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Presence of the Hemolysin Gene of *Vibrio mimicus* in Fish and Seafood Products in Sonora, México

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Abstract

Vibrio mimicus causes diseases in humans in many countries, and it is highly abundant in aquatic environments. The present study evaluated the presence of *V. mimicus* in commonly consumed fish and seafood products in Sonora, México. A total of 262 samples of fish and seafood products were analyzed using PCR to identify the presence of the hemolysin (*vmh*) gene of *V. mimicus*, which was detected in 32 food samples. The positive food samples included raw (14%) and ready to eat fish and seafood (9%). The leading raw products in which *V. mimicus* was detected were crustaceans (57%), but mollusks represented 78% of the positive ready-to-eat products (RTE). Therefore, the presence of the *V. mimicus* hemolysin gene in raw and RTE seafood may represent a potential health risk to consumers in northwest México.

Keywords: hemolysin *vmh*, seafood safety, *Vibrio mimicus*

1. Introduction

The *Vibrionaceae* family is comprised of a variety of important microorganisms (J. Farmer III, 2006), including at least 12 species of clinical concern that are causative agents of human diseases. The primary pathogenic species to humans are *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* (Daniels & Shafaie, 2000). However, *Vibrio mimicus* has been linked to food-borne illnesses in recent years (Adeleye, Daniels, & Enyinnia, 2010; Chitov, Kirikaew, Yungyune, Ruengprapan, & Sontikun, 2009; Hlady & Klontz, 1996; Newton, Kendall, Vugia, Henao, & Mahon, 2012; Zamudio, 2005).

V. mimicus is a Gram-negative, oxidase- and catalase-positive, Voges-Proskauer-negative, mobile bacterium with a polar flagellum. It produces toxins, hemolysins, hemagglutinins, proteases and siderophores (Davis et al., 1981; J. J. Farmer III, Janda, Brenner, Cameron, & Birkhead, 2005; Hasan et al., 2010). *V. mimicus* grows at refrigeration temperatures (4 °C), survives under freezing conditions (-30 °C) and tolerates up to 6% NaCl (Chowdhury, Yamanaka, Miyoshi, Aziz, & Shinoda, 1989; Wong, Chen, & Yu, 1994).

This bacterium is associated with human disease in various countries, such as Bangladesh, Japan, Costa Rica, Mexico, Thailand, the United States, Nigeria, Brazil, Australia and Venezuela. In addition, *V. mimicus* was isolated from water, plants, sediment and food samples, such as shrimp, crabs, oysters and clams (Adeleye et al., 2010; Begum & Khan, 2001; Chitov et al., 2009; Davis et al., 1981; Muñoz, Marín, Marval, & Martínez, 2012; Saad, Edris, Ibrahim-Hemmat, & Rasha, 2013; Tercero Alburo, 2008; Vieira, Teixeira, Vicente, Momen, & Salles, 2001; Zamudio, 2005).

Various genetic and molecular methodologies are frequently used to identify bacteria, including bacteria of epidemiological interest. Many molecular methodologies are based on repetitive amplification (ERIC-PCR, GTG5-PCR), random amplified polymorphic DNA (RAPD), restriction-fragment length polymorphism (RFLP, AFLP) and several types of PCR amplification (multiplex PCR or real-time PCR). These methodologies are widely accepted because of their reproducibility, simplicity and discriminatory power (Foley & Grant, 2007; Ochman, 2001; Prakash et al., 2007; Ramamurthy & Nair, 2007; Thompson, Iida, & Swings, 2004).

