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Random amplified polymorphic DNA-PCR and ERIC PCR analysis on *Vibrio parahaemolyticus* isolated from cockles in Padang, Indonesia

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Abstract: In this study, RAPD-PCR and ERIC-PCR were used to study the epidemiology of *V. parahaemolyticus* isolated from cockles in Padang, Indonesia. The Gold Oligo OPAR3 primer produced bands ranged from 1-8 with sizes from 0.2 – 5.0 kb and the Gold Oligo OPAR8 primer produced 1-7 bands with sizes 0.7 – 1.5 kb. Both primers produced twenty five RAPD patterns with a few isolates failed to produce any products. Based on phylogenetic dendrogram, all the isolates can be divided into 6 major clusters with similarity between 0 to 52%. For the ERIC primer, it produced bands ranged from 3-15 with sizes from 0.1 – 5.0 kb and twenty seven different ERIC patterns. Construction of the phylogenetic dendogram showed the isolates can be divided into 4 major clusters with similarity between 56 to 86%. The high diversity of both processes may be due to the multiple contamination sources of *V. parahaemolyticus*.

Keywords: *V. parahaemolyticus*, seafood, RAPD-PCR, ERIC-PCR, genomic fingerprinting

Introduction

Vibrio parahaemolyticus is one of the most widely recognized pathogenic *Vibrio* species due to numerous outbreaks and it is wide occurrence in marine environment (Joseph *et al*., 1983; Chiou *et al*. 1991; Mead *et al.,* 1999). This microorganism can be found in high number during summer in the United States and Europe, but all year round in Southeast Asian. The genus *Vibrio* consists of 28 species and twelve of them are recognized as human pathogens. The major species that contributed to the pathogenesis are *V. parahaemolyticus, V. cholerae, V. vulnificus, and V. alginolyticus.* In recent years, an increased awareness of the infections of some other *Vibrio* spp., including *V. mimiscus, V. fluvialis, V. hollisae, and V. damsela* have been witnessed (Baffone *et al*., 2000). Totally, *V. parahaemolyticus* has been implicated as a cause of at least a quarter of total food borne diseases caused by vibrios (Feldhusen, 2000).

DNA fingerprinting started when Jeffreys *et al*. (1985) developed minisatellite DNA analysis in humans that detected segments of DNA that are highly variable in human populations. The term DNA fingerprint was coined to describe these unique patterns because they can be used in a manner similar to that of a true fingerprint to identify an individual. DNA fingerprinting is the identification of individual based on DNA markers. The patterns detected in DNA fingerprinting are unique to each individual with the exception of identical twins who share the same DNA fingerprints (Fairbanks and Andersen, 1999).

Development of Random Amplified Polymorphic DNA (RAPD) or arbitrarily primed PCR fingerprinting gave an advantage in which molecular preliminary information of the species studied is not necessary (Welsh and McClelland, 1990) and polymorphism pattern obtained usually varies among the species. In the previous studies, RAPD-PCR has been successfully used for genetic fingerprinting and molecular typing for many species, including fingerprinting of man (Jeffreys *et al.,* 1985), animals (Kostia *et al*., 1996; Saez *et al*., 2004), plant (Welsh and McClelland, 1990), microorganism such as *Lactobacillus, Salmonella, E. coli*, yeast, *Bacillus* (Miyata *et al*., 1995; Giraffa *et al*., 2004; Elegado *et al*., 2004; Rengua-Mangia *et al.,* 2004; Foschino *et al*., 2004; Svensson *et al*., 2004)

