International Food Research Journal 18(3): 1183-1188 (2011)

Antibiotic resistance and plasmid profiling of *Vibrio parahaemolyticus* isolated from cockles (*Anadara granosa*) at Tanjung Karang, Kuala Selangor

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Abstract: A total of sixty *V. parahaemolyticus* strains isolated from local cockles (*Anadara granosa*) were investigated by their antibiotic resistance patterns and plasmid profiles. The isolates showed multiple resistances towards most of the antibiotics tested. All strains of *V. parahaemolyticus* isolated harbored 1-3 plasmids, with sizes ranging from 2.7 to 54 kb. All *V. parahaemolyticus* strains showed high multiple antibiotics in frequencies of 0.58 – 0.94 indicating that the strains were derived from high-risk sources. In addition, no particular plasmid profile was predictive of a particular pattern of antibiotic susceptibility. These findings are essential because of the suggested involvement of seafood especially shellfish and environment in transmission of this pathogen to human. Thus, indicating that seafood may be a source of food- acquired antibiotic resistant bacteria to consumer.

Keywords: *Vibrio parahaemolyticus*, antibiotic resistance, plasmid profiling, cockles

Introduction

A wide body of literature implicates the gram-negative organism *V. parahaemolyticus* with human disease and foodborne infections (Balter *et al*., 2006, Sujeewa *et al*., 2009). This ubiquitous microorganism, especially in water, can be isolated in clinical cases and from freshly caught fish and seafood. *V. parahaemolyticus* is associated with gastroenteritis as the most common clinical manifestation, and severe wound infection upon exposure to contaminated seafood and/ or seawater (Panicker *et al*., 2004). In recent years, vibriosis has become one of the most important bacterial diseases in marine cultured organisms, affecting a large number of species of fish and shellfish (Li *et al*., 1999; Molina-Aja *et al*., 2002). In Taiwan and several other Asian countries such as Japan and Hong Kong, *V. paraheomolyticus* is an important foodborne pathogen (Wong and Lin, 2001). The role of antibiotics in the management of human infections caused by *Vibrio* species has not yet been defined, although antimicrobial resistance could be an important problem for therapy directed against these organisms (Zanetti *et al*., 2001). Appropriate antimicrobial therapy directed is needed for a more effective management of severe infections caused by human pathogenic *Vibrio* spp. Hence, elucidation of the antimicrobial susceptibilities of potential pathogenic vibrios will be important for prophylaxis and treatment if *vibrio* infections caused by human beings and in cultured marine organisms. Plasmids have been found in vibrios, and in some cases, their involvement in resistance to many antibiotics has been proven (Molina-Aja *et al*., 2002; Manjusha *et al*., 2005; Zulkifli *et al*., 2009).

Hence, the aims of this study were to determine the antibiotic resistance patterns among *V. parahaemolyticus* isolated from cockles (*Anadara granosa*) from Tanjung Karang, Kuala Selangor; also to determine the presence of plasmids among the *V. parahaemolyticus* isolates and to correlate them with antibiotic resistance.

Materials and Methods

Bacterial strains

Sixty-two strains of *V. parahaemolyticus* were isolated from 62 out of 100 samples of local cockles (*Anadara granosa*) obtained from a harvesting site in Tanjong Karang, Kuala Selangor. The isolates were routinely grown at 35°C in Luria Bertani broth with addition of 3% (w/v) NaCl.

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Antibiotic susceptibility testing

The antimicrobial susceptibility tests were performed essentially by the disc diffusion method as described by Bauer *et al*. (1966), with disc containing antibiotic (Oxoid Ltd., England). Antibiotics tested were carbenicillin (100 µg), ceftriaxone (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), clindamycin (2 µg), doxycycline (30 µg), imipenem (10 µg), nalidixic acid (30 µg), netilmicin (30 µg), nitrofurantoine (300 µg), norfloxacin (10 µg), oflaxacin (5 µg), rifampicin (5 µg), streptomycin (10 µg), sulfamethoxazole (25 µg), teicoplanin (30 µg) and tobramycin (10 µg).

