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Association of Pandemic *Vibrio parahaemolyticus* O3:K6 Present in the Coastal Environment of Northwest Mexico with Cases of Recurrent Diarrhea between 2004 and 2010

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In 2004, more than 1,230 cases of gastroenteritis due to pandemic O3:K6 strains of *Vibrio parahaemolyticus* were reported in southern Sinaloa, a state in Northwestern Mexico. Recurrent sporadic cases arose from 2004 to 2010, spreading from the south to the north. In the present study, *Vibrio parahaemolyticus* was detected in both environmental samples and clinical cases along the Pacific coast of Sinaloa during 2004 to 2010. An evaluation was made of the serotypes, distribution of virulence genes, and presence of pandemic O3:K6 strains. A total of 144 strains were isolated from environmental samples (from sediment, seawater, and shrimp), and 154 clinical strains were isolated. A total of 10 O serogroups and 30 serovars were identified in the strains. Environmental strains ($n = 144$) belonged to 10 O serogroups and 28 serovars, while clinical strains ($n = 154$) belonged to 8 O serogroups and 14 serovars. Ten serovars were shared by both environmental and clinical strains. Among 144 environmental isolates, 4.1% (6/144) belonged to the pandemic clone, with 83.3% containing the *orf8* gene and with O3:K6 accounting for 67%. On the other hand, pathogenic strains (*tdh* and/or *trh*) accounted for 52% (75/144) of the environmental isolates. Interestingly, among 154 clinical isolates, 80.5% (124/154) were pandemic strains, with O3:K6 (*tdh*, $toxRS_{new}$, and *orf8*) representing the predominant serovar (99.2%, 123/124). Overall, our results indicate that in spite of a high serodiversity and prevalence of pathogenic *Vibrio parahaemolyticus* in the environment, the pandemic strain O3:K6 caused >79% of reported cases between 2004 and 2010 in Sinaloa, Mexico.

Vibrio parahaemolyticus is a marine bacterium which is also responsible for acute diarrheal illness in human beings. Eating raw seafish or contaminated seafood is responsible for acute gastroenteritis (49). Although the presence of *V. parahaemolyticus* is extensive in estuarine environments, not all strains of *V. parahaemolyticus* are considered pathogenic. For example, virulent strains are essentially limited to those that are positive for the *tdh* gene (encoding the thermostable direct hemolysin [TDH]) because the gene has been associated with most clinical strains but with very few environmental strains (27, 44). Some clinical isolates also carry the *trh* gene, encoding a TDH-related hemolysin (TRH).

Until 1996, infections caused by *V. parahaemolyticus* were usually associated with diverse serovars (for example, O1:K38, O3:K29, O4:K8, O3:K6, O2:K3, O4:K8, and other serotypes) (45, 55) and had shown a local distribution, emerging in different areas of the world during the warmer months of the year. In February 1996, however, a unique serovar (O3:K6) of *V. parahaemolyticus* with specific genetic markers abruptly appeared in Kolkata, India (45). In subsequent years, O3:K6 isolates similar to those isolated in Kolkata were reported from food-borne outbreaks and from sporadic cases in Southeast Asia as well as the Atlantic and Gulf coasts of the United States (16, 36, 45), and more recently, there have been such reports in Europe (35), Africa (3), and South America (24). This widespread occurrence of a single serotype of *V. parahaemolyticus* had not previously been reported, and it became evident that a pandemic strain had emerged.

Several studies reported that the *toxRS* operon encoded by these newly identified pandemic strains contains a unique sequence ($toxRS_{new}$) encoding transmembrane proteins involved in the regulation of virulence-associated genes (36). The open reading frame 8 (ORF8), derived from a filamentous phage (f237), has also been associated with pandemic *V. parahaemolyticus* strains. However, some pandemic strains lacking ORF8 were recently reported (8, 33, 46). As a result, the main features of the O3:K6 pandemic clone isolates include a distinctive *toxRS* sequence ($toxRS_{new}$) (36) with *orf8* (42) and *tdh* genes instead of the *trh* gene, the latter of which is found in some other pathogenic strains. In general, an isolate possessing both *tdh* and $toxRS_{new}$ can be considered a pandemic strain (46). Recent studies have shown that since 1996, at least 21 serovariants have emerged (40) that share genetic markers specific for the pandemic serovar O3:K6 (8, 15, 36, 40, 51, 54). It has been reported that these serotypes probably originated from the same clone as O3:K6 (8, 15, 36). On the other hand, non-O3:K6 serovars with pandemic traits are increasingly isolated worldwide, and therefore, they may possibly have pandemic potential (2).

