Determination of *Vibrio parahaemolyticus* in seafoods using direct plate counting, quantitative loop-mediated isothermal amplification and propidium monoazide-qLAMP

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Abstract: One of the most challenging aspects in culture independent methods for foodborne pathogens' detection is discrimination of dead and live microorganisms. This study aimed to determine the *Vibrio parahaemolyticus* in seafoods via direct plate counting (DPC) and *toxR*-based quantitative loop-mediated isothermal amplification (qLAMP) and to discriminate dead and live cells using propidium monoazide (PMA)-qLAMP. A total of 200 samples including finfishes (n= 100) and shrimps (n= 100), representing the Mediterranean, Black and Aegean sea were collected from supermarkets and fish markets of Konya-Turkey. qLAMP was performed in a Real-Time Turbidimeter and the time threshold (*tt*) values were yielded in 60 minutes. On DPC, the colonies grown on TCBS Agar was further confirmed by conventional PCR based from *gyrB*1 gene of *Vibrio* spp. and *toxR* gene of *V. parahaemolyticus*. Virulence property of the isolates were determined by *tdh* based qLAMP. The detection limit of the qLAMP was 1.2×10^4 CFU/g in artificially contaminated seafoods. DPC, qLAMP and PMA-qLAMP detected *V. parahaemolyticus* in 8 (4%), 12 (6%) and 12 (6%) samples, respectively. The CFUs of *V. parahaemolyticus* detected in qLAMP ($5.96 \pm 0.10 \log_{10}$ CFU/ml) and PMA-qLAMP ($4.71 \pm 0.13 \log_{10}$ CFU/ml) were higher than those of DPC ($1.99 \pm 0.44 \log_{10}$ CFU/ml) ($P < 0.05$). The mean *tt* reduction in PMA treated samples was $1.25 \pm 0.38 \log_{10}$ CFU/sample. The *tdh* gene was not detected in any of the isolates. In conclusion, the *toxR*-based PMA-qLAMP method could be an alternative to be used more widely and effective assay for the quantification of live *V. parahaemolyticus* in seafoods.

Keywords: DPC, propidium monoazide, qLAMP, VBNC, *V. parahaemolyticus*

Deniz ürünlerinde *Vibrio parahaemolyticus*'un direkt kültür yöntemi, kantitatif ilmiğe dayalı izotermal amplifikasyon ve propidium monoazide-qLAMP ile belirlenmesi

Özet: Gıda kaynaklı patojenlerin kültür bağımsız yöntemlerle tespitinde en zorlu hususlardan biri ölü ve canlı mikroorganizmaların ayırt edilmesidir. Bu çalışmada, deniz ürünlerinde *V. parahaemolyticus* varlığının direkt kültür yöntemi (DPC) ve *toxR* bazlı kantitatif ilmiğe dayalı izotermal amplifikasyon (qLAMP) ve canlı-ölü hücre ayrımı için propidium monoazide (PMA)-qLAMP yoluyla belirlenmesi amaçlandı. Akdeniz, Karadeniz ve Ege denizlerini temsil eden balık (n= 100) ve karides (n= 100) olmak üzere toplam 200 örnek, Konya-Türkiye'de bulunan süpermarketler ve balık hallerinden toplandı. Real-Time Turbidimetrede gerçekleştirilen qLAMP reaksiyonunda time threshold (*tt*) değerleri, 60 dakika içinde elde edildi. DPC metodunda TCBS Agar'da gelişen kolonilerde *Vibrio* spp.'nin doğrulanması için *gyrB*1 genini, *V. parahaemolyticus*'un doğrulanması için de *toxR* genini amplifiye eden konvansiyonel PCR yöntemi kullanıldı. İzolatların virülans özelliğinin belirlenmesinde *tdh* bazlı qLAMP kullanıldı. qLAMP'ın deneysel olarak kontamine edilen deniz ürünlerinde tespit limiti 1.2×10^4 CFU/g olarak belirlendi. DPC, qLAMP ve PMA-qLAMP yöntemleri ile sırasıyla 8 (%4), 12 (%6) ve 12 (%6) örnekte *V. parahaemolyticus* tespit edildi. qLAMP ($5.96 \pm 0.10 \log_{10}$ KOB/ml) ve PMA-qLAMP ($4.71 \pm 0.13 \log_{10}$ KOB/ml) yöntemlerinde tespit edilen *V. parahaemolyticus* sayılarının DPC yöntemine göre ($1.99 \pm 0.44 \log_{10}$ KOB/ml) daha yüksek olduğu gözlemlendi ($P < 0.05$). PMA uygulanan örneklerde ortalama *tt* azalması, $1.25 \pm 0.38 \log_{10}$ KOB/örnek olarak tespit edildi. İzolatların hiçbirinde *tdh* geni tespit edilmedi. Sonuç olarak; *toxR*-bazlı PMA-qLAMP yönteminin, deniz ürünlerinde canlı *V. parahaemolyticus*'un kantitatif tespitinde daha yaygın ve etkin kullanılabilecek alternatif bir yöntem olabileceği düşünülmektedir.

