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Antibiotic resistance and plasmid profiling of *Vibrio parahaemolyticus* isolated from cockles in Padang, Indonesia

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Abstract: *Vibrio parahaemolyticus* is one of the most widely recognized pathogenic *Vibrio* species due to numerous outbreaks and its' wide occurrence in marine environment. In this study, 32 isolates of *V. parahaemolyticus* isolated from cockles were tested for sensitivity to 16 antibiotics and the presence of plasmids. All the isolates were multi-resistance, defined as resistant to atleast three different antibiotics with multiple antibiotic resistance indexes ranging from 0.31 to 0.69, indicating the isolates originate from high risk sources of contamination where antibiotics are often used. In the plasmid profiling test, only 15 isolates (47%) harbored plasmid DNA, which ranged in size from 2.7 to 56.2 kb, separating the isolates into 14 plasmid profiles. Hence, food contaminated with antibiotic resistant *V. parahaemolyticus* could be a major threat to public health due to the distinct possibility that they can be a significant reservoir of genes encoding antibiotic resistance determinants that can be transferred intra or interspecies. As in many developing countries, raw food hygiene and antimicrobial resistance epidemiology is still in the infancy stage in the locality of the study and thus our data provide a current baseline profile of antimicrobial resistance and plasmid of *V. parahaemolyticus* from cockles in Padang, Indonesia.

Keywords: *Vibrio parahaemolyticus*, cockles, antibiotics, plasmid

Introduction

Vibrio parahaemolyticus is a bacterium in the same family as those that cause cholera. It lives in brackish saltwater and causes gastrointestinal illness in humans. It is a halophilic, or salt-requiring organism. When ingested, *V. parahaemolyticus* causes watery diarrhea often with abdominal cramping, nausea, vomiting fever and chills. Usually these symptoms occur within 24 hours of ingestion. Most people were infected through eating raw or undercooked shellfish, particularly oyster and cockles. Treatment is not necessary in most cases of *V. parahaemolyticus* infection because there is no evidence that antibiotic treatment decreases the severity or the length of the illness. Patients should drink plenty of liquids to replace fluids lost through diarrhoea but in severe or prolonged illnesses, antibiotics such as tetracycline, ampicillin or ciprofloxicin was used. The choice of antibiotics should be based on antimicrobial susceptibilities of the organism.

Plasmid profiles determination is a useful and the earliest DNA-based method applied to epidemiological studies (Meyer, 1988). The profile identifications were used as serotype-specific reference patterns for detecting certain strain with possible variation in plasmid content which is very important in epidemiological study. For *vibrio*s cases, the previous study showed that this bacteria species contained plasmid (Molina-Aja *et al*., 2002; Li *et al*., 2003). Sometimes there is a correlation between possessions of the plasmid with antibiotic resistance (Saunders, 1984; Son *et al*., 1998a; Kagiko *et al*., 2001). In *V. parahaemolyticus*, study has shown that the plasmid only exist in rare with low copy number and with sizes ranging from 9 to 123 kb (Li *et al*., 2003). The objective of this study was to determine the antibiotic resistance patterns and plasmid profiling among *V. parahaemolyticus* isolated from cockles.

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Materials and methods

Sources of V. parahaemolyticus

A total of 32 isolates of *V. parahaemolyticus* isolated from cockle samples in Padang, Indonesia, were subjected to routine culture, susceptibility to antimicrobial agents testing and plasmid profiling. The isolation and identification of the *V. parahaemolyticus* was carried out by the forwarding laboratory in University of Andalas, Padang, Indonesia.

Antibiotics susceptibility test

Susceptibility of the 32 isolates to antibiotics was determined on Mueller-Hinton agar (MHA) (Merck) by the disc diffusion tests described by Bauer *et al*. (1966). Briefly, single colony was inoculated in 1.5 ml culture media Luria-Bertani (LB) broth with 3% NaCl and grown for overnight at 37oC. The test cultures were streaked evenly on MHA plates and the antibiotics disc were placed on the surface of the agar (4 or 5 discs for each plates). The antibiotics disc used were: amoxycillin (10 µg), ampicillin (10 µg), bacitracin (10 µg), carbenicillin (100 µg), chloramphenicol (30 µg), cephalothin (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamicin (100 µg), kanamycin (30 µg), nalidixic acid (30 µg), norfloxacin (10 µg), penicillin (10 µg), streptomycin (10 µg), teicoplanin (30 µg) and tetracycline (30 µg). The plates were sealed and incubated at 37oC overnight. Then, the inhibition zone was measured and the bacteria were classified into either susceptible or resistant as referred to an interpretative table from BBL, "Sensi-Disc Antimicrobial Susceptibility Test Discs; Approved Standard, 1996".

