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International Dissemination of Epidemic *Vibrio cholerae* by Cargo Ship Ballast and Other Nonpotable Waters

SUSAN A. McCARTHY[1]* AND FARUKH M. KHAMBATY[2]

Gulf Coast Seafood Laboratory, Food and Drug Administration, Dauphin Island, Alabama 36528,[1] and Division of Microbiological Studies, Food and Drug Administration, Washington, D.C. 20204[2]

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In 1991 and 1992, toxigenic *Vibrio cholerae* O1, serotype Inaba, biotype El Tor, was recovered from nonpotable (ballast, bilge, and sewage) water from five cargo ships docked in ports of the U.S. Gulf of Mexico. Four of these ships had taken on ballast water in cholera-infected countries; the fifth took on ballast in a noninfected country. Isolates examined by pulsed-field gel electrophoresis were indistinguishable from the Latin American epidemic strain, C6707; however, they differed significantly from the endemic Gulf Coast strain (VRL 1984), the sixth-pandemic strain (569-B), and a *V. cholerae* non-O1 strain isolated from a ship arriving from a foreign port. On the basis of our findings, the Food and Drug Administration recommended that the U.S. Coast Guard issue an advisory to shipping agents and captains requesting that ballast waters be exchanged on the high seas before entry of ships into U.S. ports.

In January 1991, epidemic cholera was reported in Peru (4, 5) and rapidly spread through Latin America and into Mexico. In July and September 1991 and June 1992, toxigenic *Vibrio cholerae* O1 was isolated from five oysters and one fish collected from Mobile Bay, Ala. (6, 7, 24). Biochemical and serological characterization of the isolates showed their resemblance to the Latin American epidemic strain, *V. cholerae* O1, biotype El Tor, serotype Inaba.

Since 1973, 91 sporadic cholera cases unrelated to the Latin American epidemic have occurred in the United States. Most of these cases, which were related to the consumption of raw or undercooked shellfish from the Gulf of Mexico (2, 25), were caused by toxigenic *V. cholerae* O1 strain VRL 1984, which is endemic to the Gulf Coast (6, 17). Unlike strain VRL 1984, the Latin American strain lacks hemolysin and the VcA-3 vibriophage, and the strains differ in their cholera toxin gene patterns on Southern blots (31).

We investigated gas exploration and marine transportation to determine if these activities might serve to introduce the Latin American strain locally. The toxigenic strains isolated during this investigation were characterized by biochemical and serological tests, PCR, and pulsed-field gel electrophoresis (PFGE). Our results showed that cargo ships are vehicles for the transmission of epidemic cholera. In order to manage the problem, it was recommended that ships exchange their ballast water while on the high seas. (Preliminary accounts of this work were presented elsewhere [19, 20].)

MATERIALS AND METHODS

Sample collection and analysis. Along with U.S. Coast Guard authorities, Food and Drug Administration (FDA) investigators boarded vessels to collect samples from 19 cargo ships at the ports of Mobile, Ala., and Gulfport and Pascagoula, Miss. (Table 1). Samples (50 to 100 ml) from ballast tanks, fire mains, bilge tanks, oily-water separators, holding tanks, or marine sanitation devices were collected into sterile polyethylene Falcon containers and maintained on ice until processing within 18 h. The salinity and pH of ballast waters were determined. Salinity was measured with a Reichert refractometer (Cambridge Instruments, Inc., Buffalo, N.Y.); pH was measured with ColorpHast Store paper (EM Science, Cherry Hill, N.J.).

To detect the presence of *V. cholerae*, 10-fold serial dilutions of each test sample were inoculated into alkaline peptone water (APW); duplicate sets of APW enrichments were incubated at 35 and 42°C for 18 to 24 h. From evident pellicles, 3- to 5-mm loopfuls of inoculum were transferred to thiosulfate-citrate-bile salts-sucrose (TCBS) agar and incubated at 35°C for 18 to 24 h; suspect colonies on TCBS were streaked to tryptone salt (T_1N_1 [1% tryptone, 1% NaCl]) (9) agar. If no pellicles were evident in APW enrichments at 24 h, incubation was extended an additional 48 to 72 h at room temperature. Numbers of fecal coliforms per 100 ml were determined by the membrane filter method (8).

