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Vibrio Alginolyticus: An Emerging Pathogen of Foodborne Diseases

Sabir Mustapha[1,2], Ennaji Moulay Mustapha[1], Cohen Nozha[2]
[1] Laboratory de Virology et Hygiène & Microbiology, Faculty of Science and Technology - Mohammedia- Morocco
[2]Laboratoiry of Microbiology and Food Hygiene and Environment, Institut Pasteur Morocco, Casablanca- Morocco

ABSTRACT

Vibrio alginolyticus is a natural host of estuarine and coastal waters as well as seafood and cannot be eradicated in these niches. This bacterium capable of carrying the pathogenic gene trh is a threat to public health. There are, however, despite the increased complexity related to the particular ecology of these bacteria, many ways to prevent these infections from the harvest site to the consumer's plate without forgetting bathing waters that can cause serious septicemia. The identification of these risks can not be sure without a molecular characterization of virulence genes.

Keywords: *Environment - food poisoning - trh - Vibrio alginolyticus - virulence gene.*

1. INTRODUCTION

Despite the efforts made on the food safety and the environment, 2.1 million adults and three million children, including two million in developing countries, die each year from water consumption or contaminated food. Between 2000 and 2004, 7118 cases of food poisoning have been reported in Morocco, more than 86% of bacterial several cases were linked to the consumption of fishery products (Cohen *et al*., 2006). While the etiologic diagnosis is not known for many gastroenteritis, available data are often highly piecemeal, never complete, and the recovery rate of different observation networks is difficult to estimate. In addition, there is no specific information available on the severity of each case, and they are likely to be related Vibrios (Cohen, 2008).

Over a length of 3500 km of coastline, Morocco produced in 2011, 908258 tons of fish and seafood (anonyme, 2011). In application of European regulation EEC No 2073/2005, the search for pathogen *Vibrio* in fish products for export is required. For this purpose, the application of PCR is necessary both for the confirmation of the identification of the species *V. alginolyticus*, given the risk of cross-reaction with *V. parahaemolyticus* as the search for genes encoding the hemolysin.

Indeed, the species *V. alginolyticus* was isolated in 1997 from patients during an outbreak of acute enteric illness in Vladivostok, Russia (Smolikova et al., 2001). Some foodborne illness caused by *V. alginolyticus* were identified in 96 cases after eating brine shrimp in Chifeng Hongshan, China in 2004 (Xie *et al*., 2005).

The aim of this paper is to summarize the literature on *V. alginolyticus* responsible of enteric diseases, its ecology, pathogenicity and viability with a description of methods for identification and characterization frequently used and finally to give a summary of preventive measures to fight against foodborne illness associated with *V. alginolyticus*.

2. TAXONOMY

Bacteria of the genera *Vibrio* and Photobacterium, are among the most ancient bacteria described. It was left to Robert Koch after observing Pacini in 1854 vibrionnées forms in the feces of cholera cases, isolate and criminalize *Vibrio Comma* as agent of the disease cholera in 1883 in Alexandria, then to Calcutta (Howard-Jones, 1984). It was the first disease described attached to *Vibrio*. It was then necessary wait 67 years for Fujino in 1950 authenticates a food-borne illness caused by other species of *Vibrio* : *Vibrio parahaemolyticus*. But *V. parahaemolyticus* included two biotypes, biotype 1 and 2 (Sakazaki, 1990) [. Zen-Yoji *et al*. (1965) suggested transferring to a new biotype 2 species by taxonomic numerical study. Sakazaki et al., (1968) proposed the name of the new species for biotype 2 *Vibrio alginolyticus*. *Vibrio alginolyticus* is so named because of taxonomic rules. A clear difference between the two biochemical species is found in the ability of sucrose fermentation which is negative for *V. parahaemolyticus* while it is positive for *V. alginolyticus* (Shinoda, 2011).

