American Journal of Immunology 1 (1): 31-36, 2005
ISSN 1553-619X

Random Amplified Polymorphic Dna-PCR Typing of *Vibrio parahaemolyticus* Isolated from Local Cockles (*Anadara granosa*)

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Abstract: Randomly amplified polymorphic DNA (RAPD) was used in this study to examine the genetic relatedness among the *Vibrio parahaemolyticus* strains. In the analysis by RAPD-PCR, the size for RAPD fragments ranged from 0.25 to 10.0 kb with average number of ten bands. The RAPD profiles revealed a high level of DNA sequence diversity within the *Vibrio parahaemolyticus* strains tested. Hence, this study, demonstrated that the local cockles (*Anadara granosa*) in the study area are populated by genetically polymorphic strains of *V. parahaemolyticus.* In addition, RAPD-PCR is simple, robust and sensitive typing methods to differentiate the *V. parahaemolyticus* strains.

Key words: RAPD-PCR, cockles, *Vibrio parahaemolyticus*

INTRODUCTION

Vibrio parahaemolyticus is a Gram-negative marine bacterium that can cause seafood-borne gastroenteritis and traveler's diarrhea in humans after they consumed contaminated raw or partially cooked fish or shellfish, particularly oysters[1] or exposure to a marine environment. The organism is widely disseminated in estuarine environments throughout the world. Outbreaks of *V. parahaemolyticus* food poisoning are most common in Japan and Southeast Asia, although they occurred occasionally in other parts of the world[2], and are associated with diverse serovars. Recent studies[3,4] have shown the emergence of O3:K6 that appears to have the potential to spread and to be associated with infections more than other serovars.

Epidemiological typing of pathogens such as *Listeria monocytogenes*, *V. parahaemolyticus* and *Salmonella enteriditis* has become more and more important in public health control. Molecular typing procedures are applied to show clonal and close relationship between isolates of one species. Thus, it is possible to identify pathogen reservoirs and to follow up the regional and global spread of pathogens. Some typing methods also give an insight into the evolutionary dynamics of the bacterial genome. The classical and microbiological methods, based on the identification of phenotypic markers, while perfectly adequate to identify microorganisms on the level of species, are often not reliable enough when it comes to differentiating further into individual strains. This is where DNA-based procedures like Polymerase Chain Reaction (PCR) or DNA-sequencing approaches helped epidemiological investigations to be conducted more rapidly and thoroughly[5]. RAPD analysis is a commonly used method in PCR. This fingerprint has been used for typing and differentiation of bacteria and, increasingly, for the study of genetic relationships between strains and species of microorganisms, plants and animals[6]. Nowadays, molecular typing methods are necessary for proving the similarity between the isolates. The aim of the present investigation was to use RAPD-PCR assay to generate polymorphism in DNA patterns amenable to the differentiation of the *V. parahaemolyticus* strains isolated from cockles.

MATERIALS AND METHODS

Bacterial strains: The sixty-two isolates of *V. parahaemolyticus* isolated from local cockles obtained from a harvesting site in Tanjong Karang, Kuala Selangor were examined. These strains were confirmed by using specific-PCR targeting the species-specific *ToxR* region in *V. parahaemolyticus* as mentioned by Kim *et al*.[7] and Dileep *et al*.[8].

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Genomic DNA isolation: Prior to amplification, genomic DNA of the *V. parahaemolyticus* strains were extracted by mini-preparation method of Ausubel *et al.*[9].

Randomly amplified polymorphic DNA (RAPD)

RAPD-PCR amplification: Amplification reactions were performed in 25 µl volume containing 2.5 mM $MgCl_2$, 200 µM each dATP, dCTP, dGTP and dTTP (Promega), 0.5 µM primer, 1.25 Units of *Taq* polymerase, 10-20 ng of genomic DNA. Amplifications were carried out in the thermal cycler (Perkin Elmer Cetus 2400) for 35 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C. A final elongation step at 72°C for 5 min was included. Amplification products were fractionated by electrophoresis through 1.5% agarose gel and detected by staining with ethidium bromide. DNA ladder (Promega) was used as DNA size marker.

Primers: Ten random primers having the 50% G+C contents gene sequence (10-mer) were screened and three primers, the Gen 1-50-03 (5'-CTT GAG TGG A-3'), Gen 1-50-04 (5'-TCC TCAAGA C-3') and Gen 1-50-08 (5'-GAG ATG ACG A-3') were selected for further study as they provide reproducible and discriminatory patterns.

