International Food Research Journal 18(4): 1523-1530 (2011)

Antibiotic resistance and biosafety of *Vibrio cholerae* and *Vibrio parahaemolyticus* from freshwater fish at retail level

[1,2*]Noorlis, A., [1]Ghazali, F. M., [4]Cheah, Y. K., [1,3]Tuan Zainazor, T. C., [1]Wong, W. C., [1]Tunung, R., [1]Pui, C. F., [5]Nishibuchi, M., [5]Nakaguchi, Y. and [1]Son, R.

[1]*Center of Excellence for Food Safety Research, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia*
[2]*Universiti Teknologi MARA Pahang, 26400 Bandar Tun Abdul Razak Jengka, Pahang Darul Makmur, Malaysia*
[3]*National Public Health Laboratory, Ministry of Health, Lot 1853 Kampung Melayu, 47000 Sungai Buloh, Selangor Darul Ehsan, Malaysia*
[4]*Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia*
[5]*Center of Southeast Asian Studies, Kyoto University, Kyoto 606-8501, Japan*

Abstract: A total of 49 isolates of *V. parahaemolyticus* and 8 isolates of *V. cholerae* isolated from freshwater fish of patin (*Pangasius hypopthalmus*) and red tilapia (*Oreochromis* sp.) were purchased from different retail level in Selangor, Malaysia. All of the isolates showed a multiple resistances towards all 15 antibiotics tested. Some of the isolates show a high resistance to different antibiotics including bacitracin, vancomycin, tetracycline, furazididone, cephalothin and erythromycin. However, both species was susceptible towards imipenem. Overall antibiotics resistance patterns of all isolates were resistant from 2 to 14 resistance patterns with multiple antibiotic resistance (MAR) index ranging from 0.13 to 0.93 respectively. As the results obtained in the dendrogram produced from both species had indicates that these antibiotics were intensively used whether in the aquaculture farm through feeds during culture or at the hatchery production of seed. Thus, this study will provides an essential information of the MAR index and also the clustering analysis in order to determine the biosafety of *Vibrio* spp. in freshwater aquaculture fish sold at different retail level in Malaysia.

Keywords: *Vibrio* spp., *Vibrio parahaemolyticus*, *V. cholerae*, multiple antibiotic resistance, freshwater fish

Introduction

Antibiotic in a broader sense is a chemotherapeutics agent that capable of inhibit or abolishes the growth of microorganisms such as bacteria, fungi and protozoa (Kummerer, 2009). Penicillin, the first antibiotics ever discover by the Scottish scientist and Nobel Laureate, Alexander Fleming in 1928, was of natural origin by fungi in the genus *Penicillium*.

Currently, antibiotics are obtained by chemical synthesis and it can be grouped by either chemical structure or its action mechanism. Antibiotic has been used extensively in human, veterinary medicine, agriculture and aquaculture business and it has steadily increased especially in the developing countries (Kumar *et al*., 2009). It is one of the most common drugs prescribed in hospitals today and Lim *et al.*, 1993 had reported that, up to third of all patients receive at least one antibiotic during hospitalisation. There have been numerous studies on patterns of antibiotic usage in hospitals. However, international comparable data on antibiotic consumptions is scarce and the information available due to the emergence of bacterial resistant to antibiotics is heterogeneous because of the usage patterns may be vary in different countries.

Nowadays, antimicrobial resistance is a growing public health threat and has been designated by the WHO as an emerging public health problem (Chai *et al*., 2008). Chinabut *et al*., 2011 stated that only the importation of some Asian aquaculture products were banned as residue of chloramphenicol was detected, even though at a low concentration, it may be toxic or carcinogenic for humans. We believe that the increased application of antibiotics especially in aquatic environments has to be largely responsible for the emergence of drug resistance bacteria. In the field of aquaculture in Malaysia, there is a rapid growth in the production of freshwater aquaculture fish. According to the department of fisheries (DOF) Malaysia, the production from aquaculture in 2009 for food increased to 333 451 tonnes which was

*Corresponding author.
Email: *noorlisahmad@yahoo.com*
Tel: +603 8946 8368; Fax: +603 89423552

increase of 37.2% compare to 243 129 tonnes in the year 2008. The production value was also increased from RM 1 717.79 million in 2008 to RM 2 295.16 million in the next following year of 2009. Although, the brackish water aquaculture remains as the main contributor to this sub-sector at 54.2% or 181 820 tonnes, freshwater aquaculture on the other hand are still contributed to 45.8% or 152 630 tonnes to the national food fish production (Anon, 2011). It seems that consumers in Malaysia have began to accept aquaculture fish as an alternative to sea fish since the production of sea fish was depleted recently due to the threat of marine pollution and climate uncertainty. Ibrahim *et al*., 2001 also concluded that beside brackish water fish, freshwater fish are also the main aquaculture products in Malaysia especially for the red tilapia fish (*Oreochromis* sp.), patin (*Pangasius* sp.) and keli (*Clarius* sp.).