Anahtar sözcükler: DPC, propidium monoazide, qLAMP, VBNC, *V. parahaemolyticus*.

Introduction

Turkey as a country with sea on three sides has a significant potential on fishing, conribute to the quality of human nutrition, provide raw materials for industry and for export. According to the data of Turkish Statistical Institue (36), total aquaculture production and the amount of export are 630, 820 and 177.539 tons, respectively.

Vibrio spp. are known as the most common cause of seafood-borne infection and intoxication cases worldwide. Seafood-borne vibriosis is commonly caused by the consumption of fish and shellfish with inadequate heat treatment (24). Some species (e.g. *V. anguillarum* and *V. tapetis*) are pathogenic for aquatic animals, whereas other species (e.g. *V. cholerae*) are only pathogenic for humans. In addition, species such as *V. parahaemolyticus* and *V. vulnificus* are considered pathogenic for both human and aquatic animals (12). Vibriosis has caused pandemics in Japan, the United States, Korea, Denmark, the Philippines and China (2, 5, 23, 24).

Intoxications caused by *V. parahaemolyticus* are characterised by severe cramping, abdominal pain, vomiting and bloody diarrhoea. The microorganism is naturally found in marine sources and has been isolated from cod, sardines, mackerel, broodstock, oysters, octopus, shrimps, crabs, lobsters, crayfish, sea bass and oysters (30).

The discrimination between dead and viable forms of microorganisms in food is important in determining the disease-making potential. A large number of microorganisms transform to a viable but nonculturable (VBNC) form under adverse environmental conditions, such as starvation, low temperature or pH conditions that are unsuitable for reproduction (10). Foodborne VBNC pathogens may pose a risk for human health, as they can be cultured when stress conditions are eliminated (3). In the same way, cells that do not exhibit virulence in the VBNC form exhibit re-virulence under appropriate conditions (9).

The culture-dependent methods applied to isolate and identify microorganisms are aimed at determining the viable and culturable forms. The DNA-based and immunological methods used in culture-independent isolation and identification carry out the detection of cells that have not lost their DNA integrity despite cellular damage. For this reason, DNA-based microbiological methods do not give an idea of whether the target cells are dead or alive. Certain groups of chemicals, such as ethidium monoazide (EMA) and propidium monoazide (PMA), provide the ability to discriminate DNA from dead cells with high selectivity as penetrating in disrupted regions of cell membrane integrity (19). Therefore, these chemicals allow a highly selective discrimination in a mixed population of dead and living cells.

The recently developed loop-mediated isothermal amplification (LAMP) method is a DNA-based, rapid and specific molecular method used in the detection of many microorganisms as well as in *Vibrio* species. It is a rapid method that does not only require professional laboratory equipment or advanced technical skill but also achieves reliable results (17, 20). Unlike the PCR method, LAMP occurs at isothermal temperatures. Positive samples are visible in the LAMP method, characterised by the formation of a white precipitate. The source of the white precipitate is magnesium pyrophosphate, which is distinguishable by the naked eye (35). It is also possible to visualise the LAMP products by agarose gel electrophoresis via the addition of fluorescence-specific dyes or by quantitative measurements based on turbidity or fluorescence. The LAMP method has been used in many microorganisms, including *Vibrio* species (14, 15, 22, 25, 37- 39).

This study aimed to determine the *V. parahaemolyticus* in seafoods via DPC and *toxR*-based qLAMP and PMA-qLAMP to discriminate dead and live cells.

Material and Methods

Sample collection: Finfish and shrimp samples were collected during spring and summer seasons (between March 2016 and September 2016) due to the higher incidence of the *V. parahaemolyticus* as indicated by many researchers (8, 18, 21, 28)

A total of 200 samples including frozen finfishes (n= 50), frozen shrimps (n= 50), fresh finfishes (n= 50) and fresh shrimps (n= 50) (representing the Mediterranean, Black sea and Aegean sea of Turkey) were collected from the supermarkets and fish markets in Konya, Turkey at independent time arrivals. Samples were transferred to the laboratory under cold chain and analyzed within 2 hours.

