# Emergence of a New Cholera Pandemic: Molecular Analysis of Virulence Determinants in *Vibrio cholerae* O139 and Development of a Live Vaccine Prototype

**Matthew K. Waldor and John J. Mekalanos**

*Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts*

**In October 1992, a non-O1 strain of *Vibrio cholerae* emerged as a cause of epidemic cholera in India and Bangladesh. This antigenically novel clone has been designated serogroup O139 synonym Bengal. Since its emergence, *V. cholerae* O139 has caused a massive cholera epidemic throughout and beyond the Indian subcontinent. Molecular analysis of virulence determinants in clinical isolates suggests that O139 strains are highly related to El Tor O1 strains. Unlike other non-O1 strains, O139 strains carry multiple copies of the cholera toxin genetic element and also genes for the toxin-coregulated pilus. These results guided construction of a live *V. cholerae* O139 vaccine prototype through deletion of genes for at least four specific virulence determinants (*ctxA, ace, zot,* and *cep*) as well as other factors involved in site-specific and homologous recombination (RS1, attRS1, and *recA*). It is hoped that this attenuated live vaccine will help control the pandemic spread of *V. cholerae* O139.**

Two letters published in the 13 March 1993 issue of *Lancet* [1, 2] reported a dramatic change in the cause of cholera on the Indian subcontinent. Large outbreaks of cholera-like diarrheal disease in India and Bangladesh were caused by a strain of *Vibrio cholerae* that produced cholera toxin but was untypeable with a panel of antisera to all 138 known serogroups of *V. cholerae* [1, 2]. Before these reports, only the O1 serogroup of *V. cholerae* had been associated with cholera; some of the 137 non-O1 serogroups of *V. cholerae* have been associated with sporadic cases of diarrhea and extraintestinal infections, but not with cholera [3]. Shimada et al. [4] prepared a rabbit antiserum against the novel non-O1 *V. cholerae* strain and designated the new serogroup O139 with the suggested synonym "Bengal" to indicate its origin in the region surrounding the Bay of Bengal.

## Spread of *V. cholerae* O139

Since the first identification of *V. cholerae* O139 strains from outbreaks in Madras, India, in October 1992 and in southern Bangladesh in December 1992, there has been an explosive spread of this organism throughout the Indian subcontinent with a concomitant major cholera epidemic. In only the first 3 months of 1993, there were 107,297 cases of diarrhea and 1473 deaths from *V. cholerae* O139 strains in Bangladesh alone [5]. *V. cholerae* O139 strains have replaced the resident El Tor O1 strains of *V. cholerae* as the predominant cause of cholera in India [6] and Bangladesh [5]. Also, *V. cholerae* O139 strains have been isolated from surface water samples in Bangladesh at a much higher frequency than *V. cholerae* O1 strains, suggesting that O139 strains have an advantage in the environment over O1 strains [7].

*V. cholerae* O139 has now spread outside of the Indian subcontinent. Recently there have been reports of O139 cholera cases in Thailand [8], Nepal, western China, Pakistan, and Malaysia. There has been one imported case of O139 cholera in the United States in a traveler returning from India [9]. Given the rapid spread of *V. cholerae* O139 throughout and beyond the Indian subcontinent, the pandemic potential of these strains seems assured.

## Clinical and Microbiologic Features of *Vibrio cholerae* O139

The clinical features of disease produced by *V. cholerae* O139 are essentially identical to those of disease induced by *V. cholerae* O1. Thus, most patients present with severe watery diarrhea, vomiting, dehydration, and the absence of fever [5, 10]. There may be a higher frequency of abdominal cramps with O139 disease [10]. Treatment of O139 cholera cases with intravenous and oral rehydration fluids along with tetracycline, an antibiotic to which O139 strains are sensitive, has been very effective [5]. Of importance, O139 strains are resistant to trimethoprim-sulfamethoxazole and furazoli-

Received 1 February 1994; revised 17 March 1994.

Presented: Infectious Diseases Society of America meeting, October 1993, New Orleans.

Animal experimentation was done in accordance with the guidelines of Harvard Medical School and the National Institutes of Health.