In Malaysia, antibiotic and other chemotherapeutics agents and also pesticides were commonly used in fish farms either as a feed additives or immersion baths to achieve either prophylaxis or therapy, also as a common practices to avoid the overgrowth of herbal plants and fish diseases beside promoting the fast growth of the fish (Majusha *et al*., 2005 ; Ibrahim *et al*., 2010). Bacteria such as the genus of *Vibrio* spp. are commonly found in coastal, estuarine waters, brackish water, and freshwater (Li *et al*., 1999; Imziln and Hassani, 1994; Majusha *et al*., 2005; Zulkifli *et al*., 2009; Ibrahim *et al*., 2010). In the Asian region, *Vibrio* spp. have been recognized as the leading cause of foodborne outbreaks in many countries including Japan, India, China, Taiwan, Korea and Malaysia (Noorlis *et al*., 2011). Some *Vibrio* isolates are pathogenic and can cause Vibriosis, a serious infection disease in wild, cultured and shell fish (Papadopoulou *et al*., 2008). According to Roque *et al*. (2001), the most common way in Mexico to resolve the Vibriosis problem is by the use of feed plus antibiotics in shrimps aquaculture freshwater farms or directly applied to the water in case of the hatcheries ponds. Ibrahim *et al*. (2010) also reported that the chemical residue from the antibiotics or pesticides used at the farm level can be accumulated in fish and could cause a chronic health effects to consumers and potentially to cause certain organ or system malfunction such as cancer, nerve problems and immunological problems in human.

Even though, there are several work done on the assessment of aquaculture product in Malaysia most of the studies did not include freshwater aquaculture fish especially the popular red tilapia, patin and keli. As supported by the recent researcher, Ibrahim *et al*. (2010), there are no studies so far on the chemical risk assessment including the antibiotics susceptibility testing involving the freshwater aquaculture fish in Malaysia.

So, generally this study will provides important information regarding the dissemination of multiple antibiotics resistance (MAR) index and also the clustering analysis to determine the biosafety of the *vibrio* spp. in freshwater aquaculture fish sold at wet market and hypermarket in Malaysia.

Materials and Methods

Bacterial isolates, media and propagation

A total of 49 isolates of *V. parahaemolyticus* and 8 isolates of *V. cholerae* were obtained from 300 samples of freshwater fish (*Pangasius hypopthalmus* and *Oreochromis* spp.) purchased from retail levels in Selangor, Malaysia. It comprised of 48 *V. parahaemolyticus* isolates from hypermarket and only one isolate from wet market (VP23). Whereas, for all 8 isolates of *V. cholerae* was isolated from hypermarket samples and none was found at the wet markets level. All isolates were revived from glycerol stocks using Trytic Soy broth (TSB) (BactoTM, France) and 1-3% NaCl (Merck, Germany). They were incubated at 37^{0}C for 18 to 24 hours in an orbital shaker (Barnstead International, Iowa, USA).

Antibiotic susceptibility

All *vibrios* isolates under study were tested for susceptibility to various antibiotics using the disk diffusion method according to guidelines set by the National Committee for Clinical Laboratory standard (2004) and the previously described by Bauer *et al*. (1966) and Zulkifli *et al*. (2009).

All isolates were grown in TSB (Bacto™, France) with 1-3% NaCl (Merck, Germany) and were incubated at 37^{0}C for 18 to 24 hours. The cultures were swabbed evenly using sterile non-toxic swab on Mueller-Hinton (MH) agar plates (Merck, Germany), which were then left to dry for 2-5 minutes before placing the antimicrobial sensitivity discs onto the agar using a Disk Diffusion Dispenser (Oxoid Ltd., Hamshire, England). The culture of *E. coli* ATCC 25922 was included as a control test in the susceptibility testing.