RESULTS

Of the ten random primers, Gen 1-50-03, Gen 1-50-04 and Gen 1-50-08 were chosen for differentiation of *V. parahaemolyticus* isolates. The representative RAPD fingerprinting profiles obtained were displayed in Fig. 1-3. The size for RAPD fragments ranged from 0.25 to 10.0 kb with average number of ten bands for each primer used. All results from RAPD-PCR were analyzed with the GelCompare software (version 4.1). The overall achievable patterns were used to construct dendrograms using the UPGMA (unweighted pair group average method) clustering algorithms.

DISCUSSION

The application of PCR-based techniques has had a revolutionary impact on the diagnosis of infectious disease. A rapid typing method based on the random amplification of polymorphic DNA segments (RAPD-PCR) has been described[10,11]. This method has been successfully used in the characterization of several organisms including *V. parahaemolyticus* and other vibrios[1,12-14]. In this study, a library of 10 primers (50% G+C) was examined for suitability. Of these 10 primers, three (GEN1-50-03, GEN1-50-08 and GEN1-50-08) proved to be useful primers for the RAPD analysis of strains of *V. parahaemolyticus.* In order to obtain a sufficient number of polymorphic bands that will permit reliable comparison, the three different 10-mer primers were used in amplification reactions. Previously, Shangkuan *et al.*[12] demonstrated the importance of using a minimum of three different primers to discriminate different isolates of toxigenic *V. cholerae*. The results of using three primers in this study indicate that RAPD provides a high degree of discrimination between *V. parahaemolyticus* strains, with amplified products ranging from 0.25 to 10.0 kb. To determine the reproducibility of the RAPD analysis procedure, RAPD-PCR was repeated at least three times for DNA from each strain, so that the amplification profiles for each attempt could be compared. We found that profiles for a given strain to be highly reproducible, with very little variation from one RAPD analysis to another.

Our results show that all strains of *V. parahaemolyticus* isolates generated appropriate fragments. Averagely ten amplified DNA bands were resolved by agarose gel electrophoresis in these isolates. Using primer GEN1-50-03, *V. parahaemolyticus* presented bands with common sizes in the range of 1000 bp – 1500 bp (VP1-VP16, VP18 – VP21, VP23 - VP38) and 1500 bp – 2000 bp (VP39 – VP62). The isolates examined gave bands with molecular sizes of 500 bp – 750 bp (VP1 – VP17, VP43 – VP62), 750 bp – 1000 bp (VP18 – VP27, VP29 – VP62), 1000 bp (VP1 – VP17), 1000 bp – 1500 bp (VP33 – VP42) and 1500 bp – 2000 bp (VP1 – VP32, VP43 – VP62) using primer GEN1-50-04. Distinct band at the range size of 2000 bp – 2500 bp were detected for six *V. parahaemolyticus* isolates (VP4, VP8, VP12, VP21, VP24 and VP26). Dominant bands of the molecular size in the range of 750 bp – 1000 bp (VP1 – VP18) and 1000 bp – 1500 bp (VP1 – VP62) were observed using primer GEN1-50-08. However, two isolates (VP17 and VP22) and one isolate (VP28) was not typeable using primer GEN1-50-03 and GEN1-50-04, respectively, but all strains were able to be amplified using primer GEN1-50-08.

RAPD-PCR was used as a mean to determine the clonal relatedness of the isolates in this study by their chromosomal polymorphism. The data from the primer GEN1-50-03, GEN1-50-04 and GEN1-50-08 was used to generate a distance matrix. By using Gel Compar version 4.1 software, typable *V. parahaemolyticus* isolates were placed in 7, 6 and 10 clusters for primer GEN1-50-03, GEN1-50-04 and GEN1-50-08, respectively (Fig. 1-3). At 24% and 10% similarity, the isolates were classified into 2 major clusters (R1 and R2) and one major cluster (R3) with a single isolate (VP59) using primer GEN1-50-03 and GEN1-50-04, respectively. These main clusters labeled as R1, R2 and R3 were sub-divided into 6, 5 and 9 subclusters, respectively. Our RAPD analysis revealed