V. mimicus produces several extracellular toxic factors, but the most common factor is a heat-labile hemolytic/cytolytic toxin known as *V. mimicus* hemolysin or VHM (Mizuno et al., 2009; Mizuno, Nanko, Maehara, Shinoda, & Miyoshi, 2014). The VHM gene, *vmh,* is common to clinical and environmental *V. mimicus* strains, and it is a species-specific identifier (Shinoda et al., 2004; Sultan et al., 2007). Currently, *V. mimicus* is recognized as a human pathogen in the United States by the FDA (Food and Drug Administration), and it is described briefly in their Bacteriology Analytical Manual (Kaysner & DePaola, 2004). In Mexico, only *V. cholerae* is under a surveillance program by health authorities, but they recognize an average of suspected cholera cases between 3 000 and 4 000 with a minimal positive cases, probably caused by other *Vibrio* species (León Robles et al., 2013). Hence, *V. mimicus* is not reportable in Mexico by the National System of Epidemiological Surveillance (SINAVE), and there are no official statistical information about its incidence. Consequently, *V. mimicus* may go unnoticed as the causal agent of some of the food-borne diseases in Mexico (Campos et al., 1996; Gonzalez Vazquez, Tercero Alburo, Quiñones-Ramírez, & Vazquez Salinas, 2005; Tercero Alburo, 2008).

Therefore, the present study focused on the detection of the VMH gene (*vmh*) in fish and seafood products from Sonora, Mexico, to provide information on the abundance of this bacterium in seafood products and help elucidate whether this bacterium is a microbiological risk for consumers.

2. Materials and Methods

2.1 Sample Processing

The collection and initial processing of samples were conducted by personnel of the Sonora State Public Health Laboratory (LESP), which is part of the Mexican Ministry of Health. Sampling was conducted according official procedures that were adopted as part of the State *Vibrio*-surveillance program. Samples were collected from April to October 2011 and transported to the LESP for analysis. A total of 262 food samples (shrimp, scallops, oysters, fish, octopus, and clams, among others) were analyzed. Samples were collected in major cities from 14 different counties of Sonora, Mexico that were divided for statistical purposes in coastal and non-coastal counties (Figure 1). Samples were homogenized in alkaline peptone water (APW) and incubated at 37 °C for 6 h. For the molecular detection of the *vmh* gene of *V. mimicus,* 50 mL of the incubated APW were taken to the Microbiology Laboratory of the Research Center for Food and Development (CIAD) for DNA extraction.

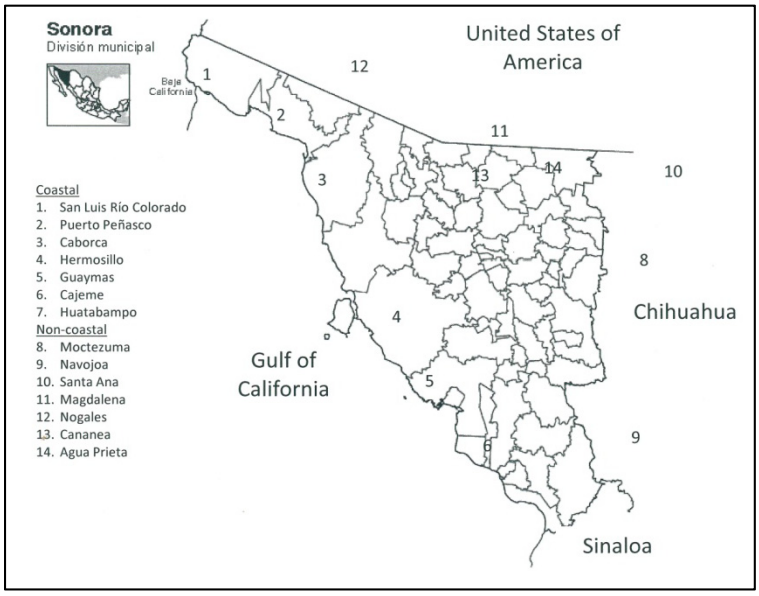

Figure 1. Localization of the seven coastal and seven non-coastal sampled counties in Sonora, Mexico (Instituto Nacional de Estadistica y Geografía [INEGI], 2010)

