巢式 PCR 快速检测海产品中的副溶血弧菌

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摘要：副溶血弧菌是一种世界范围性的食源性致病菌，食用了该菌污染的海产品可导致胃肠炎等疾病。为了建立一种可快速、特异地检测海产品中副溶血弧菌的方法，通过把副溶血弧菌基因组序列和其它不同种类弧菌的基因组序列进行比较分析，筛选出了一个副溶血弧菌特异性的标记基因-VP1331，根据该基因建立了副溶血弧菌的巢式 PCR 快速检测方法，并评估了其特异性、敏感性和稳定性。实验结果表明，该方法只有在以副溶血弧菌基因组 DNA 为模板时才能扩增出目的片段，而其它 11 种弧菌和非弧菌均不能扩增出目的片段。该方法的最低检测限为副溶血弧菌基因组 DNA 10 fg、纯培养物 6.6 CFU。人工污染实验表明，初始菌液浓度为 25.7 CFU/100 mL 时只需经过 2 h 的增菌培养即可检出。上述结果表明，VP1331 基因可以作为副溶血弧菌种特异性标记，本方法可以用于污染海产品中该菌的检测与鉴定。

关键词：副溶血弧菌；食源性致病菌；PCR；检测；鉴定；海产品
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Rapid Identification of *Vibrio parahaemolyticus* in Seafood by Nested PCR

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Abstract: *Vibrio parahaemolyticus* is a food-borne pathogen that causes gastroenteritis and other diseases worldwide, with consumption of contaminated raw or undercooked seafood. To develop a method for rapid and specific detection of *V. parahaemolyticus* in seafood, its genomic sequence was analyzed and compared with those of other *Vibrio* species, a *V. parahaemolyticus*-specific genetic marker was screened, and a nested-PCR-based method to rapid detect *V. parahaemolyticus* was established based on this gene. Additionally, the specificity, sensitivity, and reproducibility of this method were assessed. The results showed that the target amplicon only appeared with *V. parahaemolyticus* genomic DNA as the template, but not the other 11 *Vibrio* species and non-*Vibrio* species. The limits of detection of this method were 10 fg *V. parahaemolyticus* genomic DNA and 6.6 colony-forming units (CFU) for purified culture. Artificial contamination experiments indicated that *V. parahaemolyticus* could be detected after two hours of enrichment with an initial concentration of 25.7 CFU/100 mL. In conclusion, the VP1331 gene can be used as a *V. parahaemolyticus*-specific marker, and this PCR method can be used for the detection and identification of *V. parahaemolyticus* in contaminated seafood.

Key words: *Vibrio parahaemolyticus*; foodborne pathogens; polymerase chain reaction (PCR); detection; identification; seafood

Vibrio parahaemolyticus is a gram-negative halophilic bacterium naturally inhabiting warm marine and estuarine environments worldwide[1,2]. It is recognized as the leading cause of human gastroenteritis associated with consumption of raw or undercooked seafood in many countries[2~4]. With the sustained increase in international trade of seafood and reported outbreaks of foodborne disease caused by *V. parahaemolyticus* worldwide[5,6], this bacterium has become a significant concern for seafood safety and risk of infection. Typical clinical symptoms of patients

