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Antibiotic Resistance of *Vibrio parahaemolyticus* Isolated from Cockles and Shrimp Sea Food Marketed in Selangor, Malaysia

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Abstract

Introduction: The main aim of this study is to determine the antibiotic profile of *V. parahaemolyticus* gastroenteritis associated with the consumption of contaminated shrimp and cockles marketed in Selangor Malaysia. *V. parahaemolyticus* is the leading cause of seafood-associated gastroenteritis in Asian Countries typically is associated with the consumption of raw shellfish and oysters specially shrimp and cockles. Rapid, sensitive and specific detection methods are needed to control *V. parahaemolyticus* infections. We describe a recognized the pathogenic *V. parahaemolyticus* in shrimp and cockles that will be the risk of gastroenteritis associated with the consumption of seafood marketed in Malaysia.

Methods: This study was carried out between July 2011 and August 2013 at the Center of Excellence for Food Safety Research, Faculty of Food Science and Technology, Faculty of Medicine and Health Sciences, Department of Biomedical Sciences, and Faculty of Biotechnology, Dep. of Cell and Molecular Biology, University Putra Malaysia and other centers as collaboration. The seafood samples were collected from different markets and more than 400 samples from shrimp and cockles were investigated for detection and isolation of *V. parahaemolyticus*. CHROMagar Vibrio and TCBS agar media were used for fast detection and isolation of *V. parahaemolyticus* isolates. PCR based methods targeted to *toxR* regulatory gene, *tlh* the species and family gene, *tdh* and *trh* the virulence genes were extensively used. The antibiotic susceptibility testing of 65 *V. parahaemolyticus* isolates recovered from retail shrimp and cockles seafood were determined with four types of E-test antibiotic strips.

Results: All the 65 isolates were positive to *toxR* and *tlh* genes. Out of 65 isolates, only eight isolates (12.31%) were positive for *tdh* virulence gene isolated form cockles and shrimp (3 isolates from shrimp and 5 isolates from cockles), whereas twenty six (40%) isolates were positive for *trh* virulence gene isolated from shrimp and cockles (9 from shrimp and 17 from cockles). This result indicates high occurrence of *tdh*+ and *trh*+ isolates in shrimp and cockles marketed in Malaysia. None of the isolates tested possess both virulence genes. For the antibiotic *E-test* susceptibility test, overall, *V. parahaemolyticus* is remained susceptible to tetracycline (97%). A slight increase in the susceptibility of tetracycline is observed from 2011 to 2013. While reduced susceptibility was detected only in *V. parahaemolyticus* for ampicillin. The mean of MIC of the isolates toward ampicillin is increased from 64 µg/ml in 2011 to 128 µg/ml in year 2013. The current study demonstrates a high risk of pathogenic *V. parahaemolyticus* in the shrimp and cockles marketed in Selangor Malaysia.

Conclusions: The potential risk of *V. parahaemolyticus* infection due to the consumption of contaminated seafood in Malaysia should not be neglected. The increased resistance of ampicilin from our studies in Malaysia since 2004 to 2013 could be in indication of antibiotic abuse in clinical and agricultural used of ampicilin in Malaysia.

Keywords: *Vibrio parahaemolyticus;* Antibiotic E-test; *toxR; tlh; tdh; trh*; CHRO magar vibrio

Introduction

V. parahaemolyticus, a gram-negative marine bacterium, is a major food-borne pathogen that causes acute human gastroenteritis associated with the consumption of seafood. *V. parahaemolyticus* is a gram-negative halophilic bacterium and is responsible for human gastroenteritis worldwide. Sporadic cases and outbreaks of *V. parahaemolyticus* occur regularly in Asia and as well as in other countries [1-5]. Cases of *V. parahaemolyticus* were mostly sporadic and associated with diverse serovars. However, the emergence of a pandemic serovar O3:K6 in 1996 has changed the epidemiology abruptly and has since been accounted for many cases of *V. parahaemolyticus* outbreak worldwide [6-8].

