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Serologic and Molecular Characterization of *Vibrio parahaemolyticus* Strains Isolated from Seawater and Fish Products of the Gulf of Mexico

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The thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) are the main virulence factors of *Vibrio parahaemolyticus*. We isolated *V. parahaemolyticus* from seawater, fish, and oysters obtained from the Pueblo Viejo Lagoon in Veracruz, determined the serogroups, phenotypically and genotypically characterized TDH and TRH, and investigated the presence of the *toxR* gene. A total of 46 *V. parahaemolyticus* strains were isolated, and all of them amplified the 368-bp *toxR* gene fragment. The *trh* gene was not identified in any of the strains; 4 of the 46 strains were Kanagawa phenomenon (KP) positive and amplified the 251-bp *tdh* gene fragment. The most frequent serogroup was serogroup O3. This is the first report of the presence of KP-positive *tdh*-positive environmental *V. parahaemolyticus* strains in Mexico.

Vibrio parahaemolyticus is a halophilic gram-negative bacterium that is widely distributed in coastal waters worldwide and is associated with gastroenteritis, wound infections, and septicemia (5). *V. parahaemolyticus* infections are frequently reported in coastal areas, apparently because of the high consumption of sea products and direct contact with estuarine waters (19).

Epidemiological studies have revealed an association between the Kanagawa phenomenon (KP) and gastroenteritis (23, 25). KP is a type of beta-hemolysis induced by the thermostable direct hemolysin (TDH) in Wagatsuma agar. Most (90%) of the strains isolated from clinical cases show this type of hemolysis, while only 1 to 2% of the strains of environmental origin are KP positive (20).

Several cases of gastroenteritis caused by hemolytic but KP-negative TDH-negative *V. parahaemolyticus* strains were reported in the 1980s (11), which led to identification of a new hemolysin known as TDH-related hemolysin (TRH) (12, 13, 30). TDH and TRH are encoded by the *tdh* and *trh* genes, respectively; these two genes both have 567-bp open reading frames, and they show 68.6% sequence similarity. These hemolysins are considered the main virulence factors of this microorganism (20).

In the present study we determined the prevalence of *V. parahaemolyticus* in seawater, oyster, and fish samples collected from the Pueblo Viejo Lagoon in Veracruz, an important estuary on the coastline of the Gulf of Mexico. The strains isolated were serotyped and screened for hemolytic activity and for the presence of the *toxR*, *tdh*, and *trh* genes.

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MATERIALS AND METHODS

A total of 266 seawater, oyster, and fish samples were collected from 12 different sites in the Pueblo Viejo Lagoon (Fig. 1) in June, August, September, and November 2001, as well as in February; March, April, May, and June 2002.

The fish and oysters were transported in individually labeled and sealed plastic bags to avoid contamination. Seawater samples were collected in labeled plastic jars. The samples were placed in sealed containers with dry ice and transported frozen to the laboratory for analysis. The time between sample collection and analysis was approximately 24 h.

***V. parahaemolyticus* strain isolation and identification.** *V. parahaemolyticus* was isolated and identified as described in the *Bacteriological Analytical Manual* of the Food and Drug Administration (8). All commercial reagents used were obtained from Difco unless indicated otherwise. The oysters were shucked in aseptic conditions. The heads and tails of the fish were cut off, and the guts were removed. The oyster and fish samples were then homogenized in blenders, and 50 g of each homogenate was placed in 450 ml of alkaline peptone water (APW) to obtain a 10^{-1} dilution. Seawater samples (25 ml) were added to 225 ml of APW, and 10^{-2} and 10^{-3} APW dilutions were prepared in duplicate and incubated at 37 and 42°C for 6 to 24 h. Enrichment broth was streaked onto thiosulfate-citrate-bile salts-sucrose agar plates and incubated at 37°C for 18 to 24 h. Two or three suspect *V. parahaemolyticus* colonies (round, 1 to 2 mm in diameter, humid, shiny, sucrose negative) were selected. Halophilism tests were performed on NaCl-tryptone agar (T_1N_0, T_1N_3, T_1N_6, T_1N_8, and T_1N_{10}). Additional characterization tests included Gram staining, cytochrome oxidase activity tests, ornithine indole motility biochemical tests, triple sugar iron tests, lysine iron agar tests, arginine dehydrolase tests, urea tests, tests for glucose oxidation-fermentation in Hugh-Leifson broth, and tests for arabinose, lactose, mannitol, mannose, and sucrose fermentation.

