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ORIGINAL ARTICLE

RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS TO DIFFERENTIATE STRAINS OF *VIBRIO VULNIFICUS* ISOLATED FROM COCKLES AND SHRIMPS.

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A random amplified polymorphic DNA (RAPD) fingerprinting method has been developed to differentiate *Vibrio vulnificus* strains isolated. Twenty-nine strains isolated from cockles and twenty-one strains isolated from shrimps were analyzed. A total of 10 primers were screened with *Vibrio vulnificus* strains to identify those capable of generating DNA polymorphisms and two primers were selected. Primer GEN 1-50-01 and GEN 1-50-08 produced polymorphisms in most strains tested, with the band sizes ranging from 10.0 to 0.25 kb pair. Dendrogram analysis showed that primer GEN 1-50-01 produced 10 clusters and 24 single strains at a 40% similarity, whereas primer GEN 1-50-08 produced 11 clusters and 20 single strains at a 40% similarity. This study revealed the potential use of PCR fingerprinting in epidemiological studies.

Key Words: *Vibrio vulnificus, RAPD-PCR, cockle, shrimp*

Introduction

Vibrio vulnificus was first described by Riechelt *et al*. in 1976 (1) as *Beneckea vulnifica*. This organism is a member of the genus *Vibrio* which are defined as gram-negative, non-sporing rods that are straight or have a single, rigid curve. They are motile and most have a single polar flagellum (2-4). It is a marine bacterium that can cause three types of human infections such as primary septicaemia, gastroenteritis and wound infection (5).

The presence of *Vibrio vulnificus* is not associated with pollution. These bacteria are naturally marine organism that thrives in shallow, coastal waters in temperate climates throughout the world (6). Raw seafood such as oysters, eels, shrimps and fish are example of sources of these bacteria (7). A variety of DNA-based typing methods have been applied to identify *Vibrio vulnificus* species, including plasmid profiles and ribotyping analysis. Each of these approaches has provided useful insights into evolutionary and epidemiological relationships of several *Vibrio* species (8). However, while a variety of molecular subtyping approaches are available, the most general procedure for the comparison of genomes is RAPD analysis. RAPD

technique, developed by William *et al*. (9) produces reproducible and often distinctive sets of DNA fragments by subjecting genomic DNA to PCR primed by short (10 mer) oligonucleotide primers of arbitrary sequences.

In this study we determined the DNA diversity of *Vibrio vulnificus* strains by RAPD-PCR which allows rapid and sensitive differentiation between the strains.