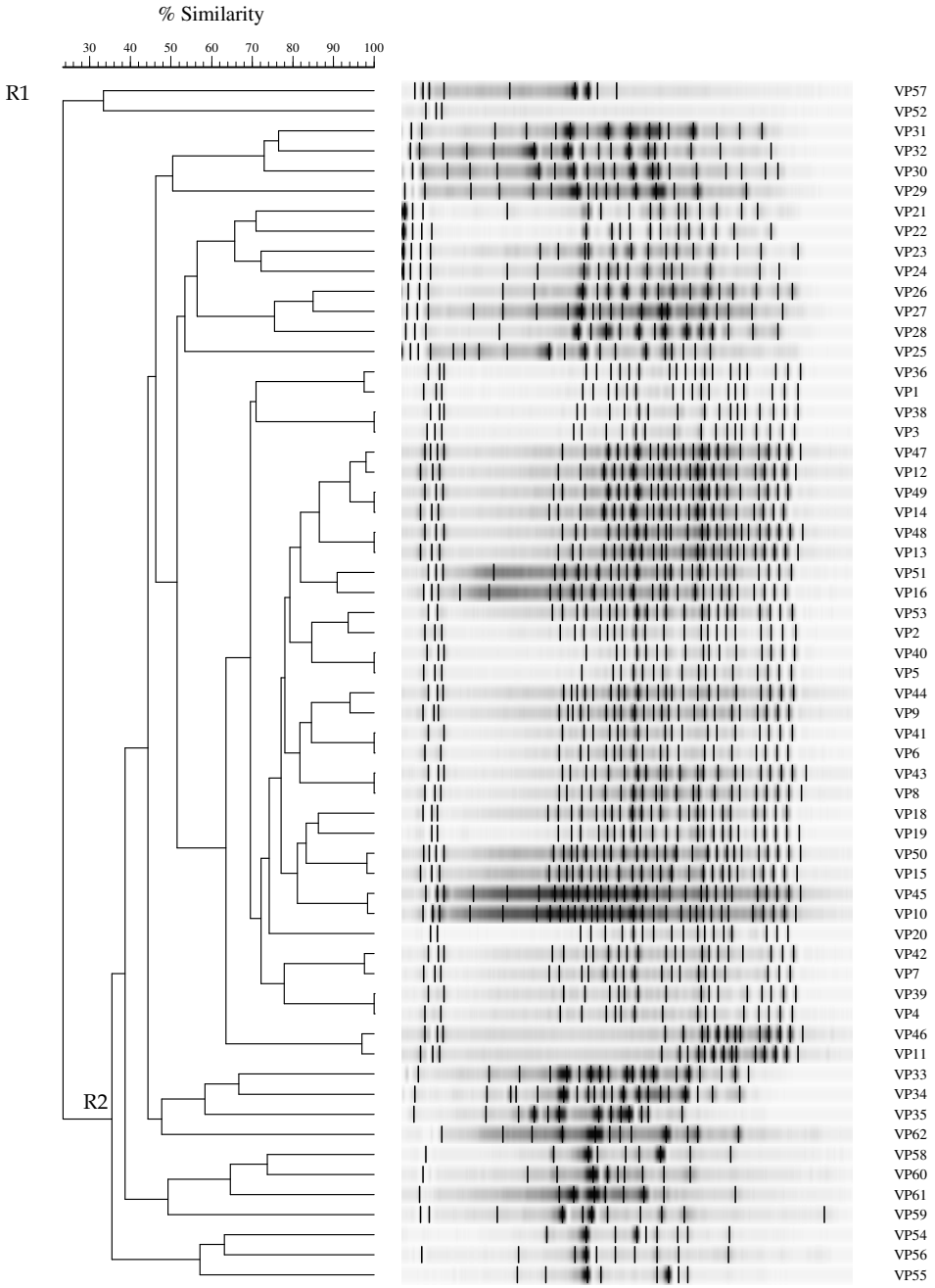

Fig. 1: Dendrogram showing RAPD profiles of typeable *V. parahaemoyticus* isolates using GEN1-50-03

heterogeneous isolates of *V. parahaemolyticus* in cockles by using these two primers (GEN1-50-03 and GEN1-50-04). On the other hand, the isolates were observed as homogeneous when typed using primer GEN1-50-08, as the cluster analysis shows that at 52% similarity the isolates were clustered into 1 major cluster and a single isolate (VP25). Our results are supported by others who observed homogeneous or heterogeneous isolates of *V. parahaemolyticus* or *Vibrio* spp. in cockles or shellfish by RAPD-PCR assays[15-18]. The high diversity among the RAPD-types probably means that contamination of cockles by *V. parahaemolyticus* is derived from wide spectra sources of contamination. It is also likely that different strains of *V. parahaemolyticus* predominate in cockles in the study area.

