

## Antibiotic Resistance and Plasmid Profiling of Clinically Significant *Vibrio vulnificus* Isolated from Coastal Water in Eastern Province of Saudi Arabia

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**Abstract:** *Vibrio vulnificus* is an estuarine bacterium that is capable of causing a rapidly fatal infection in humans. The aim of this study to investigate the occurrence, antibiotic resistance and plasmid profiles of *V. vulnificus* in Eastern Province of Saudi Arabia coastal water. A total of 10 isolates of *V. vulnificus* isolated from coastal water of Arabian Gulf in Eastern Province of Saudi Arabia. All isolates showed a multiple resistance towards all 10 antibiotics tested. Some of the isolates showed a high resistance to different antibiotics including gentamicin, ticarcillin, cefaclor, amikacin, augmentin and ampicillin. However, all isolates of *V. vulnificus* was susceptible to tetracycline and ampicillin. Overall antibiotics resistance patterns of all isolates were resistant from 2 to 3 patterns with Multiple Antibiotic Resistance (MAR) index ranging from 0.2 to 0.5 respectively. 4 out of 10 isolates of *V. vulnificus* harbored plasmid DNA with size ranging from 3.7 to 4.9 kb. These findings are very important because of the involvement of seawater in transmission of this pathogen to human. *V. vulnificus* is responsible of causing severe wound infection and is often characterized by necrotizing skin and soft-tissue infection, primary sepsis in certain high-risk populations, including patients with chronic liver disease, immunodeficiency, iron storage disorders, end-stage renal disease and diabetes mellitus.

**Key words:** Antibiotic resistance, coastal water, plasmid DNA, *Vibrio vulnificus*

### INTRODUCTION

*Vibrio vulnificus* is one of several species of the family *Vibrionaceae*, is a halophilic, motile, Gram-negative bacillus and capable of causing severe to life-threatening infection in susceptible individuals. The spectrum of illness can vary from gastroenteritis to primary sepsis and necrotizing fasciitis. Infection may result from exposing open wounds or broken skin to contaminated salt or brackish water or handling or consuming contaminated seafood (Dechet *et al.*, 2008; Jones and Oliver, 2009). Three biotypes of *V. vulnificus* are known to cause severe human disease. Biotype 1 is found worldwide in salt or brackish water. Biotype 2 occupies a more specific niche and is found in saltwater used for eel (genus *Anguilla*) farming in the Far East and Western Europe (Oliver, 2005). Biotype 3 is the strain associated with freshwater fish farming in Israel. Biotype 1 is the most common strain and is responsible for the entire spectrum of illness, including the primary sepsis associated with the often quoted fatality rate in excess of 50%. Biotype 2 is usually a serious pathogen of eels, but on rare occasions may cause wound infections in humans (Jones and Oliver, 2009). Although biotype 3 can cause severe soft-tissue infections requiring amputation, the mortality rate appears to be less than 8% (Zaidenstein *et al.*, 2008).

Most reported cases of infection with *V. vulnificus* have occurred in patients with chronic liver disease,

immunodeficiencies, or hematological disorders characterized by elevated iron levels (Zaidenstein *et al.*, 2008; World Health Organization, 2005). The spectrum of illness caused by *V. vulnificus* essentially manifests in three syndromes: gastroenteritis, 'severe sepsis' and skin and soft-tissue infection. As mentioned previously, 'sepsis or severe sepsis' appears to have the worst outcome (>50% mortality) and may be the most common presentation of infection. Gastroenteritis and primary septicemia usually result from the consumption of contaminated seafood, especially raw oysters. Skin and soft-tissue infection typically results from handling contaminated seafood or from exposure of open wounds to water in which the organism thrives. The prominent risk factor for *V. vulnificus* infection is chronic liver disease, especially cirrhosis due to alcoholism or chronic hepatitis such as hepatitis B or C (Janda *et al.*, 1988). Alcohol consumption of as little as 30 mL/day was shown to increase the risk of infection in one study (Morris, 2003). Other risk factors include immunodeficiencies involving macrophage and neutrophil dysfunction, such as those due to underlying cancers or immunosuppressive chemotherapy (e.g., for cancer and arthritis), acquired immunodeficiency syndrome, end-stage renal disease (especially patients receiving parenteral iron), gastrointestinal disorders (e.g., surgery, ulcers, achlorhydria), diabetes mellitus and hematological disorders characterized by elevated iron levels such as hemochromatosis (Barton and Acton, 2009). Other

potential contributing factors include chronic disease, especially heart disease and advanced age (>60 years).

Despite the importance of the emergence of *V. vulnificus* infections worldwide in the coastal areas, little is known about the presence of these organism in the marine environments of the region since the Arabian Gulf coast is one of the most important recreational areas in Gulf region. In the present study, we examined the presence of *V. vulnificus* in the five main sites in eastern coastal areas of this temperate coast of eastern province of Saudi Arabia.

## MATERIALS AND METHODS

**Isolation and identification of bacterial strains:** 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 in all water samples. 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.