Plasmid isolation

Minipreparations of plasmid DNA of *V. parahaemolyticus* were done by alkaline lysis method as described by Birnboin and Doly (1979) with modification. Briefly, a single bacterial colony was picked-up and grown in 3.0 ml Luria-Bertani broth (37oC, 220 rpm) overnight. The cell suspension was transferred to 1.5 ml microfuge tube and centrifuged at 10,000 rpm for 2 min (Eppendorf, Model 5415C). The cell pellets obtained were resuspended in 150 µl glucose-EDTA-Tris (GET) buffer (pH 8.0) and vortexed to mix. Then, this cell suspension was added with 175 µl of 2% SDS and 175 µl of 0.4 N NaOH. The tube was mixed vigorously and was kept at -20oC freezer for 10 min. 250 µl of cold 5M potassium acetate was added, mixed vigorously, and left to stand at room temperature for 10 min. The tube was centrifuged at 12,000 rpm for 5 min and the supernatant was transferred to a sterile 1.5 ml microfuge tube and equal volume of cold isopropanol was added. After inverting gently, the mixture was immediately centrifuge at 12,000 rpm for 10 min and the DNA pellet was washed with 650 µl of cold (4oC) 70% ethanol by centrifuging at 12,000 rpm for 5 min. The supernatant was discarded and pellet was dried for 30 min and resuspended in 40 µl of sterile deionized water. The isolated plasmid was electrophoresed on 0.8% agarose gel for 1 h at 90V, stained in ethidium bromide (0.5 µg/ml) solution for 1 min. The gel was then visualized on a 302 nm UV transilluminator (Model TM-36, UV products, Inc.). The molecular weight of plasmid DNA of the *V. parahaemolyticus* isolates were determined using the graphical method relating the logarithm of the molecular weight of plasmids of *E. coli* V517 (Macrina *et al*., 1978).

Results and discussion

An increase in the emergence of multi-drug resistant bacteria in recent years is worrying and that the presence of antibiotic resistance genes on bacterial plasmids has further helped in the transmission and spread of drug resistance among pathogenic bacteria. The growing problems with antimicrobial drug resistance are beginning to erode our antibiotic armamentarium to combat antibiotic resistance and thus limiting therapeutic options to present-day clinicians. The results demonstrate the high individual and multiple resistance to antibiotics among the 32 *V. parahaemolyticus* isolates from Padang, Indonesia (Table 1). Even in neighboring country, there are reports on multi-drug resistant of other pathogenic bacteria isolated from raw foods (Yoke-Kqueen *et al*., 2007; Robin *et al*., 2007; Chai *et al*., 2008). Thus, foods contaminated with antibiotic resistant bacteria is a threat to public health as the antibiotic resistant determinants may be transferred to other bacteria of clinical significance; and *V. parahaemolyticus* is a candidate vehicle for such transfer because of its diversity and also because it can survives in the gastrointestinal of tracts of both human and animals.

Previous studies have shown that streptomycin, rifampicin, kanamycin, tetracycline, polymyxin B were active against *Vibrio* spp. (Li *et al*., 1998; Ottaviani *et al*., 1997). However, Ottaviani *et al*. (2001) showed that *V. parahaemolyticus* were resistant to penicillin, carbenicillin, ampicillin, cephalothin, kanamycin and rifampicin. Besides, their results