Cultures derived from a single colony were grown at 35°C. Overnight cultures grown at 35°C were then spread over the Mueller Hinton agar plates using a sterile cotton bud and were allowed to dry for 2 to 5 minutes. Antibiotic discs were spaced out onto the plates and were incubated at 37°C for 24 hours. Diameter of the clear zone for each antibiotic disc was measured and interpreted as susceptible or resistant categories according to the guideline recommended by the National Committee for Clinical Laboratory Standards (1997).

The multiple antibiotic resistance index of the isolates was defined as a/b where 'a' represents the number of antibiotics to which the particular isolate was resistant and 'b' the number of antibiotics to which the isolate was exposed to (Krumperman, 1985).

Plasmid DNA extraction

All bacterial strains were grown overnight in LB broth (addition of 3% (w/v) NaCl) at 37°C. The plasmid in *Vibrio parahaemolyticus* isolates was extracted by the alkaline denaturation procedure described by Sambrook *et al*. (1989) followed by gel electrophoresis. In estimations of molecular weight of plasmids in *Vibrio paraheamolyticus* isolates, *Escherichia coli* strain V517 was used as molecular size markers. *Escherichia coli* strain V517 harbors 8 plasmids with molecular sizes of 2.1, 2.7, 3.0, 3.9, 5.1, 5.6, 7.2 and 54 kb (Macrina *et al*., 1978).

Results and Discussion

The percentage of resistance of *V. parahaemolyticus* strains to various antibiotics is given in Table 1. Isolates were resistant to carbenicillin (100%), cephalothin (100%), clindamycin (100%), oflaxacin (100%), rifampicin (100%), streptomycin (100%), sulfamethoxazole (100%), teicoplanin (100%), tobramycin (100%), ceftriaxone (85%), imipenem (79%), netilmicin (74%), nitrofurantoine (63%), doxycycline (60%), nalidixic acid (60%) and chloramphenicol (24%). However, norfloxacin was sensitive towards the isolates apart from one isolate that shows resistance to the antibiotic. Our results concur with data among the *V. parahaemolyticus* and *Vibrio* spp. reported elsewhere (Lesmana *et al*., 2001; Roque *et al*., 2001; Zanetti *et al*., 2001; Molina-Aja *et al*., 2002; Manjusha *et al*., 2005; Zulkifli *et al*., 2009), who isolated *V. parahaemolyticus* with resistance to these antibiotics.

Table 1. Distribution of antimicrobial resistance of *V. parahaemolyticus* (n=62) isolates from local cockles (*Anadara granosa*)

Antibiotics	No. (%) of *V. parahaemolyticus* resistant to selected antibiotics
Aminoglycosides	
Netilmicin (NET30)	46 (74)
Streptomycin (S10)	62 (100)
Tobramycin (TOB10)	62 (100)
Beta-lactams	
Carbenicillin (CB100)	62 (100)
Imipenem (IPM10)	49 (79)
Teicoplanin (TEC30)	62 (100)
Cephalosporins	
Ceftriaxone (CRO30)	53 (85)
Cephalothin (KF30)	62 (100)
Quinolones	
Nalidixic acid (NA30)	37 (60)
Norfloxacin (NOR10)	1 (2)
Tetracycline	
Doxycycline (DO30)	37 (60)
Others	
Chloramphenicol (C30)	15 (24)
Clindamycin (DA2)	62 (100)
Nitrofurantoine (F300)	39 (63)
Rifampicin (RD5)	62 (100)
Sulfamethoxazole (SMT25)	62 (100)
Oflaxacin(OB5)	62 (100)