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Another PCR-fingerprinting technique is ERIC-PCR but it is not arbitrary because the primer was designed to known target sequence. The primers will be complementary to repetitive sequence that highly conserved in the genome DNA. A few repetitive sequences have been reported in bacteria genome include enterobacterial repetitive intergenic consensus (ERIC) sequences, repetitive extragenic palindromic (REP) sequences, and BOX elements. The enterobacterial repetitive intergenic consensus (ERIC) sequences, also known as intergenic repeat units (IRUs) are present in many copies in the genomes of *Escherichia coli*, *Salmonella typhimurium*, and other enterobacteria (Hulton *et al*., 1991). These elements are 126 bp long, highly conserved at the nucleotide level, and include a central core inverted repeat. The position of ERIC elements in enterobacterial genomes varies between different species and has been used as a genetic marker to characterize isolates within a bacterial species (Versalovic *et al*., 1991; Son *et al.,* 2002). In ERIC-PCR a band pattern is obtained by amplification of genomic DNA located between ERIC elements or between ERIC elements and other repetitive DNA sequences. This technique uses consensus primers in the PCR to amplify DNA sequences located between successive repetitive elements such as the 126-bp enterobacterial repetitive intergenic consensus (ERIC) sequence for subtyping Gram-negative enteric bacteria (Hulton *et al.*).

The occurrence of vibrios in Indonesia is not well documented and the occurrence of vibrios in seafood is not well understood. The true incidence of *V. parahaemolyticus* in Indonesia transmitted by seafood is not known, probably due to underreporting of cases and lack of proper study on this issue. In 2001, Lesmana *et al*. started to conduct studies about the infection of *V. parahaemolyticus* in Indonesia but no genetic diversity information is reported. The objective of this study is to determine the relationship between *V. parahaemolyticus* isolated strains using RAPD-PCR and ERIC-PCR based on genomic fingerprinting.

Materials and Methods

Sources of V. parahaemolyticus

Fifty (50) cockle samples (species: *Faunus ates, Carbiculla molktiana, Batissa violaceae*) were brought from Padang, Indonesia. The samples were from lakes and rivers in West Sumatra. All the 50 cockle samples from Padang, Indonesia were tested on the CHROMagar™ *Vibrio* to detect the presence of *V. parahaemolyticus*. The *V. parahaemolyticus* isolated were then grown in Luria-Bertani broth overnight at 37°C with shaking at 220 rpm in an orbital shaker (Lab-line Incubator-shaker).

DNA extraction

Both RAPD-PCR and ERIC-PCR needs DNA template to perform the analysis. In this study, PCI (phenol-chloroform-isoamyl) based method was selected for preparation of template DNA in both analysis. The method used was the mini preparation method of Ausubel *et al*. (1987).

The cell suspension of *V.parahaemolyticus* isolates was transferred to 1.5 ml microfuge tube and was centrifuged at 10,000 rpm for 2 minutes in a Eppendorf centrifuge (Model 5415C) to get the cell pellet. The pellet then was resuspended in 700 µl GET (Glucose-EDTA-TrisHCl) buffer (17 mg/ml) and vortexed to mix. 10 µl 25% SDS (Sodium Dodecyl Sulphate) plus 5 µl Proteinase K (25 mg/ml) (as an additional to rupture the cells) were then added to the tube and mixed gently. The tube then incubated in water bath at 60ºC for 20 minutes to lyse the cell or until the solution mixture become clear. Then, 500 µl PCI mix (phenol-chloroform-isoamylalcohol) solutions were added and mixed gently. The tube then centrifuged at 12000 rpm for 1 minute. 200 µl of the upper aqueous layer (clear layer) was carefully transferred into a new sterile eppendorf tube. After that, 200 µl KAc (potassium acetate) and 400 µl cold isopropanol (100%) were added followed by incubation at -20^{o} C for 5-10 minutes. The tube then centrifuged at 12000 rpm for 7 minutes. The supernatant was discarded and the pellet was washed with 500 µl of cold 70% ethanol. After centrifugation at 12000 rpm for 5 minutes, the ethanol solution was discarded and the pellet was air-dried and dissolved in 30 µl sterile distilled water.

RAPD-PCR

A random primer 10-mer oligonucleotide set was obtained from New England Biolabs Inc., containing primers Gold Oligo OPAR1 to Gold Oligo OPAR10. This ten random primers having the 50% G+C contents gene sequence (10-mer) were used in screening process and the only primers Gold Oligo OPAR3 (5'-CTT GAG TGG A-3') and Gold Oligo OPAR8 (5'-GAG ATG ACG A-3') were selected as they gave clear and reproducible patterns.