Biochemical and serological characterization of suspect *V. cholerae* isolates. *V. cholerae* isolates were characterized by the oxidase reaction, the string test in 0.5% sodium desoxycholate (29), growth in 1% tryptone broth, and biochemical reactions in API 20E strips (Analytab Products, Plainview, N.Y.).

Biotype determinations were based on Voges-Proskauer reaction and resistance to polymyxin B on Mueller-Hinton agar (9). *V. cholerae* antigen was typed by serological agglutination with *V. cholerae* polyvalent antiserum (Difco Laboratories, Detroit, Mich.) and by slide latex agglutination with the *V. cholerae* AD Seiken kit (Denka Seiken Co., Ltd., Tokyo, Japan).

Determination of toxigenicity. *V. cholerae* enterotoxin was identified by reversed passive latex agglutination (RPLA) (VET-RPLA; Oxoid, Basingstoke, Hampshire, United Kingdom); cultures were grown in AKI medium before testing (9). The PCR was used to detect the A subunit of the cholera toxin gene from 1-ml APW enrichments (10). CTX2 and CTX3 primers were obtained from the Enteric Diseases Branch, Division of Bacterial and Mycotic Diseases, Centers for Disease Control, *Taq* polymerase, deoxynucleoside triphosphates, and 10× PCR buffer were obtained from Perkin-Elmer Cetus, Norwalk, Conn. The PCR was performed with a Hybaid thermal reactor (National Labnet Co., Woodbridge, N.J.).

* Corresponding author. Mailing address: FDA Gulf Coast Seafood Laboratory, P.O. Box 158, Dauphin Island, AL 36528. Phone: (205) 694-4480. Fax: (205) 694-4477. Electronic mail address: SAM@FDACF.SSW.DHHS.GOV.

TABLE 1. Description of ships sampled

Ship	Date[a]	Location	LPC[b]
1	10/30/91	Mobile, Ala.	Japan
2	10/30/91	Mobile, Ala.	South Africa
3	10/31/91	Mobile, Ala.	Mexico
4	11/01/91	Mobile, Ala.	Nova Scotia
5	11/01/91	Mobile, Ala.	Venezuela
6	11/05/91	Mobile, Ala.	Louisiana
7	11/05/91	Mobile, Ala.	Puerto Rico
8	11/06/91	Mobile, Ala.	Brazil
9	11/07/91	Mobile, Ala.	Colombia
10	11/19/91	Pascagoula, Miss.	Chile
11	11/25/91	Gulfport, Miss.	Venezuela
12	11/25/91	Gulfport, Miss.	Venezuela
13	11/26/91	Mobile, Ala.	Venezuela
14	11/26/91	Mobile, Ala.	Venezuela
15	12/02/91	Mobile, Ala.	Venezuela
16	12/03/91	Mobile, Ala.	Venezuela
17	12/03/91	Mobile, Ala.	Venezuela
18	06/11/92	Mobile, Ala.	Puerto Rico
19	06/12/92	Mobile, Ala.	Brazil

[a] Month/day/year.
[b] LPC, last port of call.

Amplified DNA was analyzed by electrophoresis through a 0.8% agarose gel in Tris-borate-EDTA buffer at 90 V for 1.5 to 2 h. The gel was stained for 10 min in a solution of ethidium bromide (2.0 μg/ml) and photographed under UV light. Control organisms used were *V. cholerae* O1 El Tor strains C6706 and C6707 from the Latin American epidemic, *V. cholerae* O1 El Tor strain VRL 1984 (the Gulf Coast strain), *V. cholerae* O1 classical strain 569-B, and *V. cholerae* non-O1 strain 566-454 isolated from sewage from a ship that had a last port of call in Mexico.

