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Occurrence of Potentially Human Pathogenic *Vibrio* Species in the Coastal Water of the Eastern Province of Saudi Arabia

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ABSTRACT

Non-cholera *Vibrio* infections are an important public health problem worldwide. The aim of this study was to investigate the presence of potentially pathogenic non-cholera *Vibrio* species in coastal seawater of Eastern Province of Saudi Arabia. A total of 300 seawater samples were collected from five locations along coast environment and examined for the presence or absence of pathogenic *Vibrio* species during eleven months between February and December, 2010. In this study five potentially pathogenic *Vibrio* species were detected, with overall incidence in the samples being at 38% for *V. alginolyticus* was the predominant species, 13.3 % for *V. parahaemolyticus*, 7.6% for *V. vulnificus*, 5.6% for *V. cholerae* non-O/non-O139 and 0.33 % for *V. mimicus*. All isolates of *V. cholerae* non-O/non-O139 were negative for cholera toxin (*ctx*) gene and only three isolates were positive for toxin-coregulated pilus gene (*tcp*A) out of 27 isolates. From the results of this study, coastal water of Arabian Gulf is concluded to be a reservoir for *Vibrio* species in Eastern Province of Saudi Arabia. To the best of our knowledge, this is the first study done on determining potentially pathogenic *Vibrio* in the Saudi Arabia and Arabian Gulf region. As the study has found that pathogenic *Vibrio* species are present in the study area, it is recommended that the possibility of infection caused by non cholera *Vibrio* should be considered whenever diagnosis is done on any patient who had recently been exposed to sea water or that had manipulated or consumed seafood and presenting infected wounds, ear infection, conjunctivitis, gastroenteritis or septic shock.

Key words: *Vibrio* species, seawater, arabian gulf, eastern province of Saudi Arabia

INTRODUCTION

Ample studies have shown that *Vibrio* species are highly abundant in aquatic environments, including estuaries, marine coastal waters and sediments and aquaculture settings worldwide (Ortigosa *et al*., 1989; Barbieri *et al*., 1999; Heidelberg *et al*., 2002). Members of *Vibrios* are classified as Gram-negative, curved and rod-shaped bacteria. They are known to be ubiquitous bacteria; they naturally habituate of the estuarine and marine environments and are commonly present in or on shellfish and other seafood (Farmer and Hickman-Brenner, 1992). The genus *Vibrio* includes more than 35 currently recognized species, 12 of which are considered to be human pathogens. *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are universally recognized as the major significant human pathogens. On the contrary, *V. mimicus*, *V. hollisae*, *V. alginolyticus*, *V. fluvalis*, *V. furnissi*, *V. metschnikovii*, *V. carchariae*, *V. cincinnatiensis* and *V. damsela*, considered to be responsible for cases for minor pathogenicity, are usually or frequently isolated from blood, wounds of arms and legs, infected eyes and ears (Farmer and Hickman-Brenner, 1992). The linkages between the estuarine environment and human disease are common among *Vibrios*, with each species having distinctive features (Janda *et al*., 1988).

Pathogenic *Vibrio* species are able to cause human diseases and threat public health as the causative agents of both sporadic and epidemic human infections. *V. parahaemolyticus* is a leading cause of gastro-enteritis and usually associated with the consumption of raw or undercooked shellfish and seafood. *V. cholerae*, continues to spread globally in a seventh pandemic (O1 El Tor biotype) and the emergence of a non-O1 serogroup (O139 Bengal) has led to a new pandemic (Faruque and Mekalanos, 2003; Sack *et al.*, 2004). There are more than 150 O serotypes among *V. cholerae* strains. But only the strains belonging to O1 and O139 serotypes are considered virulent because they usually carry the cholera toxin gene (*ctx*), the toxin-coregulated pilus gene (*tcp*) and other virulence genes. *V. cholerae* non-O1/non-O139 may cause sporadic episodes and occasional outbreaks of diarrheal disease and could lead to extra-intestinal diseases, such as bacteremia, invasive soft tissue infections cholecystitis and peritonitis (Hughes *et al.*, 1978; Morris *et al.*, 1981; Morris, 1994; West *et al.*, 1998; Farmachidi *et al.*, 2003).

