Simultaneous isolation and enumeration of virulent *Vibrio cholerae* and *Vibrio vulnificus* using an advanced MPN-PCR method

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**Abstract**

*Vibrio cholerae* and *Vibrio vulnificus* are critical foodborne pathogens that need to be intensively controlled for their infection due to 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 detecting *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 with processing PCR 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*.

**Keywords** Virulent *Vibrio* spp. · MPN-PCR · Enumeration · Isolation · Low cost

**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. 2004a, b). 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. vibrio that are known to evoke disease include hemolysin, protease, and siderophore (iron chelator). In addition, some of the genes such as vvh (V. vibrio hemolysin), viub (vibriobactin utilization), and vgc (virulence-correlated gene C sequence) in this species are involved in the synthesis of these factors (Gulig et al. 2005; Han et al. 2011; Jones et al. 2009).

Some of the Vibrio spp. such as V. cholerae serotype O1 and serotype O139 are known to cause cholera. Although, currently, these serotypes are no longer fatal waterborne infections 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 (Ali 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 ctaA (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 than the actual circumstances 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. (Roscè 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 occurs 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 compared to simple qPCR (quantitative PCR) with SYBR-Green (Qvarnström 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 combined 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 also had problems with isolation because these bacteria have weak viability when stored 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.

Materials and methods

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 the reference strain were used in this study (Table 1). The reference strains such as V. alginolyticus (KCTC 2472), V. cholerae O139 (MFDS-2002810), and Vibrio vulnificus (KCCM 41,665) 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
same time, TCBS (Difco, Detroit, MI; pH 8.6 ± 0.2) and NaCl (LB-2, with additional supplementation of 10% (w/v) (enumeration of bacterial colonies), the LBA with 2% (w/v) (LB-0) were used as a selective-differential growth media. However, for the comparison of viable cell count methods Luria–Bertani agar (LBA) without supplementation of NaCl was used 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, Gangneung, 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% (w/v) NaCl) has been used.

### 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 less than 10 °C. 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 °C for 14–16 h that was also continuously circulated. ASW was prepared by dissolving with 17.5 g/L of artificial sea salt (Reef Salt Mix; KENT Marine, Long Beach, CA) and 17.5 g/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 41,665 and V. cholerae O139 MFDS-2003487 were inoculated in the tank at the cell population of 10⁶ CFU/mL. The bioaccumulation of these Vibrio spp. was conducted for 6 h following the previous report (Martins et al. 2006).

### 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 s. Seawater samples were directly used without any pretreatment.

### 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-tube 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).

**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 100 bp 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. The primer designed in the present study and previously reported primer and the PCR conditions are provided in the supplementary information (Table S1 and S2). 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).

**Statistical analysis**

The simultaneous cell enumeration of *V. cholerae* and *V. vulnificus* using artificially bioaccumulated oysters 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 Inc., Chicago, USA).
IL) after performing analysis of variance (ANOVA) for significance verification (Duncan, 1955).

Results

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 3. 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.

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

The amplification of virulence-related genes of V. cholerae using PCR primer is an effective way for its identification, as suggested previously by Gao et al. (2019) and Singh et al. (2001). 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), was 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.

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 4, 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/100 g 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 the LB-0 agar and 6.64 ± 0.22 log CFU/g on the LB-2 agar, and 8.99 ± 0.38 log MPN/100 g 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.

**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 can simultaneously isolate and enumerate *V. cholerae* and *V. vulnificus* cells (Table 5). With the help of the modified MPN-PCR method, the *V. vulnificus* has been detected in the ranges of <30–36 MPN/100 g (or 100 mL) from...
environmental samples. In addition, two strains of virulent \textit{V. vulnificus} were isolated from Gizzard shad (\textit{Konosirus punctatus}) samples which were sampled from different places. And these two environmental isolates have the same virulence-related gene, \textit{vvh}. At the same time, \textit{V. cholerae} was detected to < 30 MPN/100 g, which is considered no detection (since the detection limit is 30 MPN/100 g or 100 mL).

