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A Real-Time PCR with Melting Curve Analysis for Molecular Typing of *Vibrio parahaemolyticus*

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Abstract
Foodborne disease caused by *Vibrio parahaemolyticus* is a serious public health problem in many countries. Molecular typing has a great scientific significance and application value for epidemiological research of *V. parahaemolyticus*. In this study, a real-time PCR with melting curve analysis was established for molecular typing of *V. parahaemolyticus*. Eighteen large variably presented gene clusters (LVPCs) of *V. parahaemolyticus* which have different distributions in the genome of different strains were selected as targets. Primer pairs of 18 LVPCs were distributed into three tubes. To validate this newly developed assay, we tested 53 *Vibrio parahaemolyticus* strains, which were classified in 13 different types. Furthermore, cluster analysis using NTSYS PC 2.02 software could divide 53 *V. parahaemolyticus* strains into six clusters at a relative similarity coefficient of 0.85. This method is fast, simple, and conveniently for molecular typing of *V. parahaemolyticus*.

Introduction

Vibrio parahaemolyticus is a Gram-negative halophilic bacterium that thrives in seawater and seafood, and eating food contaminated by this bacterium can cause food poisoning. In coastal countries such as China [1], the United States [2], and Japan [3], this organism has become a crucial foodborne pathogen. In China's coastal provinces, most of bacterial food poisoning cases are caused by *V. parahaemolyticus*. With the increase in reported cases of food poisoning caused by *V. parahaemolyticus*, its epidemiological investigation has attracted more and more attention. Bacterial typing is an important method of epidemiological research and it is of great scientific significance and application value to judge the genetic relationship between strains, trace the source of pathogens, cut off the route of transmission, and draw up effective countermeasures.

Currently, the typing methods of *V. parahaemolyticus* can be classified into two types: phenotypic typing and molecular typing. The method of phenotypic typing has been unable to meet the needs of *V. parahaemolyticus* epidemiological research because it contains tedious and time-consuming operations. Furthermore, these operations require experience for interpretation and are limited by subjectivity and low specificity. In recent years, with the development of molecular biology technology, molecular typing assays have played an increasingly important role in epidemiological research, such as pulsed-field gel electrophoresis (PFGE), multilocus sequencing typing (MLST) and multilocus variable number tandem repeat analysis (MLVA), random amplified polymorphic DNA (RAPD), ribotyping, arbitrarily primed PCR (AP-PCR), amplified fragment length polymorphism (AFLP), and enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) [4–6]. Compared with the phenotypic typing method, these methods have advantages of rapid and high specificity, but they all are still laborious because of the need for electrophoretic analysis.

Real-time PCR can detect samples by monitoring the fluorescence changes and has advantages of simple operation and high degree of automation due to no tedious electrophoretic analysis. It has been widely used in the detection of *V. parahaemolyticus* and other pathogens [7–9], but there is too little research on molecular typing using real-time PCR. Genomic polymorphisms analysis and epidemiological research of *V. parahaemolyticus* by microarray-based comparative genome hybridization (M-CGH) have disclosed 18 large variably presented gene clusters (LVPCs), which

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is variably distributed within the genomes of different *V. parahaemolyticus* strains [10, 11]. Xiao et al., applied these 18 LVPCs to group 251 global strains of *V. parahaemolyticus* using conventional PCR and gel electrophoresis and the results demonstrated checking LVPCs presence or absence gave a resolution for discriminating *V. parahaemolyticus* strains [12]. The aim of this study was to establish a method by using real-time PCR to check LVPCs presence or absence for molecular typing of *V. parahaemolyticus*.

Materials and Methods

Bacterial Strains

A total of 53 *V. parahaemolyticus* strains were used in this study, all strains were isolated by our laboratory from food or patients with diarrhea in Jiaxing, China (Table 1) and confirmed by biochemical characterization. All strains were cultured and maintained on thiosulfate citrate bile salts sucrose (TCBS) plates. Genomic DNA of *V. parahaemolyticus* strains was extracted with KAPA Express Extract Kits (KAPA Biosystems, Woburn, USA) according to the manufacturer's instruction.

