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Evaluation of a Multiplex Polymerase Chain Reaction for the Simultaneous Detection of *Vibrio spp*. in Vegetables and Water

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Abstract

BACKGROUND: Several foodborne outbreaks associated with the consumption of vegetables have been reported which involved *Vibrio spp*. as causative agents. Conventional methods of detecting these microorganisms are time-consuming. Therefore, the development of techniques for rapid detection seems to be of paramount importance.

OBJECTIVES: The present study recommends a rapid and reliable method for the detection of *Vibrio cholera (V. cholera)*, *V. parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus*. Moreover, the results are compared with the conventional plate culture method.

METHODS: The conventional bacteriological tests were conducted to detect *Vibrio spp.* in vegetables and their surrounding water. The samples were also subjected to a newly developed multiplex polymerase chain reaction (PCR) using five specific genes, including *VC-Rmm* of *V. cholerae*, *VP-MmR* of *V. parahaemolyticus*, *VV-Rmm* of *V. vulnificus, V.al2-MmR* of *V. alginolyticus,* and *VM-F* for all the four isolates.

RESULTS: The presence of *V. alginolyticus* and *V. vulnificus* was confirmed by amplifying the specific regions of 412 bp for *V. vulnificus* and 144 bp for *V. alginolyticus*. The results demonstrated that *V. cholerae* and *V. parahaemolyticus* were not detected in multiplex PCR, which was consistent with the findings of conventional plating methods.

CONCLUSIONS: Obtained results revealed that the designed multiplex PCR assay is a reliable, rapid, and cost-effective method for the simultaneous detection of *Vibrio spp*.

KEYWORDS: Foodborne outbreaks, Multiplex PCR, Simultaneous detection, Vegetables, *Vibrio* spp

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Introduction

Due to the increasing tendency toward healthy lifestyles in recent decades, there has been a growing demand for fresh products (Amao, 2018; Elgueta *et al.*, 2019). Vegetables are known to play an essential role in a healthy diet. Vegetables are usually eaten raw and undergo no process, such as cooking or peeling. As a result, they are likely to act as the vehicles of human pathogens (Chen *et al.*, 2019; Balali *et al.*, 2020). In some countries, vegetables are irrigated using wastewater leading to the possibility of being contaminated with enteric pathogenic microorganisms (Steele and Odumeru, 2004; Uyttendaele *et al.*, 2015; Gudda *et al.*, 2020). In recent years, there have been reports of foodborne outbreaks associated with the consumption of vegetables (de Oliveira Elias *et al.*, 2018). *Vibrio* spp. are among the pathogens which have been noted in raw vegetables (Igbinosa and Odjadjare, 2019).

The most commonly used method for the analysis of *Vibrio* spp. is a two-step protocol consisting of selective enrichment with Alkaline Peptone Water (APW) medium and then culturing on thiosulfate citrate bile salts (TCBS) agar. The main drawbacks of this conventional method are being labor-intensive and time-consuming (Tunung *et al.*, 2011; Anupama *et al.*, 2019). The sensitivity of the conventional method is low and the detection of the bacteria, when sample concentration is not adequate, is sometimes difficult. Therefore, methods based on polymerase chain reaction (PCR) are suggested to be an effective alternative for the identification of these organisms (di Pinto *et al.*, 2005; Tao *et al.*, 2020).

Several PCR-based methods have been used for identifying *Vibrio* spp. in recent years, one of which is the multiplex PCR method (Tsai *et al.*, 2019). Multiplex PCR method has been previously reported as a useful and cost-effective tool for the detection of *Vibrio* species (Awasthi *et al.*, 2019; Han *et al.*, 2019). The objective of this study was to establish a multiplex PCR method to identify *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus*. Furthermore, we compared the results of the latter technique with the conventional plate culturing method.

Materials and Methods

Sample Collection

The samples used in this study consisted of five groups of green vegetables, namely group A: spinach and onion, group B: cress and radish, group C: tarragon and basil, group D: mint and dill, group E: parsley and coriander, and their surrounding water. The vegetables were collected from the southern parts of Tehran and Varamin, Iran, and were transferred to the laboratory.