Serotyping. Serogroups were determined as described by Elliot et al. (8) by using commercial antisera (Denka Seiken, Co., Ltd., Tokyo Japan).

Hemolytic activity. *V. parahaemolyticus* strains were seeded in Wagatsuma agar prepared as described by Elliot et al. (9); this agar contained 3 g of yeast extract, 10 g of peptone, 70 g of NaCl, 5 g of K_2HPO_4, 10 g of mannitol, 0.001 g of violet crystal, 15 g of agar, 1 liter of distilled water, and 50 ml of human anticoagulated blood. The bacteria were incubated at 37°C for 18 h. In order to characterize the TRH phenotype, strains were seeded in heart infusion broth and SPP broth (5 g of NaCl, 5 g of $NaHPO_4$, and 2 g of glucose in 1 liter of distilled water; pH 7.6). Hemolytic activity was determined as described by Honda et al. (13) and Kishishita et al. (18). Positive and negative controls were included in all assays (Table 1).

DNA purification. *V. parahaemolyticus* strains were seeded in Luria-Bertani agar supplemented with 1% NaCl and incubated at 37°C for 24 h. Five colonies were selected, resuspended in 100 μl of filtered sterile distilled water, and boiled for 15 to 20 min to liberate the nucleic acid.

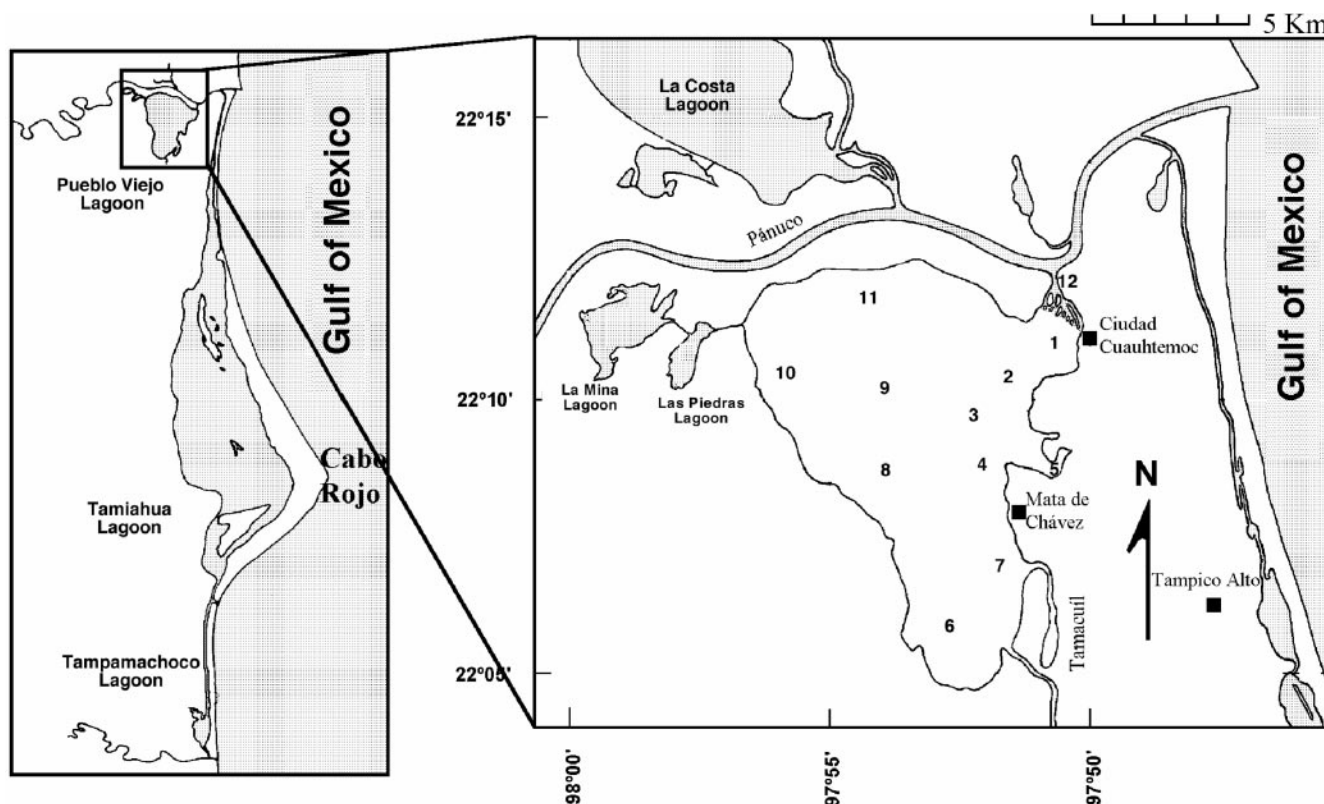

FIG. 1. Distribution of the 12 sampling sites in the Pueblo Viejo Lagoon, Veracruz. Site 1, El Bajo; site 2, Punto de Buda; site 3, Santa Clara; site 4, Ensenada; site 5, El Ciruelo; site 6, Cruz de Piedra; site 7, Isleta de Coralillo; site 8, Isleta Tomate; site 9, Medianía; site 10, Tamales; site 11, Punta de Mala Gana; site 12, Puente Hondo. Modified from reference 3.

PCR. The presence of the *toxR* gene was determined by using the following primers described by Kim et al. (17): 5′-GTCTTCTGACGCAATCGTTG-3′ (forward) and 5′-ATACGAGTGGTTGCTGTCATG-3′ (reverse), which produced a 368-bp amplicon. The PCR conditions were as follows: the reaction mixture (final volume, 25 μl) consisted of 3 μl of the solution containing DNA, 2.5 μl of 10× reaction buffer (Applied Biosystems), 6 μl of 25 mM $MgCl_2$, 1 μl of *Taq* polymerase (5 U/μl), 4 μl of deoxynucleoside triphosphates (1 μM), 1 μl of each primer (0.0125 ng/μl), and 6.5 μl of distilled water. The reactions were performed with a Gene Amp PCR system 2700 thermocycler (Applied Biosystems) as follows: 1 min of initial denaturation at 94°C, followed by 20 cycles of denaturation at 94°C for 1 min, alignment at 63°C for 1.5 min, and extension at 72°C for 1.5 min and a final extension at 72°C for 7 min.