Higher levels of resistance to carbenicillin, cephalothin, clindamycin, oflaxacin, rifampicin, streptomycin, sulfamethoxazole, teicoplanin, tobramycin, ceftriaxone, imipenem, netilmicin, nitrofurantoine, doxycycline, nalidixic acid and chloramphenicol among other bacteria from food and environmental sources have been reported (Mills-Robertson *et al*., 2002; Vivekanandhan *et al*., 2002; Thayumanaan *et al*., 2003; Gonzalez-Ray *et al*., 2004; Nayak *et al.*, 2004). Both Zanetti *et al.* (2001) and Ottaviani *et al.* (2001) found high susceptibility of *V. parahaemolyticus* strains and other *Vibrio* spp. to norfloxacin in concordance to the findings in our study. In contrast to the high resistance of isolates towards most of the antibiotics tested in our study, several reports elsewhere mentioned the susceptibility of *Vibrio* spp. to beta-lactam antibiotics and nalidixic acid (Li *et al*., 1999; Bhattacharya *et al*., 2000; Wong *et al*., 2000; Ottaviani *et al*., 2001). This suggests that geographical locations and selective pressure influence the antibiotic resistance levels. Our results showed that the second generation quinolones (norfloxacin) is the drug with the best antimicrobial effect against *V. parahaemolyticus* in agreement with data reported by other authors (Zanetti *et al*., 2001; Ottaviani *et al.*, 2001).

Susceptibility to tetracyclines in vibrios and other

Table 2. The antibiotic resistance profile patterns and Multiple Antibiotic Resistance (MAR) index of *V. parahaemolyticus* from local cockles (*Anadara granosa*)

Pattern	Strain No.	Antibiotic Profiles[a]	MAR	No of isolate/Total Isolates (% of Occurrence)
I	VP1, VP3, VP4, VP10, VP48, VP51, VP52, VP53, VP60, VP61, VP62	C, CB, CRO, DA, DO, IPM, KF, NA, NET, OB, S, TOB, TEC, SMT	0.94	11/62 (17.7)
II	VP5, VP7, VP12, VP13, VP15, VP28, VP33, VP35, VP38, VP40, VP41, VP42, VP43, VP45, VP50	CB, CRO, DA, DO, IPM, KF, NA, NET, OB, S, TOB, TEC, SMT	0.88	15/62 (24.2)
III	VP14, VP16, VP26, VP30, VP34, VP36, VP39, VP44, VP46	CB, CRO, DA, IPM, KF, NA, NET, OB, S, TOB, TEC, SMT	0.82	9/62 (14.5)
IV	VP2, VP6, VP8, VP11, VP17, VP18, VP19, VP24, VP27, VP29, VP32, VP37, VP56, VP58, VP59	CB, CRO, DA, IPM, KF, NET, OB, S, TOB, TEC, SMT	0.76	15/62 (24.2)
V	VP9, VP20, VP23, VP31, VP49	CB, CRO, DA, IPM, KF, OB, S, TOB, TEC, SMT	0.71	5/62 (8.1)
VI	VP21, VP22, VP25, VP47, VP55, VP57	CB, DA, IPM, KF, OB, S, TOB, TEC, SMT	0.65	6/62 (9.7)
VII	VP54	CB, DA, KF, OB, S, TOB, TEC, SMT	0.58	1/62 (1.6)

[a]Tested for C, chloramphenicol; CB, carbenicillin; CRO, ceftriaxone; DA, clindamycin; DO, doxycycline; F, nitrofurantoine; IPM, imipenem; KF, cephalothin; NA, nalidixic acid; NET, netilmicin; NOR, norfloxacin; OB, oflaxacin; RD, rifampin; S, streptomycin; SMT, sulfamethoxazole; TEC, teicoplanin; TOB, tobramycin.

