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Isolation, Antibiotic Resistance and Plasmid Profiling of *Vibrio alginolyticus* from Cockles (*Anadara granosa*)

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Abstract: A total of forty-three *Vibrio alginolyticus* strains isolated from cockles collected from wet markets were studied for their resistance to antibiotics and plasmid profiles. All isolates were resistant to one or more of the fifteen antibiotics tested. Twenty different antimicrobial resistance patterns were obtained, suggesting that they do not share a common resistance pattern. The multiple antibiotics resistance indexes indicate that most of the *V. alginolyticus* isolates originated from high-risk sources. The isolates showed high-resistance towards vancomycin (100%), penicillin G (97.7%), bacitracin (95.4%), cephalothin (86.1%), carbenicillin (83.7%), ampicillin (79.1%), erythromycin (79.1%) and tetracycline (55.8%), and low-resistance towards streptomycin (27.9%), chloramphenicol (11.6%), moxalactam/latomoxef (4.7%) and nalidixic acid (2.3%). However, all were susceptible to gentamicin, kanamycin and norfloxacin. Twenty-seven (62.8%) isolates were plasmidless, while sixteen (37.2%) harboured either one or two low molecular weight plasmid(s) ranging between 2.1 and 3.74 MDa. The antibiotic resistance test was more discriminating compared to plasmid profiling and is well suited to characterised the *V. alginolyticus* strains.

Key words: Cockles, *Vibrio alginolyticus*, antibiotic resistance, plasmid

Introduction

Vibrio alginolyticus, from the family *Vibrionaceae*, are aquatic organisms (Lee and Donovan, 1985). This Gram-negative, halophilic vibrio can be isolated from coastal waters and sediments all over the world (Rubin and Tilton, 1975; Gjerde and Böe, 1981; Larsen *et al.*, 1981; Janda *et al.*, 1988 and Wong *et al.*, 1992), preferring temperatures between 17 and 35°C and a salinity of 5-25% (Janda *et al.*, 1988; Patterson *et al.*, 1988 and Hornstrup and Gahrn-Hansen, 1993). *V. alginolyticus* are extremely common in seafood and known to cause non-enteric diseases in man and vibriosis in animals (Lee and Donovan, 1985 and Hörmansdorfer *et al.*, 2000). Infection is acquired by exposure to organisms in contaminated, undercooked, or raw crustaceans or shellfish. Studies by Hörmansdorfer *et al.* (2000) have reported the involvement of *V. alginolyticus* in bleaching and dying of stony corals.

In recent years, vibriosis has become one of the main concerns in the aquaculture industry. Many efforts, including usage of antibiotics, have been made in order to eradicate vibriosis, but were unsuccessful due to the presence of multiple antibiotic resistant pathogenic *Vibrio*. Antibiotics work by either actively killing bacteria (bacteriocidal) or interfering with bacterial processes, halting growth or slowing growth (bacteriostatic). However, current reports have shown an alarming increase of antibiotic resistance in food-poisoning bacteria due to non-rational and excessive uses of antibiotics as therapeutic agents or growth promoters in livestock (Collard, 1999).

The ability of bacteria to show resistance towards antibiotics resides in the resistance genes, which can be found in the bacteria's plasmid DNA and/or chromosome.

This study was carried out to isolate and identify *V. alginolyticus* from cockle samples collected from various fresh markets, and to determine the antibiotic resistance patterns and plasmid profiles among the *V. alginolyticus* isolates. The discrimination index of each analysis was also calculated to determine which analysis can be used as a tool to characterise the *V. alginolyticus*.

Materials and Methods

Samples Collection and Bacterial Isolation: Cockle samples (n = 20) were collected from wet markets at Port Klang (n = 8), Hulu Kelang (n = 4) and Sungai Besi (n = 8) in Selangor, Malaysia. *V. alginolyticus* were isolated from the

cockle samples using the MPN method described by Hara-Kudo *et al.* (2001). CHROMagar™ Vibrio (CHROMagar Microbiology, Paris, France) was used as a selective agar medium to isolate the *V. alginolyticus*. A total of forty-three (2-3 colonies per sample) *V. alginolyticus* isolates were randomly selected for further analysis.

Antibiotics Susceptibility Test: Antibiotic susceptibility tests were performed by the disc diffusion method (Bauer *et al.*, 1966) on Mueller-Hinton agar (Oxoid Ltd., England) according to the National Committee for Clinical Laboratory Standards (1997) against fifteen antibiotics. The antibiotics used included ampicillin (10 μg), bacitracin (10 μg), chloramphenicol (30 μg), carbenicillin (100 μg), cephalothin (30 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), moxalactam/latomoxef (30 μg), nalidixic acid (30 μg), norfloxacin (10 μg), penicillin G (10 μg), streptomycin (10 μg), tetracycline (30 μg) and vancomycin (10 μg) (Oxoid Ltd., England). The multiple antibiotic resistance (MAR) index of each isolate was calculated. MAR index of a single isolate is defined as *a/b*, where *a* represents the number of antibiotics to which the isolate was resistant, and *b* represents the number of antibiotics to which the isolate was exposed to (Krumperman, 1983).