Oligonucleotide Primers

Sequences of 18 LVPCs were downloaded from Genbank (http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html) and primers of target genes were designed using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA). The sequences of primer pairs of target LVPCs used in this study are shown in Table 2.

Optimization of Multiplex Real-Time PCR

Primer pairs of 18 LVPCs were distributed into three tubes and the distribution of primer pairs is also listed in Table 2. The multiplex real-time PCR was optimized by varying a single parameter while other parameters were maintained constant. Briefly, the parameters evaluated include target primer concentration from 0.1 to 0.8 µM and annealing/extension temperature from 55 to 65 °C.

Multiplex real-time PCR was performed in a 20 µl reaction mixture for each tube containing 1 µl DNA template, 3 µl EvaGreen dye, 10 µl KAPA 2G Fast Multiplex PCR Mix, moderate target primers, and corresponding distilled water. The PCR amplification was performed in a Bio-Rad CFX 96™ real-time PCR system and the program was as follows: 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 15 s, annealing temperature for 30 s, and extension at 72 °C for 5 s. Fluorescence signals were measured after extension step of each cycle.

Melting Temperature Curve Analysis

Fluorescence melting temperature curve analysis was performed after PCR amplification. The PCR products were kept to 77 °C and were then heated to 93 °C at a rate of 0.1 °C/s. Fluorescence signals were continuously monitored during the whole process of melting temperature curve analysis.

Data Analysis

Scores of '0' and '1' were attributed to the absence or the presence of a LVPC, respectively, for data obtained from multiplex PCR amplification and melting temperature curve analysis. A cluster analysis was performed by NTSYS PC 2.02 software (Exeter Software, East Setauket, NY) and the unweighted-pair group method using arithmetic averages (UPGMA).

Results

Optimization of Multiplex Real-Time PCR

In order to develop a multiplex real-time RT-PCR that can identify the presence of *V. parahaemolyticus* LVPCs, a systematic study was performed to optimize the conditions. The optimal primer combination was 0.5 µM of VP0383, 0.04 µM of VP1091, 0.12 µM of VP1778, 0.25 µM of VP2902, 0.26 µM of VPA0074, and 0.16 µM of VPA0716 for tube 1; 0.12 µM of VP0635, 0.6 µM of VP1393, 0.15 µM of VP1563, 0.18 µM of VPA0895, 0.12 µM of VPA 1336, and 0.18 µM of VPA1708 for tube 2; 0.02 µM of VP

Table 1 Strains used in this study

Origin	Strain number
Food	VP6, VP26, VP29, VP32, VP34, VP43, VP44
Patient	VP1, VP2, VP3, VP4, VP5, VP7, VP8, VP9, VP10, VP11, VP12, VP13, VP14, VP15, VP16, VP17, VP18, VP19, VP20, VP21, VP22, VP23, VP24, VP25, VP27, VP28, VP30, VP31, VP33, VP35, VP36, VP37, VP38, VP39, VP40, VP41, VP42, VP45, VP46, VP47, VP48, VP49, VP50, VP51, VP52, VP53

