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Differentiation of Pathogenic *Vibrio* Species by RAPD

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DNA polymorphism in genomic fingerprinting generated by randomly amplified primed polymorphic DNA markers (RAPD) can distinguish between strains of almost any organism. The RAPD technique which is an application of AP-PCR was applied to 25 isolates of two different *Vibrio* species, *V. cholerae* and *V. harveyi* with 20 different primers. Fourteen oligonucleotide primers yielded clear and reproducible bands corresponding to amplified products and were separable by agarose gel electrophoresis. All the primers used yielded multiple DNA markers with markers ranging in size from 250 – 6,000 nucleotide base pairs. The overall percentages of similarity ranged from 32.5 to 99.4 % with a mean 74.8 % for *V. cholerae* and 32.5 to 73.0 % with a mean 43.7 % for *V. harveyi*. The dendrogram produced from the RAPD fingerprint analysis showed that the *V. harveyi* isolates were grouped into one cluster, whereas *V. cholerae* isolates were grouped into 2 clusters. Results demonstrated that RAPD analysis is a simple and fast reliable technique that can clearly type *Vibrio* spp. and therefore can be applied for phylogenetic studies.

Key words: RAPD, random primer, polymorphism, dendrogram, *Vibrio cholerae*, *Vibrio harveyi*

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Introduction

The genus *Vibrio* is the most extensively characterized bacterial genus and it is one of the medically important bacteria within the family Vibronaceae. Several members of this genus are implicated in both human (*Vibrio cholerae*) and in marine diseases (*Vibrio harveyi*). *Vibrio cholerae* is a noninvasive gram negative bacterium which is the etiological agent for the severe diarrheal disease cholera (Xu *et al.*, 1982). On the other hand, *Vibrio harveyi* is one of the six luminous species of the genus *Vibrio* that is considered to be an opportunistic or facultative pathogen causing clinical vibriosis. In shrimp, vibriosis is the usual consequence of suboptimal environmental factors or poor management procedures in shrimp farming (Lightner, 1988; Brock, 1991).

Epidemiologically, it is important to identify bacterial species and strains accurately. Phenotypic identification of *Vibrio* spp. relies time consuming techniques such as studies on the morphology, nutrition requirement, antibiotic resistance, isoenzyme comparisons, phage sensitivity (Aber and Mackel, 1981 and Selander *et al.*, 1987) that have limited discriminatory powers. Recent advancements in the field of biotechnology have paved several approaches for the identification and typing of *Vibrio* species such as PFGE (Albert *et al.*, 1997), RFLP (Wachsmuth *et al.*, 1993), ribotyping (Faruque *et al.*, 1993), and ERIC-PCR (Rivera *et al.*, 1995). Although these techniques for strain identification and typing are highly reproducible and discriminatory, they are not widely used, as they are time consuming, tedious and technically complicated (Liu *et al.*, 1999). Therefore for studying the epidemiology and pathogenicity, reliable and rapid tools for the strain and species differentiation is necessary. The randomly amplified polymorphic DNA (RAPD) is a technique employing the PCR (Polymerase chain reaction) technique. The application of arbitrarily primers in the PCR (AP-PCR) produces DNA markers that can be used to type microorganism. This method employs a single, short oligonucleotide primer that binds to any region in the genome bearing the complementary sequence producing high discriminatory results in a short time (Caetano-Anolles *et al.*, 1991; Welsh and McClelland, 1990; Williams *et al.*, 1990). The advantage of rapid differentiation with respect to species is to provide the rapid and appropriate treatment to the problems, such as an infectious disease. Clonal similarity within many isolates would mean similar treatment can be used for all the strains.

The aim of this study is to report optimized conditions for RAPD analysis used in the characterization of *Vibrio cholerae* and *Vibrio harveyi* at the molecular level.

Materials and Methods

Source of bacteria: Out of twenty five isolates of different *Vibrio* species, twenty *V. cholerae* clinical isolates were obtained during epidemic (1995-1998) in Malaysia from the patients with cholera. Eighteen of the isolates belong to the Ogawa serotype, while one strain, Inaba and the other was 0139 Bengal serotype (Table 1). Four isolates of *Vibrio harveyi* were isolated from shrimp and pond water of different shrimp farms namely Banting, Kerpan, Pulau Carey and Serkam and these farms were known to be infected with *V. Harveyi*. In this study, an ATCC strain 141216 was also included as a reference.

Genomic DNA extraction: The total genomic DNA was extracted using the QIAGEN QIAamp Tissue kit according to manufacturer's instructions. The genomic DNA was checked for the concentrations and purifies using a UV light spectrophotometer (Shimadzu 1601).