The PCR technique was carried out in 0.2 µl microfuge tubes. The total volume consisting of reaction mixture was 25 µl consisting of 19.8 µl sterile distilled water, 2.5 µl 10x PCR optimize buffer, 0.5 µl 25 mM deoxyribonucleotide phosphate, 10 pmol primers, 0.2 µl of 0.5 units *Taq* DNA polymerase and template DNA 1.0 µl. As the negative control, one of the reaction mixtures without DNA template was

used. The solution mixture were placed in the thermal cycler (Perkin Elmer type 2400) and subjected to 45 cycles. The cycling conditions were as follows; predenaturation at 94°C for 3 minutes, denaturation at 94°C for 1 minute, annealing at 36°C for 1 minute, and extension at 72°C for 5 minutes, with a final extension at 72°C for 7 minutes at the end of 45 cycles.

ERIC PCR

Two specific primers were used to correlate to ERIC sequence (Versalovic *et al*., 1991; Son *et al*., 1998); ERIC 1 (R): 5' ATGTAAGCTCCTGGGGATTCA- 3' and ERIC 2 (F): 5' AAGTAAGTGACTGGGGTGAGC- 3'. The PCR technique was carried out in 0.5 ml microfuge tubes. The total volume consisting of reaction mixtures was 25.0 µl. Consisting of 17.5 µl sterile distilled water, 2.5 µl 10x PCR buffer with $MgCl_2$, 0.5 µl 25 mM deoxyribonucleotide phosphate, 1.0 µl of each primer, 0.5 of 0.5 units *Taq* DNA polymerase and 2 µl template DNA. The cycling conditions were as follows; predenaturation at 95°C for 7 minutes, denaturation at 90°C for 30 seconds, annealing at 58°C for 1 minute, and extension at 65°C for 8 minutes, with a final extension at 68°C for 16 minutes at the end of 30 cycles.

Agarose gel electrophoresis

After the PCR assay, the PCR products were run on 1.2% agarose gel (Sigma) in 1x Tris-Borate-EDTA (TBE). About 15 – 20 µl PCR products were loaded into sample wells and voltage at 100 volt was used for 1 hour. The gel was stained with ethidium bromide (0.5µg/ml) solution for 1 min and de-stained in water for 30 min. The gel was visualized under UV transilluminator and photographed.

Cluster analysis of the RAPD-PCR using RAPD Distance software

Before using the RAPD Distance software, scoring process must be done. For each band sizes, no. 1 was given to the strains that produced the band and 0 to the strains that failed to produced it. After that, the data was input in the software and a NJT format files was created. Automatically the dendrogram was generated by this software. The generated dendrogram was then edited by using Corel Draw software.

Cluster analysis of the ERIC-PCR using Gel Compar software

The gel picture was scanned to the computer. After the picture was input to Gel Compar software, the picture format was change from JPEG to TIFF format. Then, the software was used to analyze the picture and a dendrogram was constructed.

Results and Discussion

Studies about the origin and fingerprint of *V. parahaemolyticus* have become very important because of increasing incidence of infections caused by this organism worldwide (Feldhusen, 2000). With very small quantity of template DNA, RAPD-PCR can generate various fingerprint profiles with unlimited number of primers. It is also fast, simple and less labor than the usual fingerprinting method with non radioactive isotopes used. The technique uses oligonucleotide primers with arbitrary sequence and under standardized conditions, it gives distinct pattern for each sample analysed and the relationship between samples may be determined by comparing their unique fingerprint information (Leal *et al.*, 2004.

