RESEARCH ARTICLE **(Open Access)**

Simple DNA Extraction Method with Alkaline PEG and Rapid Detection of *Vibrio parahaemolyticus* with Real-Time PCR in Molluscan Shellfish

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Abstract

As Albanian shellfish farming is increasingly becoming an essential component of food industry, ensuring the microbial safety of the products for the consumers has been a significant concern. Vibrio species are naturally present in brackish waters. A subpopulation of *V. parahaemolyticus* can cause infection in humans with serious complications. The objective of this study was to evaluate the occurrence of *V. parahaemolyticus* in molluscan shellfish production zone such as Butrinti lagoon, cape of Rodon and bay of Shëngjin. PCR is a gold standard for the detection/confirmation of pathogenic Vibrio *spp*. in presumptive positive isolates and *toxR* gene is considered a standard marker to genotype environmental or clinical strains. DNA of Vibrio *spp*. were extracted by using the composition and protocol for Alkaline PEG. Sixty samples of live bivalve mollsuscs (*M. galloprovincialis*) were collected in the year 2020 (February-September) in three production area. Results showed that 6.7% of environmental *V. parahaemolytcicus* were positive (4/60). The highest percentage of occurence was found at Butrint lagoon (3/36) (8.3%), followed by cape of Rodon (1/14)(7.1%) positivity. No presence of *V. parahaemolyticus*was found at Shëngjin bay (0/10). Concern about foodborne illnesses from *V. parahaemolyticus*, especially when bivalve mollsucs remains as a vehicle of transmission of Vibrio, are likely to continue in future.

Keywords: *M. galloprovincialis*; real-time PCR; gold standard; *Vibrio parahaemoliticus*

1. Introduction

M. galloprovincialis (Mediteranean mussel) is a very popular shellfish within the Albanian domestic market and abroad as a result its cultivation plays an important role in the aquaculture industry and consequently constitutes a prominent economic development in this sector.On the other side, they are consideredbioindicator organisms because they have the capacity to accumulate microorganisms from surrounding waters because of their filter-feeding strategy.(Desenclos et al., 1996)A study reported the relation between the concentration of *V. parahaemolyticus* in seawater by collecting statistical data that include number of *V. parahaemolyticus*in 100 mL of seawater, seawater temperature t (°C) and seawater salinity s (%). As a result the concentration of *V. parahaemolyticus* in raw oyster was found to be 11.1 times greater than the concentration in seawater.(Ogawa et al., 1989)

Live bivalve moluscs may be a vehicle for most known pathogen bacteria.(Huss, 1997)Non-indigenous pathogen bacteria (e.g. Salmonella and Shigella) are introduced into seawater by infected animals and humans, while native bacteria are naturally occurring organisms in the marine environment, mainly belonging to the family *Vibrionaceae*.(Potasman et al., 2002)The genus Vibrio is endemic in marine and estuarine ecosystems, and regroups more than 63 species. Since many of them were pathogenic for humans, they had been associated with frequent food-borne infections. (Chakraborty et al., 1997)Among these species, 12 might cause gastrointestinal diseases or, in some cases, septicemia. Most of them were caused by *Vibrio parahaemolyticus* and *V. vulnificus*.(Oliver & Kaper, 1997)

Like other members of the genus, potentially pathogenic species are most abundant in warm waters and exhibit strong seasonality, with most infections occurring during the summer months.(Gilbert et al., 2012; Givens et al., 2014)

Vibrio parahaemolyticus is a gram-negative halophilic bacteria with the ability to cause gastrointestinal

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Special Issue of the Conference: Agriculture a Life Science with Roots in Applied Biology; 3-4 Dec. 2020. (Accepted for publication 11.01.2020)
***ISSN**: 2218-2020, © Agricultural University of Tirana*

infections and pathogenic symptoms such as diarrhea, vomiting, acute gastroenteritis, dehydration, shock, and even death after the ingestion of the bacteria.(Alonso-Hernando et al., 2013; Hartwick et al., 2019) It is principally distributed within seawater(DePaola et al., 1990), coastal areas, and river-sea junctures(Yang et al., 2017) and it can be found in contaminated seafood(Rincé et al., 2018; Twedt, 1989)pousing a global public health issue.