The *tdh* and *trh* genes were amplified with the following primer sets: 5′-GGTACTAAATGGCTGACATC-3′ (forward) and 5′-CCACTACCACTCTCATATGC-3′ (reverse) for *tdh*; and 5′-GGCTCAAAATGGTTAAGCG-3′ (forward) and 5′-CATTTCCGCTCTCATATGC-3′ (reverse) for *trh* (26). These primer sets produced 251- and 250-bp amplicons, respectively. The reaction mixtures (final volume, 25 μl) contained 3 μl of the solution containing DNA, 2.5 μl of 10× reaction buffer (Applied Biosystems), 4 μl of 25 mM $MgCl_2$, 1 μl of *Taq* polymerase (5 U/μl), 4 μl of deoxynucleoside triphosphates (1 μmol), 1 μl of each primer (0.025 ng/μl), and 8.5 μl of distilled water. The reactions were performed with a Gene Amp PCR system 2700 thermocycler (Applied Biosystems) as follows: 1 min of initial denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, alignment at 55°C for 1 min, and extension at 72°C for 1 min and a final extension at 72°C for 7 min.

Positive and negative DNA controls were included in all assays (Table 1). Amplified products were separated by electrophoresis in ethidium bromide-stained 2% agarose gels in Tris-borate-EDTA buffer at 120 V for 30 min. The gels were visualized with a UV transilluminator (Eagle Eye; Stratagene).

RESULTS

A total of 266 samples were analyzed; these samples included 103 seawater samples, 75 oyster samples, and 88 fish samples. Overall, *V. parahaemolyticus* strains were isolated from 15% of the samples (Fig. 2 and Table 2). Of the 46 strains

TABLE 1. Reference strains

Strain	Serotype	Genes			Reference or source
		tdh	*trh1*	*trh2*	
V. parahaemolyticus VPAQ4037	O3:K6	−	+	−	22
V. parahaemolyticus VPAT4	O4:K37	−	−	+	18
V. parahaemolyticus VPWP1	O4:K12	+	−	−	21
V. parahaemolyticus VP17802	O1	−	−	−	ATCC[a]
V. alginolyticus VA-219		−	−	−	Mitsuaki Nishibuchi, Kyoto, Japan

[a] ATCC, American Type Culture Collection.

