Molecular Typing and Antimicrobial Susceptibility of *Vibrio parahaemolyticus* Strains

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ABSTRACT

The aim of the present study was to use three PCR-based techniques for the analysis of genetic variability among *Vibrio parahaemolyticus* strains isolated from the Philippines. Seventeen strains of *V. parahaemolyticus* isolated from shrimps and from the environments where these shrimps are being cultivated were analyzed by RAPD, ERIC and REP-PCR. Antimicrobial susceptibility of these strains to selected compounds was investigated using broth microdilution method. Results of this work and analysis of similarity among strains using Dice coefficient and unweighted average pair group method have demonstrated genetic variability within the *V. parahaemolyticus* strains. The RAPD, ERIC and REP-PCR were found to be suitable typing methods for *V. parahaemolyticus*. They have good discriminative ability and can be used as rapid means of comparing these strains for epidemiological investigation. However, the REP-PCR analysis yielded a relatively small number of products suggesting that the REP sequences may not be widely distributed in the *V. parahaemolyticus* genome. Results of antimicrobial susceptibility revealed that resistance among the strains was rare. In conclusion, RAPD, ERIC and REP-PCR techniques are useful methods for molecular typing of *V. parahaemolyticus* strains. To our knowledge this is the first study of this kind carried out on *V. parahaemolyticus* strains isolated from the Philippines.

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INTRODUCTION

Vibrio parahaemolyticus belongs to the expanding group of water and food-borne pathogens. It is widely distributed in the aquatic environment and is increasingly regarded as an important pathogen of shrimp causing significant economic problems within the aquaculture industry worldwide (Lightner, 1996; Goarant *et al.*, 1999). In recent years, *V. parahaemolyticus* has been implicated as an opportunistic pathogen, mainly causing gastroenteritis in humans due to consumption of contaminated seafood (DePaola *et al*., 2003). These isolates tend to possess the tdh or trh genes which have only been identified in a minority of the environmental isolates. Therefore, due to increasing reports of disease outbreaks in humans and with the great economic loss in aquaculture, the correct identification and classification of this organism is of great importance.

The development of molecular biology has allowed the typing of organisms on the basis of analysis of their nucleic acids. Molecular typing techniques are specifically useful for microbial epidemiological purposes as they can give information on the genetic relatedness of strains, the source of infection and detection of particularly virulent strains, as well as the study of the geographical and host distribution of possible variants of a specific pathogen (Olive and Bean, 1999). Included are several PCR-based methods such as the Random Amplified Polymorphic DNA PCR (RAPD-PCR), Enterobacterial Repetitive Intergenic Consencus Sequence PCR (ERIC-PCR) and Repetitive Extragenic Palindromic PCR (REP-PCR). The RAPD method involves the use of short random sequence primers, usually 9 to 10 nucleotides long, and low-stringency primer annealing conditions to amplify arbitrary fragments of template DNA. The single primer anneals anywhere on the genome where a near-complementary sequence exists, and amplification occurs if two priming sites are sufficiently close (Welsh and McClelland, 1990). On the other hand, ERIC and REP-PCR methods, respectively, utilize primers complementary to specific sequences in the bacterial genome. ERIC sequences are 126 bp long and appear to be restricted to transcribed regions of the genome, whereas the REP sequences consist of a highly conserved 33 bp inverted repeat sequence (Versalovic *et al.,* 1991).

The main aim of this study was to use and compare three PCR-based techniques for the analysis of genetic variability among *V. parahaemolyticus* strains isolated from the Philippines. These strains were recovered from shrimp (*Penaeus monodon*) and from the environments where these shrimps are being cultivated. RAPD-PCR, ERIC-PCR and REP-PCR were used to establish the DNA fingerprints of *V. parahaemolyticus* strains, with the aim of evaluating the applicability of these techniques in epidemiological studies. In addition, the antimicrobial susceptibility profile and the presence of extended-spectrum β-lactamases (ESBLs) among *V. parahaemolyticus* strains were determined. To our knowledge this is the first study of this kind carried out on *V. parahaemolyticus* strains isolated from the Philippines.

MATERIALS AND METHODS

Bacterial strains

Seventeen *V. parahaemolyticus* strains were analyzed in this study including a strain from the USA and two American Type Culture Collection (ATCC) reference strains. These strains, identified by phenotypic characterisation using classical tube and plate tests (West and Colwell, 1984) and API 20E system (BioMerieux, France), matched perfectly the *V. parahaemolyticus* species description. In addition, identification was confirmed by DNA hybridisation using a thermolabile haemolysin (*tlh*) gene probe (McCarthy *et al*. 1999). The sources of these strains are shown in Table 1. Stock cultures were maintained frozen at -80ºC in tryptone soy broth (Difco, Madrid, Spain) with 15% glycerol.

Table 1. The sources of *V. parahaemolyticus* strains used in this study.