Not all strains of *V. parahaemolyticus* cause illness in humans; in fact, the majority of strains isolated from the environment or seafood are not pathogenic. The pathogenic strains of *V. parahaemolyticus* are those that produce Thermostable Direct Haemolysin (*TDH*) toxin [9,10]. *TDH* is an enzyme that lyses human red blood cells on Wagatsuma blood agar plates, which is referred to as the Kanagawa phenomenon positive (KP+). KP is a type of beta-hemolysis induced by *TDH* toxin encoded by the *tdh* gene. The role of the toxin in illness is not known and 90% of the *tdh* positive strains isolated from clinical cases show hemolysis, while only 1 to 2% of the strains of environmental origin are KP positive [11]. Another toxin produced by KP-negative *V. parahaemolyticus* strains [12] is the *TDH*-Related hemolysin (*TRH*) toxin encoded by *trh* gene. These isolates which are urease positive

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can cause skin infection when the injured skin is exposed into sea water leading to wound infections and septicemia [11,13,14]. To date pathogenic strains containing *tdh* and/or *trh* genes have been detected with low frequency (usually 0.3 to 3%) in the total *V. parahaemolyticus* environmental population [15,16].

Vibrio is generally considered to be highly susceptible to most clinically used antimicrobials [17]. However, during the past few decades, antimicrobial resistance has emerged and evolved in many bacterial genera due to the excessive use of antimicrobials in human, agriculture, and aquaculture systems [18,19]. This emerging issue has gain great concern due to increase resistance of pathogenic *V. parahaemolyticus* towards clinically used antimicrobials. Tetracycline [20] and an alternative treatments of combinations of expanded-spectrum cephalosporins (e.g., ceftazidime) and doxycycline or a fluoroquinolone alone [21] have been recommended as the antimicrobial of choice for treatment of severe Vibrio infections.

In Malaysia, *V. parahaemolyticus* is naturally occurring in the marine coastal region of Malaysia. It is prevalent in the tropical marine environment in all seasons and can cause seafood-borne gastroenteritis. *V. parahaemolyticus* has been recognized as one of the causative agents in the frequent institutional food poisoning incidences in Malaysia [22]. The occurrence of *V. parahaemolyticus* in seafood is getting intense attention in Malaysia due to frequent rejection of seafood export to EU countries [22]. Due to this, the first national risk assessment of *V. parahaemolyticus* in seafood was initiated and carried out to control and manage this seafood-borne pathogen in Malaysia [22,23]. Surveillance study carried out in Malaysia showed high prevalent contamination of pathogenic *Vibrio* spp. in retail seafoods in the country throughout the year and suggest that there is a need for adequate consumer protection measures [24].

The *V. parahaemolyticus* food poisoning incidence in Malaysia is considerably high. However, the occurrence of pathogenic strains in shrimp and cockles, and antibiotic resistance of *V. parahaemolyticus* strains is not well documented and studied. This study aims to provide an insight into the prevalence of *V. parahaemolyticus* strains (*TDH* and/or *TRH*) in the marine environment and retail seafood and the antibiotic resistance profile of *V. parahaemolyticus* isolated from 2004 and recently for this study from 2011 to 2013.

The presence of thermostable direct hemolysin *TDH* which coded by *tdh* gene is a proven virulence factor [25] [26], and *TDH* occurs in over 90% of clinical strains of *V. parahaemolyticus* [4,27,28]. Most literatures reported a low prevalence of (less than 1%) *tdh* gene in the isolates from environmental and seafood samples [29-31], except in a limited study in Grays Harbor, Washington [32]. A proposed virulence factor, the *TDH*-related hemolysin (*TRH*), encoded by the gene *trh* has been discovered also in clinical stains of *V. parahaemolyticus* lacking *tdh* [12,33]. Most clinical isolates from the U.S. Pacific Coastal have been reported to possess both *tdh* and *trh* [13]. Our findings are in agreement with Wong [4] in which some environmental isolates were found to possess *trh* genes only but very low *tdh* positive isolates. None of the isolates collected in this study possess both *tdh* and *trh* genes suggests *tdh* gene is mainly contained within clinical strains of *V. parahaemolyticus*. In the previous studies, t**he** prevalence of pathogenic *V. parahaemolyticus* in the marine environment and retail seafood is relatively low. Nonetheless, there is still a potential risk of *V. parahaemolyticus* outbreak or infection through consumption of the contaminated seafood.