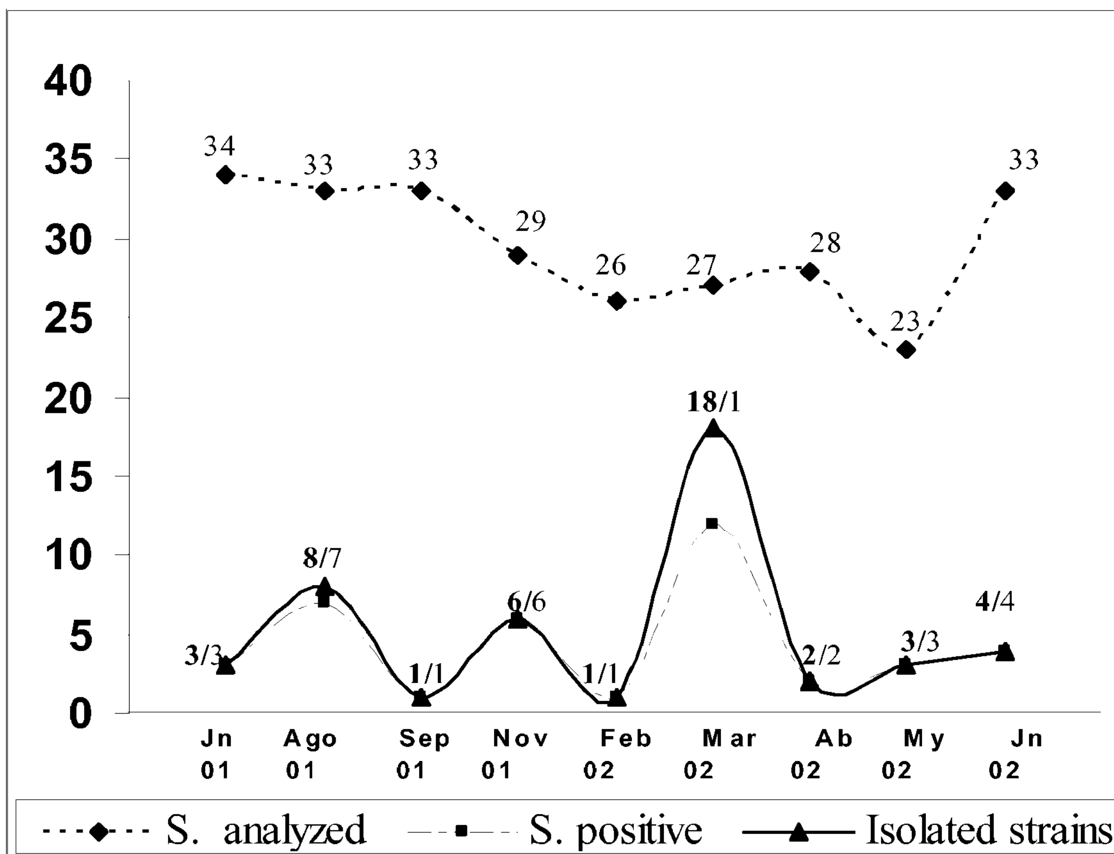

FIG. 2. Number of samples (S) from the Pueblo Viejo Lagoon positive for *V. parahaemolyticus* over the study period. Jn, June; Ago, August; Sep, September; Nov, November; Feb, February; Mar, March; Ab, April; My, May.

isolated, 20 (43%) were obtained from seawater samples, 17 (37%) were obtained from fish, and 9 (20%) were obtained from oysters.

V. parahaemolyticus serotyping was performed for epidemiological purposes. The 46 strains were identified as members of 6 of the 11 acknowledged serogroups; serotype O3 was the most frequent serotype (30%) (Table 3).

Only four strains showed hemolytic activity in Wagatsuma agar (KP^+), the TRH phenotype was not identified in any of the 46 strains, and all strains were positive for the *toxR* gene amplifying the 368-bp fragment (Fig. 3). The presence of the *tdh* and *trh* genes is indicative of the pathogenic potential of *V. parahaemolyticus*. Only 4 of the 46 strains (9%) amplified the 251-bp *tdh* gene fragment, which correlated with positive hemolytic activity (KP^+ strains) (Fig. 4). These four strains were serotype O3. The *trh* gene fragment was not amplified in any of the strains analyzed.

