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Molecular characterization, antibiotic resistance pattern and biofilm formation of *Vibrio parahaemolyticus* and *V. cholerae* isolated from crustaceans and humans

Heba A. Ahmed[a,*], Rasha M. El Bayomi[b], Mohamed A. Hussein[b], Mariam H.E. Khedr[c], Etab M. Abo Remela[d,e], Ahmed M.M. El-Ashram[f]

[a] Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, 44511, Sharkia Governorate, Egypt
[b] Department of Food Control, Faculty of Veterinary Medicine, Zagazig University, 44511 Sharkia Governorate, Egypt
[c] Department of Veterinary Public Health, Faculty of Veterinary Medicine, Zagazig University, 44511 Sharkia Governorate, Egypt
[d] Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt
[e] Department of Biology, Faculty of Science, Taibah University, Al Madina Al Munawarah, Saudi Arabia
[f] Department of Fish Diseases and Health, Faculty of Fish Resources, Suez University, Egypt

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ABSTRACT

Human infection with pathogenic vibrios is associated with contaminated seafood consumption. In the present study, we examined 225 crustaceans collected from retail markets in Egypt. Stool samples from gastroenteritis patients were also examined. Bacteriological and molecular examinations revealed 34 (15.1%) *V. parahaemolyticus* and 2 (0.9%) *V. cholerae* from crustaceans, while *V. parahaemolyticus* isolates were identified in 3 (3%) of the human samples. The virulence-associated genes *tdh* and/or *trh* were detected in 5.9% and 100% of the crustacean and human samples, respectively, whereas the two *V. cholerae* isolates were positive for the *ctx* and *hly*A genes. Antibiotic sensitivity revealed high resistance of the isolates to the used antibiotics and an average MAR index of 0.77. Biofilm formation at different temperatures indicated significantly higher biofilm formation at 37 °C and 25 °C compared with 4 °C. Frequent monitoring of seafood for *Vibrio* species and their antibiotic, molecular and biofilm characteristics is essential to improve seafood safety.

1. Introduction

Seafood is a nutritious element of healthy diets in many countries, but it is also a potential source of a wide range of foodborne pathogens (Lund, 2013). *Vibrio* species are naturally occurring autochthonous microbes that are abundant in marine and estuarine water worldwide. Several foodborne outbreaks caused by the consumption of shellfish and fish contaminated with *Vibrio* spp. have been reported in different countries (Jones and Oliver, 2009; Kirs et al., 2011).

Infection with *Vibrio* spp. occurs more frequently during summer and early fall because higher water temperatures favor the growth of these organisms (Colwell, 1984). The most important *Vibrio* spp. are *V. parahaemolyticus* and *V. vulnificus,* which are most frequently associated with the consumption of raw or partially cooked shellfish, whereas *V. cholerae* infection is mainly associated with waterborne outbreaks and, to a lesser extent, can be transmitted via shellfish consumption (Baker-Austin et al., 2010; Caburlotto et al., 2016). The maximum level of *V. parahaemolyticus* recommended by the Food and Drug Administration (FDA) is 1–10^4 organisms/mL in raw shellfish (FDA, 2001).

Human infection with *V. parahaemolyticus* is characterized by gastroenteritis with diarrhea (bloody or watery), low-grade fever, headache, abdominal pain, and vomiting (Honda and Iida, 1993). These symptoms are usually self-limiting; however, septicemia may develop in people with chronic debilitating conditions such as liver diseases, immune disorders and diabetes (Potasman et al., 2002). *V. cholerae* have been reported to cause cholera epidemics, especially in developing countries (del Refugio Castañeda Chávez et al., 2005). Out of 200 *V. cholerae* serogroups, most epidemics are caused by the toxigenic *V. cholerae* serotypes O1 and O139 (Nishibuchi and DePaola, 2005). Non-O1/non-O139 strains are natural inhabitants of water environments and have recently been shown to cause sporadic cases of diarrhea due to the ingestion of contaminated seafood (Robert-Pillot et al., 2014).