For the antibiotics, treatment of severe Vibrio infections, Tetracycline has been recommended as the antimicrobial of choice [20,34], and alternative treatments are combinations of expanded-spectrum cephalosporins (e.g., ceftazidime) and doxycycline or a fluoroquinolone alone [21,34]. Trimethoprim-sulfamethoxazole plus an aminoglycoside is used to treat children in whom doxycycline and fluoroquinolones are contraindicated [14,35]. Traditionally, Vibrio is considered highly susceptible to virtually all antimicrobials [17]. During the past few decades, however, antimicrobial resistance has emerged and evolved in many bacterial genera due to the excessive use of antimicrobials in human, agriculture, and aquaculture systems [18,19,34].

E-test and Minimum Inhibitory Concentrations (MIC) of antibiotics are routinely determined by broth or agar serial dilution methods or by agar diffusion methods. More recently, e-test was developed to reduce the time, labor, and materials used in MIC determination assays. E-test is based on arraying a concentration gradient of each antibiotic on a polymer strip. Concentration values are marked on the other side of the strip so that one can easily locate corresponding concentrations. E-strips, also known as "epsilometers", are commercially prepared by micro dispersing robotic machines that can deliver nanoliter volumes of antibiotic concentration along the strip. Each antibiotic strip is laid on the surface of an inoculated agar plate. An elliptical zone of inhibition develops with the broad end at the top of the strip with the highest antibiotic concentration and the narrowest end at the lowest amount of antibiotic that can inhibit bacterial growth, i.e., minimum inhibitory concentration. Several different antibiotic e-strips can be tested simultaneously on the same agar plate. Therefore MIC's can be determined for many antibiotics in a single step with no need for dilution in broth or agar. Also, e-test is applied routinely as a "culture sensitivity test" in some medical laboratories in place of the traditional Kirby-Bauer method. In addition to reduction of time and effort, e-test yields sensitivity test results in quantitative terms which make interpretation of results more precise and easier than routine methods [36].

Methodology

We used the rapid methods to detect and isolate *V. parahaemolyticus* from the expected and selected contaminated seafood and any environmental or (clinical) samples by using CHROMagar Vibrio medium, the highest selective medium for Vibrio, and PCR based method targeted to VP-*toxR* species-specific regulatory gene and *tdh*/*trh* the virulence genes to detect the pathogenic *V. parahaemolyticus* isolates.

The seafood samples were collected from different markets and more than 400 samples from shrimp and cockles seafood were investigated for detection and isolation of *V. parahaemolyticus*. CHROMagar Vibrio and TCBS agar media were used for fast detection and isolation of *V. parahaemolyticus* isolates. A total of 65 *V. parahaemolyticus* isolates were obtained from shrimp and cockles (27 isolates from shrimp and 38 isolates from cockles). Three reference strains were used in this study as positive and negative controls, namely VP2053 and PV1808 (tdh^+/trh^-) and VP1896 (tdh^-/trh^+) as positive control and *Escherichia coli* ATCC25922 as non-vibrios (negative control). All *V. parahaemolyticus* strains were maintained on cryogenic beads at -80°C. The working cultures of *V. parahaemolyticus* were maintained on Luria Bertani (LB) broth culture with 15% Glycerol at -30°C for no longer than 3 months or at -80°C for longer.

All the isolates were grown overnight on Luria-Bertani (LB) agar plates supplemented with 3% NaCl for *tdh* and *trh* genes detection. Three