Direct plate counting (DPC): The shrimp and finfish samples were scrubbed according to American Public Health Association Guidelines (APHA) (1). Twenty five g of sample and 225 ml of alkaline saline peptone water (ASPW, Liofilchem, 610377) were homogenized into a sterile stomacher bag (VWR, 432-3119) using a stomacher lab blender (Interscience, France). The homogenate was 10 fold diluted with phosphate buffered saline (PBS, Sigma, P5119) and then was streaked onto thiosulfate citrate bile sucrose (TCBS, Merck, 110263). TCBS plates were incubated for 24-48 h at 37°C. Suspicious *Vibrio* colonies grown on TCBS Agar were chosen and streaked onto nutrient agar (Merck 105450) supplemented with 3% NaCl (w/v). Then the isolates were tested for oxidase, catalase, Gram staining, motility test under microscope, growth in 0% and 6% NaCl. Conventional PCR assay based on primers designed by Teh et al. (33) from *gyrB*1 gene region (Table 1) of *Vibrio*

Table 1. The primers used in the study.

Primer	Sequence (5' to 3')	Reference
toxR-FIP	TGAGATTCCGCAGGGTTTGTAATTATTTTTGGCACTATTACTACCG	(6)
toxR-BIP	GTTCCGTCAGATTGGTGAGTATCTAGAAGGCAACCAGTTGTT	
toxR-F3	TTGGATTCCACGCGTTAT	
toxR-B3	CGTTCAATGCACTGCTCA	
toxR-Loop	AGAACGTACCAGTGATGACACC	
tdh-FIP	GTACCTGACGTTGTGAATACTGATTGTCTCTGACTTTTGGACAAAC	(38)
tdh-BIP	TGACATCCTACATGACTGTGAACACTTATAGCCAGACACCGC	
tdh-F3	AGATATTGTTTGTTGTTCGAGAT	
tdh-B3	AACACAGCAGAATGACCG	
tdh-LF	GTACGGTTTTCTTTTTACATTACG	
tdh-LB	AAGACTATACAATGGCAGCG	
*gyr*B1	AGCCAAACNAAAGAYAARYT	(33)
*gyr*B2	CGYARYTTRTCYGGRTTRTRYTC	

spp. and *toxR* (F3 and B3 primers of LAMP primers) region of *V. parahaemolyticus* (6) was carried out for confirmation of the isolates.

PMA treatment: Propidium monoazide (Biotium, 40013), was dissolved in 98 μL dH$_2$O and then stored at -20°C. The optimal PMA used in the study was 50 μM, which was obtained in the previous study (34) by inoculation experiments of pure *V. parahaemolyticus* cells to seafoods. Following addition of PMA to the samples, penetration was considered to be occured at room temperature and in the dark after 10 minutes of incubation. The samples were then exposed to a PMA-Lite LED Photolysis Device (Biotium 89427-066) for 15 min to allow photo-activation. Photo-activated cells were then subjected to DNA extraction procedures.

DNA extraction: The samples homogenated in ASPW were transferred as 200 μl into two sterile microsantrifuge tubes. A commercial DNA extraction kit (Qiagen DNEasy Blood and Tissue Kit, Lot No: 148019696) was used for DNA extraction in PMA treated and non-treated samples.

qLAMP and PMA-qLAMP: The LAMP primers of the *toxR* (6) gene to determine *V. parahaemolyticus* at species level, and the *tdh* gene (38) to determine the presence of the pathogenic gene region, were used (Table 1.)

A LAMP reaction mixture was prepared from spiked samples of *V. parahaemolyticus,* shrimp and finfish homogenates as serial dilutions (10^8 to 10^0 CFU/ml) for optimization of the qLAMP reaction in a Real Time Turbidimeter (MVL300 Loopamp Realtime Turbidimeter LA-500, Eiken, Japan). Quantitative bacterial counts were determined according to the linear curve formula obtained from serial dilutions of *V. parahaemolyticus* ATCC 17802 inoculated finfish and shrimp homogenate versus time

threshold (*tt*) value. The linear equation for the curve is shown in Figure 1. All experiments were run at least as triplicate and negative control samples were included in each run.