Detection of *Vibrio spp.* and other Bacteria in the Water Surrounding the Vegetables

In order to detect bacteria in the surrounding water of vegetables, two methods of membrane filtration and the most probable number (MPN) were applied.

Membrane Filtration Method

A volume of 1 L of the sample water was filtered onto 0.45 µm filter paper. The filter paper was then put in APW enrichment broth and was incubated at 35°C for 6-8 h. Following incubation, 250 mL of the sample was filtered onto a 0.45 µm cellulose acetate membrane filter which was later placed on MacConkey agar and CHROMagar vibrio plates and was incubated at 37°C for 24 h.

MPN Method

A three- tube MPN procedure was used. The sample was decimally diluted from 1/10 to 1/1000 utilizing sterilized saline and 1 mL of each dilution for 9 mL of culture broth medium in plastic tubes as triplicates for each dilution. Afterwards, the nine tubes were incubated at 35°C for 24 h. An MPN estimate was performed using standard MPN tables.

Detection of *Vibrio spp.* and Other Bacteria in Vegetables by Plate Culture

The samples were divided into portions of 10 g and were placed in separate stomacher bags.

A volume of 90 mL of APW enrichment broth was added to each sample, mixed for 1 min in a stomacher, and the samples were incubated at 35°C for 5-8 h. Following decimal serial dilution in sterilized peptone water, 1 mL of diluted samples was pour plated in TCBS agar and incubated at 35°C for 18-24 h. Because TCBS is not a highly selective media for *V. vulnifus*, CHROMagar vibrio plates were used as well with the same procedure as TCBS plates. Mannitol salt agar and cetrimide agar were applied to detect *Staphylococcus spp.* and *Pseudomonas spp.*, respectively.

Detection of *Vibrio spp.* and Other Bacteria in Vegetables by Multiplex PCR

Genomic DNA was extracted using CinnaGen genomic DNA extraction kit following the procedure provided by the company. The used oligonucleotide primer sets, melting temperatures (T_m), and the size of amplicons used in this study are listed in Table 1. Multiplex PCR was optimized in a 25 µL reaction mixture consisting of 3 µL of DNA template, 3 µL of 10 µM forward primer, 3 µL of 10 µM reverse primer, 12.5 µL of PCR Master Mix, and 3.5 µL of nuclease-free water. A three-step PCR amplification process was carried out in a DNA thermal cycler. Multiplex PCR conditions were optimized using the following temperature-cycling parameters: initial denaturation at 94°C for 180 min followed by 30 cycles of amplification with denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec, and primer extension at 72°C for 1 min. Following the amplification cycles, samples were subjected to 72°C for 420 min for the final extension of incompletely synthesized DNA. Next, the PCR products were analyzed using agarose gel electrophoresis 2% (w/v). Agarose gel was stained by erythrogel. Electrophoresis was performed using 1x TBE buffer. All products were visualized and documented with a gel documentation and analysis system.

Results

Detection of Bacteria in Vegetables and Water by Culture

The plates were examined following 24 h incubation. Shiny yellow colonies of 2-3 mm on TCBS were initially considered as *V. cholorae* and *V. alginolyticus.* The salt tolerance of the isolates was tested to differentiate *V. cholorae* and *V. alginolyticus* as they can grow in 0% and 6%-10% of NaCl, respectively. Green or blue-green colonies on TCBS were considered as *V. vulnificus*. The results of cultures for different specimens are summarized in Table 2. *Vibrio spp.* and *Staphylococcus aureus* were present in all the tested vegetables but not in the surrounding water. *Escherichia coli*, *Pseudomonas aeruginosa*, and *Streptococcus faecalis* were not detected in any of the samples.

Multiplex PCR Results

Representative multiplex PCR gel is shown in Figure 1. The presence of *V. alginolyticus* and *V. vulnificus* was confirmed by amplifying the specific regions of 412 bp for *V. vulnificus* and 144 bp for *V. alginolyticus*. *V. cholerae* and *V. parahaemolyticus* were not detected in multiplex PCR. The annealing temperature, extension time, and primer concentrations used in this multiplex PCR assay were optimized.