In Mexico, there had been few reports of environmental *tdh*-positive strains isolated from water and fish (11), but recently, between September and October of 2004, more than 1,230 cases of gastroenteritis were reported in a relatively small geographic area, the southern part of Sinaloa, a state located in Northwest Mexico. These cases were associated with consumption of raw or under-

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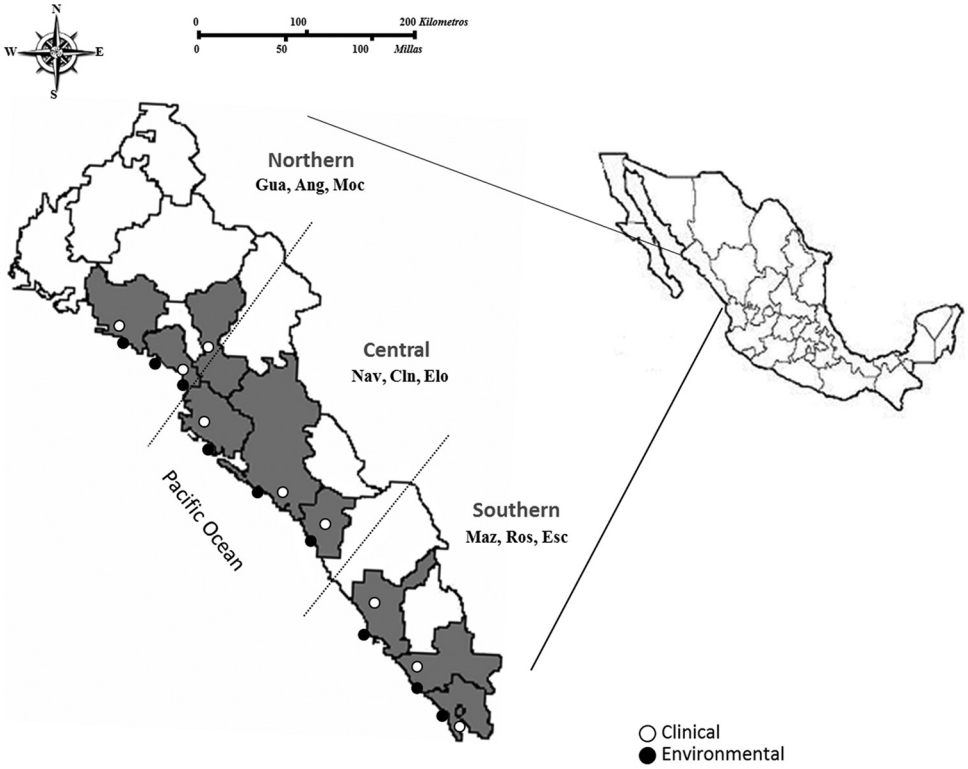

FIG 1 Map showing the geographical locations of sampling sites. Clinical cases (stools specimens or rectal swabs; $n = 3{,}023$) and environmental samples (seawater, sediment, and shrimp; $n = 2{,}266$) were collected between 2004 and 2010 in the coastal regions of northern (Gua, Guasave; Ang, Angostura; Moc, Mocorito), central (Nav, Navolato; Cln, Culiacán; Elo, Elota), and southern (Maz, Mazatlán; Ros, Rosario; Esc, Escuinapa) Sinaloa.

cooked shrimp collected in a lagoonal system. In Mexico, this was the first outbreak of gastroenteritis caused by the pandemic strain of O3:K6 *V. parahaemolyticus* (9). In subsequent years, recurrent sporadic cases were present in this state; however, the cases occurred between south and north areas in the state. The present study focuses on the dissemination of this pandemic strain in the state of Sinaloa from 2004 to 2010 and, within this context, identifies the serotypes, serodiversity, and virulence genes of *Vibrio parahaemolyticus* strains that were isolated from environmental (from shrimp, seawater, and sediment) and clinical samples. To our knowledge, this is the first report on the prevalence of *V. parahaemolyticus* strains (toxigenic and pandemic O3:K6) isolated in clinical and environmental samples over an extended period of time in Mexico.

MATERIALS AND METHODS

Area of study and sample collection. This study was performed in the state of Sinaloa, located in Northwest Mexico. Sinaloa has over 650 km of coastline, with most (75%) of it facing the Sea of Cortez and some (25%) bordering the Pacific Ocean. Sample collection was performed in three regions within the Sinaloa state that were divided into nine sites (three sites for each region) between September 2004 and December 2010. The sites represent leading shrimp producers in the state, and clinical cases were also detected near these regions. A total of 2,266 environmental samples (380 shrimp, 65 sediment, and 1,821 seawater) were collected in the northern region (Guasave, Angostura, and Mocorito), the center region (Navolato, Culiacán, and Elota), and the southern region (Mazatlán, Rosario, and Escuinapa) of Sinaloa (Fig. 1). Stool and environmental samples were not collected in 2005. Stool specimens or rectal swabs ($n = 3{,}023$) were collected from persons with gastroenteritis who had eaten seafood and requested attention in public-sector health care agencies between September 2004 and December 2010 (Table 1). Written informed consent was obtained, and donors of human samples were selected as recommended by the ethics committee of the Faculty of Medicine-UAS and the Sinaloa State Public Health Laboratory.

Bacteriological analyses. All samples were processed as described in the *Bacteriological Analytical Manual* of the Food and Drug Administration (32) and also as described by Canizalez-Roman et al. (13). Briefly, 50 g of shrimp and sediment samples or 50 ml of seawater was weighed and added to 450 ml of sterile alkaline peptone water (APW) (pH 8.6) and homogenized in a Stomacher 400 circulator. The APW homogenate was incubated at 37°C for 6 to 8 h. The nonbloody stool samples were collected in Cary-Blair transport medium and transported at room temperature (RT) to the laboratory within 2 h. These specimens were also enriched in APW (pH 8.6) for 6 to 8 h at 37°C. After incubation, the enrichment broths (APW) were streaked onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates and/or CHROMagar Vibrio (CV) medium (CHROMagar, Paris, France) and incubated at 37°C for 18 to 24 h. At least three typical colonies of *V. parahaemolyticus* were isolated from each plate and subjected to identification by biochemical tests and PCR as mentioned below. After identification of *Vibrio parahaemolyticus*, a single colony from each sample was used to continue the analysis (serotyping and virulence genes).