Materials and methods

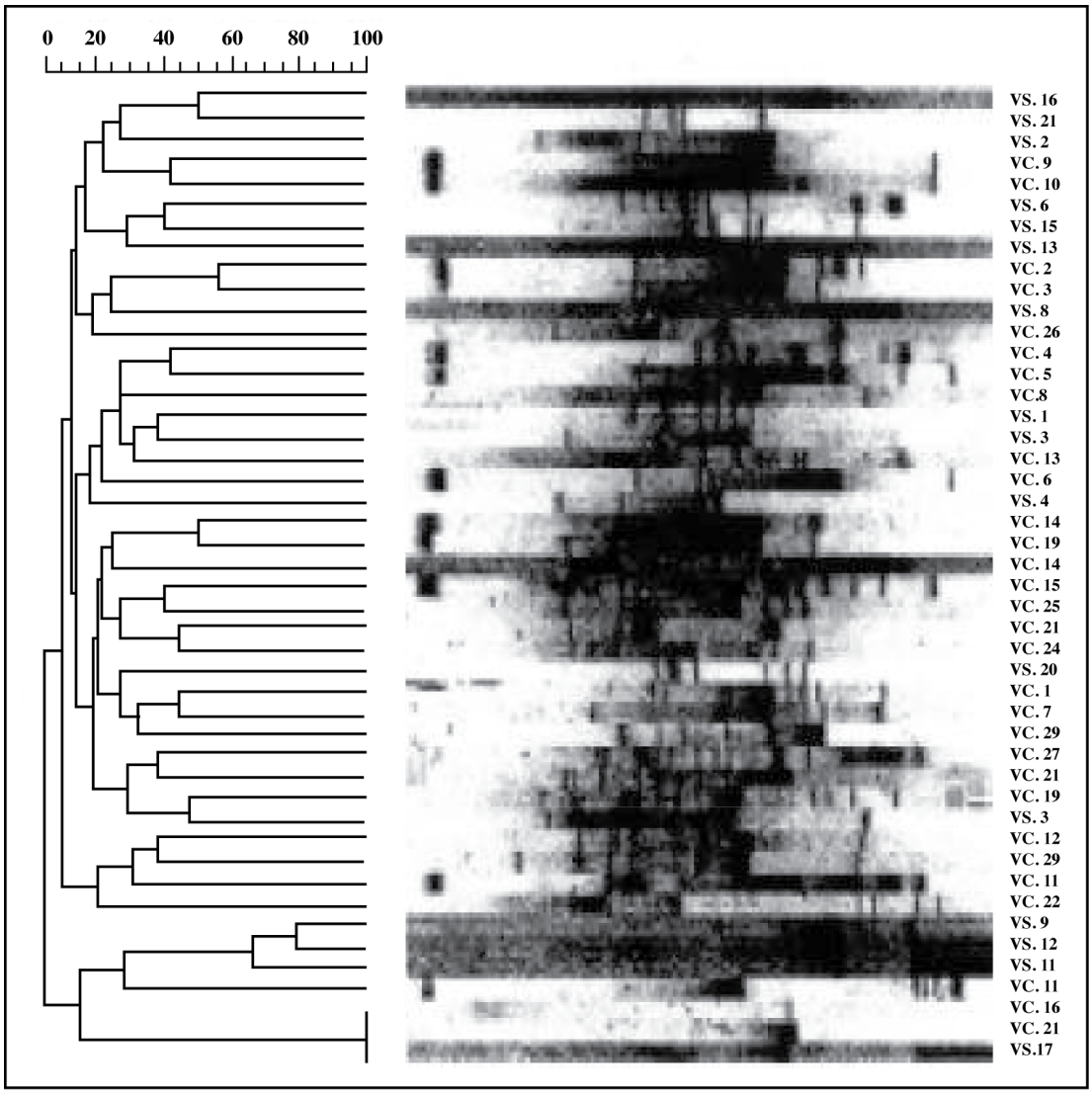
Bacterial isolates

Twenty-nine and twenty-two isolates of *Vibrio vulnificus* from cockles and shrimps purchased from wet markets and supermarkets in Seri Serdang, Seri Kembangan, Subang Jaya, Seremban, Penang and Kuching were examined. All isolates were grown on Thiosulfate Citrate Bile Salts Agar plates (TCBS) and were characterized by standard biochemical tests (4).

Genomic DNA isolation

Genomic DNA extraction was done according to the method by Ausubel *et al*. (10). About 1.5 ml of the bacterial culture was decanted into a sterile eppendorf tube and was centrifuged for 1 minute, at 10,000 rpm. The supernatant was decanted completely and the pellet was resuspended in 700 ml Glucose-EDTA-Tris-HCl buffer. Subsequently, 50 ml of 10% sodium dodecyl sulphate and 5 ml of 20 mg ml^{-1} proteinase K were added to the

Figure 1: Dendrogram produced from cluster analysis of Vibrio vulnificus DNA fingerprints with primer GEN 1-50-01. The similarity index is indicated on top of the plot. Strains VS.5, VS.10, VS.18 and VS.19 were untypeable. VC = Vibrio vulnificus from cockle, VS = Vibrio vulnificus from shrimp.



suspension and the cells were lysed for 20 minutes at 60ºC in a water bath shaker. After the incubation, 500 ml of Phenol-Cheoroform-Isoamyl Alcohol mixture were added and mixed gently for 5 minutes. The mixture was centrifuged for 1 minute at 12,000 rpm. About 200 ml of the aqueous upper layer was collected in an eppendorf tube and then 200 ml of 3 M sodium acetate and 800 ml of isopropanol were added. The precipitated DNA was recovered by centrifugation for 10 minutes at 12,000 rpm. The pellet was washed with 600 ml of 70% ethanol. After centrifuging for 10 minutes at 12,000 rpm the pellet was dried at room temperature for 30 minutes. The dried pellet was resuspended in 50 ml of sterile water and used immediately for PCR analysis.

