RESEARCH ARTICLE (Open Access)

Evaluation of the Presence of *Vibrio spp.* in Shellfish Using the International Standard ISO 21872-1:2017

ALESSIO MANFREDI[1*], PATRIZIO LORUSSO[1], ANNAMARIA PANDISCIA[1], GIUSEPPINA MARILIA TANTILLO[2], GIANCARLO BOZZO[1], VALENTINA TERIO[1].

[1]Department of Veterinary Medicine, University of Bari, Provincial Road to Casamassima Km 3, 70010 Valenzano, Italy.

[2] Department of Interdisciplinary Medicine, University of Bari, Place Giulio Cesare 11, 70124 Bari.

* Corresponding author; E-mail: a.manfredi21@studenti.uniba.it

Abstract

Vibrio spp. are gram-negative bacteria responsible for foodborne illness associated with consumption of raw or undercooked shellfish. In particular, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae* are common human-pathogenic vibrios that are associated with clinical syndromes that can range from mild gastroenteritis to life-threatening primary septicemia.Therefore, the aim of the study is the evaluation of the presence of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* in shellfish marketed in south Italy.During the period between December 2022 and January 2023, 36 samples of purified live bivalve molluscs were collected. Then, the culture method described in International Standard ISO 21872-1:2017 was applied for the detection of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*. The characteristic colonies, isolated on TCBS and CHROMagar™ Vibrio, were confirmed by PCR through the detection of toxR gene for *V. parahaemolyticus* (Vp-toxR), prVC region for *V. cholerae* and VVH region for *V. vulnificus*. 5/36 samples tested positive for *Vibrio parahaemolyticus* with a 13,9% positivity rate, while no samples tested positive for *Vibrio cholerae* and *Vibrio vulnificus*. Although some samples were positive for *Vibrio parahaemolyticus*, the pathogenicity of the strains cannot be established because of the absence of analysis of virulence genes such as "tdh" and "trh"; thus, additional analyses are needed. However, the presence of *Vibrio spp.* during times of the year when temperatures should be lower and unsuitable for the growth of these microorganisms emphasizes both how climate change influences the presence of these microorganisms in seafood and how the current purification methods are ineffective in eradicating shellfish from these pathogens. In addition, Commission Regulation (CE) 2073/2005, which lays down the microbiological criteria for shellfish placed on the market, don't provide any kind of criteria for the presence of these pathogens.Thus, the presence of *Vibrio spp.* in winter season, the ineffectiveness of current purification methods and the lack of microbiological limits suggest how the detection of *Vibrio spp.* in shellfish samples is very important to prevent emerging health risks.

Keywords: shellfish; *Vibrio spp*.; food safety.

1. Introduction

***Vibrio* spp. are a group of gram-negative, rod-shaped bacteria that live in freshwater, estuarine and marine environments [1]. Species belonging to this genus are oxidase positive -except for *Vibrio metschnikovii*- and they are facultative anaerobes [2]. In addition, Vibrio spp. are non-sporulating bacteria, therefore they can respond to environmental changes (such as pH and temperature) by entering in a quiescent phase in which they are viable but not cultivable by traditional laboratory methods [3]. *Vibrio* spp. require salt for their growth, whose concentration varies according to the species. This feature allows to separate these bacteria into non-halophilic groups such as *V. cholerae* and *V. mimicus* that grow on nutrient agar and halophilic groups that require salt supplementation in their growth media [4]. Moreover, these bacteria have an optimal growth**

*Corresponding author: Alessio Manfredi; E-mail: a.manfredi21@studenti.uniba.it
(Accepted for publication 10.05.2023)
***ISSN**: 2218-2020, © Agricultural University of Tirana*