Indeed, the first criteria for taxonomic characterization was based on several criteria such as morphology phenotypic strains (presence of flagella, bending cells, appearance of crops) and the use of different compound as carbon nutrient sources, setting evidence of enzyme activity (arginine dihydrolase, gelatinase, chitinase, oxidase, catalase, lysine and ornithine decarboxylase), salt tolerance and growth at different temperatures. To date, the genus *Vibrio* species has 91 well identified but the four species most frequently isolated in clinical microbiology laboratories are *V. cholerae*, *V.*

parahaemolyticus, *V. vulnificus* and *V. alginolyticus* (Fournier and Quilici, 2002).

3. ECOLOGY

V. alginolyticus has a large geographic distribution in marine and estuarine waters especially in bathing areas (Baffone et al., 2000 ; Barbieri *et al*., 1999). It is normal host mussels and was also isolated fish and seafood variety. Indeed, the vibrios support high concentrations of salt and water to survive in different environments (seawater, brackish water). Resistance to sea salt which is explained by their character is more pronounced halophilic *V. alginolyticus* than *V. choleae* with which it shares many characters (Denis *et al*., 2007). Several studies have shown a wide distribution of genes in *V. cholerae* and *V. parhaemolyticus* between strains of *V. alginolyticus* into the environment (Snoussi *et al*. 2008). Studies have shown that *V. alginolyticus* is considered the most frequent species living freely in water and sediments (Harriague *et al*., 2008) and can survive in sea water even under conditions of nutrient stress while maintaining their virulence (Ben Kahla-Nakbi *et al*., 2007).

Studies on the prevalence of vibrios in seafood Morocco, showed a prevalence of *V. alginolyticus* exceeds 50% (Bouchriti *et al.,* 2001). While other studies have reported a prevalence of *V. alginolyticus* 72% in fishery products marketed in Casablanca (Cohen *et al.*, 2007) and 71% in the marine environment of the Bay area Tamouda with highest concentrations during the warmer seasons. Which showed that the temperature is the main factor influencing the concentration of *V. alginolyticus* (Sabir *et al*., 2011).

4. PATHOGENICITY OF *VIBRIO ALGINOLYTICUS*

Vibrio alginolyticus is a halophilic associated with several diseases of marine animals including fish, crustaceans and molluscs (Balebona *et al*., 1998 ; Ben kahla-Nakbi *et al*., 2006 ; Gay, 2004 and Gómez-León *et al*., 2005). This reflects both the increasing pressure of human-induced coastal areas and the genetic and phenotypic plasticity of *Vibrio* quickly adapt to changing environmental conditions (Fischer Le Saux *et al*., 2002).

The mode of infection and transmission of this species remains to be studied, a transmission paths is probably seawater. we do not know if this bacterium is endemic in the marine environment and if it behaves as an opportunistic pathogen of fish (Ben Kahla-Nakbi et al., 2007).

Studies have considered strains of *V. alginolyticus* as a potential reservoir of many virulence genes known in other *Vibrio* species in the aquatic environment. Which may contribute to the development of wound infections, enteric diseases and sepsis in humans by exposure to sea water (Lafisca *et al*., 2008 ; Masini *et al*., 2007) . The first reports showed that identification of *V. alginolyticus* has the trh gene occurred in Alaska (Narjol *et al*., 2006) and Tunisia (Ben kahla-Nakbi et al., 2006). Other studies have highlighted the virulence of *V. alginolyticus* in China (Xie *et al*., 2005), confirming reports from other countries in Europe and America (Barbieri *et al*., 1999; Hervio-Heath *et al*., 2002; Matte *et al*., 1994; Robert-Pillot *et al*., 2002; Sun˜en *et al*., 1995). In addition, it has been shown that in Morocco, strains of *V. alginolyticus* carry the trh gene is a virulence gene associated with a positive test Kanagawa phenomenon (Sabir *et al.*, 2012).

Indeed, in humans, *V. alginolyticus* can be isolated from skin infections, often as a result of contact with sea water (Scheftel *et al*.2006). Previous work has shown the potential risks to health associated with the use of non-sterile products such as alginate gels derived from seaweed prepared at home for cleaning wounds that could incriminate *V. alginolyticus* infections as a cause serious wounds especially among vulnerable groups such as older people and those with conditions underlying risk (Reilly *et al*., 2011). Similarly, *V. alginolyticus* was isolated from the pus of ear and spitting, while being responsible for conjunctivitis infection and tissue necrosis and opacification of the sphenoid sinus (Lopes et al., 1993).