V. parahaemolyticus originating from seafood or the environment are mostly nonpathogenic; the pathogenicity of this species is determined by the production of thermostable toxin (TDH) and/or TDH-thermostable hemolysin (TRH), encoded by the *tdh* and *trh* genes, respectively

* Corresponding author.
E-mail address: heba_ahmed@zu.edu.eg (H.A. Ahmed).

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(Dileep et al., 2003; Honda and Iida, 1993). The two genes are associated with hemolysis and cytotoxic activity of *V. parahaemolyticus* in the host cell. In *V. cholerae*, cholera toxin (CT) is encoded by the *ctx*AB gene; some strains that lack *ctx* gene can cause less severe and rarely life-threatening illness (Anderson et al., 2004; Karunasagar et al., 1995).

Antibiotics are used intensively in aquaculture for therapy and prophylaxis (Jerbi et al., 2011), resulting in the selection of resistant strains and an increase in antibiotic resistance among *Vibrio* species (Tendencia and de la Peña, 2001). This has a potential risk to human health due to the direct transmission of resistant bacteria to consumers via food or the transfer of resistance genes to other human pathogens by mobile genetic elements (Duran and Marshall, 2005; Guglielmetti et al., 2009).

Biofilms are complex assemblies of bacteria on biotic or abiotic surfaces and embedded within a matrix of extracellular polymeric substances that allow the organisms to survive as self-organized, three-dimensional structures (Han et al., 2016; Mizan et al., 2015). The Centers for Disease Control and Prevention estimate that nearly 65% of all reported infections are caused by bacterial biofilms (Lewis, 2007). *Vibrio* spp. are capable of producing adherence factors that allow them to adhere to surfaces and initiate biofilm formation (Donlan, 2002). The effect of temperature on modulating virulence factors and biofilm formation in different micro-organisms has been reported (Han et al., 2016). Bacterial biofilms have heightened resistance to disinfectants, antibodies and antibiotics (Elexson et al., 2014a; Sharma et al., 2010). In Mansoura, Egypt, the prevalence of *Vibrio* spp. in retail shellfish has been reported, however, biofilm formation ability of the isolates was not addressed (Abd-Elghany and Sallam, 2013). To the best of our knowledge, no studies are available on the biofilm formation ability of *Vibrio* spp. isolated from seafood in Egypt.

The correct identification and classification of *Vibrio* spp. in seafood sold at outlets is of utmost importance due to the associated burden on human health and the consequent economic loss for aquaculture (Chen et al., 2012; Goarant et al., 1999). Therefore, this study was designed to estimate the prevalence of *Vibrio* spp. in shrimp and crab sold at fish markets in Sharkia Governorate, Egypt. Diarrheal stool samples from gastroenteritis patients were also examined. The virulence determinants, antibiotic resistance profile and biofilm formation of *Vibrio* isolates were investigated.

2. Material and methods

2.1. Samples

Two hundred twenty-five crustacean samples comprising 132 shrimps (*Penaeus semisulcatus*) and 93 crabs (*Portunus pelagicus*) were aseptically collected from fish markets in Sharkia Governorate, Egypt, during June–September 2016. The shrimp samples originated from the brackish water of Gulf of Suez, while the crab samples were caught off the coast of the Mediterranean Sea at Damietta City. In addition, 100 stool samples were collected from diarrheic patients attending the Outpatient Clinic at Al-Ahrar Hospital. Informed verbal/written consent was obtained from the human participants, and the study was approved by the Committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University, Egypt.

2.2. Isolation and biochemical identification

Isolation of *Vibrio* spp. was performed following the FDA's Bacteriological Analytical Manual (BAM) instructions (FDA, 2001). Ten grams of crustacean flesh were homogenized in 90 mL of sterile alkaline peptone water (HiMedia, M618) and incubated at 35 ± 2 °C for 24–48 h (ISO-TS-21872-1, 2007). A loopful of the enriched culture was streaked onto Vibrio chromogenic agar (Condalab, Pronadisa, 2054), and the plates were incubated at 37 °C for 24 h. Presumptive colonies were then purified and identified using different biochemical tests.