**Culture procedure:** All samples were analyzed for potentially pathogenic Vibrios. 25 mL of test water sample was mixed with 225 mL Alkaline Peptone Water (APW) enrichment broth (1% peptone, 3% NaCl, pH 8.6) to isolate *V. vulnificus* (Elhadi *et al.*, 2004). All samples enriched in APW were incubated for 18 to 24 h at 37°C and from this enrichment culture was transferred with a loop on 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. After incubation 3 to 5 five colonies suspected to be *V. vulnificus* were picked up from the TCBS agar and CV plates and cultured on Tryptic Soy Agar (TSA) to obtain pure the colonies. Organism showing oxidase positive reactions were identified by using conventional biochemical methods described previously (Elhadi *et al.*, 2004). Series number of biochemical tests and API 20 E system (BioMerieux, France) were used to identify *V. vulnificus* to species level.

**Antimicrobial susceptibility tests:** The antimicrobial susceptibility tests were performed essentially by the disc diffusion method as described elsewhere (Bauer *et al.*, 1966), with disc containing antibiotic (Oxoid Ltd.,

England). Antibiotics tested were gentamicin (10 µg), cefotaxime (30 µg), ticarcillin (75 µg), trimethoprim/sulfamethoxazole (25 µg), norfloxacin (10 µg), cefaclor (30 µg), tetracycline (30 µg), amikacin (30 µg), augmentin (30 µg) and ampicillin (10 µg). Cultures derived from a single colony were grown at 35°C. Overnight cultures grown at 35°C were then spread over the Mueller Hinton agar plates using a sterile cotton bud and were allowed to dry for 2 to 5 min. Antibiotic discs were spaced out onto the plates and were incubated at 37°C for 24 h. Diameter of the clear zone for each antibiotic disc was measured and interpreted as susceptible or resistant categories according to the guideline recommended by the Clinical Laboratory and Standards Institute (CLSI, 2007). The multiple antibiotic resistance index of the isolates was defined as a/b where 'a' represents the number of antibiotics to which the particular isolate was resistant and 'b' the number of antibiotics to which the isolate was exposed to (Krumperman, 1985).

**Plasmid DNA extraction:** The Covalently Closed Circular (CCC) plasmid in *V. vulnificus* isolates were extracted by the alkaline denaturation procedure (Sambrook *et al.*, 1989). The whole procedure was performed at room temperature. All bacterial strains were grown overnight in LB broth at 37°C in an orbital shaker. The bacterial cells were pellets by centrifugation at 10,000 rpm for 1 min in a 1.5 mL micro centrifuge. The supernatant was carefully discarded and the cell pellet thoroughly resuspended in 150 µL of glucose-EDTA-Tris buffer (GET buffer). After 10 min's incubation on ice, 300 µL of lysis buffer solution was added and the mixture was gently vortexed. The extracted plasmid DNA was precipitated and concentrated by using iso-propanol and alcohol respectively. The extracted plasmid in *V. vulnificus* isolates were followed by gel electrophoresis. In estimations of molecular weight of plasmids in *V. vulnificus* isolates, *Escherichia coli* strain V517 was used as molecular size markers. *Escherichia coli* strain V517 harbors 8 plasmids with molecular sizes of 2.1, 2.7, 3.0, 3.9, 5.1, 5.6, 7.2 and 54 kb (Marcina *et al.*, 1978) respectively.

## RESULTS

A total of 300 water samples were obtained from five different location of Arabian Gulf. 90, 75, 60, 45 and 30 water samples were collected from Al Khobar, Half moon, Al Qatif, Al-Azziziah and Dammam beaches respectively. The highest numbers of *V. vulnificus* were isolated from Half moon and Al-Azziziah sites during the month of November and December, where the seawater temperature during sampling collection was 24 and 18°C, respectively. The lowest numbers of *V. vulnificus* were isolated from Al-Khobar beach. All the isolates of



Fig.1: Appearance of *V. vulnificus* green blue colonies on CHROMagar vibrio (CV) medium

Table 1: *V. vulnificus* strains used in this study

Strain	Antibiotic resistance*	Multiple antibiotic resistance index (MAR)	Plasmid DNA size (Kb)
VV8	CN, TIC, AMP,	0.3	4.8
VV13	CN, TIC, AK, AMC, AMP	0.5	3.7
VV14	CN, TIC, AMP	0.3	-
VV23	CN, TIC, AK, AMC, AMP	0.5	-
VV26	CN, TIC, AK, AMC, AMP	0.5	4.8
VV27	TIC, AMP	0.2	-
VV28	CN, TIC, CEC, AMC, AMP	0.5	-
VV29	CN, TIC, AMC, AMP	0.4	-
VV30	CN, TIC, AK, AMC, AMP	0.5	4.9
VV31	CN, TIC, AK, AMC, AMP	0.5	-

\*: Tested for gentamicin (CN), ticarcillin (TIC), cefaclor (CEC), amikacin (AK), augmentin (AMC) and ampicillin (AMP)

