* **116**:258–263.
18. Swerdlow, D. L., G. Malenga, G. Begkoyian, D. Nyangulu, M. Toole, R. J. Waldman, D. N. Puh, and R. V. Tauxe. 1997. Epidemic cholera among refugees in Malawi, Africa: treatment and transmission. *Epidemiol. Infect.* **118**:207–214.
19. Verenkar, M., R. Savio, N. Venkatesh, M. J. Pinto, and I. Singh. 1994. Cholera in Goa. *Indian J. Pathol. Microbiol.* **37**:289–292.
20. Yamasaki, S., G. B. Nair, S. K. Bhattacharya, S. Yamamoto, H. Kurazono, and Y. Takeda. 1997. Cryptic appearance of a new clone of *Vibrio cholerae* O1 biotype El Tor in Calcutta, India. *Microbiol. Immunol.* **41**:1–6.