Isolation, molecular characterization, and antibiotic resistance patterns of *Vibrio parahaemolyticus* isolated from coastal water in the Eastern Province of Saudi Arabia

Lubna Ghenem and Nasreldin Elhadi

ABSTRACT

Vibrio parahaemolyticus is a Gram-negative halophilic marine microbe that causes gastroenteritis, wound infections, and septicemia in humans. Since the emergence of the pandemic clone O3:K6, *V. parahaemolyticus* has become a globally well-known pathogen. In this study, 375 seawater samples collected from the Eastern coast of Saudi Arabia were tested for the presence of *V. parahaemolyticus*. Three hundred and forty samples were determined positive for *V. parahaemolyticus* using traditional microbiological techniques. The genes *toxR* and *tlh* were detected via polymerase chain reaction (PCR) in 41 isolates from 23 samples (6%). Thermostable direct hemolysin (*tdh*) and thermostable direct hemolysin-related hemolysin (*tdh*) are the most common virulence genes associated with *V. parahaemolyticus*. As such, four isolates were *tdh* + (1%) and another four were *trh*+ (1%). No evidence of pandemic clones was detected using group-specific PCR (GS-PCR). Samples were tested for antibiotic susceptibility against 28 agents. The vast majority of samples exhibited high resistance to carbenicillin (98%), ampicillin (88%), and cephalothin (76%). The multiple antibiotics resistance index was >0.2 for 35% of the isolates. The results of this study confirm the presence of *V. parahaemolyticus* in the Eastern coast of Saudi Arabia. This is the first report of *tdh*+ and *trh*+ isolates from this area.

Key words | antibiotic resistance, coastal water, GS-PCR, *Vibrio parahaemolyticus*, virulence genes

Lubna Ghenem
Nasreldin Elhadi (corresponding author)
Department of Clinical Laboratory Science, College of Applied Medical Sciences,
Imam Abdulrahman Bin Faisal University,
P.O. Box 2435, Dammam 31441,
Kingdom of Saudi Arabia
E-mail: *nmohammed@iau.edu.sa*

INTRODUCTION

Vibriosis is a human illness caused by pathogenic species of the family *Vibrionaceae* (CDC 2016). The aquatic bacterial species *Vibrio parahaemolyticus* is the most frequently reported cause of vibriosis in the USA (Fabbro *et al.* 2010; CDC 2016). Several studies have found that *V. parahaemolyticus* isolation is positively correlated with mean water temperature (Blackwell & Oliver 2008; Rosec *et al.* 2009; Urquhart *et al.* 2016). The most common clinical manifestation of *V. parahaemolyticus* is gastroenteritis following ingestion of raw seafood contaminated by virulent strains (Quilici *et al.* 2005). In patients suffering from pre-existing medical conditions, vibriosis can progress to life-threatening septicemia (Ceccarelli *et al.* 2013). In addition, *V. parahaemolyticus* may also cause skin infections (Fabbro *et al.* 2010).

Molecular techniques have become important for the detection of *V. parahaemolyticus* in water samples. The species-specific markers *toxR* and *tlh* have been proposed to confirm *V. parahaemolyticus* strains identity to the species level (Brasher *et al.* 1998; Bej *et al.* 1999; Kim *et al.* 1999). The thermostable direct hemolysin gene (*tdh*) and the thermostable direct hemolysin related-hemolysin gene (*trh*) are the most common virulence factors associated with gastrointestinal symptoms (Roque *et al.* 2009). Consequently, polymerase chain reaction (PCR) protocols for the

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detection of these virulence genes have been established (Tada *et al.* 1992).

Historically, *V. parahaemolyticus* cases were sporadically reported (de Jesús Hernández-Díaz *et al.* 2015) until there was a sudden surge of cases in India in 1996 caused by the novel serotype O3:K6 (Okuda *et al.* 1997). Since then, reports of gastroenteritis caused by O3:K6 have spread to Africa (Ansaruzzaman *et al.* 2008), Asia (Li *et al.* 2016), Europe (Martinez-Urtaza *et al.* 2005), and the American continent (Velazquez-Roman *et al.* 2014). The rapid spread of O3:K6 marked the first pandemic of *V. parahaemolyticus* and placed this pathogen at the front of the global public health agenda (Ceccarelli *et al.* 2013). The clonality of the pandemic O3:K6 isolates can be confirmed using group-specific PCR (GS-PCR) targeting the *toxRS* gene (Okuda *et al.* 1997).