J.J.M. is a stockholder in Virus Research Institute Inc., Cambridge, Massachusetts. This company has licensed the vaccine constructs described here from Harvard University.

Grant support: National Institutes of Health (AI-18045). M.K.W. is a postdoctoral fellow of the Howard Hughes Medical Institute.

Reprints or correspondence: Dr. Matthew Waldor, Dept. of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Ave., Boston, MA 02115.

**The Journal of Infectious Diseases 1994;170:278–83**

0022–1899/94/7002–0004$01.00

done, two antibiotics commonly used to treat O1 strains of *V. cholerae*.

Epidemiologic data have revealed an important difference in the age distributions of O139 and O1 cholera cases: The majority of O139 cholera cases have occurred in adults, while the majority of O1 cases occur in children [5, 11]. The susceptibility of the adult population to O139 strains in a region where O1 strains are endemic suggests that prior immunity to *V. cholerae* O1 does not protect against O139 [5].

The initial microbiologic assessments of *V. cholerae* O139 revealed that these strains shared many characteristics with O1 strains. O139 strains are motile gram-negative rods [5]. Like O1 strains, O139 strains produce yellow colonies on thiosulfate citrate bile salts sucrose agar, a growth medium traditionally used in the isolation of *V. cholerae*. O139 strains do not type with any of the phages used for typing O1 strains [5]. Two biotypes of the O1 serogroup are distinguished, El Tor and classical. Like El Tor O1 strains, O139 strains agglutinate chicken red blood cells and are resistant to polymyxin B [5]. This finding, along with other genetic data discussed below, suggests that O139 strains are closely related to El Tor O1 strains.

After the initial outbreaks of O139 cholera cases were reported from India and Bangladesh, Yoshifumi Takeda (Kyoto University, Japan) and G. Balakrish Nair (National Institute of Cholera and Endemic Diseases, Calcutta) generously sent our laboratory a collection of clinical isolates of *V. cholerae* O139 strains for molecular analysis of the genes encoding some of the known virulence factors of *V. cholerae* O1.

## Virulence genes in *V. cholerae* O139

Molecular genetic studies of *V. cholerae* O1 strains have revealed that the genes encoding cholera toxin, the *ctxAB* operon, are part of a larger genetic element termed the cholera toxin genetic element (figure 1). The CTX genetic element consists of a core region flanked by two or more copies of a 2.7-kb repetitive sequence called RS1. The core region is a 4.2-kb DNA segment that carries the *ctxAB* operon, *zot* (which encodes the zonula occludens toxin [12]), *ace* (which encodes accessory cholera enterotoxin [13]), and *cep* (which encodes a pilin-like intestinal colonization factor [14]). The RS1 sequences encode a site-specific recombination system that mediates recombination between the CTX genetic element and an 18-bp target sequence on the *V. cholerae* chromosome termed attRS1 (figure 1). In composite, the CTX genetic element can be thought of as a site-specific transposon [14].

Using a series of Southern blots on chromosomal DNA purified from 3 clinical isolates of O139 strains (MO10, MO45, and MDO14) from the 1993 epidemic in India, we ascertained the structure of the CTX genetic element in these strains. Several representative *V. cholerae* O1 and non-O1 strains were used as controls. Probes for *ctxA* (CT-A1), *zot* (L-1), and RS1 (RS1*) were used for these analyses. As shown in figure 2 (top), all O1 and O139 strains reacted strongly with the L-1, RS1*, and CT-A1 probes. In contrast, 3 other non-O1 strains from India that were not associated with epidemic disease (VO7, SG38, and SG33) did not react with any of these probes. Additional structural analysis using other restriction enzyme digests allowed us to deduce the probable structures of the CTX genetic element in these strains (figure 2, bottom). An isolate from the 1991 Peruvian cholera epidemic, C6709, contained one copy of the CTX element core region flanked by two RS1 copies. In contrast, all of the O139 strains carried at least two copies of the core region flanked by variable numbers of RS1 copies (figure 2). These copies were tandemly arranged as evidenced by *Xba*I fragment numbers 4 and 6, which hybridized to both CT-A1 and L-1 probes [15]. The intensity with which some of these tandem repeat bands hybridized to the probes (i.e., band 4 vs. band 8 for MO10), along with other data not shown, indicate that some of these isolates (e.g., MO10) carry three or more copies of the CTX genetic element. The finding of multiple copies of the CTX virulence cassette in O139 strains suggests that duplication and amplification of the CTX genetic element may play a role in the virulence of O139 strains as has been previously suggested for O1 strains [15].