In addition, *Vibrio alginolyticus* has been shown responsible for gastroenteritis (Darbas *et al*., 1992 ; Reina *et al*., 1995) and peritonitis (Taylor *et al*., 1981) in humans. Studies have repport infection with *Vibrio alginolyticus* have caused mortality in immunocompromised patients (Campanelli *et al*., 2008). The first case of septic shock due to *Vibrio alginolyticus* in a cirrhotic patient has been reported in Korea after eating seafood (Dong-Young *et al*., 2008).

In Morocco, several cases of gastroenteritis linked to the consumption of fish products are listed each year. The etiological diagnosis is not known for many gastroenteritis, they are likely to be associated with these vibrios. On the other hand, it was reported that foodborne *Vibrio* in Morocco are all due to the consumption of bivalve molluscs harvested in a clandestine way (Bouchriti *et al*., 2001). Indeed the bivalve molluscs and lamellibranchs are invertebrates that filter the water and concentrate the particles and micro-organisms. These foods often consumed raw or undercooked explain the increase in the density of vibrios in water and therefore in seafood freshly collected, but also to multiply in improperly preserved foods, raw or contaminated after cooking . In addition, a remarkable growth of vibrios in seafood raw, even at low temperatures was observed if the conditions of harvesting, processing, distribution and storage are unhygienic. Other studies have shown a correlation between the load of *V. alginolyticus* and the surface temperature of sea water with a clear distinction between the cold season (November-April) and warm

season (May-October) (Sabir *et al.*, 2011) which suggests that the number of infections may increase with warming coastal attributed to climate change (Baker-Austin et al., 2010).

Several studies about the quality of fishery products in Morocco showed more or less variable prevalence of vibrios with a frequency of isolation particularly higher in crustacea and bivalve molluscs (Bouchriti *et al*., 1997; Bouchriti *et al*., 2001; Cohen *et al*., 2007; Sabir *et al.*, 2011).

5. METHODS OF IDENTIFICATION

Bacteriological monitoring of fishery products and the marine environment is necessary to prevent *Vibrio* infections. This requires use of reliable analytical methods and standardized. But currently there is no reference methods really effective for detection and enumeration of vibrios in foods. In addition, using biochemical tests can not always identify the species level and it is often necessary to use molecular tools (Hirsch, 2002).

5.1 Biochemical Characterization

Conventional bacteriological procedures based on biochemical characteristics for the isolation of vibrios from natural samples, including water, were generally unsuccessful, long and fastidious because the vibrios requirent salt for growth and are entering a phase of dormant when conditions are unfavorable to its growth and reproduction.

5.1.1 Microscopic Appearance

Vibrios have a form of gram-negative bacilli purposes, curved, 2 to 3 microns in length. They are characterized by high mobility due to the presence of a single flagellum. Thus their movements are very fast, and maintain a straight path.

5.1.2 Macroscopic Appearance

Bacteria of the genus *Vibrio* grown on selective medium thiosulfate citrate bile sucrose (TCBS) (Oxoid Ltd, Basingstoke, England) give after 18-24 hours at 37 ° C colonies 2 to 3 mm in diameter, circular with regular edges, slightly convex, color varies according to the species of the genus *Vibrio* (Table 1).

Table 1: Typical morphology of *Vibrio* colonies on TCBS agar.

Organisms	Growth	Color of colonies
Vibrio alginolyticus	+ + +	Large yellow colonies
Vibrio parahaemolyticus	+ + +	Blue colonies with green centers
Vibrio cholerae	+ + +	Large yellow colonies
Vibrio fluvialis	+ + +	yellow colonies
Vibrio vulnificus	+ / + +	yellowGreenish yellow colonies
V. harvey / V. fischeri	+ / + +	Gray to bluish green colonies

5.1.3 Culture Conditions

Aerobic preferably *V.alginolyticus* develops little or not under anaerobic conditions. It grows well on ordinary culture media at 10 ° C and 40 °C (mesophilic and psychrophilic), a pH slightly above 7 is optimal for their culture (neutrophilic and alkalophilic). On medium peptone single culture is fast and abundant.