Fortunately, *V. parahaemolyticus*-associated gastroenteritis is self-limiting in most patients (Bennett *et al.* 2015). However, when needed, CDC recommends that patients receive tetracycline or ciprofloxacin antibiotic treatment (Elmahdi *et al.* 2016). As a result of the excessive use of antibiotics in human and aquaculture systems, vibrios have begun to acquire antibiotic resistance genes similar to many other bacterial genera (Elmahdi *et al.* 2016). Several studies have reported a high rate of *V. parahaemolyticus* resistance to ampicillin (Ottaviani *et al.* 2013; Shaw *et al.* 2014; Silvester *et al.* 2015; He *et al.* 2016). Furthermore, one concerning report characterized a multidrug resistance (MDR) conjugative plasmid, acquired by a strain of *V. parahaemolyticus*, that mediates resistance to multiple antibiotics, including ampicillin, ceftriaxone, cefotaxime, nalidixic acid, kanamycin, chloramphenicol, and streptomycin (Liu *et al.* 2013). The transmission of MDR genes via conjugative plasmids jeopardizes the effectiveness of disease control and treatment and poses a significant threat to public health (Liu *et al.* 2013; Li *et al.* 2015). Since the status and occurrence of *V. parahaemolyticus* in the Eastern coastal environment of Saudi Arabia is not well characterized, the objectives of this study were: (i) to determine the incidence of *V. parahaemolyticus* in seawater samples collected from the Arabian Gulf coast; (ii) to confirm their species identity using PCR targeting *toxR* and *tlh*; (iii) to determine their potential pathogenicity using PCR assays targeting *tdh* and *trh* genes; (iv) to screen for pandemic clones of *V. parahaemolyticus* using GS-PCR; and (v) to study the antibiotic susceptibility patterns of the *V. parahaemolyticus* strains isolated from the Arabian Gulf.

MATERIALS AND METHODS

Site of study

The Arabian Gulf is a semi-enclosed marine environment that covers an area of about 240,000 km^2. It has high levels of salinity and experiences intense fluctuations in water temperatures (Sheppard *et al.* 2010). Since the Arabian Gulf serves as a major global hub for oil transportation, its ecosystem is continuously stressed by the discharge of hydrocarbon pollutants and crude oil spills (Mahmoud *et al.* 2009). The industrial and sewage discharges combined with the Gulf's low water exchange rates has made this sea one of the most anthropogenically impacted regions in the world (Naser 2013). In this study, seawater was collected from 17 different locations along the coastline of the Arabian Gulf, as illustrated in Figure 1. The sample locations include public beaches, fishing areas, and recreational water sources. During sample collection, each location was divided into two to three equidistant sampling sites using GPS.

Water sample collection and transportation

Throughout a one-year time period (February 2015 to February 2016), a total of 375 surface water samples were collected using sterile 500 mL screw-cap bottles (Fischer, UK). The number of samples collected from the surface water of each location is listed in Table 1. The water pH and temperature of each sampling site were measured using a multi-parameter water quality meter (YSI-50 series, Horiba, USA). The samples were transported in portable coolers at ambient temperature and immediately analyzed on arrival at the laboratory.

Sample treatment

Sample treatment was based on the US FDA Bacteriological Analytical Manual (BAM) method for *V. parahaemolyticus* isolation with a few modifications (Kaysner & DePaola 2004). Briefly, samples were enriched in both 1% and 3% NaCl enriched alkaline peptone water (APW). To prepare the APW (pH ± 8.5), Peptone and NaCl were dissolved in

Figure 1 | Locations of the 17 sampling sites in the Eastern Province of Saudi Arabia.

water and dispensed into screw-cap bottles. Then, 25 mL of each seawater sample was added to 225 mL of the prepared APW and incubated for 18 hours at 35 °C.

Cultivation and identification

Enriched samples were streaked on both thiosulfate-citrate-bile salts-sucrose agar (TCBS) (Oxoid, UK) and CHROM Vibrio agar (CHROM, France) and incubated at 37 °C for 18–24 hours. A minimum of three to five typical colonies of *V. parahaemolyticus* were purified on tryptic soy agar supplemented with 2% NaCl and incubated at 37 °C overnight. Colonies were biochemically confirmed as *V. parahaemolyticus* using Gram stains, oxidase tests, string tests, urease tests, and Kligler iron agar. Phenotypic characterization was achieved by

Table 1 | Total number of seawater samples collected from each location

Location	Number of samples
Alaziziyah Beach (AZB)	25
Corniche Tiba Jubail (CTJ)	5
Dammam Corniche (DMC)	10
Dammam Marina Front (DMF)	20
Fanateer Corniche (FNC)	35
Half-Moon Beach (HMF)	50
Alkhubar Corniche (KBC)	35
Alkubar Marina Front (KBF)	15
Albuhairah Beach (LAK)	40
Almorjan Island (MOI)	5
Palm Beach Jubail (PBJ)	30
Qatif Corniche (QTC)	20
Ras Tanura Corniche (RTC)	5
Sayhat Corniche (SEC)	15
Alshibaly (SHB)	25
Tarout Corniche (TRC)	30
University of Dammam (OUD)	10
Total	375

using API 20 E, API NE, and API 10 S strip tests (Bio-Mérieux, France). Glycerol stocks of pure colonies were prepared and stored at −80 °C for subsequent molecular testing.

