J. Trop. Agric. and Fd. Sc. 30(1)(2002): 31–37

Characterization of *Vibrio vulnificus* isolated from retail cockle and shrimp by plasmid profiling and antibiotic susceptibility test

(Pencirian *Vibrio vulnificus* yang dipencilkan daripada kerang dan udang dengan profil plasmid dan ujian kerentanan antibiotik)

S. Radu*, T.A.F.T. Ahmad* and A.M. Sahilah**

Key words: *Vibrio vulnificus*, shrimp, cockle, antibiotic resistance, plasmid

Abstrak

Daripada 148 sampel kerang *(Anadara granosa)* dan 433 sampel udang *(Paneus indicus)* yang dikaji, 27% dan 6.9% masing-masing didapati positif mengandungi *Vibrio vulnificus*. Sebanyak 29 pencilan daripada kerang dan 21 pencilan daripada udang dikaji untuk kerintangan terhadap antibiotik. Semua pencilan menunjukkan kerintangan kepada satu atau lebih antibiotik yang diuji. Dalam ujian transkonjugasi, tiada kaitan didapati antara plasmid dengan berat molikul yang tinggi (35.8 Mda) yang dikesan di dalam beberapa pencilan, dengan fenotip kerintangan. Ini menunjukkan kerintangan antibiotik adalah berasaskan kromosom. Profil plasmid dan corak kerintangan antibiotik digunakan sebagai pendekatan awal untuk mencirikan, pada tahap pencilan, kesemua pencilan daripada kerang dan udang yang dikaji. Analisis corak kerintangan antibiotik menunjukkan polimorfisma fenotipik yang tinggi. Walau bagaimanapun oleh sebab banyak pencilan yang tidak ada plasmid, teknik ini kurang berguna dalam pencirian. Keputusan ini menunjukkan bahawa pencilan *V. vulnificus* yang mempunyai kerintangan pelbagai dan mempamerkan variasi genotip dan fenotip, amat mudah diperoleh daripada kerang dan udang. Keadaan ini berpotensi membahayakan kesihatan awam.

Abstract

Of the 148 cockle *(Anadara granosa)* and 433 shrimp *(Paneus indicus)* samples examined, 27% and 6.9% were positive for *Vibrio vulnificus*, respectively. Twenty-nine and 21 isolates from cockles and shrimps were examined for their antibiotic resistance. All isolates showed resistance to one or more of the antibiotics tested. In transconjugation tests, no relationship was found between the high molecular weight plasmid (35.8 MDa) detected in several isolates and their resistance phenotypes, indicating that their antibiotic resistance is chromosomal. Plasmid profiles and antibiotic resistance patterns were used as a preliminary approach to type, at strain level, the isolates from cockles and shrimps. Analysis by antibiotic resistance patterns showed a high phenotypic polymorphism. However, the high number of isolates devoid of plasmid rendered this technique less useful. These results indicate that multiresistant *V. vulnificus*

*Department of Biotechnology, Faculty of Food Science and Biotechnology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

**Strategic Resources Research Centre, MARDI Headquarters, Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia

Authors' full names: Son Radu, Tg Ahbrizal Farizal Tg Ahmad and Sahilah Abdul Mutalib

E-mail: son@fsb.upm.edu.my

isolates exhibiting genotypic or phenotypic variations are easily recovered from cockles and shrimps in the study area, posing a potential public health risk.

Introduction

Vibrio vulnificus is a naturally occurring, free-living inhabitant of brackish water and salt water. It prefers tropical to subtropical climates and proliferates in areas or during months where the water temperature exceeds 18 °C (Oliver 1989; Kasper and Tamplin 1993; Hoi et al. 1998). Though the number of people infected with *V. vulnificus* is low when compared to other vibrio-related illnesses, this bacterium has the ability to cause serious and often fatal infections (Morris and Black 1985; Hlady 1997). Human infection is generally acquired through consumption of contaminated raw or undercooked seafood, especially shellfish, or through contamination of wounds by seawater or marine animals (Hlady and Klontz 1996). Most cases of *V. vulnificus* infections are sporadic and characterized by three major clinical syndromes which include gastroenteritis, wound infection and primary septicaemia.