Strain Code	Origin	Year	Donor
Phillipine isolates			
MF-0107-1	Creek water	2001	SEAFDEC
MF-0107-4	Pond water	2001	SEAFDEC
MF-0107-7	Reservoir water	2001	SEAFDEC
CLM3-0108	Crab haemolymph	2001	SEAFDEC
IA3	Pond water	2002	SEAFDEC
IA13	Creek water	2002	SEAFDEC
IA16	Creek water	2002	SEAFDEC
IM12	Source water	2002	SEAFDEC
03S1a-04-5	Rearing water of *P. monodon*	2003	SEAFDEC
03S3-01-2	P. monodon	2003	SEAFDEC
03S3-01-4	P. monodon	2003	SEAFDEC
03S3-07-2	Crab haemolymph	2003	SEAFDEC
03S3-07-5	P. monodon	2003	SEAFDEC
03S3-07-6	P. monodon	2003	SEAFDEC
Reference strains			
IV – 86	Chesapeake Bay, USA	1986	R.R. Colwell
ATCC 43996	Cockles, England	1972	ATCC
ATCC 17802^T	Shirasu food poisoning, Japan	1951	ATCC

SEAFDEC, Southeast Asian Fisheries Development Center, Tigbauan (Philippines)(Dr. C. Lavilla-Pitogo collection); R.R. Colwell, Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore (MD), USA; ATCC, American Type Culture Collection, Manassas (VA), USA.

Isolation of DNA

Isolation of DNA was performed using InstaGene Matrix (Bio-Rad, Madrid, Spain). Strains were routinely grown on Trypticase soy agar (TSA, Oxoid Ltd., Madrid, Spain) plates with 1% NaCl at 25ºC for 24 to 48 hr, after which colonies were scraped off and suspended in 1 ml of autoclaved water and centrifuged at 13,200 x g for 1 min. The supernatants were

removed and the remaining pellets were resuspended in 200µl of InstaGene Matrix and incubated at 56ºC for 30 min. They were then vortexed at high speed for 10 sec and boiled in a water bath for 8 min. The lysates were vortexed again at high speed and centrifuged at 13,200 x g for 3 min. The InstaGene DNA preparations were stored at -20ºC until used for PCR amplifications.

RAPD-PCR typing

The RAPD-PCR amplifications were performed using Ready-to-Go RAPD analysis beads (Amersham Biosciences) as previously described (Romalde *et al*., 1999). Six distinct random 10-mer primers (Amersham Pharmacia Biotech) were used: primers P1 (GGTGCGGGAA), P2 (GTTTCGCTCC), P3 (GTAGACCCGT), P4 (AAGAGCCCGT), P5 (AACGCGCAAC), and P6 (CCCGTCAGCA). Amplifications were performed in a T-Gradient thermocycler (Biometra) programmed as follows: an initial denaturation step at 95ºC for 5 min followed by 30 cycles of denaturation (95ºC for 1 min), annealing (35ºC for 1 min), and extension (72ºC for 2 min), with a final extension step at 72ºC for 5 min.

ERIC-PCR typing

The ERIC-PCR amplifications were performed using Ready-to-Go PCR analysis beads (Amersham Biosciences). A pair of 22-mer primers (Sigma): ERIC 1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC 2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') were used as previously reported by Versalovic *et al.* (1991). After bead rehydratation, each 25 µl ERIC-PCR reaction mixture contained 2.5 U of *Taq* polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate, 20 ng/µl of the respective primer, and 1 µl of DNA solution. Amplifications were carried out in a T-Gradient thermocycler using an initial denaturation step at 95ºC for 5 min followed by 35 cycles of denaturation (92ºC for 45 secs), annealing (52ºC for 1 min), and extension (70ºC for 10 min), with a final extension step at 70ºC for 20 min.

REP-PCR typing

The same Ready-to-Go PCR analysis beads were used for REP-PCR amplifications. For REP-PCR the following 18-mer primers (Sigma) were utilized: REP 1D (5'-NNN RCG YCG NCA TCM GGC-3') and REP 2D (5'-RCG YCT TAT CMG GCC TAC -3'), where M is A or C, R is A or G, Y is C or T, and N is any nucleotide (Stern *et al*., 1984). Each 25 µl REP-PCR reaction mixture contained the same components as with ERIC-PCR mixture except that here REP primers were used. Amplifications were also performed in a T-Gradient thermocycler programmed as follows: an initial denaturation step at 95ºC for 7 min followed by 35 cycles of denaturation (92ºC for 45 secs), annealing (40ºC for 1 min), and extension (72ºC for 8 min), with a final extension step at 72ºC for 15 min.

Gel electrophoresis

The RAPD, ERIC and REP-PCR products were separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide (Bio-Rad). A negative control, consisting

of the same reaction mixture but with distilled water instead of DNA template DNA, was included in each run. The gels were photographed under UV light. A 50- to 2000-bp ladder (Sigma, St. Louis, Mo.) was used as a molecular mass marker.

To determine significant differences in the patterns, the reproducibility of results was assessed by repetition of at least three independent RAPD, ERIC and REP-PCR assays.

Computer data analysis

All the gels were also scanned and the images were captured by a Gel Doc-2000 gel documentation system (Bio-Rad). The data analysis was performed by using Diversity Database software (Bio-Rad), and the computed similarities among strains were estimated by means of the Dice coefficient (S_d) (Dice, 1945). Dendrograms were produced on the basis of the unweighted average pair group method (UPGMA) (Sneath and Sokal, 1973).

Annealing temperature gradient-PCR

To confirm the presence of ERIC and REP sequences in the genome of the investigated strains, representative samples were subjected to PCR amplification using 10 different annealing temperature gradients. The method used was patterned with the work of Gillings and Holley (1997). If ERIC and REP sequences are not present on the genome, bands will fail to amplify at higher temperatures, specifically the T_m of ERIC and REP primers.