Table 2 The primer sequences of 18 LVPCs

	Genomic locus	PCR target	Primers	
			Name	Nucleotide sequence (5′-3′)
Tube 1	LVPC01 (VP0380-0403)	VP0383	VP0383F VP0383R	CTCTCCTCGTCGCAAGCATTTAGC GAGCGGGTGTGCAACGGTGT
	LVPC03 (VP1071-1095)	VP1091	VP1091F VP1091R	GAACCACGCTATCGCTGCTG CGTTACCTGTGGGCGGACTT
	LVPC07 (VP1771-1864)	VP1778	VP1778F VP1778R	CTAAAATGTGGTTGGCGTATGC TGACGAATGGCGACAAAGATC
	LVPC09 (VP2900-2910)	VP2902	VP2902F VP2902R	TCAGCAACTAACAGCAAACATAAAATC GCCATCCACAATCAAAGTGTCTAAA
	LVPC10(VPA0074-0089)	VPA0074	VPA0074F VPA0074R	CGCACGCTAAGGCATCACTG CCAAGTAGAGGATTTCCCCGC
	LVPC12(VPA0713-0732)	VPA0716	VPA0716F VPA0716R	AAACCAATAAGAAAAACCTCCGTGT CTGATCCCCCCTCGACCAA
Tube 2	LVPC02 (VP0634-0643)	VP0635	VP0635F VP0635R	GCCATAAAATCGGGCAGCA GGCAACACCAACCTACCAAAAC
	LVPC05 (VP1386-1420)	VP1393	VP1393F VP1393R	GTCTATCAACGGTGAAACTCAAGGT AGAAACGAATCAAACACTCAGGAAG
	LVPC06 (VP1549-1590)	VP1563	VP1563F VP1563R	ATCATTCCGCACACTCTTTTGG AGTTTTTCTGCCATTGGATTGATAG
	LVPC13(VPA0887-0914)	VPA0895	VPA0895F VPA0895R	GTGTCACCCGCCGTGCCTG CGCCGCAACCCGTGAATG
	LVPC16(VPA1334-1370)	VPA1336	VPA1336F VPA1336R	TTTCTAATCTAACTTTCACCCCCTCT CCGTCATCCTACGAAAAATGTCTAT
	LVPC17(VPA1700-1709)	VPA1708	VPA1708F VPA1708R	GTCATCCCTGTTATCGCCATCG TGCGGCTTCAACCGTGCT
Tube 3	LVPC04 (VP1351-1368)	VP1351	VP1351F VP1351R	GCAACCAAGAAAAATGGGACTCGTG CGACATGGTTGACATAAGCACTGCC
	LVPC08 (VP2131-2144)	VP2132	VP2132F VP2132R	AGGCTCTTGGATACGAGGTTGATAC AGTACGGTGTATTACGGCGGTCT
	LVPC11(VPA0434-0458)	VPA0440	VPA0440F VPA0440R	ACACAACTTCAAACGACACATCTATTACA GCCAGTCCTGCGAACCGAG
	LVPC14(VPA1194-1210)	VPA1199	VPA1199F VPA1199R	TAGCGTTGGTATGTTTGGCTCTGG CAACTCGAATGAACGGTGGTAGTAAGTT
	LVPC15(VPA1253-1270)	VPA1256	VPA1256F VPA1256R	TACAAGCCTATCGCTGCCAAT CGAAGAAGAAGCACCAGAAACC
	LVPC18(RPI01-74)	RPI08	RPI08F RPI08R	TCCACTTTTGGCAGCATACCTTTG GCCCTGGCATCACCCGCTC

1351, 0.4 μM of VP2132, 0.1 μM of VPA0440, 0.1 μM of VPA1199, 0.26 μM of VPA1256, and 0.4 μM of RPI08 for tube 3. The optimal annealing/extension temperature was 62 °C.

PCR Amplification and Melting Temperature Curve Analysis

The PCR analyses were performed in five different runs. The VP0383 primer pairs produced PCR products with melting peak at 81.8 ± 0.5 °C, the VP1091 primer pairs produced PCR products with melting peak at 89.4 ± 0.1 °C, the VP1778 primer pairs produced PCR products with melting peak at 87.1 ± 0.3 °C, the VP2902 primer pairs produced PCR products with melting peak at 84.1 ± 0.3 °C, the VPA0074 primer pairs produced PCR products with melting peak at 88.5 ± 0.3 °C, the VPA0716 primer pairs produced PCR products with melting peak at 85.6 ± 0.4 °C, the VP0635 primer pairs produced PCR products with melting peak at 87.4 ± 0.4 °C, the VP1393 primer pairs produced PCR products with melting peak at 85.5 ± 0.5 °C, the VP1563 primer pairs produced PCR products with melting peak at 84.1 ± 0.6 °C, the VPA0895 primer pairs produced PCR products with melting peak at 90.5 ± 0.1 °C, the VPA1336 primer pairs produced PCR products with melting peak at 81.3 ± 0.4 °C, the VPA1708 primer pairs produced PCR products with melting peak at 89.5 ± 0.2 °C, the VP1351 primer pairs produced PCR products with

melting peak at 89.3 ± 0.2 °C, the VP2132 primer pairs produced PCR products with melting peak at 81.6 ± 0.6 °C, the VPA0440 primer pairs produced PCR products with melting peak at 86.9 ± 0.4 °C, the VPA1199 primer pairs produced PCR products with melting peak at 88.3 ± 0.4 °C, the VPA1256 primer pairs produced PCR products with melting peak at 83.3 ± 0.5 °C, and the RPI08 primer pairs produced PCR products with melting peak at 85.3 ± 0.6 °C (Fig. 1). All PCR products were further confirmed by gel electrophoresis (Online Resource 1).