Primers: The twenty random primers were obtained from Operon 10-mer Kits (Operon Technologies Inc.) containing 10-base oligonucleotide primers. These primers include OPAE-1, OPAE-2, OPAE-3, OPAE-4, OPAE-5, OPAE-6, OPAE-7, OPAE-8, OPAE-9, OPAE-10, OPAE-11, OPAE-12, OPAE-13, OPAE-14, OPAE-15, OPAE-16, OPAE-17, OPAE-18, OPAE-19 and OPAE-20. However, only fourteen primers gave satisfactory result, which are OPAE-1, OPAE-2, OPAE-4, OPAE-6, OPAE-7, OPAE-8, OPAE-10, OPAE-12, OPAE-13, OPAE-14, OPAE-15, OPAE-16, OPAE-17, and OPAE-18.

Table 1: The isolates of *Vibrio cholerae* and *Vibrio harveyi* used in the study

Type of species	Serotype/places	Outbreak
V. cholerae	Ogawa	1996
V. cholerae	Ogawa	1996
V. cholerae	Ogawa	1995
V. cholerae	Ogawa	1995
V. cholerae	Ogawa	1998
V. cholerae	Ogawa	1998
V. cholerae	Ogawa	1998
V. cholerae	Ogawa	1998
V. cholerae	Ogawa	1998
V. cholerae	Ogawa	1998
V. cholerae	Ogawa	1998
V. cholerae	Inaba	Clinical isolate
V. cholerae	Ogawa	1998
V. cholerae	Ogawa	1998
V. cholerae	Bengal	Clinical isolate
V. cholerae	Ogawa	1998
V. cholerae	Ogawa	1998
V. cholerae	Ogawa	1998
V. cholerae	Ogawa	1998
V. harveyi	ATCC 141216	Reference strain
V. harveyi	Banting	water
V. harveyi	Kerpan	water
V. harveyi	Pulau Carey	water
V. harveyi	Serkam	water

DNA amplification: The RAPD amplification was performed according to Van Coppenolle *et al.* (1993) with minor modification (Williams *et al.*, 1990). The amplification was carried out in a total volume of 25 μl containing 1 μl of DNA template (different concentrations, 100, 200 and 300 ng μl^{-1}), 1 X reaction buffer, 1.8 mM $MgCl_2$, 200 μM dNTP mix, 1 μl of 5 - 10 pmole of any one of the primers and 1U μl^{-1} of taq polymerase. The DNA amplification was performed with 2 different primer concentrations (5 and 10 pmole) to optimize the exact concentration to amplify the DNA. The cycling profile were: initial denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 34 °C for 1 min, and elongation at 72 °C for 2 min with final extension at 72 °C for 7 min. Amplification was performed in a DNA thermal cycler (Biometra-TRIO Thermoblock). The PCR product was separated on a 1.0 % agarose gel electrophoresis and the gel was then stained with ethidium bromide. The amplified PCR products were visualized and photographed under UV using Polaroid 322 camera with film type 667.

RAPD analysis: The data of 1' and 0's (presence and absence of RAPD bands) was recorded manually and analyzed by RAPDistance Software program (Armstrong *et al.*, 1998). A dendrogram was constructed from the combined results (14 primers) and derived from the distance matrices by using NJTREE (Neighbour-Joining Tree) algorithm (Li and Ferguson, 1998). The genetic distance and percent of similarity among the strains were determined based on Nei and Li (1979) distance. Calculation of the percentage of similarity was done manually from the bands scored from the DNA fingerprints. It was calculated based on the following formula:

Percentage of similarity, $F = 2N_{xy} / N_x + N_y \times 100\%$

Where

N_{xy} = Number of shared bands
N_x = Total number of bands in lane x
N_y = Total number of bands in lane y

Results

Reliability of RAPD primers: Different DNA fingerprints were obtained when different primers were used on the same isolate. The number of DNA fragments amplified from a given sample ranged from 1 to 10 depending on the primer used. All primers

