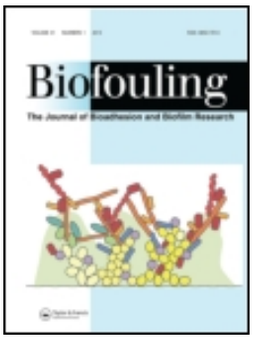

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Biofouling
The Journal of Bioadhesion and Biofilm Research

ISSN: 0892-7014 (Print) 1029-2454 (Online) Journal homepage: www.tandfonline.com/journals/gbif20

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To cite this article: Valeria J. Revilla-Castellanos, Abraham Guerrero, Bruno Gomez-Gil, Erick Navarro-Barrón & Marcial L. Lizárraga-Partida (2015) Pathogenic *Vibrio parahaemolyticus* isolated from biofouling on commercial vessels and harbor structures, Biofouling, 31:3, 275-282, DOI: 10.1080/08927014.2015.1038526

To link to this article: https://doi.org/10.1080/08927014.2015.1038526

Biofouling, 2015
Vol. 31, No. 3, 275–282, http://dx.doi.org/10.1080/08927014.2015.1038526

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Pathogenic *Vibrio parahaemolyticus* isolated from biofouling on commercial vessels and harbor structures

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(*Received 13 October 2014; accepted 1 April 2015*)

Ballast water is a significant vector of microbial dissemination; however, biofouling on commercial vessel hulls has been poorly studied with regard to pathogenic bacteria transport. Biofouling on three commercial vessels and seven port structures in Ensenada, Baja California, Mexico, was examined by qPCR to identify and quantify *Vibrio parahaemolyticus*, a worldwide recognized food-borne human pathogen. Pathogenic variants (*trh*+, *tdh*+) of *V. parahaemolyticus* were detected in biofouling homogenates samples from several docks in Ensenada and on the hulls of ships with Japanese and South Korean homeports, but not in reference sampling stations. A total of 26 *tdh*+ *V. parahaemolyticus* colonies and 1 ORF8+/O3:K6 strain were also isolated from enriched biofouling homogenate samples confirming the qPCR analysis. Our results suggest that biofouling is an important reservoir of pathogenic vibrios. Thus, ship biofouling might be an overlooked vector with regard to the dissemination of pathogens, primarily pathogenic *V. parahaemolyticus*.

Keywords: *Vibrio* biofilms; ship hull fouling; microbial biofouling

Introduction

Human activity is now considered the primary cause of the global spread of non-native marine species (Carlton 1985, 1989; Gollasch 2002; Leppäkoski et al. 2002). The transoceanic transfer of microorganisms has serious implications due to their high numbers in natural waters, ability to enter a dormant or resistant state, and their pathogenic potential (Drake et al. 2007). Commercial vessels have been reported to mediate pathogenic transportation in ballast water (Ruiz et al. 2000; Rivera et al. 2013) and on their hulls due to biofouling communities (Shikuma & Hadfield 2010), creating significant reservoirs of invasive and potentially pathogenic organisms. Moreover, the introduction of pathogens to a new host, population, or territory has been directly linked to the magnitude and frequency of global transportation (Smith & Guégan 2010). Therefore, biofouling on the surface of ships' hulls is an invasive mechanism by which potentially unsuitable species can be transported and thus colonize new environments (Apte et al. 2000).

A biofilm, which forms in the first step of biofouling, is an assembly of microbial cells that are strongly associated with a surface and are enclosed in a matrix of polysaccharides (Zobell & Allen 1935). Biofilms can form on many surfaces, including living tissues, certain medical instruments, piping systems, and natural or man-made aquatic surfaces (Donlan 2002). Furthermore, biofilms provide an important mechanism that allows *Vibrio* spp. to persist in the environment (Alam et al. 2007). Zettler et al. (2013) reported that by forming tight biofilm associations on abiotic surfaces, a member of the genus *Vibrio* constituted ~24% of a plastic marine debris microbial community, in comparison with seawater samples that make up < 1%.