A simple short primer is used without the need of prior knowledge of the template DNA. The selection of an appropriate primer and optimization of PCR conditions are the important factor in RAPD analysis (Blixt *et al*., 2003). This method is different from previous PCR in the use of a single primer instead of two, and used low annealing temperature. The primers that work for some bacteria may fail for others (Shangkuan and Lin, 1998) and because of that, the screening process need to determine the appropriate primers. In the present study, ten primers (50% G+C) were screened and two primers (Gold Oligo OPAR3 and Gold Oligo OPAR8; New England Biolabs Inc.) were chosen because they yielded more clearer band patterns than those obtained with other primers.

In this study, out of the 50 cockle samples tested on the CHROMagar, twenty five samples were found positive and 32 isolates were selected for further characterization by RAPD-PCR and ERIC-PCR. The selection of the isolates was based on the appearance of purple coloured strong colonies on CHROMagar that indicate presence of *V. parahaemolyticus*. The results in this study showed that RAPD provides a high diversity of polymorphism between *V. parahaemolyticus* isolates isolated from Padang, Indonesia, with the PCR products ranging from 0.2 to 15 kb. Representative results of the RAPD patterns obtained are shown in Figure 1 (a) – (c) as examples. For the Gold Oligo OPAR3 primer, it produced bands ranged from 1-8 with sizes from 0.2 – 5.0 kb. For Gold Oligo OPAR8, it produced 1-7 bands with sizes 0.7 – 15.0 kb. OPAR3 primer produced twenty five RAPD patterns and OPAR8 also produced twenty five RAPD patterns (results not shown). For certain isolates they failed to produce any products with primers used and referred as untypeable. The results were analyzed using RAPD-Distance software to construct the phylogenetic dendrogram and then the

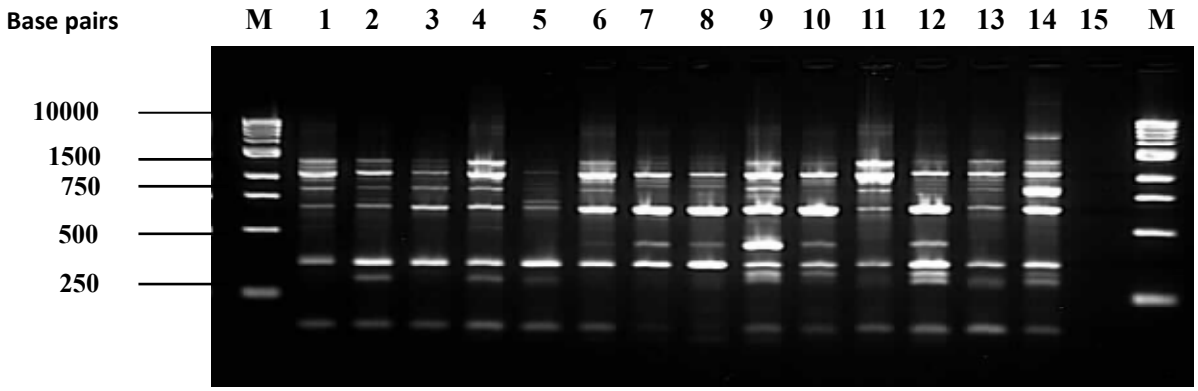

Figure 1. (a) Agarose (1.2%) gel electrophoresis of RAPD-PCR products for primer Gold Oligo OPAR3 of *V. parahaemolyticus* isolates. Lanes : M, 1 kb ladder; 1, VP1; 2, VP2; 3, VP3; 4, VP4; 5, VP5; 6, VP6; 7, VP7; 8, VP8; 9, VP9; 10, VP10; 11, VP11; 12, VP12; 13, VP13; 14, VP14; 15, VP15

