Rapid detection and E-test antimicrobial susceptibility testing of *Vibrio parahaemolyticus* isolated from seafood and environmental sources in Malaysia

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ABSTRACT

الأهداف: دراسة مدى انتشار بكتيريا الباراهيموليتيكاس الضمية الموجودة في الأطعمة البحرية والمصادر الطبيعية الأخرى، بالإضافة إلى تقييم حساسية البكتيريا للمضادات الحيوية.

الطريقة: أُجريت هذه الدراسة في مركز ومختبرات مستشفى الجامعة الوطنية الطبي ومعهد الأبحاث الحيوية الجزيئية الطبي، الجامعة الوطنية العالمية، وفي مركز أبحاث سلامة الأطعمة وجودتها التابع لجامعة بوترا، ماليزيا. وقد استمرت الدراسة من يناير 2006م إلى أغسطس 2008م. شملت الدراسة 144 عزلة بكتيرية تم جمعها من 400 عينة من الأطعمة البحرية (122 عزلة)، بالإضافة إلى العزلات التي تم جُمعت من مياه البحر (22 عزلة). ولقد تم تحليل مثل هذه العزلات للتحري عن ظهور جينات كلاً من: الهيمولايسين المباشر والثابت الحرارة، والهيمولايسين المرتبط بهذا الجين وذلك باستخدام طريقة التفاعل التسلسلي المبلمر. وتم اللجوء إلى اختبار إي من أجل تقييم حساسية البكتيريا للمضادات الحيوية.

النتائج: أشارت الدراسة إلى أن نسبة ظهور الهيمولايسين المباشر والثابت الحرارة (0.69%)، والهيمولايسين المرتبط بهذا الجين (8.3%) كانت قليلة نسبياً لكنها ذات أهمية. لوحظ وجود حساسية عالية ضد المضاد الحيوي التتراسيكيلين (98%)، وقد زاد معدل التركيز المثبط الأدنى للعزلات تجاه الأمبيسيلين من 4 ميكروغرام/مل في عام 2004م إلى 24 ميكروغرام/مل في عام 2007م، وهذا يعني ظهور سلالات مقاومة للمضاد الحيوي الأمبيسيلين.

خاتمة: أظهرت الدراسة الحالية بأن نسبة ظهور بكتيريا الباراهيموليتيكاس المسببة للأمراض تعد ضئيلة في الأطعمة البحرية والمصادر المائية، ولكن يجب أن نضع في الحسبان احتمالية الإصابة بعدوى هذا الميكروب إثر تناول الأطعمة البحرية الملوثة في ماليزيا.

Objectives: To find out the prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* in seafoods and environmental sources.

Methods: The study was carried out at the Center of Excellence for Food Safety Research, University Putra Malaysia; Universiti Kebangsaan Malaysia; Medical Molecular Biology Institute; and University Kebansaan Malaysia Hospital, Malaysia between January 2006 and August 2008. One hundred and forty-four isolates from 400 samples of seafood (122 isolates) and seawater sources (22 isolates) were investigated for the presence of thermostable direct hemolysin (tdh^+) and TDH-related hemolysin (trh^+) genes using the standard methods. The E-test method was used to test the antimicrobial susceptibility.

Results: The study indicates low occurrence of tdh^+ (0.69%) and trh^+ isolates (8.3%). None of the isolates tested posses both virulence genes. High sensitivity was observed against tetracycline (98%). The mean minimum inhibitory concentration (MIC) of the isolates toward ampicillin increased from 4 ug/ml in 2004 to 24 ug/ml in 2007.

Conclusions: The current study demonstrates a low occurrence of pathogenic *Vibrio parahaemolyticus* in the marine environment and seafood. Nonetheless, the potential risk of vibrio infection due to consumption of *Vibrio parahaemolyticus* contaminated seafood in Malaysia should not be neglected.

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Vibrio parahaemolyticus (*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 many parts of the world. In most of the studies of *V. parahaemolyticus* were sporadic and caused by diverse serovars.[1-5] However, the emergence of a pandemic serovar O3:K6 in 1996 has changed the epidemiology abruptly and has 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] Thermostable direct haemolysin is an enzyme that lyses human red blood cells on Wagatsuma blood agar plates, which is referred to as the Kanagawa phenomenon positive (KP+). Kanagawa phenomenon 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 1-2% 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 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 and pandemic strains of *V. parahaemolyticus* containing TDH and/or TRH genes have been detected with low frequency (usually 0.3-3%).[15,16]