Genomic DNA extraction

To subculture *V. parahaemolyticus* colonies, 1 mL of glycerol stock was transferred to Luria-Bertani (LB) broth (2% NaCl) and incubated overnight in a thermal shaker (Stuart shaking incubator S1500, UK). A modified version of the boiling extract method was used to extract genomic DNA (Silvester *et al.* 2015). Briefly, 1.5 mL of LB culture was transferred to Eppendorf tubes and centrifuged (10,000 rpm, 4 °C, 1 minute). The remaining pellets were diluted 1:10-fold in sterile distilled water and vortexed for 1 to 2 minutes. The resulting suspension was boiled at 100 °C for 15 minutes to lyse the cells and free crude DNA. The tubes were immediately stored at −20 °C for further use.

Species identity confirmation of *V. parahaemolyticus* isolates

PCR targeting *toxR* and *tlh* genes was used to confirm the identity of *V. parahaemolyticus* to the species level. Positive (ATCC 17802) and negative (*V. alginolyticus*, ATCC 17749) controls were included in each run. A 368 bp region of the *toxR* gene (F-5′-GTC TTC TGA CGC AAT CGT TG-′3 and R-5′-ATA CGA GTG GTT GCT GTC ATG-′3) was amplified with the following thermocycler conditions: 20 cycles of 1 minute denaturation at 94 °C, 1.5 minutes of annealing at 63 °C, and 1.5 minute extension at 72 °C, followed by a final extension at 72 °C for 7 minutes as described by Kim *et al.* (1999). The thermocycler conditions for the 450 bp region of the *tlh* gene (F-5′-AAA GCG GAT GTA TCA GAA GCA CTG-3′ and R-5′-GCT ACT TTC TAG CAT TTT CTC TGC-3′) included 30 cycles of 1 minute at 94 °C, 1 minute of annealing at 58 °C, and 1 minute of extension at 72 °C, followed by a final extension at 72 °C for 3 minutes (Brasher *et al.* 1998; Bej *et al.* 1999). Finally, 10 μL of the final PCR mixture were mixed with 1 μL of dye solution Ethidium bromide (Promega, USA) and resolved on a 1% agarose gel via electrophoresis.

Virulence detection

The virulence of all *toxR* + and *tlh* + isolates was further assayed via PCR amplification of the *tdh* and *trh* genes. *V. alginolyticus* (ATCC 17749) was used as a negative control for both *tdh* and *trh* genes. *V. parahaemolyticus* (ATCC 17802) was the positive control for the *tdh* gene and *V. parahaemolyticus* (AQ 4037) was used as a positive control for *trh*. A 251 bp region of the *tdh* gene (F-5′-CCA CTA CCA CTC TCA TAT GC-3′and R-5′-GGTACTAAATGGCTGACATC-3′) were performed as outlined by Tada *et al.* (1992) with minor changes: 35 cycles of denaturation at 94 °C for 1 minute, annealing at 59 °C for 1 minute, and extension at 72 °C for 1 minute, followed by a final extension at 72 °C for 7 minutes. A 251 bp region of the *trh* gene (F-5′ GGC TCA AAA TGG TTA AGC G-3′ and R-5′-CAT TTC CGC TCT CAT ATG C-3′) was amplified using previously published methods (Tada *et al.* 1992). Lastly, 10 μL of the final mixture were mixed with 1 μL of dye solution ethidium bromide (Promega, USA) and resolved on a 1% agarose gel via electrophoresis.

Group-specific PCR

All *V. parahaemolyticus* isolates that are *tdh* + or *trh* + were tested for the presence of *toxRS* gene using GS-PCR (Matsumoto *et al.* 2000). The sequence of the forward primer was 5′-TAATGAGGTAGAAACA-3′ and the reverse primer sequence was 5′-ACGTAACGGGCCTACA-3′. A positive (*V. parahaemolyticus* ATCC 17802) and a negative control (*V. alginolyticus*, ATCC 17749) were included in each run. The amplification program began at 96 °C for 5 minutes, followed by 25 cycles of 1 minute of denaturation at 94 °C, 2 minute of annealing at 45 °C, and 3 minutes of extension at 72 °C. The 25 cycles were followed by a final extension at 72 °C for 7 minutes. Finally, 10 μL of the final mixture were mixed with 1 μL of dye solution ethidium bromide (Promega, USA) and resolved in 1% agarose gel electrophoresis.