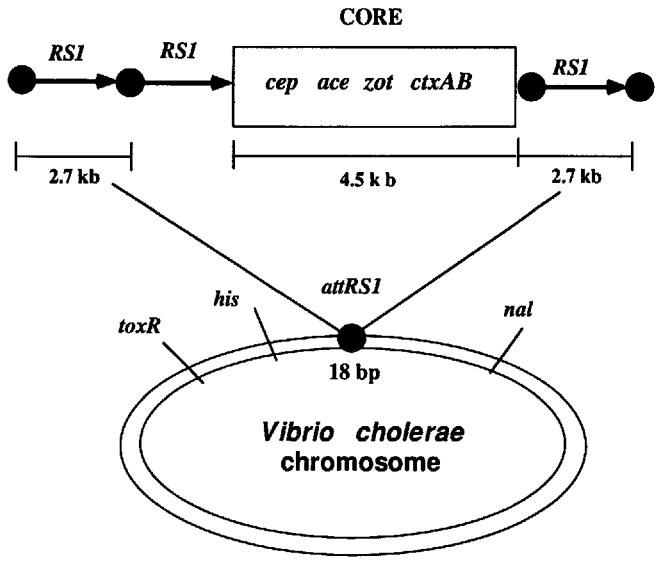


**Figure 1.** Schematic depiction of CTX genetic element, a site-specific transposon. Arrows depict RS1 sequences; box, core region; circle, attRS1 sequences.

Expression of several of the virulence factors in *V. cholerae* O1 strains is coordinately regulated by the transcriptional activator ToxR [19]. ToxR-regulated virulence factors include cholera toxin and toxin-coregulated pilus (TCP), an essential factor for colonization of the small intestine [20, 21]. Initial Southern blots on the collection of O139 strains showed that all of these strains contained sequences that hy-