Analysis of *V. parahaemolyticus* Strains

Fifty-three *V. parahaemolyticus* strains were tested by the newly developed assays. Genotyping of tested *V. parahaemolyticus* strains with PCR amplification and melting temperature curve analysis revealed 13 different types. Figure 2 showed the analysis results of VP23. Furthermore, cluster analysis using NTSYS PC 2.02 software could classify 53 *V. parahaemolyticus* strains into six clusters at a relative similarity coefficient of 0.85 (Fig. 3).

Discussion

In many countries, intestinal infectious diseases caused by *V. parahaemolyticus* is a serious public health problem and need to be primarily monitored; virulence gene detection and molecular typing analysis in *V. parahaemolyticus* isolates are important parts of the monitoring work [13–15]. Because real-time PCR was applied in virulence genes detection [16–18], the degree of automation for virulence gene detection has been markedly improved. However, the molecular typing analysis for *V. parahaemolyticus* isolates still uses some traditional methods that are commonly dependent on electrophoresis analysis, thus it is low degree of automation, and time-consuming and laborious. Recently, some researchers have already devoted to using real-time PCR to conduct molecular typing analysis of pathogens. For example, Pang et al. performed the molecular typing analysis of Mycobacterium tuberculosis using real-time PCR combined with high-resolution melt (HRM) technology [19]. But the method used in their study requires HRM technology that has many limitations. For example, HRM is very sensitive, however, results of HRM can be influenced by initial nucleic acid template or amplimer concentrations [20]. Moreover, not all real-time PCR instruments are suitable for HRM analysis. Pang et al. [19] recommended the use of Roche 480 to ensure the credibility of experimental results. In this study, we developed a method for molecular typing of *V. parahaemolyticus* based on conventional resolution melt technology applied in real- time PCR. This method has the advantage that does not require HRM technology, as applied in the study of Pang et al. [16].

In the long evolutionary process, microorganisms obtain new genes through the transfer of genes, and stably preserve new genes that are beneficial to adaptive microevolution in the population under natural selection pressures, for example, *V. parahaemolyticus* acquires pathogenicity islands (VPAIs) by horizontal gene transfer [11] thereby causing diseases to humans. Meanwhile, *V. parahaemolyticus* deletes some DNA regions that are not conducive to microbial survival and reproduction and enables it to be stably inherited in the population. Thus, the acquisition and deletion of genes as a main molecular evolution strategy of bacterial genomes greatly increases the genetic polymorphism of *V. parahaemolyticus* [21]. Previous studies using M-CGH have screened 18 large LVPCs of *V. parahaemolyticus* [10, 11]. LVPC refers to large fragments of gene clusters containing at least 10 consecutive genes, these large fragments of gene clusters have different distributions in the genomes of different strains, for example, they are absent in some strains but present in other strains. The study of Xiao et al. has shown that genomic analysis of *V. parahaemolyticus* based on LVPCs is a very effective method for the genotyping of *V. parahaemolyticus* [12]. However, the analysis of *V. parahaemolyticus* LVPCs in their study still depended on the conventional PCR and gel electrophoresis, that was time-consuming and labor-intensive. In this study, we analyzed the LVPCs of *V. parahaemolyticus* by using real-time PCR combined with melting curve analysis, determining whether the corresponding LVPCs existed according to the presence or absence of melting curve peak. As a result, it eliminates the cumberse electrophoresis process, compared with the method used by Xiao et al., this method is simple, and time- and effort-saving. In addition, it is different from the method used by Pang et al., which used the HRM technique to analyze the molecular typing analysis based on the changes of HRM profiles. We use conventional resolution melt technology and conduct molecular typing analysis according to the presence or absence of melting curve peak, thus, the results are not influenced by initial nucleic acid template or amplimer concentrations and it could be applied in most commercially available real-time PCR instruments.