Plasmid Profiling: The isolates were grown overnight at 37°C in LB broth. Plasmid DNA was extracted from each isolates by the alkaline lysis method described by Birnboim and Doly (1979). The number of plasmid DNA obtained were determine by performing horizontal gel electrophoresis using 0.7% agarose gel submerged in 1X TBE (Tris Base-Borate-EDTA) for 1 hour at 150 volts. The gels were stained with ethidium bromide (0.5 μg/ml) and the plasmid DNA bands were visualised and photographed under uv illumination. 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).

Discrimination Index (DI): The discriminatory power of a typing method is its ability to distinguish between unrelated strains. It is determined by the number of types defined by the test method and the relative frequencies of these types (Hunter and Gaston, 1988). It is based on the probability of two strains being characterised as the same type. This index may be used to compare typing methods and select the most discriminatory system.
The DI can be calculated by using the following equation:

$$DI = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j (n_j - 1)$$

where N is the total number of strains in sample population, s is the total number of type described and n_j is the number of strains belonging to j^{th} type.

Results

V. alginolyticus was detected and isolated from all the cockle samples collected from different wet markets. The strains were easily recognised by the milk-white colour exhibited from the colony on the chromogenic agar containing substrates for beta-galactosidase. The forty-three *V. alginolyticus* strains representing the samples examined were found to be susceptible to gentamicin, kanamycin and norfloxacin, but exhibited various degree of resistance towards the other twelve antibiotics tested: vancomycin (100%), penicillin G (97.7%), bacitracin (95.4%), cephalotin (86.1%), carbenicillin (83.7%), ampicillin (79.1%), erythromycin (79.1%), tetracycline (55.8%), streptomycin (27.9%), chloramphenicol (11.6%), moxalactam/latomoxef (4.7%) and nalidixic acid (2.3%) (Fig. 1). A total of twenty different resistance patterns were observed among the *V. alginolyticus* strains (Table 1). Sixty-five percent (65%) of the resistance patterns were represented by single isolates. Resistance patterns A and G were two of the most common patterns occurring with equal frequency (18.6%). Isolates exhibiting pattern A resisted ampicillin, bacitracin, carbenicillin, cephalothin, erythromycin, penicillin G, tetracycline and vancomycin, while those that exhibited pattern G resisted all of the antibiotics as in pattern A, excluding tetracycline. Five (11.6%) isolates exhibited resistance pattern B, the next-most-common pattern, resisting the same type of antibiotics as in pattern A, including streptomycin. The MAR indexes obtained from the *V. alginolyticus* strains were ranging between 0.07 and 0.73 (Table 1).
All forty-three *V. alginolyticus* strains were screened for the presence of plasmid DNA twice. Twenty-seven (62.8%) isolates were devoid for the presence of plasmid(s). Sixteen (37.2%) isolates harboured either one or two low molecular weight plasmid(s) ranging between 2.1 and 3.74 MDa (Table 1). High molecular weight plasmid DNA was not found in any of the isolates.
The DI value calculated for antibiotic susceptibility tests and plasmid profiling was 0.94 and 0.60, respectively.

Discussion

Establishment of the MPN method by Hara-Kudo *et al.* (2001) was for the isolation of *V. parahaemolyticus*, but has proved to be useful for the isolation of other *Vibrio* species, in particular *V. alginolyticus*. This method uses two-step enrichment and plating onto chromogenic agar medium, which is much more