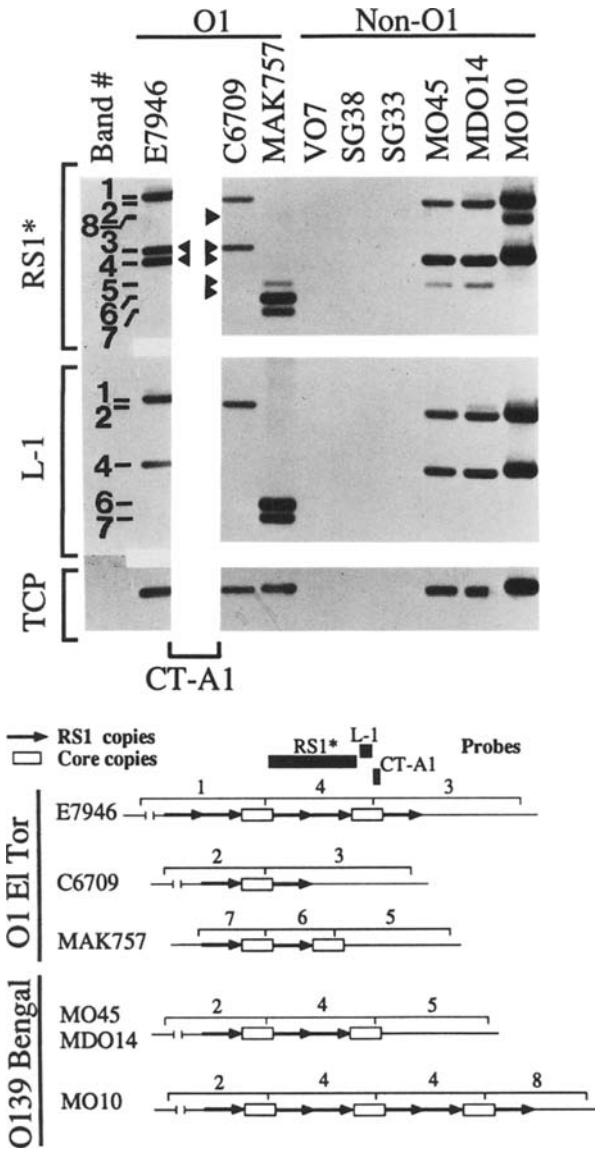


**Figure 2.** Top, Southern blot analysis of virulence genes of O1 (E7946, C6709, and MAK757 [El Tor O1], isolated in Bahrain, Peru, and Sulawesi, respectively) and non-O1 (VO7, SG38, SG33 [serogroups O37, O74, and O39, respectively] and MO45, MDO14 and MO10 [all serogroup O139], isolated in India) strains. Chromosomal DNA was purified, digested with *Xba*I, and analyzed by Southern blot hybridization as described [15]. Four probes were used: RS1*, corresponding to plasmid pGP20 [14]; L-1, corresponding to 2.7-kb *Pst*I-*Xba*I fragment of pJM17 [16]; TCP, corresponding to plasmid pRTG7H3 [17]; and CT-A1, corresponding to 0.55-kb *Xba*I-*Cla*I fragment of pJM17. Arrowheads = specific bands that also hybridized to the CT-A1 probe. Band numbers correspond to schematic diagram below. Bottom, schematic diagram (not to scale) of CTX element structure deduced from Southern blot analysis. Horizontal arrows and open boxes correspond to copies of RS1 and core region of CTX genetic element, respectively [15, 18]. Structures shown for E7946 and MAK757 (same as RV79) [15] have been previously reported and confirmed by molecular cloning and restriction mapping [15]. Locations of probes RS1*, L-1, and CT-A1 are shown. Numbered brackets correspond to bands in top part of figure.

bridized with a *toxR* probe [22]. We then probed the collection of O139 strains for the presence of sequences homologous to the *tcp* operon. All O139 strains examined displayed the same characteristic 5-kb *Xba*I fragment that hybridized to the *tcp* probe (figure 2). In contrast, the 3 other non-O1 strains, VO7, SG38, and SG33, were similar to previously examined non-O1 strains [23] in that they lacked detectable sequences hybridizing to the *tcp* probe. Additional Southern analysis showed that all Bengal O139 strains, like all El Tor O1 strains examined, displayed a 2.1-kb *Pst*I fragment that hybridized to the *tcp* probe; in contrast, classical O1 strains displayed a 1.4-kb *Pst*I hybridizing fragment [22, 23]. This restriction fragment polymorphism supports an El Tor origin for the O139 strains. Hall et al. [24] also observed the similarity of *tcp* in O139 and El Tor O1 strains using polymerase chain reaction amplification of *tcp* sequences in an El Tor O1 and an O139 strain.

We have shown that the *tcp*-homologous sequences present in O139 strains express TCP antigen with similar immunologic reactivity to that produced by O1 strains [22]. The environmental conditions that lead to the expression of cholera toxin and TCP in the laboratory are different for El Tor and classical strains of *V. cholerae* O1. We have recently found that the laboratory conditions that enhance expression of these virulence factors in El Tor strains are the same for O139 strains [22]. This is another indication that O139 and El Tor O1 strains are closely related. TCP antigen is expressed by O139 strains in a ToxR-dependent manner, and *toxR*-null derivatives of O139 strains are defective in intestinal colonization of experimental animals [22]. These results suggest that as in O1 strains [20, 21], TCP is an essential intestinal colonization factor for the novel O139 strains.