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infected with *V. parahaemolyticus* consist of headache, watery or bloody diarrhea, dehydration, abdominal cramps, vomiting, nausea, fever, and chills within 24 h after consumption of seafood[7]. *V. parahaemolyticus* can also cause life-threatening septicemia, wound infection, otitis, and necrotizing fasciitis when skin lesions are exposed to contaminated seafood or water[5]. Considering the sanitary significance, the establishment of specific, sensitive, and rapid detection methods is important to public health. However, conventional cultural and biochemical-based approaches for isolation and identification of *V. parahaemolyticus* are labor intensive and time consuming, usually requiring 4~7 days for enrichment, plating onto selective agar plates, and biochemical tests to differentiate from other *Vibrio* species of similar colony morphology and characteristics[4]. Alternatively, nucleic acid-based rapid detection methods, represented by the polymerase chain reaction (PCR), have been routinely used owing to the advantages of efficiency, low cost, high specificity and sensitivity[8,9]. In the past few decades, a large number of PCR assays with various targeting genes have been evaluated for rapid detection of *V. parahaemolyticus*[8,9]. However, false-positive amplifications giving unexpected PCR products are still commonly encountered problems due to primers binding to incorrect regions of target DNA. Therefore, it is essential to identify distinct and more specific molecular markers to establish an advanced assay for accurate identification of *V. parahaemolyticus*. Nested PCR was developed to reduce non-specific products generated by amplification of unexpected primer binding sites. Nested PCR involves two sets of primers, used in two successive rounds of PCR; the second set is intended to amplify a secondary target within the first-round product. It is very unlikely that any of the undesired PCR products contain binding sites for the second-round primers, thus ensuring that the product of the second PCR has negligible contamination from unwanted products of primer dimers, hairpins, and alternative primer target sequences.

This study describes the development and validation of a novel single-tube nested PCR that is more sensitive, specific, rapid, and cost-effective for detection of *V. parahaemolyticus* in seafood than traditional PCR.

1 Materials and Methods

1.1 Bacterial strains

Two reference strains of *V. parahaemolyticus* were used to optimize the nested PCR assay, and six other *Vibrio* reference strains and seven other common foodborne bacteria were used as positive and negative controls, respectively, to evaluate the specificity of the nested PCR method (listed in Table 1). An additional 126 isolates, including 114 *V. parahaemolyticus* from seafood and 12 from clinical samples collected during 2012 and 2013 were utilized to confirm the efficiency of the nested PCR assay. The 126 putative *V. parahaemolyticus* isolates were cultured in alkaline peptone water (APW, 1% peptone, 1% NaCl, pH 8.5) at 28 ℃ overnight and identified by their colony morphology on thiosulfate citrate bile salt sucrose (TCBS) agar and CHROMagar Vibrio chromogenic medium (CHROMagar, Paris, France), biochemical tests according to Bergey's Manual of Systematic Bacteriology, and 16S rRNA gene sequencing. The reference strains and isolates were stored in APW for *Vibrio* species or brain-heart infusion broth (Beijing Land Bridge Technology Co. Ltd, Beijing, P. R. China) for other strains with 15% glycerol at -80 ℃.

1.2 DNA extraction

Genomic DNA was extracted from 1.5 mL overnight cultures of reference strains at about 10^8 CFU/mL, isolate strains, and enriched artificially infected seafood. Briefly, the aliquots of cell suspension were centrifuged at 15,000 × *g* for 5 min (Eppendorf 5810R, Hamburg, Germany), and the cell pellets were rinsed three times with 200 μL of ddH_2O. The final suspension in 200 μL ddH_2O was boiled at 100 ℃ for 5 min and then cooled on ice for 10 min. The cell lysate was centrifuged, and the supernatant was transferred to a new tube and stored at -20 ℃ until use.

1.3 Oligonucleotide primers

The genomic sequences of *Vibrio* species were compared and analyzed with other bacteria (ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/) using the Basic Local Alignment Search Tool (BLAST) and Mauve 2.3.1 software (http://gel.ahabs.wisc.edu/mauve/). The VP1331 gene (GenBank Accession Number: NC_004603), which encodes the small subunit of

D-amino acid dehydrogenase, was screened as a specific marker. Subsequently, two sets of primers were designed using Primer Premier 5 software (Premier Biosoft International, Palo Alto, CA, USA) targeting VP1331. The sequences of external primers (Outer F and R) and internal primers (Inner F and R) and a schematic diagram of primer binding sites are summarized in Table 1 and Figure 1, respectively. All primers were synthesized commercially by Sangon Biotech, Shanghai, P. R. China.