Vibrios are free-living, Gram-negative aquatic bacteria, of which many species have strains that are human pathogens, such as *Vibrio cholerae*, *V. vulnificus*, and *V. parahaemolyticus*. *Vibrio* infection is usually related to seawater exposure or contaminated raw/undercooked seafood (Dechet et al. 2008), and it is estimated to cause 80,000 illnesses, 500 hospitalizations, and 100 deaths each year in the US (Newton et al. 2012). Two virulence factors have been identified in clinical isolates of *V. parahaemolyticus*, the Thermostable Direct Hemolysin (TDH) and TDH-Related Hemolysin (TRH). Organisms of the serotype O3:K6 with the *tdh* gene, but not the *trh* gene, have been identified as the main pathogenic serotype worldwide (Nair et al. 2007).

Gastroenteritis is the most common clinical manifestation of *V. parahaemolyticus*, and on rare occasions it could progress to septicemia in individuals with underlying medical conditions (FAO/WHO 2011). *V. parahaemolyticus* was first identified as the cause of foodborne diseases in Japan in 1950, and since 1996 the O3:K6 serotype and its serovariants have been implicated in several outbreaks (Nair et al. 2007). In

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Asia, *V. parahaemolyticus* has been considered a pandemic strain since 1996 and caused half of the food poisoning outbreaks in Taiwan, Japan and several Southeast Asian countries (Wong et al. 2000). In the US, the pandemic strain O3:K6 was isolated from a clinical case in Peru in 1996 (Gil et al. 2007) and thereafter outbreaks of *V. parahaemolyticus* O3:K6 were reported in Chile, the US and Mexico (Daniels et al. 2000; Cabanillas-Beltrán et al. 2006; Fuenzalida et al. 2006).

Newton et al. (2012), indicate that the crude *V. parahaemolyticus* incidence per 100,000 population increased in the US from 1996 to 2010, from 0.01 to 0.13 according to the Cholera and Other *Vibrio* Illness Surveillance system (COVIS), and from 0.06 to 0.23 according to the Foodborne Diseases Active Surveillance Network (Food-Net). Although the reason for the increment of *V. parahaemolyticus* infections remains unclear, Baker-Austin et al. (2010) suggest that warming of marine waters as a result of climate change and the increasing consumption and trade in seafood increase the incidence of infections.

The port of Ensenada is one of the most important Mexican ports on the Pacific coast with increasing arrivals of touristic and commercial ships. The number of commercial ships that arrived from Asia and other countries in the port of Ensenada increased from 32,202 in 2004 to 132,727 in 2011 (Secretaría de Comunicaciones y Transportes 2011). This increase has rendered the port a suitable area to research the presence of *V. parahaemolyticus* in biofouling communities on the hulls of transoceanic commercial vessels. In this study, the authors examined whether ship biofouling is a reservoir and potential dissemination vector for *V. parahaemolyticus*.

Materials and methods

Sampling sites

Biofouling samples were collected from several docks located in Bahía Todos Santos, Ensenada, Baja California, Mexico (Figure 1). The Ensenada International Terminal (EIT) lies in the harbor, where a docking area for cargo vessels is located. The samples from this dock area were designated as follows: EIT-B1: on arrival of Ship B1 from Yokohama, Japan; EIT-B2: on arrival of Ship B2 from Busan, South Korea; and EIT-B3: on arrival of Ship B3 from Fukuyama, Japan. The Ensenada Cruise Port Village dock (ECP), where tourist cruise ships arrive weekly, and the Local Tours Port dock (LTP), also lie within the harbor. Two sampling sites situated outside the harbor and not influenced by cargo or cruise vessel activity were included as reference stations: Coral Marina (CM), a docking facility used by small private boats, and the oceanographic buoy FLUCAR, situated outside the Bahía Todos Santos.