Antibiotic susceptibility testing

The isolates of *V. parahaemolyticus* were tested against 28 antimicrobial agents (Oxoid, UK). Susceptibility testing was performed using the disk diffusion method according to CLSI protocols. The following antibiotic agents were tested: ampicillin (AMP: 25 μg), ticarcillin (TE: 75 μg), carbenicillin (CAR: 100 μg), piperacillin (PRL: 100 μg), amoxycillin/clavulanic acid 'Augmentin' (AMC: 30 μg), piperacillin/tazobactam (TZP: 110 μg), cephalothin (KF: 30 μg), cefoxitin (FOX: 30 μg), cefaclor (CEC: 30 μg), ceftizoxime (ZOX: 30 μg), cefotaxime (CTX: 30 μg), ceftriaxone (CRO: 30 μg), ceftazidime (CAZ: 30 μg), cefepime (FEP: 30 μg), chloramphenicol (C: 30 μg), kanamycin (K: 30 μg), gentamicin (CN: 10 μg), streptomycin (S: 10 μg), amikacin (AK: 30 μg), tetracycline (TE: 30 μg), imipenem (IPM: 10 μ), meropenem (MEM: 10 μg), nalidixic acid (NA: 30 μg), ciprofloxacin (CIP: 5 μg), sulfamethoxazole/trimethoprim (SXT: 25 μg), aztreonam (ATM: 30 μg), and nitrofurantoin (F: 300 μg). The reference strain *Escherichia coli* (ATCC11775) was used as a control during antibiogram testing.

Multiple antibiotic resistance index and MDR definition

The multiple antibiotics resistance (MAR) index was calculated as described by Krumperman (1983) using the formula a/b where 'a' represents the number of antibiotics to which the particular isolate is resistant and 'b' represents the total number of multiple antibiotics to which the particular isolate has been exposed. MDR was defined as the non-susceptibility of the organism to at least one agent in three or more categories of antimicrobials (Magiorakos *et al.* 2012).

RESULTS

Water physical parameters

Water temperatures ranged from 14.6 °C to 32.4 °C throughout the sampling period (February 2015 to February 2016). The lowest seawater temperature from which *V. parahaemolyticus* was isolated was 16.9 °C and the highest was 32.4 °C. In regards to the pH, the lowest and highest values at which *V. parahaemolyticus* was detected was pH 8.2 and 8.8, respectively.

Presumptive *V. parahaemolyticus* identification

Using traditional microbiological techniques (i.e., culturing and biochemical tests), 84 of the 375 samples (22%) were determined to be positive for the presence of *V. parahaemolyticus*.

Confirmation of *V. parahaemolyticus* identity by *toxR* and *tlh* PCR

Forty-one isolates from 23 samples (6%) were found to harbor both *toxR* and *tlh* genes. They were isolated from 23 samples (6%). The results are summarized in Table 2.

Virulence characterization

Four (1%) *V. parahaemolyticus* isolates were positive for the *tdh* gene and four (1%) other isolates harbored the *trh* gene. None of the isolates were positive for both *tdh* and *trh* genes. The urease test also serves as a method for determining virulence, for which two (0.5%) isolates produced weak positive results.

Table 2 | Number of *V. parahaemolyticus* isolates confirmed by PCR targeting *toxR* and *tlh* genes

Location	No. of water samples	Presumptive isolates (culture and biochemical)	*toxR*	*tlh*	No. of positive water samples confirmed by PCR
AZB	25	21	0	0	0
CTJ	5	6	0	0	0
DMC	10	2	1	1	1
DMF	20	8	4	4	2
FNC	35	35	6	6	6
HMF	50	24	0	0	0
KBC	35	40	1	1	1
KBF	15	16	0	0	0
LAK	40	50	0	0	0
MOI	5	15	15	15	5
PBJ	30	53	1	1	1
QTC	20	12	10	10	4
RTC	5	4	0	0	0
SEC	15	4	2	2	2
SHB	25	36	0	0	0
TRC	30	10	1	1	1
UOD	10	4	0	0	0
Total	375	340	41 (12%)	41 (12%)	23 (6.13%)

Group-specific PCR

All isolates positive for *tdh* and *trh* were further assayed for the *toxRS* gene using GS-PCR, but none was positive. Detailed information regarding the characterization of *V. parahaemolyticus* isolates ($n = 41$) from the coast of the Eastern Province of Saudi Arabia is listed in Table 3.