to five colonies were scraped from the agar plates and re-suspended in 400µl of filtered sterile Milli-Q-distilled water, and boiled for 10 min to liberate the nucleic acid as described elsewhere [11]. The tubes were then incubated on ice for 20 min followed by centrifugation at 9000 x g. The supernatant that contained the DNA template was transferred into new labeled sterile tubes and stored at -30°C until used for PCR amplification. The *tdh* and *trh* genes were amplified with the following primer sets: 5′-GGTA CTAA ATGG CTGA CATC-3′ (forward) and 5′-CCAC TACC ACTC TCAT ATGC-3′ (reverse) [21]; and 5′-GGCT CAAA ATGG TTAA GCG-3′ (forward) and 5′-CATT TCCG CTCT CATA TGC-3′ (reverse) [23], respectively. The reaction mixtures (final volume, 25 µl) contained µl of DNA template (50 ng/µl con.), 2.5 µl of 10x reaction buffer (1st BASE Laboratories), 4 µl of 50 mM MgCl2, 0.25 µl of Taq polymerase (5 U/µl), 0.5 µl of deoxynucleoside triphosphates (10 mmol), 0.5 µl of each primer (10 µM/µl), and 15.75 µl of distilled water. The reactions were performed with a Gene Amp PCR system 2700 thermocycler (Bio-Rad) as follows: 4 min of initial denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 30s, alignment at 58°C for 30s, and extension at 72°C for 30s and a final extension at 72°C for 7 min. Positive and negative DNA controls were included in all assays. Amplified products were separated by electrophoresis in ethidium bromidestained 1.5% agarose gels in Tris-borate-EDTA (0.5x TBE) buffer at 90V for 40 min. A 100- to 1500-bp ladder (Sigma) was used as a molecular mass marker. The gels were visualized for 251bp (*tdh* gene amplicon) and 250bp (*trh* gene amplicon) with a UV transilluminator system and software (Bio-Rad).

All the strains were randomly selected for antibiotic susceptibility testing by using E-test strips. Five reference strains namely, VP2053, VP1896, VP1808, *V. alginolyticus* 2341 and *Escherichia coli* ATCC25922 were included in the analysis. Susceptibility testing was performed using the *E-test* gradient technology recommended by the National Committee for Clinical Laboratory Standard Institute (CLSI). The measurements were interpreted as resistant (R), intermediate (I) and susceptible (S) to the antibiotics according to the CLSI [37]. The antibiotic being tested in this study are Tetracycline (Tc) (MIC 0.5-2 µg/ml), Cefalexin (Cx) (MIC 4-16 µg/ml), Ciprofloxacin (Ci), (MIC 0.0125-0.5 µg/ml), and Ampicillin (Am) (MIC 2-8 µg/ml) (AB BIODISK). Using a sterile cotton swabs, 3 to 5 pure colonies were picked up from fresh LB agar plate overnight cultures and inserted into a tube containing 3 ml sterile normal saline (0.85%) and the turbidity is adjusted to 0.5 McFarland turbidity level. The suspension was then surface inoculated onto Mueller Hinton agar plates. The inoculated plates were allowed to air dry in laminar airflow for 10min before the E-test antibiotic strips were placed on the surface carefully with sterile forceps. The plates were incubated at 37°C for 18- 24 h. The MIC was read at the point where the zone of growth inhibition intersected the strip.

Statistical Design

Statistical design and data analysis; A Completely Randomized Design (CRD) was considered to create different experimental treatments. In the current study, the effect of four different types of antibiotics (i.e. Tetracycline, Ampicilin, Cefalexin and Ciprofloxacin) as independent variables on total count of different strains of *V. parahaemolyticus* isolated from retail shrimp and cockle seafood was investigated. In the present study, total count of different strains of *V. parahaemolyticus* isolated from retail shrimp and cockles as percentage of sensitive and resistance *V. parahaemolyticus* were considered as response variable. In this study, cluster random sampling was employed to collect more than 400 samples from shrimp and cockles from different market in Malaysia between July 2011 and August 2013.

Results

All the 65 isolates were positive to *toxR* environmental regulatory gene and *tlh* family gene. Out of 65 isolates, only eight isolates (12.31%) were positive for *tdh* virulence gene isolated form cockles and shrimp (3 isolates from shrimp and 5 isolates from cockles), whereas twenty six (40%) isolates were positive for *trh* virulence gene isolated from shrimp and cockles (9 from shrimp and 17 from cockles). This result indicates high occurrence of *tdh*+ and *trh*+ isolates in shrimp and cockles marketed in Selangor, Malaysia. None of the isolates tested possess both virulence genes.

For the E-test antibiotic susceptible testing of the selected 65 isolates of *V. parahaemolyticus* isolated from cockles and shrimp in this study revealed a high resistant in Ampicillin (63.1%) and Cefalexin (35.4%). In general, the isolates showed the highest susceptibility to Tetracycline (97%) followed by Ciprofloxacin (49.3%), (Table 1 and Figure 1). The isolates originated from retail cockles purchased over 2011 to 2013 showed the highest resistance level toward Ampicillin, Cefalexin and Ciprofloxacin compared to isolates collected from shrimp (Figure 1).