The qLAMP reaction mixture was consisted of F3 and B3 primers (0.5 μM), FIP and BIP primers (0.8 μM), Loop Primers (0.4 μM), Reaction Buffer (10X) (2.5 μl), MgSO$_4$ (6 mM), Bst DNA Polymerase (8U/μL, New England Biolab) and the target DNA from PMA treated and non-treated samples (2 μl).

Statistical analyses: Time threshold (*tt*) values of qLAMP, PMA-qLAMP and colony counts obtained from DPC of the positive isolates were converted to logarithmic values. Standart linear curve for quantification of *V. parahaemolyticus* were plotted according to the logaritmic counts of serial bacterial dilutions versus *tt* values. The mean logarithmic values were compared with the paired samples T test in SPSS package program version 21.00.

Results

Standard curve and detection sensitivity: The detection limit of the *toxR* based qLAMP reaction was 1.2×10^4 CFU/g in artificially contaminated samples without enrichment in independent triplicate experiments (Figure 1).

Direct plate counting: According to DPC, 36 (18%) samples were isolated as *Vibrio* spp.. Culturally isolated *Vibrio* spp. colonies were confirmed by a conventional PCR assay based on primers designed by Teh et al. (33) from the *gyr*B1 gene region of *Vibrio* spp.. Eight (4%) of the samples were identified as *V. parahaemolyticus* according to biochemical tests then confirmed by a *toxR*-based (F3 and B3 primers of LAMP primers) conventional PCR assay (5).
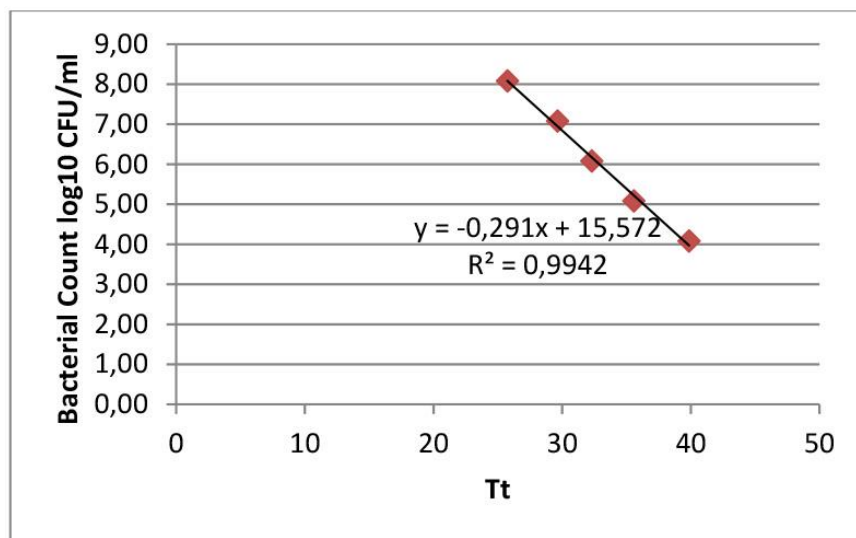
Figure 1. Standart curve of linear relationship between *tt* values of Log CFU/qLAMP of *V. parahaemolyticus* in artificially contaminated samples.

$$y = -0{,}291x + 15{,}572$$
$$R^2 = 0{,}9942$$
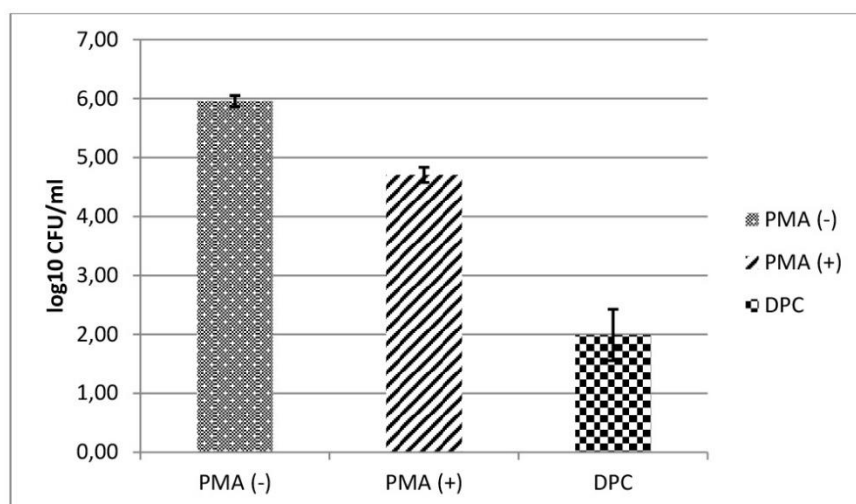
Figure 2. Logarithmic mean values of qLAMP, PMA-qLAMP and DPC.