Fifteen type antibiotics were selected for the tests which selected randomly from the main group such as Aminoglycosides, Beta-lactams, Cephalosporins, Macrolides, Nitrofurantoins, Phenols, Tetracyclines, Quinolones and others. Antibiotics tested were Amikacin (AK, 30 µg), Gentamicin (CN, 10 µg), Kanamycin (K, 30 µg), Streptomycin (S, 10 µg), Imipenem (IPM, 10 µg), Cephalothin (KF, 30 µg),

Ceftazidime (CAZ, 30 µg), Erythromycin (E, 15 µg), Furazididone (FR, 100 µg), Chloramphenicol (C, 30 µg), Tetracycline (TE, 30 µg), Bacitracin (B, 10U), Ciprofloxacin (CIP, 5 µg), Norfloxacin (NOR, 10 µg) and Vancomycin (VA, 5 µg). The antibiotic cartridges with commercially prepared antibiotic discs were purchased from Oxoid (Hamphire, United Kingdom) and BBL (Becton-Dickinson Microbiology Systems, Maryland, USA). Each antibiotic test was run in duplicate on freshly prepared Mueller Hinton agar (MHA) plates.

All plates were incubated at 37^0C for 24 hours. After incubation, the size of the inhibition zones was recorded and the levels of susceptibility (sensitivity, intermediates and resistant) were determined according to the National Committee for Clinical laboratory Standards (NCCLS) (2004).

MAR indexing isolation

Based on the occurrence of the multiple resistance of isolates from each of the sampling sites, the multiple antibiotic resistance index of the isolates is 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, 1983).

Bionumerics analysis method

Association between the resistance profiles obtained for each isolates were analysed using the hierarchic numerical methods. In this study, the numerical matrix obtained was computed using the Software package version 4.5 (Applied Maths, Kortrijk, Belgium) employing the Pearson correlation coefficient and UPGMA to determine the relatedness of each isolates based on the dendrogram produced. All the results obtained were coded using '0' for sensitive and intermediate whereas '1' resistant phenotypes for each 15 type of antimicrobial drugs tested.

Results and Discussion

An increase in the emergence of multi-drug resistant bacteria in recent years is worrying and begins to erode our antibiotics armamentarium to combat antibiotic resistance and thus limiting therapeutics options to present-day clinician (Zulkifli *et al*., 2009). Fish farming has encounter disease problems similar to other sectors of intensive husbandry and the used of antimicrobial agents has increased significantly (Spanggaard *et al*., 1993). These antibiotics and other chemotherapeutic agents are commonly used in fish farms either as feed additives or immersion baths to achieve either prophylaxis or therapy (Li *et al*., 1999). The results demonstrate in Table 1 and 2 show a high individual and multiple resistance to antibiotics among the 49 isolates of *V. parahaemolyticus* and 8 isolates of *V. cholerae* isolates after tested against 15 types of antibiotics isolated from several hypermarket and wet market in Selangor, Malaysia.

Table 1. Distribution of antimicrobial resistance, intermediate and susceptible of *V. cholerae* from freshwater fish

Antibiotics	No.(%) of ***Vibrio cholerae*** to selected antibiotics		
	Resistance(R)	Intermediate(I)	Susceptible(S)
Aminoglycosides			
Amikacin (AK30)	0(0)	1(13)	7(88)
Gentamicin (CN10)	2(25)	1(13)	5(63)
Kanamycin (K30)	1(13)	0(0)	7(88)
Streptomycin (S10)	2(25)	0(0)	6(75)
Beta-lactams			
Imipenem (IPM10)	0(0)	2(25)	6(75)
Cephalosporins			
Cephalothin (KF30)	6(75)	1(13)	1(13)
Ceftazidime (CAZ30)	1(13)	0(0)	7(88)
Macrolides			
Erythromycin (E15)	5(63)	1(13)	2(25)
Nitrofurantoins			
Furazididone (FR100)	8(100)	0(0)	0(0)
Phenols			
Chloramphenicols (C30)	2(25)	2(25)	4(50)
Tetracyclines			
Tetracycline (TE30)	7(88)	0(0)	1(13)
Quinolones			
Ciprofloxacin (CIP5)	1(13)	1(13)	6(75)
Norfloxacin (NOR10)	0(0)	2(25)	6(75)
Others			
Bacitracin (B10)	8(100)	0(0)	0(0)
Vancomycin (VA5)	8(100)	0(0)	0(0)

Table 2. Distribution of antimicrobial resistance, intermediate and susceptible of *V. parahaemolyticus* from freshwater fish