A study was conducted in the year 2011 as a result 84 samples were analyzed. All analyzes were carried out in collaboration with the FSVI by using the cultural method. Out of 84 samples screened, 4 samples (4.8%) harbored Vibrio *spp*. And such genius was isolated from samples collected from three sampling points of Butrint lagoon; north (SP1), south(SP7) and west(SP5). All positive samples of Vibrio *spp*. were generally isolated in the summer months (July-September 2011).(Çoçoli et al., 2012)

In a previous study, the prevalence of enteropathogenic*V. parahaemolyticus*at theButrint Lagoonwas estimatedduring the period June 2013 till December 2013 through confirmation with conventional PCR of *toxR, tdh* and *trh* gene. From the analyzes performed on 50 samples collectedatthe Butrint lagoon, 45 samples were testedpositive for *V. parahaemolyticus*(90% positivity). 15/45 samples harbored the virulence factor TDH, while the TRH factor was not emphasized at all.(Shehu et al., 2014)

The real-time PCR is used in the diagnosis of a single gene and provides a rapid, sensitive, and highly specific means for the detection of*Vibrio parahaemolyticus*. In recent years scientists have developed real-time PCR-based assays that target one or more of the genes simultaneously in a multiplexed format. However, real-time PCR assays in which fluorescently labeled TaqMan probes are used to provide greater specificity than conventional or SYBR green assays. Successful real-time PCR for *V. parahaemolyticus* using a TaqMan probe has targeted a single locus *tdh*or dual loci in a multiplexed format *tdh* and *toxR*. (Blackstone et al., 2003; Iijima et al., 2004)

2. Material and Methods

This study was conducted based on samples taken under the monitoring plan of bivalve molluscs and their production areas for wthin a period of one year. Samples were taken in three production areas: Butrint lagoon, Shëngjin bay and cape of Rodon.

Table 1. Geographical coordinates of sampling points for the production area of *M. galloprovincialis*.

Location	*Site*	*Latitude*	*Longitude*
Butrint lagoon	PK1	39.80614	20.01609
	PK5	39.78816	20.02051
	PK7	39.75483	20.03181
Shëngjin bay	P1	41.7814421	19.5920895
	P2	41.7845713	19.5913431
Cape of Rodon	PK1	41.57855	19.4992
	PK2	41.57653	19.50655

2.1. Sample preparation, bacterial strains and growth media

After scrubbing, washing, and rinsing with sterile distilled water 10 individual live bivalve mollusks were shucked and homogenized in a stomacher for 2 min. Ten-gram portion of the homogenate (one sample) were inoculated into 225± 5 ml ASPW and subject to primary enrichment at 41.5± 1 °C for 6± 1 h. One milliliter aliquots of each enrichment were subject to secondary enrichment in 10± 0.5 ml fresh ASPW at 41.5± 1 °C for 18± 3 h. Following primary and secondary enrichment, one mL of each enrichment broth was streaked onto the surface of TCBS and HiCrome Vibrio agar. TCBS plates and HiCrome Vibrio agar were incubated at 37± 1 °C for 24± 3 h. Subsequent cultures were checked visually for purity and subject to oxidase tests, urease test, and sugar fermentation. Suspected isolates of Vibrio spp. were codified and stored at -80 °C with glycerol.

2.2. DNA extraction

Vibrio *spp*.DNA were extracted from a single colony by using the composition and protocol for alkaline

polyethylene glycolReagent. We combined 60 g PEG 200 reagent with 0.93 mL 2 M KOH, and 39 mL DI water. We executed the three steps protocol as follows: First, mix 1-10 mg bacterial cell or 1-10 µL bacterial suspension with 0.1 mL of the reagent. The amount of alkaline PEG reagent used to process was 10 times higher than the sample volume; Second, lyse sample by incubation in the reagent for 15 min at room temperature. Although 15 min was preferred most samplescould be processed after 1-3 min.Finally,the lysates were mixed and transferred in 1-5 µL aliquot directly into a 20µL PCR mixture. The preferred amount of the DNA for a 20 µL PCR was considered from 1 ng to 20 ng(measured with spectrometry, data not shown).