Extraction and purification of chromosomal DNA. Chromosomal DNA was extracted using the Wizard genomic DNA purification kit (Promega Corp.) according to the manufacturer's instructions. Briefly, 3 ml of a 16- to 18-h culture in LB broth containing 3% NaCl was harvested by centrifugation at 13,000 × *g* to 16,000 × *g* for 5 min. Cells were lysed at 80°C in nuclei lysis solution (Promega Corp.). RNase solution was added to the cell lysate, followed by incubation at 37°C for 1 h and cooling at room temperature. Protein precipitation solution (Promega Corp.) was

TABLE 1 Distribution of *Vibrio parahaemolyticus* strains isolated from clinical (stool) and environmental (seawater, sediment, and shrimp) samples between 2004 and 2010

	No. of isolates collected[a]													
					Environmental									
	Clinical				Shrimp			Seawater			Sediment			
Yr	S	C	N	Total	S	C	N	S	C	N	S	C	N	Total
2004	67			67	8			3						11
2006	17	34	10	61	15		4	20			10			49
2007								2			3			5
2008	9	1		10	32			4			4			40
2009					6	1		6			1			14
2010	12	4		16	3		1	13	2		6			25
Total				154										144

[a] S, southern region of the state of Sinaloa; C, central region of Sinaloa; N, northern region of Sinaloa. The total number of clinical samples was 3,023, and the total number of environmental samples was 2,266 (380 shrimp, 65 sediment, and 1,821 seawater samples).

added to the RNase-treated cell lysate and vortexed vigorously. After incubation and centrifugation at 13,000 × *g* to 16,000 × *g* for 3 min, the DNA was precipitated by adding 0.6 volumes of isopropanol at room temperature. The DNA pellet was washed with 70% ethanol, air dried, and dissolved in DNA rehydration solution (Promega Corp.). Prehydrated DNA was stored at 2 to 8°C until use.

PCR assays. PCR amplification was performed in a 25-μl volume consisting of 1× GoTaq green master mix (Promega), primers targeting the *tl* gene (7), the pR72H plasmid (34, 50), and the *tdh* and *trh* genes (7), and 0.5 μg of purified genomic DNA template, with the remaining volume consisting of molecular biology grade water. PCR was routinely carried out in a Thermal Cycler C1000 (Bio-Rad Laboratories, Hercules, California) under the following cycling conditions: an initial period of DNA denaturation at 94°C for 3 min, followed by 35 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C, and a final extension of 5 min at 72°C. PCR assays for the amplification of the *toxRS* and *orf8* pandemic marker genes were performed using specific primers previously reported to detect $toxRS_{new}$ (36) sequences unique to the pandemic clone of *V. parahaemolyticus* and the *orf8* (39) sequence of phage f237, respectively. Briefly, the PCR conditions were as follows: for the $toxRS_{new}$ gene, initial denaturation at 94°C for 3 min, followed by 25 cycles of 30 s at 94°C, 30 s at 45°C, and 1 min at 72°C, with a final extension of 5 min at 72°C, and for the *orf8* gene, denaturation at 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, with a final extension of 5 min at 72°C. Negative-control reactions were performed simultaneously by replacing the template DNA with sterilized water in the PCR mixture. Ten-microliter aliquots of each amplification product were separated by electrophoresis in a 2% agarose gel. Ethidium bromide staining (0.5 mg/ml) allowed for the visualization of DNA fragments with a digital imaging system (model E1 logia 100 imaging system; Kodak). The sizes of the PCR fragments were compared against a 50-bp DNA ladder (Promega DNA step ladder).

Serotyping. Serotyping of the *V. parahaemolyticus* isolates was done by using a commercially available *V. parahaemolyticus* antiserum test kit (Denka Seiken, Tokyo, Japan) with O1 to O11 antisera and 71 K antisera according to the manufacturer's instructions. Briefly, overnight strains were first grown on LB agar containing 3% NaCl and incubated at 37°C; a pool of colonies was collected and resuspended in 1 ml normal saline, an aliquot (0.5 ml) was boiled for 2 h, and these lysates were used for serotyping based on the O antigen. The remaining cell suspension that was not boiled was used for serotyping based on the K antigen.

RESULTS

Detection of *V. parahaemolyticus*. A total of 2,266 environmental samples (380 samples from shrimp, 1,821 from seawater, and 65 from sediment) collected from nine sites in northern, central, and southern Sinaloa between September 2004 and December 2010 (Fig. 1) were analyzed for the presence of *V. parahaemolyticus* strains. These strains were isolated from 6.3% (144) of the samples. Of the 144 strains isolated, 70 (49%) were obtained from shrimp samples, 50 (35%) from seawater, and 24 (16%) from sediment (Table 1). In clinical samples taken during the same period, *Vibrio parahaemolyticus* was detected in 154 of 3,023 (5.09%) stool specimens or rectal swabs from persons with gastroenteritis who had eaten seafood (Table 1). Regarding environmental samples, the presence of *V. parahaemolyticus* was detected mainly in southern sites, whereas in cases of diarrhea, this bacterium was commonly isolated from patients from both southern and northern Sinaloa (Table 1 and Fig. 1).