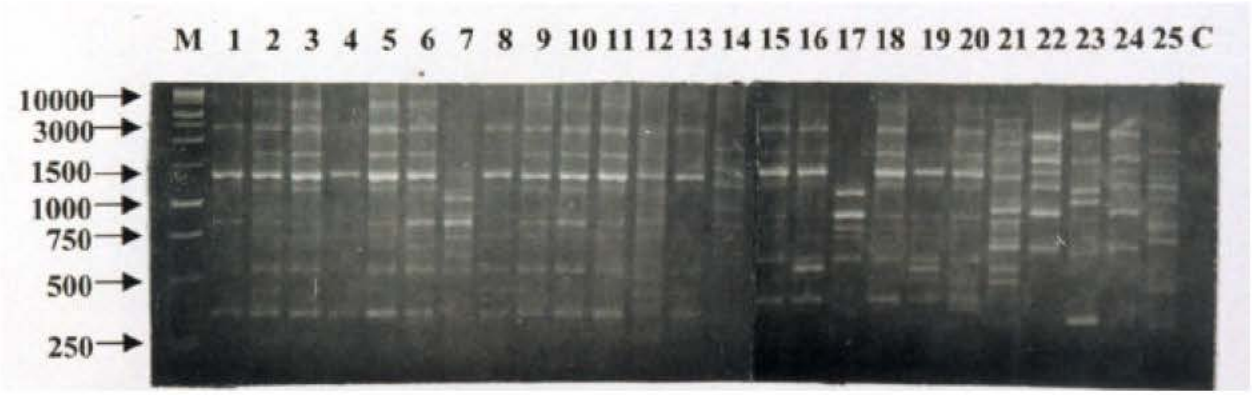

Fig. 1: RAPD patterns obtained with primer OPAE-01. Lanes 1-20 are isolates of *V. cholerae* whilst lanes 21-25 are *V. harveyi* isolates. Lane M is 1 kb molecular weight marker and lane C is the negative control

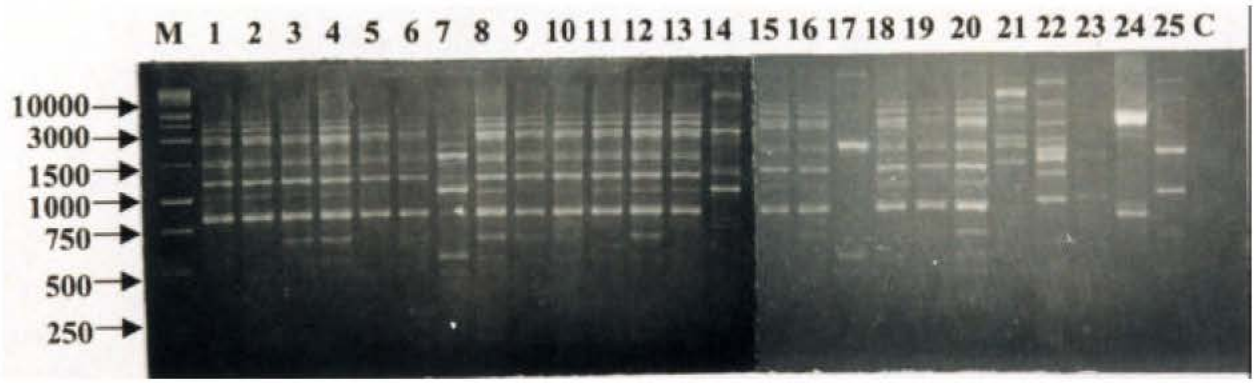

Fig. 2: RAPD patterns obtained with primer OPAE-12. Lanes 1-20 are isolates of *V. cholerae* whilst lanes 21-25 are *V. harveyi* isolates. Lane M is 1 kb molecular weight marker and lane C is the negative control. (250-10,000 Nucleotide base pairs)

were used produced multibanded fingerprints with bands ranging in size from about 250-6,000 nucleotide base pairs (Fig. 1 and Fig. 2). Polymorphism among *V. cholerae* isolates were similar whilst in *V. harveyi* isolates, the pattern of the bands were quite different. Out of twenty primers tested, only fourteen primers gave satisfactory DNA patterns. Six primers (OPAE-3, OPAE-4, OPAE-5, OPAE-11 OPAE-19 and OPAE-20) produced poor RAPD profiles or exhibited no polymorphism. In this study, most of the *Vibrio cholerae* isolates gave similar RAPD profiles with the fourteen primers that gave reproducible bands. All the 25 isolates possessed a 750 bp fragment when amplified with OPAE-15. Thus, this primer can be used as a marker for the identification of *Vibrio* species, for which more number of *Vibrio* species have to be characterized using OPAE-15 to confirm this primer as a genetic marker in order to be applied in the diagnosis of *Vibrio* sp. infection. The RAPD fingerprints generated were found to be unique for each isolates but none of 14 primers can be chosen as a diagnostic marker to distinguish between *V. cholerae* and *V. harveyi* isolates.