2.2 DNA Extraction

DNA extraction was based on the method described by Noriega-Orozco, Acedo-Félix, Higuera-Ciapara, Jiménez-Flores, and Cano (2007), with modifications. A 1.5-mL aliquot of APW was suspended in a trypticase soy broth (TSB) containing 1% NaCl, and the solution was centrifuged (17,100 g for 5 min). Then, 500 µL of buffer (500 mM Tris-HCl, 100 mM NaCl, 1 mM sodium citrate), 50 µL of lysozyme (20 mg/mL) and 5 µL of mutanolysin (5 U) were added, and the samples were incubated at 37 °C for 2 h. A total of 50 µL of proteinase K (20 mg/mL) was added, and the solution was incubated at 50 °C for 2 h. Subsequently, 500 µL of lysis solution (200 mM Tris-HCl, 100 mM NaCl, 4% sodium dodecyl sulfate, 1.5% polyvinylpolypyrrolidone) and 600 µL of phenol-chloroform-isoamyl alcohol (25:24:1) were added prior to centrifugation for 25 min at 11,300 × g. The top layer was recovered, and 500 µL of cold isopropanol was added and maintained at -20 °C for 18 h before centrifugation (15 min at 17,100 g). The supernatant was decanted, and the pellet was allowed to dry at room temperature. Finally, 50 µL of RNase (20 mg/mL) was added, and the solution was incubated at 37 °C for 1 h. DNA extraction was confirmed by agarose gel electrophoresis (0.8% agarose) at 100 V for 30 min. DNA extracts were stored at -20°C until used as templates for the PCR reaction.

2.3 Primers

Species identification was based on amplification of the hemolysin gene of *V. mimicus* (*vmh*) (Shinoda et al., 2004; Sultan et al., 2007). A 390-bp region of *vmh* was used as amplification primers in this study, *Vmh390F:* GGTAGCCATCAGTCTTATCACG and *Vmh390R:* ATCGTGTCCCAATACTTCACCG. These primers were previously reported as species specific (Shi et al., 2000).

2.4 PCR conditions

The amplification-reaction mixture contained the following components: 10 µL of DNA template, 4 µL of dNTPs (200 µM each), 2 µL of $MgCl_2$ (25 mM), 1 µL of each primer (50 pM), 10 µL of 5× Buffer, and 0.5 µL of Taq DNA polymerase (5 U) in a final volume of 57.5 µL. The PCR reaction was performed in a Perkin Elmer thermal cycler under the following conditions: 1 cycle at 94 °C for 2 min; 35 cycles of 95 °C for 45 sec, 54 °C for 45 sec and 72 °C for 35 sec; and 1 cycle at 72 °C for 5 min. *V. mimicus* CAIM 602 (ATCC 33653) and *Escherichia coli* (K88) were used as positive and negative controls, respectively. Additionally, *V. cholerae* CAIM 1410, *V. cholerae* CAIM 1409 and *V. parahaemolyticu*s CAIM 1772 were tested to confirm primer specificity. PCR products were visualized in 1.8% agarose gel electrophoresis (95 V for 80 min). Samples that showed the 390-bp fragment corresponding to the *vmh* gene region were reported as *V. mimicus*-positive.

2.5 Statistical Analysis

The percentage of *V. mimicus*-positive samples was calculated, and the result was referred to as the *V. mimicus* incidence. The incidence for statistical purposes was calculated for total fish and seafood samples, type of product (crustaceans, fish and mollusks), sample condition (raw and ready to eat–RTE), county (coastal and non-coastal) and sampling month. Results were analyzed using s JMP® V 9.0.2 (2010 SAS Institute Inc) program for multivariate analysis to compare all calculated incidences ($P < 0.05$).

3. Results

3.1 PCR Specificity

Specificity for the detection of the *vmh* gene of *V. mimicus* is shown in Figure 2. Negative results were obtained for *V. cholerae*, *V. parahaemolyticus* and the negative control *E. coli.* Only *V. mimicus* produced the expected 390-bp fragment of the *vmh* gene, which corroborate the specificity of these primers.