Earlier studies indicate that *V. parahaemolyticus* isolates were susceptible to most of the antimicrobial agents.[17] However, in the last 3 decades, antimicrobial resistance has been reported in different studies.[18-20] In the treatment of severe Vibrio infections, tetracycline has been recommended as the antimicrobial of choice,[18,21] Trimethoprim-sulfamethoxazole plus an aminoglycoside is used to treat children in whom doxycycline and fluoroquinolones are contraindicated.[18,22]

In Malaysia, *V. parahaemolyticus* is naturally occurring in the marine coastal region. 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.[23] The occurrence of *V. parahaemolyticus* in seafood is getting intense attention in Malaysia due to frequent rejection of seafood export to European countries.[23] In this reason, 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.[23,24] The surveillance study that was carried out in Malaysia showed a 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.[25]

The *V. parahaemolyticus* food poisoning incidence in Malaysia is considerably high. However, the occurrence of pathogenic strains in the environment and antibiotic resistance of environmental strains is not well documented and studied. This study aims to provide an insight into the prevalence of pathogenic strains (TDH and/or TRH positive strains) in the marine environment and retail seafood, and the antibiotic resistance profile of the bacterium isolated from 2004-2007.

Methods. This study was carried out at the Universiti Kebangsaan Malaysia, Medical Center and Medical Molecular Biology Institute, University Kebansaan Malaysia and Center of Excellence for Food Safety Research, Faculty of Food Sciences and Biotechnology, University Putra Malaysia from January 2006 to August 2008. This study was approved by the Ethical Committee of UKM Medical Center, Malaysia. The 3 states covered were Selangor, Penang and Perak. No specific criteria were used to select specimens.

We used the rapid methods to detect and isolate *V. parahaemolyticus* from the expected contaminated seafood and any environmental 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 the virulence genes *tdh*/*trh* to detect the pathogenic *V. parahaemolyticus* isolates.

On hundred and forty-four *V. parahaemolyticus* isolates was obtained from various resources in Malaysia for the current study. All of these isolates were isolated from various retail seafood (47 isolates from shrimp and 75 isolates from cockles) and coastal seawater (22 isolates) from 3 states in Malaysia namely Penang, Selangor, and Perak for a period of 3 years (2004 to 2007). Three reference strains were used in this study as positive and negative controls, namely VP2053 (tdh^+/trh^-), VP1896 (tdh^-/trh^+), VP1808 (tdh^+/trh^-). All *V. parahaemolyticus* strains were maintained on cryogenic beads at -80°C. The working cultures of *V. parahaemolyticus* were maintained on Luria Bertani slant agar supplemented with 3% sodium chloride (NaCl) at room temperature for no longer than one week.

All *V. parahaemolyticus* isolates were maintained and grown overnight on Luria-Bertani agar plates supplemented with 3% NaCl for *tdh* and *trh* genes detection. Three to five colonies were scraped from the agar plates and resuspended in 400µl of filtered sterile Milli-Q-distilled water, and boiled for 10 min to liberate

the nucleic acid.[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);[26] and 5'-GGCT CAAA ATGG TTAA GCG-3' (forward) and 5'-CATT TCCG CTCT CATA TGC-3' (reverse),[24] respectively. The reaction mixtures (final volume, 25 µl) contained 1µl of DNA template (50ng/µl con), 2.5 µl of 10x reaction buffer (first 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 GeneAmp PCR system 2700 thermocycler (Bio-Rad) as follows: 4 minutes of initial denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 30 seconds, alignment at 58°C for 30 seconds, and extension at 72°C for 30 seconds and a final extension at 72°C for 7 minutes. Positive and negative DNA controls were included in all assays. Amplified products were separated by electrophoresis in ethidium bromide stained 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).

A total of 94 strains of *V. parahaemolyticus* in the collection were randomly selected for antibiotic susceptibility testing. 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 Clinical and Laboratory Standards Institute (CLSI). The measurements were interpreted as resistant (R), intermediate (I) and susceptible (S) to the antibiotics according to the CLSI.[27] The antibiotics being tested in this study are tetracycline (Tc), cefalexin (CX), ciprofloxacin (CI), and ampicillin (Am) (AB BIODISK). Using a sterile cotton swab, 3-5 pure colonies were picked up from fresh Luria Bertani agar plate overnight cultures and inserted into a tube containing 3 ml sterile saline (0.85%) and the turbidity is adjusted to 0.5 McFarland turbidity level. The suspension was then inoculated onto Mueller Hinton agar with sterile cotton swabs. The inoculated plates were allowed to air dry in laminar airflow for 10 min before the E-test antibiotic strips were placed on the agar surface carefully with sterile forceps. The plates were incubated at 37°C for 18-24 hours. The minimum inhibitory concentration (MIC) was read at the point where the zone of growth inhibition intersected the strip.