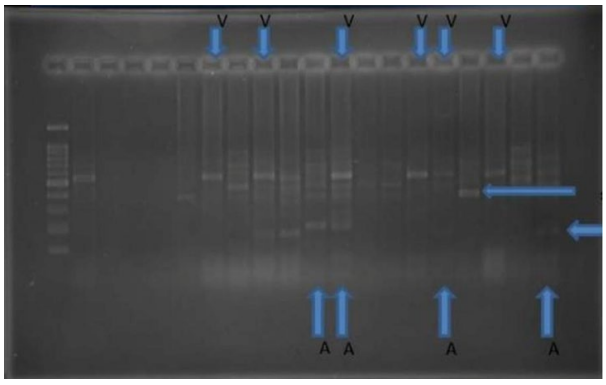

Figure 1. Result of the multiplex PCR

Discussion

Low-quality water is being used for agricultural purposes in some countries (Farhadkhani *et al.*, 2020). People who use food products irrigated by this water are exposed to potential foodborne hazards, one of which is *Vibrio spp.* as the most common bacteria in surface waters (Adeleye *et al.*, 2010)

Vibrio spp. can be transmitted to humans via ingesting raw, undercooked, or ready-to-eat products (Daniels and Shafaie, 2000; Beshiru *et al.*, 2020). *Vibrio spp.* have been recognized as a common cause of foodborne outbreaks in a wide range of Asian countries (Tunung *et al.*, 2011; Letchumanan, Chan, and Lee, 2014). Due to the fact that sewage water is being used for irrigating vegetables in Iran, people in this country are potentially exposed to cholera infections. In recent years, many outbreaks have been reported from Iran, especially during 2009-2014 (Asl *et al.*, no date; Marashi *et al.*, 2012).

In spite of the efforts by health officials to control cholera in Iran, the incidence is still high (Mousavi *et al.*, 2008). An effective way to prevent a cholera outbreak is to rapidly diagnose the infection. Conventional techniques used for this purpose are usually time-consuming and labor-intensive (Singh *et al.*, 2002). In recent years, PCR techniques have been used extensively in various studies to detect pathogenic bacteria, including *vibrio spp.* because they are more rapid and sensitive than conventional plate culturing methods (Bonnin-Jusserand *et al.*, 2019). We aimed to confirm the isolates detected by culturing methods using multiplex PCR. In the present study, five specific genes were used, namely *VC-Rmm* of *V. cholerae*, *VP-MmR* of *V. parahaemolyticus*, *VV-Rmm* of *V.vulnificus, V.al2-MmR* of *V.alginolyticus,* and *VM-F* for all the four isolates.

Hossain *et al.* (2013) developed a *groEL* gene-based multiplex PCR to detect *V. cholera*, *V. parahaemolyticus*, and *V. vulnificus* simultaneously. The latter authors revealed that the multiplex PCR technique is highly cost-effective and useful for the detection of *Vibrio spp.* Alishahi *et al.* (2013) similarly concluded that multiplex PCR is a rapid, easy to use, reliable, and cost-effective tool to detect *V. cholera.*

Bej *et al.* (1999) developed a multiplex PCR assay for detecting total and virulent strains of *V. parahaemolyticus* utilizing *tl*, *tdh*, and *trh* genes. Their study indicated that multiplex PCR can be a highly successful method for detecting *V. parahaemolyticus* strains in shellfish. In addition, these authors observed that multiplex PCR was more reliable and more rapid, compared to the conventional methods. The advantage of the present study over the mentioned investigation is that we detected four species of *Vibrio* instead of one. Moreover, we showed that multiplex PCR can be considerably more reliable for detecting *Vibrio spp*., in comparison with the conventional methods because *V. vulnificus* was detected in more samples using multiplex PCR.

In another study, real-time PCR was used to detect the *tdh* gene of *V. parahaemolyticus* in oyster enrichments. It was concluded that real-time PCR was more reliable, less time-consuming, and less labor-intensive, in comparison to the streak plate/probe method. The superiority of the current study over the latter study is that qualitative multiplex PCR is less costly and easier to use than quantitative real-time PCR (Blackstone *et al.*, 2003).