For ERIC-PCR amplifications the annealing temperature gradient ranged from 52.3 to 65.6ºC, whereas for REP-PCR a temperature gradient between 40.5 and 63.4ºC was employed.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

A broth microdilution method (VetMIC™, National Veterinary Institute (SVA), Uppsala, Sweden) was used for susceptibility testing of the 14 *V. parahaemolyticus* strains isolated from the Philippines. The standards of the National Committee for Clinical Laboratory Standards (NCCLS) M31-A were followed with some modifications (NCCLS, 1999). The direct inoculum method, with Mueller-Hinton broth (MHB, Difco, MD, USA) as test medium, was used and the microdilution panels were incubated at 28°C for 18 h. For isolates that did not grow, 2% NaCl was added to MHB. *Escherichia coli* ATCC 25922 was included as a quality control strain

Minimum inhibitory concentration (MIC) was registered as the lowest concentration of an antimicrobial agent completely inhibiting bacterial growth. As no criteria were available for these bacteria, the Swedish Veterinary Antimicrobial Resistance Monitoring (SVA, Uppsala, Sweden) susceptibility criterion for enterobacteriaceae was used (SVARM, 2002). The MIC results were interpreted using the following resistance breakpoints (mg/L): ampicillin: >8; ceftiofur: >2; gentamicin: >8; neomycin: >8; streptomycin: >32; enrofloxacin: >0.5; florfenicol: >16; oxytetracycline: >8; and trimethoprim-sulphamethoxazole (TMPS) : >2/38.

Detection of extended-spectrum β-lactamases (ESBLs)

Strains resistant to ampicillin (n=12) were tested for the presence of ESBLs by combination disc diffusion method. Discs of cefpodoxime (10 μg), ceftazidime (30 μg), cefotaxime (30 μg) and imipenem (10 μg) were used in the detection, along with discs in combination with clavulanic acid: cefpodoxime (10/1 μg), ceftazidime (30/10 μg), cefotaxime (30/10 μg) (Oxoid, Hampshire, England). The tests were conducted according to NCCLS standard M100-S9 with some modifications (NCCLS, 1995). Mueller-Hinton agar (Oxoid, Hampshire, England) was used and the plates were incubated at 28°C for 18 h. An ESBLs-producing organism was confirmed if there was a ≥5 mm increase in zone diameter for the antimicrobial agent tested in combination with clavulanic acid compared with the zone when tested without clavulanic acid.

RESULTS

RAPD fingerprinting

RAPD analysis of the *V. parahaemolyticus* strain MF-0107-1 was initially performed using each of the six primers provided in the commercial kit. Only two of them, oligonucleotides P4 and P6, generated reproducible patterns with an appropriate number of amplified products suitable for an accurate analysis. Analysis with the remaining primers did not provide a good amplification of the strain or only gave a small number of PCR products. Primers P4 and P6 were then used to analyse all the 17 strains of *V. parahaemolyticus*. A set of reproducible bands produced for a particular primer was defined as a pattern or profile. The reproducibility of the RAPD method was tested by repeating the RAPD assays at least three times for each primer tested. Results revealed that apart from some minor variations in the band intensity, no significant differences were observed between the profiles obtained, which demonstrated the reproducibility of the method.

The nine distinct patterns obtained with primer 4 , designated RAPD types A to I exhibited between 2 and 6 bands ranging from 300 to 1,500 bp in size (Figs 1 and 5). Three strains recovered from water were shown to form RAPD profile A, while three isolates from *P. monodon* and the type strain ATCC 17802 comprised profile F. Reference strain ATCC 43996 clustered together with three isolates from different sources forming profile G. The remaining profiles contained a unique strain. Regardless of the RAPD profile, all the strains shared a common band at 750 bp (Figure 1).

When primer 6 was used, six distinct patterns were observed and were designated RAPD types A to F, comprising of two to four bands with sizes ranging between 300 and 2,000 bp (Figures 2 and 6). Eight strains from aquatic environment formed RAPD types A and D, while four strains from *P. monodon* formed profile F. Profile E was composed of two strains coming from aquatic environment and from *P. monodon*. Finally, profiles B and C contained a unique strain. Using primer 6, all strains showed a common band at approximately 600 bp (Figure 2).

The results of the analysis of similarity among the different profiles with the Diversity database software employing the S_d and the UPGMA allowed us to identify at least 3 genetic groups or clusters (I-III), with at least 70% similarity level. Thus, with primer

4, the three genetic groups were defined at S_d values of 70% for cluster I (profiles A, C, H, I), 99% for cluster II (profile F), and 90% for cluster III (patterns B and G) (Figure 5). At 80% similarity level all the strains in cluster I were from aquatic environments. The second cluster was composed of two strains from *P. monodon* that are genetically identical sharing the same common bands at 750, 800 and 1,000 bp. Lastly cluster III was composed of strains coming from both aquatic environment and *P. monodon.* The RAPD patterns of all remaining strains were diverse, with similarities below 70% and were considered genetically unrelated by this method.