Table 1. Antibiotic and plasmid profiles of the *V. parahaemolyticus* isolates

Strain No.	Antibiogram	Multiple antibiotic resistance index (MAR)	Plasmid (kb)
VP17	Ba,E,K,Na,Tec	0.31	2.7, 7.2
VP25	A,Ap,Ba,Pg,S,Tec	0.38	7.2, 42.4
VP6	A,Ap,Ba,Py,Kf,Pg,Tec	0.44	None detected
VP11	A,Ap,Ba,Py,Kf,Pg,Tec	0.44	None detected
VP10	A,Ap,Ba,Py,K,Pg,Tec	0.44	None detected
VP24	A,Ap,Ba,Py,K,Pg,Tec	0.44	6.0, 7.2, 54.0
VP26	A,Ap,Ba,Py,Cip,Pg,Tec	0.44	7.2
VP27	A,Ap,Py,E,Na,Pg,Tet	0.44	42.4, 54.0
VP1	A,Ap,Ba,Py,Kf,Pg,S,Tec	0.50	5.6, 8.2, 42.4, 54.0
VP4	A,Ap,Ba,Py,Kf,Pg,S,Tec	0.50	None detected
VP5	A,Ap,Ba,Py,Kf,Pg,S,Tec	0.50	5.6, 7.2, 42.4, 54.0
VP9	A,Ap,Ba,Py,Kf,Pg,S,Tec	0.50	3.8
VP14	A,Ap,Ba,Py,Kf,Pg,S,Tec	0.50	3.9, 5.6, 54.0
VP22	A,Ap,Ba,Py,Kf,Pg,S,Tec	0.50	5.6, 7.2, 42.4, 54.0
VP32	A,Ap,Ba,Py,Kf,Pg,S,Tec	0.50	None detected
VP7	A,Ap,Ba,Py,Kf,K,Pg,Tec	0.50	None detected
VP23	A,Ap,Ba,Py,Kf,K,Pg,Tec	0.50	5.6, 7.2, 42.4, 54.0
VP8	A,Ap,Ba,Py,Na,Pg,S,Tec	0.50	None detected
VP12	A,Ap,Ba,Py,Kf,K,Pg,S,Tec	0.56	None detected
VP13	A,Ap,Ba,Py,Kf,K,Pg,S,Tec	0.56	5.6, 42.4
VP18	A,Ap,Ba,Py,Kf,K,Pg,S,Tec	0.56	2.7
VP20	A,Ap,Ba,Py,Kf,K,Pg,S,Tec	0.56	None detected
VP15	A,Ap,Ba,Py,Kf,E,Pg,S,Tec	0.56	3.8, 54.0
VP16	A,Ap,Ba,Py,Kf,Na,Pg,S,Tec	0.56	None detected
VP21	A,Ba,Kf,C,E,Na,Pg,T,Tec,	0.56	None detected
VP30	A,Ap,Ba,Py,Kf,Cip,Na,Pg,Tec	0.56	None detected
VP31	A,Ap,Ba,Py,Kf,C,Pg,S,Tec	0.56	None detected
VP2	A,Ap,Ba,Py,Kf,K,Na,Pg,S,Tec	0.63	None detected
VP3	A,Ap,Ba,Py,Kf,E,Na,Pg,T,Tec,	0.63	None detected
VP28	A,Ap,Ba,Py,Kf,Cip,Na,Pg,S,Tec	0.63	5.6, 7.2, 42.4, 54.0, 56.2
VP19	A,Ap,Ba,Py,Kf,E,K,Na,Pg,S,Tec	0.69	None detected
VP29	A,Ap,Ba,Py,Cip,E,Na,Nor,Pg,S,Tec	0.69	None detected

also showed that increase of salt concentration cause the change of sensitivity toward antibiotics from the resistant to susceptible phenotype. In our study, of the 32 strains tested, more than 50% of strains were resistant to amoxicillin, bacitracin, penicillin, teicoplanin, ampicillin, carbenicillin, cephalothin and streptomycin. All the isolates were susceptible to gentamicin. Overall, the multi-drug resistant of the *V. parahaemolyticus* were resistant to five and up to eleven antibiotics tested generating twenty resistotype with multiple antibiotic resistance index (MAR) ranged from 0.31 to 0.69 (Table 1).

In this study, resistance to ampicillin was observed in 93.8% of the analyzed isolates similar to other studies that have been reported, which was ranging from 44.4% to 98% (Son *et al*., 1998; Lesmana *et al*., 2001). These results are also similar to those of French *et al*. (1989) who reported similar antibiotic susceptibility profiles for *V. parahaemolyticus*. In their study, most isolates were resistant to ampicillin but most were susceptible to chloramphenicol and tetracycline. Low percentage of resistance has also been reported against cephalothin (Roque *et al*., 2001) and cephalothin (Pedersen *et al*., 1996; Lesmana *et al*.,

2001). Generally, the susceptibility to tetracyclines of the *V. parahaemolyticus* examined in this study is similar to those in *Vibrio*s reported elsewhere (Wong *et al*., 2000; Zanetti *et al*., 2001; Gonzalez-Ray *et al*., 2004). Our data also demonstrated that 31 of the 32 *V. parahaemolyticus* isolates were observed to be sensitive toward norfloxacin, an observation that concur with data among the *V. parahaemolyticus* and *Vibrio* spp. reported in various countries (Lesmana *et al*., 2001; Roque *et al*., 2001; Zanetti *et al*. 2001; Molina-Aja *et al*., 2002).

