Simultaneous Isolation and Enumeration of Virulent Vibrio Cholerae and Vibrio Vulnificus using an Advanced MPN-PCR Method

Jae-Hwa Lee
Pukyong National University
Seul-Ki Park
Pukyong National University
Fazlurrahman Khan
Pukyong National University
Du-Min Jo
Pukyong National University
Do-ha Lee
Pukyong National University
Min-Gyun Kang
Pukyong National University
Young-Mog Kim (ymkim@pknu.ac.kr)
Pukyong National University https://orcid.org/0000-0002-2465-8013

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Abstract

Vibrio cholerae and *Vibrio vulnificus* are one of the critical foodborne pathogens that need to be intensively controlled their infection as a result of the intake and distribution of seafood, especially raw oysters. For this reason, various methods have already been developed for the detection and enumeration of these bacteria. The most probable number (MPN)-PCR (polymerase chain reaction) method is commonly used with the selective-differential medium for the efficiency and convenience of cell enumeration. One of the most frequently used for the detection of *Vibrio* spp. is Thiosulfate-Citrate-Bile salts-Sucrose (TCBS) agar. But this selective-differential medium can fail to distinguish between *V. cholerae*, *V. vulnificus*, and *Vibrio alginolyticus.* For this reason, the conventional MPN-PCR method with TCBS medium for the detection of *Vibrio* spp. has a problem that processing PCR to the two-times. This study suggests a simple and minimized detection method using one-time PCR and non-NaCl Luria-Bertani (LB-0) medium culture. This detection method is based on the difference in salt requirement between *V. cholerae* and *V. vulnificus*. Employing the developed methodology, the simultaneous cell enumeration of *V. cholerae* and *V. vulnificus* can be possible at a low cost. Furthermore, this study proposes a new specific primer to detect virulence-related genes from *V. cholerae* and *V. vulnificus*. This advanced MPN-PCR method was verified using bioaccumulated pacific oysters (*Crassostrea gigas*) by *V. cholerae* and *V. vulnificus*.

1. Introduction

Various *Vibrio* spp. such as *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* are known as foodborne pathogens and have been reported to cause bacterial enteritis and sepsis (Khan et al., 2020). The illnesses caused by these bacteria commonly occur in the intake of raw or undercooked fishery products and wounds that are created during fish processing (Panicker et al., 2004). Among these species, *V. vulnificus* is one of the most deadly infectious halophilic species known to cause septicemia. In immunocompromised patients, the lethality of *V. vulnificus* septicemia has been reported more than 50% within 1–2 days after onset of symptoms (Starks et al., 2000). Virulence factors of *V. vulnificus* that are known to evoke disease include hemolysin, protease, and siderophore (iron chelator). In addition, some of the genes such as *vvh* (*V. vulnificus* hemolysin), *viuB* (vibriobactin utilization), and *vcgC* (virulence-correlated gene C sequence) in this species are involved in the synthesis of these factors (Guling et al., 2005; Han et al., 2011; Jones et al., 2009).

Some of the *Vibrio* sp. such as *V. cholerae* serotype O1 and serotype O139 are known to cause cholera. Although, currently, these serotypes are no longer a fatal waterborne infection in countries where there is safe drinking water and advanced sanitation systems, such as Europe and North America. However, there is still a fatal disease caused by *V. cholerae* in many countries, particularly in at least 47 countries, where 2.9 million cases with 95,000 death occurred by its infections (Ail et al., 2015; WHO, 2017). The virulence factors of *V. cholerae* are highly diverse, and some of these include cholera toxin (CT), toxin coregulated pilus (TCP), zonula occludens toxin (ZOT), accessory cholera toxin (Ace), etc. (Singh et al., 2001; Gao et al., 2019). The genes associated with these virulence factors, such as *ctxA* (subunit A of cholera toxin),

tcpI (toxin coregulated pilus inhibitor), and *zot* (zonula occludens toxin) are reported to get upregulated during the time of its infections (Singh et al., 2001; Gao et al., 2019).

According to the World Health Organization (WHO), the number of reported cholera cases is less as compared to the actual circumstances, which is due to the limitations of the disease observation system, inaccurate case definitions, and limits of laboratory analysis capabilities (WHO, 2016). Various culture-based methods using selective-differential media, such as thiosulfate-citrate-bile-sucrose (TCBS) agar and CHROMagar™Vibrio (CHROMagar, Paris, France) have been already developed for the convenience and high accuracy detection of pathogenic *Vibrio* spp. (Rosec et al., 2012; Zavala-Norzagaray et al., 2015; Stuart et al., 2016). However, due to the strong selective properties of these media, the culture-based approaches are challenging to isolate and detect the damaged cells (Alam et al., 2003). Furthermore, these selective-differential media are also not suitable for the isolation of the pathogenic *V. cholerae* and *V. vulnificus* from the environment. In the case of TCBS, the colonies of *V. cholerae* (yellow color) can be distinguished from other *Vibrio* spp. (olive or green color) based on the appearance of the colored cells which is occurred due to the changes in pH of the media through sucrose fermentation (Nigro et al., 2011). Similarly, the colonies of other *Vibrio* species such as *Vibrio alginolyticus* and *V. vulnificus* also form yellow color colonies after growing in TCBS media (Bunpa et al., 2016; Passalacqua et al., 2016). However, blue color colonies of *V. cholerae* (light blue) and *V. vulnificus* (dark blue) have been reported to occur after growing on CHROMagar ™ Vibrio (Deeb et al., 2018).

In the current trends, the polymerase chain reaction (PCR) based detection of the bacterial culture has become an effective and advanced technology to solve problems that arise in the culture-based methods. Although it has certain advantages such as rapid analysis and high accuracy, some of the PCR methods (real-time PCR) are expensive for the enumeration of bacteria (Levin, 2004; Gyawali et al., 2015). Furthermore, for the enumeration of more than two species of pathogenic *Vibrio* spp. at the same time, a real-time multiplex PCR using fluorescence oligonucleotide probe is required, which is also costly as compared to simple qPCR (quantitative PCR) with SYBR-Green (Qvarnstrom et al., 2005; Kim et al., 2012). Hence, due to the high experimental cost, the above PCR-based detection and management of pathogenic bacteria might be one of its limitations in developing countries (Chan et al., 2016; Nyaruaba et al, 2019). To solve the above problem, the MPN-PCR method combining with the most probable number (MPN) method has been developed (Barrera et al., 2016). However, the MPN-PCR method is not suitable for the detection and isolation of different pathogenic *Vibrio* spp. and strains, though this method is suitable only for bacterial enumeration (Andrews et al., 2000; Rivera et al., 2001; Shaw et al., 2014; Bonny et al., 2018). In addition, some strains of *V. vulnificus* are also had problems with isolation because these bacteria have weak viability when storing at low temperatures without preservation treatment (Burnham, et al., 2009). To overcome the limitation of the MPN-PCR method employed for the enumeration of the *Vibrio* spp. we developed a low-cost and accurate detection method, which is the combination of MPN-PCR and cultural methods. The developed advanced MPN-PCR method in the present study is very helpful for the enumeration and isolation of pathogenic *V. cholerae* and *V. vulnificus* as tested and verified for their detection from the environmental samples.