Table 1 Nucleotide sequences of primers designed in this study

Primer	Sequence (5'-3')	Primer Tm/℃	Amplicon size /bp
Outer F	GGAATTCGCAGTGATCGCCGCTTGAG	75.3	1164
Outer R	CGCGTCGACAGCATCGCGGTTATAGG	75.8	
Inner F	CGCAGAACTGATAGGAAACGGCAATG	70	403
Inner R	TGATGCGTTAGCGGAACAAGGTG	67	

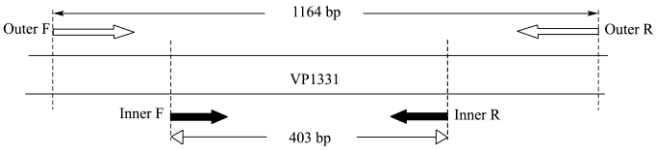

Fig.1 Schematic diagram of primer biding sites in the VP1331 nucleotide sequence (GenBank accession number NC_004603)

Note: The first round of nested PCR yields a 1164-bp fragment product from the external primers (Outer F and R). A second nested PCR cycle incorporates the external primers (Inner F and R) to yield a 403-bp product.

1.4 Specificity test of primers

Table 2 Bacterial strains used to assess the specificity of the nested PCR assay

	Species	Source of reference	PCR
1	*V. parahaemolyticus*	ATCC 17802	+
2	*V. parahaemolyticus*	ATCC 33847	+
3	*V. alginolyticus*	ATCC 33787	-
4	*V. cholerae*	ATCC 14035	-
5	*V. fluvialis*	ATCC 33810	-
6	*V. harveyi*	ATCC 33842	-
7	*V. mimicus*	ATCC 33653	-
8	*V. vulnificus*	ATCC 27562	-
9	*Enterococcus faecium*	ATCC 27270	-
10	*Escherichia coli*	ATCC 43888	-
11	*Staphylococcus aureus*	ATCC 6538	-
12	*Salmonella typhimurium*	ATCC 13311	-
13	*Shigella flexneri*	ATCC 1333	-
14	*Listeria monocytogenes*	ATCC 7644	-
15	*Pseudomonas aeruginosa*	ATCC 15442	-

The specificity of the primers was examined using the National Center for Biotechnology Information (NCBI) GenBank database Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and then confirmed using 2 *V. parahaemolyticus*, 6 other closely related *Vibrio* species, 7 non-*Vibrio* spp. listed in Table 2, and 126 isolates, including 114 seafood and 12 clinical isolates, which are not listed.

1.5 Sensitivity and reproducibility test

A sensitivity test was undertaken with bacterial cultures and purified genomic DNA. Reference strain *V. parahaemolyticus* ATCC 17802 was cultured overnight at 37 ℃ in APW. It was then 10-fold diluted, and the CFU number was determined by plate count. Meanwhile, 1 mL of each dilution was collected to extract DNA as described above.

The concentration of chromosomal DNA (10 ng) in the original solution extracted from a pure culture of *V. parahaemolyticus* ATCC 17802 was measured using a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) and then the DNA was 10-fold serially diluted (10 ng/μL to 10 fg/μL) in sterile distilled water. PCR amplification was performed with optimized parameters as described below.

The reproducibility of the entire nested PCR assay was examined by performing in triplicate and with three biological replicates on different days.

1.6 PCR strategy

The PCR assay consisted of two rounds of reactions and was performed and optimized using an Eppendorf PCR system in 30-μL containing 2× *Taq* PCR Master Mix (Sangon Biotech). In the first round, each 30-μL reaction contained 1.25 U *Taq* DNA polymerase, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L each of dATP, dGTP, dCTP, and dTTP, 0.3 μmol/L of each external primer, and

approximately 1 ng genomic DNA. An identical reaction mixture without genomic DNA was performed as a negative control.

The nested PCR thermal cycling conditions were: initial pre-denaturation at 94 ℃ for 3 min, followed by 30 amplification cycles of denaturation at 94 ℃ for 30 s, annealing at 59.6 ℃ for 1 min, extension at 72 ℃ for 75 s, and a final extension for 8 min at 72 ℃.