Antibiotic susceptibility testing

Based on the antibiotic susceptibility tests, the resistance rate of the 41 *V. parahaemolyticus* isolates in our study was 98% to carbenicillin, 88% to ampicillin, and 76% to cephalothin. In contrast, all *V. parahaemolyticus* isolates were sensitive to piperacillin/tazobactam, ceftazidime, chloramphenicol, gentamicin, imipenem, meropenem, nalidixic acid, levofloxacin, and sulfamethoxazole/trimethoprim. The results of the antibiotic susceptibility tests are listed in Table 4.

Multiple antibiotic resistance index and MDR

The values of the MAR index ranged from 0.03 to 2.8. Ten different resistance patterns had a significant MAR value >0.2. Collectively, they were exhibited by 35% of the *V. parahaemolyticus* isolates. The MAR index of one of the *tdh* + and two of the *trh* + isolates was >0.2. The present study found 15 out of 41 (37%) isolates of *V. parahaemolyticus* to be MDR. One of the *tdh* + isolates and two of the *trh* + isolates were MDR.

DISCUSSION

Physical parameters

The strong association between the occurrence of *V. parahaemolyticus* and the increased temperature of the environment is well documented in the literature (Blackwell & Oliver 2008; Rosec *et al.* 2009; Urquhart *et al.* 2016). In accordance with the previous reports, we were able to isolate *V. parahaemolyticus* from water samples with temperatures between 16.9 °C and 32 °C (Deepanjali *et al.* 2005). Previous studies have found that the cultivation of *V. parahaemolyticus* during the winter months is difficult because they enter a viable but non-culturable state (Igbinosa & Okoh 2008). In contrast, our study was able to isolate *V. parahaemolyticus* from the Arabian Gulf during both summer and winter seasons. This may be explained by the temperate nature of the Arabian Gulf. Six out of the eight pathogenic isolates of *V. parahaemolyticus* (*tdh* + or *trh* +) were recovered from seawater samples of temperatures above 29 °C. Our findings contrast with a previous report that demonstrated that the ratio of pathogenic to total *V. parahaemolyticus* is higher when temperatures are lower (Johnson *et al.* 2010).

The presence of *V. parahaemolyticus* in the coastal water

V. parahaemolyticus inhabits marine and estuarine environments (Fabbro *et al.* 2010). Its presence imposes potential health risks to the public due to its association with gastroenteritis, wound infection, and septicemia (Tena *et al.*

Table 3 | Characterization of *V. parahaemolyticus* isolates ($n = 41$) collected from the coastal water environment of the Eastern Province of Saudi Arabia

				Water parameters			PCR				
Isolate no.	**Sample location**	**Isolate code**	**Date of isolation**	**Temp (°C)**	**pH**	**Urease test**	***tox*R**	***tlh***	***tdh***	***trh***	***tox*RS**
1	KBC	VP116	24/02/2015	19.9	8.8	−	+	+	+	−	−
2	FNC	VP10	07/04/2015	24.8	8.35	−	+	+	−	−	−
3	FNC	VP55	07/04/2015	24.8	8.35	−	+	+	−	−	−
4	FNC	VP56	07/04/2015	24.8	8.35	−	+	+	−	−	−
5	FNC	VP57	07/04/2015	24.8	8.35	−	+	+	−	−	−
6	FNC	VP58	07/04/2015	24.8	8.35	−	+	+	−	−	−
7	FNC	VP59	07/04/2015	24.8	8.35	−	+	+	−	−	−
8	DMF	VP22	26/05/2015	30.4	8.36	−	+	+	−	+	−
9	DMF	VP23	26/05/2015	30.4	8.36	−	+	+	−	+	−
10	DMF	VP24	26/05/2015	30.4	8.36	−	+	+	−	+	−
11	DMC	VP25	26/05/2015	30.4	8.41	−	+	+	−	−	−
12	DMF	VP68	27/05/2015	29.4	8.45	−	+	+	−	−	−
13	QTC	VP27	19/10/2015	32.4	8.2	−	+	+	−	−	−
14	QTC	VP28	19/10/2015	32.4	8.2	−	+	+	+	−	−
15	QTC	VP29	19/10/2015	32.4	8.2	−	+	+	−	−	−
16	QTC	VP30	19/10/2015	32.3	8.26	−	+	+	−	−	−
17	QTC	VP31	19/10/2015	32.3	8.26	−	+	+	−	−	−
18	QTC	VP32	19/10/2015	32.3	8.26	−	+	+	−	−	−
19	SEC	VP26	19/10/2015	31.8	8.49	−	+	+	−	−	−
20	TRC	VP34	19/10/2015	32.4	8.36	−	+	+	+	−	−
21	PBJ	VP36	28/10/2015	29.4	8.44	−	+	+	+	−	−
22	MOI	VP125	30/12/2015	16.9	8.76	−	+	+	−	−	−
23	MOI	VP126	30/12/2015	16.9	8.76	−	+	+	−	−	—-
24	MOI	VP127	30/12/2015	16.9	8.76	−	+	+	−	−	−
25	MOI	VP128	30/12/2015	16.9	8.76	−	+	+	−	−	−
26	MOI	VP129	30/12/2015	16.9	8.76	−	+	+	−	−	−
27	MOI	VP130	30/12/2015	16.9	8.76	−	+	+	−	−	−
28	MOI	VP131	30/12/2015	16.9	8.76	−	+	+	−	−	−
29	MOI	VP132	30/12/2015	16.9	8.76	−	+	+	−	−	−
30	MOI	VP133	30/12/2015	16.9	8.76	−	+	+	−	−	−
31	MOI	VP134	30/12/2015	16.9	8.76	−	+	+	−	−	−
32	MOI	VP135	30/12/2015	16.9	8.76	−	+	+	−	−	−
33	MOI	VP136	30/12/2015	16.9	8.76	−	+	+	−	−	−
34	MOI	VP137	30/12/2015	16.9	8.76	−	+	+	−	−	−
35	MOI	VP138	30/12/2015	16.9	8.76	−	+	+	−	−	−
36	MOI	VP139	30/12/2015	16.9	8.76	−	+	+	−	+	−
37	QTC	VP140	24/01/2016	21.1	8.85	−	+	+	−	−	−
38	QTC	VP141	24/01/2016	21.1	8.85	−	+	+	−	−	−