The results obtained by RAPD-PCR indicated that multiple clones of *V. parahaemolyticus* were present in the environment. These observations are in agreement with those of others who demonstrated heterogeneity

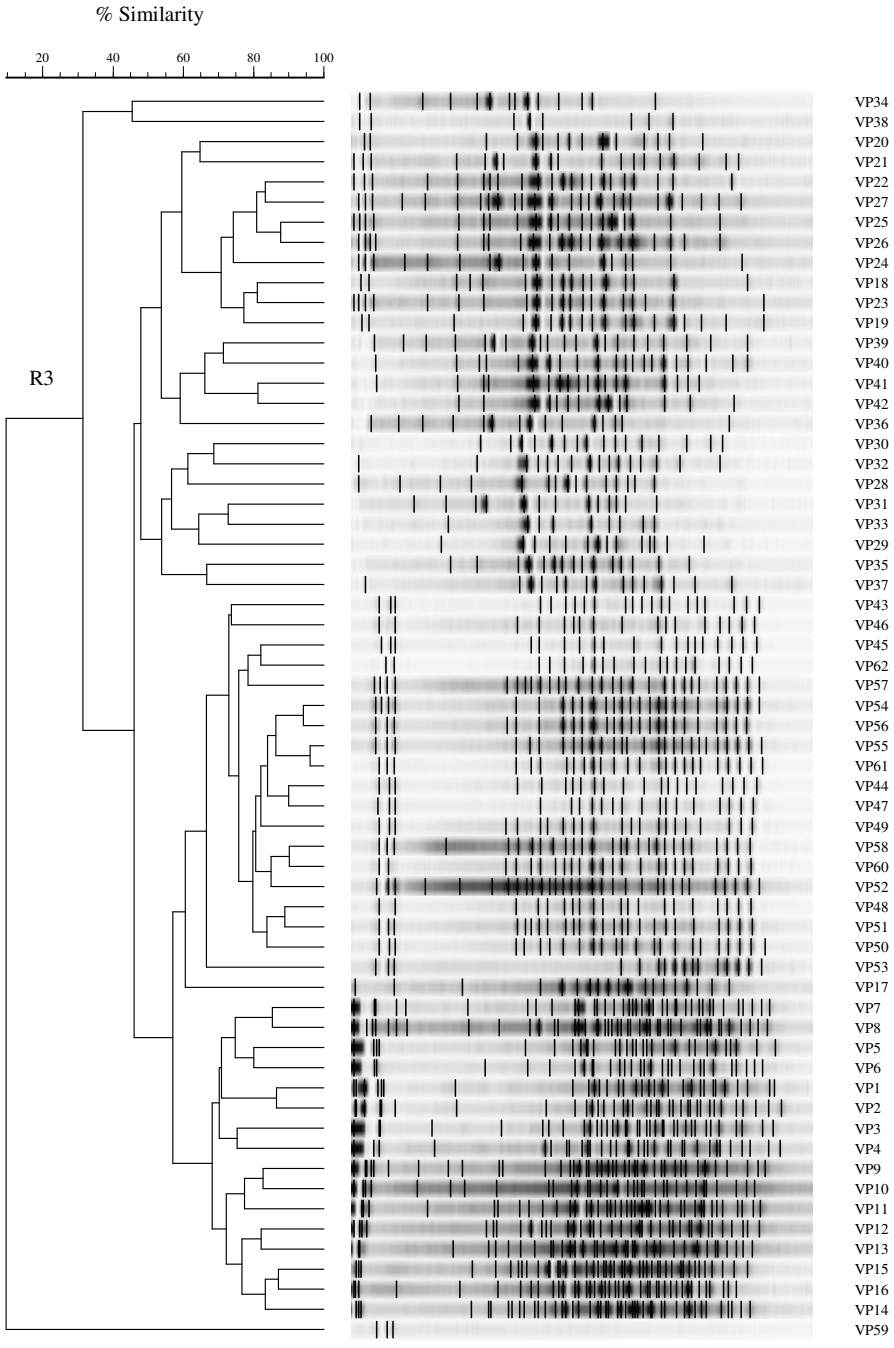

Fig. 2: Dendrogram showing RAPD profiles of typeable *V. parahaemoyticus* isolates using GEN1-50-04

in *V. parahaemolyticus* or *Vibrio* spp.[15,19]. Many researches reported the abundance of *V. parahaemolyticus* during summer in temperate zone when temperature was above 25°C[1,20,21], whereas the organism is expected to be prevalent throughout the year in the tropical zone like Malaysia[22]. The hot climate in Malaysia and other tropical countries make conditions more favourable for the growth of *V. parahaemolyticus,* increasing the risk of outbreaks associated with this organism.