PFGE. Agarose plugs containing DNA were prepared by a modification of the method of Smith and Cantor (28). Cells were grown aerobically at 35°C in Luria-Bertani broth to an A_{610} of about 0.6. From each culture, 5 ml was centrifuged (12,000 × *g* for 10 min); the cells were washed once and resuspended in 5 ml of an ice-cold solution of 10 mM Tris-Cl, 1.0 M NaCl, and 50 mM EDTA (pH 8.0). A 1-ml aliquot of the cell suspension was briefly warmed to 52°C, mixed with an equal volume of molten 1% PFGE-grade agarose (tempered to 52°C), and dispensed into molds. After the plugs solidified, they were transferred to sterile tubes containing 2.0 ml of a lysozyme solution (1 mg/ml in 1 M NaCl–0.5% sarcosyl–0.2% sodium deoxycholate–6 mM Tris-Cl–100 mM EDTA, pH 8.0) and incubated at 37°C for 1 h. The lysozyme solution was removed, and 2.0 ml of a proteinase K solution (2 mg/ml in 0.1% sarcosyl–0.5 M EDTA, pH 9.0) was added. The plugs were incubated at 55°C for 8 to 12 h. The proteinase K solution was then replaced with 2.0 ml of a phenylmethylsulfonyl fluoride solution (1.5 mM in 10 mM Tris-Cl–1 mM EDTA, pH 8.0). The tubes were agitated gently at room temperature for 1 h; the solution was discarded and the phenylmethylsulfonyl fluoride rinse was repeated. Finally, the plugs were rinsed three times (1 h each) with 10 mM Tris-Cl–50 mM EDTA (pH 8.0) with gentle agitation. Plugs were stored at 4°C in the final wash solution.

Slices (1 to 2 mm thick) from the plugs were aseptically cut and transferred to 1.5-ml microcentrifuge tubes. The slices were first rinsed with 0.5 ml of sterile distilled water for 5 min and then for 10 min with the digestion buffer recommended for the restriction enzyme. Finally, the slices were incubated at the recommended temperature for 8 to 12 h in 150 μl of digestion buffer containing 20 U of either *Apa*I or *Not*I. After incubation, the digestion buffer was aspirated and the slices were rinsed for 10 min with 0.5 ml of gel running buffer (45 mM Tris-borate–1 mM EDTA, pH 8.0). Slices were then transferred to a 1% agarose gel and analyzed by electrophoresis, by using the CHEF DR II system (Bio-Rad, Hercules, Calif.). The DNA in the gels was visualized and photographed after staining with ethidium bromide (1 μg/ml) for 30 min.

TABLE 2. Recovery of fecal coliforms and *V. cholerae* O1 from nonpotable water

Ship	Water source	pH[a]	Salinity (ppt)[a]	Concn of fecal coliforms (CFU/100 ml)	Presence of *V. cholerae*
1	Bilge			1.2×10^2	–
	Sewage			2.7×10^4	–
2	Bilge			ND[b]	–
	Sewage			0	–
3	Bilge			ND	–
	Sewage			2.8×10^4	–
4	Ballast	7	32	0	–
	Sewage			0	–
5	Ballast	7	17	0	–
	Sewage			0	–
6	Ballast	7	30	1.2×10^1	–
	Bilge			ND	–
	Sewage			3.0×10^6	–
7	Ballast	6	32	3.0	–
	Bilge			ND	–
8	Ballast	7	13	2.0×10^1	+
	Fire main	7	14	5.8×10^2	+
9	Ballast	6	14	0	+
	Bilge			0	+
	Sewage			1.5×10^5	+
10	Ballast	7	32	0	+
	Bilge			0	+
11	Ballast	7	31	0	–
	Sewage			0	–
12	Ballast	7	28	0	–
	Sewage			0	–
13	Ballast	7	26	0	–
	Sewage			0	–
14	Ballast	7	26	0	–
15	Ballast	7	32	0	–
16	Ballast	7	18	9.0×10^1	–
17	Ballast	7	21	5.1×10^2	–
18	Ballast	6	20	0	+
19	Ballast	6	12	0	+

[a] Determined only for ballast water.
[b] ND, not done.

TABLE 3. Toxigenicity of *V. cholerae* isolates

Ship or organism	Water source	RPLA titer	*ctxA* detection by PCR
8	Ballast	1:64	+
	Fire main	1:64	+
9	Ballast	1:32	+
	Bilge	1:4	+
	Sewage	1:32	+
10	Ballast	1:64	+
	Bilge	1:64	+
18	Ballast	1:64	+
19	Ballast	1:64	+
V. cholerae O1			
C6706		1:16	+
C6707		1:32	+
569B		1:64	+
VRL1984		1:64	+
V. cholerae non-O1		0	–