Factors that affecting reproducible of RAPD profiles: Reproducibility of the RAPD profiles was tested by repeating the PCR amplifications for all the isolates for more than two times. Exact reproducibility was observed when similar conditions and parameters were utilized. In this study, primers at the concentration of 5 pmoles μl^{-1} failed to amplify any band and resulted in smearing on the agarose gel, whereas primers with higher concentrations about 10 pmoles μl^{-1} were able to produced clear bands without smearing. On the other hand, it was found that only DNA concentration about 300 ng μl^{-1} was able to amplify the DNA template. In order to confirm that the observed bands were amplified by the genomic DNA and not by the primer artifacts, a control PCR reaction for each primer was carried out without the template (Fig. 1 and 2). There was no amplification product produced for any primer.

DNA polymorphism between *V. cholerae* and *V. harveyi* isolates: Polymorphism rates of RAPD bands were much higher between *V. cholerae* and *V. harveyi* than those found among *V. cholerae* isolates. Low levels of polymorphism was observed among the *V. cholerae* isolates which consisted of different serotypes Ogawa, Inaba and Bengal. However, high levels of polymorphism were observed in *V. harveyi* isolates. From the data (Fig. 1 and 2), it can be seen that some of the polymorphism are clear and easy

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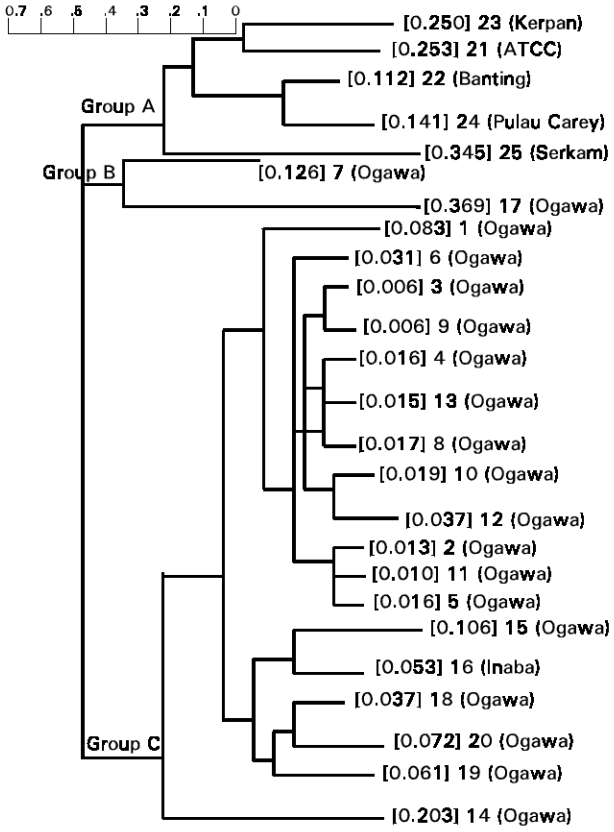

Fig. 3: The clustering between *Vibrio cholerae* and *V. harveyi* species. The dendrogram was generated using the RAPDistance package neighbor-joining tree designed by Li Jin and Ferguson (1990). It was constructed from a combined data from all the primers.

to score, others appear ambiguous and are not useful as genetic marker.

RAPD analysis: The genetic relationships between the species, *Vibrio cholerae* and *Vibrio harveyi* respectively are represented graphically by the dendrogram (Fig. 3). Based on the dendrogram, there are three major clusters. Most of the *V. cholerae* isolates, 18 out of 20 isolates are in the same group (group C) and only two isolates, V7 and V17 are in group B. While, all the *Vibrio harveyi* isolates were in the same group (Group A). The isolates from two different locations that are close in vicinity were clustered together. Within cluster C, the *V. cholerae* isolates were found to be highly related. The values of percentage similarities between *V. cholerae* isolates in this cluster ranged from 44.44 to 99.4 % with a mean of 82.1%. These results indicate that the *V. cholerae* isolates are genetically similar to one another or are closer to one another. However the percentage of similarity between all *V. cholerae* isolates ranged 32.5 to 99.4 %. Whilst, the percentage of similarity between *V. harveyi* isolates ranged from 32.5 to 73.0 % with a mean 43.7 %. The Nei and Li's genetic distance obtained from this study ranged from 0.024691-0.644860 and 0.253968– 0.633028 among *V. cholerae* and *V. harveyi* isolates, respectively. The highest percentage of similarity is between V10 and V9 (*V. cholerae*) isolates, while in *V. harveyi*, it is between Banting and Pulau Carey isolates. However, the highest percentage of similarity between *V. cholerae* and *V. harveyi* isolate is 52.3% which is between V5 and Kerpan isolate. Banting and Pulau Carey are in close vicinity and thus bacterial isolates from these sites are close in genetic distance. On the other hand, the lowest percentage of similarity is between Kerpan and Serkam. Accordingly, these two places are far in geographical location.