Mean: ± SE log10 CFU/ml, PMA (-): 5.96±0.10, PMA (+): 4.71±0.13, DPC: 1.99±0.44.

Table 2. Rates of *V. parahaemolyticus* positive samples using DPC, qLAMP and PMA-qLAMP.

| Sample	DPC		qLAMP		PMA-qLAMP	
	n	**%**	**n**	**%**	**n**	**%**
Fresh finfish (n= 50)	5	10	6	12	6	12
Frozen finfish (n= 50)	ND		1	2	1	2
Fresh shrimp (n= 50)	2	4	3	6	3	6
Frozen finfish (n= 50)	1	2	2	4	2	4
Total (n= 200)	8	4	12	6	12	6

ND: Not detected.

In the comparison of logarithmic bacterial counts using DPC, qLAMP and PMA-qLAMP methods using a paired sample t-test, each methods were statistically different from the other (P<0.05). Descriptive statistics of bacterial counts are shown in Figure 2.

Comparison of bacterial counts using qLAMP and PMA-qLAMP: The *tt* values were obtained from the *V. parahaemolyticus*-positive samples (n=12, 6%) (Table 2), using qLAMP and PMA-qLAMP and their graphs are shown in Figure 3. The mean signal reduction in the optimum PMA dose applied samples was 1.25±0.38 $\log_{10}$ CFU/sample. Bacterial counts of fresh and frozen samples detected using DPC, qLAMP and PMA-qLAMP were not statistically different (P>0.05).

None of the samples identified as *V. parahaemolyticus* were found to have the *tdh* gene according to the qLAMP reaction.
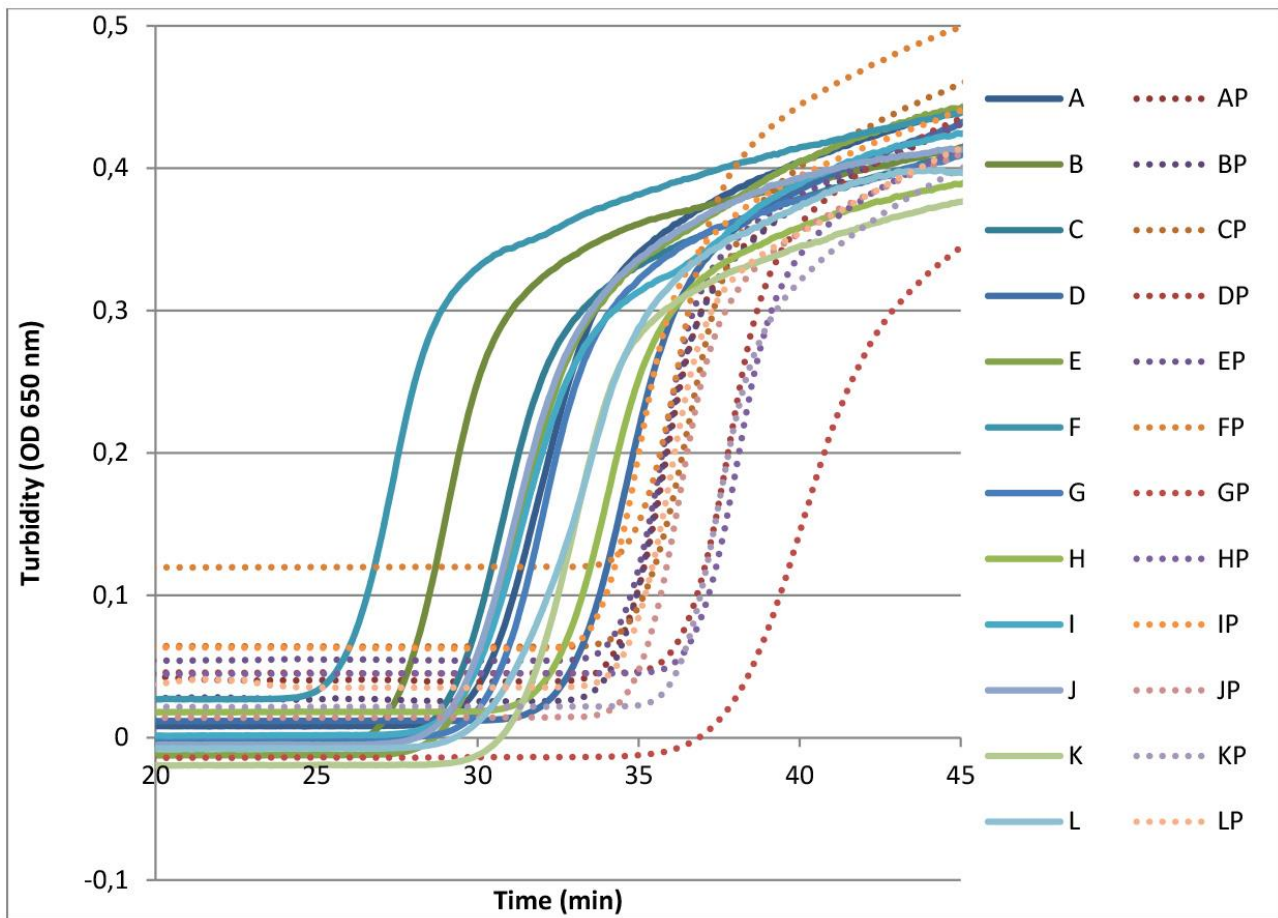
Figure 3. A representative graph of the positive samples generated using real-time turbidimeter.
A-L: qLAMP *tt* values, AP-LP: PMA-qLAMP *tt* values.