2.3. Selection of oligonucleotide primers and probes

The primers and probes used in this study are shown in Table 2

Table 2. Target genes and oligonucleotide primers and probes used for simplex PCR detection of total *V. parahaemolyticus.*

Target gene	*Primer or probe*	*Sequence(5'-3')*	*Reference*
toxR	VpToxR (FW)	GAACCAGAAGCGCCAGTAGT	(Powell et al., 2012)
	VpToxR (REV)	AAACAGCAGTACGCAAATCG	
	VpToxR Probe	[FAM]-TCACAGCAGAAGCCACAGGTGC-[TAMRA]	

2.1. Real-time PCR

Amplification was performed using the qPCRStratagene Mx3000P (Agilent Technologies). Aliquots of 1uL of extracted DNA were added to a mastermix containing 10 µLForget-Me-Not qPCR Master Mix, 1 µL primer (0.1 µM) forward and reverse and 1 µL probe (0.5 µM), 1 µLRox reference dye (30 nM) and 3 µLnuclease-free water. All samples were subjected to real-time PCR according to the cycling parameters described in annex D of ISO 21872-1:2017. All samples were subjected to PCR according to the cycling parameters included an initial denaturation step of 95°C for 10 min, followed by 45 cycles of amplification. Each cycle consisted of template DNA denaturation at 95°C for 15 s, primer annealing at 60°C for 60 s, and primer extension at 72°C for 30 s. The increase in fluorescence for FAM channel was measured and recorded during the annealing step of each cycle.(Anonymous, 2017)

3. Results and Discussion

Out of 60 samples screened, 4 samples (6.7%) harbored *V. parahaemolyticus* and the organism was isolated from samples collected from twoproduction zone under study during biweekly sampling.

Table 3. Summary of real-time PCR detection of *V. parahaemolyticus* in natural live bivalve molluscs samples.

Locus	*Location*	*No. tested*	*No. poitive*	**%**
toxR gene of Vibrio parahaemolyticus	Butrint lagoon	36	3	8.3
	Shëngjinbay	14	1	7.1
	Cape of Rodon	10	0	0.0

No, number; %, percengage

Bacterial isolates that exhibited similar biochemical reactions to that of *V. parahaemolyticus* for oxidase, catalase, sugar fermentation, and chromogenic properties were selected for PCR investigation. Out of 84 suspected isolates were subjected to *toxR* gene amplification and 4 isolates that produced Ct value less than 45 were confirmed as *V. parahaemolyticus*. The presence of *V. parahaemolyticus*atButrint lagoon

was confirmed in 3 out of 36 shellfish samples (8.3%), 1 out of 14cape of Rodon (7.1%). No presence of *V. parahaemolyticus* was found at Shëngjin bay (0/10).