Although the exact mechanism of virulence factors of *V. vulnificus* has yet to be properly identified, a study of the molecular diversity of *V. vulnificus* strains should offer insight into their ecology and epidemiology. In spite of the rapid advancements in the molecular epidemiology of *V. vulnificus* as a result of availability of various molecular techniques (Arias et al. 1997; Vickery et al. 1998; Ryang et al. 1999), relatively little effort has been made to study the occurrence and to characterize this bacterial species in Malaysia. Thus, in the study reported here, the techniques of antibiotic resistance patterns and plasmid profiling were used to characterize the *V. vulnificus* isolated from cockles (*Anadara granosa*) and shrimps (*Paneus indicus*).

Materials and methods

Sample collection, isolation and identification

A total of 148 cockle and 433 shrimp samples obtained from wet markets were examined. The samples were washed with distilled water and the cockle shells were scrubbed free of dirt. Both cockle and shrimp samples were shucked with sterile scalpel and the muscles together with intravulvar fluid were collected in individual sterile stomacher bags. The samples (50 g) were enriched in 450 mL of alkaline peptone water (APW) with 1% NaCl and the samples were homogenized for 2 min (Stomacher Lab-Blender 400). After 18 h of incubation at 37 °C, the samples were serially diluted with alkaline peptone water and 0.1 mL of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions were plated onto thiosulfate citrate bile salts agar (TCBS) plates (Oxoid Ltd, Basingstoke, England). Plates were incubated for 18–24 h at 37 °C. Five non-fermenting sucrose colonies (green coloured colonies) per samples were transferred onto duplicate plates of TCBS agar to obtain pure colonies. The green colonies obtained on TCBS agar medium were identified by standard biochemical tests as described by Tison et al. (1982).

Antibiotic susceptibility tests

Disc diffusion tests were performed three times with antibiotic-containing discs obtained from BBL Microbiology System, Cockeysville, MD, on Mueller Hinton agar following the method described by the National Committee for Clinical Laboratory Standards (1997). All strains were tested against 15 antibiotics: bacitracin (10 µg), carbenicillin (100 µg), cefoperazone (75 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), nalidixic acid (30 µg), norfloxacin

(10 µg), penicillin (10 µg), kanamycin (30 µg), streptomycin (10 µg) and tetracycline (30 µg).

Plasmid DNA isolation

Plasmid DNA of the *V. vulnificus* strains was extracted three times from each isolate by the mini-preparation method of Sambrook et al. (1989). Extracted plasmid was electrophoresed in 0.7% agarose gel. The gel was stained with ethidium bromide and photographed under UV transillumination. Plasmids of *Escherichia coli* V517 were used to determine molecular weight of each plasmid (Macrina et al. 1978).

Conjugation tests

Multiresistant *V. vulnificus* isolates harbouring high molecular weight plasmid (35.8 MDa) were selected for conjugation studies. The selected resistant isolates, as donors, were incubated overnight at 37 °C with a nalidixic acid-resistant *E. coli* K12 as the recipient on nutrient agar plate. The mating mixtures were resuspended and serially diluted with 0.85% saline. Transconjugants were selected by plating the diluted mating mixtures (10^{-1} to 10^{-4}) on nutrient agar plates containing 50 µg/mL of nalidixic acid and inhibiting concentration of agent to which the donor isolates had been resistant.

Results

Forty of the 148 cockle samples were positive for *V. vulnificus*, yielding 80 isolates. Among the 433 shrimp samples only 30 were positive for *V. vulnificus*, yielding 60 isolates. For further characterization, 29 and 21 isolates of *V. vulnificus* were randomly selected from the isolates from cockles and shrimps, respectively. Biotyping of the isolates showed the presence of both *V. vulnificus* biotype 1 and 2 from both sources *(Table 1)*. Isolates were resistant to one or more of the 15 antibiotics tested; exhibiting a wide range of multiresistance distribution and resistance to as many as 12 antibiotics *(Table 1)*.