Antibiotics	No.(%) of ***Vibrio parahaemolyticus*** to selected antibiotics		
	Resistance(R)	Intermediate(I)	Susceptible(S)
Aminoglycosides			
Amikacin (AK30)	22(45)	0(0)	27(55)
Gentamicin (CN10)	25(47)	2(4)	24(39)
Kanamycin (K30)	26(53)	0(0)	23(47)
Streptomycin (S10)	25(51)	3(7)	21(43)
Beta-lactams			
Imipenem (IPM10)	6(12)	0(0)	43(88)
Cephalosporins			
Cephalothin (KF30)	37(76)	7(14)	10(20)
Ceftazidime (CAZ30)	24(49)	0(0)	25(51)
Macrolides			
Erythromycin (E15)	33(68)	13(27)	3(6)
Nitrofurantoins			
Furazididone (FR100)	42(86)	0(0)	7(15)
Phenols			
Chloramphenicols (C30)	18(37)	6(13)	25(51)
Tetracyclines			
Tetracycline (TE30)	40(82)	2(4)	7(14)
Quinolones			
Ciprofloxacin (CIP5)	23(47)	6(13)	20(41)
Norfloxacin (NOR10)	19(39)	2(4)	28(57)
Others			
Bacitracin (B10)	48(98)	1(2)	0(0)
Vancomycin (VA5)	32(65)	4(9)	13(27)

The prevalence of resistance to antimicrobial agents among *V. cholerae* isolates are shown in Table 3 and Figure 1. The resistance to Furazididone, Bacitracin and Vamcomycin was observed in 100% of the analysed *V. cholerae* isolates followed by Tetracycline (88%), Cephalothin (75%) and Erythromycin at 63%. The resistance towards other antibiotics was found to be considerably lower towards Gentamicin (25%), Streptomycin (25%), Chloramphenicol (25%), Kanamycin (13%), Ceftazidime (13%) and Ciprofloxacin (13%). None of the *V. cholerae* isolates were resistant against Amikacin, Imipenem and Norfloxacin.

Table 3. Prevalence of resistance to antimicrobial agents among *Vibrio cholerae* and *Vibrio parahaemolyticus* isolated from freshwater fish

Antibiotics (µg/ml)	No. of resistant (%) *Vibrio* spp. isolates		TOTAL
	V. cholerae	*V. parahaemolyticus*	
Amikacin (AK30)	0(0)	22(45)	22(39)
Gentamicin (CN10)	2(25)	23(47)	26(46)
Kanamycin (K30)	1(13)	26(53)	28(49)
Streptomycin (S10)	2(25)	25(51)	29(51)
Imipenem (IPM10)	0(0)	6(12)	6(11)
Cephalothin (KF30)	6(75)	37(76)	43(75)
Ceftazidime (CAZ30)	1(13)	24(49)	25(44)
Erythromycin (E15)	5(63)	33(68)	38(67)
Furazididone (FR100)	8(100)	42(86)	50(88)
Chloramphenicols (C30)	2(25)	18(37)	20(35)
Tetracycline (TE30)	7(88)	40(82)	47(83)
Bacitracin (B10)	8(100)	48(98)	56(98)
Ciprofloxacin (CIP5)	1(13)	23(47)	24(43)
Norfloxacin (NOR10)	0(0)	19(39)	20(35)
Vancomycin (VA5)	8(100)	32(65)	40(70)

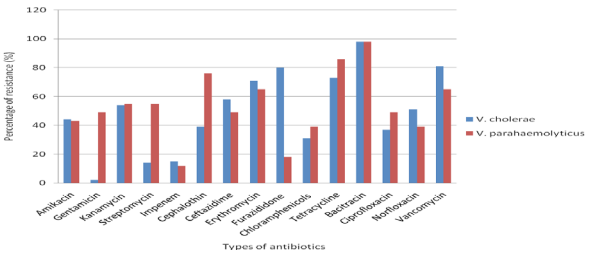


Figure 1. Prevalence of resistance to antimicrobial agents among *V. cholerae* and *V. parahaemolyticus* isolated from freshwater fish

Table 2 and Figure 1 shows the distribution of antimicrobial resistance of 49 *V. parahaemolyticus* isolate with the highest prevalence of resistance was towards Bacitracin (98%), Tetracycline (82%), Furazididone (82%), Cephalothin (76%), Erythromycin (68%) and Vancomycin with 65% resistance level. Whereas, the other antibiotics was found to be considerably lower; Kanamycin (53%), Streptomycin (51%), Ceptazidime (49%), Gentamicin and Ciprofloxacin with 47% equally, Amikacin (45%), Norfloxacin (39%), Chloramphenicol (37%) and the least resistant toward Imipenem with only 12%.