2. Materials And Methods

2.1. Bacterial strain and growth media

For the development and verification of the advanced MPN-PCR method, three reference strains, six environmental isolates, and one DNA from reference strain were used in this study (Table 1). The reference strains such as *V. alginolyticus* (KCTC 2472), *V. cholerae* O139 (MFDS-2003487), and *Vibrio vulnificus* (KCCM 41662) were purchased from the Korean Culture Center of Microorganism (KCCM; Seoul, Korea), Korean Collection for Type Culture (KCTC; Jeongeup, Korea), and Korean Culture Collection for Foodborne Pathogens (the Ministry of Food and Drug Safety, Cheongju, Korea). The chromosomal DNA of *V. cholerae* O1 was obtained from the Korean Culture Center of Microorganisms (KCCM), and it was stored at -20℃ and using for the experiment. In addition, the environmental isolates of *Vibrio* spp. such as *V. cholerae* (VCGS-1 strain) and *V. vulnificus* (VVBS-1, VVBS-2, VVGN, VVGS-1, and VVGS-2 strains) have also been used in this study. These strains were obtained from the Laboratory of food hygiene and microbiology (Gangneung-wonju national university, Gangneug, Korea), Laboratory of food hygiene (Gunsan national university, Gunsan, Korea), and isolated from Geoje in 2018. For the enrichment of bacteria, alkaline peptone water (Merck Millipore, Germany, APW, pH 8.6 ± 0.2) and double concentration APW (2× APW) were used. At the same time, TCBS (Difco, Detroit, MI; pH 8.6 ± 0.2) and Luria-Bertani agar (LBA) without supplementation of NaCl (LB-0) were used as a selective-differential growth media. However, for the comparison of viable cell count methods (enumeration of bacterial colonies), the LBA with 2%(w/v) NaCl (LB-2, with additional supplementation of 10 g/L NaCl) has been used.

Table 1
List of bacterial strains used in this study

Strains	Virulence-related gene	Sources
Vibrio alginolyticus KCTC 2472	-	Korean Collection for Type Cultures
Vibrio cholerae O1 KCCM 41626 (chromosomal DNA)	*ctxA*, *tcpI*, *zot*	Korean Culture Center of Microorganism
V. cholerae O139 MFDS-2003487	*tcpI*, *zot*	Korean Culture Collection for Foodborne Pathogens
V. cholerae O139 VCGS-1	*tcpI*, *zot*	Laboratory of food hygiene, Gunsan national university, Gunsan, Korea
Vibrio vulnificus KCCM 41665	*vvh, viuB*	Korean Culture Center of Microorganism
V. vulnificus VVBS-1	*vvh, vcgC*	Isolated from seawater at Geoje, Korea
V. vulnificus VVBS-2	*vvh, viuB, vcgC*	Isolated from seawater at Geoje, Korea
V. vulnificus VVGN	*vvh, viuB, vcgC*	Laboratory of food hygiene and microbiology, Gangneung-wonju national university, Gangneung, Korea
V. vulnificus VVGS-1	*vvh, vcgC*	Laboratory of food hygiene, Gunsan national university, Gunsan, Korea
V. vulnificus VVGS-2	*vvh, viuB, vcgC*	Laboratory of food hygiene, Gunsan national university, Gunsan, Korea

ctxA, Cholera toxin A. *tcpI*, Toxin coregulated pilus inhibitor. *zot*, Zonula occludens toxin. *vvh, Vibrio vulnificus* hemolysin. *viuB*, Vibriobactin utilization. *vcgC*, Virulence-correlated gene C sequence

2.2. Bioaccumulation of V. cholerae and V. vulnificus in live oyster

Alive oyster (*Crassostrea gigas*) samples were purchased from an aquaculture farm located in Tong-Yeong, Gyeongnam, Korea. Oyster samples were harvested in February 2019 and transported to the laboratory within 4 h under keeping less than 10℃. Oysters were washed briefly with tap water to remove bows from shells. To remove the debris from inside of the oysters, the samples were placed in a tank (high-density polyethylene with a dimension of 50 × 55 × 60 cm) containing 100 L of artificial seawater (ASW; salinity 35 psu) at 15℃ for 14–16 h that was also continuously circulated. ASW was prepared by dissolving with 17.5g/L of artificial sea salt (Reef Salt Mix; KENT Marine, Long Beach, CA) and 17.5g/L of sea salt (Hanju Salt., Ulsan, Korea). The recirculation of ASW was carried out using a pump with a flow rate of 480 L/h (8 L/min) to ensure sufficient dissolved oxygen in the water. The *V. vulnificus* KCCM 41665 and *V. cholerae* O139 MFDS-2003487 were inoculated in the tank at the cell population of 10^6 CFU/mL.

The bioaccumulation of these *Vibrio* spp. was conducted for 6 h following the previous report (Martins et al., 2006).

2.3. Sample collection and preparation

Seawater and seafood samples were collected from February 2019 to October 2019 at the southeast coastal area and from a local market and consignment market at Tong-Yeong, Gyeongnam, Korea (Fig. 1). The details of sample information were shown in Table 2. The samples were prepared according to the procedure described by the U.S. Food and Drug Administration Bacteriological Analytical Manual (FDA-BAM): *Vibrio* (Bonnin-Jusserand et al., 2019). The samples were washed with tap water to remove the muds present on the shell. After removing shells, 50 g of meat (including liquid) was mixed with 450 g of 0.1 M phosphate-buffered saline (PBS, pH 7.2 ± 0.2) and blended for 90 sec. Seawater samples were directly used without any pretreatment.