Twenty-nine of the 50 isolates have been found to harbour 1–7 plasmids with sizes ranging from 1.5 to 35.8 megadalton (MDa) *(Table 1)*. Plasmid analysis grouped the plasmid-containing isolates into 28 groups, with three isolates showing the same plasmid pattern, while 27 isolates have unique plasmid profiles. In genetic transfer study, none of the isolates tested (VC9, VC26, VS6, VS18 and VS19) showed transfer of their antibiotic resistance phenotypes or plasmid. Antibiotic resistance patterns allowed division of the 50 isolates into 47 antibiotic resistance patterns *(Table 1)*.

Discussion

Although occurrence of foodborne *V. vulnificus* infection has not been recognized in Malaysia, it has been widely recognized in other countries in association with consumption of various *V. vulnificus*-contaminated seafoods (Hlady 1997; Morena and Landgraf 1998; Austin and Austin 1999). The high percentage of cockles positive for *V. vulnificus* is not surprising as it has been reported that filter-feeding molluscs such as oysters, clams, mussels and scallops have high concentrations of the bacteria in their guts and other tissues (Strom and Paranjpye 2000). Although the numbers of positive shrimp positive (30/433) were small, shrimp as a primary source of *V. vulnificus* contamination cannot be ruled out. Therefore, more research is needed to establish the role of shrimp and cockle contamination with *V. vulnificus*.

Vibrio vulnificus includes two biotypes that have been defined on the basis of differences in biochemical property (Tison et al. 1982). In this study, *V. vulnificus* biotype 1 and 2 were isolated from both sources. Only biotype 1 has been associated with human disease while biotype 2 previously identified as only pathogenic for eels or fish has now been shown to cause opportunistic infections in humans (Amaro et al. 1992; Veenstra et al. 1992). Of particular interest is the report on the

emergence of the Israeli clone of *V. vulnificus* biotype 3, a newly identified virulent clone of *V. vulnificus* causing outbreak of wound infection and bacteremia associated with exposure to pond-cultured fish (Bisharat et al. 1999).

There is no National Surveillance system for *V. vulnificus* in Malaysia. The ingrained tradition of consuming foods and beverages sold by street vendors will increase the risk of infection, especially when raw shellfish is involved. This situation is of special interest risk, since there are certain practices that encourage consumption of half-cooked cockles by street vendors. In previous studies, the survival of *Vibrio* spp. during heat-treatment of cockles and in street foods indicate their potential as sources of infection (Rusul et al. 1997; Liew et al. 1998). Regardless of the source of the contamination, additional tests should be conducted in packing houses, because of the possibility for sample-to-sample transmission. However, the sources of *V. vulnificus* and the routes of contamination of the product examined are still unknown.

Vibrio vulnificus has been reported to be sensitive to tetracycline, chloramphenicol, aminoglycoside, and third-generation cephalosporins (Tacket et al. 1984; Morris and Black 1985). These results ran contrary to our finding of *V. vulnificus* showing resistance to these antibiotics *(Table 1)*. Such resistance might be due to the indiscriminate use of these antibiotics. Despite the fact that the usage patterns of antibiotics in animals or humans in the study area is still not clear, it cannot be disputed as a contributing factor. The origin of