Study of the immune response in convalescent cholera patients have shown that TCP is weakly immunogenic [25]. This is in contrast to the prominent, protective immune response induced against other pilus colonization factor antigens produced by, for example, enterotoxigenic *Escherichia coli* [26]. These observations suggest that the low immunogenicity of TCP may contribute to the ability of O139 strains to cause disease in populations already immune to endemic $TCP^+$ *V. cholerae* O1 strains. The apparent amplification of the CTX genetic element, with the expected concomitant increase in *ctx*, *zot*, *ace*, and *cep* expression, might also contribute to the virulence of O139 strains in populations living in cholera-endemic areas, in which immune responses to these gene products are probably prevalent because of prior exposure to *V. cholerae* O1 strains. These results support the concept that immune responses directed against the major virulence determinants of an organism may not be protective if these determinants have evolved to be weakly immunogenic or, alternatively, if the organism simply overproduces the target antigens through regulatory or genetic strategies. This conclusion is also consistent with the notion that the immune response to the major somatic antigen of *V. cholerae*

(presumably the O antigen of lipopolysaccharide) is the most significant factor in immunity to cholera rather than immune responses to TCP, cholera toxin, or other virulence determinants shared by O1 and O139 strains of *V. cholerae.*

## Development of a Live *V. cholerae* O139 Vaccine Prototype

Our results suggest that deletion of the virulence determinants associated with the CTX genetic element might not adversely affect the immunogenicity of live oral cholera vaccines providing that they still express the O139 antigen and still colonize the intestinal mucosa. Ideally, such a deletion should interfere with reacquisition of the CTX genetic element by site-specific recombination. Accordingly, we have adopted a strategy for development of live attenuated O139 vaccine strains that has been previously used in the development of live attenuated El Tor vaccine strains [27] (unpublished data). Plasmid pAR62 carries a defined deletion encompassing the entire CTX element (including RS1 sequences and the attRS1 integration site) flanked by adjacent chromosomal DNA [14]. When recombined onto the chromosome of the El Tor strain E7946, this deletion eliminated site-specific recombination associated with the CTX genetic element [14]. Plasmid pAR62 was transformed into MO10, a *V. cholerae* O139 clinical isolate from Madras, India, and then colony hybridization was used to find recombinants that had lost chromosomal sequences hybridizing to the RS1* probe. Figure 3A is a schematic depicting the construction of one such recombinant derivative of MO10, Bengal-2. DNA from Bengal-2 did not hybridize with the RS1* probe or with the CT-A1 or L-1 probes in Southern blot analysis (not shown). Additional analysis confirmed that the deletion present on pAR62 had indeed been recombined into the chromosome of Bengal-2. This result indicates that the copies of the CTX element present in the O139 strain MO10 are inserted into the same chromosomal site, attRS1, occupied by the CTX genetic element in other El Tor strains such as E7946 [14]. This provides another indication that O139 strains are closely related to El Tor O1 strains.

The large attRS1 deletion [14] introduced into MO10 with pAR62 to create the vaccine prototype strain Bengal-2 removes the genes for the virulence factors encoded by *ctxAB, zot, cep,* and *ace.* However, the O139 somatic antigen of Bengal-2 remains intact, and rabbits immunized with Bengal-2 mount a strong anti-O139 antibody response. Previous studies have shown a correlation of intestinal colonization in experimental animals with immunogenicity of live cholera vaccines [28]. Bengal-2 colonizes the small intestines of suckling mice almost as well as the parental strain MO10 (data not shown). Therefore, the colonization properties of Bengal-2 are consistent with its use as an effective live vaccine.