However, when primer 6 was used three genomic clusters with S_d values ranging from 85 to 99% were formed (Figure 6). Cluster I has an 85% similarity and was formed by RAPD profiles D and E. Majority of the strains (4/7) from this cluster were recovered from aquatic environment. Clusters II (profile A) and III (profile F) were composed of strains that have an identical RAPD type. Both clusters had a 99% similarity. Cluster II was composed of strains coming from aquatic environments whereas cluster III was composed of strains recovered from affected *P. monodon*. In addition, genetic clusters I and II showed a close relationship with a similarity of 80%. Lastly, RAPD types B and C were more diverse with similarities below 50% and were considered genetically unrelated to any of the three clusters.

ERIC-PCR fingerprinting

The fingerprints of *V. parahaemolyticus* strains consisted of 6 to 8 amplification bands, ranging in size from 50 to 2,500 bp (Figure 3). All strains were typeable by ERIC-PCR. The same fingerprints were observed when ERIC-PCR was repeated at least three times, demonstrating the reproducibility of this technique. Almost each strain gave a different ERIC profile although a common band was observed in all 17 strains around 500 bp (Figure 3). Analysing the similarity among the different profiles with the Diversity database software identified three genomic clusters; although there are only few strains that formed these clusters as majority of the strains were genetically unrelated (Figure 7). The first group included strains CLM3-0108 and reference strain ATCC 43996 with 85% similarity. The second cluster consisted of strains IA13 and 03S1a-04-5 with a S_d of 90%. Both strains were recovered from the aquatic environment. Lastly, the third cluster with 80% similarity was composed of strains MF-0107-4 and 03S3-01-2 recovered from pond water and *P. monodon*, respectively. The remaining strains were distinct with similarity of lower than 80%.

REP-PCR fingerprinting

Analysis of the strains with REP-PCR yielded one to four bands depending on the strains (Figure 4). The size of these bands ranged from 150 to 2,000 bp and it was evident that the majority of the strains exhibited patterns with small number of REP-PCR products. As with ERIC-PCR, each strain gave a REP-PCR profile that was different from each other. However, all strains demonstrated a common band at approximately 400 bp (Figure 4). The REP-PCR amplifications were also repeated at least three times. Results revealed that some of the minor light amplification bands were inconsistent making the analysis more difficult.

Similar to ERIC-PCR, only a few strains formed clusters as most strains had unique RAPD profile. Nevertheless, three genomic clusters were identified among the 17 strains of *V. parahaemolyticus* using the Dice coefficient and the UPGMA (Figure 8). The first cluster with 99% similarity was composed of only two strains (MF-0107-4 and MF-0107-7) isolated from the aquatic environment and strain number 03S3-01-2 from *P. monodon*. The second genomic group was composed of two strains (IA13 and IM12) that were both recovered from creek water. The two strains were considered to be genetically related as demonstrated by a 90% similarity. The first and second group showed a closer relationship, with a similarity of 85%. Lastly, the third cluster with a S_d of 90% was composed of strains (03S3-07-5 and 03S3-07-6) isolated from *P. monodon.* The REP-PCR patterns of the majority of the strains demonstrated similarities below 90%.

Annealing temperature gradient-PCR

For both ERIC and REP-PCR, band formation was observed at the highest temperature gradient (T_m) (data not shown). This confirmed the presence of ERIC and REP sequences in the genome of the investigated strains.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

The results of MIC determination and antimicrobial susceptibility revealed that 12/14 (86%) of *V. parahaemolyticus* were resistant to ampicillin. In the case of the remaining antimicrobials, all strains were susceptible. In addition, none of the ampicillin-resistant strains were ESBLs producing and all were susceptible to imipenem.

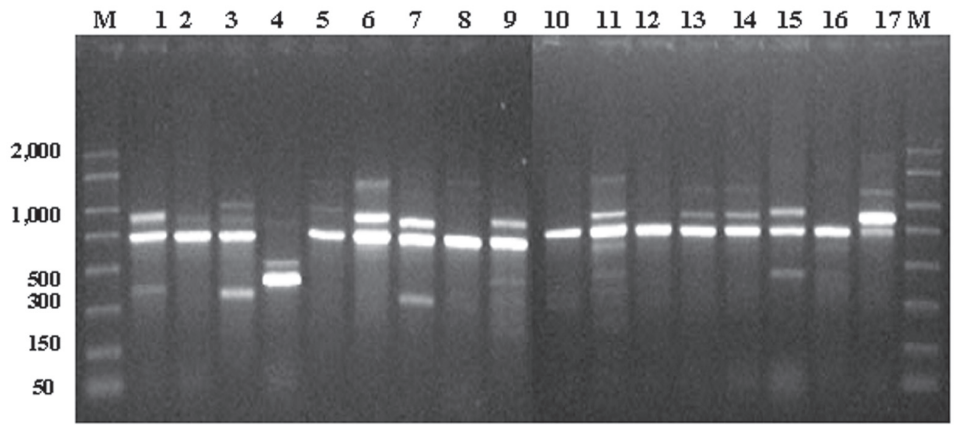

Figure 1. RAPD fingerprints obtained for the *V. parahaemolyticus* strains with primer 4. Lanes: M, AmpliSize Molecular Ruler (50-2000-bp; Sigma); 1, MF-0107-1; 2, MF-0107-4; 3, MF-0107-7; 4, CLM3-0108; 5, IA3; 6, IA13; 7, IA16; 8, IM12; 9, 03S1a-04-5; 10, 03S3-01-2; 11, 03S3-01-4; 12, 03S3-07-2; 13, 03S3-07-5; 14, 03S3-07-6; 15, IV-86; 16, ATCC 43996; 17, ATCC 17802^T. The molecular sizes (in base pairs) are indicated on the left.