Masini *et al.* (2007) studied the occurrence and pathogenicity of *Vibrio spp.* in Conero Riviera. They used TCBS by the membrane filter method and three target genes, namely *ctx*, *trh*, and *tdh* were detected by PCR. In the present study, water samples were subject to the membrane filter method and were cultured on TCBS medium. Afterwards, five target genes, including *VC-Rmm*, *VP-MmR*, *VV-Rmm, V.al2-MmR*, and *VM-F* were detected using PCR. Masini *et al.* reported that one isolate of *V. algynoliticus* and one isolate of *V. harveyi* possessed the *trh*

gene. The other isolate of *V. harveyi* and one isolate of *V. parahaemolyticus* had the *ctx* gene. In our study, *V. vulnificus,* and *V. alginolyticus* were detected to possess *VV-Rmm* and *V.al2-MmR* genes, respectively. In addition, they both had the *VM-F* gene. The results of the study performed by Masini *et al.* indicated the presence of potentially pathogenic *Vibrio spp.* in water, which is in agreement with the present study.

Neogi *et al.* (2010) targeted *toxR* for detecting *V. cholerae* and *V. parahaemolyticus*. Moreover, these authors targeted *vvhA* for the detection of *V. vulnificus.* In the current investigation, five target genes were used to detect four species indicating the good potential of our method.

Conclusion

In conclusion, the multiplex PCR assay developed in the present study is rapid, simple, and convenient. This technique provides the possibility of successful detection and differentiation of *Vibrio spp.* Therefore, it could be considered as an efficient method for detecting *Vibrio spp.*

Acknowledgments

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Conflict of Interest

The authors declared no conflict of interest.

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ارزیابی واکنش زنجیره‌ای پلیمراز چندگانه برای تشخیص همزمان گونه‌های ویبریو در سبزیجات و آب

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زمینه مطالعه: طغیان‌های غذازاد متعددی مرتبط با مصرف سبزیجات گزارش شده‌اند که گونه‌های ویبریو، عامل ایجاد آنها بوده‌اند. به دلیل اینکه روش‌های رایج تشخیصی، زمان‌بر هستند، تشخیص سریع این میکروارگانیسم‌ها، اهمیت بالایی دارد.

هدف: مطالعه حاضر، روشی سریع و قابل اعتماد را برای شناسایی *ویبریو کلرا*، *ویبریو پاراهمولیتیکوس*، *ویبریو وولنیفکوس* و *ویبریو آلجینولیتیکوس* ارائه می‌دهد و نتایج این روش را با نتایج روش‌های رایج کشت، مقایسه می‌کند.

روش کار: تست‌های رایج باکتریولوژیک انجام شدند تا گونه‌های ویبریو را در سبزیجات و آبی که آنها را احاطه می‌کند مورد بررسی قرار دهند. نمونه‌های همچنین تحت مالتیپلکس پی سی آر قرار گرفتند که در این روش از پنج ژن استفاده شد که شامل VC-Rmm برای *ویبریو کلرا*، VP-MmRبرای *ویبریو پاراهمولیتیکوس*، VV-Rmm برای *ویبریو وولنیفکوس*، V.al2-Mmr برای *ویبریو آلجینولیتیکوس* و VM-F برای هر چهار ایزوله بودند.

نتایج: حضور *ویبریو آلجینولیتیکوس* و *ویبریو وولنیفکوس* توسط محل‌های اختصاصی تقویت‌کنندگی (412bp برای *ویبریو وولنیفکوس* و ۱۴۴ bp برای *ویبریو آلجینولیتیکوس*) تأیید شدند. *ویبریو کلرا* و *ویبریو پاراهمولیتیکوس* توسط مالتیپلکس پی سی آر شناسایی نشدند که این یافته با نتایج روش‌های کشت متداول، مطابقت داشت.

نتیجه گیری نهایی: نتایج به دست آمده، نشان داد که مالتیپلکس پی سی آر طراحی شده در این مطالعه، روشی قابل اعتماد، سریع و مقرون به صرفه برای شناسایی هم زمان گونه‌های ویبریو است.

واژه‌های کلیدی: طغیان‌های غذازاد، مالتیپلکس پی سی آر، تشخیص همزمان، سبزیجات، سویه‌های ویبریو

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