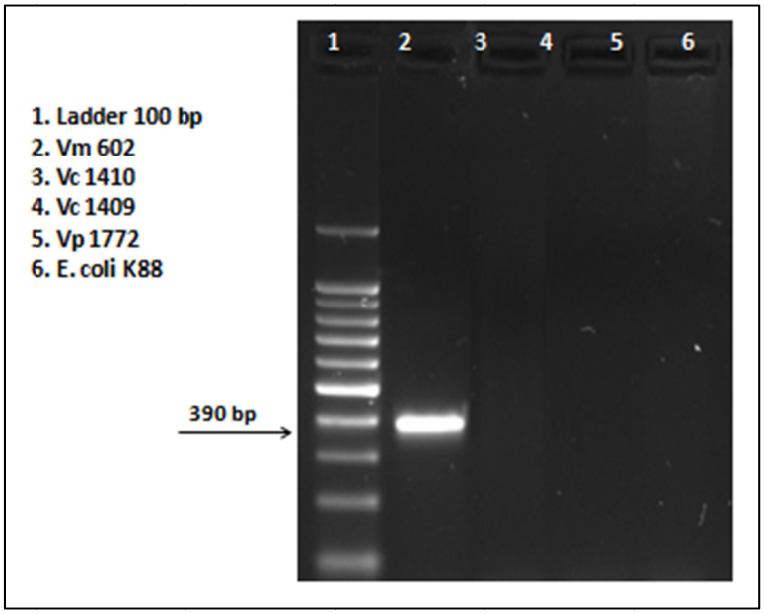

Figure 2. Amplification of the 390-bp fragment of the hemolysin gene (*vmh*) of *V. mimicus*: 1. Ladder 100bp; 2. *V. mimicus* CAIM 602; 3. *V. cholerae* CAIM 1410; 4. *V. cholerae* CAIM 1409; 5. *V. parahaemolyticus* CAIM 1772; 6. *E. coli* K88

3.2 Detection of V. mimicus Hemolysin Gene Using PCR

Sample frequency by product type and detection of the *vmh* gene of *V. mimicus* in fish and seafood samples is show in Table 1. Thirty-two of the 262 samples analyzed were positive (12%), and a higher percentage was observed in raw (14%) than ready-to-eat products (9%). Crustaceans (20%) had the highest detection rate, followed by fish (11%) and mollusks (8%). Positive correlations were observed between the total incidence of *V. mimicus* and RTE ($P < 0.0016$), raw ($P < 0.0052$), crustaceans ($P < 0.0076$) and mollusks ($P < 0.0018$) groups, but not fish products. Table 2 shows the distribution of positive raw and RTE seafood samples by product type. From the 23 positive *V. mimicus* raw samples, 57% corresponded to crustaceans, and 7 out of 9 positive RTE samples were mollusks (78%).

Month and county distributions of positive samples are presented in Figure 3 and Table 3, respectively. The highest incidence of *V. mimicus* was detected in July, and a positive correlation with crustaceans and mollusks was observed. The incidence of fish products was most associated to June, which was not significantly correlated with any other product. The county analysis showed no differences between coastal (12%) and non-coastal (14%) counties. Guaymas and Cajeme were the coastal counties with the highest incidences, and Santa Ana and Agua Prieta were the non-coastal counties with the highest incidences.

Table 1. Percentages of positive samples for the *vmh* gene of *V. mimicus* by product type as detected by PCR

	Samples	Positives Samples	
		No.	(%)
Total Samples	262	32	12
Raw Products	164	23	14
RTE[1]	98	9	9
Crustaceans	74	15	20
Fish	66	7	11
Mollusks	122	10	8

[1] RTE: ready-to-eat products, including fully cooked or seafood intended for raw consumption.

Table 2. Distribution of *V. mimicus*-positive raw and RTE seafood samples by product type

	Raw		RTE	
	Positive samples	(%)	Positive samples	(%)
V. mimicus (+) samples	23		9	
Crustaceans (%)	13	57	2	22
Fish (%)	7	30	0	0
Mollusks (%)	3	13	7	78

RTE: ready-to-eat fish or seafood products, includes fully, partially cooked or seafood intended for raw consumption.