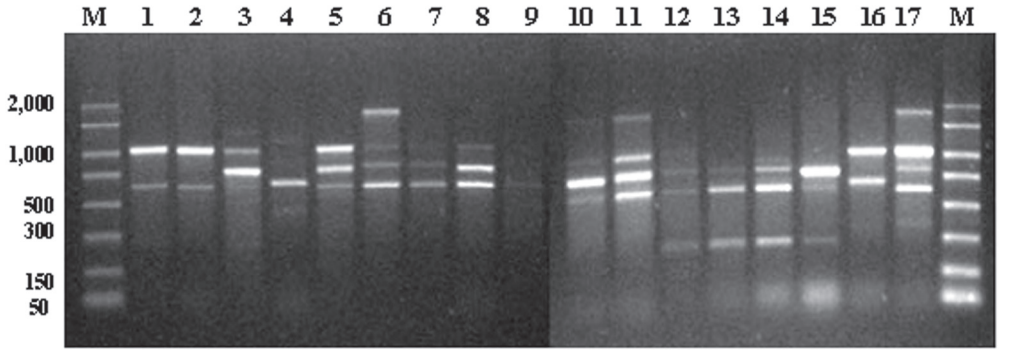

Figure 2. RAPD fingerprints obtained for the *V. parahaemolyticus* strains with primer 6. Lanes as in Figure 1. The molecular sizes (in base pairs) are indicated on the left.

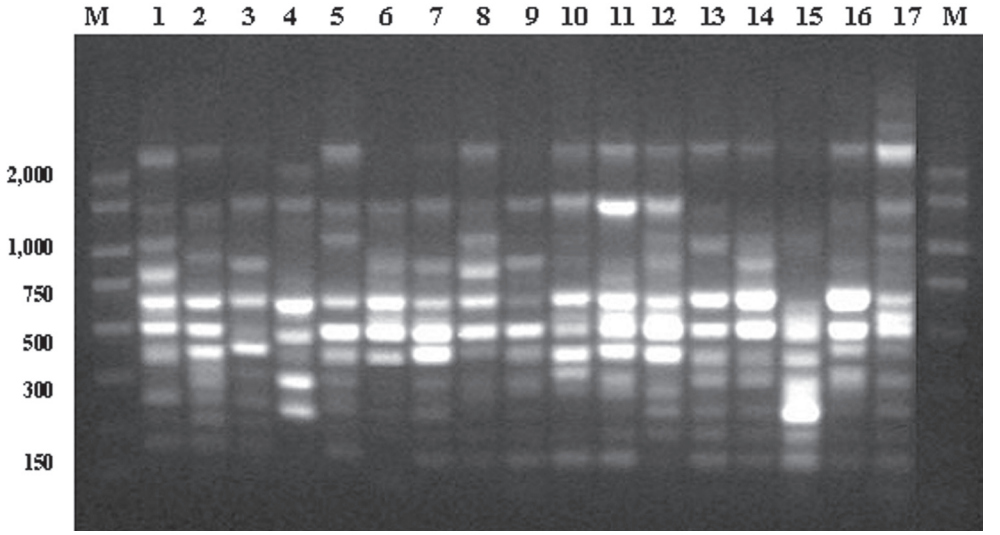

Figure 3. ERIC-PCR fingerprints obtained for the *V. parahaemolyticus* strains. Lanes as in Figure 1. The molecular sizes (in base pairs) are indicated on the left.

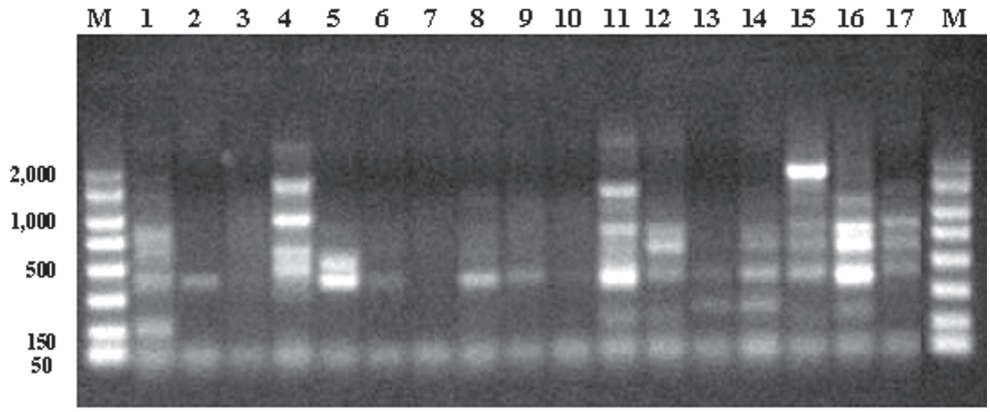

Figure 4. REP-PCR fingerprints obtained for the *V. parahaemolyticus* strains. Lanes as in Figure 1. The molecular sizes (in base pairs) are indicated on the left.

