DETECTION AND MOLECULAR CHARACTERIZATION OF *VIBRIO VULNIFICUS* FROM COASTAL WATERS OF MALAYSIA

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Abstract. A total of 57 *Vibrio vulnificus* isolates from coastal water were characterized for their antimicrobial resistance, plasmid profiles and were typed by the PCR-based techniques: a random amplification of polymorphic DNA (RAPD) method and the enterobacterial repetitive intergenic consensus sequence (ERIC) method. All isolates were susceptible to chloramphenicol, nalidixic acid, tetracycline and trimethoprim-sulfamethoxazole. Fifty-one isolates were resistant to one or more of the other antibiotics tested. Plasmid analysis indicated that only 18 isolates carried small plasmids of 1.6 to 16 megadaltons. Analysis of the RAPD and ERIC DNA fingerprints of the *V. vulnificus* isolates with Gel Compare and cluster analysis software revealed significant genetic heterogeneity among these isolates. The combination of RAPD and ERIC analysis allowed us to distinguish all isolates. Thus, the combination of the two techniques is recommended for epidemiological investigation.

INTRODUCTION

Vibrio vulnificus has emerged as an important pathogen responsible for septicemia and severe wound infections in immunocompromised patients (Oliver, 1989). *V. vulnificus* is an estuarine bacterium commonly found in coastal waters and has been detected in shellfish and the intestinal contents of fish (Oliver, 1989; O'Neill *et al*, 1992; DePaola *et al*, 1994; Amaro *et al*, 1995). Molecular typing is being used for epidemiological study of this potential pathogen in the environment. Loi *et al*, (1997) showed that it is necessary to characterize several isolates from the same source because it could harbor different clones of *V. vulnificus*. Thus, the aim of this study was to characterize and compare the strains of *V. vulnificus* isolated from coastal water. The characterization was done by plasmid profiling, antibiotic resistance pattern, randomly amplified polymorphic DNA (RAPD)- and enterobacterial repetitive intergenic consensus (ERIC)-PCR analysis.

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MATERIALS AND METHODS

Bacterial strains

Fifty-seven strains of *Vibrio vulnificus* isolated from seawater along the coast of Pulau Kapas, Terengganu, Malaysia were examined. Seawater samples were serially diluted in 10-fold increments of 100 ml of 0.5% peptone water. One ml of serially diluted samples was transferred into 9 ml alkaline peptone broth, incubated overnight at 37ºC and were streaked onto thiosulfate-citrate-bile salts-sucrose agar (TCBS: Oxoid). The inoculated plates were incubated for 24 hours at 37ºC and green colonies were picked and maintained on trypticase soya agar (TSA, Oxoid). Colonies were inoculated into API 20E biochemical strips (Analytab Products) for species identification.

Antimicrobial susceptibility testing

The *V. vulnificus* isolates were tested against 13 antimicrobial agents [ampicillin (10 μg), carbenicillin (100 μg), cefuroxime (30 μg), ceftazidime (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), neomycin (30 μg), streptomycin (10 μg), trimethoprim-sulfamethoxazole (25 μg) and tetracycline (30 μg)] by a standard disk diffusion method using antibiotic discs (BBL,

Becton Dickinson Microbiology System, Maryland, USA) and Mueller Hinton agar plates as recommended by the National Committee for Clinical Laboratory Standards (1997).

Extraction of genomic and plasmid DNA

Genomic DNA of the *V. vulnificus* strains was extracted by a mini-preparation method as described previously (Son *et al*, 1998). Plasmid DNA was isolated as described by Sambrook *et al* (1989). The approximate molecular mass of each plasmid was determined by comparison with plasmids of known molecular mass of *Escherichia coli* V517 (Macrina *et al*, 1978).