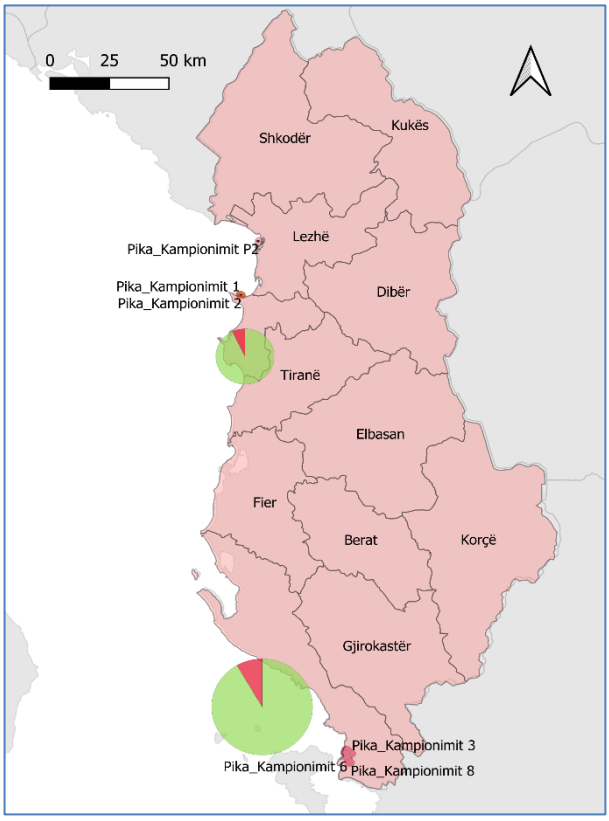

Figure 1. Sampling location in the Shëngjin bay, cape of Rodon and Burint lagoon. Geographical location of genotype *toxR* of *V. parahaemolyticus*. Map visualization of geospatial data with pie charts. (Maci et al. 2020)

In this study, *V. parahaemolyticus* was detected during the summer season (July) in the environments with higher temperatures located in two production zone(Butrint lagoon and cape of Rodon) as shown in Figure 2

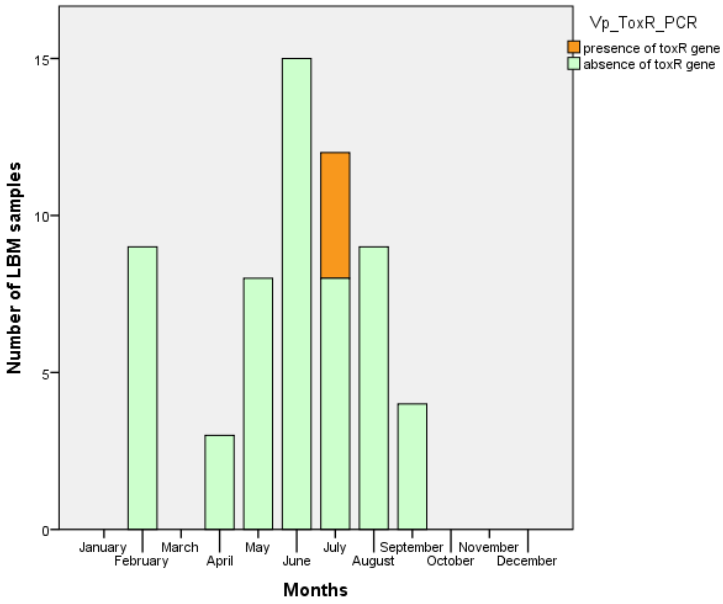

Figure 2. Seasonal distribution of Vibrio parahaemolyticus in Mylitusgalloprovincialis.

4. Conclusions

In this study, we optimized extraction protocol with alkaline PEG as well as singleplexTaqMan probe-based real-time PCR assay for detection of toxR gene of V. parahaemolyticus in *Mylitusgalloprovincialis*. All the strains that we have isolate originate from the environment. We targeted *toxR* gene in order to obtain comprehensive detection of pathogenic and nonpathogenic *V. parahaemolyticus* in live bivalve mollusks. The primers used for the *toxR* DNA were equal to primers previously reported by other studies and all the strains were isolate bothButrint lagoonand cape of Rodon.

The results of our study indicate that our simple extraction protocol with alkaline PEG and real-time TaqMan probe-based PCR system can successfully detect *toxR* gene in natural shellfish samples.This assay can provide information about the presence of total *V. parahaemolyticus*tha can be clinical or environmental isolates. Rapid detection like real-time PCR should provide the aquaculture industry with early warning of potential health risks associated with potentially contaminated shellfish and allow appropriate measures to prevent disease outbreaks to be swiftly undertaken. The use of an assay such as this assay should be beneficial to both industry and consumer health.

5. Acknowledgements

This work was supported byDepartment of Food Microbiology, Food Safety and Veterinary Institute (FSVI) in collaboration with Department of Veterinary Public Health, Faculty of Veterinary Medicine (FVM) in Albania.

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Special Issue of the Conference: Agriculture a Life Science with Roots in Applied Biology; 3-4 Dec. 2020. (Accepted for publication 11.01.2020)
***ISSN**: 2218-2020, © Agricultural University of Tirana*

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