RESULTS AND DISCUSSION

The salinity of the ballast waters from the ships examined ranged from 12 to 32 ppt, whereas the pH values were either 6 or 7 (Table 2). Culturable *V. cholerae* O1 cells were isolated from ballast water (ballast tanks and fire main), bilge water (bilge and oily-water separator), and sewage from 5 of the 19 ships. On the basis of growth in APW, viable counts of *V. cholerae* were 10^6/ml or greater. The five contaminated ships had last ports of call in Brazil, Colombia, Chile, and Puerto Rico (Table 1). Fecal coliforms were found in two of the eight samples from which *V. cholerae* was isolated (Table 2). This result suggests that in these types of samples, fecal coliforms are not a reliable indicator of *V. cholerae*. *V. cholerae* was recovered from ballast water with salinities of 12, 13, 14, 20, and 32 ppt, indicating its ability to survive in a variety of estuarine and marine environments.

The isolates were identified as El Tor biotype, Inaba serotype. All isolates were positive by PCR for the *ctxA* gene and by RPLA for *V. cholerae* enterotoxin production, with titers of 1:4 to 1:64 (Table 3). Of the control *V. cholerae* O1 strains, C6706, C6707, 569-B, and VRL 1984 also tested positive by RPLA and PCR; the *V. cholerae* non-O1 strain was negative in both tests.

It is possible that viable nonculturable *V. cholerae* cells were also present in ballast water. Since we detected an alarming number of culturable cells and identified the source of the strain by conventional methods, we did not attempt to determine the presence of viable nonculturable cells. However, if viable nonculturable cells constituted a significant portion of the *V. cholerae* population, they could pose a potential threat under appropriate conditions.

PFGE analysis showed that the toxigenic isolates from ships shared an essentially identical *Apa*I restriction pattern (Fig. 1, lanes 1 through 7). This pattern was indistinguishable from that of the Latin American strain C6707 (Fig. 1, lane 8), a strain reported to have two copies of the *ctxA* gene region (16). The other Latin American strain tested (C6706) had a single copy of the toxin gene region (16) and an *Apa*I pattern that differed from that of C6707 by a single band (Fig. 1, lane 9). These bands in C6706 and C6707, approximately 100 and 108 kb, respectively, differed in size by about 8 kb. This value matches that of a reported tandem duplication observed in the genome of other El Tor strains (21). Thus, it is likely that the toxin gene operon is located on these *Apa*I fragments. The results also suggest that the Latin American strains may be able to switch between having either one or multiple copies of the toxin operon.

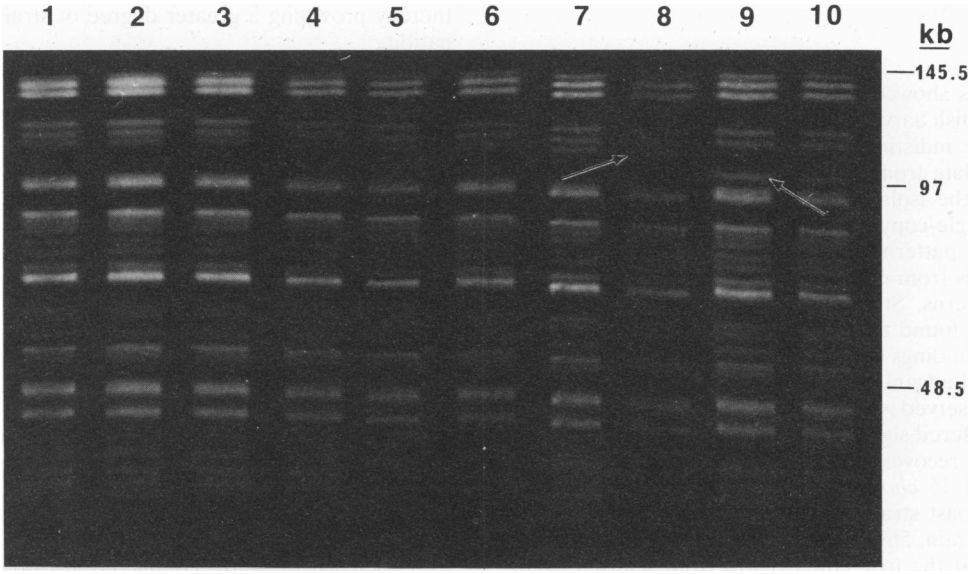

FIG. 1. PFGE of *Apa*I restriction digests of total DNA. Isolates from ships were as follows: lane 1, ballast water, ship 8; lane 2, fire main, ship 8; lane 3, ballast water, ship 9; lane 4, bilge, ship 9; lane 5, ballast water, ship 10; lane 6, ballast water, ship 18; and lane 7, ballast water, ship 19. Other isolates were as follows: lane 8, *V. cholerae* C6707 (Latin American epidemic strain carrying two copies of the toxin gene region); lane 9, *V. cholerae* C6706 (Latin American epidemic strain carrying one copy of the toxin gene region); and lane 10, *V. cholerae* 134-9 (toxigenic strain from a Mobile Bay oyster). Arrows point to bands by which patterns in lanes 8 and 9 differ.