Bengal-2 is expected to be incapable of reacquiring the

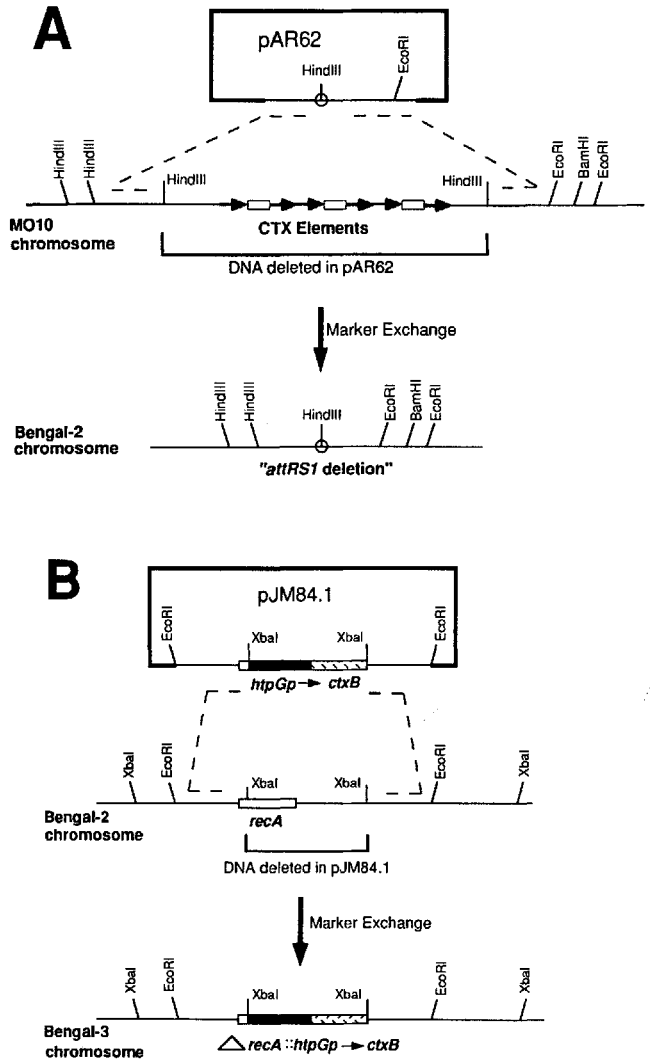


**Figure 3.** Schematic depiction of constructions of Bengal-2 (**A**) and Bengal-3 (**B**), vaccine derivatives of O139 strain MO10. Plasmid pAR62, which carries defined deletion of entire CTX element [14], was transferred into strain MO10 by conjugation. Recombination depicted by dashed lines resulted in attRS1 deletion in MO10. This MO10 derivative was named Bengal-2. Plasmid pJM84.1 [27], which contains *ctxB* under control of htpG promoter (*htpGp*) cloned into *Xba*I deletion in *recA* locus, was then transferred into Bengal-2 by conjugation. Recombination depicted by dashed lines gave rise to Bengal-3. Chromosomal structures of Bengal-2 and Bengal-3 were confirmed by Southern analysis.

CTX genetic element by site-specific recombination because it lacks RS1 and the attRS1 site, two critical components in CTX element transposition [14]. However, because *V. cholerae* is capable of gene transfer by transduction, conjugation, and transformation, Bengal-2 could potentially reacquire the CTX genetic element from toxigenic strains in nature by homologous recombination. Therefore we have constructed *recA* derivatives of Bengal-2. We used a strategy that not only introduced a deletion into the *recA* gene but

also simultaneously inserted a construct encoding *ctxB* under control of the *htpG* heat-shock promoter [29] (figure 3B). The resultant derivative, Bengal-3, when grown in vitro, produces levels of the B subunit of cholera toxin that exceed those produced by the parental strain MO10 by 25-fold. The B subunit of cholera toxin is nontoxic but has been shown to induce some short-term immunity to cholera and enterotoxigenic *E. coli* [30]. The combination of the attRS1 deletion with *recA* provides an unprecedented level of safety from possible reversion to enterotoxicity by reacquisition of the transposable CTX genetic element. Also, the *recA* mutation in Bengal-3 renders this strain highly sensitive to uv light. Since O139 strains seem particularly adapted to survival in surface water [7], the *recA* mutation in Bengal-3 should help ensure that this live vaccine strain will be short lived in the environment. Bengal-3, like Bengal-2, expresses the O139 antigen and colonizes suckling mice (data not shown). Thus, Bengal-3 has many properties that recommend it as a live vaccine candidate. These properties include the deletion of all of the CTX genetic element–encoded virulence genes (*cep*, *zot*, *ace*, and *ctxAB*), the deletion of the recombination systems that could potentially allow the strain to revert to enterotoxicity (RS1, attRS1, and *recA*), the deletion of a locus that should render the strain less fit to survive in the environment (*recA*), the continued expression of the presumed major protective antigen (O139), the ability to colonize the small intestine, and the overexpression of a protein that should enhance the protective response of the vaccine against both cholera and enterotoxic *E. coli* (B subunit of cholera toxin). Human volunteer studies to evaluate the safety and efficacy of Bengal-3 are ongoing.