2.3. Molecular identification

Biochemically suspected colonies were confirmed with PCR using 16S rRNA primers specific to *Vibrio* species (Tarr et al., 2007). Bacterial DNA was extracted using the QIAamp DNA Mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's guidelines.

Specific primers targeting the *tox*R gene were used to identify *V. parahaemolyticus* (Kim et al., 1999) and *V. cholerae* (Miller et al., 1987). To serotype *V. cholerae* isolates, PCR amplifying O1-*rfb* and O139-*rfb* genes were used (Hoshino et al., 1998). Molecular identification of *tdh* and *trh* virulence-associated genes in *V. parahaemolyticus* isolates was performed using SYBR green real-time PCR (Rizvi and Bej, 2010). Virulence-associated genes in *V. cholerae* isolates were identified using primers targeting the *ctx* gene (Mousavi et al., 2009) and the *hly*A gene (Shangkuan et al., 1995). Positive controls for *V. parahaemolyticus* and *V. cholerae* were run alongside the tested isolates and were generously supplied by the Biotechnology Unit, Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza, Egypt.

Isolates identified as *V. cholerae* were further confirmed by sequencing of the *ctx* gene. QIAquick Gel Extraction Kits (Qiagen, S. A. Courtaboeuf, France) were used for amplicon extraction from gel according to the manufacturer's guidelines. DNASTAR software (Lasergene version 7.2; DNASTAR, Madison, WI) was used to analyze two sequences that were then submitted to the GenBank, which provided the two accession numbers KY228382.1 and KY228383. The identity of the two isolates with other *V. cholerae* isolates in the GenBank was determined.

2.4. Kanagawa phenomenon

Isolates identified as *V. parahaemolyticus* and *V. cholerae* were examined for the phenotypic determination of thermostable direct hemolysin (TDH) virulence factor. For each isolate, a drop of Tryptone soya broth culture with 3% NaCl was spotted on duplicate plates of Wagatsuma agar (HiMedia, M626) containing fresh human red blood cells and then incubated at 37 °C for 24 h. Positive results were indicated by a β-hemolysis zone. Plates containing positive *V. parahaemolyticus* isolate and negative controls were included in the test. The positive *V. parahaemolyticus* isolate was generously supplied by the Microbiology Department, Faculty of Veterinary Medicine, Zagazig University, Egypt.

2.5. Antibiotic susceptibility testing

The antibiotic susceptibility of the *Vibrio* isolates was determined with the Kirby–Bauer disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS), and the zones of inhibition were measured according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2010) when available; for ampicillin/sulbactam, kanamycin and nalidixic acid, the interpretation criteria for *Enterobacteriaceae* were used (Table S1). The 12 antibiotic discs (Oxoid) used were ampicillin (AMP, 10 μg), nalidixic acid (NA 30 μg), kanamycin (K, 30 μg), ciprofloxacin (CIP, 5 μg), chloramphenicol (C, 30 μg), amikacin (AK, 30 μg), gentamicin (CN, 10 μg), tetracycline (TE, 30 μg), trimethoprim/sulfamethoxazole (SXT, 25 μg), cefotaxime (CTX, 30 μg), ampicillin/sulbactam (SAM, 20 μg), and ceftazidime (CAZ, 30 μg). *E. coli* ATCC 25922 was used as the quality control organism. The multiple antibiotic resistance (MAR) index was determined as the ratio of the number of antibiotics to which the *Vibrio* isolates displayed resistance to the number of drugs to which the *Vibrio* isolates were exposed (Krumperman, 1983). Multidrug resistance (MDR) was defined as the resistance of an isolate to at least one agent in three or more antibiotic classes (Magiorakos et al., 2012).