Vibrio have properties which allow them to "elective" in different environments:

- They grow at an alkaline pH between 7.5 and 9.
- They are grown to high NaCl concentration, due to their character halophilic. Indeed the sodium chloride is required for the growth of *V. alginolyticus* and strengthen the growth of other species such as *V. cholorae*. The culture media used for identification must at least contain a percentage of 1% NaCl (Robert-Pillot *et al.*, 2002). *Vibrio alginolyticus* has similar properties to those of *V. parahaemolyticus* but differs in the production of acetone, the fermentation of sucrose and arabinose, NaCl needs and growth at 40 ° C.
- Their growth is not inhibited by the addition of various inhibitors such as bile salts, sodium citrate, sodium thiosulfate. This makes them potential sources of misidentification with enterobacteria, which are selective culture media for the development of *Vibrio*.

5.1.4 Analysis Method

According to the protocol proposed by the National Reference Center for Vibrios and Cholera (Pasteur Paris) and adopted by all laboratories of the International Network of Pasteur Institutes, research vibrios in food and water is made in several steps:

1. Pre-enrichment performed according to the sample type as follows:
- 25 g samples (in the case of bivalves or sediment) diluted in 225 ml of alkaline peptone water (APW) 2% NaCl, and then incubated for 24 h at 37 ° C.

- 2L of water (in the case of sea water) concentrated using 0.20µm membrane filters (Millipore Corp., Bedford, MA, USA). Then the filters were washed with 20 ml APW to obtain a final volume corresponding to 100x concentrated.

2. Isolation on selective agar: thiosulfate, citrate, the ball and sucrose (TCBS) (Oxoid Ltd, Basingstoke, England), from the enrichment broth.
3. Reisolation colonies on nutrient agar (GN) (Bio-Rad, Marnes la Coquette, France), 2% NaCl.
4. Identification of isolated colonies by the search oxidase using the disk oxidase (Bio-Rad, Marnes la Coquette, France), arginine dihydrolase (ADH) (Bio-Rad, Marnes la Coquette, France) and of lysine decarboxylase (LDC) (Bio-Rad, Marnes la Coquette, France).

Strains with the following characteristics: oxidase positive (appearance of a purple color within 10 seconds), ADH negative (yellow middle) and LDC positive (violet middle) followed by seeding a gallery Enterobacteriaceae API 20E (Biomerieux, Marcy l'Etoile, France). This identification made from colonies of GN is complemented by a gallery of growth in salt consists of a series of tubes alkaline peptone water containing 0, 3, 6, 8 and 10% NaCl.

5.1.5 Highlighted Character Hemolytic of Strains

To detect strains of *V. alginolyticus* with the hemolytic character, reports have published that the production of β-hemolysis on Wagatsuma blood agar is termed the Kanagawa phenomenon (KP) (Elliot *et al.*, 1992). This reaction is regarded as a characteristic of virulent strains (Nishibuchi *et al.*, 1986). Wagatsuma agar containing 5% of washed rabbit erythrocytes, yeast extract 0.5%, peptone 1%, 7% NaCl, 0.0001% crystal violet, and 1.5% agar (pH 7.5). The inoculation is done by measurement of 20 µ l (optical density OD = 0.5) of the supernatant APW tubes into wells provided for this purpose. After 24 hours of incubation at 37 ℃, the light areas of β-hemolysis around wells on Wagatsuma blood agar are considered positive Kanagawa reaction (Sabir *et al*., 2012).

5.2 Molecular Characteristics

Studies on the effects of adaptation of *V. alginolyticus* and *V. parahaemolyticus* with a deficiency of nutrients in seawater showed morphological changes in the appearance of the coccoid form, changes in extracellular proteins and gelatinase models. This same study noted the change of expression of virulence genes. This prompts reflection on genetic instability and the virulence of bacteria stressed (Ben Abdallah *et al*., 2011). These variations can be a serious problem for the characterization of the strains isolated from the environment. Indeed, the method of molecular biology became an important tool in the identification and characterization of bacteria from the marine environment and fishery products.