	Tetracycline		Ampicillin		Cefalexin		Ciprofloxacin	
	%	# isolates	%	# isolates	%	# isolates	%	# isolates
Sensitive	97	63	20	13	23.1	15	49.3	32
Intermediate	3.0	2	16.9	11	41.5	27	29.2	19
Resistance	0.0	0	63.1	41	35.4	23	21.5	14
TOTAL		**65**		**65**		**65**		**65**

Table 1: Antibiotic susceptibility E-test assays for sixty five strains of *V. parahaemolyticus* isolated from shrimp and cockles marketed in Selangor Malaysia.

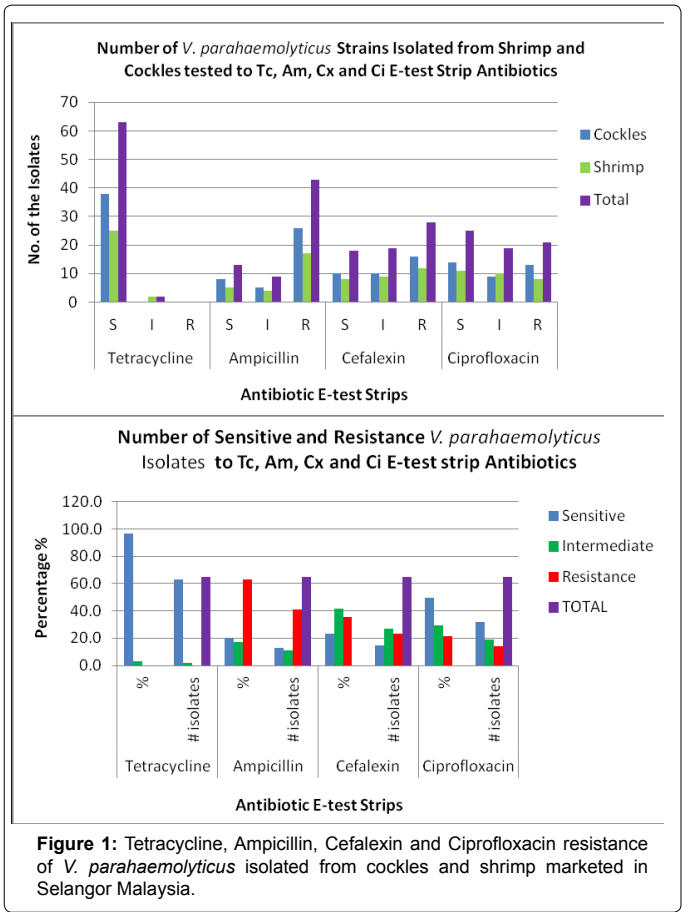

Figure 1: Tetracycline, Ampicillin, Cefalexin and Ciprofloxacin resistance of *V. parahaemolyticus* isolated from cockles and shrimp marketed in Selangor Malaysia.