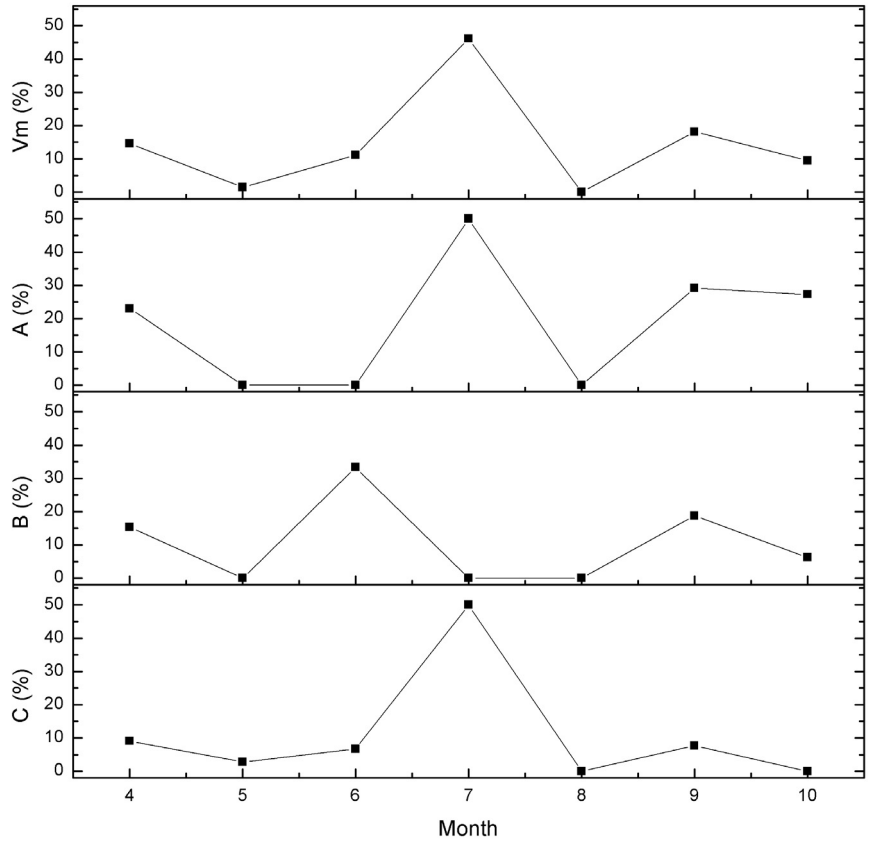

Figure 3. Percentage of monthly detection of *V. mimicus* in seafood samples using PCR of the *vmh* gene in different seafood samples. Vm: total seafood samples positive to *V. mimicus*, A: crustacean samples positive to *V. mimicus*, B: fish samples positive to *V. mimicus* and, C: mollusks samples positive to *V. mimicus*

Table 3. Total of *V. mimicus*-positive samples by location type: coastal or non-coastal county

Location	Counties	No. of Samples	No. Positives	% Positives
Coastal		**182**	**23**	**12.6**
	Cajeme	22	4	18.2
	Guaymas	50	9	18
	San Luis Río Colorado	19	3	15.8
	Pto. Peñasco	17	2	11.8
	Hermosillo	39	4	10.3
	Huatabampo	19	1	5.3
	Caborca	16	0	0
Non-coastal counties		**80**	**9**	**11.2**
	Santa Ana	9	2	22.2
	Agua Prieta	15	3	20
	Magdalena de Kino	12	2	16.7
	Nogales	13	2	15.4
	Cananea	11	0	0
	Navojoa	14	0	0
	Moctezuma	6	0	0