Conventional methods of bacterial identification are less specific and can not differentiate between species, particularly *V. parahaemolyticus*, which is biochemically very similar to *V. alginolyticus.*

So the use of reactions (PCR) provides not only a better specificity than conventional bacteriological techniques for the determination of the speciesbut also enables the identification of different virulence genes of both species primarily associated risk *Vibrio* in seafood the presence of representatives of the genus *Vibrio* in seafood, especially bivalves, are naturally related to the ecology of this bacterium, the adoption of PCR techniques for the detection *Vibrio* strains carrying virulence genes is therefore essential to determine the safety of fishery products intended for consumption (Cohen *et al*., 2007).

Indeed, molecular biology techniques provide new tools for the study of relationships between organisms including classification, nomenclature and identification, and help define fingerprints and the singularity of a bacterial strain. Many typing methods have been developed to differentiate strains of *Vibrio*. Some, phenotypic include serotyping and biotyping and phagetyping patterns, including other molecular sequencing of ribosomal genes has rewrite the classification of *Vibrio* (Kita-Tsukamoto *et al*. 1993).

In contrast, the phenotypic variability of isolates from environmental and food makes it difficult to distinguish precisely between *V. alginolyticus* and other members of this genus, especially *V. parahaemolyticus* by biochemical tests. Robert-Pillot showed that amplification of the fragment R72H, if the amplicon is 320 bp or 387, is a powerful tool for accurate and reliable identification of *V. parahaemolyticus*. Hence the biochemical identification of strains of *V. alginolyticus* can be confirmed by the absence of the sequence R72H. The application of this study by PCR of strains biochemically identified as eliminates *V.alginolyticus* strains of *V. parahaemolyticus* atypical sucrose (Robert-Pillot *et al*., 2002).

5.2.1 Molecular Identification Method

The extraction of genomic DNA of *V. alginolyticus* is performed according to the technique Wizard adapted to Gram-negative and Gram-positive (Wizard Genomic - Promega, France) (Sabir *et al*., 2012). Primers, 320 or 387

bp in size, used for gene amplification for gene research R72H chromosome *V. alginolyticus* are defined VP32 (5-AATCCTTGAACATACGCAGC-3) and antisense VP33 (5 - TGCGAATTCGATAGGGTGTTAACC-3) (Lee *et al.*, 1995). Gene amplification of the gene for the R72H research is performed in a thermocycler DNA (Bio-Rad, DyadDisciple) using the following parameters: initial denaturation at 94 ℃ for 5 min followed by 35 amplification cycles, each cycle consisting of denaturation of 1 min at 94 ℃, primer annealing for 1 min at 60 ℃ and primer extension for 1 min at 72 ℃. After the last cycle, the PCR mixtures were incubated for 10 min at 72 ℃ (Sabir *et al*., 2012). PCR products were visualized by staining with ethidium bromide after electrophoretic migration in agarose gel 2% (Invitrogen).

6. METHOD OF RESEARCH OF THE VIRULENCE GENE

The search for virulence genes coding for two hemolysins, thermostable direct hemolysin (tdh) and thermostable related hemolysin (*trh)* is applied to all identified strains of *V. alginolyticus* PCR. DNA extraction from *V. alginolyticus* is made according to the protocol described in the commercial kit DNA purification (Wizard Genomic - Promega, France). The primers used for amplification of virulence genes in *V. alginolyticus* are *trh* (250 bp): sense L.trh (5 - GGCTCAAAATGGTTAAGCG -3) and antisense R.*trh* (5 - CATTTCCGCTCTCATATGC -3). *tdh* (373 bp): sense L.*tdh* (5 - CCATCTGTCCCTTTTCCTGC -3) and antisense R.tdh (5 - CCAAATACATTTTACTTGG -3) (Cohen *et al.*, 2007). PCR amplification was performed in a thermocycler DNA (Bio-Rad, DyadDisciple) using the following parameters: initial denaturation at 94 ℃ for 5 min followed by 30 amplification cycles, each cycle consisted of denaturation at 94 ℃ for 1 minute, primer annealing at 54 ℃ for 1 min, and a primer extension at 72 ℃ for 1 min. After the amplification cycles, the samples were stored at 72 ℃ for 10 min to allow a final extension of DNA synthesized incomplete. PCR products were visualized by ethidium bromide staining after electrophoretic migration in agarose gel 2% (Invitrogen).