V. vulnificus distributed in the marine environment can infect the humans either by consumption of contaminated seafood or by invasion through a skin lesion, which can cause sepsis and serious wound infections during the consumption of raw or undercooked shellfish and seafood or exposure of skin wounds to seawater (Morris, 2003). *V. vulnificus* can cause fatal infection in the individuals with underlying diseases (diabetes, liver diseases, etc.) or the individuals treated with immunosuppresive agents. Opportunistic infections with *V. vulnificus* can cause severe wound infections (Oliver, 2005) and fulminant septicaemia, with highly virulent strains causing extensive host-tissue damage and producing mortality rates of up to 60% (Linkous and Oliver, 1999). Other *Vibrio* species, including *V. alginolyticus, V. fluvialis, V. furnissii, V. mimicus, V. hollisae, V. metschnikovii* and *V. damsela* are halophilic *Vibrios* also sporadically involved in human diseases (Davis *et al.*, 1981; Brenner *et al.*, 1983; Abott and Janda, 1994; Carnahan *et al.*, 1994; Farmer *et al.*, 2003; Yamane *et al.*, 2004).

The aim of this study was to determine the incidence of clinically important *Vibrio* species in sea water samples collected from coastal areas of the Arabian Gulf of Eastern Province of Saudi Arabia. A standardized protocol was developed for the isolation of *Vibrio* species from environmental samples following enrichment. The identification of *Vibrio* isolates was confirmed by using CHROMagar *Vibrio* (CV) agar, API 20E and serotyping of *Vibrio cholerae* non-O1/non-O139 isolates. All isolates of non-O1/non-O139 *V. cholerae* were screened using Polymerase Chain Reaction (PCR) analysis to examine the virulence genes *ctxA* and *tcpA*. Despite the importance of the emergence of *Vibrio* infections in the coastal areas of Arabian, little is known about the presence of these organisms in the marine environments of the region. Arabian Gulf coast is one of the most important recreational and fish and shrimp-producing areas in Gulf region. In the present study, presence or absence of potentially pathogenic *Vibrio* species were investigated from five locations of this temperate coast of Eastern Province of Saudi Arabia.

MATERIALS AND METHODS

Study sites: The Arabian Gulf is a shallow semi-enclosed marginal sea, with less than 100 m in depth over its entire extent and with a mean of only 35 m (Reynolds, 1993). It covers an area of about 240,000 km^2, with 1000 km in length and widths ranging from 185 to 370 km, with a mean of 240 km. The shallowness of the Arabian Gulf water leads to the formation of a very high saline and dense water, with maximum salinities being as high as 57 gram per litre along the southern coast due to the highly evaporation rate (John *et al.*, 1990). The coastal environment of the Eastern Province of Saudi Arabia, including Abu Ali Island, is continuously subjected to oil pollution

incidents as a result of damaged oil wells, oil pipeline leaks, or ballast water discharge from nearby loading terminals (Al-Thukair *et al.*, 2007). This frequent oil pollution represents an additional stress factor to already harsh environmental conditions of high temperature, salinity, rapid rate of evaporation and desiccation (Al-Thukair *et al.*, 2007).

Sample collection and preparation: A total of 300 water samples were collected from the Arabian Gulf coast at five different locations from February to December, 2010. A total of 90, 75, 60, 45 and 30 water samples were collected from Al Khobar, Half Moon, Al Qatif, Al Azziziah and Dammam beaches, respectively. All water samples were collected in sterile 500 mL glass bottles from a depth of approximately 10 to 15 cm and 10 to 15 m beyond the seashores. Water temperature was measured at all collection sites using temperature sensor and pH values ranged from 8.0 to 8.6. All water samples were transported to the Microbiology Laboratory (University of Dammam) in insulated cooler and examined immediately after their arrival. The transportation time arranged from 3 to 5 h. Inoculations into selective medium were made within 24 h after the collection of water samples. Number of water samples and incidence of pathogenic *Vibrio* species are summarized in Table 1.

Culture procedure: All samples were analyzed for potentially pathogenic *Vibrio* species. The 25 mL test water sample was mixed with 225 mL alkaline-peptone water enrichment broth (APW: 1% peptone, 1% NaCl, pH 8.6) to isolate *V. cholerae* or salt APW (1% peptone, 3% NaCl, pH 8.6) to isolate all pathogenic *Vibrio* species (Smith, 1970; Elhadi *et al.*, 2004). All samples enriched in APW were incubated for six to eight hours to isolate *V. cholerae* and 18 to 24 h at 37°C to isolate other pathogenic *Vibrio* species. A sample from this enrichment culture was transferred with a loop to Thiosulphate citrate bile-salt sucrose agar (TCBS; Difco Laboratories, USA) and CHROMagar *Vibrio* (CV; Paris, France) and incubated at 37°C for 24 h. In this study the CHROMagar *Vibrio* medium was used as a selective medium for *Vibrios* and allowed good recovery for *V. colerae, V. parahaemolyticus* and *V. vulnificus* (Fig. 1) compared with TCBS medium.