Table 1: Analysis results for the forty-three *Vibrio alginolyticus* strains

Strain	Antibiotic resistance pattern [a]	Resistance pattern	MAR index	Plasmid(s) size, (Mda)
S1	AmpBCbCfEPTeVa	A	0.53	n.d.[b]
S2	AmpBCbCfEPSTeVa	B	0.6	3.52
S3	AmpBCbCfEPSTeVa	B	0.6	3.52
S4	AmpBCbCfEPSTeVa	B	0.6	3.52, 3.74
S5	AmpBCbCfEPSTeVa	B	0.6	n.d.
S6	AmpBCCbCfEPSTeVa	C	0.67	2.83
S7	AmpBCCbCfEMoxPSTeVa	D	0.73	2.83
S8	Va	E	0.07	2.89, 3.2
S9	AmpBCbPSVa	F	0.4	3.22
S10	AmpBCbCfEPVa	G	0.47	2.1, 2.59
S11	AmpBCbCfEPVa	G	0.47	2.1
S12	AmpBCbCfEPVa	G	0.47	2.03, 2.86
S13	BcfEPVa	H	0.33	n.d.
S14	AmpBCbCfPVa	I	0.4	n.d.
S15	AmpBCCbCfEPSTeVa	J	0.67	2.78
S16	AmpBCbCfEPSTeVa	B	0.6	2.78
H1	AmpBCCbCfEPSTeVa	C	0.67	2.78
H2	BCCbCfEPTeVa	K	0.53	n.d.
H3	AmpCbPVa	L	0.27	n.d.
H4	BcbCfEPTeVa	M	0.47	n.d.
H5	BcfEPTeVa	N	0.4	n.d.
H6	AmpBCbCfPVa	I	0.4	n.d.
H7	AmpBCbCfEPTeVa	A	0.53	n.d.
H8	AmpBCbPVa	O	0.33	n.d.
H9	BcfPVa	P	0.27	n.d.
H10	AmpBCbCfEPVa	G	0.47	n.d.
H11	AmpBCbCfEPVa	G	0.47	n.d.
P1	AmpBCbCfEPSVa	Q	0.53	n.d.
P2	AmpBCbCfEPVa	G	0.47	n.d.
P3	AmpBCbCfEPTeVa	A	0.53	n.d.
P4	AmpBCbCfEPVa	G	0.47	2.6
P5	AmpBCbCfEPVa	G	0.47	2.6
P6	AmpBCbCfEPTeVa	A	0.53	n.d.
P7	AmpBCbCfEPTeVa	A	0.53	n.d.
P8	AmpBCbCfEPTeVa	A	0.53	n.d.
P9	AmpBCbCfEPTeVa	A	0.53	n.d.
P10	BcfEPTeVa	N	0.4	n.d.
P11	AmpBCbEKNaPSTeVa	R	0.67	n.d.
P12	AmpBCbCfEPTeVa	A	0.53	n.d.
P13	BEPTeVa	S	0.33	3.18, 3.31
P14	AmpBCbCfPVa	I	0.4	n.d.
P15	BcfPVa	P	0.27	n.d.
P16	AmpBCbCfEMoxPTeVa	T	0.6	n.d.

[a]Tested for ampicillin (Amp), bacitracin (B), chloramphenicol (C), carbenicillin (Cb), cephalothin (Cf), erythromycin (E), gentamicin (Gm), kanamycin (K), moxalactam/latomoxef (Mox), nalidixic acid (Na), norfloxacin (Nor), penicillin G (P), streptomycin (S), tetracycline (Te) and vancomycin (Va).

[b]n.d., non-detected for presence of plasmid(s)

Strains P1-P16 were isolated from Port Klang, strains S1-S16 are from Sungai Besi and strains H1-H11 are from Hulu Kelang in Selangor, Malaysia.

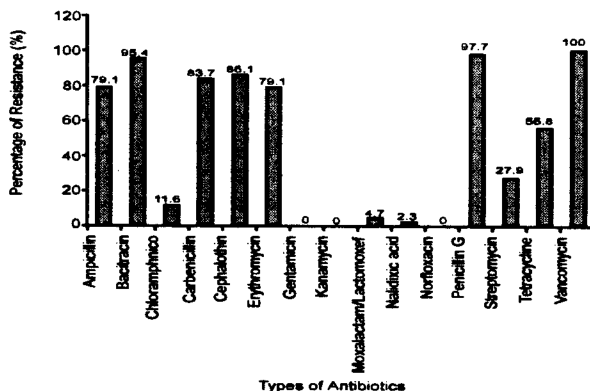

Fig. 1: The percentage of resistance of *Vibrio alginolyticus* against different types of antibiotics used

efficient and sensitive in isolating *Vibrio* species in seafood compared to the currently used method, i.e. using thiosulphate-citrate-bile-salt-sucrose (TCBS) agar (Kobayashi *et al.*, 1963). Each *Vibrio* species grown on CHROMagar™ Vibrio can be distinguished through the colour exhibited from the colonies. *V. parahaemolyticus* will form mauve colonies, *V. alginolyticus* form milk-white/colourless colonies and turquoise-blue colonies for *V. cholerae*. On TCBS agar, *V. alginolyticus* and *V. cholerae* cannot be distinguished from each other, as both bacteria will form yellow colonies (fermentation of sucrose).