RAPD- and ERIC-PCR amplification

All PCR amplification reactions were performed in a reaction mixture of 25 µl. RAPD-PCR reactions consisted of 2.5 µl 10x reaction buffer, 1 mM of each dNTP, 2 µM primer, 2.5 mM $MgCl_2$, 20-30 ng genomic DNA and one unit of *Taq* DNA polymerase, made up to 25 µl with sterile distilled water. Thirty cycles of amplification were performed at 94ºC for 2 minutes, 36ºC for 1 minute and 72ºC for 2 minutes. A final elongation step at 72ºC for 5 minutes was included. ERIC-PCR amplification reactions consisted of 25 µl volume containing 2.5 µl of 10x reaction buffer, 10 mM each of dNTP, 5 µM each of the forward (ERICR, 5′-ATG TAAGCTCCTGGGGATCAC-3′) and reverse (ERIC2, 5′-AAGTAAGTGACTGGGGTGAGCG-3′) primers (Versalovic *et al*, 1991), 2.5 unit of *Taq* DNA polymerase, 2 mM $MgCl_2$ and 10 ng of genomic DNA. Amplification was done as follows: 92ºC for 45 seconds, 52ºC for 1 minute, 70ºC for 10 minutes and a final elongation step at 70ºC for 20 minutes at the end of the 35th cycle. The RAPD- and ERIC-PCR amplification products were fractionated by electrophoresis through 1.2% agarose gel and detected by staining with ethidium bromide. A DNA ladder (Promega, USA) was used as DNA size markers.

RAPD and ERIC patterns analysis

The RAPD and ERIC patterns obtained were analyzed by using the PC-Windows software package Gel Compare, version 4.1 and cluster analysis was performed by using the UPGMA algorithm.

RESULTS AND DISCUSSION

V. vulnificus is a normal inhabitant of seawater. This organism includes two biotypes that have been defined on the basis of differences in biochemical and serological properties (Tison *et al*, 1982). Biotype 1 is widespread in seawater and is an opportunistic pathogen capable of producing fatal disease after ingestion of raw shellfish or wound infection (Oliver, 1989; Vickery *et al*, 2000). Though biotype 2 has been isolated only from diseased eels, in our previous report we demonstrated that biotype 2 was isolated from cockles (*Anadara granosa*) (Biosca *et al*, 1991; Son *et al*, 1998). Literature on the isolation and epidemiological characteristics of *V. vulnificus* in Malaysia is scanty. In this study, we isolated 57 strains of *V. vulnificus* biotype 1 from seawater used for recreational purposes (Table 1).

The results of the antibiotic susceptibility of the strains are shown in Table 1, which revealed that chloramphenicol, nalidixic acid, neomycin, tetracycline and trimethoprim-sulfamethoxazole are effective against the isolates tested. In the treatment of severe infection caused by *V. vulnificus* biotype 1, tetracycline is normally the drug of choice, as it has demonstrated excellent bactericidal effects *in vivo* (Bowdre *et al*, 1983; Morris and Tenney, 1985). Our results therefore support the choice of tetracycline for therapeutic purposes as all the isolates tested were uniformly susceptible to this antibiotic. As has been previously shown, *V. vulnificus* from aquatic sources were susceptible to gentamicin, kanamycin, carbenicillin, ampicillin, erythromycin and nalidixic acid (Biosca *et al*, 1996; Dalsgaard and Hoi, 1997). With the exception of nalidixic acid, these observations are in contrast to the data obtained in the present study and thus indicate differences in antibiotic resistance among *V. vulnificus* strains in different geographical regions.

Several independent studies have shown that *V. vulnificus* biotype 1 generally lacked plasmids, with only a limited number of strains reported to carry plasmids of between 6 to 52 megadaltons (Amaro and Biosca, 1996; Biosca *et al*, 1996; Dalsgaard and Hoi, 1997). In contrast, this study showed that 28% (16/57) of the *V. vulnificus* isolates carried small plasmids of 1.6 to 16 megadaltons (Table 1). The incidence of plasmids conferring resistance to a particular antibiotic(s) and/or to other harmful agents among aquatic bacterial population has been studied (Hada and Sizemore, 1981; Burton *et al*, 1982; Frederickson *et al*, 1988; Jobling *et al*, 1988; Tamani-Shacoori *et al*, 1995). However, to date the possible role of plasmid in

Table 1
Vibrio vulnificus strains examined in this study.