bacteria has been reported by others (Gonzalez *et al*., 1999; Wong *et al*., 2000; Zanetti *et al*., 2001; Gonzalez-Ray *et al*., 2004). Our study revealed high occurrence of tetracycline-resistant *V. parahaemolyticus*. This observation is of importance to public health because doxycycline (one of the tetracyclines) is the primary antibiotic used in the treatment of gastrointestinal infection caused by *V. cholera* in hospitals in Malaysia (HUKM, 2000). There are also reported studies of other bacteria such as *Escherichia coli* (Apun *et al*., 2008) and *Campylobacter jejuni* (Chai *et al*., 2008) which are tetracycline-resistant. Lesmana *et al*. (2001) suggested that tetracycline or trimethoprim-sulfamethoxazole is effective for the treatment of infection caused by *V. parahaemolyticus*, and Wong *et al*., (2000) noticed that cephalothin and tobramycin were active against most *V. parahaemolyticus* isolates from patients as partially in agreement with Wenzel *et al*. (2003), results which ran contrary to the findings of our study where high level of resistance of *V. parahaemolyticus* towards these antibiotics is observed. Tetracycline has been widely used in the farming and agriculture sector as growth promoters and pesticides. It is also a common antibiotic used in human medicine. Therefore, the usage of this antibiotic must be carefully monitored to avoid development of stronger resistant strains (Rahman *et al*., 2008). Alarming rate of resistance is observed among *V. parahaemolyticus* isolates from cockles in the present study towards rifampicin, doxycycline and chloramphenicol.

All strains of *V. parahaemolyticus* isolated from cockles in the current study showed multiple antibiotic resistances (MAR), which has been reported before in other vibrios (Yuherman, 2000; Nasreldin, 2001; Micky *et al*., 2002). The MAR indices for the *V. parahaemolyticus* are given in Table 2. Such high level of multiple resistances may arise from selective pressure due to the indiscriminate use of antibiotics. Animal farms release their effluent and water following antibiotic therapy on the farms (Schmidt *et al*., 2000); residual water from the industries plants where products containing microbial agents have been prepared and washed, and the residual water is released directly into the sewage system (Guardabassi *et al*., 1998), which ultimately flows into the sea. MAR of *V. parahaemolyticus* that is introduced into the marine environments readily contaminates the shellfish, which concentrate the marine microflora via filter feeding, subsequently causing a mutation in the cellular DNA (Zulkifli *et al*., 2009) These observations have been seen to contribute to the occurrence of MAR *Salmonella* and *Aeromonas hydrpohila,* repectively, in marine environment and seafood (Vivekanandhan *et al*., 2002; Matinez-Urtaza *et al*., 2004).

Plasmid analysis of all the isolates as shown in Table 3 provided a general picture that revealed *V. parahaemolyticus* contains plasmids. This finding correlates with results of previous studies, which have shown that *V. parahaemolyticus* harboured plasmids (Kagiko *et al*., 2001; Kaufman *et al*., 2002; Molina-Aja *et al*., 2002; Manjusha *et al*., 2005; Zulkifli *et al*., 2009). Molina-Aja *et al*. (2002) noted that 80% of the *Vibrio* strains analyzed harbour plasmids and 11 different plasmid profiles were observed. The plasmid profiles in our study revealed that 54.8% of the investigated *V. parahaemolyticus* strains carried more than 2 plasmids with diverse sizes. The molecular weight of the plasmids ranged from 2.7 to more than 54 kb. Of the 62 strains analyzed, 31 (50%) strains had 2 plasmids and 4 (6%) strains had 3 plasmids. These results are in concordance with Li *et al*. (1999) and Molina-Aja *et al*. (2002) which

Table 3. The plasmid profile patterns of *V. parahaemolyticus* from local cockles (*Anadara granosa*)