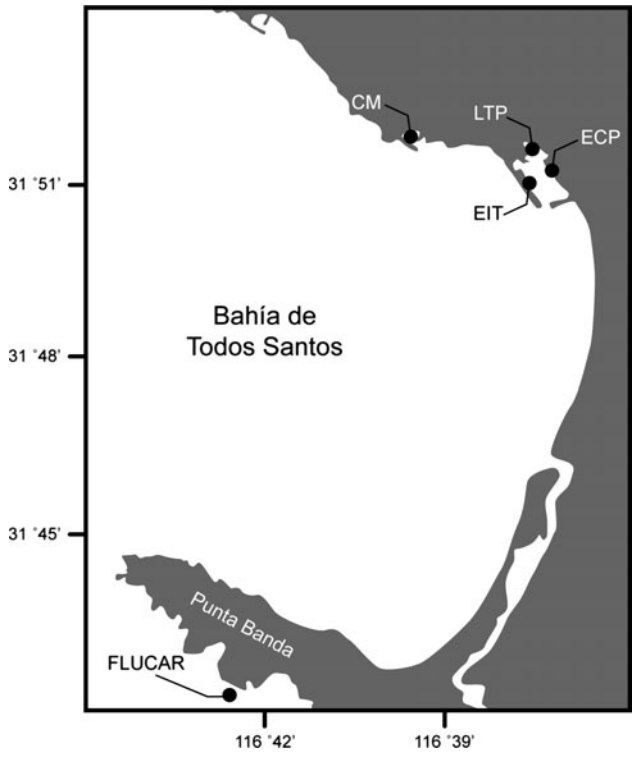

Figure 1. Location of sampling docks at Bahía Todos Santos in Ensenada, B.C. (EIT) Ensenada International Terminal for cargo ships; (LTP) dock local tours port; (ECP) dock Ensenada Cruise Port Village; (CM) dock Coral Marina for small boats; (FLUCAR) oceanographic buoy.

Biofouling collection from vessel hulls

A diver collected samples from three ships by scraping biofouling material underwater with a spatula into airtight bags from randomly selected areas of the hull. The samples were then filtered through a 0.22 µm nitrocellulose membrane to remove seawater, and the biofouling that was retained in the filter was placed in 9 ml of alkaline peptone water (APW).

Biofouling collection from harbor structures

Samples from six docks and the buoy were collected into airtight bags, after which 200 g of biofouling material from each location were weighed and homogenized with 200 ml of phosphate-buffered saline (PBS); 1 ml of this homogenate was diluted with 9 ml of APW (1:10). The biofouling samples from the vessels and harbor structures in APW were incubated at 35°C for 12–18 h, for an enrichment PCR technique as per the Bacteriological Analytical Manual (FDA 2004).

DNA extraction and real-time PCR (qPCR)

Biofouling samples from the commercial vessels and port structures were analyzed by real-time polymerase chain reaction (qPCR) to detect *V. parahaemolyticus*.

The genomic DNA for the qPCR was extracted from 200 µl of APW broth by thermal shock (Fukui & Sawabe 2007). The DNA of two reference strains, CICESE-595/CAIM 1400 (*tlh*+, *tdh*+, *trh*−, *orf8*+) and CICESE-596/CAIM 1772 (*tlh*+, *tdh*+, *trh*+, *orf8*−), was extracted using the Qiagen DNeasy® Tissue Kit as per the manufacturer's instructions, (CAIM, www.ciad.mx/caim/Catalog.html).

The samples were diluted 1:20 for analysis using the Applied Biosystems StepOnePlus™ Real-Time PCR apparatus (Life Technologies, Paisley, UK). *V. parahaemolyticus* and specific pathogenic genes were identified using MicroAmp® Fast Optical 96-Well Reaction Plates (Life Technologies) in a final volume of 10 µl. The PCR reaction comprised of 5 µl of SYBR® Green PCR master mix (Applied Biosystems), 0.6 µl of forward (f) and 0.6 µl of reverse (r) primers at 20 µM, and 4.4 µl of DNA template at 10 ng μl^{-1}.

qPCR primers were designed to amplify a region of the species specific *tlh* gene of *V. parahaemolyticus* (Bej et al. 1999). qPCR primers were: tlh-636f- CGC GCT AAC AGG GGT TGG TGA and tlh-831r- CTT CGC ACC TGC GTC CGT CA. The sensitivity of these primers was determined by serially diluting a 10^8 CFU ml^{-1} bacterial suspension of the *V. parahaemolyticus* reference strains CAIM 1400 (*tlh*+, *tdh*+, *trh*–) and CAIM 1772 (*tlh*+, *tdh*+, *trh*+). DNA was extracted from each dilution (10^8 to 10^0) with a commercial kit (Promega Wizard [Promega Corporation, Madison, WI, USA]) and used for detection of the *tlh* gene with the qPCR primers.