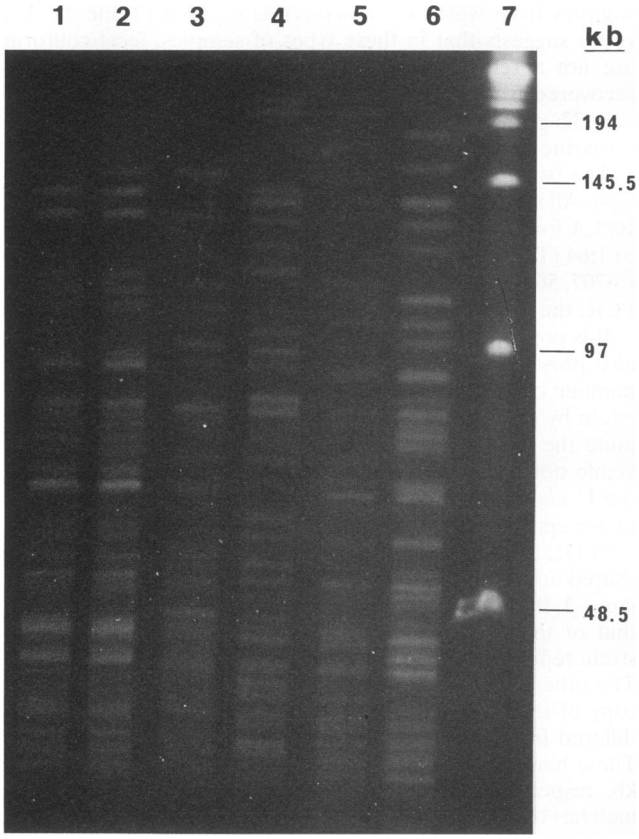

FIG. 2. PFGE of *Apa*I restriction digests of total DNA. Lane 1, toxigenic *V. cholerae* O1 isolate from ship 8; lane 2, *V. cholerae* C6707 (Latin American epidemic strain); lane 3, *V. cholerae* 409-1C2i (non-O1, cholera toxin-negative isolate from shrimp); lane 4, *V. cholerae* 566-454 (non-O1, cholera toxin-negative isolate from ship); lane 5, *V. cholerae* VRL 1984 (toxigenic Gulf Coast strain); lane 6, *V. cholerae* 569-B (sixth-pandemic strain, classical biotype); lane 7, 48.5-kb lambda ladder (size standards).

PFGE analyses showed that the patterns of eight isolates from oysters and fish harvested near ship traffic lanes in Mobile Bay (7, 24) were indistinguishable from that of C6706. The pattern of an isolate from an oyster is seen in Fig. 1, lane 10. Although all of the isolates from seafoods examined in this study had the single-copy pattern, all of those from ships had the double-copy pattern. However, in another study (23), additional isolates from oysters revealed both the single- and double-copy patterns. Strains with higher numbers of toxin gene copies were found to have growth advantages in vivo (1, 13, 14, 22). Our findings suggest that the double-copy version may have survival advantages in seawater as well.

The highly conserved *Apa*I patterns of the strains described above (Fig. 2) differed significantly from those of (i) a non-O1 *V. cholerae* strain recovered from shrimp from Ecuador (lane 3); (ii) a non-O1 *V. cholerae* ship isolate (lane 4); (iii) the toxigenic Gulf Coast strain VRL 1984 (lane 5); and (iv) the sixth-pandemic strain, 569-B (lane 6). Thus, the data rule out the possibility that the toxigenic isolates from seafood examined in this study were derived from either the endemic Gulf Coast strain or from the sixth-pandemic strain. It points to the Latin American area as the most likely source of the isolates and strongly implicates cargo ships as the vectors for transmission of these strains to U.S. coastal areas.