## Conclusions

The notion that only strains in the O1 serogroup of *V. cholerae* can give rise to epidemic and pandemic cholera has been firmly contradicted by the rapid spread of the O139 cholera epidemic originating in southeastern India. The emergence of *V. cholerae* O139 poses a challenge to contemporary microbiologists to explain the molecular bases of the origin and selective advantage of this new variant of an ancient pathogen. Investigation of the genetic structures and expression of the virulence factors of O139 strains suggest that these strains are closely related to El Tor O1 strains of *V. cholerae*. This raises the possibility that *V. cholerae* O139 arose from a *V. cholerae* El Tor O1 strain that had adquired novel genes encoding the biosynthesis of the O139 antigen through recombination. The human immune response against the O1 O antigen or some environmental factor may have selected for such a recombinant strain. Identification of the genes encoding the O139 antigen and the chemical structure of this antigen will help to further clarify the origins of *V. cholerae* O139. The factors that have allowed O139 strains to replace O1 strains both as a cause of cholera and in the environment are not yet clearly defined.

Though important questions concerning the evolution and spread of O139 strains remain, our analysis of the CTX genetic element in these strains has served to guide the construction of the first live *V. cholerae* O139 vaccine prototypes. A derivative of an O139 strain was constructed through the deletion of the CTX genetic element–associated virulence determinants as well as other factors involved in CTX genetic element site-specific and homologous recombination. We hope that this live vaccine strain will aid in controlling the pandemic spread of *V. cholerae* O139.

## Acknowledgments

We thank Y. Takeda and G. B. Nair for providing us with O139 and non-O1 *V. cholerae* strains and T. Shimada for the gift of O139 antisera; Gregory Pearson, Ronald Taylor, and Angela Woods for providing valuable plasmid constructs; and Jerald Sadoff for his encouragement and helpful discussions.

## References

1. Albert MJ, Siddique AK, Islam MS, et al. Large outbreak of clinical cholera due to *Vibrio cholerae* non-O1 in Bangladesh. Lancet 1993;341:704.
2. Ramamurthy T, Garg S, Sharma R, et al. Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. Lancet 1993;341:703–4.
3. Morris JG. Non-O group 1 *Vibrio cholerae:* a look at the epidemiology of an occasional pathogen. Epidemiol Rev 1990;12:179–91.
4. Shimada T, Nair GB, Deb BC, Albert MJ, Sack RB, Takeda Y. Outbreak of *Vibrio cholerae* non-O1 in India and Bangladesh. Lancet 1993;341:1347.
5. Cholera Working Group. Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. Lancet 1993;342:387–90.
6. Garg S, Saha PK, Ramamurthy T, Nair GN, Shimada T, Takeda Y. Nationwide prevalence of the new epidemic strain of *Vibrio cholerae* O139 Bengal in India. J Infect 1993;27:108–9.
7. Islam MS, Hasan MK, Miah MA, et al. Isolation of *Vibrio cholerae* O139 Bengal from water in Bangladesh. Lancet 1993;342:430.
8. Chongsa-nguan M, Chaicumpa W, Moolasart P, et al. *Vibrio cholerae* O139 Bengal in Bangkok. Lancet 1993;342:430–1.
9. Centers for Disease Control and Prevention. Imported cholera associated with a newly described toxigenic *Vibrio cholerae* O139 strain—California, 1993. MMWR Morb Mortal Wkly Rep 1993;42:501–2.
10. Bhattacharya SK, Bhattacharya MK, Nair GB, et al. Clinical profile of acute diarrhoea cases infected with the new epidemic strain of *Vibrio cholerae* O139: designation of the disease as cholera. J Infect 1993;27:11–5.
11. Siddique AK, Zaman K, Baqui AH, et al. Cholera epidemics in Bangladesh: 1985–1991. J Diarrhoeal Dis Res 1992;10:79–86.
12. Baudry B, Fasano A, Ketley J, Kaper JB. Cloning of a gene (*zot*) encoding a new toxin produced by *Vibrio cholerae*. Infect Immun 1992;60:428–34.