DISCUSSION

V. parahaemolyticus is distributed in water environments and is associated with gastroenteritis, wound infections, and septicemia. Diseases caused by this pathogen are frequently reported in coastal states and are caused by seafood consumption and direct contact with estuarine waters (19).

In the present study we demonstrated the presence of *V. parahaemolyticus* in 15% of the seawater, fish, and oyster samples analyzed. This study was the first research study to investigate isolation and the distribution of this pathogen in a Mexican coastal lagoon. There have been reports of *V. parahaemolyticus* isolation in Canada (16), France (10), Asia (2, 28), the United States (6), and Mexico (27). The factors affecting the incidence and distribution of *V. parahaemolyticus* in the environment include water temperature, salt and oxygen concentrations, interactions with the plankton, the presence of sediment, the organic matter in suspension, fish, and seafood, as well as the incorporation and tidal action of estuarine waters. The presence of *V. parahaemolyticus* seems to be constant in the Gulf of Mexico because the temperature does not drop below 11.6°C, unlike what occurs in Japan, Europe, Australia, and the United States, where isolation of this pathogen decreases during the winter months (14).

We found *V. parahaemolyticus* in 11 of the 12 sampling sites; the only site at which we did not find this pathogen was the Tamales site, which was located in the northeast part of the lagoon. Strains were serotyped for epidemiological purposes, and serogroup O3 was the most frequent serogroup (30%) (Table 2). Several researchers have pointed out that certain serogroups are constant in some geographical areas (1, 24). Serotypes O3:K6, O4:K68, and O1:KUT (K untypeable) have been related to the majority of the infections caused by this pathogen in Asia and the United States during the last 5 years (4, 5).

We observed positive *tdh* gene amplification in 4 of the 46

TABLE 2. Origins and serotypes of the *V. parahaemolyticus* strains isolated at different times

Month	No. of samples analyzed	No. of samples positive	No. of colonies assessed	No. of colonies confirmed	Sampling site	Sample type	No. of strains	Serogroup
June 2001	34	3	99	3	Santa Clara	Fish	1	O3
					El Ciruelo	Fish	1	O3
					El Bajo	Seawater	1	O3
August 2001	33	7	102	8	Isleta de Coralillo	Fish	2	O4; O4
					El Ciruelo	Fish	1	O3
					Mediania	Fish	1	O1
						Seawater	1	O10
					Cruz de Piedra	Seawater	1	O10
					Punta de Buda	Seawater	1	O3
					Santa Clara	Seawater	1	O10
September 2001	33	1	69	1	Ensenada	Seawater	1	O3
November 2001	29	6	81	6	Isleta Tomates	Oyster	1	O1
						Seawater	1	O3
					Ensenada	Fish	1	O1
					El Bajo	Fish	1	O1
					El Ciruelo	Fish	1	O4
					Cruz de Piedra	Seawater	1	O10
February 2002	26	1	104	1	Mediania	Seawater	1	O4
March 2002	27	12	122	18	Punta de Malagana	Seawater	1	O2
					Puente Hondo	Seawater	1	O5
					El Bajo	Seawater	3	O5; O3; O3
						Fish	1	O1
					El Ciruelo	Seawater	1	O1
						Fish	1	O3
					Cruz de Piedra	Oyster	1	O3
					Punta de Buda	Fish	3	O2; O3; O5
						Oyster	1	O1
					Mediania	Fish	1	O3
					Santa Clara	Oyster	3	O1; O1; O10
					Isleta Tomates	Seawater	1	O2
April 2002	28	2	96	2	Punta de Buda	Oyster	1	O1
					Santa Clara	Oyster	1	O1
May 2002	23	3	108	3	Mediania	Seawater	1	O10
					Punta de Malagana	Seawater	1	O2
					Santa Clara	Oyster	1	O5
June 2002	33	4	101	4	Santa Clara	Fish	1	O5
					Punta de Buda	Seawater	1	O3
					El Bajo	Fish	1	O5
					Ensenada	Seawater	1	O5
Total	266	39	882	46			46	

TABLE 3. Serotyping of the 46 strains isolated

Serogroup	No. of strains
O1	11
O2	4
O3	14
O4	4
O5	7
O10	6

strains analyzed, which correlated with positive hemolytic activity in Wagatsuma agar (KP^+). Two of these strains were isolated from fish, and two were isolated from water samples. Most environmental strains are known to be KP^-, and only 1 to 2% are KP^+ (14, 20). This implies that there is a source of human fecal contamination in the estuarine waters of Pueblo Viejo Lagoon. The lagoon is surrounded by small rural populations, some of which use latrines that discharge feces into the lagoon. Furthermore, the water is very shallow (only 1.5 m deep during the rainy season), so fisherman, dogs, and other domestic animals can easily walk through it, which is an additional source of contamination.