Higher percentages of resistance to carbenicillin, cephalothin, streptomycin, teicoplanin, nalidixic acid and chloramphenicol among other bacteria from food and environmental sources have been reported (Overmann and Janda, 1999; Stock *et al*., 1999; Szych *et al*., 2001; Vivekanandhan *et al*., 2002). But in contrast to the high resistance of isolates towards nalidixic acid and chloramphenicol, our result showed the low resistance level to chloramphenicol and nalidixic acid which are in general agreement with several reports elsewhere regarding the susceptibility of *V. parahaemolyticus* to both antibiotics (Wong *et al*., 2000; Ottaviani *et al*., 2001; Li *et al*., 2003). It is also interesting to note that our findings on resistance of the *V. parahaemolyticus* isolated from cockles towards chloramphenicol were also in agreement to the data provided by a local hospital in Malaysia from 1998 to 1999 on the antibiotic resistance of clinical isolates of this human pathogen (HUKM, 2000). Previous report suggested that cephalothin is effective for the treatment of infection caused by *V. parahaemolyticus* (Wong *et al*., 2000), but Lesmana *et al*. (2001) noticed that tetracycline was active against most *V. parahaemolyticus* infection. Karlowsky *et al*. (2003) suggested that geographical locations and selective pressure influence the antibiotic resistance levels. Thus, more studies on the elucidation of the antimicrobial susceptibilities of potential pathogenic *V. parahaemolyticus* are important for identification of more suitable and effective treatment of *V. parahaemolyticus* infections in human beings and in cultured marine organisms.

There are few factors that may contribute to the *Vibrio* spp. antibiotic resistant. Firstly, a mutation in cellular DNA could modify the antibiotic target site or transport mechanism, causing to a decreased action of the antibiotic on the cell. Other factor was an extra gene product could inactive the antibiotic, or modify the antibiotic target site or transport mechanism. However, extensive use of these antibiotics has resulted in an increase of antibiotic-resistant bacteria (Saitanu *et al*., 1994; Son *et al*., 1997). The multiple antibiotic resistance (MAR) indexes of the V. *parahaemolyticus* are shown in Table 1. In this study, all the antibiotic resistant isolates have MAR index of 0.31 or more which can be group under the origination from high risk sources of contamination like humans and commercial industry where antibiotics are often used (Krumperman, 1983). This may be due to the cockles under study have high risk contamination from areas where antibiotics are often used where the samples were collected from locations in Indonesia (Maninjau Lake, Singkarak Lake and Indrapura River) that were near to the city and industrial area where many antibiotics are often used. As new antibiotics are developed and used, resistant strain may be develop because there is a tendency to assume that antibiotic resistance genes appeared only when antibiotics are used widely in medicine and fields industry.

Among the 32 *V. parahaemolyticus* isolates obtained from cockles in Padang, Indonesia, only 15 isolates (47%) harbored between one to five plasmid DNA bands, which range in size from 2.7 to 56.2 kb and the rest does not exhibit the present of plasmid DNA (Table 1). This study correlates with results from the previous studies which have reported that *V. parahaemolyticus* harbored plasmids with sizes from 9 to 123 kb (Li *et al*., 2003; Kagiko *et al*., 2001; Molina-Aja *et al*., 2002; Kaufman *et al*., 2002). The plasmid profiles in *vibrio*s have also been studied in some other *Vibrio* spp. such as *Vibrio ordalli, Vibrio vulnificus* and *Vibrio salmonicida* (Hanninen *et al*., 1995; Tiainen *et al*., 1995; Son *et al*., 1998). However, the present study showed higher percentage of plasmid containing isolates compare to other findings (Son *et al*., 1998; Nasreldin *et al*., 2001; Li *et al*., 2003). In this study, 14 plasmid profiles were detected which indicates that plasmid profiling is can be use as an epidemiological tool for typing *V. parahaemolyticus* as described by Meyer (1988). Son *et al*. (1998) stated that generally epidemiologically unrelated isolates contains different plasmid profiles whereas related isolates could also display variation in plasmid profiles. The more plasmids exist in an organism, the more specific is the plasmid profile as a marker for a single isolates.

Large sizes of plasmid were detected in all plasmid positive isolates of *V. parahaemolyticus*. Bacterial antibiotics resistance patterns sometimes associated with the presence of large plasmids and ability of plasmids for conjugation process. Generally, plasmids which can be transconjugated usually possess a high molecular weight so the presence of plasmids that may harbor the antibiotic resistance genes in these isolates may increase their capacity to

threaten human consumers since foodborne strains carrying resistant genes qualified them as potential human pathogens. However, for others isolates that were plasmidless (53% of isolates), they also showed the multiple antibiotics resistances pattern with high number of antibiotic which indicates that resistance to most of these antibiotics is of chromosomal origin or on mobile genetic elements that may help in the disseminations of the resistant genes to other bacteria of human clinical significance (Son *et al*., 1998). In this study, the results could not correlate the antibiotic resistance among the *V. parahaemolyticus* with a specific plasmid detected because no genetic transfer study was performed. Although in most cases antimicrobial resistance in *Vibrio* spp. is intrinsic to the species rather than acquired through plasmid transfer or through antibiotic exposure (Ottaviani, 2001), the role of plasmids in multiple antimicrobial resistances still should be investigated further.

In summary, the prevalence of multi-drug resistant *V. parahaemolyticus* is quite high in the locality of study and that the bacterial population is rather diverse based on the phenotypic and genotypic characterization of the isolates.

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