In summary, results from this study demonstrated that genotyping *V. parahaemolyticus* isolates by using RAPD -PCR is feasible for differentiation of various strains. RAPD-PCR has shown to be rapid, sensitive, discriminative and cost effective in typing the *V. parahaemolyticus* isolates from cockles caught in Tanjong Karang, Kuala Selangor.

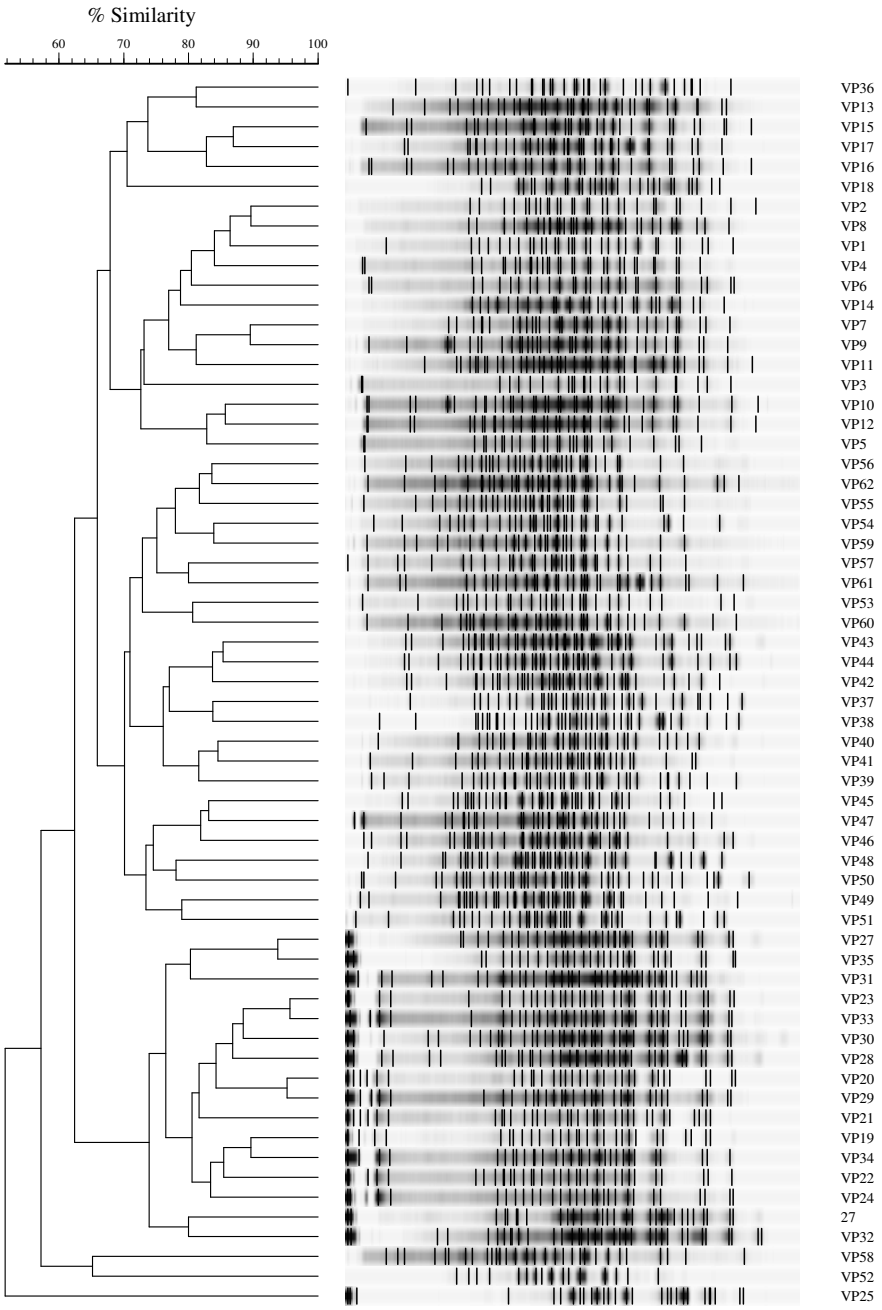

Fig. 3: Dendrogram showing RAPD profiles of typeable *V. parahaemoyticus* isolates using GEN1-50-08

ACKNOWLEDGEMENTS

Funding for this research was made possible by the IRPA grant from Malaysian Government.

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