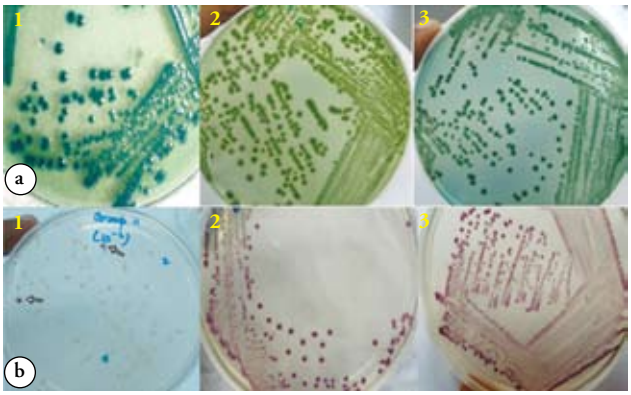

Figure 1 - **a**) Plates 1-3: Pure colonies plated on Thiosulphate citrate bile salt (TCBS) agar medium incubated at 37°C for 24 hours. Green colonies on TCBS agar with different colors making confusing, but only one color mauve colonies on high selected CHROMagar Vibrio medium. **b**) Plates 1-3 were identified as *Vibrio parahaemolyticus.* B1 plate is CHROMagar Vibrio medium inoculated directly from the clinical samples that showed rapid detection during 15-18 hours of incubation time, B2 and B3 plates of CHROMarag vibrio inoculated from TCBS agar with *Vibrio parahaemolyticus* pure colonies for confirmation.

Results. The rapid methods using CHROMagar Vibrio and compared by TCBS agar medium (**Figures 1a & 1b**) in this study was the best to give pure colonies of *V. parahaemolyticus* isolates and to save the time. The result showed a low prevalence of pathogenic strains in the environmental isolates of *V. parahaemolyticus*. Approximately 0.69% (1 out of 144 strains) and 8.3% (12 out of 144 strains) were positive for *tdh* and *trh* gene, respectively. The presence of thermostable direct hemolysin (TDH) is a proven virulence factor,[28] and TDH occurs in over 90% of clinical strains of *V. parahaemolyticus.*[29-31] Most literatures reported a low prevalence of (<1%) tdh gene in the isolates from environmental and seafood samples,[32-34] except in a study carried out at Grays Harbor, Washington.[35] A proposed virulence factor, the TDH-related hemolysin (TRH), encoded by the gene trh, has been discovered in clinical stains of *V. parahaemolyticus* lacking tdh.[12,36] Most clinical isolates from the US Pacific Coast have been reported to possess both *tdh* and *trh*.[13] Our findings are in agreement with Caburlotto et al[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*. The prevalence of pathogenic

Table 1 - Antibiotic susceptibility E-test assays for 94 *Vibrio parahaemolyticus* isolate.

Antibiotic susceptibility	Tetracycline	Ampicillin	Cefalexin	Ciprofloxacin
Sensitive	93 (98.9)	58 (61.7)	32 (34.0)	24 (25.5)
Intermediate	1 (1.1)	11 (11.7)	10 (10.6)	26 (27.7)
Resistant	0 (0.0)	25 (26.6)	52 (55.3)	44 (46.8)
Total	**94 (100)**	**94 (100)**	**94 (100)**	**94**

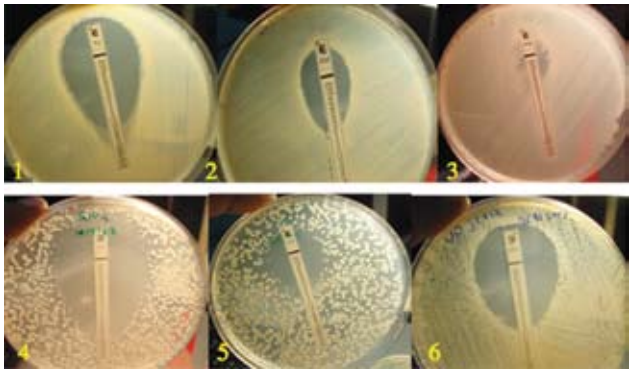

Figure 2 - Epsilometer E-test antibiotic susceptibility assay of *Vibrio parahaemolyticus* performed on Muller Hinton agar medium. Plates 1 & 6: tetracycline, Plates 2 & 3: amoxicillin, Plate 4: ampicillin and Plate 5: cephalexin.

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 contaminated seafood. This result demonstrated the presence of pathogenic *V. parahaemolyticus* in cockles and shrimp harvested in the study area and revealed the potential risk of illness associated with their consumption.