Strain no.	Antibiotic resistance patterns	Plasmid (s) sizes (MDa)	ERIC-PCR patterns	RAPD patterns with primer: GEN15003	GEN15009
Vv1	ApCarCxmErSm	-[b]	43	7	15
Vv2	ApCarCxm	-	44	6	16
Vv3	ApCarCazCxmErKmSm	-	30	6	13
Vv4	ApCarCazCxmKmSm	-	30	8	14
Vv5	CarCazCxmSm	-	35	2	31
Vv6	Sm	-	40	11	1
Vv7	CarCxmKmSm	-	37	14	24
Vv8	CarCxmKm	-	38	15	25
Vv9	CxmSm	2, 2.8, 7.6	41	16	28
Vv10	CarCazCxmEr	2, 2.8	39	12	26
Vv11	ApCarCazCxmGmErSm	7.6	40	13	27
Vv12	CxmSm	-	15	25	17
Vv13	ApCarCxmErSm	-	34	23	19
Vv14	ApCarCxmErSm	-	34	24	20
Vv15	ApCarCazCxm	-	36	26	18
Vv16	ApCarCazCxm	-	32	9	22
Vv17	ApCarCazCxmGmErSm	-	33	10	39
Vv18	ApCarCaz	7.6	31	18	21
Vv19	ApCarCxm	-	42	21	23
Vv20	ApCarCazCxmKmSm	7.6	25	41	43
Vv21	ApCarCxm	-	21	39	41
Vv22	ApCarCxm	-	23	40	44
Vv23	ApCar	-	21	UT	51
Vv24	ApCarCazCxm	-	16	49	45
Vv25	CarCxm	7.6	19	42	42
Vv26	ApCar	2, 2.8	21	50	47
Vv27	ApCar	7.6	19	44	47
Vv28	ApCarCazSm	-	17	47	48
Vv29	ApCar	-	24	45	49
Vv30	Sm	1.9, 2.3, 16	UT	43	50
Vv31	ApCarCaz	-	18	46	52
Vv32	ApCarCazCxm	-	20	48	46
Vv33	CarCxm	-	UT	37	40
Vv34	Car	-	27	35	37
Vv35	-[a]	-	26	UT	38
Vv36	Sm	-	28	36	37
Vv37	-	-	29	33	35
Vv38	Sm	-	26	34	36
Vv39	-	2.3	9	22	9
Vv40	Sm	2, 2.8	10	17	10
Vv41	-	16	UT	19	3
Vv42	CarCxm	2.2, 16	8	20	29
Vv43	CarCxm	-	7	4	30
Vv44	Cxm	16	7	5	4
Vv45	Cxm	16	1	3	5
Vv46	CarCazCxm	2.3, 16	12	UT	11
Vv47	CarCazCxm	16	7	1	2
Vv48	ApCarCxm	-	11	38	34
Vv49	ApCalCazCxm	-	4	3	32
Vv50	-	-	2	3	32
Vv51	Cxm	-	5	30	33
Vv52	ApCarCazCxm	2.2, 2.4, 13	3	27	7
Vv53	-	-	6	31	6
Vv54	ApCarCazCxm	-	13	UT	12
Vv55	Cxm	-	14	32	11
Vv56	CarCazCxm	-	UT	29	8
Vv57	ACarCazCxm	-	UT	28	8

Tested for resistance against ampicillin (Ap), carbenicillin (Car), cefuroxime (Cxm), ceftazidime (Caz), chloramphenicol, erythromycin (Er), gentamicin (Gm), kanamycin (Km), nalidixic acid, neomycin, streptomycin (Sm), trimethoprim-sulfamethoxazole and tetracycline; [a] and [b], none detected; UT, untypeable.

mediating resistance to antibiotics among *V. vulnificus* biotype 1 has not been established. Likewise, it is not possible from our results to correlate the presence of the small plasmids with the resistance to a particular antibiotics tested. The correlation, if any, needs to be investigated by genetic transfer or curing studies.