In the second round of amplification, 1 μL of the first-round PCR and 0.3 μmol/L of each internal primer were added to the reaction mixture instead of genomic DNA and external primers, respectively, and the reaction was performed with another 30 amplification cycles of denaturation at 94 ℃ for 30 s, annealing at 56 ℃ for 30 s, extension at 72 ℃ for 30 s, and a final extension at 72 ℃ for 8 min.

The PCR products were electrophoresed in 1% (*m/V*) agarose gels, stained with 0.5 μg/mL of ethidium bromide, and visualized under UV light.

1.7 Detection of *V. parahaemolyticus* in artificially inoculated seafood and samples purchased from a supermarket

In each of four groups, 25 g of oysters without *V. parahaemolyticus* (confirmed by biochemical tests) were homogenized and mixed with 225 mL of 3% NaCl (*m/V*) APW and then seeded with *V. parahaemolyticus* at initial cell densities ranging from 2.57×10^{-1} to 2.57×10^{3} CFU/mL. Each group was incubated overnight for enrichment at 180 r/min and 37 ℃. Every hour during incubation, 1 mL of the culture was obtained to extract DNA and perform nested PCR.

1.8 Prevalence of *V. parahaemolyticus* in natural samples purchased from a local supermarket

One hundred seafood samples (shellfish and fish) were purchased from a local supermarket to detect *V. parahaemolyticus* after homogenization and enrichment as described above. The nested PCR was compared with a recently reported conventional PCR method[9]. The results of both assays were confirmed by biochemical tests according to the Bergey's Manual of Systematic Bacteriology and amplifying the 16S rRNA of PCR-positive samples.

2 Results

2.1 Specificity of primers

DNA fragments of 1100 bp and 400 bp in length were amplified using external (Outer F and Outer R) and internal primers (Inner F and Inner R), respectively (Figs. 2 and 3). These two fragments were detected in *V. parahaemolyticus* strains ATCC 17802 and ATCC 33847, and 126 isolated strains after PCR, but no amplification was observed for the other *Vibrio* and non-*Vibrio* species.

Fig. 2. Agarose (1%) gel electrophoresis of PCR products amplified using primers Outer F and Outer R

Note: M, DL2000 DNA ladder marker; lane 1, *V. parahaemolyticus* ATCC 17802; lane 2, *V. parahaemolyticus* ATCC 33847; lane 3, *V. alginolyticus* ATCC 33787; lane 4, *V. cholerae* ATCC 14035; lane 5, *V. fluvialis* ATCC 33810; lane 6, *V. harveyi* ATCC 33842; lane 7, *V. mimicus* ATCC 33653; lane 8, *V. vulnificus* ATCC 27562; lane 9, *Enterococcus faecium* ATCC 27270; lane 10, *Escherichia coli* ATCC 43888; lane 11, *Staphylococcus aureus* ATCC 6538; lane 12, *Salmonella typhimurium* ATCC 13311; lane 13, *Shigella flexneri* ATCC 1333; lane 14, *Listeria monocytogenes* ATCC 7644; lane 15, *Pseudomonas aeruginosa* ATCC 15442; lane 16, negative control.