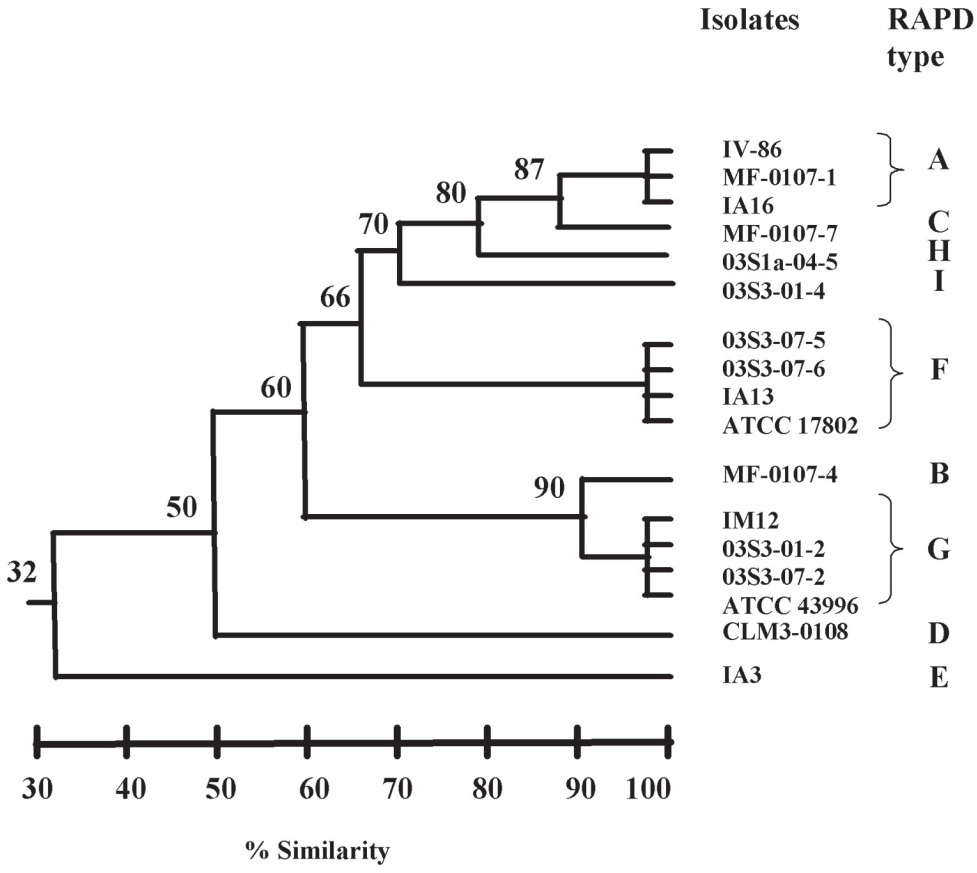

Figure 5. Dendrogram established by the Diversity Database software package (Bio-Rad) using the Dice similarity coefficient and UPGMA on the basis of the RAPD profiles of *V. parahaemolyticus* strains obtained with primer 4.

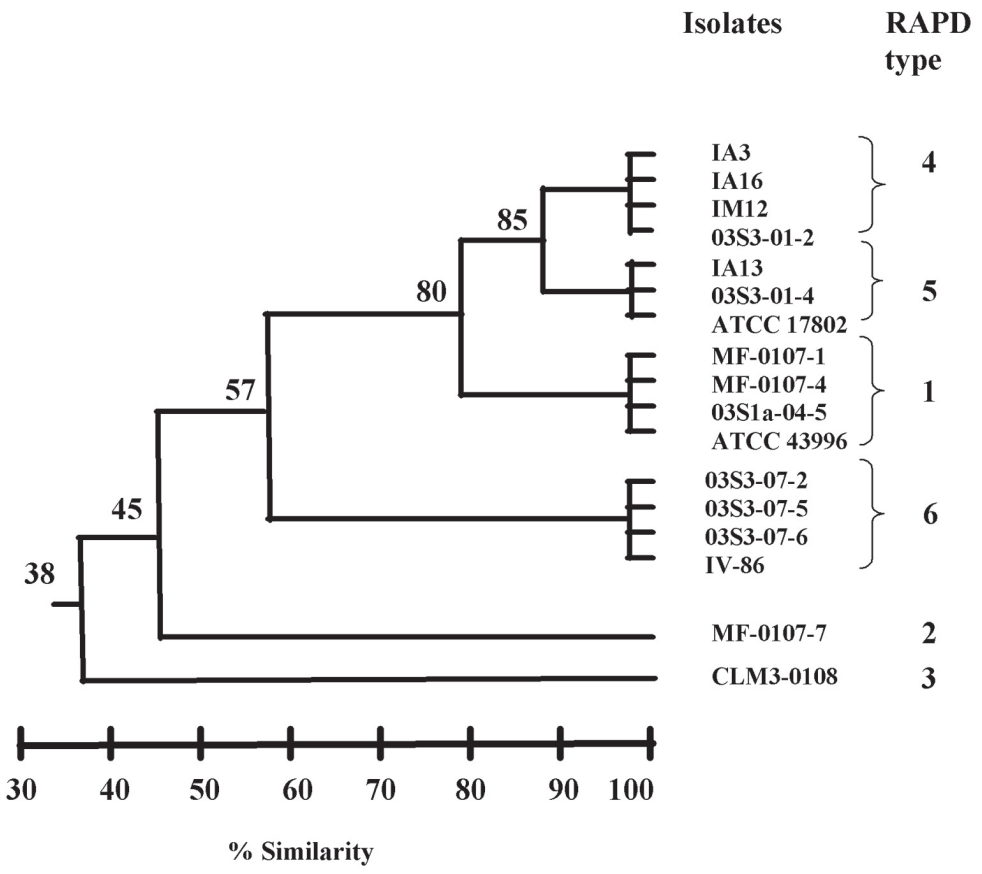

Figure 6. Dendrogram established by the Diversity Database software package (Bio-Rad) using the Dice similarity coefficient and UPGMA on the basis of the RAPD profiles of *V. parahaemolyticus* strains obtained with primer 6.