Table 2
Sample information collected from local-market, consignment market and south-east coastal area of Korea

Sampling month	Sample	Number of samples
February	Swimming crab (*Portunus trituberculatus*)	15
	Longarm octopus (*Octopus minor*)	15
March	Swimming crab (*Portunus trituberculatus*)	15
	Longarm octopus (*Octopus minor*)	15
April	Swimming crab (*Portunus trituberculatus*)	15
	Longarm octopus (*Octopus minor*)	15
	Seawater	9
May	Swimming crab (*Portunus trituberculatus*)	15
	Longarm octopus (*Octopus minor*)	15
	Seawater	9
June	Swimming crab (*Portunus trituberculatus*)	15
	Longarm octopus (*Octopus minor*)	15
	Seawater	9
July	Swimming crab (*Portunus trituberculatus*)	15
	Longarm octopus (*Octopus minor*)	15
	Seawater	9
August	Gizzard shad (*Konosirus punctatus*)	18
September	Gizzard shad (*Konosirus punctatus*)	18
	Seawater	9
October	Gizzard shad (*Konosirus punctatus*)	18
	Seawater	9
Total	-	288

2.4. Isolation of Vibrio spp. from samples

APW enrichment was performed to recover the damaged cells(Humphries et al., 2015) and to enumerate the cell population using an MPN method. The enrichment procedure was performed according to the 3-tubes APW enrichment procedure from FDA-BAM: *Vibrio* (Bonnin-Jusserand et al., 2019). The inoculate procedure, as shown in Fig. 2, and the inoculated culture media were incubated for 18 h at 35 ± 2°C. After

APW enrichment, positive tubes were recognized based on turbidity. Further, to confirm *Vibrio* spp. from the positive tubes, the cell culture was streaked on a TCBS agar plate and incubated at 35°C for 18 h. The green-colored colony on the 1st TCBS agar plate was sub-cultured on the 2nd TCBS agar plate using streaking. Also, the yellow-colored colony was sub-cultured on the LB-0 agar plate and incubated under the same conditions (Fig. 3).

2.5. Identification of Vibrio spp. using PCR with specific primer

Genomic DNA (gDNA) for PCR analysis was extracted using AccuPrep® Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea) according to the instruction given in the manual as provided by the manufacturer. The AccuPower® PCR premix (Bioneer, Daejeon, Korea), PCR primers, and the 100bp Plus DNA ladder (Bioneer, Daejeon, Korea) used in this study were purchased from Bioneer (Daejeon, Korea). The PCR analysis was performed for the identification between *Vibrio* spp., *V. cholerae* serotype, and virulence-related genes. PCR conditions and primers used in the present study were shown in Table 3, 4, and the PCR was carried using TaKaRa PCR Thermal Cycler Dice® Gradient (Takara-bio, Kusatsu, Japan). The primers suggested by this study were newly designed based on the analysis of Primer-BLAST® (National Center for Biotechnology Information, Bethesda, MD).

Table 3
Primers and conditions used in the PCR for the confirmation of *Vibrio vulnificus*, *Vibrio cholerae*, and pathogenic serotype of *Vibrio cholerae*

PCR Target	Oligonucleotide sequence	PCR conditions		Product Size (bp)	References
V. vulnificus	5'- CAGCCGGACGTCGTCCATTTTG – 3'	94°C 5 min	1 cycles	484	(Jang et al., 2018)
		94°C 30 sec	25 cycles		
	5'- ATGAGTAAGCGTCCGACGCGT – 3'	60°C 30 sec			
		72°C 30 sec			
		72°C 4 min	1 cycles		
V. cholerae (*ompW*)	5'- CACCAAGAAGGTGACTTTATTGTG – 3'	94°C 5 min	1 cycles	304	(Bisweswar et al., 2000)
		94°C 30 sec	30 cycles		
	5'- GGTTTGTCGAATTAGCTTCACC – 3'	64°C 30 sec			
		72°C 30 sec			
		72°C 4 min	1 cycles		
O1 *rfb*	5'- GTTTCACTGAACAGATGGG – 3'	94°C 5 min	1 cycles	192	(Shimada et al., 2006)
		94°C 30 sec	35 cycles		
	5'- GGTCATCTGTAAGTACAAC – 3'	60°C 30 sec			
		72°C 30 sec			
		72°C 4 min	1 cycles		

**rfb*, antigen gene

PCR Target	Oligonucleotide sequence	PCR conditions		Product Size (bp)	References
O139 *rfb*	5'- AGCCTCTTTATTACGGGTGG –3'	94°C 5 min	1 cycles	449	
		94°C 30 sec 60°C 30 sec 72°C 30 sec	35 cycles		
	5'- GTCAAACCCGATCGTAAAGG –3'				
		72°C 4 min	1 cycles		

**rfb*, antigen gene

Table 4
Primers and conditions used in the PCR for detecting virulence-related genes of *Vibrio* spp.

Species	PCR Target	Oligonucleotide sequence	PCR conditions		Product Size (bp)	References
V. cholerae	*ctxA*	5'-CTCAGACGGGATTTGTTAGGCACG – 3'	94°C 5 min	1 cycles	302	(Jiang et al., 2018)
			94°C 30 sec	30 cycles		
			55°C 30 sec			
		5'-TCTATCTCTGTAGCCCCTATTACG – 3'	72°C 30 sec			
	tcpI	5'- TAGCCTTAGTTCTCAGCAGGCA – 3'	94°C 5 min	1 cycles	862	(Singh et al., 2001)
			94°C 30 sec	30 cycles		
			60°C 30 sec			
		5'- GGCAATAGTGTCGAGCTCGTTA – 3'	72°C 55 sec			
		5'- GCCTTAGTTCTCAGCAGGCA – 3'	94°C 5 min	1 cycles	439	This study
			94°C 30 sec	30 cycles		
			56.2°C 30 sec			
		5'- AACATCCCACTGCCGTTAGG – 3'	72°C 55 sec			
			72°C 4 min	1 cycles		
	zot	5'-CACTGTTGGTGATGAGCGTTATCG – 3'	94°C 5 min	1 cycles	243	(Gao et al., 2019)
			94°C 30 sec	30 cycles		
			55°C 30 sec			
		5'-TTTCACTTCTACCCACAGCGCTTG – 3'	72°C 55 sec			
		5'- TAACGATGGCGCGTTTTTGG – 3'	94°C 5 min	1 cycles	257	This study

Species	PCR Target	Oligonucleotide sequence	PCR conditions		Product Size (bp)	References
		5'- TATCTCCGCCGCCTCTCTTA – 3'	94°C 30 sec 56.2°C 30 sec 72°C 30 sec	35 cycles		
			72°C 4 min	1 cycles		

Table 4
Primers and conditions used in the PCR for detecting virulence-related genes of *Vibrio* spp. (continue)