Serovars of *V. parahaemolyticus* isolates. *V. parahaemolyticus* serotyping was performed for epidemiological purposes and serves as an important marker for both diarrheagenic and pandemic strains. As shown in Table 2, 30 serovars were identified among 200 isolates that were serotyped and recognized with O and K antisera from the 298 isolates (144 environmental isolates and 154 clinical strains). Approximately 38% (55/144) of the environmental isolates could be typed by both O and K antisera, resulting in 10 different O groups, 23 different K types, and 28 serovars. A total of 19.4% (28/144) of the strains were not recognized by O antisera, while 61% (88/144) were not recognized by K antisera, and 27 of these latter strains did not react to O:K antisera (OUT:KUT). The serogroups O1, O2, O3, O4, O5, O6, O7, O8, O10, and O11 accounted for 26% (37/144), 6.25% (9/144), 15.3% (22/144), 6.25% (9/144), 10.42% (15/144), 5.6% (8/144), 0.69% (1/144), 3.5% (5/144), 6.25% (9/144), and 0.69% (1/144) of all the environmental isolates, respectively. Hence, the most prevalent O serogroups were O1, O3, and O5. Isolates that could be serotyped by O antisera but not by any of the available K antisera represented 42.3% (61/144) of the samples. Twenty-three were serogroup O1:KUT (untyped), one was O2:KUT, eleven were O3:KUT, five were O4:KUT, eight were O5:KUT, two were O6:KUT, one was O7:KUT, eight were O10:KUT, and one was O11:KUT. The most frequent serovars were O1:KUT, O5:KUT, and O3:KUT, comprising 23, 8, and 11 strains, respectively. Among serotypes recognized by the O and K antisera, the most frequent were O2:K28, O3:K6, and O5:K17, comprising 7, 4, and 3 strains, respectively. One

TABLE 2 Serovar, virulence, and pandemic characteristics of 298 isolates collected between 2004 and 2010

O serogroup and serovar	Total no. of isolates	Presence of each virulence gene				No. of clinical isolates (from feces)	No. of environmental isolates from[a]:		
		tdh	*trh*	*toxRS*$_{new}$	*orf8*		Sh	SW	S
O1									
O1:KUT	1	+	−	−	+		1		
	1	+	+	+	−	1			
	4	+	+	−	−		4		
	7	+	−	−	−		4	3	
	1	−	+	−	−		1		
	11	−	−	−	−		3	3	5
O1:K9	1	+	+	+	−	1			
O1:K25	1	+	−	−	−		1		
O1:K26	1	+	−	−	−		1		
	1	−	−	−	−			1	
O1:K32	1	+	+	−	−				1
	1	−	−	+	−			1	
	1	−	−	−	−				1
O1:K33	2	+	−	−	−	1	1		
	1	−	−	−	−				1
O1:K41	2	+	+	−	−		1	1	
O1:K56	1	+	−	−	−			1	
	1	−	−	−	−				1
O2									
O2:KUT	1	−	−	−	−		1		
O2:K28	1	−	−	−	+		1		
	1	−	−	+	−			1	
	4	+	−	−	−		1	3	
	2	−	−	−	−			2	
O3									
O3:KUT	1	+	−	+	+			1	
	3	+	+	−	−		1	2	
	1	−	−	+	−			1	
	5	+	−	−	−		3	1	1
	1	−	−	−	−				1
O3:K4	1	+	+	−	−		1		
O3:K6	3	+	−	−	+	3			
	127	+	−	+	+	123	1	2	1
O3:K29	1	+	−	−	−			1	
O3:K32	1	+	−	−	−		1		
O3:K33	1	−	−	−	+		1		
O3:K58	2	+	+	−	−	1	1		
	1	+	−	−	−	1			
O3:K59	1	+	−	−	−		1		
O3:K68	2	+	+	−	−	1	1		
	1	+	−	−	−				1
O4									
O4:KUT	1	−	−	−	+		1		
	3	+	+	−	−	1		2	
	1	+	−	−	−			1	
	1	−	−	−	−		1		
O4:K8	1	+	+	−	−	1			
O4:K10	2	+	+	−	−	2			
	1	−	−	−	−		1		
O4:K12	1	+	−	+	+	1			
	1	−	−	−	−			1	
O4:K53	1	−	−	−	−			1	
O4:K63	1	+	+	−	−	1			
O4:K68	1	+	−	−	+		1		

(Continued on following page)

TABLE 2 (Continued)