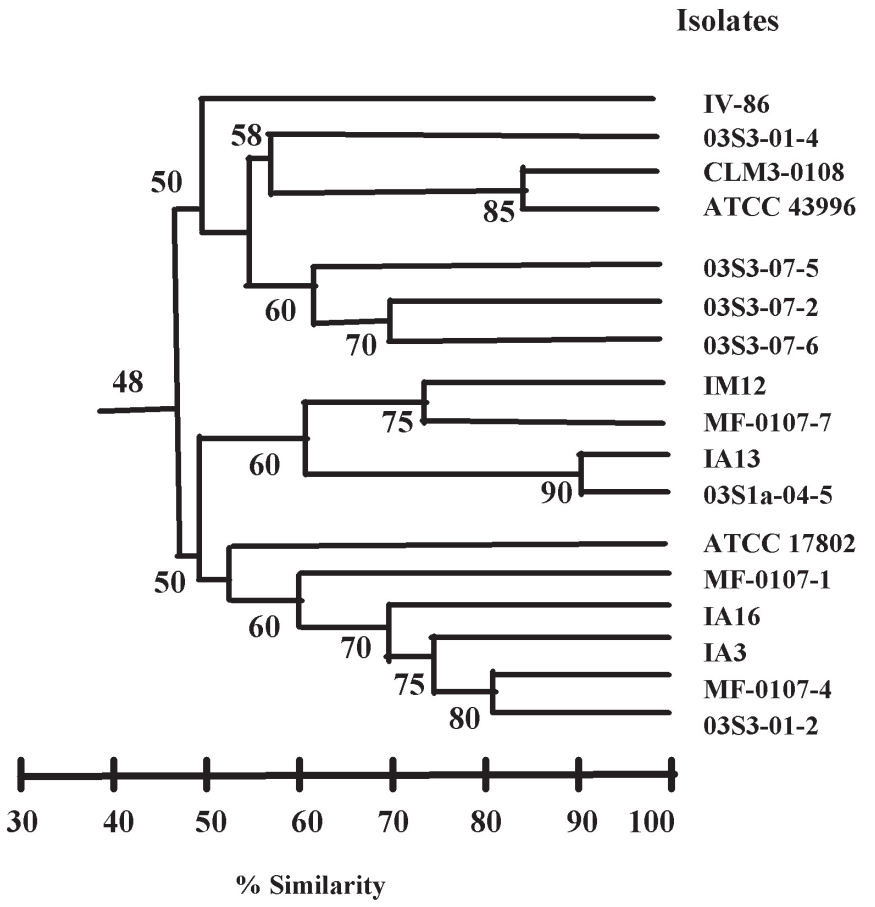

Figure 7. Dendrogram established by the Diversity Database software package (Bio-Rad) using the Dice similarity coefficient and UPGMA on the basis of the ERIC-PCR profiles of *V. parahaemolyticus* strains.