Species	PCR Target	Oligonucleotide sequence	PCR conditions		Product Size (bp)	Reference
V. vulnificus	*vvh*	5'-TTCCAACTTCAAACCGAACTATGAC – 3'	94°C 5 min	1 cycles	205	(Cañigral et al., 2010)
			94°C 30 sec	35 cycles		
		5'-ATTCCAGTCGATGCGAATACGTTG – 3'	65°C 30 sec			
			72°C 30 sec			
			72°C 4 min	1 cycles		
	viuB	5'-GGTTGGGCACTAAAGGCAGATATA – 3'	94°C 5 min	1 cycles	504	(Panicker et al., 2004)
			94°C 30 sec	30 cycles		
		5'- CGGCAGTGGACTAATACGCAGC – 3'	65°C 30 sec			
			72°C 35 sec			
			72°C 4 min	1 cycles		
	vcgC	5'- AGCTGCCGATAGCGATCT – 3'	94°C 5 min	1 cycles	278	(Yokochi et al., 2013)
			94°C 30 sec	35 cycles		
		5'- CGCTTAGGATGATCGGTG – 3'	56°C 30 sec			
			72°C 30 sec			
			72°C 4 min	1 cycles		

2.6. Statistical analysis

The simultaneous cell enumeration of *V. cholerae* and *V. vulnificus* using artificially bioaccumulated oyster was carried out in triplicate. The MPN table listed in FDA-BAM Appendix 2 (Bonnin-Jusserand et al., 2019) was used for the cell enumeration. Results are expressed as mean ± standard deviation. Statistics were processed using Duncan's multiple range test ($P < 0.05$) with SPSS (v.23.0, SPSS Ine., Chicago, IL) after performing analysis of variance (ANOVA) for significance-verification (Duncan, 1955).

3. Results

3.1. Isolation and identification of V. cholerae using non-NaCl growth media and advanced MPN-PCR method

The LB-0 agar was formulated as an effective selection media for the sub-culturing of *V. cholerae* that were isolated by growing on TCBS agar plate. For the selection of *V. cholerae* using LB-0 agar selection media, a total of nine strains of *Vibrio* spp. were inoculated to the LB-0 agar plate that was pre-cultured on the TCBS agar plate, and results are shown in Table 5. The results showed that only *V. cholerae* strains were found to grow on the LB-0 agar plate until 24 h. This result indicates that LB-0 agar is an effective selection media for the isolation of *V. cholerae* that were initially grown on a TCBS agar plate.

Table 5
The cultural response of various *Vibrio* spp. inoculate from TCBS agar

Strains	Recovery by incubation time at 35°C	
	12 hours	24 hours
Vibrio alginolyticus KCTC 2472	None	None
V. cholerae O139 MFDS-2003487	Good	Good
V. cholerae O139 VCGS-1	Good	Good
Vibrio vulnificus KCCM 41665	None	None
V. vulnificus VVBS-1	None	None
V. vulnificus VVBS-2	None	None
V. vulnificus VVGN	None	None
V. vulnificus VVGS-1	None	None
V. vulnificus VVGS-2	None	None

3.2. Improvement of the accuracy and specificity of the advanced MPN-PCR method

The amplification of virulence-related genes of *V. cholerae* is an effective way for its identification. Previous research showed that the amplification of virulence-related genes using PCR primer for the identification of *V. cholerae* as suggested by Gao et al. (2019) and Singh et al.(2004). These PCR primers were designed to detect the *tcpI* and *zot* virulence genes, but it gives a weak amplification by PCR along with amplification of non-specific products (Fig. 4). To solve the above problem, in the present study, new primers for *tcpI* and *zot* genes have been designed using Primer-BLAST®. As shown in Fig. 4, a good PCR product has been amplified using newly designed primers for the detection of target genes as compared to the previous studies. The *tcpI* gene-specific primer, as suggested by Singh et al. (2001) were reported to amplify 862 bp PCR product, however, a non-specific PCR product of about 600 bp was also amplified. However, in the present study, the PCR amplification using a newly designed primer was found to exhibit a 439 bp PCR product. In the case of *zot* gene detection, the previous PCR process, as suggested by Gao et al. (2019) showed 243 bp PCR product of target along with few non-specific PCR products. However, the amplification of *zot* gene using the newly designed primers resulted in 257 bp amplification.

3.3 Verification of the advanced MPN-PCR method on enumerating oyster bioaccumulated V. cholerae and V. vulnificus cells

The bioaccumulated oysters were used to verify the accuracy and specificity of the modified MPN-PCR method on the enumeration of *Vibrio* spp.. The obtained results in the present study have also been compared with the results of other *Vibrio* spp. enumeration methods using different media. As shown in Table 6, no significant difference was observed in the number of *Vibrio* spp. cell count according to the enumeration method using different media (LB-0 medium, LB-2 medium, and APW medium for MPN-PCR), but not for *V. vulnificus* on LB-0 agar as described above. The viable cell count of *V. vulnificus* in the oyster was found to be 6.64 ± 0.13 log CFU/g on the LB-2 agar plate and 8.55 ± 0.47 log MPN/100g as determined using the MPN-PCR method. In the case of *V. cholerae*, the viable cell count was found to be 6.56 ± 0.13 log CFU/g on LB-0 agar and 6.64 ± 0.22 log CFU/g on LB-2 agar, and 8.99 ± 0.38 log MPN/100g as determined using the MPN-PCR method. These results strongly indicate that the modified MPN-PCR method in the present study can be successfully applied to enumerate *Vibrio* spp. cells in the shellfish samples.

Table 6
Comparison of various methods for viable cell quantification

Species	Method for viable cell quantification		
	Spread-plate method with LB-0 agar	Spread-plate method with LB-2 agar	MPN-PCR method
V. cholerae	6.56 ± 0.13[a] (log CFU/g)	6.64 ± 0.22[a] (log CFU/g)	8.99 ± 0.38[a] (log MPN/100g)
V. vulnificus	-	6.64 ± 0.13[b] (log CFU/g)	8.55 ± 0.47[b] (log MPN/100g)

LB-0 agar without supplementation of NaCl. LB-2 agar, Luria-Bertani agar total containing 2% NaCl. TCBS, thiosulfate citrate bile salts sucrose medium. MPN-PCR, Bacterial cells were cultivated using alkaline peptone water for 12 ± 2 h at 35°C and the viable cell numbers were counted using the most probable number method combined with PCR amplification. a-b, Means with different superscripts within each column indicate significant differences by Duncan's multiple range test ($P < 0.05$)

3.3. Simultaneous isolation and enumeration of V. cholerae and V. vulnificus from environmental samples

The development of the advanced MPN-PCR method in the present study has also been tested for the isolation and enumeration of the *Vibrio* spp. (both *V. cholerae* and *V. vulnificus*) using environmental samples (Fig. 5). Total 288 seawater and seafood samples were collected from the local market, consignment market, and the coastal area of Korea for over 9 months. As suggested in this study, this method is capable of simultaneously isolating and enumerating *V. cholerae* and *V. vulnificus* cells (Table 7). With the help of the modified MPN-PCR method, the *V. vulnificus* has been detected in the ranges of < 30–36 MPN/100g (or 100 mL) from environmental samples. In addition, two strains of virulent *V. vulnificus* were isolated from Gizzard shad (*Konosirus punctatus*) samples which were sampled from different places. And these two environmental isolates have the same virulence-related gene, *vvh*. Whereas *V. cholerae* was detected to < 30 MPN/100g, which is considered as no detection (since the limit of detection is 30 MPN/100g or 100mL).