temperature between 20°C and 40°C under alkaline conditions [4], in fact *Vibrio* spp. can be more abundant in seawater with a temperature higher than 15 °C. Nowadays, more than 100 species of these bacteria have been described, however, only 12 species cause infections in humans [5]; among these *V. cholerae*, *V. parahaemolyticus* and *Vibrio vulnificus* are the most epidemiologically important. *Vibrio cholerae* is one of the most clinically studied bacteria as the etiologic agent of cholera. This species is divided into various serotypes by the somatic antigen O, a thermostable polysaccharide of the lipopolysaccharide layer. The serotypes O1 - that includes Classical and El Tor Biotypes- and O139 are recognized as "cholera Vibrio" with epidemic/pandemic character [6]. Transmission of this bacterium occurs mainly by the oro-fecal route, through ingestion of contaminated water or raw or sligthly cooked food [5]. Symptoms of the disease occur 12-72h after infection and include: profuse diarrhea, nausea, vomiting, abdominal pain. If adequate therapy is not administered, hypovolemia, hypokalemia, metabolic acidosis, arrhythmias and death may occur [7]. This clinical setting is due to cholera toxin (CTX) that is released by Vibrio cholerae when it adheres to the intestinal wall via the polar flagellum. CTX is a binomial exotoxin of type A-B and consists of two protein fractions: an active subunit (A) and a binding subunit (B). This toxin acts by altering the intracellular concentration of c-AMP, leading to an imbalance in salt secretions. Sodium absorption is blocked and chloride secretion is promoted. The electrolyte imbalance causes severe profuse diarrhea with subsequent dehydration, followed by weakness and muscle pain due to potassium leakage [8]. At the epidemiological level, the World Health Organization (WHO) reported 323.369 cases and 857 deaths in 24 countries in 2020. However, these data are often underestimated. In fact, according to WHO, cholera cases each year are around 1.4 million with about 143000 deaths [9]. Vibrio parahaemolyticus, commonly isolated in seawater and sediments [11], marine animals, plankton [12] and bivalve molluscs (13), was identified for the first time in 1951 in Osaka, Japan. Cases of illness caused by this bacterium have been found worldwide, with a higher occurrence in Japan where it is estimated to be responsible for 20 to 30 percent of gastroenteritis [10]. The most important virulence factors are: direct hermostable hemolysin (TDH)", "TDH- related hemolysin (TRH)" and "Type III Secretion Systems" [14]. According to the data reported by EFSA, in 2020 *V. parahaemolyticus* was responsible for 4 outbreaks with no cases of hospitalization or death [15]. Despite the absence of cases of hospitalization and death, it is necessary to constantly monitor the presence of *Vibrio parahaemolyticus* in food matrices. Moreover, it could become dangerous and difficult to eradicate because recently, there have been cases of antibiotic resistance linked to this bacterium [10]. Finally, *V. vulnificus* is a bacterium commonly isolated from water, sediments and a variety of seafood such as oysters and shellfish [16]. There are three biotypes of *V. vulnificus*: (i) biotype I strains are responsible for most human infections; (ii) biotype II strains are mainly pathogenic to eels; (iii) biotype III was recently identified and it has characteristics intermediate between biotypes I and II [17]. Open wounds, from contact with infected seawater, and consumption of raw or undercooked bivalve mollusks are the main causes of infection with this bacterium [18]. The disease caused by *V. vulnificus* manifests itself through gastroenteritis, septicemia (especially if there are previous diseases such as diabetes, liver or kidney disease in the patient) and wound infections [19]. This pathogen has several virulence factors including a capsular polysaccharide (CPS), endotoxins and exotoxins [5]. In the United States, 45 hospitalizations and 16 deaths occur every year out of 50 infections [20]. These data suggest that monitoring and surveillance measures need to be constantly applied due to the impact of the bacterium on human health. Based on the above, bivalve mollusks represent a very important source associated with Vibrio spp. infection. Indeed, bivalve mollusks feed on small food particles in water or sediment through intense filtration activity during which they can retain in their hepatopancreas bacteria -such as Vibrio spp. They can be transmitted to humans especially when they are eaten raw or undercooked [21], [22]. Therefore, the study evaluated the presence of V. parahaemolyticus, *V. vulnificus* and *V. cholerae* in retail mussels (*Mytilus galloprovincialis*) in south Italy.

2. Material and Methods

2.1 Sampling

Thirty-six samples of retail mussels (*Mytilus galloprovincialis*) reared in South Italy, were analyzed. The samples from different dispatch centers of Bari (Italy) were collected between December 2022 and January 2023; the samples were stored at 6°C and then, they were transported to the laboratory and processed immediately.

2.2 Cultural isolation of V. parahaemolyticus, V. vulnificus and V. cholerae (ISO 21872:1-2017)

According to International standard ISO 21872:1-2017, the samples were cleaned of various impurities by vigorous brushing and rinsed under sterile water. 25 grams of flesh and intra-valvular liquid were omogenized by stomacher (Seward Ltd.) under sterility condition and then, two selective enrichments were carried out using Alkaline Saline Peptone Water (Oxoid, Hampshire, UK) as a liquid medium. Two separate platings of enrichment culture onto thiosulphate-citrate-bile salt-sucrose (TCBS) (Oxoid, Hampshire, UK) and CHROMagar™ Vibrio were carried out. The characteristic colonies were transferred to Nutrient Agar (Oxoid, Hampshire, UK) with 3% of NaCl. After incubation at 37°C for 24h, colonies were transferred to Tryptone Soya Broth (Oxoid, Hampshire, UK) with 3% of NaCl and incubated for 24h at 37°C.