Table 1. *Vibrio vulnificus* isolates examined in this study

Strain[a]	Antibiotic resistance[b]	Plasmid size (MDa)
VC1 (1)	BCbCfpCazCroCfCmErNaPSmTe	4.8,2.2,1.6
VC2 (1)	BCbCfCmErPSmTe	3.6,2.2,2.1,2.0
VC3 (1)	BCbCroCfCmErPTe	ND
VC4 (1)	BCbCfpCazCroCfErPSmTe	ND
VC5 (1)	BCbCazCroCfErPSmTe	2.1,2.0,1.5
VC6 (1)	BCfpErPTe	ND
VC7 (1)	BCbCroCfCmErPTe	9.8,5.6,4.3,4.0,4.3,2.8,2.2
VC8 (1)	BCbCfP	4.8,3.6,2.6,2.2,2.0,1.7
VC9 (1)	BCbCazCroCfCmErPSmTe	35.8,6.4,4.8,3.1,2.2
VC10 (1)	BCbCfpCazCroCfCmErPTe	10.1,7.4,5.6,2.4
VC11 (2)	BCbCazErNaPSmTe	ND
VC12 (2)	CbCazCroCfGmNaNorPKmSmTe	ND
VC13 (2)	BCbErP	ND
VC14 (1)	BCbCazCroCfCmErPSm	ND
VC15 (2)	BErPTe	2.3
VC16 (1)	BCbCfCmErSmTe	10.5,2.7,2.5,2.4
VC17 (1)	BCfCmErPSmTe	ND
VC18 (2)	CbPSmTe	7.4,5.4,4.4,4.1
VC19 (1)	BCbCfCmErPTe	ND
VC20 (2)	BCbCfpCazErPTe	ND
VC21 (2)	CbCfpCazCroCfErNaPKmSm	ND
VC22 (2)	BErPTe	ND
VC23 (1)	BCbCazCfCmErPTe	2.4,2.3
VC24 (2)	CbP	ND
VC25 (2)	BCbCazPTe	ND
VC26 (2)	BCbCazPTe	35.8,3.4,2.8,2.4

(cont.)

Table 1. *(cont.)*

Strain[a]	Antibiotic resistance[b]	Plasmid size (MDa)
VC27 (1)	BCbCroCmErNaPKmTe	10.2,4.8,4.4,2.9
VC28 (1)	BCbCfpCazCroErGmNaPSmTe	2.0,1.5
VC29 (2)	CbCfPSm	1.5
VS1 (1)	CbCmErPTe	1.5
VS2 (2)	BCroErPTe	1.5
VS3 (2)	BErPSmTe	2.0
VS4 (2)	BCbCazCroErNorPSmTe	ND
VS5 (1)	BCbCfP	ND
VS6 (2)	BCbP	35.8,2.0
VS7 (2)	BCbErP	5.4,4.0,2.3,2.0
VS8 (2)	CbP	6.6,2.0,1.6
VS9 (1)	ErNaPSmTe	ND
VS10 (2)	BCfP	9.0,3.7,3.5,3.2,2.7,2.4,2.2
VS11 (2)	BCbErPSm	2.3,2.1
VS12 (1)	BCbErPSm	2.1,1.6
VS13 (2)	CbCroCfCmErNaNorPKm	ND
VS14 (2)	CfP	7.6,2.1,1.6
VS15 (2)	CbCfP	ND
VS16 (2)	BCbCfErP	ND
VS17 (1)	BCbCfpCazCfCmErNaPSmTe	2.7,2.1,2.0
VS18 (1)	BCbP	35.8,2.1,1.6
VS19 (2)	CbP	35.8
VS20 (2)	CbCfErP	10.7,7.6
VS21 (2)	P	6.2,2.0,1.7

[a]Number in parenthesis indicate biotypes of the isolates. VC and VS denote isolates from cockle and shrimp, respectively
[b]Tested for bacitracin (B), carbenicillin (Cb), cefoperazone (Cfp), ceftazidime (Caz), ceftriaxone (Cro), cephalothin (Cf), chloramphenicol (Cm), erythromycin (Er), gentamicin (Gm), kanamycin (Km), nalidixic acid (Na), norfloxacin (Nor), penicillin (P), streptomycin (Sm) and tetracycline (Te)

multiresistant *V. vulnificus* strains in cockles and shrimps requires explanation and prompts the need to evaluate their potential roles in human infections where antimicrobial therapy would be required. Multiresistance to antibiotics is therefore a phenotypic character of clinical and epidemiological interest, but of overriding concern is the efficacy of these antibiotics in the treatment of multiresistant *V. vulnificus* infections. Every source of resistance must be controlled as well as possible to safeguard the efficiency of antibiotics in the treatment of infections.