To ascertain the origin of the Latin American epidemic strain, we compared the *Apa*I restriction pattern of C6707 with those of clinical El Tor isolates from Bangladesh, India, Indonesia, Saudi Arabia, Thailand, and Vietnam and of El Tor isolates from food from Korea. All of the El Tor isolates examined had highly conserved patterns, although no two were identical (data not shown). This conservation confirmed the findings of others (32) that the Latin American outbreak is an extension of the seventh pandemic. The strain is, however, evolving over time, as indicated by the distinct differences seen among the various patterns. The pattern of C6707 most closely matched that of an Indonesian strain. The fact that Indonesia is a maritime country and the Latin American epidemic started in a coastal region of Peru (4) suggests that transmission of the epidemic to this hemisphere also may have involved ships.

Comparison by PFGE of the *Not*I restriction patterns of the strains showed that patterns of all the toxigenic isolates from ships, both Latin American strains, and the toxigenic isolates from seafoods were indistinguishable from one another (data not shown). Those of the other control strains differed significantly from one another and from the isolates from ships and seafood. This finding reiterates the *Apa*I digest findings that the Latin American strains and the isolates from ships and seafood are clonal. However, the inability of *Not*I to distinguish C6706 from C6707 underscores the importance of using more than one enzyme for comparative studies and marks *Apa*I as the enzyme of choice for studying *V. cholerae* even though the patterns obtained are more crowded than those of *Not*I.

The diversity in restriction patterns among *V. cholerae* strains increases the value of PFGE as an epidemiological tool. Previously, Kaper et al. (15) used Southern blots and restriction digest patterns to study the molecular epidemiology of *V. cholerae* non-O1 and *Vibrio mimicus* in the U.S. Gulf Coast region. Our study illustrates the advantage of using the remarkable resolving ability of the PFGE method to conduct comparative restriction fragment analysis. The need for blotting and probing is eliminated, and, unlike ribotyping (26), this approach allows essentially the entire genome to be examined, thereby providing a greater degree of strain-specific discrimination.

Ballast discharge is a major factor in the transmission of aquatic organisms worldwide; it has been implicated in the introduction of several exotic aquatic organisms into new areas, e.g., the European cladoceran into the Great Lakes (3), the Asian clam into San Francisco Bay (3), and toxic dinoflagellates into Australian waters (12, 27). Our findings show that ballast and other nonpotable waters from cargo ships are likely vehicles for the transmission of epidemic cholera as well. In July 1991, the Marine Environmental Protection Committee of the International Maritime Organization adopted a resolution: the *International Guidelines for Preventing the Introduction of Unwanted Pathogens from Ships' Ballast Water and Sediment Discharges*. Because of our findings, these guidelines were published in the *Federal Register* on December 12, 1991 (30), and the U.S. Coast Guard requested that shipping agents and ships' captains comply with the recommendation that ballast water be exchanged twice on the high seas to reduce the possibility of introducing pathogens into U.S. coastal waters.

In a nationwide effort conducted in 1991 to 1992, the FDA, with the U.S. Coast Guard and quarantine officers from the Centers for Disease Control, sampled nonpotable water from an additional 90 ships arriving in U. S. ports from Latin America (18). Six of the 109 ships sampled were contaminated with the Latin American strain of *V. cholerae* O1. The FDA, in

collaboration with the Alabama Department of Public Health and the Mobile County Health Department, monitored the occurrence of *V. cholerae* O1 in oysters and the environment until October 1992. No additional contaminated seafood was recovered after June 1992 (24). At that point, the problem appeared to have been resolved and surveillance was terminated. No cases of cholera have been reported in the immediate area. The detection of *V. cholerae* O1 by our research surveys and the measures implemented as a result of our finding the pathogen in the nonpotable waters of cargo ships may have averted further contamination of coastal seafood resources and thus prevented human illnesses.

The recent advent and pandemic spread of a non-O1 strain of *V. cholerae* has caused much concern. This strain, which was serogrouped as O139, genetically and physiologically resembles the seventh-pandemic O1 El Tor strains (11). We suggest that control measures effective against the seventh-pandemic strain, including ballast water surveillance and exchange advisories, may help to contain the spread of *V. cholerae* O139 as well.

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