The *tdh and trh* genes were detected using the PCR primers in Bej et al. (1999), yielding a qPCR melting peak of 78.12°C and 74.79°C, respectively (Supplemental material, Figure S-1). The reaction program was: 95°C for 5 min and 40 cycles of 95°C for 30 s, 56°C for 1 min, and 72°C for 1 min. The open reading frame 8 (ORF8) primers in Myers et al. (2003) were used to amplify the predominant pandemic serovar O3:K6, yielding a melting peak of 81.15°C with the following PCR program: 95°C for 5 min and 40 cycles of 95°C for 30 s, 57.5°C for 1 min, and 72°C for 1 min. Each qPCR program had a melting curve stage with the following conditions: 95°C for 15 s, 60°C for 1 min with a 0.3°C increment in each cycle, and 95°C for 15 s.

Linear regression and DNA quantification

Standard curves for qPCR were established using DNA from CICESE 595 (*V. parahaemolyticus* ORF8+, *tlh*+, *tdh*+, *trh*−). Four dilutions of DNA were used in triplicates (10 ng μl^{-1}, 1 ng μl^{-1}, 0.1 ng μl^{-1}, 0.01 ng μl^{-1}), measured using a nanodrop NanoVue™ (General Electric, GE Healthcare Biosciences, Pittsburgh, PA, USA). The qPCR reaction and program conditions used were the same as those mentioned above for the *tlh* gene. The logarithmic linear regression coefficient was calculated with cycle threshold (C_T) on the X axis and DNA concentration on the Y axis:

$$Y = -14.155x + 18.902$$

$$r^2 : 0.99$$

The number of genome copies was determined using the molecular weight of *V. parahaemolyticus* (5.17 Mb) (Makino et al. 2003), as per Hien et al. (2001), Staroscik (2004), and Shikuma and Hadfield (2010), where 1 ng of *V. parahaemolyticus* DNA represents 1.79×10^5 copies.

Isolation of *V. parahaemolyticus*

The biofouling samples enriched in APW that were positive for *tlh* were streaked onto thiosulfate-citrate-bile-sucrose agar (Difco™ TCBS, Becton, Dickinson & Co., Sparks, MD, USA). Green colonies were then isolated and streaked on *Vibrio* CHROMagar™ to confirm the presence of *V. parahaemolyticus*. Mauve colonies on CHROMagar™ were isolated; DNA was extracted by thermal shock, and screened for the presence of *tlh*, *tdh*, and *trh* or ORF8. Positive strains were deposited into the Microorganism Collection of Centro de Investigación Científica y de Educación Superior de Ensenada, B.C. (CICESE), Ensenada, Baja California, Mexico (Table 1).

Serotyping

The *V. parahaemolyticus* ORF8+strains were additionally serotyped with commercial antigens for O3 and K6 (Denka Seiken 2014) as per the manufacturer's instructions. Briefly, a dense suspension of overnight colonies of *V. parahaemolyticus* were suspended in saline solution and tested directly with the K6 antigen; part of the suspension was boiled for 2 h and tested using the O3 antigen. Agglutination was performed using saline suspension as a blank; the *V. parahaemolyticus* O5:K12 strain CICESE 204/CAIM 728 was used as a negative control, and CICESE 595/CAIM 1400, O3:K6 was used as a positive control.

Results and discussion

Detection of V. parahaemolyticus

To determine whether *V. parahaemolyticus* was present in biofouling samples from sampled ships and dock structures, qPCR analysis was performed using primers for species-specific detection. Globally, *V. parahaemolyticus* is a leading cause of seafood-associated gastroenteritis and has increased significantly in the US over the past 15 years (Newton et al. 2012). The reasons for this increase remain unknown, but its presence in this study

Table 1. *V. parahaemolyticus* strain list.