2.3 Detection of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* from broth culture

The "boiling method" described by the Italian National Institute of Health was used for bacterial DNA extraction [23]. The PCRs were performed in a total volume of 25 µl using 12.5 µl HotStarTaq Master Mix (Qiagen, GmbH, Hilden, Germany), and 1 µM primer pairs (Eurofins Genomics) (Table 1). The PCR reactions, performed with 10 µl of template, were processed in a ProFlex PCR System (Applied biosystems, Waltham, Massachusetts, USA) as described in Table 2.

2.4 Detection of amplified products

PCR amplified products were analyzed by electrophoresis on 1.8% (w/v) agarose NA (Pharmacia, Uppsala, Sweden) gel in 1X TBE buffer containing 0.89 M tris, 0.89 M boric acid, 0.02 M EDTA, pH 8.0 (USB, Cleveland, OH, USA) and stained with Green Gel Safe 10,000X Nucleic Acid Stain (5 ll/100 ml) (Fisher Molecular Biology, USA). The Gene RulerTM 100 bp DNA Ladder molecular weight marker (MBI Fermentas, Vilnius, Lithuania) was used. Image acquisition was performed with Gel Doc™ EZ imager Bio-rad.

Table 1. Oligonucleotide primers

Species	Target	primers	Sequence (5'-3')	Amplicon size	Reference
V. parahaemolyticus	ToxR	VpToxR- F	gtc ttc tga cgc aat cgt tg	368pb	(24)
		VpToxR-R	ata cga gtg gtt gct gtc atg		
V. vulnificus	VVH	VVH-F	ccg gcg gta cag gtt ggc gc	519pb	(25)
		VVH-L	cgc cac cca ctt tcg ggc c		
V. cholerae	prVC	prVC-F	tta agc stt ttc rct gag aat g	295-310 pb	(26)
		prVC-R	Agt cac tta acc ata caa ccc g		

Table 2. Amplification profiles

Target	Initial denaturation	Denaturation	Annealing	Extension	Cycles	Post amplification
toxR	95 °C for 15'	94 °C for 1'	63°C for 90''	72°C for 90''	30	72°C for 7'

VVH	95 °C for 15’	94 °C for 1’	63°C for 90’’	72°C for 90’’	30	72°C for 7’
prVC	95 °C for 15’	94 °C for 1’	50°C for 1’	72°C for 90’’	30	72°C for 10’

3. Results and Discussion

Molecular analysis, carried out on the thirty-six samples, showed that five samples were positive for the presence of *Vibrio parahaemolyticus*, with a positivity rate of 13.9% (5/36) (Table 3).

No samples tested positive for Vibrio cholerae and Vibrio vulnificus.

Table 3. Positivity rate

	positive samples/ total samples	percentage of positivity
V. parahaemolyticus	5/36	13.9 %
V. cholerae	0/36	0 %
V. vulnificus	0/36	0 %

The results of this analysis underscore a significant positivity rate for *Vibrio parahaemolyticus*; however, although ToxR gene detection suggests the presence of *Vibrio parahemolytius*, it is not possible to assess the pathogenicity of the analyzed bacteria.Therefore, further analysis will be needed to detect the virulence genes of this bacterium such as the TDH and TRH genes. Moreover, it is necessary to underline that the sampling was done in winter season, a time of year when sea temperatures should be lower than 15°C and consequently unsuitable for the growth of these microorganisms [11]. Indeed, at low temperatures these bacteria enter in a phase in which they are viable but not cultivable by traditional laboratory methods, so their detection is very difficult [27].

Thus, the high positivity of the samples for *V. parahaemolyticus* may be attributed to the progressive increase in sea surface temperatures as a result of climate change [5].

In addition to seawater temperature, another factor that may have contributed to the high positivity of Vibrio parahaemoltycus in the samples is the ineffectiveness of commonly used purification methods of shellfish such as ozonation, chlorination and UV treatment of the water.

This evidence is in line with studies conducted on the purification efficiency on mussels experimentally contaminated with *E. coli*, *V. cholerae* O1 and *V. parahaemolyticus* and purified in a pilot plant using ozone. After 44 hours of treatment, the reduction of *E.coli* was significant (about 3 logs), while the reduction of both vibrio species was very low (about 1 log) [28].