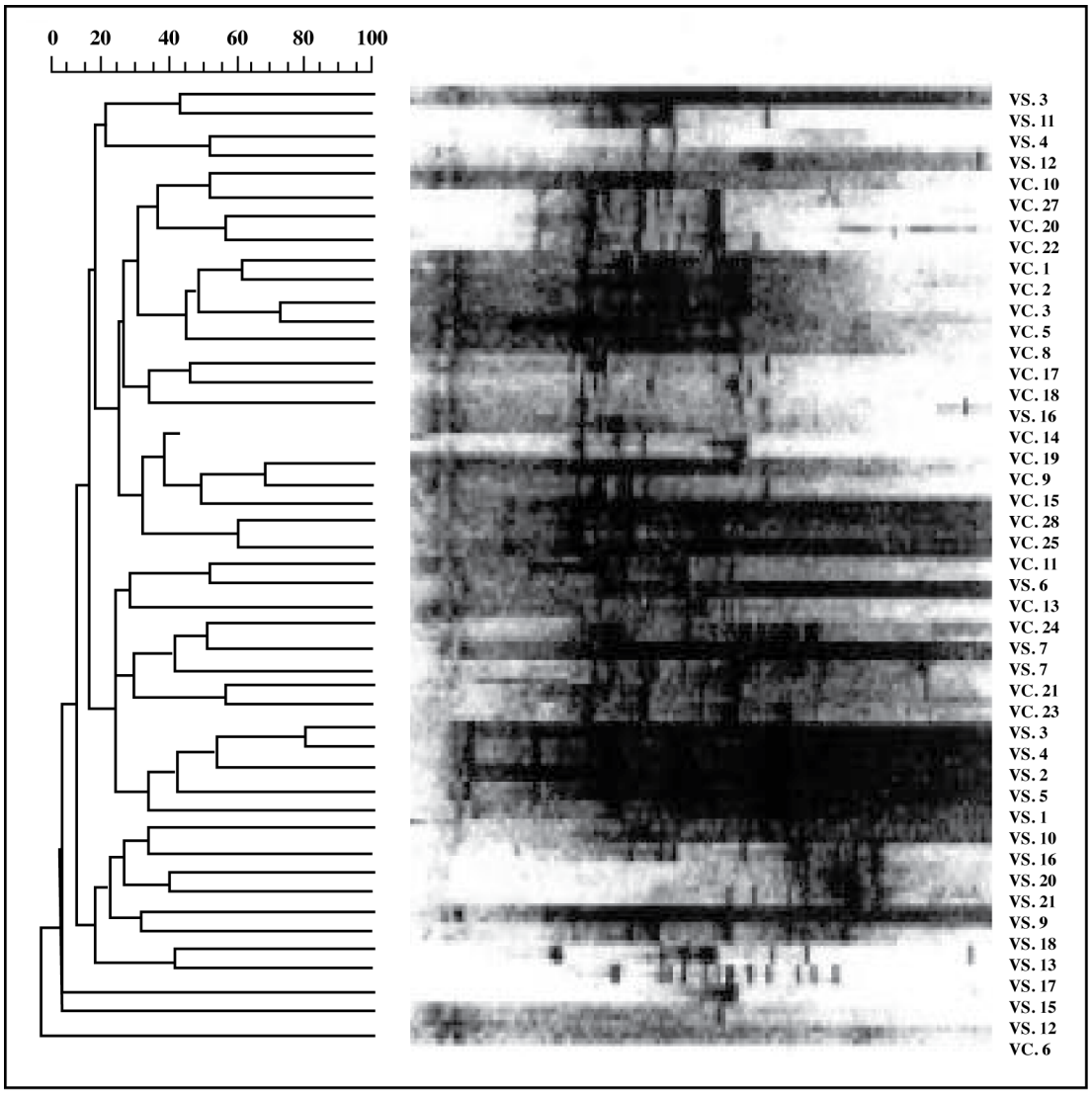
DNA primer

One set of randomly designed 10-mer oligonucleotides (with G+C content of 50%) was obtained from Genosys Biotechnologies., Inc., USA. All primers were screened to determine the ones that can generate clear polymorphic bands.

RAPD-PCR fingerprinting

Reaction mixtures consisted of 2.5 ml 10x reaction buffer, 0.5 ml of 10 mM dNTP mix, 2 mM of primer (Genosys Biotechnologies Inc., USA), 2 ml of 25 mM MgCl2, 0.5 ml of *Taq* polymerase (Promega, USA), 1 ml (20-40 ng)genomic DNA and made up to 25 ml with sterile distilled water. A thermal cycler (Perkin Elmer Model 2400) was used

Figure 2: Dendrogram produced from cluster analysis of Vibrio vulnificus DNA fingerprints with primer GEN 1-50-08. The similarity index is indicated on top of the plot. Strains VC.29, VS.14 and VS.19 were untypeable.
VC = Vibrio vulnificus from cockle, VS = Vibrio vulnificus from shrimp.



for amplification. The reaction was subjected to 45 cycles at 94 ^{0}C for 2 min, 36 ^{0}C for 1 min and 72˚C for 5 min. A final elongation step of 72 ^{0}C for 5 min was included. The PCR amplification products were visualized by running 10 ml of the reaction mixture on a 1.2% agarose gel, which was then stained with 5 mg ml^{-1} ethidium bromide and examined over UV light. A 1 kb DNA ladder (Promega, USA) was used as DNA size markers.