Among the plasmid-containing isolates, five isolates harbouring high molecular weight plasmid of 35.8 MDa were tested for their abilities in transconjugation. In general, plasmids are associated with resistance phenotypes in bacteria and usually the high molecular weight plasmid has ability for transconjugation. However, in three independent experiments the five isolates tested showed no ability for transconjugation, an observation indicating that resistance phenotypes of these isolates were chromosomal.

In the two types of samples examined, more than one genotype or phenotype could be detected by plasmid and antibiotic resistance patterns. It is also worth noting that the two different types of samples examined shared some related genotypes or phenotypes. Jackson et al. (1997) reported on the *V. vulnificus* infections caused by single strains among heterogeneous

populations in shellfish. Therefore, multiple isolates from a single sample type should be typed in epidemiological and contamination studies of *V. vulnificus*.

In spite of the low number of *V. vulnificus* strains tested, the present results indicate that there were only a limited number of isolates recovered from cockles and shrimps sharing the same genotypes or phenotypes *(Table 1)* and they were probably spread between samples through trading, but other ways of contamination could not be excluded. Looking through the antibiotic resistance patterns and plasmid profiles, it was observed that there were some disagreements between the results obtained from these two techniques. For example, VC29, VS1 and VS2 isolates had similar plasmid profiles, but had different antibiotic resistance patterns. These results suggested that antibiotic resistance pattern is not influenced by the presence of plasmids, hence supporting the results of the conjugation studies that the antibiotic resistant phenotypes of the isolates tested were not plasmid-mediated. These results showed that it is important to select carefully the typing technique used as they could provide a different insight into the diversity composition of isolates.

Conclusion

The results of this work showed that characterization of *V. vulnificus* strain using phenotypic (antibiotic resistance patterns) and genotypic (plasmid profiles) polymorphism analysis are convenient methods to differentiate *V. vulnificus* of the same or different biotypes. The strains from the same biotype had different profiles which allowed for a simple determination of inter- and intra-biotype genetic differences. The possibility of using antibiotic resistance patterns, and to a less extent the plasmid profiles as an adjunct, for differentiation of *V. vulnificus* strains can provide knowledge of their diversity in epidemiological studies.

Acknowledgement

This study was supported by the Malaysian Government through the Intensification of Research in Priority Areas (IRPA) grant. The authors gratefully acknowledge Dora Bulan in the preparation of the manuscript.

References

Amaro, C., Biosca, E.G., Esteve, C., Fonz, B. and Toranzo, A.E. (1992). Comparative study of phenotypic and virulence properties of *V. vulnificus* biotypes 1 and 2 obtained from a European eel farm experiencing mortalities. *Dis. Aqua. Organ.* ***13:*** 29–35

Arias, C.R., Verdonck, L., Swings, J., Aznar, R. and Garay, E. (1997). A polyphasic approach to study the intraspecific diversity amongst *Vibrio vulnificus* isolates. *Syst. Appl. Microbiol.* ***20:*** 622–33

Austin, B. and Austin, D.A. (1999). *Bacterial fish pathogens: disease of farmed and wild fish*. 3rd ed. New York: Springer

Bisharat, N., Agmon, V., Finkelstein, R., Raz, R., Ben-Dror, G., Lerner, L., Soboh, S., Colodner, R., Cameron, D.N., Wykstra, D.L., Swerdlow, D.L., Farmer III, J.J. and for the Israeli Vibrio Study Group. (1999). Clinical, epidemiological and microbiological features of *Vibrio vulnificus* biogroup 3 causing outbreaks of wound infections and bacteraemia in Israel. *The Lancet* ***354:*** 1421–4

Hlady, W.G. (1997). *Vibrio* infections associated with raw oyster consumption in Florida, 1981–1994. *J. Food Prot.* ***60:*** 353–7

Hlady, W.G. and Klontz, K. (1996). The epidemiology of vibrio infections in Florida, 1981–1993. *J. Infect. Dis.* ***173:*** 1176–83