Table 7
Result of simultaneous cell quantification and virulence-related gene detection

Species	Sampling month	Collected location	Sample	Cell count (MPN/100g)	Detected Virulence-related gene
Vibrio vulnificus	August	Consignment market B	Gizzard shad (*Konosirus punctatus*)	36	*vvh*
Vibrio vulnificus	August	Consignment market D	Gizzard shad (*Konosirus punctatus*)	36	*vvh*

* Table only shown results up to LOD (limit of detection, 30 MPN/100g, or 100 mL).

4. Discussion

It has been reported that a few strains of *V. vulnificus* form a yellow colony after growing on TCBS agar plate, which is similar to the yellow-colored colony of *V. cholerae* (Passalacqua et al., 2016). Hence, the appearance of the yellow color colonies of both species on the TCBS agar plate makes a false identification and enumeration of *Vibrio* spp.. Thus, due to this problem, the application of the MPN-PCR method will require unnecessary additional time for confirming the *V. cholerae* and *V. vulnificus*, which also makes it difficult to isolates these *Vibrio* spp.. Similarly, the same problem has also occurred for the identification and isolation of another marine bacterium i.e. *V. alginolyticus*, since this bacterium also forms a yellow colony on TCBS agar plate (Bunpa et al., 2016). Thus, in the MPN-PCR method, unnecessary PCR requirements lead to an increase in the experimental cost and time for the simultaneous enumeration of *V. cholerae* and *V. vulnificus*.

In this study, the application of LB-0 agar supplementation becomes an advantage for the selective culturing and distinguishing *V. cholerae* from the *V. vulnificus*. The reason behind this is due to the different Na^+ concentrations requirements of different *Vibrio* spp.. The minimal requirement of Na^+ concentrations for *V. alginolyticus* is 200 mM, *V. cholerae* is 5 mM, and *V. vulnificus* is 140 mM (Farmer lii et al., 2015). Hence, formulating a selective culture media such as LB-0 agar make it possible to easy identification *V. cholerae* and *V. vulnificus*. This approach with such an advantage can reduce the unnecessary PCR based-identification and is also helpful for simultaneous viable cell enumeration of *V. cholerae* and *V. vulnificus* with low cost. In addition, some strains of *V. vulnificus* are possible to losing activity easily before preservation because some strains have weak viability when storing at low temperatures (Burnham et al., 2009). However, the culture process with LB-0 and TCBS agar also meaning subculture to prevent losing activity of isolates before preservation treatment.

Reports showed that there is a high chance of microbial contamination of bivalve molluscs during its cultivation as compared to the natural environment such as seawater (Campos et al., 2013; Ronald et al., 2008). Due to the highly prone to get contamination, there are many reports showed the food poisoning associated with consuming shellfish that are contaminated with microbial pathogens (Ronald et al., 2008; Anacleto et al., 2014). Depuration is an approach to control the microbial contamination of shellfish using ultra-violet (UV) light treated seawater, and also the Food and Agriculture Organization of the United Nations (UN FAO) recommended that for the same purpose (Lee et al., 2008). Furthermore, to check the efficiency of the depuration, several studies showed the artificial bioaccumulation of the shellfish with the microbial pathogen followed by its enumeration of the cells during the process of depuration (Croci et al., 2002; Marino et al., 2005; Schneider et al., 2009). Martins et al. (2006) and Kwon et al. (2011) reported a clam *Thylacodes decussatus* and a mussel *Mytilus edulis* bioaccumulated by *Escherichia coli*. In this study, we selected pacific oysters for the bioaccumulation of *V. cholerae* and *V. vulnificus*, respectively (detail procedure described in Materials and Methods).

5. Conclusion

In the present study, the advanced MPN-PCR method was developed, which adding a culture process using a non-NaCl medium for simultaneous isolation and enumeration of *V. cholerae* and *V. vulnificus*. The non-NaCl medium was effectively distinguished *V. cholerae* and *V. vulnificus* which are unable to identify using TCBS agar. This simple selective culture process makes it possible to reduce the experimental cost required in the confirmation of *V. cholerae* and *V. vulnificus* using additional PCR analysis. Furthermore, the effectiveness of this advanced MPN-PCR method has also been verified by comparing it with other *Vibrio* spp. cell enumeration methods. To apply this method in the enumeration of *V. cholerae* and *V. vulnificus* from the environmental samples, these bacteria were bioaccumulated using pacific oyster. Furthermore, this MPN-PCR method has also been applied for bacterial analysis from the environmental samples. From the environmental samples, two virulent *V. vulnificus* have been isolated, which have the characteristic virulence-related gene as evidenced by the PCR amplification. Hence, the present study provides a low-cost MPN-PCR method for the simultaneous isolation and enumeration of *V. cholerae* and *V. vulnificus* that may be helpful for those countries that have low research budgets. This low-cost MPN-PCR method will also contribute to the control of microbial risks by intaking seafood that is contaminated with *V. cholerae* and *V. vulnificus*.

Declarations

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of

the authors.

Consent for publication

The authors give their consent to publish this original article.

Availability of data and material

Please contact author for data requests.

Competing interests

Jae-Hwa Lee, Seul-Ki Park, Fazlurrahman Khan, Du-Min Jo, Do-ha Lee, Min-Gyun Kang, and Young-Mog Kim declare that they have no conflict of interest.

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Author contribution

JHL and YMK designed and lead the writing and analysis of data. JHL, MGK, DHL, and DMJ were performed in the sampling, experiment, and analyzed data. JHL, SKP, FK, and YMK were involved in the data analysis, writing, and editing of the manuscript.

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Authors' information

Not applicable.