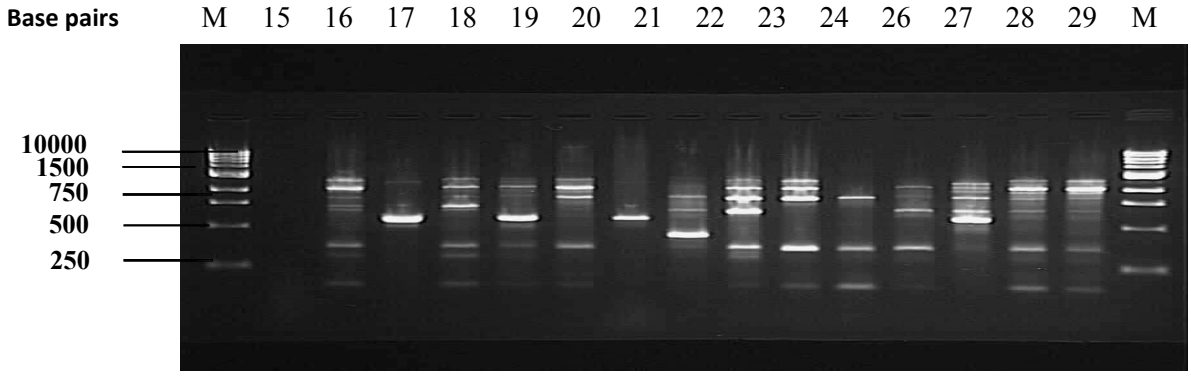

Figure 1. (b) Agarose (1.2%) gel electrophoresis of RAPD-PCR products for primer Gold Oligo OPAR3 of *V. parahaemolyticus* isolates. Lanes : M, 1kb ladder ; 15, VP15; 16, VP16; 17, VP17; 18, VP18; 19, VP19; 20, VP20; 21, VP21; 22, VP22; 23, VP23; 24, VP24; 25, VP25; 26, VP26; 27, VP27; 28, VP28; 29, VP29

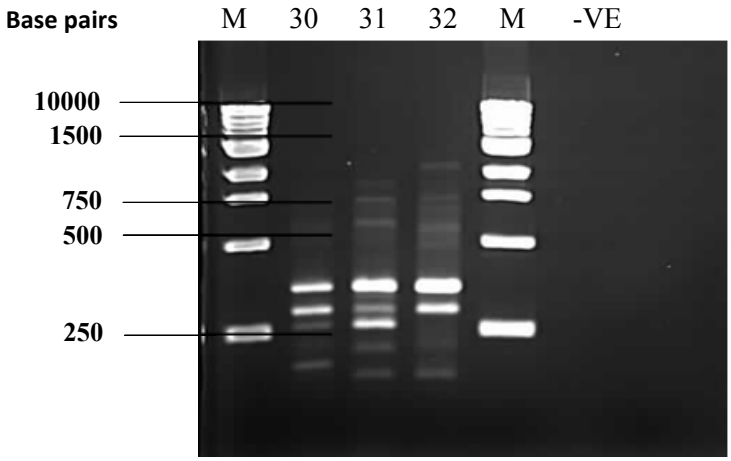

Figure 1. (c) Agarose (1.2%) gel electrophoresis of RAPD-PCR product for primer Gold Oligo OPAR3 of *V. parahaemolyticus* isolates. Lanes: M, 1 kb ladder; 30, VP30; 31, VP31; 32, VP32; -VE, negative control