Plasmid Pattern	Plasmid Size (kb)	Strain No.	No of isolate/Total Isolates (% of Occurrence)
A	-	VP18, VP26, VP30, VP31, VP46, VP56, VP60	7/62 (11.3)
B	2.7	VP28	1/62 (1.6)
C	3.0	VP24	1/62 (1.6)
D	5.1	VP27	1/62 (1.6)
E	5.6	VP54	1/62 (1.6)
F	7.2	VP36, VP37, VP39, VP40, VP45, VP47, VP55 VP57, VP58, VP59, VP61	11/62 (17.7)
G	54	VP1, VP4, VP7, VP10, VP14	5/62 (8.1)
H	2.7, 7.2	VP25	1/62 (1.6)
I	2.7, >54	VP2	1/62 (1.6)
J	3.0, 7.2	VP43	1/62 (1.6)
K	3.0, 54	VP8, VP16	2/62 (3.2)
L	3.8, 7.2	VP22, VP42	2/62 (3.2)
M	5.1, 7.2	VP23, VP33, VP34, VP41, VP44, VP51, VP52	7/62 (11.3)
N	5.6, 7.2	VP20, VP21, VP29, VP32, VP35, VP38, VP48, VP49, VP50, VP53, VP62	11/62 (17.7)
O	54, >54	VP3, VP5, VP6, VP9, VP11, VP12	6/62 (9.7)
P	3.0, 54, >54	VP15, VP17	2/62 (3.2)
Q	5.6, 54, >54	VP19	1/62 (1.6)
R	7.2, 54, >54	VP13	1/62 (1.6)

suggested the high occurrence of multiple plasmids in *V. parahaemolyticus* isolates as shown in our data of plasmid profile.

The plasmid profiles in vibrios have been studied in some species such as *Vibrio ordalli*, *Vibrio vulnificus*, *Vibrio salmonicida* and most extensively in *Vibrio anguillarum* (Tiatinen *et al.*, 1995; Livesly *et al*., 1997; Radu *et al*., 1998; Pedersen, 1999; DePaola *et al*., 2003), where a high diversity of profiles was observed (Pedersen *et al*., 1996). Plasmid profiling has been proven as a useful tool to differentiate between *Vibrio salmonicida* strains (Sorum *et al*., 1990). In this study, the plasmid profile varied within the *V. parahaemolyticus* isolates where 18 plasmid profiles were identified. This result indicates that plasmid profiling is still considered a capable tool for typing *V. parahaemolyticus* or other *Vibrio* spp. isolates.

Antibiotic resistance in *V. parahaemolyticus* in this study is presumably an innate rather than an acquired trait through plasmid transfer or antibiotic selection. This is proven by the presence of MAR *V. parahaemolyticus* isolates but no plasmid was detected among these isolates. Nevertheless, the linkage of the presence of plasmids in the isolates to antibiotic resistance has not been determined. Molina-Aja *et al*. (2002) noted that the most common profile that they obtained had only 21.2 kb plasmid and a significant correlation was found between the presence of this plasmid and resistance to carbenicillin, although some exceptions could be detected (three strains had the plasmid and were resistant to carbenicillin, three strains had the plasmid but were not resistant), it is suggested that resistance can be encoded in some strains in plasmids and in others in the chromosome.

In Malaysia, plasmid profiling is not a new tool to be used as an epidemiological tool. Several reports have been cited to utilize plasmid profiling as a phenotypic method to type environmental, food or clinical isolates in our country (Gwendelynne, 2004; Jurin, 2004; Zulkifli *et al*., 2009). High percentage of strains in our study contained plasmids in comparison with the previous local findings (Micky, 2001; Nasreldin, 2001; Son *et al*., 2002). Thus, it appears that *V. parahaemolyticus* strains isolated from cockles in Tanjong Karang, Kuala Selangor is very diverse and one possible reason is because the cockles are caught from wild populations of the study area.

Acknowledgements

This work was supported in-part by grant-in-aid of Ministry of Health, Labor and Welfare, Japan and by Grant-in-Aid for Scientific Research (KAKENHI 19101010) from Japan Society for the Promotion of Sciences.

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