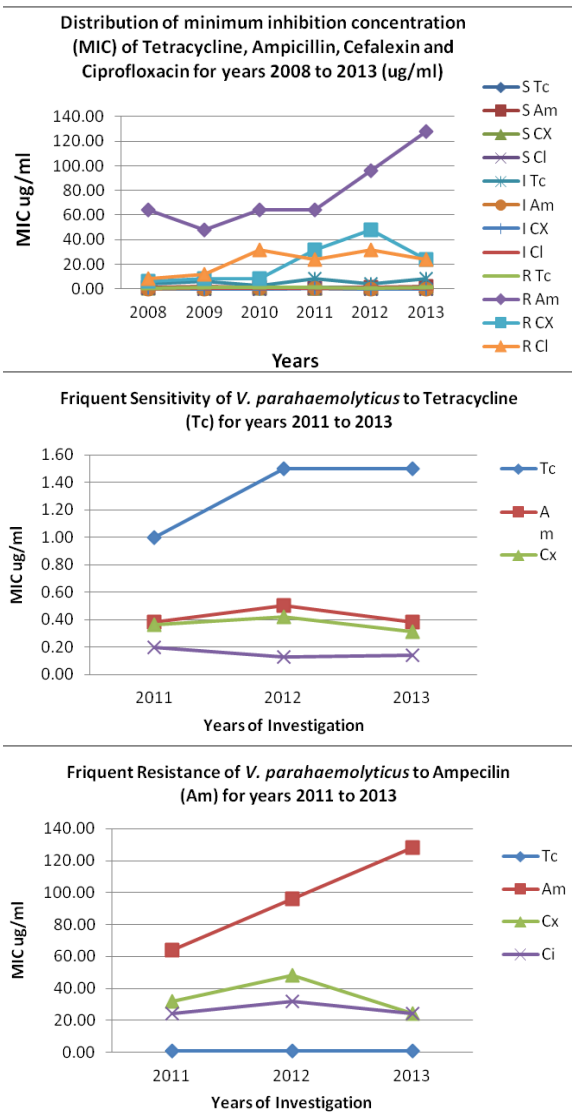

Figure 2: Distribution of Minimum Inhibition Concentration (MIC) of Tetracycline, Ampicillin, Cefalexin and Ciprofloxacin in year 2011 to 2013.

Figure 1 shows the distribution of antibiotic resistant profiles of *V. parahaemolyticus* for the four tested antibiotics from 2004 then from 2011 to 2013. The distribution showed a clear development of ampicillin resistance from maximum MIC of 64 µg/ml in 2011 to maximum MIC of 128 µg/ml in 2013 (Figure 2). The magnitude of resistance development is rapid with about 3 fold increase in the mean MIC over more than four years and continues tracing since 2004 and from this study from2011 to 2013 (Figure 3). The current study demonstrates a high risk of pathogenic *V. parahaemolyticus* in the shrimp and cockles marketed in Selangor, Malaysia.

The antibiogram obtained in current study clearly indicates that the first-line drug-tetracycline still remained highly effective against *V. parahaemolyticus*. The results showed slight decrease in the MIC of tetracycline from 2011 to 2013 suggesting the outcome of the ban of tetracycline used as a growth promoting in animal feed. Excess use of antibiotics encourages the development of antibiotic resistance and that of reduction may consequence the decrease in antibiotic resistance [37,38].