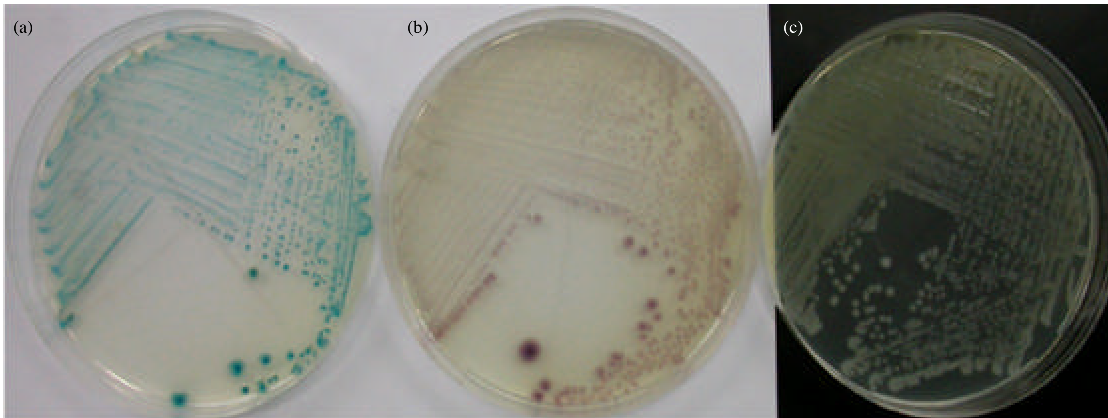

Fig. 1(a-c): (a), Appearance of *Vibrio cholerae* and *V. vulnificus* green blue colonies, (b), *V. parahaemolyticus* mauve (violet) colonies; and (c), *V. alginolyticus* colourless colonies on CHROagar Vibrio (CV) medium

Identification procedures for *Vibrios*: Five to seven colonies suspected to be *Vibrio* species per sample were picked up from the TCBS agar and CV plates and cultured on Tryptic Soy Agar (TSA) to purify the colonies. These colonies were first examined for oxidase reaction (Kovacs method) and the oxidase-positive isolates were also tested for string reaction (Smith, 1970). Organisms showing oxidase positive reactions were identified by using conventional biochemical methods described previously (Elhadi *et al.*, 2004). Biochemical tests analyses were carried out from pure isolated colonies grown on TSA for 24 h at 37°C. Salt tolerance was determined by growth of the isolates at 37°C in 1% peptone broth without NaCl or supplemented with either 1, 3, 6, 8 and 10% NaCl. Further identification of *Vibrio* spp. were carried by using API 20 E system (BioMerieux, France). Standard strains of *Vibrio* species, obtained from the Microbiology Laboratory of the King Fahad Hospital of the University (KFHU) were used for quality control of the biochemical tests.

Serotyping: Strains presumptive of *V. cholerae* were grown at 37°C on nutrient agar plates and serological confirmation of *V. cholerae* strains was performed by an agglutination test with polyvalent anti-O1, mono-specific Ogawa-Inaba antisera and with specific anti-O139 antisera obtained commercially (Denka Seiken Co. Ltd., Tokyo, Japan).