Discussion

The classification of bacterial species or strains is an area of active research in population biology and medicine. PCR-based DNA fingerprinting techniques with single, short and random oligonucleotide primers have successfully been used to analyze genomic DNA's of viral, bacterial, fungus, plant and animal origin (Bansal and McDonell, 1997; Welsh and McClelland, 1990). The results (Fig. 1 and 2) of this comprehensive study showed that, RAPD generated a large number of polymorphic DNA bands between *V. cholerae* and *V. harveyi* isolates but showed monomorphic bands among 85% of *V. cholerae* isolates. From this study, it is observed that no relationship between RAPD pattern and serotype could be established since both of the Inaba and Bengal isolates were clustered together with Ogawa isolates (Fig. 3). This study revealed that no relationship between RAPD pattern and serotype could be established since both of the Inaba and Bengal isolates were grouped together however in different subcluster. On the other hand, 16 out of 18 isolates from Ogawa serotype were in the same group. According to the Bando *et al.* (1998), most of the *Shigella* and enteroinvasive *Escherichia coli* (EIEC) strains that belong to the same serotype were clustered together but each serotype was placed in a different subcluster and the geographical origin did not determine clonal relationship. Nakashima *et al.* (2001) on the penicillin non-susceptible *Streptococcus pneumonia* (PNSSP) suggested that samples within the same branch might share similar origins. In addition, strains of the same serotype showed similar susceptibility to several antimicrobial agents and also had a close proximity in genotyping. However Hall (1998) reported that serotypes do not always reflect genetic background, because of the horizontal gene transfer of a determinant of the serotypes. Perhaps using more samples from Inaba and Bengal serotypes in this work would distinguish these serotypes better.

This study (Fig. 1 and 2) demonstrated that short primers (10-mer) of random sequences could be used to amplify segments of genomic DNA from a wide variety of species. Different DNA fingerprints were obtained when different primers were used on the same isolates (Dziva *et al.*, 2001). According to Liu *et al.* (1999), primers that show high levels of reproducibility will produce more polymorphic bands. Williams *et al.* (1990) have been reported that a small change in a primer sequence will cause a complete change in the pattern of amplified DNA segments. According to Liu *et al.* (1999), variation in numbers of amplified bands was observed with drastic changes in DNA template and primer concentrations respectively. They also noted, higher concentrations of DNA template and primer led to amplification of more bands and making the scoring more difficult. From this study, there is no primer that could be used as a genetic marker to distinguish between the *V. cholerae* and *V. harveyi* isolates. More number of primers should be screened with higher sample size to find the genetic marker that could be used for differentiating these species. However, Tinker *et al.* (1993) was able to distinguish 27 inbred lines of barley with only seven primers. Based on genetic polymorphism, these two species were found to be distinctly separated into different groups. According to Foo *et al.* (1995), the high degree of similarity among the RAPD profiles of individuals could be an indication of their close relatedness and low genetic variability among the individuals. Ambiguous polymorphism may result from poor discrimination by a primer between alternative priming sites of slight changes in nucleotide sequences (Williams *et al.*, 1990). In this study (Fig. 3), all the *V. cholerae* isolates were grouped together with the exception of two isolates, V7 and V17 whilst the *V. harveyi* isolates were also grouped into their own cluster. The clustering pattern provides the best estimates of relationships between close

relatives and a poorer representation of relationships between far relatives (Heun *et al.*, 1994).

In conclusion, RAPD technique is highly reproducible and sensitive enough to discriminate the two *Vibrio* species. Previous data suggested that RAPD results were comparable and may offer a degree of sensitivity higher than those of other DNA fingerprinting techniques (Williams *et al.*, 1990). The RAPD technique also enabled to process a large number of samples simultaneously, which may be essential in the event of a large outbreak. Recently, the RAPD assay has been utilized as a simple and rapid alternative method of DNA fingerprinting and has been used successfully to differentiate among strains including *Vibrio vulnificus* (Warner and Oliver, 1998), *Lactococcus lactis* (Cancilla *et al.*, 1992), *Haemophilus somnus* (Myers *et al.*, 1993) and *Streptococcus uberis* (Jayarao *et al.*, 1992). Lastly, RAPD also tended to separate the *V. cholerae* and *V. harveyi* isolates respectively.

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