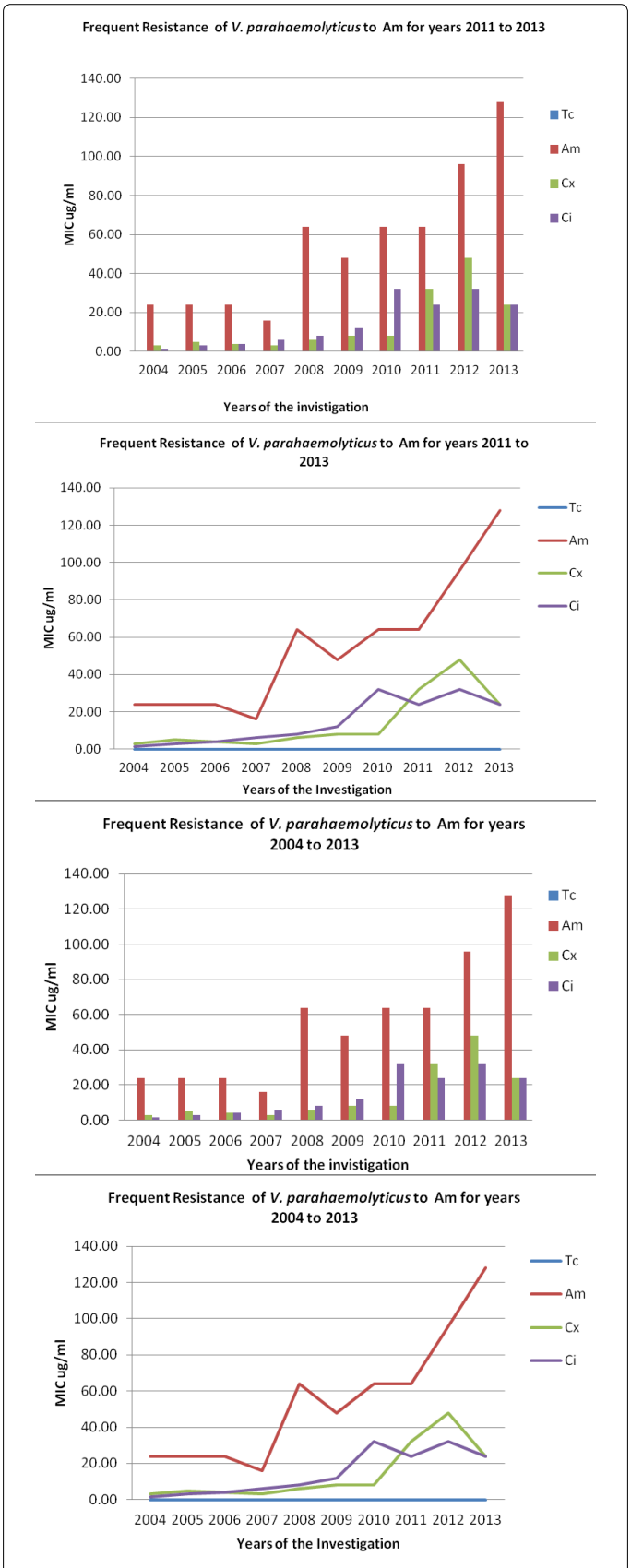

Figure 3: The increase of Ampicilin resistance in *V. parahaemolyticus* from 2004 and for this study from 2011 to 2013.

Discussion

This study is a preliminary examination of the antimicrobial susceptibilities of *V. parahaemolyticus* for retail seafood (shrimp and cockle) marketed in Selangor, Malaysia. Aquatic bacteria, including vibrios, live in the coastal and estuarine waters, an open area particularly subject to environmental contaminations by agricultural runoff or wastewater treatment plants [39], which may contain various levels of antimicrobials and heavy metals and act as selective pressure for antimicrobial-resistant aquatic bacteria [26,34,40]. These findings indicated that the *V. parahaemolyticus* strains isolated from local shrimp and cockles collected from several markets remained susceptible to the majority of antimicrobials tested; however, the observed high percentage of *V. parahaemolyticus* isolates with reduced susceptibilities to ampicillin suggests that ampicillin has a potentially low efficiency in empirical treatment of *V. parahaemolyticus* infections. Therefore, continued monitoring of both the prevalence and the antimicrobial susceptibility profile of *V. parahaemolyticus* is important to better ensure seafood safety.

From these results, the rapid method used in this study using CHROMagar Vibrio (Figure 4) compared by conventional TCBS agar medium (Figure 5) was the best to give pure colonies of *V. parahaemolyticus* within 12 to 24 hours that decrease the time wasting, cost, and efforts.

Our findings are in agreement with Wong [4] in which some environmental isolates were found to possess *trh* genes only but very low *tdh* positive isolates. None of the isolates collected in this study possess both *tdh* and *trh* genes suggests *tdh* gene is mainly contained within clinical strains of *V. parahaemolyticus*. In the previous studies, the prevalence of pathogenic *V. parahaemolyticus* in the marine environment and retail seafood is relatively low. Nonetheless, there is still a potential risk of *V. parahaemolyticus* outbreak or infection through consumption of the contaminated seafood.

The *V. parahaemolyticus* food poisoning incidence in Malaysia is considerably high. However, the occurrence of pathogenic strains in shrimp and cockles, and antibiotic resistance of *V. parahaemolyticus* strains is not well documented and studied. This study aims to provide an insight into the prevalence of *V. parahaemolyticus* strains (*TDH* and/or *TRH*) in the marine environment and retail seafood and the antibiotic resistance profile of *V. parahaemolyticus* isolated from 2004 and recently for this study from 2011 to 2013.