Resistance of marine fish and shrimp pathogenic bacteria to commonly used antibiotics has been reported throughout the world (Vaseeharan *et al*., 2005). Previous studies have shown that Streptomycin, Rifampicin, Kanamycin, Tetracycline, Polymyxin B were active against *Vibrio* spp. (Zulkifli *et al*., 2009). However, Ottaviani *et al*., (2001) showed that *V. parahaemolyticus* were resistance to Penicillin, Carbenicillin, Ampicillin, Cephalothin, Kanamycin and Rifampicin. Different bacteria vary in their susceptibility to antibiotics. Thus, antibiotics resistance may cause economic losses as the outbreak of different disease may not be treated efficiently. Other problems may also arise from the intensive use of these antimicrobial drugs. Bacteria which are pathogenic to humans may occur naturally on farmed fish and in the aquatic environment. As the fish are used for human consumption, the development of antibiotic resistance in pathogens could pose a health risk to the consumer (Spanggaard *et al*., 1993).

Table 4. Antibiotics resistance patterns and multiple antibiotic resistances (MAR) index of *V. cholerae* from freshwater fish in hypermarket and wet market level

Isolates no.	Samples location	Sample source	[a]Resistance patterns	[b]MAR index
C52	Wet market	Gill	VaFrBTe	0.27
C53	Wet market	Gill	CVaFrKfNorEBTe	0.53
C54	Wet market	Gill	VaFrKfEBTeCaz	0.47
C55	Wet market	Gill	VaFrB	0.20
C56	Wet market	Gill	CVaFrKfBAkTeK	0.53
C57	Wet market	Gill	CVaFrKfEBCipTe	0.53
C58	Wet market	Gill	VaFrKfEBTe	0.40
C59	Wet market	Int. tract	CVaFrKfEBTe	0.47

[a]Tested for Chloramphemicol (C), Vancomycin (Va), Furazolidone (Fr), Cephalothin (Kf), Norfloxacin (Nor), Erythromycin (E), Streptomycin (S), Bacitracin (B), Ciprofloxacin(Cip), Gentamicin (Cn), Imipenem (Ipm), Amikacin (Ak), Tetracycline (Te), Ceftazidime (Caz), Kanamycin (K).
[b]MAR Index = $\frac{\text{The number of antibiotic agents resisted}}{\text{Total number of antibiotic agents used}}$

Table 4 showed the antibiotics resistance patterns and multiple antibiotic resistance (MAR) index of all 8 isolates of *V. cholerae* isolates which were found to be resistant to a quite high number of 3 to 8 antibiotic tested with MAR indices ranging from 0.20 to 0.53. Whereas for all *V. parahaemolyticus* isolates under study, antibiotic resistance pattern and multiple antibiotic resistance (MAR) index are shown in Table 5 was found to be resistant from 2 to 4 antibiotics tested. The MAR index value shown was in the range of 0.13 to 0.93 respectively.

With the high indices values detected in this study, we can say that there was a mix of isolates originated from a sources in which seldom or never been exposed to antibiotics. This is because isolates with a MAR index values of more than 0.2 were considered to have originated from the higher risk sources of contamination like humans, commercial poultry farms, swine and dairy cattle where antibiotics are often used. For the MAR index values lower than 0.2 were considered to have originated from animals in which antibiotics are seldom or never used. It is well known that the wide use and abuse of antibiotics in human therapy has produced MAR pathogenic microorganisms in the faeces of human as well. Release of pathogenic bacteria in the faeces results in dispersal into aquatic systems was where they contaminate these aquatic environments, where genetic exchange between bacteria is readily facilitated and account for a higher frequency of MAR forms (Krumperman *et al*., 1983).

A dendrogram of *V. cholera* in Figure 3 shows the clustering of the 15 antimicrobial agents tested based on the resistance or susceptible of the isolates obtained using the Bionumerics version 4.5 software package. From the dendrogram, all 8 isolates were clustered into 2 major clusters, Cluster A with 6 isolates and Cluster B with only 2 isolates. *V. cholerae* resistance profiles clustering into G1 was formed at 87% similarity, consist of VC52 and VC55 isolates which was isolated from the gill samples. Whereas, at 93% similarity, G2 was clustered consist of VC54, VC58 and VC59 isolates. As for the last groups

Table 5. Antibiotics resistance patterns and multiple antibiotic resistances (MAR) index of *V. parahaemolyticus* from freshwater fish in hypermarket and wet market level