Although it is known that 0 to 5% of the environmental

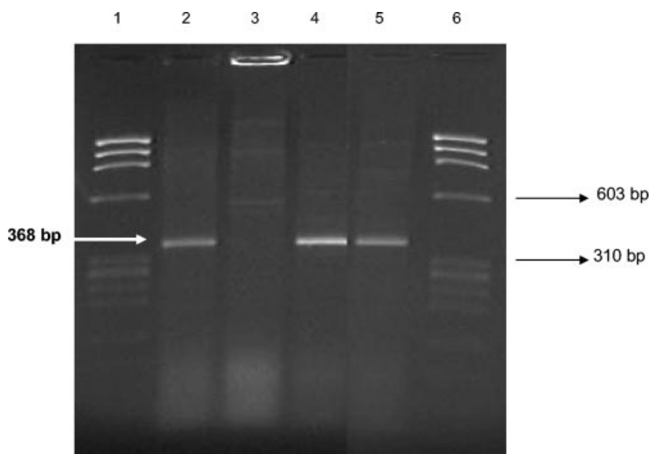

FIG. 3. Amplification of the *toxR* gene by PCR. The primers amplified a 368-bp fragment. Lanes 1 and 6, molecular weight marker φX147/HaeIII; lane 2, control strain *V. parahaemolyticus* ATCC 17802; lane 3, negative control strain Va-219; lanes 4 and 5, experimental strains.

strains produce TRH (10, 29), the *trh* gene and the TRH phenotype were not identified in any of the strains isolated here. The possibility of false-negative results is unlikely, as the positive control strain (ATCC 17802) (Table 1) amplified the 368-bp *trh* fragment in all assays performed. There have been few reports of the presence of the *tdh* and *trh* genes in *V. parahaemolyticus* strains of environmental origin; only 0 to 6% of the samples analyzed from the coasts of the United States (6, 7, 15), Europe (10), and Asia (2, 28) contained *tdh*-positive *V. parahaemolyticus* strains. The *toxR* gene fragment was amplified in all the strains isolated here, confirming that they were in fact *V. parahaemolyticus*, as this gene can be used to identify the species (17).

The data here presented are part of the first studies on the isolation, distribution, and detection of virulence factors of *V. parahaemolyticus* in Mexico. The presence of potentially pathogenic *V. parahaemolyticus* strains in the Pueblo Viejo Lagoon is matter of concern for sanitary authorities, because this organism has not been considered a health problem in spite of the information on infection outbreaks along the coastline of the Gulf of Mexico. This information may be important for preventing sanitary problems that might affect the health of the population.

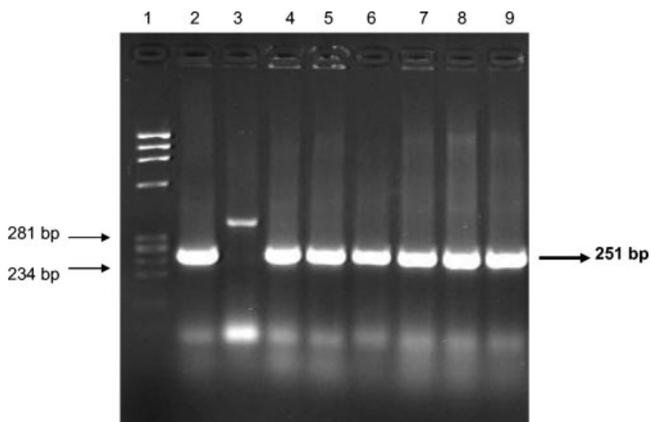

FIG. 4. Amplification of the *tdh* gene by PCR. The primers amplified a 251-bp fragment. Lane 1, molecular weight marker φX174/HaeIII; lane 2, control strain *V. parahaemolyticus* WP1; lane 3, negative control strain Va-219; lanes 4 to 9, experimental strains.

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