Antibiotic susceptible testing of the selected 94 environmental isolates of *V. parahaemolyticus* in this study revealed a high resistance to Cefalexin (55.3%) and ciprofloxacin (46.8%). In general, the isolates showed the highest susceptibility to tetracycline (98.9%) followed by ampicillin (61.7%) (**Table 1**) (**Figure 2**). The isolates originated from retail cockles purchased from 1st of July 2004 to 25th of August 2007 showed the high resistance to ampicillin, cefalexin and ciprofloxacin compared to isolates collected from shrimp and seawater (**Figure 2**). **Figure 3** shows the distribution of antibiotic resistant profiles of *V. parahaemolyticus* for the four tested antibiotics from 2004 to 2007. The distribution showed a clear development of ampicillin resistance from maximum MIC of 24 ug/ml in 2004 to maximum MIC of 64 ug/ml in 2007 (**Figure 4**). The magnitude

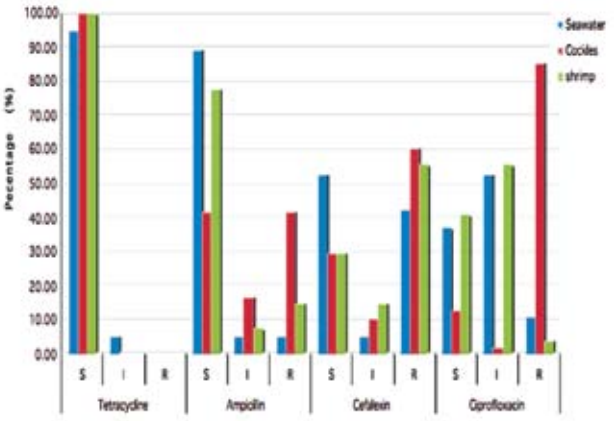

Figure 3 - Tetracycline, ampicillin, cefalexin and ciprofloxacin resistance of *Vibrio parahaemolyticus* isolates from seawater, cockles and shrimp from 2004-2007.

of resistance development is rapid with about 3 fold increase in the mean MIC over 3 years (**Figure 5**).

Discussion. Vibrio organisms are 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.[19,20] This emerging issue has gained great concern due to the development of resistance of pathogenic *V. parahaemolyticus* towards clinically used antimicrobials. Tetracycline and an alternative treatment of combinations of expanded-spectrum cephalosporins (namely, ceftazidime) and doxycycline or a fluoroquinolone alone have been recommended as the antimicrobial of choice for treatment of severe Vibrio infections.[16,26] 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 2004-2007 suggesting the outcome of the banned of tetracycline in animal feed. Excessive use of antibiotics encourages the development of antibiotic resistance[36] and that of reduction may consequence the decrease in antibiotic resistance. However, the findings in this study showed a steady increase in ampicillin resistance in 2004-2007. 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 study in the United States reported that over 90% of *V. parahaemolyticus* isolates were resistant to ampicillin and exhibited-lactamase activity.[37] This finding was also in agreement with a number of studies in the world.[24,38] This study was a