O serogroup and serovar	Total no. of isolates	Presence of each virulence gene				No. of clinical isolates (from feces)	No. of environmental isolates from[a]:		
		tdh	*trh*	$toxRS_{new}$	*orf8*		Sh	SW	S
O5									
O5:KUT	1	−	−	−	+			1	
	1	+	+	−	−				1
	2	+	−	−	−		2		
	5	−	−	−	−	1	2		2
O5:K15	1	+	+	−	−		1		
	3	−	−	−	−	2		1	
O5:K17	3	−	−	−	−		1	1	1
	1	−	+	−	−	1			
O5:K68	1	−	−	−	+		1		
	1	−	−	−	−			1	
O6									
O6:KUT	2	−	−	−	−		2		
O6:K6	1	−	−	−	+		1		
	1	+	−	−	−			1	
O6:K46	1	+	−	−	−		1		
	1	+	+	−	+	1			
	3	−	−	−	−		2	1	
O7									
O7:KUT	1	+	−	−	−		1		
O8									
O8:KUT	1	−	−	−	−	1			
O8:K8	1	+	−	−	−			1	
	1	−	−	−	−			1	
O8:K20	1	+	−	−	+	1			
	1	−	−	−	+			1	
O8:K21	2	+	−	−	−		1	1	
O10									
O10:KUT	1	+	−	+	−				1
	2	+	+	−	−		1		1
	3	+	−	−	−		1	1	1
	2	−	−	−	−		1		1
O10:K52	2	+	−	−	−	1	1		
	4	−	−	−	−	4			
O11									
O11:KUT	1	+	+	−	−		1		
	2	−	−	−	−	2			
OUT									
OUT:K8	1	−	−	−	−		1		
OUT:KUT	1	+	−	−	+	1			
	1	−	−	−	+			1	
	2	−	−	+	−			2	
	3	+	+	−	−		1	2	
	13	+	−	−	−	1	10	1	1
	9	−	−	−	−		3	4	2
Total	298					154			

[a] Sh, shrimp; SW, seawater; S, sediment.

strain was recognized by K antisera but not by O antisera (OUT:K8) (Table 2).

In clinical isolates, there were 8 O serogroups, 14 different K types, and 14 serovars that could be identified in the 146 strains recognized by both O and K currently available antisera. These 146 strains accounted for 94.8% of 154 clinical strains found with *V. parahaemolyticus*. Six clinical strains (3.9%) could be recognized by O antisera but not by K antisera (one was O1:KUT, one was

TABLE 3 O3:K6 pandemic clone and pathogenic strains in isolates of *Vibrio parahaemolyticus* from environmental and clinical samples collected between 2004 and 2010

Yr	% of isolates from each group (no. of isolates from that group/total no. of isolates)			
	O3:K6 pandemic clone[a]		Pathogenic strains[b]	
	Clinical	Environmental	Clinical	Environmental
2004	88% (59/67)	0% (0/11)	2.98% (2/67)	0% (0/11)
2006	70.5% (43/61)	0% (0/49)	21.3% (13/61)	36.7% (18/49)
2007	–[c]	0% (0/5)	–	60% (3/5)
2008	80% (8/10)	7.5% (3/40)	20% (2/10)	92.5% (37/40)
2009	–	0% (0/14)	–	85.7% (12/14)
2010	100% (16/16)	4% (1/25)	0% (0/16)	20% (5/25)
Total	81.8% (126/154)	2.7% (4/144)	11% (17/154)	52% (75/144)

[a] Pandemic clone serotype O3:K6 is *tdh* positive, *toxRS*$_{new}$ positive, and *orf8* positive or negative.
[b] Pathogenic strains are *tdh* positive and/or *trh* positive.
[c] –, absence of *Vibrio parahaemolyticus* isolates.

O4:KUT, one was O5:KUT, one was O8:KUT, and two were O11:KUT). Serogroups O1 and O3 were the most commonly recognized, accounting for 89% (137/154) of all clinical strains. Importantly, O3:K6 was the most predominant serovar of the clinical strains throughout the period of the study, accounting for 79.8% (123/154) of strains. Other serotypes were found besides O3:K6, including O1:K9, O1:K33, O3:K58, O3:K68, O4:K8, O4:K10, O4:K12, O4:K63, O5:K15, O5:K17, O6:K46, O8:K20, and O10:K52 (Table 2).

Detection of virulence and pandemic marker genes by PCR. *V. parahaemolyticus* strains isolated from environmental and clinical samples were screened by PCR for the virulence and pandemic marker genes *orf8*, *toxRS*$_{new}$, *tdh*, and *trh*. Based on the presence or absence of virulence genes, we classified the isolates into three groups: pandemic, pathogenic (nonpandemic), and nonpathogenic strains. The features of pandemic strains include *tdh* positive, *toxRS*$_{new}$ positive, *orf8* positive or negative, and *trh* negative. Pathogenic strains are *tdh* and/or *trh* positive. Nonpathogenic strains are characteristically both *tdh* and *trh* negative.

Among environmental *V. parahaemolyticus* strains, 4.1% (6/144) of the isolates were identified as pandemic: four were pandemic O3:K6 strains containing the *orf8* gene (two detected in seawater, one in shrimp, and one in sediment samples), one was a pandemic O3:KUT strain (detected in seawater), and one was a pandemic O10:KUT strain without *orf8* (detected in sediment). A total of 52% (75/144) of environmental isolates were found to carry the virulence genes (*tdh* and/or *trh*). Of these, 68% (51/75) had the *tdh* gene and belonged to 22 different serotypes, most of which were OUT:KUT ($n = 22$). Other serotypes included O1:K25, O1:K26, O1:K33, O1:K56, O2:K28, O3:K59, O4:K68, O8:K21, and O10:K52. The 30.6% (23/75) of environmental isolates with both *tdh* and *trh* genes included O1:K32, O1:K41, O3:K58, O5:K15, and OUT:KUT serotypes. We detected only 1 isolate with the *trh* gene that had the O1:KUT serotype. Nonpathogenic strains, belonging to serotypes O1:K26, O1:K32, O1:K33, O1:K56, O2:K28, O4:K10, O4:K12, O4:K53, O5:K15, O5:K17, O8:K20, and O10:K52, with a high frequency of the O1:KUT (11) serovar, were found in 43.8% (63/144) of environmental isolates (Table 2).