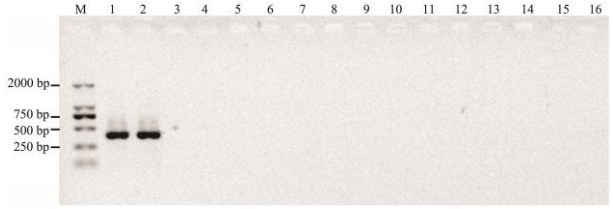

Fig.3 Agarose (1%) gel electrophoresis of PCR products using internal primers

Note: M, DL2000 DNA ladder marker; lane 1, *V. parahaemolyticus* ATCC 17802; lane 2, *V. parahaemolyticus* ATCC 33847; lane 3, *V. alginolyticus* ATCC 33787; lane 4, *V. cholerae* ATCC 14035; lane 5, *V. fluvialis* ATCC 33810; lane 6, *V. harveyi* ATCC 33842; lane 7, *V. mimicus* ATCC 33653; lane 8, *V. vulnificus* ATCC 27562; lane 9, *Enterococcus faecium* ATCC 27270; lane 10, *Escherichia coli* ATCC 43888; lane 11, *Staphylococcus aureus* ATCC 6538; lane 12, *Salmonella typhimurium* ATCC 13311; lane 13, *Shigella flexneri* ATCC 1333; lane 14, *Listeria monocytogenes* ATCC 7644; lane 15, *Pseudomonas aeruginosa* ATCC 15442; lane 16, negative control.

2.2 Sensitivity of nested PCR protocol

The sensitivity of the nested PCR assay was investigated using 10-fold serially diluted purified DNA and bacterial cultures. The detection limit was as little as 100 fg of total DNA (Fig. 4) and 6.62 CFU (Fig. 5). The nested PCR assay displayed excellent reproducibility.

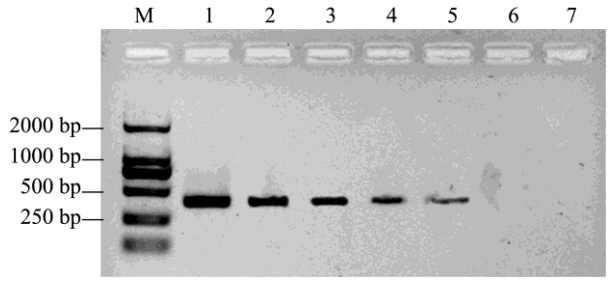

Fig.4 Sensitivity evaluation of nested PCR primers for the VP1331 gene as a *V. parahaemolyticus*-specific marker at various amounts of genomic DNA

Note: M, D2000 DNA ladder marker; lane 1, 100 pg; lane 2, 10 pg; lane 3, 1 pg; lane 4, 100 fg; lane 5, 10 fg; lane 6, 1 fg; lane 7, negative control.

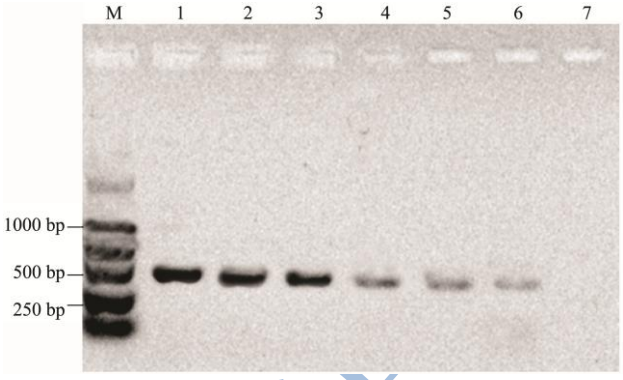

Fig.5 Sensitivity of the nested PCR assay for the amplification of target amplicons at various concentrations of pure *V. parahaemolyticus* cultures

Note: M, D2000 DNA ladder marker; lane 1, 6.62×10^5 CFU/mL; lane 2, 6.62×10^4 CFU/mL; lane 3, 6.62×10^3 CFU/mL; lane 4, 6.62×10^2 CFU/mL; lane 5, 6.62×10^1 CFU/mL; lane 6, 6.62 CFU/mL; lane 7, 6.62×10^{-1} CFU/mL.

2.3 Detection of *V. parahaemolyticus* in artificially inoculated seafood

The nested PCR assay established for this study could accurately and specifically detect *V. parahaemolyticus* in all artificially inoculated seafood. The 510 bp amplification product could be produced after enrichment for 3 h in the homogenized oysters seeded with 26 CFU per 100 mL of *V. parahaemolyticus* (Fig. 6).