Hoi, L., Larsen, J.L., Dalsgaard, I. and Dalsgaard, A. (1998). Occurrence of *Vibrio vulnificus* biotypes in Danish marine environments. *Appl. Environ. Microbiol.* ***64:*** 7–13

Jackson, J.K., Murphree, R.L. and Tamplin, M.L. (1997). Evidence that mortality from *Vibrio vulnificus* infection results from single strains among heterogeneous population in shellfish. *J. Clin. Microbiol.* ***35:*** 2098–101

Kasper, C.W. and Tamplin, M.L. (1993). Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. *Appl. Environ. Microbiol.* ***59:*** 2425–9

Liew, W.S., Leisner, J.J., Rusul, G., Son, R. and Rasip, A. (1998). Survival of *Vibrio* spp. including inoculated *V. cholerae* O139 during heat-treatment of cockles (*Anadara granosa*). *Internat. J. Food Microbiol.* ***42:*** 167–73

Macrina, F.L., Kopecko, D.J., Jones, K.R., Ayers, D.J. and McCowen, S.M. (1978). A multiple-plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. *Plasmid* ***1:*** 417–20

Morena, M.L. and Landgraf, M. (1998). Virulence factors and pathogenicity of *Vibrio vulnificus* strains isolated from seafood. *J. Appl. Microbiol.* ***84:*** 747–51

Morris, Jr. J.G. and Black, R.E. (1985). Cholera and other vibrioses in the United States. *New Engl. J. Med.* ***312:*** 343–50

National Committee for Clinical Laboratory Standards. (1997). *Approved standards M2–A6. Performance standards for antimicrobial disc susceptibility tests*. 6th ed. Wayne, Pennsylvania, U.S.A.: NCCLS

Oliver, D. (1989). *Vibrio vulnificus*. In: *Foodborne bacterial pathogens*. p. 569 – 99. (Doyle, M.P., ed.). New York: Marcel Dekker

Rusul, G., Chun, C.K. and Son, R. (1997). Survival and growth of *Vibrio cholerae* O139 in selected Malaysian street foods. *J. Food Prot.* ***60:*** 644 – 8

Ryang, D.W., Koo, S.B., Shin, M.G., Shin, J.H. and Suh, S.P. (1999). Molecular typing of *Vibrio vulnificus* isolated from clinical specimens by pulsed-field gel electrophoresis and random amplified polymorphic DNA analysis. *Japanese J. Infect. Dis.* ***52:*** 38 – 41

Sambrook, J., Fritsch, E.F. and Maniatis, M. (1989). *Molecular cloning: a laboratory manual*. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press

Strom, M.S. and Paranjpye, R.N. (2000). Epidemiology and pathogenesis of *Vibrio vulnificus*. *Microb. Infect.* ***2:*** 177– 88

Tacket, C.O., Breener, F. and Black, P.A. (1984). Clinical features and an epidemiological study of *Vibrio vulnificus*. *J. Infect. Dis.* ***149:*** 558 – 61

Tison, D.L., Nishibuchi, M., Greenwood, J.D. and Seidler, R.J. (1982). *Vibrio vulnificus* biogroup 2: a new biogroup pathogenic for eels. *Appl. Environ. Microbiol.* ***44:*** 640 – 6

Veenstra, P.J., Rietra, J.P.G.M., Stoutenbeck, C.P., Coster, J.M., De Hier, H.H.W. and Dirks-Go, S. (1992). Infection by an indole-negative variants of *Vibrio vulnificus* transmitted by eels. *J. Infect. Dis.* ***16:*** 209 –10

Vickery, M.C.L., Smith, A.L., DePaola, A., Jones, D.D., Steffan, R.J. and Bej, A.K. (1998). Optimization of the arbitrarily-primed polymerase chain reaction (AP-PCR) for intra-species differentiation of *Vibrio vulnificus*. *J. Microbiol. Meth.* ***33:*** 181– 9

Accepted for publication on 20 February 2002