Stable and discriminatory bacterial typing methods which allow determination of the relatedness among the isolated strains are required in epidemiological investigation of infection by *V. vulnificus*. Among the methods to detect DNA polymorphism by PCR, RAPD (Williams *et al*, 1990) is one of the easiest. This technique uses arbitrary GC-rich decamers as single primers. Another technique is ERIC-PCR (Versalovic *et al*, 1991). The reproducibility of RAPD and ERIC fingerprints were determined by repeated examinations. DNA was extracted from each isolate and amplified at least three times and reproducible and stable profiles were obtained for both techniques.

Ten oligonucleotide decamers (Genosys Biotechnologies Inc, USA) were tested for their ability to generate RAPD profiles from genomic DNA of a subset of five isolates. Two primers gave clear and reproducible profiles, and were chosen for further analysis of all 57 *V. vulnificus* isolates. All isolates tested were typeable by primer GEN15009 (5′-AGAAGCGATG-3′), whereas two isolates (Vv46 and Vv54) were untypeable by primer GEN 15003 (5′-AGGATACGTG-3′) (Table 1). This indicate the value of using more than one primer in RAPD analysis. Dendrograms based on the profiles obtained by RAPD typing with primers GEN 15003 and GEN 15009 are shown in Figs 1 and 2 respectively. Primer GEN15003 generated 50 different profiles, forming six clusters and 40 single isolates while primer GEN15009 generated 52 different profiles forming 11 clusters and 30 single isolates at a similarity level of 90% respectively. ERIC-PCR produced 44 distinct DNA profiles with five isolates (Vv30, Vv33, Vv41, Vv56 and Vv57) untypeable by this technique. The 44 profiles of the typeable isolates generated 13 clusters and 16 single isolates at a similarity level of 90%. A dendrogram based on the profiles obtained by ERIC typing is shown in Fig 3. This is in agreement with the findings of other groups of researchers who reported that different PCR typing strategies may result in detection of different amounts of genetic diversity (van Belkum *et al*, 1993; van Belkum 1994).

Because there are no generally accepted

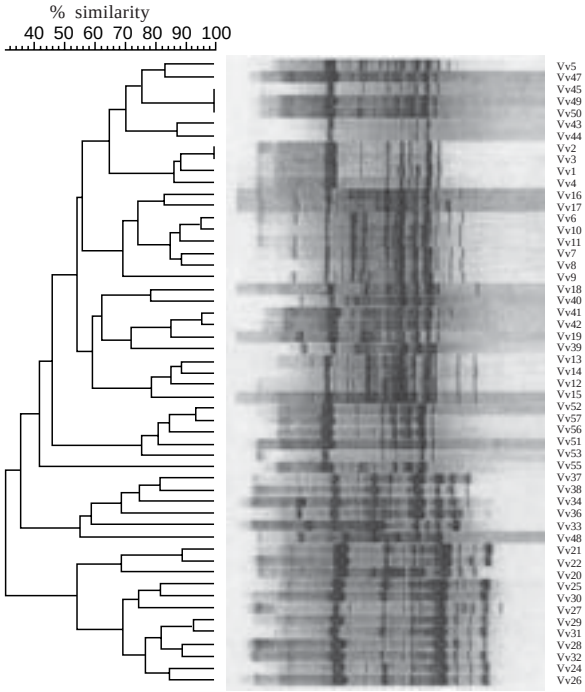

Fig 1–Dendrogram of typeable strains of *V. vulnificus* based on RAPD patterns by primer GN 15003. The RAPD type of each isolate is also included.