PCR analysis of virulence genes: Amplification of the target cholera toxin gene was carried out by PCR assay using bacterial cell lysate as the source of template DNA (Shirai *et al.*, 1991). In brief, cells from an 18 h LB culture were collected and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), treated with 10% (wt/vol) sodium dodecyl sulfate and freshly prepared proteinase K and incubated at 37°C for 1 h. After incubation, 10% cetyl trimethyl ammonium bromide in 0.7 M NaCl was added and incubated at 65°C for 10 min. The aqueous phase was treated with phenol-chloroform and the DNA pellet was washed with 70% ethanol. The extracted nucleic acid was suspended in TE and treated with RNase at 37°C for 30 min. Amplification was performed in a thermal cycler (Applied Biosystems, USA) using 200 µL PCR tubes with a reaction mixture volume of 25 µL. Each of the reaction mixtures contained 3 µL of template DNA (lysate), 2.5 µL of each primer (10 pmol μL^{-1}), 2.5 µL of 2.5 mM deoxynucleoside triphosphates, 0.3 µL (5 U μL^{-1}) of *Taq* DNA polymerase (promega, USA), 2.5 µL of 10× reaction buffer containing 20 mM $MgCl_2$ (Promega, USA) and 11.8 µL of sterilized deionized water. PCR for detecting the cholera toxin and *tcp*A gene primer are as follow: For *ctx*A, F-5'-TCAGACGGGATTTGTTAGGCACG-3' and *ctx*A, R-5'-TCTATCTCTG TAGCCC CT ATTACG-3' (Li *et al.*, 2003) and for *tcp*A, F- 5'-CACGATAAGAAAACC GGTCAAGAG-3' and R, 5' ACCAAATGCACGCCGAATG GAGC-3'(R) (Mukhopadhyay *et al.*, 2001). The amplification program began with denaturation at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 94°C for 1.5 min, annealing at 60°C for 1.5 min and extension at 72°C for 1.5 min and a final extension at 72°C for 7 min. A negative control (sterile distilled water) and a positive control (*V. cholerae* serogroup O1 strain from the King Fahad Hospital of the University) were run in each amplification. The PCR products were electrophoresed through a 1.5% agarose gel to resolve the amplified products, which were visualized under UV light after ethidium bromide staining.

RESULTS

Incidence of potentially pathogenic *Vibrio* species: The presence and abundance of *Vibrio* species were detected at all five sites throughout the entire period of the study. In this study

Table 1: Incidence of potentially pathogenic *Vibrio* species in water samples obtained from different locations

		No. of positive sample (%)			
Location	No. of samples examined	*V. cholerae* non-O1/ non-O139 (%)	*V. parahaemolyticus* (%)	*V. vulnificus* (%)	*V. mimicus* (%)
Al Khobar	90	0	3 (3.3)	2 (2.2)	0
Al Azziziah	45	7 (15.5)	16 (35.5)	6 (13.3)	0
Half Moon	75	6 (8.0)	19 (25.3)	8 (10.6)	1 (1.3)
Dammam	30	2 (6.6)	1 (3.3)	2 (6.6)	0
Al Qatif	60	2 (3.3)	1 (1.6)	5 (8.3)	0
Total number (% positive)	300	17 (5.6)	40 (13.3)	23 (7.6)	1 (0.33)

Table 2: Monthly incidence of potentially pathogenic *Vibrio* species in water samples collected from Arabian Gulf between February and December, 2010

		No. of *Vibrio* isolates			
Month	Water temperature (°C)	*V. cholerae* non-O1/ non-	*V. parahaemolyticus*	*V. vulnificus*	*V. mimicus*
February	15	5	2	0	1
March	24	7	5	1	0
April	22	0	2	0	0
May	30	3	8	2	0
June	31	3	17	4	0
July	33	1	14	0	0
November	24	6	3	12	0
December	18	2	5	9	0
Total No.		27	56	28	1

Vibrio alginolyticus was the predominant and the most frequently isolated species (114 of 300; 38%), followed by *V. parahaemolyticus* (40 of 300; 13.3%) as shown in Table 1. The highest number of *V. parahaemolyticus* were isolated from Half Moon and Al Azzizah beaches during the month of June and July from Half Moon and Al Azziziah (Fig. 2). Water temperature during the month of June and July when the water samples were collected was 31 and 33°C, respectively (Table 2 and Fig. 2).

***V. cholerae* and serotyping:** All isolates of *V. cholerae* non-O1/O139 described here did not react with both of anti-O1 and anti-O139 antisera. *V. cholerae* Non-O1/non-O139 (17 of 300; 5.6%) were isolated from four locations and no *V. cholerae* were detected in water samples collected from Al Khobar beach. The highest numbers of *V. cholerae* were isolated during the month of March and November, when the seawater temperature during sampling collection was 24°C (Table 2 and Fig. 2).

PCR analysis of *V. cholerae* virulence genes: *V. cholerae* non-O1/non-O139 isolates were screened for the presence or absences of *ctxA* and *tcpA* genes using PCR and the results showed that the cholera toxin gene was absent in all the isolates. Out of the 27 isolates of *V. cholerae* non-O1/non-O139, only three isolates were positive for *tcp*A gene; these isolates were isolated from Al Azziziah (Table 1).