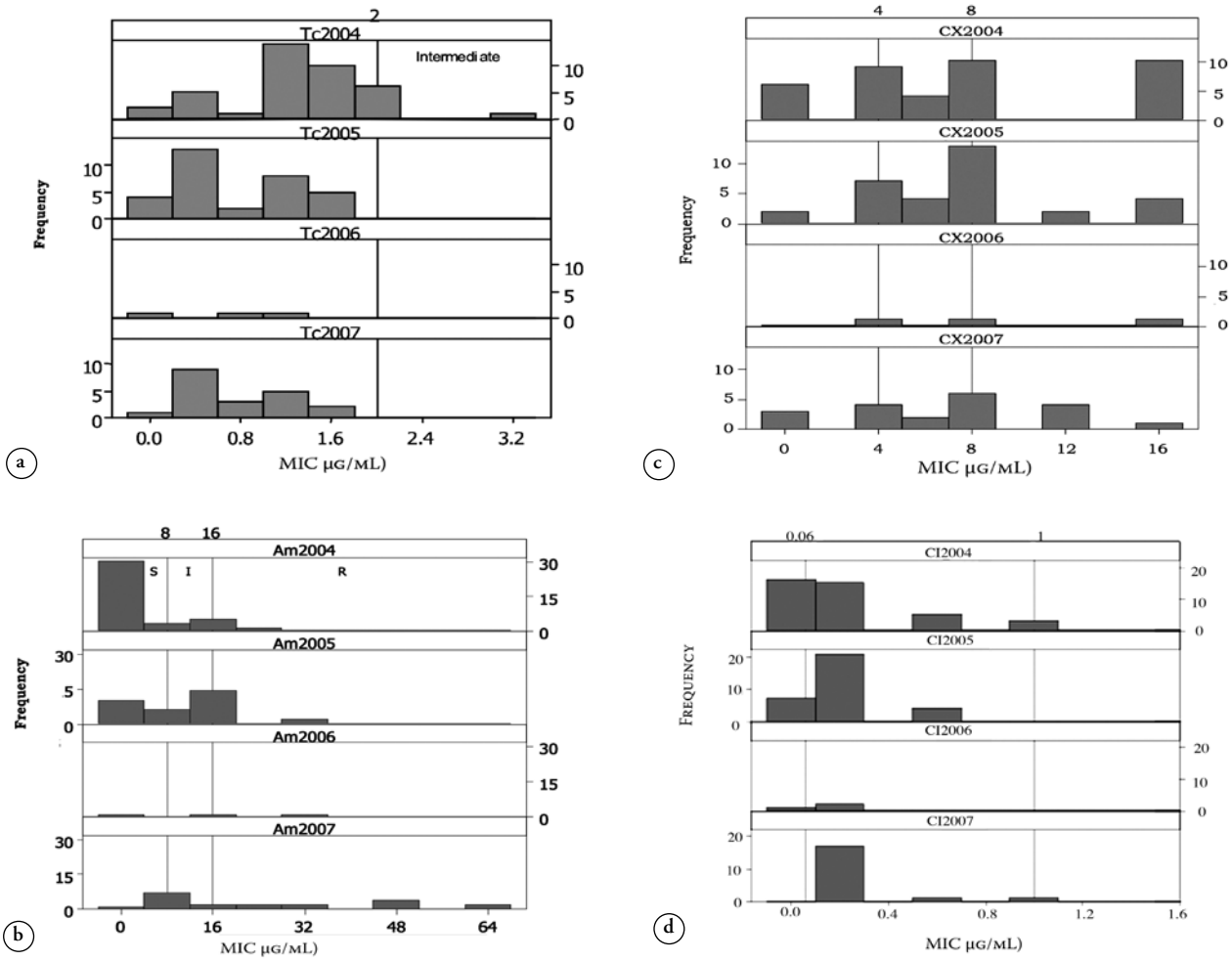

Figure 4 - Distribution of minimum inhibition concentration (MIC) of a) tetracycline (Tc), b) ampicillin (Am), c) cefalexin (Cx), and d) ciprofloxacin (Ci) in the year 2004, 2005, 2006 and 2007.

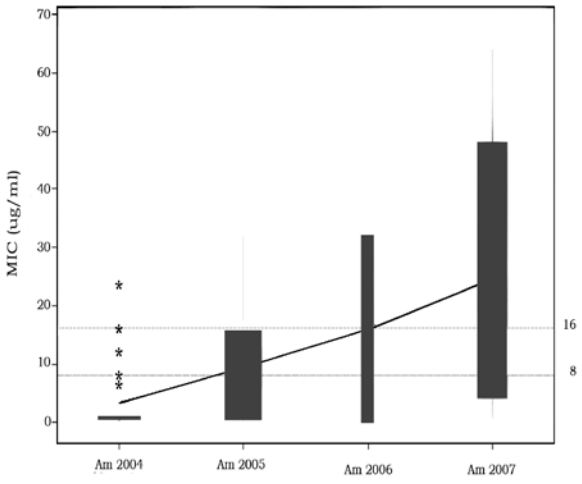

Figure 5 - Increase of ampicillin (Am) resistance in *Vibrio parahaemolyticus* from 2004-2007 .

preliminary examination of antimicrobial susceptibility pattern of *V. parahaemolyticus* isolated from retail seafood (shrimp and cockle) in the east of 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, which may contain various levels of antimicrobials and heavy metals and act as selective pressure for antimicrobial-resistant aquatic bacteria.[18,27,39] These findings indicated that the environmental strains of *V. parahaemolyticus* isolated from various sources and locations 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. The main limitation of the study is that the study was carried out covering from 3 states of Malaysia and does not represent national data.

In conclusion, the present study reveals the occurrence of pathogenic *V. parahaemolyticus* in seafood and therefore poses a threat as one of the risk factors for human gastroenteritis. The CHROMagar *Vibrio* is the best selective medium for rapid detection of *V. parahaemolyticus* in seafood sample and also clinical samples. The development of drug resistance to tetracycline and ampicillin clearly indicate the need for continuous monitoring of the development of drug resistance in *V. parahaemolyticus* isolates.

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