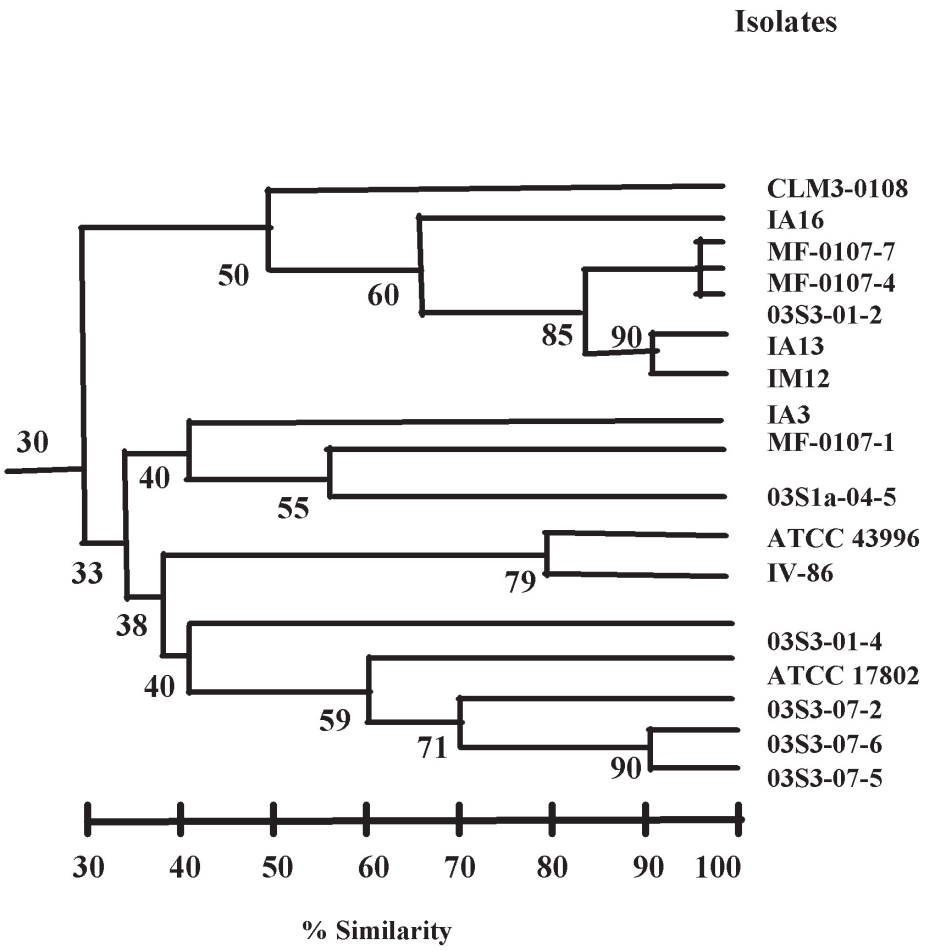

Figure 8. Dendrogram established by the Diversity Database software package (Bio-Rad) using the Dice similarity coefficient and UPGMA on the basis of the REP-PCR profiles of *V. parahaemolyticus* strains.

DISCUSSION

Results of several epidemiological studies on *V. parahaemolyticus* infections have supported the genetic similarity between clinical and environmental strains (Kelly and Stroh, 1998; Marshall *et al.*, 1999), revealing that *V. parahaemolyticus* acquired infections in humans were only reported when the organism could be detected in the local environment (Kelly and Stroh, 1998). This was particularly true when the water temperatures were greater than 14ºC. This is of great importance in tropical countries such as the Philippines, wherein environmental temperatures are usually high.

Results of RAPD fingerprinting with the appropriate primers revealed that some of the patterns obtained could be related to the origin of the strains, which indicated its potential use in epidemiological studies of this organism. Analysis of the similarity of the RAPD patterns using the Dice coefficient and UPGMA has revealed the high genetic variation among strains. As shown in the dendrograms, only few strains clustered with a similarity of 90 to 99%. In a study done by Szczuka and Kaznowski (2004), strains with similarities below 90% were considered genetically unrelated. We could say that based on similarity using the Dice coefficient, our *V. parahaemolyticus* strains are genetically diverse wherein majority have similarity coefficient of below 90%. This result agreed with other studies confirming the genetic diversity among *V. parahaemolyticus* strains (Goarant *et al.,* 1999; Sudheesh *et al.,* 2002). It was found that, regardless of the RAPD profiles observed, all strains showed a common band of 750 bp or 600 bp using both P4 and P6 respectively. These fragments would be favourable traits for the development of genetic amplification and hybridization assays for diagnostic purpose (Dalla Valle *et al*., 2002), which can be important to verify *V. parahaemolyticus* strains that are relatively difficult to identify.

Both ERIC and REP methods gave almost a unique profile for each strain. Comparison of the similarities among the different patterns using S_d and UPGMA confirmed the genetic heterogeneity among *V. parahaemolyticus* strains, as the majority of the strains gave a similarity value below 90%. Although these two methods did not allow the establishment of well defined genetic clusters because of their high discriminatory power such techniques could be useful to follow the spreading of bacterial strain responsible for a particular outbreak. In addition, a common band of 500 bp and 400 bp was shown by all the strains using ERIC and REP PCR methods, respectively. This could be useful for the tracing of the point source of infection.

As shown by Marshall *et al.* (1999), Sudheesh *et al.* (2002) and Szczuka and Kaznowski (2004), it was found that both RAPD and ERIC-PCR gave more reproducible results compared with REP-PCR. The presence of the repeatable fingerprints in ERIC and REP-PCR suggested the presence of these repetitive consensus sequences (ERIC and REP) in *V. parahaemolyticus*. However, Gillings and Holley (1997) have revealed that besides the presence of ERIC, the formation of fingerprints could be due to random amplification. Our results showed band amplifications at higher temperatures (T_m of ERIC and REP) confirming the presence of these sequences on the genome of *V. parahaemolyticus* strains.

Antibiotics resistance among *V. parahaemolyticus* strains was less prevalent in this study. The only resistance observed was to ampicillin (12/14). This finding supported

several reports on the increasing β-lactam resistance among vibrios from different sources (Zanetti *et al*., 2001; Molina-Aja *et al.,* 2002). This study also determined that *V. parahaemolyticus* strains were susceptible to the majority of antimicrobials tested, indicated by the susceptibility of all the strains to ceftiofur, aminoglycosides, florfenicol, TMPS, enrofloxacin and oxytetracycline. However, an increasing number of cases of resistance to these antimicrobials have been reported (Molina-Aja *et al.,* 2002).

Extended-spectrum β-lactamases (ESBLs) are enzymes that mediate resistance to extended-spectrum (third-generation) cephalosporins. The presence of ESBLs was reported in enterobacteriaceae and *Pseudomonas aeruginosa* (Carter *et al.,* 2000; Gheldre *et al.,* 2003). In this study, production of ESBLs among *V. parahaemolyticus* strains could not be demonstrated.

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