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Figures

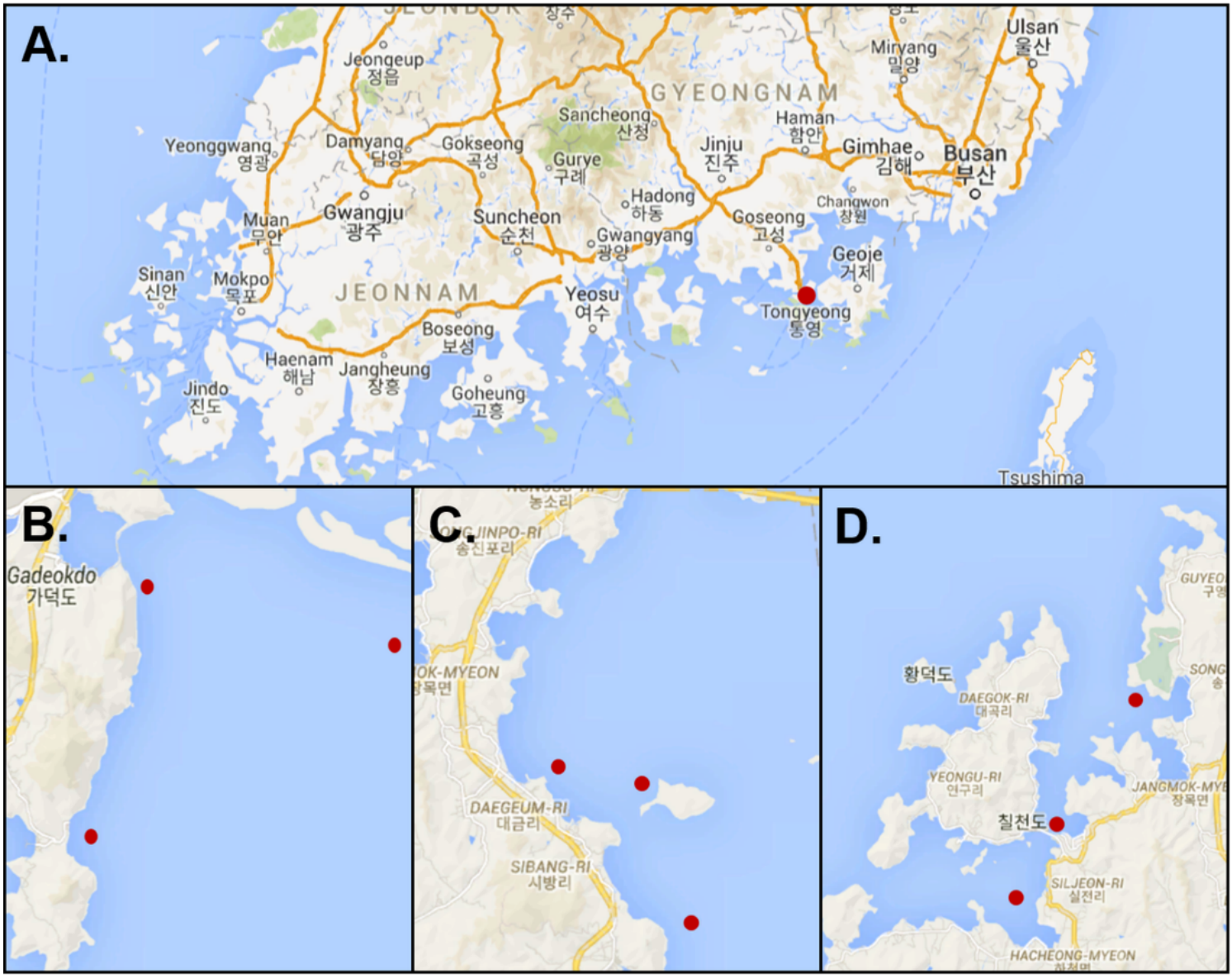

Figure 1

Sampling area of seafood and seawater in the Republic of Korea. (A) Tong young-city where local-market located. (B) Sampling points of seawater samples at the east coast of Gadeok Island. (C) Sampling points of seawater samples at the east coast of Geoje-city. (D) Sampling points of seawater samples at the coast of Chilcheon Island. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