Discussion and Conclusion

There are many studies on detection of *V. parahaemolyticus* in fresh and frozen seafood (27-29). Shanthini and Kumar (29) reported that crustaceans, gastropods, cephalopods and finfish samples were contaminated with *V. parahaemolyticus* by the most probable number (MPN) method at the rates of 62.50%, 25.50%, 3% and 51.88%, respectively in Tuticorin, India.

Sudha et al. (31) isolated *V. parahaemolyticus* from 45.1% of the 182 finfish samples collected from markets in southern India. Rosec et al. (28) isolated *V. parahaemolyticus* in 21 of 69 isolates (30.4%) in 2008 and 14 (32.6%) in 42 samples. Researchers stated that none of the isolates were found to contain the *tdh* gene (28). Robert-Pillot et al. (27) found that 34% (n= 58) of the 167 frozen marine products consumed in France were contaminated with *Vibrio* spp., and 31% of them were identified as *V. parahaemolyticus*. Unlike our study, researchers observed the presence of the *tdh* gene in 25% of *V. parahaemolyticus*-positive samples.

As a result of the paired sample test of the obtained data, the highest bacterial counts were observed using the qLAMP without PMA treatment (P<0.05) due to the overvaluation of the *tt* values without viable dead cell discrimination. There are a number of similar studies supporting that molecular methods are more sensitive than classical culture methods (4, 16, 28, 32). Furthermore, *tt* reduction in PMA-qLAMP was in agreement with that of similar studies using LAMP or PCR methods (7, 11, 13, 25, 26, 38). In this context, some of the bacterial cells were estimated to have compromised cell membranes or to have lost their culturability and pass through the VBNC form although they might have a contact membrane.

Garcia-Cayuela et al. (13) used the PMA-qPCR method to quantify living cells by mixed cultures of lactic acid bacteria (*Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus casei* subsp. *casei*, *Streptococcus thermophilus*) and *Bifidobacterium lactis* in fermented milk products. The decrease in the number of live microorganisms in the fermented milk stored at 4°C was observed with PMA-qPCR and a classical culture method. The results of the two methods were similar, and a linear correlation was observed (0.995). Rawsthorne et al. (25) used PMA as an intercalator in their investigations aimed to discriminate between live and inactive *Bacillus subtilis* spores. The

researchers observed a statistically significant difference between PMA-qPCR and qPCR when they identified survivors from thermal inactivation stress. Chen et al. (7) used PMA and LAMP methods in combination to determine the number of live *Salmonella*. Microorganism inactivation was verified by the PMA-LAMP method in suspensions containing heat-inactivated microorganisms at concentrations of up to 10^8 CFU/ml in experimentally contaminated melon, spinach and tomato samples. The researchers found that PMA-LAMP provides similar results as PMA-qPCR and is 100-fold more sensitive than PMA-PCR.