Isolates no.	Samples location	Samples source	[a]Resistance patterns	[b]MAR index
P1	Hypermarket	Flesh	CFrNorSCipCnAkTeCazK	0.67
P2	Hypermarket	Flesh	CFrKfSBCnAkCazK	0.60
P3	Hypermarket	Int. tract	CFrKfNorESBCipCnAkTeCazK	0.87
P4	Hypermarket	Gill	FrKfSBCipCnAkTeCazK	0.67
P5	Hypermarket	Gill	VaFrKfNorSBCipCnAkTeCazK	0.80
P6	Hypermarket	Flesh	VaFrEBTeCaz	0.40
P7	Wet market	Int. tract	CFrKfNorESBCipCnAkCazK	0.80
P8	Wet market	Flesh	VaFrKfNorESBCipCnTeCazK	0.80
P9	Wet market	Flesh	CVaFrNorESBCipCnAkTeCazK	0.87
P10	Hypermarket	Int. tract	CVaFrKfEBTe	0.47
P11	Hypermarket	Int. tract	CVaFrKfEBTe	0.47
P12	Hypermarket	Gill	FrNorESBCipCnIpmAkTeCazK	0.80
P13	Hypermarket	Gill	CVaFrKfNorESBCipCnAkTeCazK	0.93
P14	Hypermarket	Gill	FrNorESBCipCnAkTeCazK	0.73
P15	Hypermarket	Int. tract	BCaz	0.13
P16	Hypermarket	Gill	CFrNorESBCipCnIpmAkTeCazK	0.87
P17	Hypermarket	Gill	FrNorESBCipCnAkTeCazK	0.73
P18	Hypermarket	Flesh	CNorESBCipCnAkTeCazK	0.73
P19	Hypermarket	Gill	CVaFrKfNorSBCipCnAkTeCazK	0.87
P20	Hypermarket	Gill	VaFrKfEBCipTe	0.47
P21	Hypermarket	Gill	VaKfEB	0.27
P22	Hypermarket	Gill	CKfNorSBCipCnAkTeCazK	0.73
P23	Hypermarket	Flesh	CFrKfNorESBCipCnIpmAkTeCazK	0.93
P24	Hypermarket	Flesh	CFrKfNorESBCnAkTeCazK	0.80
P25	Hypermarket	Flesh	CVaFrKfESBCipCnAkTeCazK	0.87
P26	Hypermarket	Gill	CVaEBTe	0.33
P27	Hypermarket	Gill	VaEBTe	0.27
P28	Hypermarket	Gill	VaKfEBTe	0.33
P29	Hypermarket	Flesh	VaKfESBCipCnIpmAkTeCazK	0.80
P30	Hypermarket	Flesh	FrKfNorSBCipCnIpmAkTeCazK	0.80
P31	Hypermarket	Gill	VaFrEBTe	0.33
P32	Hypermarket	Gill	CFrESBCnIpmAkTeCazK	0.73
P33	Hypermarket	Int. tract	VaFrKfNorESBTe	0.53
P34	Hypermarket	Flesh	VaFrB	0.20
P35	Hypermarket	Flesh	FrB	0.13
P36	Hypermarket	Flesh	VaFrB	0.20
P37	Hypermarket	Flesh	VaFrKfEBTe	0.40
P38	Wet market	Int. tract	VaFrKfBTeK	0.40
P39	Wet market	Gill	VaFrKfEBTe	0.40
P40	Wet market	Gill	VaFrKfBTe	0.33
P41	Wet market	Gill	VaBTe	0.20
P42	Wet market	Gill	VaFrKfBTeK	0.40
P43	Wet market	Gill	VaFrKfEBTe	0.40
P44	Wet market	Flesh	CVaFrKfNorESBCipCnTeK	0.80
P45	Wet market	Flesh	VaFrKfESBCipTe	0.53
P46	Wet market	Flesh	VaFrKfBCipTe	0.40
P47	Wet market	Flesh	VaFrKfBCipTe	0.40
P48	Wet market	Int. tract	CVaFrKfESBTeK	0.60
P49	Wet market	Int. tract	VaFrKfEBTe	0.40

[a]Tested for Chloramphemicol (C), Vancomycin (Va), Furazolidone (Fr), Cephalothin (Kf), Norfloxacin (Nor), Erythromycin (E), Streptomycin (S), Bacitracin (B), Ciprofloxacin(Cip), Gentamicin (Cn), Impenem (Ipm), Amikacin (Ak), Tetracycline (Te), Ceftazidime (Caz), Kanamycin (K).