*V. vulnificus* in this study were identified as biotype I because all the isolates were positive for indole production. CHROMagar Vibrio (CHROMagar; Paris, France) was used in this study in conjunction with TCBS agar because of its ability to isolate and identify *V. vulnificus* compared to the TCBS agar. CHROMagar Vibrio uses chromogenic technology to allow for the isolation and detection of *V. vulnificus* resulting in development dark blue colonies which can be distinguished from other *Vibrio* species as shown in Fig. 1. Ten isolates of *V. vulnificus* were tested for antibiotic susceptibility and plasmid DNA profiles. All the ten isolates of *V. vulnificus* demonstrated resistance to two or more antibiotics tested (Table 1). Out of 10 *V. vulnificus* isolates were screened for the presence of the plasmid DNA, only 4 isolates were harboring plasmid DNA range from 3.7 to 4.9 kb as shown in Table 1.

## DISCUSSION

The main objective of this study was to determine the incidence of *V. vulnificus* in water samples collected from Arabian Gulf coastal areas that vary in water temperature and salinity. Seawater samples were analyzed during the summer months because of the greater number and

diversity of *Vibrio* species expected to occur during the warmer season (Michael and Salim, 2011). *V. vulnificus* is considered as an opportunistic infection which causes severe human infection in susceptible individuals and may cause mortality within 24-48 h of exposure and through wounds or consumption of contaminated seafood. *V. vulnificus* is a major bacterial cause of mortality associated with food-borne diseases and it results in the highest death rate (Todd, 1989). Diseases associated with *V. vulnificus* infection have been found to be present in two patterns (Harwood *et al.*, 2004). In one, primary septicemia occurs in individuals with chronic liver disease shortly after eating raw oysters, with a mortality rate of over 50%. In the other pattern, 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 (Hlady and Klontz, 1996). *V. vulnificus* has been detected in coastal and estuarine environments throughout the world, in areas with warm seawater temperatures (>20°C) (Kelly, 1989).

Most of the *V. vulnificus* isolates in this study were resistant to gentamicin, ticarcillin, amikacin, augmentin and ampicillin. However, norfoxacin, trimethoprim/sulfamethoxazole and tetracycline were susceptible towards the isolates. Our results concur with data among the *V. vulnificus* and *Vibrio* spp. reported elsewhere (Manjusha *et al.*, 2005) who isolated *V. vulnificus* with resistance to these antibiotics. Zanetti *et al.* (2001) and Ottaviani *et al.* (2001) found high susceptibility of *V. vulnificus* strains and other *Vibrio* spp to norfoxacin in concordance to the findings in our study. Our results showed that the second generation quinolones (norfoxacin) is the drug with the best antimicrobial effect against *V. vulnificus* in agreement with data reported by other authors (Zanetti *et al.*, 2001). The MAR indices for the *V. vulnificus* were very high as shown in Table 1 and such high level of multiple resistances may arise from selective pressure due to the indiscriminate use of antibiotics. Animal farms release their effluent and water following antibiotic therapy on the farms (Schmidt *et al.*, 2000) residual water from the industries plants where products containing microbial agents have been prepared and washed and the residual water is released directly into the sewage system (Guardabassi *et al.*, 1998), which ultimately flows into the sea. MAR of *V. vulnificus* that is introduced into the marine environments readily contaminates the shellfish, which concentrate the marine microflora via filter feeding, subsequently causing a mutation in the cellular DNA (Zulkifi *et al.*, 2009). These observations have been seen to contribute to the occurrence of MAR *Salmonella* and *Aeromonas hydrophila*, respectively, in marine environment and seafood (Vivekanandhan *et al.*, 2002; Martinez-Urtaza *et al.*, 2004). Plasmid analysis of all the isolates as shown in Table 1 provided a general picture that revealed

*V. vulnificus* contains plasmids. This finding correlates with results of previous studies, which have shown that *V. vulnificus* harbored plasmids (Radu *et al.*, 1998). Molina-Aja *et al.* (2002) noted that 80% of the *Vibrio* strains analyzed harbor plasmids and 11 different plasmid profiles were observed. The plasmid profiles in our study revealed that 40% of the investigated *V. vulnificus* strains carried more than plasmids with diverse sizes. Antibiotic resistance in *V. vulnificus* in this study is presumably an innate rather than an acquired trait through plasmid transfer or antibiotic selection. This is proven by the presence of MAR *V. vulnificus* isolates but no plasmid was detected among these isolates. Nevertheless, the linkage of the presence of plasmids in the isolates to antibiotic resistance has not been determined.

This study concluded that *V. vulnificus* is present in sea water of the Arabian Gulf coast of Eastern province of Saudi Arabia. Sporadic cases of *V. vulnificus* along the Arabian Gulf coast of Saudi Arabia still not reported yet but this research finding should prompt us to pay attention to the role of *Vibrio vulnificus* in wound infections. Facing any patient recently exposed to sea water and presenting infected wounds or septic shock, the possibility of infection caused by *V. vulnificus* must be thought about.

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