### Discussion

It has been reported that a few strains of \textit{V. vulnificus} form a yellow colony after growing on TCBS agar plate, which is similar to the yellow-colored colony of \textit{V. cholerae} (Passalacqua et al. 2016). Hence, the appearance of

| Strains                        | Recovery by incubation time at 35 ℃ |
|-------------------------------|-------------------------------------|
|                               | 12 h | 24 h |
| \textit{Vibrio alginolyticus} KCTC 2472 | None | None |
| \textit{V. cholerae} O139 MFDS-2002810 | Good | Good |
| \textit{V. cholerae} O139 VCGS-1       | Good | Good |
| \textit{Vibrio vulnificus} KCCM 41,665 | None | None |
| \textit{V. vulnificus} VVBS-1        | None | None |
| \textit{V. vulnificus} VVBS-2        | None | None |
| \textit{V. vulnificus} VVGN          | None | None |
| \textit{V. vulnificus} VVG-1         | None | None |
| \textit{V. vulnificus} VVG-2         | None | None |
| \textit{V. vulnificus} VVGN-1        | None | None |
| \textit{V. vulnificus} VVGN-2        | None | None |
| \textit{V. vulnificus} VVGN-3        | None | None |
| \textit{V. vulnificus} VVGN-4        | None | None |

The yellow color colonies of both species on the TCBS agar plate makes a false identification and enumeration of \textit{Vibrio} spp.. Thus, due to this problem, applying the MPN-PCR method will require unnecessary additional time for confirming the \textit{V. cholerae} and \textit{V. vulnificus}, which also makes it difficult to isolate these \textit{Vibrio} spp.. Similarly, the same problem has also occurred to identify and isolate another marine bacterium, i.e. \textit{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 increase the experimental cost and time for the simultaneous enumeration of \textit{V. cholerae} and \textit{V. vulnificus}.

In this study, the application of LB-0 agar supplementation becomes an advantage for selective culturing and distinguishing \textit{V. cholerae} from the \textit{V. vulnificus}. The reason behind this is due to the different Na$^+$ concentrations requirements of different \textit{Vibrio} spp.. The minimal requirement of Na$^+$ concentrations for \textit{V. alginolyticus} is 200 mM, \textit{V. cholerae} is 5 mM, and \textit{V. vulnificus} is 140 mM (Farmer lii et al. 2015). Hence, formulating a selective culture media such as LB-0 agar makes it possible to easily identify \textit{V. cholerae} and \textit{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 \textit{V. cholerae} and \textit{V. vulnificus} with low cost. In addition, some strains of \textit{V. vulnificus} can easily lose activity before preservation because some strains have weak viability when stored at low temperatures (Burnham et al. 2009). However, the culture process using LB-0 and TCBS agar also involve subcultures to prevent isolates from losing activity before preservation treatment.

![Fig. 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 \textit{V. cholerae} KCCM 41,662 (Positive control). VC2; \textit{V. cholerae} MFDS-2002810. VC3; Isolated \textit{V. cholerae} (VCGS-1). N Negative control.](image-url)
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). Due to the highly prone to contamination, many reports show the food poisoning associated with consuming shellfish contaminated with microbial pathogens (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 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”).

### Conclusion

The improved MPN-PCR technique was established in the current study, which included a culture procedure utilizing a non-NaCl medium for simultaneous isolation and enumeration of V. cholerae and V. vulnificus. The non-NaCl medium effectively distinguished V. cholerae and V. vulnificus, which cannot be identified using TCBS agar. This simple selective culture process makes it possible to reduce the experimental cost required to confirm 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 oysters. 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.

### Table 4

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<sup>a</sup> (log CFU/g)  | 6.64 ± 0.22<sup>a</sup> (log CFU/g) | 8.99 ± 0.38<sup>a</sup> (log MPN/100 g) |
| V. vulnificus    | –                                    | 6.64 ± 0.13<sup>b</sup> (log CFU/g) | 8.55 ± 0.47<sup>b</sup> (log MPN/100 g) |

LB-0 agar without supplementation of NaCl. LB-2 agar, Luria–Bertani agar total containing 2% NaCl. TCBS, thiosulfate-citrate-bile salt-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. <sup>a</sup>–<sup>b</sup>, Means with different superscripts within each column indicate significant differences by Duncan’s multiple range test (P < 0.05).
Fig. 5 The flowchart shows 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.

**Table 5** Result of simultaneous cell quantification and virulence-related gene detection

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

*Table only shows results up to LOD (limit of detection, 30 MPN/100 g, or 100 mL)*
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Availability of data and materials  Please contact author for data requests.

Declarations

Conflict of interest  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.

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.

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