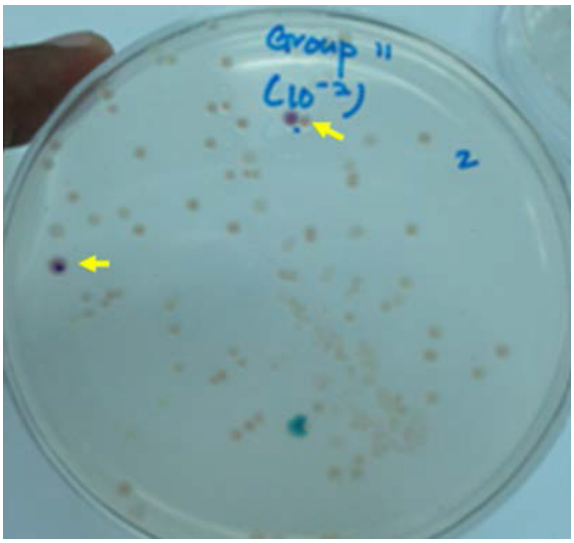

Figure 4: Colonies plated out from high selected CHROMagar Vibrio plate incubated at 37˚C for 18-24 h, direct and spread plating from cockles. Mauve colonies on CHROMagar Vibrio (indicated by arrow) were identified as *Vibrio parahaemolyticus*.

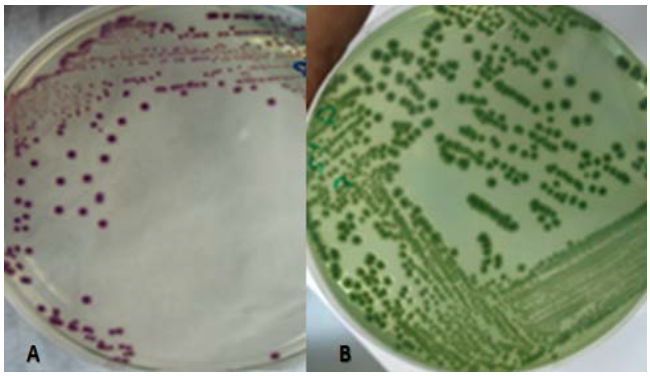

Figure 5: Pure colonies plated on CHROMagar Vibrio plates and TCBS agars Plates incubated at 37˚C for 24 h. Mauve colour colonies on CHROMagar Vibrio (A) and green colonies on TCBS agar (B) were identified as *Vibrio parahaemolyticus*.

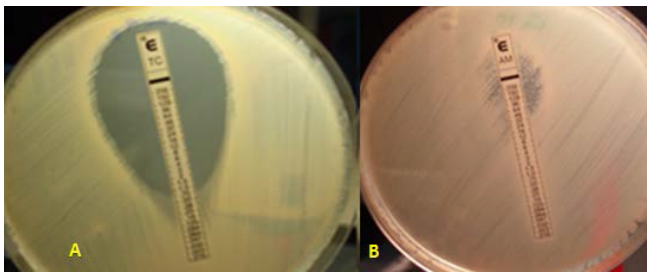

Figure 6: (A and B, the E-test plates for Tetracycline and Ampicilline antibiotics) Representative photo plates for antibiotic susceptibility E-test assay of *V. parahaemolyticus* performed on Muller Hinton agar medium showed the zoon of inhibition and MIC of the growth.

The antibiogram obtained in current study clearly indicates that the first-line drug-tetracycline still remained highly effective against *V. parahaemolyticus*. The results showed slight decrease in the MIC of tetracycline from 2011 to 2013 suggesting the outcome of the ban of tetracycline used as a growth promoting in animal feed. Excess use of antibiotics encourages the development of antibiotic resistance [37] and that of reduction may consequence the decrease in antibiotic resistance (Figure 6).

However, the findings in this study showed an increase in ampicillin resistance since 2004 as in our pervious study [41] and until this study from 2011 to 2013 and others [36,38]. Although ampicillin is not used empirically to treat *V. parahaemolyticus* infection in the hospital, the increase resistance rate has created a great concern. Ampicillin resistance in *V. parahaemolyticus* is not a new phenomenon. A 1978 study in the United States reported that over 90% of *V. parahaemolyticus* isolates were resistant to ampicillin and exhibited -lactamase activity [42,43]. This finding was also in agreement with a number of literatures from all around the world and Malaysia [23,44-46].

Conclusion

The occurrence of pathogenic *V. parahaemolyticus* in seafood and their drug resistance pattern in this study demands immediate need for paying attention. A judicious exploitation of antibiotics both for aquaculture farming and for treatment diseases should be followed to combat this drug resistance in pathogenic gram negative bacteria.

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