4. Discussion

The presence of *Vibrio* species that are associated with marine ecosystems worldwide has been widely documented (Adeleye et al., 2010; Begum & Khan, 2001; Chitov et al., 2009; Eyisi, Nwodo, & Iroegbu, 2013). In this study, 12% of the food samples were positive for the *vmh* gen of *V. mimicus*, which was more common in raw (14%) than RTE products (9%). Additionally, *V. mimicus* was more frequently detected in crustaceans (20%) followed by fish (11%) and mollusks (8%). Most of the crustacean samples were raw products and mollusks as RTE. Crustaceans, especially shrimp and mollusks, are products that are widely consumed without further heat treatment, which may present a higher risk to the consumer. The values obtained in this study are higher than the values reported for raw seafood by Franco-Monsreal et al. (2003), who detected the presence of *V. mimicus* in 7.7% of raw food samples and 1.5% of partially cooked food, but not in fully cooked foods. In contrast, Tercero Alburo (2008) isolated strains of *V. mimicus* from fish (5), oysters (9) and water samples (6) taken from the Pueblo Viejo Lagoon in Veracruz, Mexico and found that these strains were gene virulence carriers, which demonstrate their pathogenic potential. *V. mimicus* in Lagos, Nigeria was detected in 22.7% of the seafood samples analyzed (shrimp, crabs and mollusks), whereas *V. cholerae* was detected in only 6.8% of these samples. *V. mimicus* strains are not enterotoxigenic, but they have the capacity to lyse red blood cells and invade intestinal epithelial cells. Therefore, this pathogen could cause infections in humans (Adeleye et al., 2010). Recently, high loads of *Vibrio* species were found in crayfish, lobster and water samples from the southeastern Atlantic coast of Nigeria. The species identified were *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. fluvialis* and *V. mimicus*. *V. mimicus* was most often isolated in lobster (21.4%) and crayfish (21.5%) (Eyisi et al., 2013). Differences in the frequency of *V. mimicus* detection in food products between regions may be due to several factors, including the initial contamination level, product handling at inadequate temperatures and cross-contamination after capture. The initial level or origin contamination could be affected by environmental parameters because temperature, salinity, and conductivity, among others, affect *Vibrio* occurrence in marine environments (Caburlotto et al., 2012; Collin & Rehnstam-Holm, 2011; León Robles et al., 2013). However, factors such as temperature and hygienic handling must to be controlled along the productive food chain to reduce the survival and growth of *Vibrio* species and other pathogenic bacteria (Boonyawantang, Mahakarnchanakul, Rachtanapun, & Boonsupthip, 2012; Nunes et al., 2010).

The *vmh* gene of *V. mimicus* was detected at low levels in RTE seafood products (9%), but these products will not have heat treatment before eating, and the consumer health risks need to be addressed. Notably, most

crustaceans were in the raw form and represented the highest percentage of positive samples. Proper cooking of food products is sufficient to eliminate *V. mimicus* (Adeleye et al., 2010; Chitov et al., 2009). Therefore, its detection in cooked products leads us to assume that inadequate cooking or cross-contamination after heat treatment because of poor handling procedures had occurred.

The association of different species of the genus *Vibrio* with crustaceans, especially shrimp or shrimp-shells, has been widely documented (Aguirre-Guzman, Mejia Ruiz, & Ascencio, 2004; Manilal et al., 2010; Somboon, Purivirojkul, Limsuwan, & Chrchird, 2012). One of the major components of crustaceans shells, chitin, has also been reported to have a protective effect on *Vibrio* species (Pruzzo, Vezzulli, & Colwell, 2008; Vezzulli, Pruzzo, Huq, & Colwell, 2010). Therefore, it is not surprising that crustaceans were the most commonly contaminated product. The highest incidence of positive crustacean samples was found in July and September, which are initial months of aquaculture product harvesting and wild-shrimp season. These factors increase crustacean availability as a fresh product and its raw consumption.

The presence of *vmh* in fish samples was detected only in raw products, with the highest incidences in July and September. Therefore, no RTE samples were positive, and the low incidence in fish (11%) suggests that these products are low risk if the product is properly handled after catch.