(*continued*)

Table 3 | continued

				Water parameters			PCR				
Isolate no.	**Sample location**	**Isolate code**	**Date of isolation**	**Temp (°C)**	**pH**	**Urease test**	***tox*R**	***tlh***	***tdh***	***trh***	***tox*RS**
39	QTC	VP142	24/01/2016	21.1	8.85	−	+	+	−	−	−
40	QTC	VP143	24/01/2016	21.1	8.85	−	+	+	−	−	−
41	SEC	VP154	24/01/2016	21.1	8.6	−	+	+	−	−	−
Total						0	41	41	4	4	0

2010; Zhang & Orth 2013). Since the incidence and virulence of *V. parahaemolyticus* on the coast of the Eastern Province of Saudi Arabia is not well documented, the goal of this study was to assay seawater samples collected from the Arabian Gulf for *V. parahaemolyticus*. Our study confirmed the presence of *V. parahaemolyticus* in 6.13% of the collected seawater samples and nine of the 17 sample locations. The highest percentage of the isolates was recovered from Almorjan Island (MOI).

In this study, two selective media were used for the isolation of *V. parahaemolyticus* (TCBS and CHROM *Vibrio* agar). In accordance with previous studies, we found that TCBS did not effectively select for *Vibrio* spp. and that CHROM Vibrio agar was more specific and accurate than

Table 4 | Results of antibiotic susceptibility testing performed on 41 *V. parahaemolyticus* isolates

Antibiotic class	**Antibiotic name**	**No. of resistant (%)**	**No. of intermediate (%)**	**No. of sensitive (%)**
Penicillins	Ampicillin	36 (88)	5 (12)	0 (0)
	Ticarcillin	18 (44)	22 (54)	1 (2)
	Carbenicillin	40 (98)	0 (0)	1 (2)
	Piperacillin	1 (2)	7 (17)	33 (80)
	Amoxy/Clavulanic	1 (2)	16 (39)	24 (59)
	Pipera/Tazobactam	0 (0)	0 (0)	41 (100)
Cephalosporins	Cephalothin	31 (76)	6 (15)	4 (10)
	Cefoxitin	2 (5)	21 (51)	18 (44)
	Cefaclor	25 (61)	3 (7)	13 (32)
	Ceftizoxime	0 (0)	10 (24)	31 (76)
	Cefotaxime	0 (0)	17 (41)	24 (59)
	Ceftriaxone	0 (0)	12 (29)	29 (71)
	Ceftazidime	0 (0)	0 (0)	41 (100)
	Cefepime	0 (0)	2 (5)	39 (95)
Phenicols	Chloramphenicol	0 (0)	0 (0)	41 (100)
Aminoglycosides	Kanamycin	2 (5)	29 (71)	10 (24)
	Gentamicin	0 (0)	0 (0)	41 (100)
	Streptomycin	12 (29)	27 (66)	2 (5)
	Amikacin	5 (12)	12 (29)	24 (59)
Tetracycline	Tetracycline	0 (0)	1 (2)	40 (98)
Carbapenems	Imipenem	0 (0)	0 (0)	41 (100)
	Meropenem	0 (0)	0 (0)	41 (100)
Quinolones	Nalidixic acid	0 (0)	0 (0)	41 (100)
	Ciprofloxacin	0 (0)	8 (20)	33 (80)
	Levofloxacin	0 (0)	0 (0)	41 (100)
Sulfonamides	Sulf./Trimethoprim	0 (0)	0 (0)	41 (100)
Monobactams	Aztreonam	11 (27)	20 (49)	10 (24)
Nitrofurans	Nitrofurantoin	1 (2)	5 (12)	35 (85)