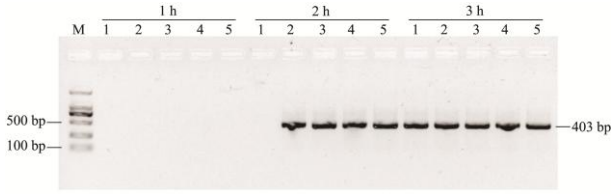

Fig. 6. Detection limit of nested PCR assay targeting the VP1331 gene of *V. parahaemolyticus* from artificially inoculated seafood after different enrichment times

Note: M, D2000 DNA ladder marker; lane 1, 2.6 CFU per 100 mL; lane 2, 2.6×10^1 CFU per 100 mL; lane 3, 2.6×10^2 CFU per 100 mL; lane 4, 2.6×10^3 CFU per 100 mL; lane 5, 2.6×10^4 CFU per 100 mL; 1 h, 2 h, and 3 h indicate the enrichment times.

2.4 Investigation of *V. parahaemolyticus* in natural samples purchased from a local supermarket

Samples from a local supermarket were used to compare the effectiveness of nested PCR and normal PCR. The results indicate that 66 samples were positively identified as *V. parahaemolyticus* by nested PCR compared to 52 positively identified samples using conventional PCR (Table 3). Finally, biochemical tests and sequence analysis both showed that these 66 positive samples all contained *V. parahaemolyticus*.

Table 3 Comparison between nested PCR and conventional PCR for detection of *V. parahaemolyticus* in seafood.

	Sea foods	Isolation rate
Total No.	100	
Positive No. with nested PCR	66	66%
Positive No. with ordinary PCR	52	52%

3 Discussion

3.1 *V. parahaemolyticus* is one of the most important foodborne pathogens that poses a severe threat to food safety, public health, and leads to substantial economic losses worldwide[10]. Several methods have been employed to detect *V. parahaemolyticus*, including PCR[8,9,11,12]. PCR has been reported to detect various pathogens for decades; it is less time consuming, much simpler, and more economical than traditional culture-based methods. Yu *et al.*[8] developed a PCR method for detecting the *irgB* gene to distinguish *V. parahaemolyticus* from other *Vibrio* species and

non-*Vibrio* bacterial strains. This PCR assay produced a 369-bp fragment, and the sensitivity was 0.17 pg purified genomic DNA from *V. parahaemolyticus*. Kang *et al*.[13] established a two-step ultrarapid real-time PCR assay with a microchip to detect *V. parahaemolyticus* carrying the *tdh* gene. This method used 6-μL reaction volumes and total run times of about 6 min. To the best of our knowledge, this is the most rapid detection approach thus far reported. Copin *et al*.[14] evaluated a PCR assay using most-probable number enrichment cultures for the detection of total and pathogenic *V. parahaemolyticus* in frozen shrimp. The detection limit was one cell per gram and can be expected to be completed within two days.

3.2 In the present study, a highly specific nested PCR assay was established to immediately detect *V. parahaemolyticus* in food samples, targeting the VP1331 gene, which was aligned as the specific gene of *V. parahaemolyticus*. The results show that this assay could detect *V. parahaemolyticus* in both pure culture broth and artificially infected seafood samples, and the detection limit was 10 fg with purified DNA and 6.6 CFU with pure culture. As shown in this study and a previous report[11], this nested PCR detection limit is lower than that of conventional PCR. This nested PCR protocol can be completed in about five hours, including half an hour for sample processing, two hours for bacterial enrichment, two hours for PCR, and half an hour for electrophoretic analysis. Therefore, the nested PCR assay is relatively time efficient. The number of nested PCR cycles from 30 to 27 and 25 to save time, but no bands were detected; thus, 30 cycles were determined to be optimal to avoid non-specifically amplified bands. As the results show in the detection of one hundred specimens purchased from a local supermarket, the isolation rate between nested PCR and conventional PCR was different and the nested PCR showed higher sensitivity and accuracy. In conclusion, this nested PCR can be invoked as a robust tool for the detection of *V. parahaemolyticus* in seafood.

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