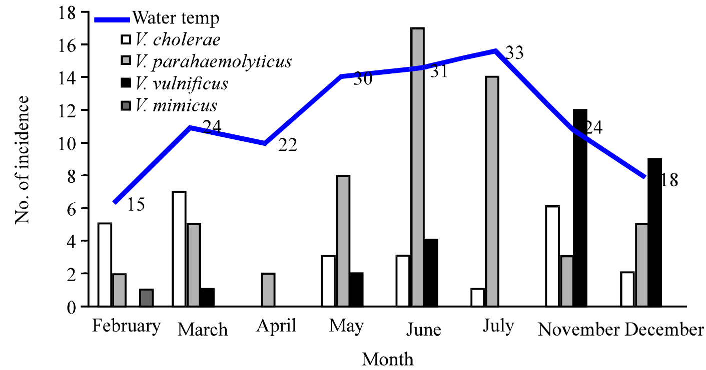

Fig 2: Monthly incidence of potentially pathogenic *Vibrio* species in water samples collected from Arabian Gulf between February and December, 2010.Y axis denotes the number of positive water samples to *Vibrio* species; X axis denotes the number of months positive for *Vibrio* species and the curve denotes the water temperature (°C) during water samples collection

Vibrio vulnificus: *V. vulnificus* was detected from all water samples sites (23 of 300; 7.6%), yielding 28 isolates as shown in (Fig. 2). The highest numbers of *V. vulnificus* were isolated during the month of November (12 isolates) and December (9 isolates), when the seawater temperature during sampling collection was 24°C and 18°C, respectively (Fig. 2).

Vibrio parahaemolyticus: *V. parahaemolyticus* was detected from all water samples sites (40 of 300; 13.3%), yielding 56 isolates as shown in Table 1. The highest numbers of *V. parahaemolyticus* were isolated during the month of June (17 isolates) and July (14 isolates), when the seawater temperature during sampling collection was 31 and 33°C, respectively (Fig. 2).

Vibrio mimicus: In this, study *V. mimicus* was isolated only in one water samples obtained from Half Moon beach (Table 1). It was isolated during the month of February, when the seawater temperature during sampling collection was 15°C as shown in Fig. 2.

DISCUSSION

The main objective of this study was to determine the incidence of potentially pathogenic *Vibrio* species in water samples collected from Arabian Gulf coastal areas that vary in water temperature and salinity. More seawater samples were analyzed during the summer months (May, June and July) because of the greater number and diversity of *Vibrio* species expected to occur during the warmer season (Huq *et al*., 1983; Baffone *et al*., 2000). *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* still remain serious human pathogens (Ortigosa *et al*., 1994; Centers for Disease Control and Prevention (CDC), 1999; Finkelstein *et al*., 2002). In the present study, the most frequently isolated *Vibrio* species from TCBS agar were *V. alginolyticus* , *V. parahaemolyticus* and *V. cholerae*. Few numbers of *V. vulnificus* were isolated using the TCBS medium since it has a very low plating efficiency for clinical and environmental strains of *V. vulnificus* (Hoi *et al*., 1998). In this study the CHROMagar *Vibrio* medium was found to be highly selectively for *V. vulnificus*, *V. cholerae* and *V. parahaemolyticus* (Fig. 1).

V. alginolyticus was the most prevalent species in the water samples collected from the five locations in this study. This species has also been recognized for many years as a pathogen of both humans and marine animals (Blacke *et al*., 1980). *V. alginolyticus* is commonly associated with ear infections (otitis media and otitis externa) and superficial wounds resulting from prior exposure to contaminated water sources (Pezzlo *et al*., 1979). The disease caused by this microorganism mainly affects individuals who are in direct contact with seawater or handle shellfish and its incidence significantly increases during warmer months (Morris and Black, 1985).