7. STABILITY AND VIABILITY

It has been shown that *Vibrio* species in particular, *V.* alginolyticus are generally resistant to penicillin and vancomycin, but they are sensitive to tetracycline, chloramphenicol, aminoglycosides and β-lactams other, it may have a β-lactamase positive activity (Ben kahla-Nakbi *et al*., 2006 ; Joseph *et al*., 1978). Other publications have confirmed that *V. alginolyticus* is generally sensitive to most antibiotics, including trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol, gentamicin, quinolones and third generation cephalosporins (Mukherjee *et al*., 2008).

While *Vibrio* is sensitive to sodium hypochlorite 1%, 70% ethanol, 2% glutaraldehyde and formaldehyde (Anonyme, 2011). But in the marine environment, studies have pointed out that the salinity is crucial for the survival and proliferation of bacteria that can survive the winter in marine sediments, and its proliferation restarts when temperatures reach at least 15 °C (Su and Liu, 2007). In addition, research conducted in the Mediterranean countries have confirmed that the highest concentrations of *V. alginolyticus* were recovered during the warmer seasons and the temperature was the main factor influencing the concentration in the marine environment (Sabir *et al.*, 2011). In addition, the temperate Mediterranean climate favors the survival of the bacteria in the waters of germ aquaculture stations while retaining its virulence is expressed in terms of different physical, chemical and biological (Ben Kahla-Nakbi *et al*., 2007).

8. PROPHYLAXIS

Given that *V. alginolyticus* is a bacterial pathogen, prevention is the only way to avoid food poisoning incidents or following contact with a contaminated environment. Indeed, the water and seafood monitoring should allow the detection of potentially pathogenic isolates, especially during the warmer months when the bacterial concentration is high.

In order to control and eliminate *V. alginolyticus* in aquaculture and oyster ponds, it is recommended the use of bacteria Bdellovibrio (Delta class Proteobacteria) with characteristic parasitize bacteria Gram negative that develop in their periplasmic space. However, systematic research vibrios can be considered if the technical analysis and enumeration are sufficiently powerful and serve to clarify the meaning of their presence in health food. Indeed, even if the level of contamination of a product is initially low, it can quickly reach the infective dose evaluated 10^5 bacteria per gram, a few hours if kept in poor conditions (CDC, 1998).

No prophylaxis is currently recognized effective, but we can avoid infections by maintaining low temperature raw shellfish for consumption and reducing the time between harvest and consumption because it has been established that the number of bacteria is 50 times higher in shellfish after 10 hours storage at 26 °C, and 760 times higher after 24 hours at the same temperature (Yeung and Boor, 2004). In addition, being extremely sensitive to heat, bacteria of the genus *Vibrio* are effectively inactivated by cooking shellfish at a temperature of 48-50 °C for 5 minutes (Yeung and Boor, 2004). Similarly, Su and Liu have shown that reducing the amount of bacteria in seafood can be achieved by cold storage (3 °C) for 7 days

by freezing or low temperature pasteurization, viable bacteria can completely inactivated at a temperature of -18 °C or -24 °C for 15 to 28 weeks (Su and liu, 2007).

9. CONCLUSION

This synthesis reveals (i) the increase in the frequency of isolation of *V. alginolyticus* carrying the trh virulence gene homologous to that described in *V. parahaemolyticus*, (ii) the consideration *V. alginolyticus* as a reservoir of potential virulence genes in the aquatic environment and (iii) the emergence of *V. alginolyticus* as a pathogen responsible for food poisoning in humans after consumption of contaminated seafood. It is therefore necessary to integrate *V. alginolyticus* in the list of pathogenic *Vibrio* sought in samples of the marine environment and fishery products to prevent food poisoning collective. However, a systematic search of *Vibrio* can be considered if PCR techniques for the detection of *V. alginolyticus* strains carrying virulence genes are adopted.

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