Although *V. alginolyticus* is known to occur within the same habitat as *V. parahaemolyticus* and often isolated from the same types of samples, limited ecological and epidemiological studies have been done that included only isolates from temperate water (Joseph *et al.*, 1983). *V. alginolyticus* has been reported in previous studies to be susceptible to chloramphenicol, nalidixic acid, streptomycin, kanamycin, gentamicin and tetracycline, and resistant to ampicillin, penicillin G, erythromycin, cephalothin, vancomycin and carbenicillin (Rubin, 1977; Joseph *et al.*,1978; Joseph *et al.*, 1983; Baumann and Schubert, 1984; Molitoris *et al.*, 1985; Lee *et al.*, 1996; Hörmansdorfer *et al.*, 2000). In this study, all forty-three *V. alginolyticus* strains proved to be resistant to one or more antibiotics. The multiple antibiotic resistant *V. alginolyticus* strains had MAR indexes ranging between 0.07 and 0.73. Forty-two (98%) of the forty-three isolates had MAR indexes above 0.2, indicating that they originated from high-risk sources of contamination such as poultry farms, swine, dairy cattle and human environments where antibiotics are frequently used. A single isolate (2%) had MAR index below 0.2 (Table 1) and thus was presumed to have originated from low-risk sources of contamination like orchard soil, wild and domestic animals environments in which antibiotics are seldom or never used. The MAR indexing is a useful tool in risk assessment as contamination from low- and high-risk environments can be identified (Krumperman, 1983).

The *V. alginolyticus* strains showed various degree of resistance toward twelve of the fifteen antibiotics used. Thus, resulting in twenty different antibiotic resistance patterns. Geographical location and physiological environmental conditions may have contributed to the differences in characteristics of resistance to antibiotics among the *V. alginolyticus* strains. A broad range of antibiotics has been used uncontrolled to eliminate vibriosis, but were unsuccessful. The usage of antibiotics and other chemotherapeutics is becoming an extremely critical issue worldwide due to the resistance, environmental (pollution) and residue problem. The emergence of antibiotic-resistant diseases is causing a human health crisis all over the world. The World Health Organisation (WHO) worries that if the current pattern of resistant development continues, the world would plunge back into the pre-antibiotic era. WHO and the U.S. Center for Disease Control and Prevention agree that the use of antibiotics by the livestock and aquaculture industries is an important cause of antibiotic resistance in food-borne illness.

The ability of *V. alginolyticus* to resist multiple antibiotics is independent on the presence of plasmid DNA. Although 62.8% of the isolates showed the absence of plasmid(s), they exhibited resistance towards the antibiotics tested. It is now well known that bacteria can acquire antibiotic resistance through the presence of resistance gene(s) encoded in the bacterial chromosome or plasmid DNA.

Sixteen (37.2%) *V. alginolyticus* strains harboured either one or two low molecular weight plasmid DNA. The presence of two bands could be the result of conformational changes in the supercoiled plasmid DNA. During plasmid extraction, mechanical shearing may cause the supercoiled plasmid to break and form open circular plasmid, which is slightly heavier than the supercoiled plasmid. Therefore, the open circular plasmid will migrate slower than the supercoiled plasmid during gel electrophoresis. To avoid this phenomenon from occurring, the plasmid analysis must be repeated until a consistent banding pattern is achieved, and minimise the mechanical

shearing of plasmid DNA, example harsh pipetting of plasmid DNA solution.
The discrimination index (DI) aids in the comparison between typing systems. In choosing a new typing scheme, one should aim for as large a DI as possible. The acceptable level of discrimination will depend on various factors, but an index of greater than 0.90 would seem to be desirable if the typing results are to be interpreted with confidence (Hunter and Gaston, 1988). The plasmid profiling had a DI of 0.60, indicating that if two strains were sampled randomly from a population, then on 60% of occasions they would fall into different types. Hence, the discriminatory power of this test is fairly poor. The antibiotic susceptibility tests is clearly much more discriminating compared to plasmid profiling because of its DI of 0.94, which indicates that if two strains were sampled randomly from a population, then on 94% of occasions they would fall into different types.

Conclusjon

In this study, the MPN method developed by Hara-Kudo *et al.* (2001) could be used in the isolation and identification of *V. alginolyticus* from the cockle samples. *V. alginolyticus* can be easily distinguished from other *Vibrio* species through its colony colour. On the other hand, additional steps, such as biochemical tests, can be carried out for confirmational purposes. Each *V. alginolyticus* strains isolated was unique and possesses different genotypic characteristics. This was shown by the different degree of resistance each strains exhibited towards various antibiotics tested and also the occurrence of plasmid DNA. Through this study, it can be concluded that there was no evidence on the linkage between the presence of plasmid DNA and the antibiotic sensitivity of a bacterium. In the absence of contemporary molecular techniques such as the polymerase chain reaction, pulsed field gel electrophoresis, the antibiotic susceptibility test has proved to be more discriminating compared to plasmid profiling and could be used as phenotypic tool to characterised the *V. alginolyticus* strains.

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