In the study, *V. parahaemolyticus*, an important public health concern, was detected in both frozen and fresh sea products. Using the LAMP method combined with PMA could be an alternative on determination of the quantitative ratios of live microorganisms and the potential risk can be evaluated more realistically in a shorter time and at a lower cost than the other molecular methods. From this point of view, it can be assumed that this method can be applied more widely and effectively within the scope of point-of-care testing in food microbiology.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **American Public Health Association (APHA)** (1992): Standard Methods for the Examination of Water and Waste Water. American Public Health Association, Washington DC, USA.
2. **Austin B** (2010): *Vibrios as causal agents of zoonoses.* Vet Microbiol, **140**, 310-317.
3. **Bates TC, Oliver JD** (2004): *The viable but nonculturable state of Kanagawa positive and negative strains of Vibrio parahaemolyticus.* J Microbiol, **42**, 74-79.
4. **Cai T, Jiang L, Yang C, et al** (2006): *Application of real-time PCR for quantitative detection of Vibrio parahaemolyticus from seafood in eastern China.* FEMS Immunol Med Microbiol, **46**, 180-186.
5. **Chao G, Jiao X, Zhou X, et al** (2009): *Distribution, prevalence, molecular typing, and virulence of Vibrio parahaemolyticus isolated from different sources in coastal province Jiangsu, China.* Food Control, **20**, 907-912.
6. **Chen S, Ge B** (2010): *Development of a toxR-based loop-mediated isothermal amplification assay for detecting Vibrio parahaemolyticus.* BMC Microbiol, **10**, 41.
7. **Chen S, Wang F, Beaulieu JC, et al** (2011): *Rapid detection of viable Salmonellae in produce by coupling propidium monoazide with loop-mediated isothermal amplification (PMA-LAMP).* Appl Environ Microbiol, **77**, 4008-4016.
8. **DePaola A, Ulaszek J, Kaysner CA, et al** (2003): *Molecular, serological, and virulence characteristics of Vibrio parahaemolyticus isolated from environmental, food, and clinical sources in North America and Asia.* Appl Environ Microbiol, **69**, 3999-4005.
9. **Du M, Chen J, Zhang X, et al** (2007): *Retention of virulence in a viable but nonculturable Edwardsiella tarda isolate.* Appl Environ Microbiol, **73**, 1349-1354.
10. **Ducret A, Chabalier M, Dukan S** (2014): *Characterization and resuscitation of 'non-culturable' cells of Legionella pneumophila.* BMC Microbiol, **14**, 3.
11. **Fang J, Wu Y, Qu D, et al** (2018): *Propidium monoazide real-time loop-mediated isothermal amplification for specific visualization of viable Salmonella in food.* Lett Appl Microbiol, **67**, 79-88.
12. **Farmer JJ** (2005): Genus I. Vibrio pacini 1854. 494-546. In: GM Garrity (Ed), Bergey's Manual of Systematic Bacteriology. Springer, New York.
13. **García-Cayuela T, Tabasco R, Peláez C, et al** (2009): *Simultaneous detection and enumeration of viable lactic acid bacteria and bifidobacteria in fermented milk by using propidium monoazide and real-time PCR.* Int Dairy J, **19**, 405-409.
14. **Han F, Ge B** (2010): *Quantitative detection of Vibrio vulnificus in raw oysters by real-time loop-mediated isothermal amplification.* Int J Food Microbiol, **142**, 60-66.
15. **Jones JL, Hara-Kudo Y, Krantz, JA, et al** (2012): *Comparison of molecular detection methods for Vibrio parahaemolyticus and Vibrio vulnificus.* Food Microbiol, **30**, 105-111.
16. **Kokkinos PA, Ziros PG, Bellou M, et al** (2014): *Loop-mediated isothermal amplification (LAMP) for the detection of Salmonella in food.* Food Anal Methods, **7**, 512-526.
17. **Nagamine K, Hase T, Notomi T** (2002): *Accelerated reaction by loop-mediated isothermal amplification using loop primers.* Mol Cell Probes, **16**, 223-229.
18. **Nigro OD, Hou A, Vithanage G, et al** (2011). *Temporal and spatial variability in culturable pathogenic Vibrio spp. in lake Pontchartrain, Louisiana, following Hurricanes Katrina and Rita.* Appl Environ Microbiol, **77**, 5384-5393.
19. **Nocker A, Cheung CY, Camper AK** (2006): *Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells.* J Microbiol Methods, **67**, 310-320.
20. **Notomi T, Okayama H, Masubuchi H, et al** (2000): *Loop-mediated isothermal amplification of DNA.* Nucleic Acids Res, **28**, e63.
21. **Oberbeckmann S, Wichels A, Wiltshire KH, et al** (2011): *Occurrence of Vibrio parahaemolyticus and Vibrio*

alginolyticus in the German Bight over a seasonal cycle. Antonie Van Leeuwenhoek, **100**, 291-307.