Collection number	Isolated from	Locality and year	Genetic characteristics
CICESE 250 to 269 and 274	Biofouling from a dock structure at the arrival of a ship from Yokohama, Japan	Ensenada harbor, Mexico, 2012	tlh/tdh (+)/trh/ORF8(-)
CICESE 270 to 272	Biofouling from the hull of a ship arriving from Fukuyama, Japan	Ensenada harbor, Mexico, 2012	tlh/tdh (+)/trh /ORF8(-)
CICESE 273	Biofouling from the hull of a ship arriving from Fukuyama, Japan	Ensenada harbor, Mexico, 2012	tlh/tdh/ORF8/ O3:K6 (+) trh (-).
CAIM 1400 CICESE 595	Clinic	Sinaloa, Mexico, 2005	tlh/tdh/ORF8/O3:K6 (+) trh (-)
CAIM 1772 CICESE 596	From shrimp	Sinaloa, Mexico, 2005	tlh/tdh/trh (+) ORF8 (-) O5:K17
CAIM 728 CICESE 204	Clinic	Japan	/tlh/tdh (+) trh/ORF8(-) O5:K12

CICESE (Center for Scientific Research and Higher Education of Ensenada, CAIM, www.ciad.mx/caim/Catalog.html).

reveals data that contribute to understanding its dispersion and persistence.

The thermolabile hemolysin gene (*tlh*), although not related to pathogenicity, has been observed in all *V. parahaemolyticus* strains identified so far and is therefore considered a signature marker for species-specific detection. The qPCR primers for the *tlh* gene produced an amplicon of 196 bp and a melt peak of 82.0–82.5°C (Figure S1). qPCR amplification could be observed from 10^3 CFU ml^{-1} up to 10^8 CFU ml^{-1} but not at the lower dilutions. 10^3 CFU ml^{-1} were detected at a Ct value of 29.9.

Biofouling samples from the oceanographic buoy (FLUCAR) and dock area CM (Figure 1) were negative for the *V. parahaemolyticus* molecular marker *tlh*. All other samples within the Ensenada harbor, however, were positive for the presence of *V. parahaemoylitcus* markers *tlh* and *tdh* as shown in Figure 2a and b and Table 2. The results suggest that its presence is closely associated with commercial or cruise vessel activity.

V. parahaemolyticus-positive samples contained 10^3 to 10^5 genomic copies per sample, as per Staroscik's formula (2004), in which 1 ng of DNA equals 1.79×10^5 copies of the *V. parahaemolyticus* genome. The biofouling sample from dock EIT-B1 contained the highest concentration of *V. parahaemolyticus* (10^5) (see Table 2), representing 19.3% of the total DNA extracted in the sample (Figure 3a). In contrast, the biofouling sample from Ship B1 from Yokohama, Japan, had 10^3 genome copies and constituted only 0.1% of total DNA in the sample. Ship B3 from Fukuyama, Japan harbored 6×10^3 genomic copies (3.2% of total DNA in the sample). The *V. parahaemolyticus* genome represented < 1% of the total DNA in the remaining samples. It is important to clarify that these results are based on DNA extraction from the enriched APW incubated sample, which favors *Vibrio* growth. The purpose of this enrichment was not only to facilitate qPCR detection, but to ensure colony isolation.