In conclusion, the method developed by us is fast, simple, and practical, in view of its advantages, it has a potential application as a useful tool for tracing infectiousness and investigating outbreaks caused by *V. parahaemolyticus*. The advantages of this new method are obvious, but we must admit that there are some aspects that need to be further improved, for example, in order to ensure that the LVPC amplifiers are clearly distinguishable, we have use 6 LVPCs as a group, and 18 LVPCs analysis is performed in 3 different tubes. In the future, we consider establishing a multi-color melting curve analysis method

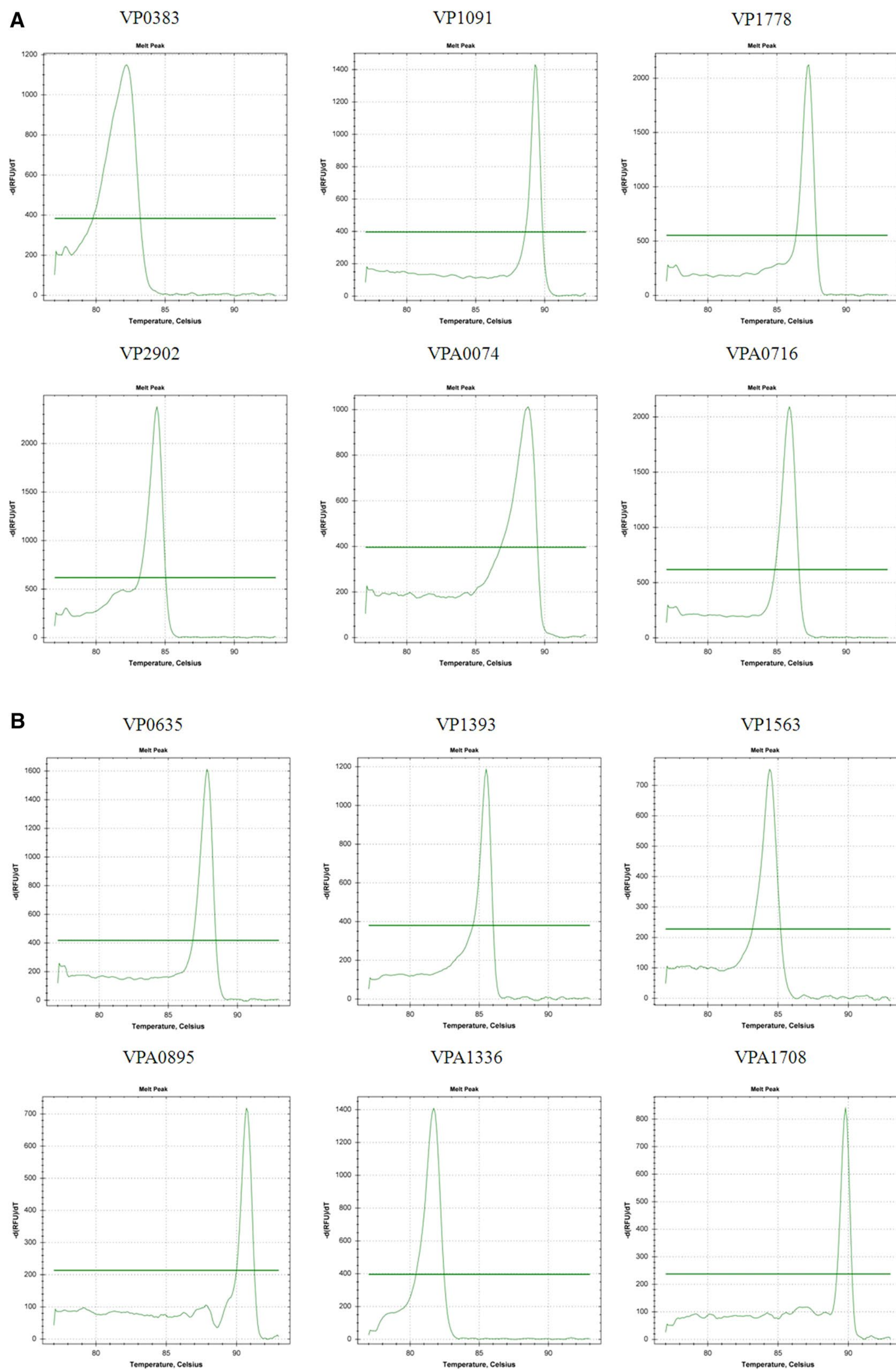

Fig. 1 Melting peaks of 18 LVPCs obtained after PCR amplification. **a** Melting peaks produced by primer pairs in tube 1, **b** melting peaks produced by primer pairs in tube 2, and **c** melting peaks produced by primer pairs in tube 3

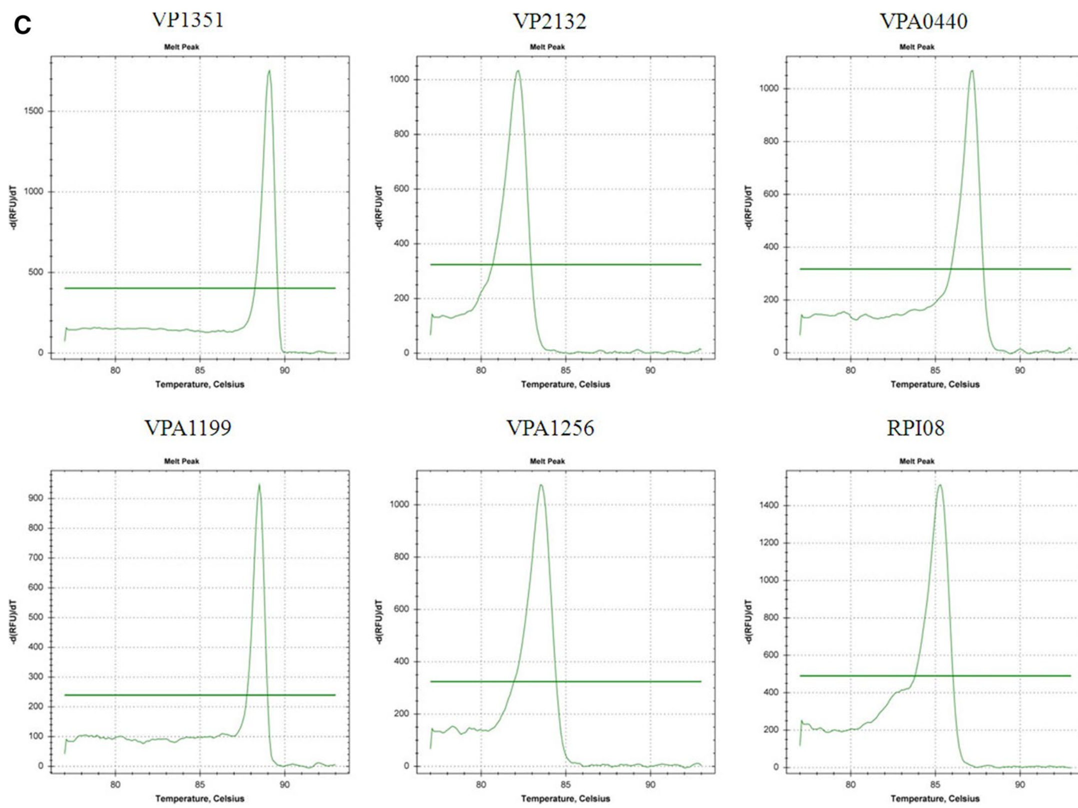

Fig. 1 (continued)