13. Trucksis M, Galen J, Michalski J, Fasano A, Kaper JB. Accessory cholera enterotoxin (Ace), the third toxin of a *Vibrio cholerae* virulence cassette. Proc Natl Acad Sci USA **1993**;90:5267–71.
14. Pearson GDN, Woods A, Chiang SL, Mekalanos JJ. CTX genetic element encodes a site-specific recombination system and an intestinal colonization factor. Proc Natl Acad Sci USA **1993**;90:3750–4.
15. Mekalanos JJ. Duplication and amplification of toxin genes in *Vibrio cholerae*. Cell **1983**;35:253–63.
16. Mekalanos JJ, Swartz DJ, Pearson GD, Harford N, Groyne F de WM. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature **1983**;306:551–7.
17. Shaw CE, Taylor RK. *Vibrio cholerae* O395 *tcpA* pilin gene sequence and comparison of predicted protein structural features to those of type 4 pilins. Infect Immun **1990**;58:3042–9.
18. Goldberg I, Mekalanos JJ. Effect of a *recA* mutation on cholera toxin gene amplification and deletion events. J Bacteriol **1986**;165:723–31.
19. DiRita VJ. Co-ordinate expression of virulence genes by ToxR in *Vibrio cholerae*. Mol Microbiol **1992**;6:451–8.
20. Taylor RK, Miller VL, Furlong DB, Mekalanos JJ. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc Natl Acad Sci USA **1987**;84:2833–7.
21. Herrington DA, Hall RH, Losonsky G, Mekalanos JJ, Taylor RK, Levine MM. Toxin, toxin-coregulated pili, and the *toxR* regulon are essential for *Vibrio cholerae* pathogenesis in humans. J Exp Med **1988**;168:1487–92.
22. Waldor MK, Mekalanos JJ. ToxR regulates virulence gene expression in non-O1 strains of *Vibrio cholerae* that cause epidemic cholera. Infect Immun **1994**;62:72–8.
23. Taylor R, Shaw C, Peterson K, Spears P, Mekalanos J. Safe, live *Vibrio cholerae* vaccines? Vaccine **1988**;6:151–4.
24. Hall RH, Khambaty FM, Kothary M, Keasler SD. Non-O1 *Vibrio cholerae*. Lancet **1993**;342:430.
25. Hall RH, Losonsky G, Silveira AP, et al. Immunogenicity of *Vibrio cholerae* O1 toxin-coregulated pili in experimental and clinical cholera. Infect Immun **1991**;59:2508–12.
26. Levine MM, Ristaino P, Marley G, et al. Coli surface antigens 1 and 3 of colonization factor antigen II–positive enterotoxigenic *Escherichia coli:* morphology, purification, and immune response in humans. Infect Immun **1984**;44:409–20.
27. Roberts A, Pearson GD, Mekalanos JJ. Cholera vaccine strains derived from a 1991 Peruvian isolate of *Vibrio cholerae* and other El Tor strains. In: Proceedings of the 28th Joint Conference, U.S.–Japan Cooperative Medical Science Program on Cholera and Related Diarrheal Diseases. Bethesda, MD: NIH, **1992**.
28. Pierce NF, Cray WJ, Kaper JB, Mekalanos JJ. Determinants of immunogenicity and mechanisms of protection by virulent and mutant *Vibrio cholerae* O1 in rabbits. Infect Immun **1988**;56:142–8.
29. Parsot C, Mekalanos JJ. Expression of *toxR*, the transcriptional activator of the virulence factors in *Vibrio cholerae*, is modulated by the heat-shock response. Proc Natl Acad Sci USA **1990**;87:9898–902.
30. Clemens JD, Sack DA, Harris JR, et al. Cross-protection by B subunit–whole cell cholera vaccine against diarrhea associated with heat-labile toxin–producing enterotoxigenic *Escherichia coli:* results of a large-scale field trial. J Infect Dis **1988**;158:372–7.