[b]MAR Index = $\frac{\text{The number of antibiotic agents resisted}}{\text{Total number of antibiotic agents used}}$

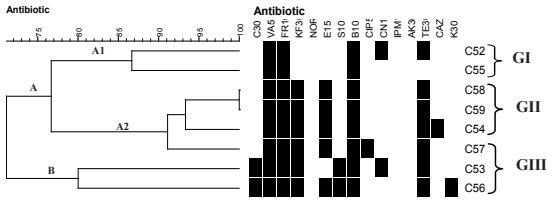
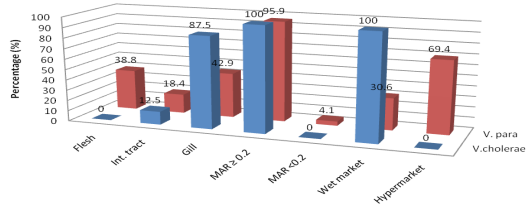


Figure 3. Dendrogram based on the hierarchic numerical analysis on the resistance profiles for 8 *V. cholerae* isolates, employing the Pearson correlation coefficient and UPGMA for clustering

form in this dendrogram was G3 at 80% similarity consist of VC53 and VC56 isolates which was previously isolated from the gill samples also. Only one isolates of VC57 comes with a unique profiles which was not groups in any 3 groups constructed in the dendrogram.

On the other hand, cluster analysis of the 49 *V. parahaemolyticus* isolates shown in Figure 3 was found to be clustered in 2 major cluster groups. For the first Cluster C which was then further subclustered into cluster C1 with several minor clusters and only one isolate was group in a single-member cluster at 63% similarity level. From the constructed dendrogram, there was 4 cluster at the highest similarity of 93% which were G1 (VP1, VP3, VP8, VP13 and VP25), G2 (VP5 and VP19), G3 (VP16 and VP23) and finally G4 where all the isolates was isolated from the gill samples (VP12, VP14 and VP17). As for the second cluster D was further subclustered into cluster D1 and D2 followed with several minor clusters. Also at the highest similarity of 93%, 4 more groups were formed in cluster D. It was G5 (VP28, VP38, VP40, VP42, VP43, VP46, VP47 and VP49), in G6 (VP20, VP26 and VP37), G7 with all isolates was isolated from fish's gills samples (VP26, VP27 and VP31) and as for the last groups was G8 withthe VP34, VP35 and VP36 was isolated from the fish's flesh samples.

The distribution of antibiotic resistance as observed in the dendrograms produced clearly indicates that antibiotics were intensively used. This is further explained when many isolates were resistant to the same antibiotics tested for both, *V. cholera* and *V. parahaemolyticus* isolates under study. This informative observation may also give us some idea on the suspect usage of antibiotics in the aquaculture farms through feeds during culture or during the hatchery production of seeds in order to reduce the potential risk of bacterial diseases. As the fish's flesh, intestinal tract and gill samples comes from 2 different retail level of wet and hypermarket, we can see in Figure 2 that *V. cholera* was 100% found or originated from the fish samples bought from the wet market and none was found in the fish samples bought from the hypermarket. As compared to the *V. parahaemolyticus* isolates with most of the isolates (69.4%) was from the hypermarket fish samples and only 30.6% come from the wet market fish samples. This scenario may be due to the cross contamination of the fish on the display bench, since fish was always covered with ice to maintain its freshness. The same display area with other seafood may also contribute to the presence of *Vibrios* in the fish samples under study. As reported by the previous researchers, the improper handling and poor hygienic practices could be the major source of contamination of food especially raw fish at the hypermarket level (Noorlis *et al*., 2011).



	Flesh	Int. tract	Gill	MAR ≥ 0.2	MAR <0.2	Wet market	Hypermarket
V.cholerae	0	12.5	87.5	100	0	100	0
V. para	38.8	18.4	42.9	95.9	4.1	30.6	69.4

Figure 2. Distribution summary of the antibiotic prevalence according to sample sources, MAR indices and the sample locations of the isolates under study

In term of sampling types, fish's gill samples was found to give the most highest prevalence reading or the most common place that we can harbour *V. cholera*

and *V. parahaemolyticus* in this study followed by flesh and intestinal tract. This is because as we know that *Vibrio* spp. was a waterborne bacteria. So, in this case, gill was a respiratory organ for a fish and of course it will interact directly with the surrounding water. So, it will be the best place for the *Vibrios* to be found as compared to the flesh and intestinal tract.