Vibrio vulnificus was recovered from all water samples collected from the five locations of the Arabian Gulf coast (Table 1). The highest numbers of *V. vulnificus* isolates were isolated from water samples collected from Half Moon beach during the month of November, when the water during the samples collection was 24°C (Fig. 2). Diseases associated with *V. vulnificus* infection have been found to be present in two patterns (Harwood *et al*., 2004). In partner one, primary septicemia occurs in individuals with chronic liver disease shortly after eating raw oysters, with a mortality rate of over 50%. In pattern two, wound infections are incurred via exposure to seawater or handling seafood products, with a death rate of approximately 25% (Blake *et al*., 1979). *V. vulnificus* infections have been reported in the USA, Europe and Asia (Park *et al*., 1991; Chuang *et al*., 1992; Dalsgaard *et al*., 1996; Hlady and Klontz, 1995). *V. vulnificus* has been detected in coastal and estuarine environments throughout the world (Oliver, 1989); it has been found in areas with warm seawater temperatures (>20°C) (Morris, 1988; Oliver, 1989; Kelly, 1989). *V. parahaemolyticus* was present at all sites but it was most prevalent in water samples collected from AlAzziziah and Half Moon beaches as shown in Table 1. The highest numbers of *V. parahaemolyticus* were isolated during the month of May, June and July, when the seawater temperature during samples collection was 30, 31 and 33°C, respectively yielding 39 isolates. The presence of the *V. parahaemolyticus* has traditionally been confined to warm and temperate geographical areas (DePaola *et al*., 2000). The emergence of infections caused by *V. parahaemolyticus* in Europe and America has revealed the presence of the organism in regions where it had never previously been reported (Gonzalez-Escalona *et al*., 2005; Martinez-Urtaza *et al*., 2005; McLaughlin *et al*., 2005; Fuenzalida *et al*., 2006). The progressive spread of *V. parahaemolyticus* and its colonization of new areas have been related to an unusual increase in seawater temperatures in coastal zones (Gonzalez-Escalona *et al*., 2005; Martinez-Urtaza *et al*., 2005; McLaughlin *et al*., 2005; Cabello *et al*., 2007).

In this study, *V. cholerae* non-O1/non-O139 were detected from all sampling sites except Al Khobar site as shown in (Table 1). The highest numbers of *V. cholerae* were recovered from Al Azziziah location during the month of March, when the water temperature during samples collection was 24°C as shown in Fig. 2. Three out of 27 isolates *V. cholerae* non-O1/ non-O139 harbored *tcp*A genes; two isolates were isolated from Al Azziziah site and one isolates was isolated from the Half Moon site. Cholera toxin and Toxin-coregulated Pilus (TCP) are two major virulence factors produced by *V. cholerae* during infection (Faruque *et al*., 1998). Toxigenic *Vibrio cholerae* belonging to the O1 and O139 serogroups cause cholera, an illness characterized by acute watery diarrhea that occurs as epidemics in many developing countries (Faruque *et al*., 1998).

In this study, *V. mimicus* in this study was detected only from one sample from Half Moon site as shown in Table 1. *Vibrio mimicus* has been established as a pathogenic member of the genus *Vibrio* (Davis *et al*., 1981) and found to be responsible for various types of human illnesses. Isolation of *V. mimicus* pathogen from clinical samples has been made in different countries including the United States, Japan, Bangladesh, New Zealand and Canada (Davis *et al*., 1981).

Shandera *et al.* (1983) isolated *V. mimicus* from patients who had recently been exposed to seawater. *V. mimicus* gastrointestinal infections are rare. *V. mimicus* has also been isolated from a number of environmental sources, including oysters, prawns, rivers and brackish waters (Sanyal *et al.*, 1983; Chowdhury *et al.*, 1987; Chowdhury *et al.*, 1989). Cholera-toxin production was demonstrated in *V. mimicus* isolates from aquatic environments. The significance of toxigenic environmental strains is unknown and specific studies are required to elucidate their epidemiological relevance and their role in the pathogenesis of diarrheal diseases. It has been shown that some *V. mimicus* strains were able to produce a heat-labile enterotoxin, functionally and immunobiologically related to the heat-labile CT (Dotevall *et al.*, 1985; Tamplin *et al.*, 1990), as well as other toxins and toxic substances that might contribute to its pathogenesis (Chowdhury *et al.*, 1991, 1994).

From this study, it is concluded that potentially pathogenic *Vibrios* are indeed present in sea water of the Arabian Gulf coast of Eastern province of Saudi Arabia. The clinical significance of these isolates is their potential association with gastroenteritis and/or invasive septicaemia, usually contracted via consumption of raw or undercooked seafood or wound infections acquired by contact with sea water, respectively. Sporadic cases of *Vibrios*is along the Arabian Gulf coast of Saudi Arabia have not yet been reported. Nonetheless, these research findings should prompt us to pay attention to the role of *Vibrio* species in local foodborne diseases and wound infections. Many reports from Europe, USA, India and Asia indicate that human infections occur wherever these pathogens have been isolated (Hlady, 1997; Hoi *et al.*, 1998; Morris, 1999; Yam *et al.*, 2000). Thus, consideration should be given to long-term monitoring programmes for potential human pathogenic *Vibrios* in the Arabian Gulf coastal areas of Saudi Arabia. Since *TcpA* gene was prevalent in the environmental isolates, intensive surveillance study recommended for environmental samples along the coastal areas will help in understanding the evolution of the *tcpA* gene.

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