TCBS (Fabbro *et al.* 2010; Di Pinto *et al.* 2011). In addition, our study came to an agreement with those of Fabbro *et al.* (2010) and Ottaviani *et al.* (2013) on the fact that the identity of *V. parahaemolyticus* may not be fully confirmed by traditional microbiological techniques. Confirming previous research, we found that the *toxR* PCR assay was a reliable method for the detection of *V. parahaemolyticus* (Kim *et al.* 1999). The results of *tlh* PCR analysis were in complete harmony with the ones achieved by *toxR* analysis. Although the *tlh* gene is similar to other genes harbored by different *Vibrio* spp. increasing the likelihood of a false positive PCR result (Klein *et al.* 2014), our study found *tlh* to be a useful marker for confirming the identity of *V. parahaemolyticus*, as reported by Rojas *et al.* (2011). In summary, PCR-based *toxR and tlh* gene detection assays are the most accurate methods of choice for confirming the presence of *V. parahaemolyticus* (Jun *et al.* 2012). The genes *tdh* and *trh* are the most commonly detected virulence factors for *V. parahaemolyticus*, so many clinically isolated strains of *V. parahaemolyticus* possess hemolytic activity that is attributed to these two genes (Ceccarelli *et al.* 2013). To the best of our knowledge, this study represents the first report of the isolation of *tdh* + and *trh*+ *V. parahaemolyticus* isolates from the coastal environment of the Arabian Gulf of the Eastern Province of Saudi Arabia. Our study reported the isolation of *tdh* + and *trh* + isolates of *V. parahaemolyticus* at a very low prevalence rate (1%), and none of the isolates harbored both genes (*tdh* + /*trh* +). Our results follow the trends of globally distributed studies that have demonstrated that it is rare to isolate virulent *V. parahaemolyticus* strains from environmental sources (Lopez-Joven *et al.* 2015; Di *et al.* 2016). The presence of *tdh* + and *trh*+ *V. parahaemolyticus* in the Arabian Gulf is a pressing concern that has several impacts. First, the fact that these isolates are potentially diarrheal entails more health facilities to assess bacterial gastroenteritis clinical samples for the presence of *V. parahaemolyticus* (Jun *et al.* 2012). Second, not only does pathogenic *V. parahaemolyticus* contaminate seafood and cause disease, but it also causes enormous economic losses in the seafood industry (Fuenzalida *et al.* 2006; Thongjun *et al.* 2013). Thus, the results of this study also emphasize the importance of monitoring seafood for bacterial contaminants. Third, several reports have suggested that the O3:K6 group emerged after the pathogenic strains acquired genetic elements that increased their fitness and ability to infect humans (Nasu *et al.* 2000; Hurley *et al.* 2006). Although our study did not detect any *toxRS*+ isolates, research to detect the emergence of any pandemic clones should continue to be performed. Importantly, some of the isolated clinical strains of *V. parahaemolyticus* do not contain *tdh* and/or *trh* (Raghunath 2014). While the main hemolysin genes were absent from their genome, *V. parahaemolyticus* was still pathogenic, indicating that there are additional virulence factors associated with *V. parahaemolyticus* (Raghunath *et al.* 2009). Likewise, early reports of infection due to urease-positive/Kanagawa-negative strains of *V. parahaemolyticus* have been documented (Kelly & Stroh 1989; Honda *et al.* 1992). Our results included two isolates with a weak urease-positive result (<1%). Suthienkul *et al.* (1995) suggested a correlation between the production of urease and TRH in *V. parahaemolyticus*. However, our study disputes this theory because one of the urease positive strains isolated was *trh* − . This finding is consistent with the results of Devi *et al.* (2009) and Alaboudi *et al.* (2016).