Moreover, in the European Union, Commission Regulation (EC) 2073/2005, which lays down microbiological criteria for shellfish placed on the market, only assesses the presence of *E.coli* and Salmonella spp.,exposing consumers to great risk (29). Indeed, the pathogens considered by the regulation are indicators of fecal contamination, so they do not correlate in any way with *Vibrio* spp. that are naturally present in the seawater instead.

Therefore, both the current purification methods of shellfish and the microbiological parameters in Commission Regulation (EC) 2073/2005 do not guarantee the absence of these pathogens in live bivalve molluscs.

Based on the above, the evaluation of more effective technologies for shellfish purification becomes essential; among these technologies, high hydrostatic pressures appear to be promising.

This method, already proven effective in inactivating viruses such as HAV in experimentally contaminated mussels (30), has been tested against Vibrio spp.

A HPP of 293 MPa for 120 s was identified capable of both achieving greater than 3.52-log reductions of *V. parahaemolyticus* in Pacific oysters and increasing the shelf life of the product [31].

Therefore, this method could be adopted in post-harvest processes in the shellfish industry to ensure consumer health and at the same time increase product quality.

4. Conclusions

The high positivity of *Vibrio parahaemolyticus* in the samples underscores how the evaluation of the presence of these bacteria should be considered even at times of the year when one would not normally expect to find them.

In addition, the inefficiency of the current purification methods against these bacteria and the lack of microbiological criteria within regulations do not guarantee consumer safety.

Therefore, from the perspective of food safety, it is necessary both to assess the presence of these pathogens in shellfish constantly and to investigate new, more effective technologies to eradicate vibrios from bivalve molluscs, especially in the absence of regulations to protect consumers.

5. References

1. Zhu S, Kojima S, Homma M: **Structure, gene regulation and environmental response of flagella in Vibrio**. Frontiers in microbiology 2013, **4**(410).
2. Malaiyan J, Balakrishnan A, Nasimuddin S, Mohan K, Meenakshi-Sundaram P, Mamandur-Devarajan S, Gnanadesikan S, Kandasamy M, Jayakumar N, Elumalai D, Ra GG: **Novel gas producing Vibrio cholerae: a case report of gastroenteritis with acute kidney injury.** Access Microbiology 2019, **1**(2): e000005.
3. Prosdocimi E.M, Arioli S, Mapelli F, Zeaiter Z, Fusi M, Daffonchio D, Borin S, Crotti E: **Cell phenotype changes and oxidative stress response in Vibrio spp. induced into viable but non-culturable (VBNC) state.** Annals of Microbiology 2023, **73**(1).
4. Steven L. Percival, David W. Williams: **Microbiology of Waterborne Diseases**: Academic Press; 2014.
5. Baker-Austin C, Oliver JD, Alam M, Ali A, Waldor MK, Qadri F, Martinez-Urtaza J: **Vibrio spp. infections**. Nature Reviews Disease Primers 2018, **4**(1):8.
6. Chatterjee SN, Chaudhuri K: **Lipopolysaccharides of Vibrio cholerae. I. Physical and chemical characterization**. Biochimica et Biophysica Acta 2003, **1639**(2):65-79.
7. Keen MF, Bujalski L. **The diagnosis and treatment of cholera**. Nurse Practice 1992, **17**(12):53-6.
8. Caianello C, **Vibrio Cholerae**, Microbiologia Italia (2020), https://www.microbiologiaitalia.it/batteriologia/vibrio-cholerae/ (accessed on: 02 April 2023).
9. **Cholera**, World Health Organization (2022), https://www.who.int/news-room/fact-sheets/detail/cholera (accessed on: 02 April 2023).
10. Letchumanan V, Loo K, Law J.W, Wong S.H, Goh B, Mutalib N.A, Lee L: **Vibrio parahaemolyticus: The protagonist of foodborne diseases**. Progress In Microbes & Molecular Biology 2019, **2**(1).
11. Alipour M, Issazadeh K, Soleimani J: **Isolation and identification of Vibrio parahaemolyticus from seawater and sediment samples in the southern coast of the Caspian Sea**. Comparative Clinical Pathology 2014, **23**(1): 129-133.
12. Hartwick MA, Berenson A, Whistler CA, Naumova EN, Jones SH: **The Seasonal Microbial Ecology of Plankton and Plankton-Associated Vibrio parahaemolyticus in the Northeast United States**. Applied and Environmental Microbiology 2021, **87**(15).
13. Mok J.S, A. Ryu A, Kwon J.Y, Kim B, Park K: **Distribution of Vibrio species isolated from bivalves and bivalve culture environments along the Gyeongnam coast in Korea: Virulence and antimicrobial resistance of Vibrio parahaemolyticus isolates**. Food Control 2019, **106**.
14. Broberg CA, Calder TJ, Orth K: **Vibrio parahaemolyticus cell biology and pathogenicity determinants**. Microbes and Infection 2011, **13**(12-13):992-1001.
15. EFSA and ECDC: **The European Union One Health 2020 Zoonoses Report**. EFSA Journal 2021, **19**(12):6971.
16. Chase E, Young S, Harwood VJ: **Sediment and vegetation as reservoirs of Vibrio vulnificus in the Tampa Bay Estuary and Gulf of Mexico**. Applied and Environmental Microbiology 2015, **81**(7).
17. Jones MK, Oliver JD: **Vibrio vulnificus: disease and pathogenesis**. Infection and Immunity 2009, **77**(5):1723-33.
18. **Vibrio vulnificus Infection (including Necrotising Fasciitis caused by V. vulnificus)**, Centre for Health Protection The Government of the Hong Kong Special Administrative Region