Among clinical *V. parahaemolyticus* strains, 80.5% (124/154) of isolates were identified as belonging to the pandemic serotype with the *orf8* gene. Of these, 123 belonged to serovar O3:K6 and one isolate belonged to serovar O4:K12. A total of 12.9% (20/154) of the clinical isolates carried *tdh* and/or *trh*. Of these, 4 isolates, with serotypes O1:K33, O3:K58, O10:K52, and OUT:KUT, carried *tdh* only. On the other hand, 5 isolates, with serotypes O3:K6 (3), O8:K20, and OUT:KUT, were positive for both the *tdh* and *orf8* genes. We also detected seven isolates, with serotypes O3:K58, O3K68, O4:K8, O4:K63, O4:K10, and O4:KUT, that were *tdh* and *trh* positive, as well as three isolates, with serotypes O1:K9, O1:KUT, and O6:K46, that were *tdh*, *trh*, and *orf8* positive. One of the clinical isolates, with the O5:K17 serotype, was only *trh* positive (*tdh* negative) (Table 2). Interestingly, 6.5% (10/154) of clinical isolates belonged to the nonpathogenic group, in which strains carry neither the *tdh* nor the *trh* gene, including serotypes O5:K15 (2), O5:KUT (1), O8:KUT (1), O10:K52 (4), and O11:KUT (2). Unlike those serovars detected in environmental isolates, in clinical samples, the most prevalent serotype was O3:K6, and the following serotypes were detected not only in environmental but also in clinical strains: O1:K33, O3:K6, O3:K58, O4:K10, O4:K12, O5:K15, O5:K17, O6:K46, O8:K20, and O10:K52.

Yearly presence of the O3:K6 pandemic clone and pathogenic strains. In the present study, the pandemic clone O3:K6 serotype was the most prevalent one isolated from clinical samples in every year. *V. parahaemolyticus* was present in high proportions of these clinical strains (70.5% to 100%) in most years. Only in 2 years (2007 and 2009) were gastroenteritis cases caused by *V. parahaemolyticus* not detected. In contrast, in environmental strains, the O3:K6 pandemic clone was detected only in 2008 and 2010, at proportions of 7.5% and 4%, respectively (Table 3).

It is noteworthy that in clinical *V. parahaemolyticus* strains, pathogenic versions (with *tdh* and/or *trh*) were detected in low proportions, between 2.98% and 21.3%, through the years (except in 2007 and 2010, when pathogenic strains were not detected). However, pathogenic strains were detected every year in the environment in high proportions, between 20% and 92.5% (except in 2004, when pathogenic strains were not detected) (Table 3).

DISCUSSION

V. parahaemolyticus, a halophilic bacterium, has in recent years emerged as a worldwide pandemic pathogen causing gastroenteritis related to consumption of seafood (5, 18, 54). Before 2004, there had been no reports of outbreaks caused by *V. parahaemolyticus* in Mexico (9) and few reports of environmental *tdh*-positive strains had been isolated from water and fish (11). The

first outbreak in Mexico (1,230 cases) was caused by the pandemic strain of serotype O3:K6 and associated with the consumption of raw or undercooked shrimp in southern Sinaloa, a state in Northwest Mexico (9). Since 2004, recurrent sporadic cases have shown up in this state. In recent years, new cases have arisen in different areas of Sinaloa (including southern and northern areas). This study includes data collected over 6 years (from September 2004 to December 2010) along the Pacific coast of Sinaloa. In both clinical cases and environmental samples, we detected the presence of pandemic and pathogenic strains belonging to different serotypes. It turns out that the pandemic clone O3:K6 (with *tdh* and $toxRS_{new}$ genes and with or without *orf8* genes) was the most prevalent serotype isolated from clinical samples in every year of the study. It was also detected in environmental samples (seawater, sediment, and shrimp), suggesting that it is endemic to this setting. To our knowledge, this is the first report of the prevalence of *V. parahaemolyticus* strains (toxigenic and pandemic) isolated in Mexico.

Our data show that as of 2004, the infections caused by the O3:K6 pandemic clone spread rapidly from the southern to the northern region in Sinaloa, over more than 650 km of coastline. This spreading of *V. parahaemolyticus* is possibly due to the movement of oceanic waters or to human activities, such as ballast water discharges (4). This spreading of pandemic *V. parahaemolyticus* is still a speculative question that requires further investigation since the pandemic clone can potentially migrate to southern Mexico and the United States.