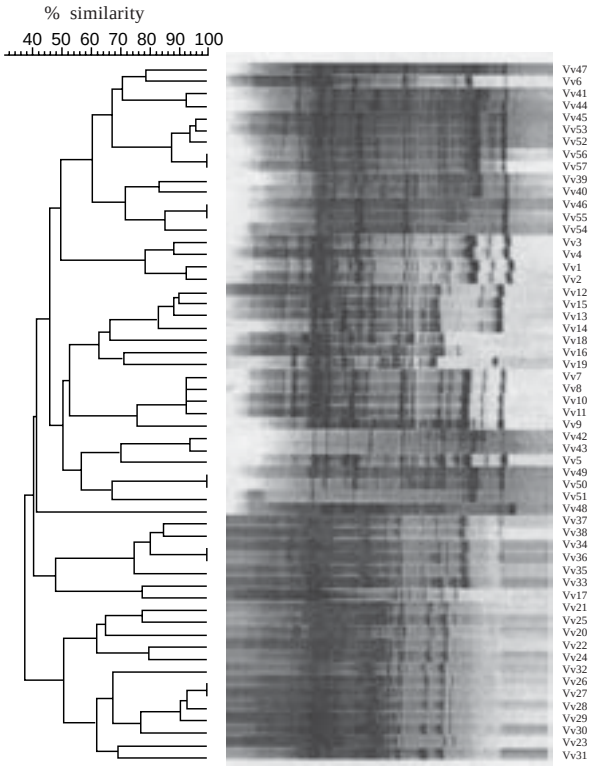

Fig 2–Dendrogram of typeable strains of *V. vulnificus* based on RAPD patterns by primer GEN 15009. The RAPD type of each isolate is also included.

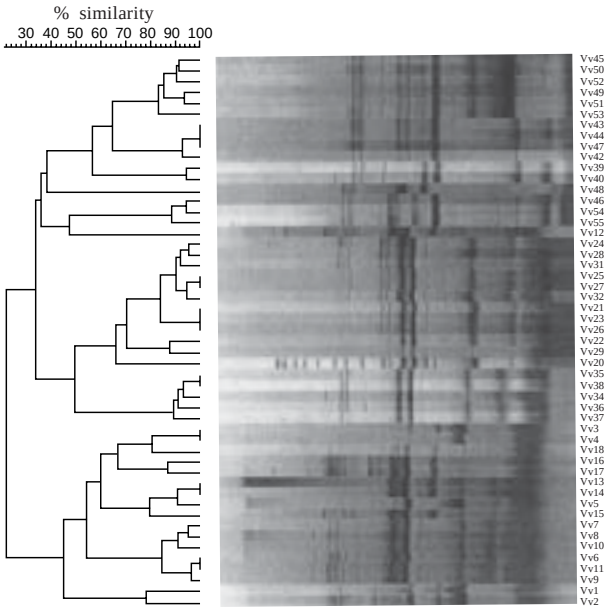

Fig 3–Dendrogram of typeable strains of *V. vulnificus* based on ERIC patterns. The ERIC type of each isolate is also included.

procedures for interpreting the differences observed between DNA patterns produced by any of these molecular methods a composite DNA type was defined on the basis of the combination of types by the two methods (Figs 1, 2 and 3). Hence, a high degree of discrimination was observed by combining the results of RAPD and ERIC techniques: 57 different types were obtained for all 57 strains (Table 1). It is evident that the RAPD analysis resolves strains not separated in ERIC analysis and vice versa. This finding is particularly encouraging as PCR genotyping has advantage of being the least labor intensive, fast and economical technique among the different typing procedures.

In conclusion, the results obtained in this study confirm the presence of multiple antibiotic resistant strains and a wide molecular genetic heterogeneity within environmental isolates of *V. vulnificus* biotype I (Arias *et al,* 1997) and that molecular typing of environmental isolates will play an important role in the epidemiological investigation of *V. vulnificus*.

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