Mollusk samples showed the lowest incidence (8%), but these samples were the main positive seafood type in RTE products (78%). More than half of the positive mollusk samples were raw bivalves on shells during July, and the rest were fully cooked mollusks. Cross-contamination plays an important role in the presence of *V. mimicus* and other pathogenic bacteria in cooked products (Center for Disease Control and Prevention [CDC], 2010); but also the ambient temperature for fresh bivalve mollusks such oysters, particularly in Sonora were the highest average temperatures occur during July and August (Instituto Nacional de Estadistica y Geografía [INEGI], 2010).

The counties with the highest percentages of *V. mimicus*-positive samples were Cajeme (18.2%) and Guaymas (18.0%) as coastal counties and Santa Ana (22.2%) and Agua Prieta (20.0%) as non-coastal counties. However, there was no difference in the incidence of *V. mimicus* between coastal (12.6%) or non-coastal (11.2%) counties. The number of samples collected per county was not identical because more samples were collected in counties with larger populations, which were normally found in the coastal area. The seasonality of some seafood products also reduces product availability in smaller or non-coastal counties throughout the year, and more data are needed for comparison between these counties.

Month distribution did not show the common warm-weather patterns that were reported for other *Vibrio* species (Centers for Disease Control and Prevention [CDC], 2012; Hlady & Klontz, 1996). This study showed three peaks of incidence for *V. mimicus,* April, July and September, with the highest incidence in July because of crustaceans and mollusks. However, the sampling period did not encompass a full year, only the warmest months. Nevertheless, there are differences in ambient temperature between July-September (>30 °C) and April (<25 °C). Analyses of the distribution of *V. mimicus* infections per month in Florida from 1981 to 1993 showed that the highest incidences occurred during July, April and March also without a seasonal pattern, like *V. vulnificus* and other *Vibrio* species (Hlady & Klontz, 1996). There are no official statistics of *V. mimicus* in Mexico, and the real impact of this bacterium on the health of Mexican population is unknown. However, it is well known that most cases of *Vibrio* infections occur during the summer months (Centers for Disease Control and Prevention [CDC], 2014). A previous study of the environmental factors affecting the abundance of some *Vibrio* species, including *V. mimicus,* in the area of Guaymas, Mexico showed good correlation between the abundance and ambient parameters (temperature, salinity, pH, among others) and some *Vibrio* species. However, environmental conditions could not fully explain the behavior of *V. mimicus* and its abundance should be influenced by others parameters (León Robles et al., 2013). Therefore, the low incidence of these bacteria in natural environments, its association by product type or other external or environmental factors could affect its frequency in fish and seafood products.

However, the State of Sonora accounts for 49% of the national fish and seafood production (catches and aquacultures), and it is one of the main shrimp-exporting regions in Mexico (Oficina Estatal de Información para el Desarrollo Rural Sustentable del Estado de Sonora [OEIDRUS], 2010, 2011). Therefore, the sanitary condition of Mexican fish and seafood products has a great impact on the health of countries other than Mexico. The need to expand this study to assess the economic and health impact for *V. mimicus* and other pathogenic *Vibrio* species is evident. The collection of more data on the consumption of marine products by species is also highly recommended to determine the potential risk to the population.

5. Conclusions

V. mimicus may represent a potential health risk because it was detected in raw and RTE products. Raw crustaceans and mollusks are the products of major concern, but fish may be considered a low-risk product because of its low incidence. Crustaceans were the most commonly contaminated product, with a high frequency at the beginning of the harvest and wild season. Mollusks were the most common RTE product associated to *V. mimicus*. The presence of total *V. mimicus* in fish and seafood showed no seasonal patterns, and no differences in coastal and non-coastal counties were found. Therefore, the presence of *V. mimicus* in seafood products may be due to several factors, including the initial contamination level, product type, product handling, food chain and cross-contamination after capture. This study demonstrated the need to understand and analyze patterns of fish and seafood consumption, and handling practices for fish and seafood. In addition to the detection of pathogenic species, various virulence factors that are associated with *V. mimicus* should be monitored to determine their infective potential and possible effects on consumer health.

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