Cluster analysis of RAPD-PCR

The gel picture was first scanned into a digital format as a tagged information file format image using the program Gelcompar ver. 4.1 (Applied Maths, Ghent, Belgium). Dendrogram based on the similarity coefficients were then generated by the unweighted pair-group method of average linkage (UPGMA).

Results

The initial experiments were performed with a subset of *Vibrio vulnificus* strains to identify primers that provide polymorphic band patterns. RAPD-PCR using primers GEN 1-50-01 and GEN 1-50-08 resulted in amplification of genomic DNA of *Vibrio vulnificus* generating fragments of DNA ranging in sizes between 0.25 to 10.0 kilobase pairs (kb). Out of 50 strains examined, 46 and 47 different RAPD patterns were generated using primer GEN 1-50-01 and GEN 1-50-08 respectively. Four and three strains showed no band with primer GEN 1-50-01 and GEN 1-50-08 respectively. Though these strains were untypeable using the respective primer, the combination of the amplification patterns of the two primers allowed all the *Vibrio vulnificus* strains to be typed. Figure 1 and Figure 2 showed the dendrogram generated from the computer cluster analysis of the DNA fingerprints for primer GEN 1-50-01 and GEN 1-50-08. With the primer GEN 1-50-01, 10 clusters and 24 single strains were observed at a similarity level of 40%. Primer GEN 1-50-08 produces 11 clusters and 20 single strains at a 40% similarity level. Some strains of *Vibrio vulnificus obtained from* cockles and shrimps were observed to cluster together, indicating their possible genetic relatedness.

Discussion

Interest in the microbiological relationship of *Vibrio vulnificus* from seafoods has been driven by the recognition that strains of these species are associated with human infections. Despite the increased interest in the *Vibrio* (11) and the rapidly expanding body of knowledge concerning the various associations of these genera with seafoods, limited studies have reported the presence of *Vibrio vulnificus* in seafoods in Malaysia (12). Biochemical characterization revealed that some of the *Vibrio vulnificus* strains isolated from cockles and shrimps in this study were of biotype1, which is known as an opportunistic human pathogen with infection resulting from the consumption of contaminated seafood or exposure to marine environment in the case of wound infections. Thus, it would obviously be of great benefit to develop rapid screening methods for seafoods for the presence of *Vibrio vulnificus* strains that are potentially pathogenic in humans.

A major obstacle in understanding the natural transmission patterns of *Vibrio vulnificus* is the lack of a simple and reliable strain typing system. Utilization of phenotypic properties of *Vibrio vulnificus* to distinguish strains from different hosts face serious limitations as the phenotypic traits of bacteria can vary under different growth conditions. In addition, recent advances in molecular biology have disclosed an enormous diversity in the microbial world, and at the same time they have pointed out the limitations of traditional typing techniques. To end this, we employed an approach that is fairly simple and which can be performed rapidly. RAPD-PCR fingerprinting has been optimized previously, primarily for environmental and cockle sources of this microorganism (12-13). Analysis of the DNA fingerprints of all the *Vibrio vulnificus* strains examined in this study with gel imaging and cluster analysis software revealed significant genetic heterogeneity among the strains. The overall grouping pattern indicated that *Vibrio vulnificus* showed a high degree of variation in its genomic organization, possibly due to transposon activity, recombination shuffling or horizontal gene transfer. This result is consistent with data from several other studies (14 -15) which demonstrated that *Vibrio vulnificus* strains isolated from environmental samples had a diverse genomic organization. Dendrogram obtained using primer GEN 1-50-01 revealed that strains VC.16, VC.23 and VS.17 were clustered together at 100% similarity level, whereas the dendrogram obtained using primer GEN 1-50-08 showed these three strains were in different clusters. Generally, clustering patterns obtained differed when different

primers were used.

Several other studies have reported on the application of various PCR-based strategies to fingerprint *Vibrio vulnificus* strains (16-19). These studies also revealed a high degree of genomic heterogeneity. Among the molecular technique used, RAPD-PCR is the most economical and has shortened the time of typing. This PCR fingerprinting method is simple and rapid, and may be a useful tool for differentiating *Vibrio vulnificus* strains in epidemiological analysis, particularly in large studies and in urgent situations. Reports suggesting that *Vibrio vulnificus* biotype 1 have pathogenic potential in humans (11) emphasizes the need for caution when dealing with seafoods contaminated with this organism.

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