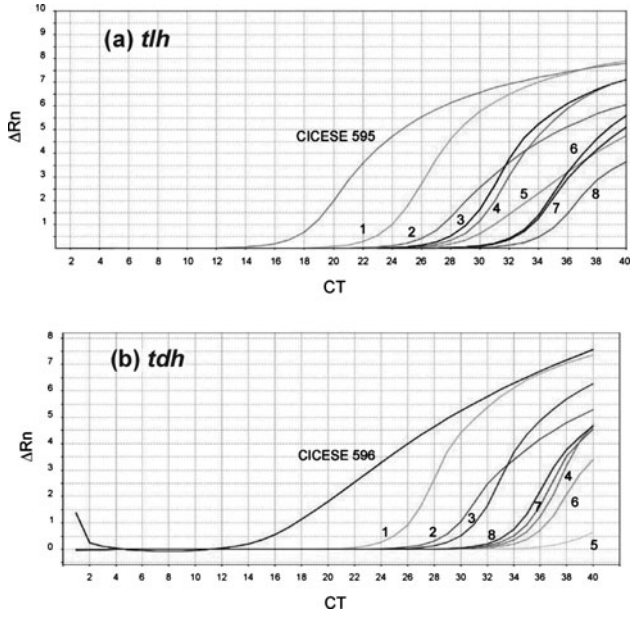

Figure 2. (a) Real-time qPCR linear amplifications for the tlh gene: CICESE 595, reference strain. (b) Real-time qPCR linear amplifications for the tdh gene: CICESE 596, reference strain. (1) EIT-B1, dock at arrival of Ship 1; (2) B3, Ship 3; (3) EIT-B3, dock at arrival of Ship 3; (4) EIT-B2, dock at arrival of Ship 2; (5) LTP, dock local Tours Port; (6) B2, Ship 2; (7) ECP, dock Ensenada Cruise Port Village; (8) B1, Ship 1.

Detection of pathogenicity genes

In this study, all positive samples for the *tlh* gene were analyzed for the presence of the hemolysins genes *tdh* and *trh*. The Thermostable Direct Hemolysin (TDH) and its homolog TDH-Related Hemolysin (TRH) are the most frequently used diagnostic indicators of pathogenicity. Their biological activities include hemolysis of various species of erythrocytes, cytotoxicity, stimulation of fluid accumulation and vascular permeability (Nishibuchi et al. 1992). *V. parahaemolyticus tdh*+ was detected in all samples within the Ensenada harbor in concentrations

Table 2. DNA concentration (ng μl^{-1}) based on cycle times (C_T) in positive *V. parahaemolyticus* samples using *tlh* primers and calculated number of genome copies.

Sample	Code	C_T	DNA ng μl^{-1}	Genome copies
Ship 1 Yokohama, Japan	B1	33.84	0.008	1.43×10^3
Dock at the arrival Ship 1	EIT-B1	22.61	2.4	4.3×10^5
Ship 2 Busan, South Korea	B2	32.2	0.016	2.87×10^3
Dock at the arrival Ship 2	EIT-B2	28.42	0.09	1.61×10^4
Ship 3 Fukuyama, Japan	B3	25.98	0.34	6.09×10^4
Dock at the arrival Ship 3	EIT-B3	27.73	0.13	2.33×10^4
Dock local tours port	LTP	29.31	0.06	1.08×10^4
Dock Ensenada cruise port	ECP	32.04	0.017	3.05×10^3