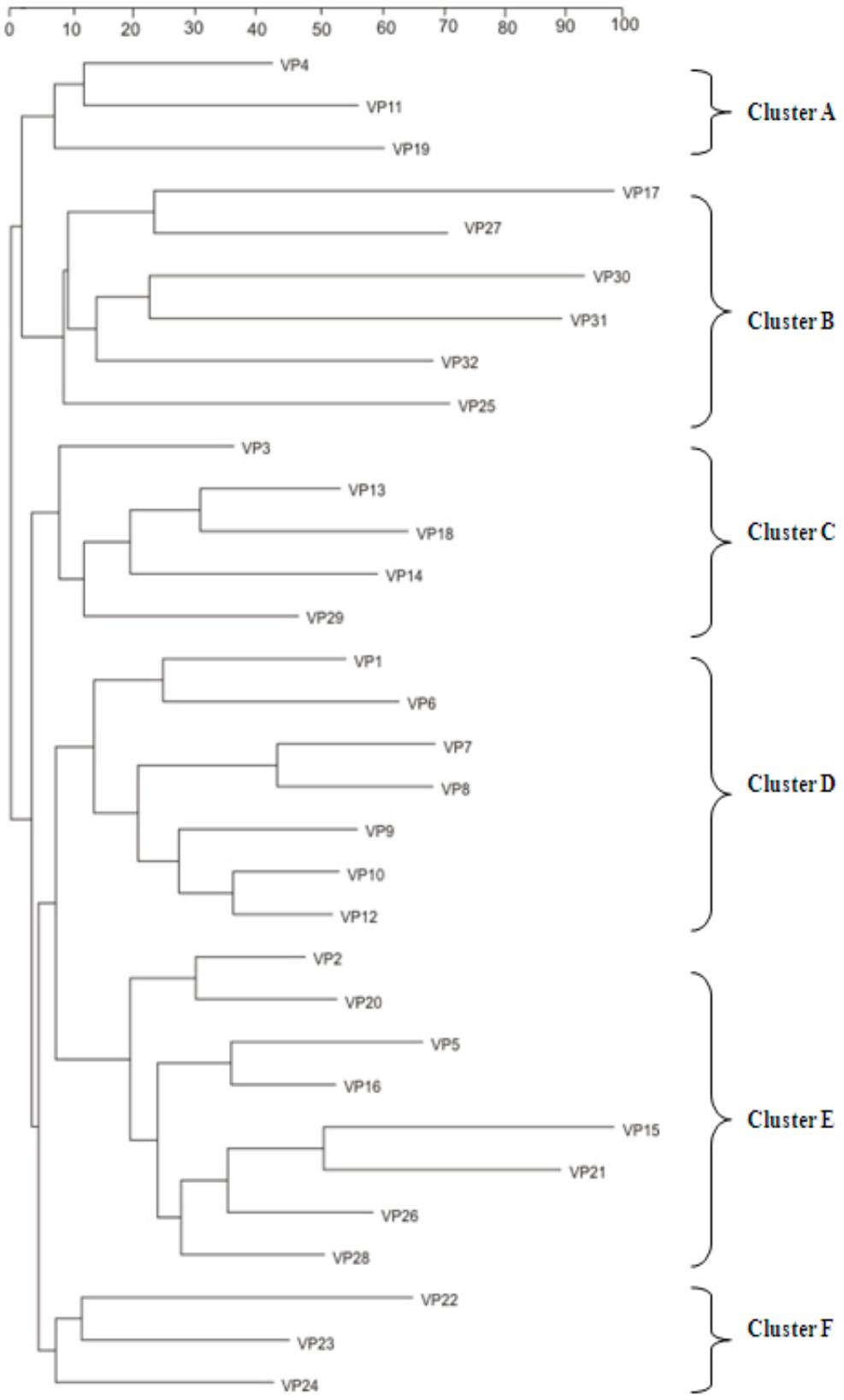

Figure 2. RAPD-Distance cluster analysis: Gold Oligo OPAR3 and OPAR8 primers

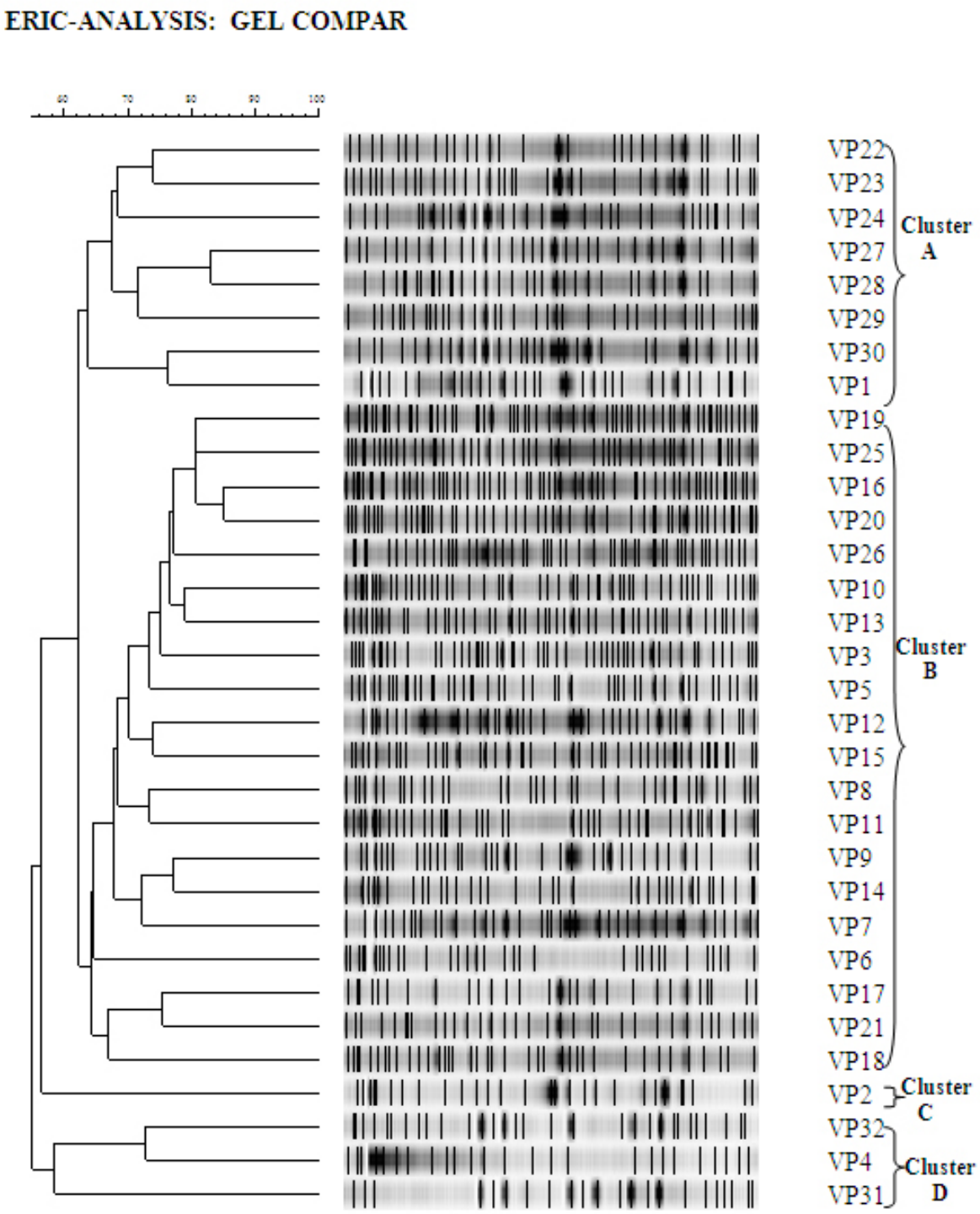

Figure 3. Dendrogram showing ERIC profiles of *V. parahaemoyticus* isolates

relationship between isolates can be estimated. The data from the two primers were used to generate a distance matrix and from the dendrogram generated, all the isolates can be divided into 6 major clusters (Figure 2). Cluster A – 3 isolates (VP4, VP11, VP19), Cluster B – 6 isolates (VP17, VP27, VP30, VP31, VP32, VP25), Cluster C – 5 isolates (VP3, VP13, VP18, VP14, VP29), Cluster D – 7 isolates (VP1, VP6, VP7, VP8, VP9, VP10, VP12), Cluster E – 8 isolates (VP2, VP16, VP20, VP15, VP21, VP26, VP28, VP5), Cluster F – 3 isolates (VP22, VP23, VP24) and the similarity is between 0% to 52%.