22. **Okada K, Chantaroj S, Taniguchi T, et al** (2010): *A rapid, simple, and sensitive loop-mediated isothermal amplification method to detect toxigenic Vibrio cholerae in rectal swab samples.* Diagn Microbiol Infect Dis, **66**, 135-139.

23. **Oliver JD, Kaper JB** (2007): Vibrio Species. 343-378. In: MP Doyle, LR Beuchat (Eds), Food Microbiology: Fundamentals and Frontiers. ASM Press, Washington DC.

24. **Peterson KM** (1999): Molecular pathogenesis of Vibrio infections. 157-190. In: JW Carry, JE Linz, D Bhatnagar (Eds), Microbial Foodborne Diseases: Mechanisms of Pathogenesis and Toxin Synthesis. Techomic Publishing, USA.

25. **Rawsthorne H, Dock CN, Jaykus LA** (2009): *PCR-based method using propidium monoazide to distinguish viable from nonviable Bacillus subtilis spores.* Appl Environ Microbiol, **75**, 2936-2939.

26. **Ren CH, Hu CQ, Luo P, et al** (2009): *Sensitive and rapid identification of Vibrio vulnificus by loop-mediated isothermal amplification.* Microbiol Res, **164**, 514-521.

27. **Robert-Pillot A, Copin S, Himber C, et al** (2014): *Occurrence of the three major Vibrio species pathogenic for human in seafood products consumed in France using real-time PCR.* Int J Food Microbiol, **189**, 75-81.

28. **Rosec JP, Causse V, Cruz B, et al** (2012): *The international standard ISO/TS 21872–1 to study the occurence of total and pathogenic Vibrio parahaemolyticus and Vibrio cholerae in seafood: ITS improvement by use of a chromogenic medium and PCR.* Int J Food Microbiol, **157**, 189-194.

29. **Shanthini CF, Kumar PA, Patterson J** (2004): *Incidence and antibiotic susceptibility of Vibrio parahaemolyticus from sea foods of Tuticorin.* Indian J Fish, **51**, 43-47.

30. **Su YC, Liu C** (2007): *Vibrio parahaemolyticus: a concern of seafood safety.* Food Microbiol, **24**, 549-558.

31. **Sudha S, Divya PS, Francis B, et al** (2012): *Prevalence and distribution of Vibrio parahaemolyticus in finfish from Cochin (south India).* Vet Ital, **48**, 269-281.

32. **Techathuvanan C, Draughon FA, D'Souza DH** (2011): *Comparison of reverse transcriptase PCR, reverse transcriptase loop-mediated isothermal amplification, and culture-based assays for Salmonella detection from pork processing environments.* J Food Prot, **74**, 294-301.

33. **Teh CSJ, Chua KH, Thong KL** (2010): *Simultaneous differential detection of human pathogenic and nonpathogenic Vibrio species using a multiplex PCR based on gyrB and pntA genes.* J Appl Microbiol, **108**, 1940-1945.

34. **Telli AE, Dogruer Y** (2019): *Discrimination of viable and dead Vibrio parahaemolyticus subjected to low temperatures using propidium monoazide- quantitative loop mediated isothermal amplification (PMA-qLAMP) and PMA-qPCR.* Microb Pathog, **132**,109-116.

35. **Tomita N, Mori Y, Kanda H, et al** (2008): *Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products.* Nat Protoc, **3**, 877-882.

36. **Turkish Statistical Institue (2019):** Fishery Statistics. Available at http://www.tuik.gov.tr/PreTablo.do?alt_id=1005. (Accessed March 30, 2019).

37. **Wan C, Yang Y, Xu H, et al** (2012): *Development of a propidium monoazide treatment combined with loop-mediated isothermal amplification (PMA-LAMP) assay for rapid detection of viable Listeria monocytogenes.* Int J Food Sci Technol, **47**, 2460-2467.

38. **Yamazaki W, Ishibashi M, Kawahara R, et al** (2008): *Development of a loop-mediated isothermal amplification assay for sensitive and rapid detection of Vibrio parahaemolyticus.* BMC Microbiol, **8**, 163.

39. **Yamazaki W, Kumeda Y, Misawa N, et al** (2010): *Development of a loop-mediated isothermal amplification assay for sensitive and rapid detection of the tdh and trh genes of Vibrio parahaemolyticus and related Vibrio species.* Appl Environ Microbiol, **76**, 820-828.