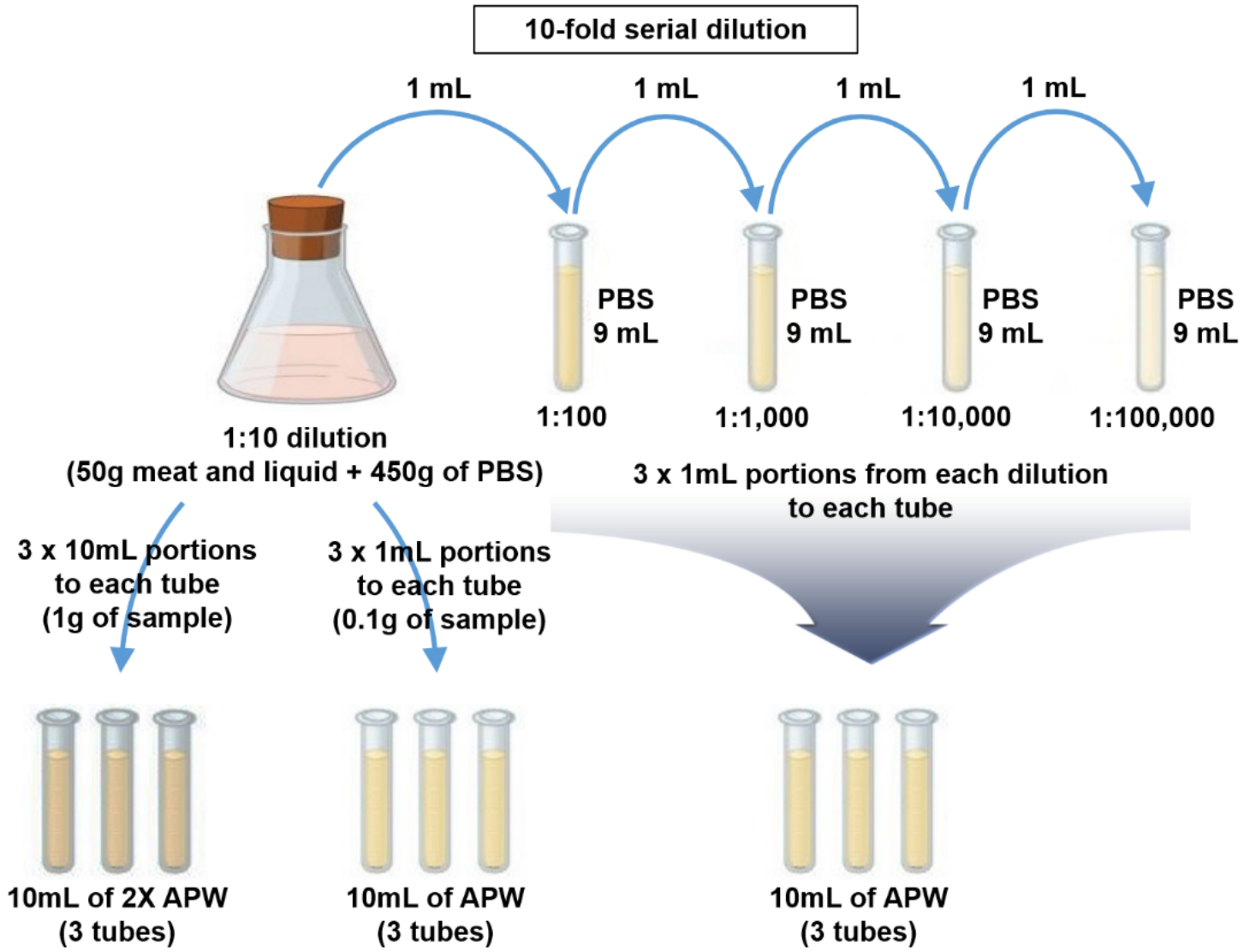

Figure 2

Schematic representation of the inoculate procedure for APW enrichment.

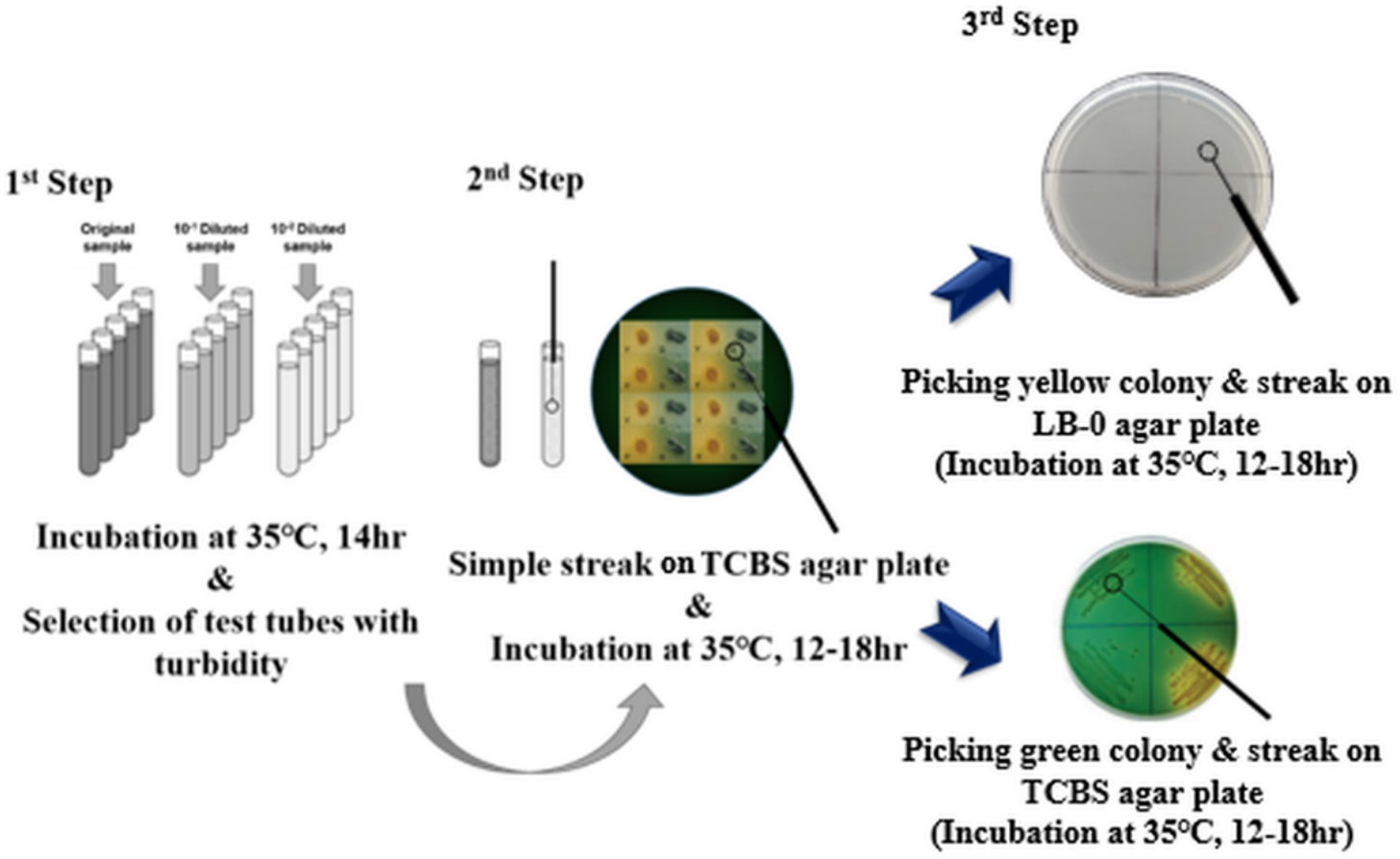

Figure 3

Schematic representation of the culture procedure before PCR.