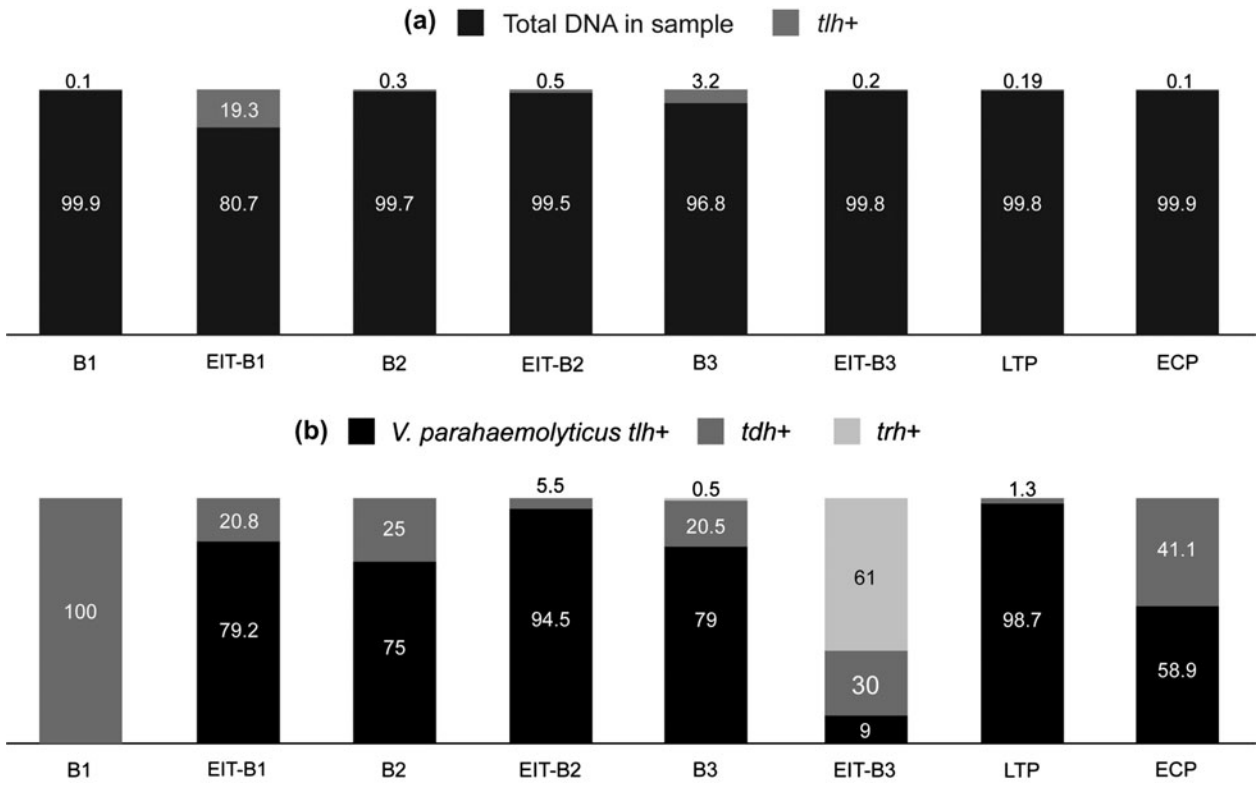

Figure 3. (a) Percentages of DNA belonging to *V. parahaemolyticus* in each positive sample. (b) Percentages of *tdh* and *trh* present in each sample with respect to the total DNA positive (tlh+) for *V. parahaemolyticus*. (B1) Ship 1; (EIT-B1) dock at arrival of Ship 1; (B2) Ship 2; (EIT-B2) dock at arrival of Ship 2; (B3) Ship 3; (EIT-B3) dock at arrival of Ship 3; (LTP) dock local Tours Port; (ECP) dock Ensenada Cruise Port Village.

that ranged between 10^2 and 10^4 genome copies per ng (Table S-1), whereas *V. parahaemolyticus trh*+ was present in two samples. The percentage of DNA positive for the molecular markers *tdh/trh* (Figure 3b), ranged from 1.3% to 100% of the previously *tlh* positive DNA percentage seen in Figure 3a (eg 0.1% of the total extracted DNA in sample B1 was positive for the *tlh* gene and the entire amount of that 0.1% was also positive for the *tdh* gene, resulting in 100%). The highest percentages of *tdh*+DNA were noted in samples from the cargo docks, cargo hulls, and cruise ship dock; the lowest percentage was observed on the local tours dock. The presence of TDH or TRH hemolysins is mostly associated with clinical isolates (McLaughlin et al. 2005), therefore their detection in these biofouling samples is extremely rare.

Pandemic serotype

V. parahaemolyticus can be classified into distinct somatic (O) and capsular (K) serotypes. Since 1996 a pandemic serotype (O3:K6/*tdh*+) has began to be

detected in different countries worldwide. This serotype, as well as its serovariants, carried the filamentous phage f237 that could be detected by a PCR method that targeted ORF8 of f237 (Nair et al. 2007). The presence of the pandemic strain O3:K6 in Mexico initiated an outbreak in 2004 (Cabanillas-Beltrán et al. 2006). In the state of Sinaloa, Mexico, Velázquez-Román et al. (2012) reported the presence of *V. parahaemolyticus tdh*+, ORF8+, and O3:K6 in 126 of 154 strains isolated from clinical cases and in four of 144 from environmental samples, showing that it could be isolated from the environment but at very low frequency. The ORF8 fragment used to identify the O3:K6 related strains was detected in the samples from Ship B3 (5×10^3 copies) and dock EIT-B1 (5.38×10^2 copies). In the sample from Ship B3, the ORF8 gene constituted 50% of the total DNA (3.2%) that was positive for *tlh* and the 20.5% positive for *tdh* *vs* 0.5% in the EIT-B1 sample.