Antibiotic susceptibility testing

The results of the antibiotic susceptibility tests placed carbenicillin at the top of the *V. parahaemolyticus* resistance scope (98%). As such, the resistance of *V. parahaemolyticus* to carbenicillin has been previously reported by Sudha *et al.* (2014) and Silvester *et al.* (2015). Also, similar to our study, they detected a high rate of ampicillin resistance (88% in our study). In fact, *V. parahaemolyticus* resistance to ampicillin is well-known in the literature (Shaw *et al.* 2014; He *et al.* 2016). Interestingly, ampicillin resistance was in 100% of the strains isolated by Devi *et al.* (2009) and Ottaviani *et al.* (2013). *V. parahaemolyticus* resistance to ampicillin and other penicillins is believed to be due to its chromosomally encoded β-lactamase (Devi *et al.* 2009). In addition, a high number of *V. parahaemolyticus* isolates were resistant to cephalothin (76%), which agrees with the results published by Igbinosa & Okoh (2008) (82%). In regards to streptomycin, the present study reported a 27% resistance rate. In accordance, Shaw *et al.* (2014), Lopatek *et al.* (2015) and He *et al.* (2016) have reported streptomycin resistance with the percentages of 4%, 45%, and 70.3%,

respectively. The CDC organization recommends that severe or prolonged *V. parahaemolyticus* infections be treated with tetracycline or ciprofloxacin antibiotics (Elmahdi *et al.* 2016). The sensitivity rate to tetracycline (98%) brings our result to an agreement with the CDC's results. However, our ciprofloxacin results did not follow the same trends as previous reports; the response of 20% of the *V. parahaemolyticus* isolates was in the intermediate range. In contrast, all of the tested isolates were sensitive to piperacillin/tazobactam, ceftazidime, gentamicin, imipenem, meropenem, and sulfamethoxazole/trimethoprim. Those results are consistent with Shaw *et al.* (2014). The MAR index values ranged from 0.03 to 0.28.

Ten different resistance patterns had a significant MAR value >0.2. Collectively, they were expressed by 35% of the *V. parahaemolyticus* isolates. Most of them were isolated from Almorjan Island (MOI). MAR indices that exceed 0.2 are rendered from high risk sources of contamination which imposes human risks (Letchumanan *et al.* 2015; Tanil *et al.* 2005). Accordingly, the present study found that 15 out of 41 (37%) isolates of *V. parahaemolyticus* were MDR, most of which originated from MOI as well. MDR has previously been reported in *V. parahaemolyticus* environmental isolates (Silvester *et al.* 2015; He *et al.* 2016). The resistance patterns observed in our study were consistent with those of Ottaviani *et al.* (2013); there was no significant difference in the distribution of the resistance patterns between virulent (*tdh* + / *trh* +) and non-virulent isolates (*tdh* − /*trh* −).

The frequency of dangerous antibiotic-resistant bacteria has been increasing over the past several decades (Fair & Tor 2014). In the case of *V. parahaemolyticus* this may be explained by the excessive use of antimicrobial agents in order to protect the industrial aquatic produce from infectious diseases and massive stock damages (Xu *et al.* 2016). Although antimicrobial drugs are essential for food security and productivity, the effects of excessive and inappropriate antimicrobial application likely outweigh the benefits (FAO 2015). The Food and Agriculture Organization of the United Nations (FAO) have recently set an action plan to improve the awareness on antimicrobial resistance and promote prudent use of antimicrobials (FAO 2015). The increase in antimicrobial resistance is also likely caused by seawater contamination from illegal dumping of medical waste or sewage containing human-consumed antibiotics (Xu *et al.* 2016). The transmission of MDR genes jeopardizes the effectiveness of vibrios infection control programs and complicates the treatment of severe *Vibrio* spp. infections (Liu *et al.* 2013; Li *et al.* 2015). Furthermore, resistant strains present in the environment act as potential reservoirs of drug resistance genes, which may be further transferred to pathogenic bacteria through horizontal gene transfer (Silvester *et al.* 2015).

CONCLUSIONS

Having used highly accurate detection and identification methods (i.e., a combination of PCR-based and culture-based detection techniques), this study confirms the presence of *V. parahaemolyticus* in the Arabian Gulf coast of the Eastern Province of Saudi Arabia. In addition, this study represents the first evidence of the presence of potentially pathogenic (*tdh* + and *trh* +) isolates of *V. parahaemolyticus* in this area. The presence of *V. parahaemolyticus* in the environment is a pressing concern because it has been associated with human infections, seafood contamination, and large disease outbreaks. The antibiotic susceptibility tests revealed high resistance of *V. parahaemolyticus* to clinically important antibiotics. These findings highlight the need for protecting aquatic environments from the effects of irresponsible consumption of antimicrobial agents. Moreover, this study serves as a baseline on which future studies can monitor any future changes in *V. parahaemolyticus* antibiotic resistance and associated human health risks.

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