2019, https://www.chp.gov.hk/en/healthtopics/content/24/3780.html (accessed on: 03 April 2023).

19. Heng SP, Letchumanan V, Deng CY, Ab Mutalib NS, Khan TM, Chuah LH, Chan KG, Goh BH, Pusparajah P, Lee LH: **Vibrio vulnificus: An Environmental and Clinical Burden**. Frontiers in Microbiology 2017, **8**.
20. Centers for Disease Control and Prevention, **Vibrio vulnificus**, CDC 2013, http://www.cdc.gov/vibrio/vibriov.html (accessed on 04 April 2023).
21. Pruzzo C, Gallo C, Canesi L: **Persistence of vibrios in marine bivalves: the role of interactions with haemolymph components**.Environmental Microbiology 2005, **7**(6): 761-772.
22. Klohmann C. A, Padilla-Gamiño J.L: **Pathogen Filtration: An Untapped Ecosystem Service**. Frontiers in Marine Science 2022, **9**.
23. Ripabelli G, Grasso G. M, Sammarco M. L, Luzzi I: **Procedure di isolamento e caratterizzazione di Vibrio spp. di importanza clinica**. Istituto Superiore di Sanità Rapporti ISTISAN 97/31 1997.
24. Kim Y.B, Okuda J, Matsumoto C, Takahashi N, Hashimoto S, Nishibuchi M: **Identification of Vibrio parahaemolyticus strains at the species level by PCR targeted to the toxR gene**. Journal of Clinical Microbiology 1999, **37**(4): 1173-7.
25. Hill WE, Keasler SP, Trucksess MW, Feng P, Kaysner CA, Lampel KA: **Polymerase chain reaction identification of Vibrio vulnificus in artificially contaminated oysters**. Applied and Environmental Microbiology 1991, **57**(3): 707-11.
26. Chun J, Huq A, Colwell RR: **Analysis of 16S-23S rRNA intergenic spacer regions of Vibrio cholerae and Vibrio mimicus**. Applied and Environmental Microbiology 1999, **65**(5): 2202-8.
27. Oliver D.J: **The Viable but Non culturable State for Bacteria: Status Update: This dormant form of bacteria was first appreciated in 1982; now skeptics recognize this state as a bacterial response to stress and a strategy for survival**. Microbe Magazine 2016, **11**(4): 159-164.
28. Croci L, Suffredini E, Cozzi L, Toti L: **Effects of depuration of molluscs experimentally contaminated with Escherichia coli, Vibrio cholerae O1 and Vibrio parahaemolyticus.** Journal of Applied Microbiology 2002, **92**(3):460-5.
29. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. OJ L 338/1.
30. Terio V, Tantillo G, Martella V, Di Pinto P, Buonavoglia C, Kingsley D.H: **"High Pressure Inactivation of HAV Within Mussels"**. Food and Environmental Virology 2010, **2**: 83–88.
31. Ma L, Su Y.C: **Validation of high pressure processing for inactivating Vibrio parahaemolyticus in Pacific oysters (Crassostrea gigas).** International Journal of Food Microbiology 2011, **144**(3):469-74.