To confirm qPCR results, all sample homogenates positive for *tlh* were spread onto TCBS; *tdh*+colonies were detected in two samples: Ship B3 and EIT-B1. A total of 20 *V. parahaemolyticus tdh*+ colonies were isolated from dock EIT-B1 *vs* 6 from Ship B3. Of these 26 colonies, one *V. parahaemolyticus* ORF8+ colony was isolated from the biofouling sample from Ship B3. These results are consistent with the calculated DNA percentages, because colony isolation was only possible in samples with a higher percentage of *V. parahaemolyticus* genome copies. The ORF8 colony was isolated from Ship B3, where it constituted 50% of the sample. Subsequent serotyping of the ORF8+ colony confirmed that it was the pandemic serotype O3:K6.

Microbial pathogens associated with cargo ships

The results from this study indicate that biofouling on the hulls of cargo and cruise vessels serves as a reservoir and transoceanic vector of microbial pathogens. All samples associated with these activities were positive for *V. parahaemolyticus* and absent in the reference stations. Ballast water and zooplankton are vectors for the transmission of *V. cholerae* (Ruiz et al. 2000; De Magny et al. 2008; Lizárraga-Partida et al. 2009; Rivera et al. 2013), but biofouling material on the hulls of ships has not been considered a significant vector of pathogenic vibrios. This statement became acute in a global economic crisis context such as that reported for 2008–2009, when 10–25% of global merchant vessels were unemployed and remained at anchor in the coastal waters of Southeast Asia, developing biofouling, and became a risk for transporting invasive species once back in service (Floerl & Coutts 2009). The merchant vessels that were sampled in Ensenada spent only a few hours in dock for container operations. Samples taken from hull areas were not in contact with dock structures, therefore contamination from the dock seems unlikely. However, in-water cleaning could explain the detection of *V. parahaemolyticus tdh*+ in all dock samples inside the harbor. According to Coutts and Taylor (2004), the Australian Environmental Protection Agency managed the biosecurity risks from hull biofouling cleaning by prohibiting in-water cleaning of vessels > 200 GT. Nevertheless in-water cleaning is a current practice in some ports, like in Ensenada.

Shikuma and Hadfield (2010) have detected by qPCR non-toxigenic *V. cholerae* in biofilms formed on microscope slides and on the hull of a ship in Ala Wai Harbor (Hawaii). Their results indicate that hulls of ships can harbor and transport microbes internationally. Microbial species that are transported by ships might persist in marine habitats, and current ballast water management techniques might be insufficient to protect against invasive species due to the presence and density of microorganisms in ships' hull biofouling communities (Gollasch 2006). The transport regulation of potential invasive species *via* biofouling has not been fully addressed. This is in part because there is no standard or commercially available method by which ships can eliminate these biofouling organisms before arriving at their destination, and because it is unknown which organisms survive the transoceanic journeys. Therefore, regulating ballast water discharge addresses only part of the problem (Coutts & Taylor 2004; Strain 2012).

In conclusion, ships' hull biofouling is a potential reservoir and dissemination vector for pathogenic microorganisms. Biofouling should be considered when discussing topics like emerging infectious diseases, as well as for additional development of protective measures, for the public and environment.

Acknowledgements

The authors wish to acknowledge the collaboration of Dr Rita Colwell and Dr Horacio de la Cueva for reviewing the manuscript and Maximiliano Lizárraga for graph editing. They would also like to thank all port authorities for sampling permission, and the Consejo Nacional de Ciencia y Tecnología (CONACyT) for scholarship 242862.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was funded by the grant Fondo Sectorial de Investigación en Salud y Seguridad Social [CONACyT/SSA S0008-2009-1114024].

Supplemental material

The supplemental material for this paper is available online at http://dx.doi.org/10.1080/08927014.2015.1038526.

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