The RAPD patterns obtained showed the various pattern among the isolates and it means that the *V. parahaemolyticus* isolates have high diversity among them. This high diversity maybe caused by the different sources of *V. parahaemolyticus* infection in the cockles under study. Some of the isolates yielded high intensity bands and others with low intensity bands. If an individual carries a mutation over the binding sites of the RAPD primer to the DNA, this mutation would prevent the primer from binding to the template DNA. The previous report also stated that the characterization and identification of environmental *Vibrio* spp. caused many problems because of their high diversity (Goarant *et al.,* 1999).

As a supporting method to RAPD–PCR, another fingerprinting method was done but with different principle. The method is ERIC-PCR uses specific primers related to ERIC (entero-bacterial repetitive intergenic consensus) sequences, the repetitive sequences that found either in gram negative or gram positive bacteria. ERIC elements have been discovered in non-coding and inter-genic regions (Sharples and Lloyd, 1990). These elements are 126 bp long, highly conserved and include a central core inverted repeat. The position of ERIC elements in enterobacterial genomes varies between different species and has been used as a genetic marker to characterize isolates within a bacterial species (Versalovic *et al*., 1991; Son *et al*., 2002). For this reason, ERIC-PCR can be used to increase the efficiency in study of genetic distribution of *V. parahaemolyticus* strains beside RAPD. In the previous studies, ERIC-PCR has been successfully used for genetic fingerprinting and molecular typing for many type of bacteria include *E. coli* (Silveira *et al*., 2003; Panutdaporn *et al*., 2004; Leung *et al*., 2004), *Bacillus* spp. (Pinna *et al.,* 2001; Herman *et al*., 2000), *Salmonella* (Millemann *et al*., 1996; Kerouanton *et al*., 1996), *V. cholerae* (Rivera *et al*., 1995; Colombo *et al.,* 1997; Son *et al*., 1998), *Pseudomonas* (Achouak *et al.,* 2000) and *V. parahaemolyticus* (Khan *et al*., 2002).

The results for ERIC-PCR in this study showed a high diversity of polymorphism between *V. parahaemolyticus* isolates but lower than RAPD. These results are in agreement with previous study that also found the heterogeneity in *V. parahaemolyticus* or *Vibrio* spp. (Son *et al.,* 1998; Brite *et al*., 2001; Khan *et al.* 2002; Bhanumathi *et al.* 2003). For the ERIC primer, it produced bands ranged from 3-15 with sizes from 0.1 – 5.0 kb. The isolates *V. parahaemolyticus* from Padang, Indonesia produced twenty seven ERIC patterns but no isolates failed to produce any products with primers used. The result was analyzed using Gel Compar Software 4.1 to construct the phylogenetic dendrogram and then the relationship between isolates can be estimated.

The result data were used to generate a distance matrix and from the dendrogram (Figure 3) generated, all the isolates can be divided into 4 major cluster. Cluster A - 8 isolates (VP22, VP23, VP24, VP27, VP28, VP29, VP30, VP1) Cluster B – 20 isolates (VP19, VP25, VP16, VP20, VP26, VP10, VP1, VP5, VP12, VP15, VP8, VP11, VP9, VP14, VP7, VP6, VP17, VP21, VP18) Cluster C – 1 isolates (VP2), Cluster D - 3 isolates (VP32, VP4, VP31). The similarity of these clusters is between 56% to 86% and it supported the results in RAPD-PCR that multiple contamination sources of *V. parahaemolyticus* were present in the cockles under study. More than 80% of the isolates are still in same cluster like as in RAPD and in general some isolates are in the same cluster using two different fingerprinting analyses (Vrioni *et al*. 2003). ERIC-PCR can be used as genome-specific markers in detection the presence of *V. parahaemolyticus* in cockles or other organisms but has to be still compared with other methods because of high level of ERIC heterogeneity detected in this study.

Conclusion

Random amplified polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus (ERIC) can be applied successfully in study of genetic distribution and epidemiology of *V. parahaemolyticus*. In this study of determination of the relatedness of *V. parahaemolyticus* isolates in cockles in Padang, Indonesia, the high diversity obtained from both processes indicate that the contamination is from different sources of *V. parahaemolyticus* in the cockles.

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