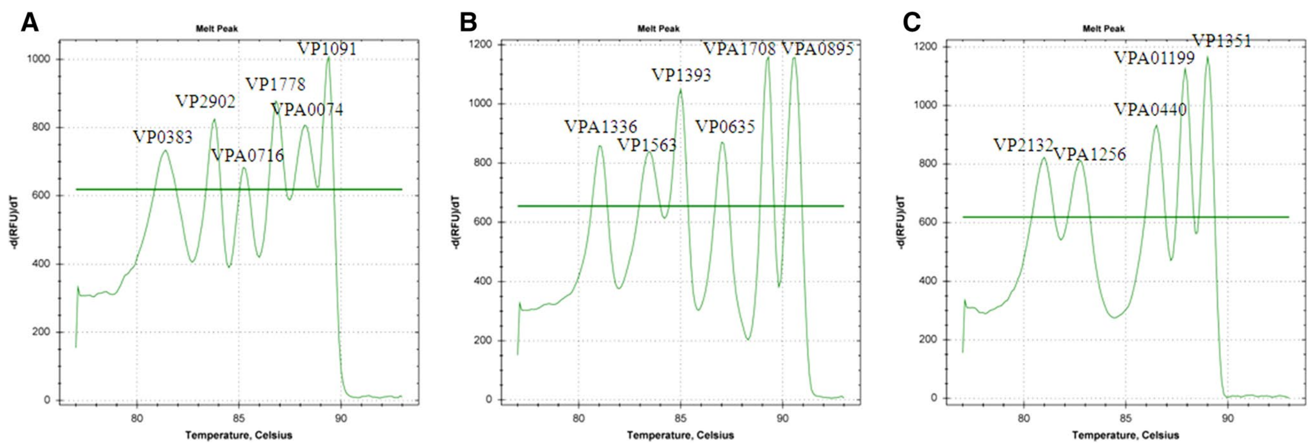

Fig. 2 Molecular typing of *Vibrio parahaemolyticus* strain VP23. **a** Melting peaks produced in tube 1, **b** melting peaks produced in tube 2, and **c** melting peaks produced in tube 3

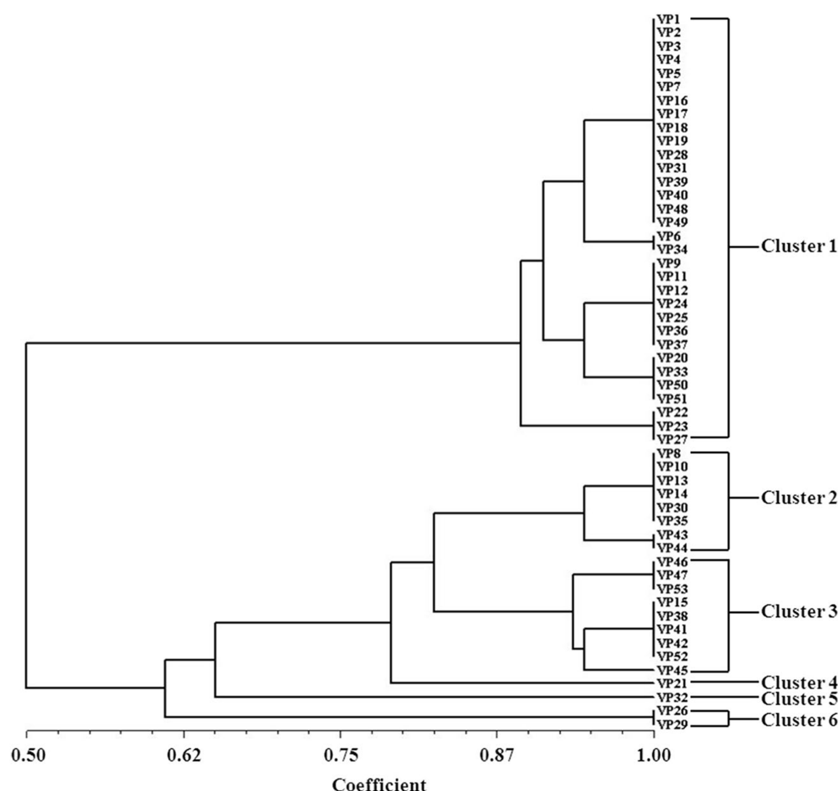

Fig. 3 Dendrogram of the 53 *Vibrio parahaemolyticus* strain

in combination with multi-color labeled probes and melting curve analysis to complete 18 LVPCs detection within a reaction tube.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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