Notably, it has been shown that in different parts of the world, the distribution of *V. parahaemolyticus* in the marine environment (seawater, sediment, and marine flora and fauna) is highly related to a combination of environmental parameters, including sea surface temperature, salinity, turbidity, and chlorophyll concentrations (12, 28–31). Kaneko and Colwell (29–31) reported the seasonal cycle of *V. parahaemolyticus* in the sediment, water, and plankton of the Chesapeake Bay (United States), determining that the bacterium might survive in sediments during the winter and be released into the water column in late spring or early summer when the temperature increases. In tropical countries, the seasonal cycle of the organism is correlated with the rainy and dry seasons; the lowest numbers are found in rainy months, and the highest numbers are found in the dry season (43). Additionally, in tropical waters, the temperature fluctuates less throughout the year, while the salinity fluctuates more (17). In the present study, the isolation rate of *V. parahaemolyticus* was low (6.3%) in the marine environment. However, we found moderate prevalence of 36% in sediment (24/65) and 18% in shrimp samples (70/380). The prevalence of *V. parahaemolyticus* in seawater samples was only 2.7% (50/1,821) despite the high number of seawater samples (1,821) collected in this study. Thus, we speculate that the observed prevalence may also be produced by seasonal variation of the *V. parahaemolyticus* population (due to salinity, turbidity, and chlorophyll concentration) by "hibernating" in the sediment or by partnership with the marine fauna during the sampling period (mainly from November to January). However, we cannot prove these hypotheses due to insufficient or absent data in this work (sea surface temperature, salinity, and chlorophyll). Additional research is needed to determine the possibility of changes in the relative abundance of pathogenic *V. parahaemolyticus* during different seasons of the year on the Pacific coast of Mexico, particularly in Sinaloa, as well as the identification of associated environmental factors (e.g., temperature or salinity effects).

Serotyping analysis demonstrates that there were fewer serogroups and serovars in clinical isolates than in environmental isolates, clearly demonstrating that clinical isolates belong to a specific group of strains, in agreement with other studies (14). Whether these strains will contain other or unknown virulence determinants remains to be investigated. Among the diversified environmental samples, O1 was the predominant O serogroup found, accounting for 26% of all isolates. This study on the Pacific coast of Mexico differs with the unique serogroup study developed on the Gulf coast of the same country in which O3 was the most frequently encountered serogroup (30%) among the environmental strains (11). Several researchers have pointed out that certain serogroups are constant in some specific geographical areas (1, 48).

On the other hand, throughout the present study, O3:K6 was the predominant serovar of the recognized clinical strains, accounting for 79.8% (123/154) of the total number of strains isolated. The regional predominance of a specific serotype of *V. parahaemolyticus* has occasionally been reported in distinct geographic areas. Along the United States Pacific coast, *V. parahaemolyticus* O4:K12 was the dominant serotype causing infections (1). Similarly, O3:K6 was the predominant serovar in Peru in 2007 (23). In Chile, the O3:K6 pandemic clone was also the predominant serovar, causing outbreaks in Puerto Montt that began in 2004 and reached a peak in 2005 with 3,600 clinical cases. Up to 2006, every analyzed case in Chile was caused by the serovar O3:K6 pandemic strain. Among the 475 cases reported in the summer of 2007, 73% corresponded to the pandemic strain (10, 21, 24). In 2009, Harth et al. (25) reported that the decrease of outbreaks caused by O3:K6 was associated with a change in serotype of many pandemic isolates to O3:K59 and with the emergence of new clinical strains. On the contrary, no change in the prevalence of the O3:K6 pandemic clone was observed in the present study. We identified eight serovars that were not listed by the Japanese committee on the serological typing of *V. parahaemolyticus*, two in clinical strains (O1:K9 and O1:K33) and six in environmental strains (O3:K32, O3:K68, O4:K68, O6:K6, O8:K8, and O10:K52). These serovars have also been found in several other studies (14, 25a, 38, 40, 41, 56).

It has been demonstrated that the existence of the *tdh* and/or *trh* gene in a strain is associated with its ability to cause gastroenteritis (44). Therefore, the presence of these genes is routinely used to determine the pathogenicity of *V. parahaemolyticus* strains (30). In molecular epidemiological studies, it has been shown that clinical isolates possess the *tdh* and/or *trh* gene in up to 90% of clinical strains (serotype O3:K6 strain has increasing prominence) (14, 22, 45), whereas their presence in environmental isolates is rare (14, 17, 26, 40, 44, 52, 57). Data on the current study are in accordance with these previous observations, as 93% of all clinical strains carried the *tdh* and/or the *trh* gene (80.5% were in the pandemic group and 12.9% were in the pathogenic group). Interestingly, we detected a high proportion (52%) of strains with pathogenic markers (*tdh* and/or *trh*) but a low proportion (4.1%) of strains belonging to the pandemic group in the environmental isolates, which were represented by many different serotypes. The high rate of detection of pathogenic *V. parahaemolyticus* in the marine environment may be due to a decreased prevalence of total *V. parahaemolyticus* and not to a greater abundance of pathogenic strains. However, data related to the prevalence of pathogenic

Vibrio parahaemolyticus has not previously been investigated at the Pacific coasts of Mexico. This study provides novel information on the abundance and characteristics of pathogenic *V. parahaemolyticus* in this setting.

We found that most of the clinical isolates belong to pandemic strains (*tdh* positive, $toxRS_{new}$ positive) with *orf8* and that O3:K6 was the dominant pandemic serovar during the 6-year period of study. Previous studies have shown that isolates belonging to the new clone O3:K6 (*tdh* positive, $toxRS_{new}$ positive) are associated with outbreaks of human gastroenteritis (36, 45, 46); however, as in our study, these strains are rarely isolated from environmental samples (14, 19, 53). In the current contribution, the few environmental isolates belonging to the pandemic serotype were O3:K6 (in seawater, shrimp, and sediment), O3:KUT (in seawater), and O10:KUT (in sediment). Moreover, the discovery of these strains in seawater and sediment suggests a complete seasonal cycle of *V. parahaemolyticus*, as has been reported by Kaneko and Colwell in the Chesapeake Bay (United States) (30, 31). Thus, this is the first report about the presence of the pandemic group in the environment in Mexico.