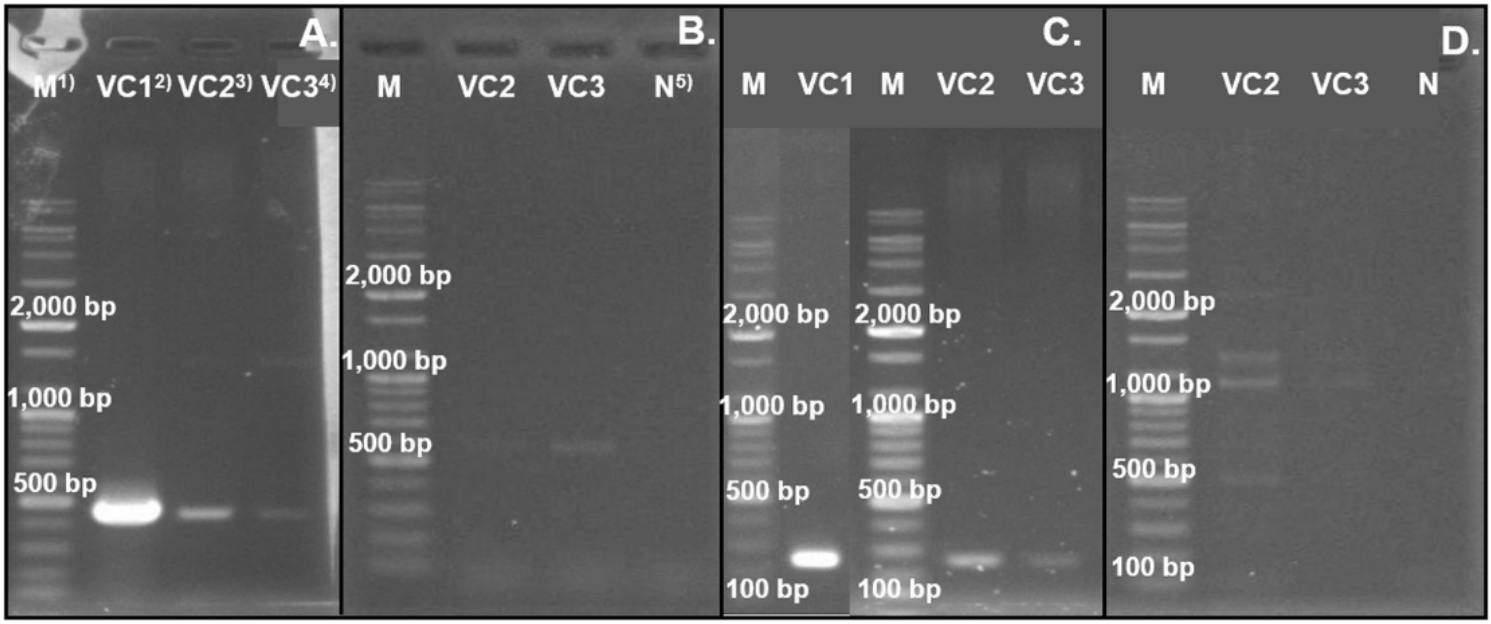

Figure 4

Result of PCR with different tcpI and zot genes. (A) Result of PCR with tcpI gene-specific primer suggested by this study. (B) Result of PCR with tcpI gene-specific primer suggested by Singh et al. (2001). (C) Result of PCR with zot gene-specific primer was suggested by this study, and (D) Result of PCR with zot gene-specific primer was suggested by Gao et al. (2019). Whereas, M; Marker. VC1; DNA of V. cholerae KCCM 41665. VC2; V. cholerae MFDS-2002810. VC3; Isolated V. cholerae (VCGS-1). N; Negative control.

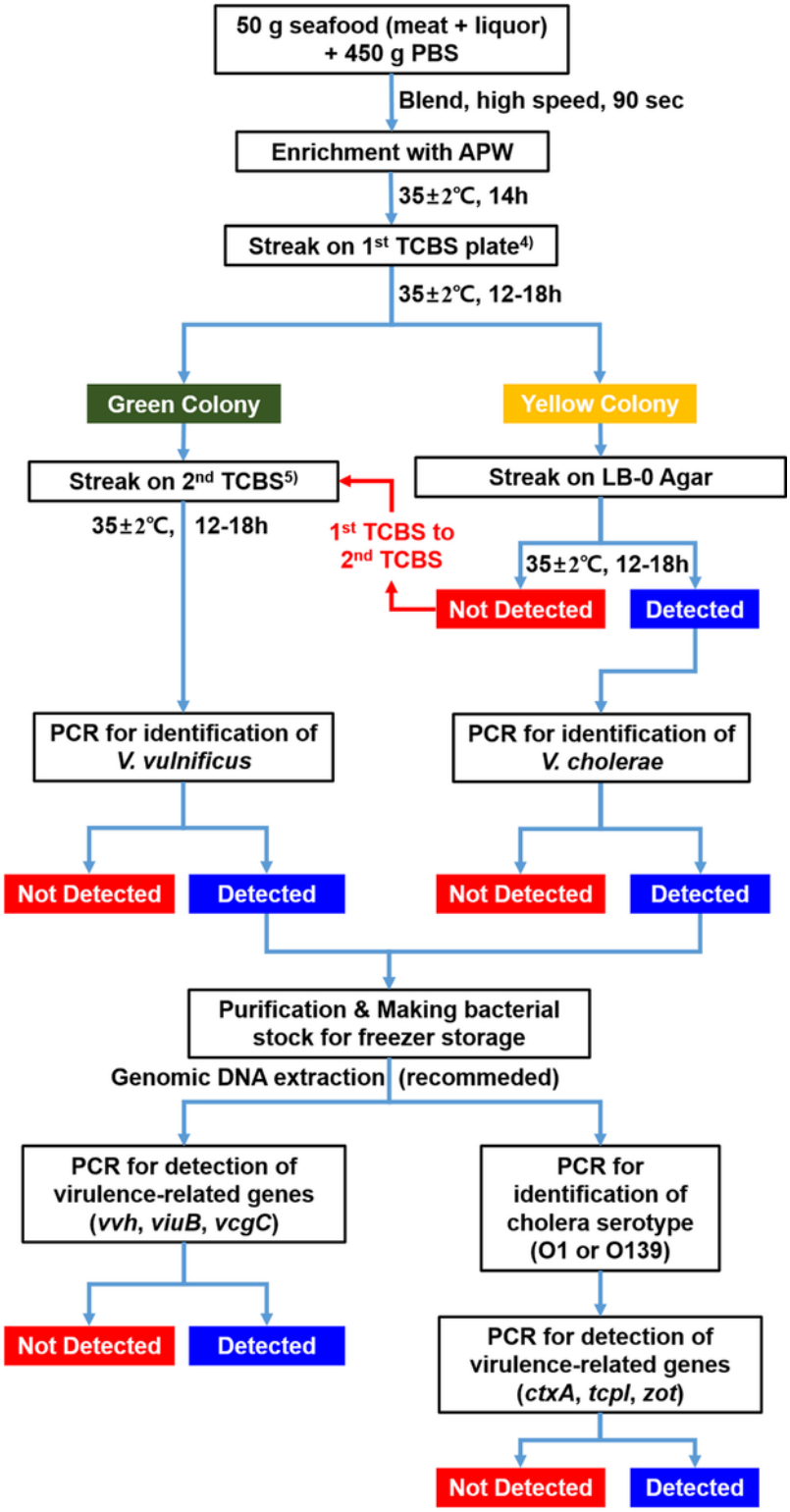

Figure 5

The flow-chart showing the steps involved in the sample preparation, isolation, and quantification of Vibrio vulnificus and Vibrio cholerae colonies using the modified MPN-PCR method as suggested in this study.