Along the approximately 650 km of the Sinaloa Pacific coastal area, seawater temperatures range from 23 to 30.8°C (47), with an average humidity of 68%. It has been reported that elevated temperatures (>30°C) are appropriate for the proliferation of this pathogen (19). The importance of water temperature in the epidemiology of infections is reflected by the fact that most outbreaks occur during the warmer months. The progressive spread of *V. parahaemolyticus* and its colonization of new areas have been related to an unusual increase in seawater temperatures in coastal zones (16, 27, 45). This might account for the outbreaks of diarrhea caused by *V. parahaemolyticus* during the summer of 2004 in Alaska (38) and also in southern Chile at Puerto Montt between 2004 and 2007, places where seawater temperatures are normally low and *V. parahaemolyticus* infection is very rare. In the latter situation, ~7,000 cases were reported, a rate which is perhaps due to climatic change, such as the warm ocean currents commonly referred to as the El Niño phenomenon (10, 20, 21, 24). The arrival of El Niño waters to South America causes a general disruption of the environmental conditions of coastal areas, displacing the native species southward (4). Besides weather changes, the spread of nonindigenous microorganisms has been attributed to the water ballast of ships (37, 39). Ballast water-mediated invasions have been proposed for the entrance of O3:K6 strains into Texas in 1998 (16) as well as into Coruña, Spain, in 2004 (35). According to Cabanillas-Beltran et al., the O3:K6 pandemic may have been transported to Mexico via the port of Mazatlan (in southern Sinaloa) (9). However, the ecology and seasonality of *V. parahaemolyticus* in the marine environment of Northwest Mexico are still unclear.

Recently, the analysis of the arrival of the O3:K6 clone at the Pacific coasts of South America has provided novel insights linking its origin to an invasion in 1997 from the Asian (Indian) populations and describing the successful establishment of the O3:K6 populations, first in Peru and subsequently in the south of Chile (4). Thus, we speculate that this Peruvian foothold of the O3:K6 clone may possibly be the origin of the arrival of this strain to the coasts of Northwestern Mexico (Sinaloa) in 2003 to 2004. However, the dynamics of dissemination of the environmental human pathogen *Vibrio parahaemolyticus* are uncertain.

Results obtained within the present study revealed the presence and persistence of *V. parahaemolyticus* (O3:K6) in marine environments in Sinaloa. Records of the Sinaloa State Public Health Laboratory indicate that from 1999 to 2003, diarrhea cases related to seafood consumption (<0.5%) were not widespread in the Sinaloa state in spite of the large consumption of raw shellfish. Since the arrival of the pandemic strain O3:K6, possibly between 2003 and 2004, cases of seafood-related diarrhea have now increased (to 5%).

Noteworthy in this study was the prevalence of a few serotypes of *V. parahaemolyticus* that were similar between heterogeneous environmental strains (especially from shrimp) and clinical strains from stools (O1:K33, O3:K6, O3:K58, O4:K10, O4:K12, O5:K15, O5:K17, O6:K46, O8:K20, and O10:K52). The fact that we isolated similar serotypes and virulence genes from patients and incriminated environmental sources during the outbreaks of several epidemics also suggests a certain association between serotypes found in the environment and those linked to gastroenteritis (6). Although the serotyping technique is good for differentiating clinical and environmental isolates of *V. parahaemolyticus*, further molecular methods for the analysis of genetic variability among isolated strains should be performed. For this purpose, in future studies, we would like to compare the phenotypic and genotypic properties, including DNA fingerprints by pulsed-field gel electrophoresis (PFGE) or multilocus sequence typing (MLST), of the Mexican *V. parahaemolyticus* (O3:K6) strains isolated from clinical sources with those from strains isolated from Mexican environments (shrimp, seawater, and sediment).

Data from the present study are in accordance with other reports in which *V. parahaemolyticus* environmental strains show a high serological variability. We demonstrate that the pandemic clone O3:K6 harboring $toxRS_{new}$ and *orf8*, along with *tdh*, is predominant in infections all along coastal areas of Sinaloa. In this same area, serotyping and virulence factors showed some similar distributions of pandemic and pathogenic *V. parahaemolyticus* strains isolated from clinical and environmental sources. The fact that certain serotypes and virulence factors were found in both clinical and environmental isolates while many others were observed only in environmental isolates implies that certain serotypes with virulence factors are more relevant for human disease. The current results provide the first available data on the distribution, prevalence, and detection of virulence factors and pandemic genes of *V. parahaemolyticus* in clinical and environmental strains in Mexico. The presence and persistence of pandemic and pathogenic environmental *V. parahaemolyticus* strains in Sinaloa are a matter of concern for public health authorities, as the potential of outbreaks along the northern Mexican coastline of the Pacific Ocean is now well established. Therefore, this information is important for preventing public health problems that not only are likely to affect the populations in Sinaloa but also might spread to other states in Mexico and to the United States.

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