Overall, 14 patterns of resistance was observed with majority of isolates were resistant to 12 antibiotics among the *V. cholerae* and *V. parahaemolyticus* isolates. When combining the antibiotics profiles of *V. cholerae* and *V. parahaemolyticus* isolates, two huge major cluster was constructed from the dendrogram (Figure 5). For this dendrogram which was constructed from the combination of both antibiotic profiles of *V. cholera* and *V. parahaemolyticus* isolates, 2 huge cluster was formed which was referred as cluster E and F. In the first cluster E, all of the isolates in cluster E were group into 1 cluster of E1 and one single isolate that is not group in any other clusters (VP15). Then, it was further subdivided into 2 minor clusters of E1a and E1b at 72% similarity. The same clustering patterns as in Figure 4, where the highest cluster formed was at 93% with 6 groups in cluster E. All isolates in the G1 to G6 was not isolated from the same source as what happened in the Figure 4 previously. As what constructed in cluster E, cluster F were also group into 1 cluster of F1 and one single isolate of VP32. Whereas other isolates was subdivided at 93% similarity into several groups of G7 until G9 with isolates comes from the mix sources of flesh, intestinal tracts and gills. Only one isolates in G10 (VP12, VP14 and VP17) was isolated from the same source of gills samples.

Figure 4. Dendrogram based on the hierarchic numerical analysis on the resistance profiles for 49 *V. parahaemolyticus* isolates, employing the Pearson correlation coefficient and UPGMA for clustering

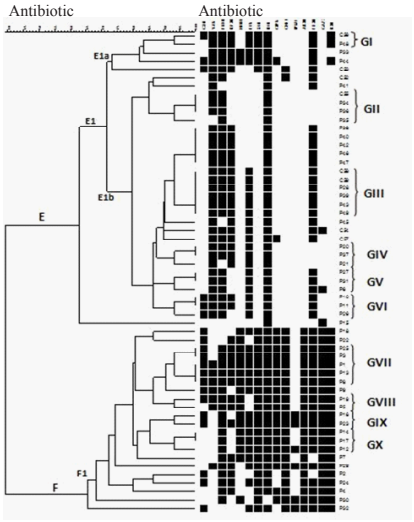


Figure 5. Dendrogram based on the hierarchic numerical analysis on the resistance profiles for *V. cholerae* and *V. parahaemolyticus* isolates, employing the Pearson correlation coefficient and UPGMA for clustering

The high incidence of foodborne diseases worldwide and the death rate from some of the foodborne illness can also surprisingly increased with one in five people estimated to become sick from the food poisoning caused by the bacteria will die from it each year (30). A food safety enhancement throughout the entire food supply chain which is from farm to fork should be observed and monitor seriously by the authority in order to improve the food safety level in every stages of the food production processes, starting from how the animal was raised and how the raw materials was handled, harvest, process and distribute until its final stage where the food reaches the consumers. This is because Kumar *et al*. (2009) had stated that most of the bacteria that are pathogenic to humans may occur naturally in farmed fish or aquatic environments and make their way to humans with the spread of resistance genes leading to health problems. For these reasons, Zulkifli *et al*. (2009) had stated previously that food contamination with antibiotics resistance bacteria is a threat to public health as the antibiotic resistance determinants may be transferred to other bacteria of clinical significance and *Vibrio* spp. is a candidate vehicle for such transfer because of its diversity and because it can survives in the gastrointestinal tracts of both human and animals.

Looking at the highest antibiotic resistance percentage and the extensively used of antibiotics in human, veterinary medicine as well as in aquaculture, a serious and frequent monitoring of the antibiotic used in the farm level for the purpose of preventing and treating of microbial infections or as the animal growth promoter is important to ensure of the freshwater fish safety particularly. Exposure of the dangerous effect of antibiotic resistance towards humans

Table 6. Antibiotic resistance patterns of *Vibrio cholerae* and *Vibrio parahaemolyticus* from hypermarket and wet market

Pattern No.	[a]Antibiotics resistance pattern	No. of antibiotic resistant pattern	Isolates numbers
1	FrB	2	P35
2	BCaz	2	P15
3	VaFrB	3	P34
4	VaBTe	3	P41
5	VaFrBTe	4	C52
6	VaKfEB	4	P21
7	VaEBTe	4	P27
8	VaFrEBTe	5	P40
9	VaEBTeK	5	P28
10	VaFrKfEBTe	6	P49,P43,P39. P37
11	VaFrKfBCipTe	6	P46, P47
12	VaFrKfBTeK	6	P38, P42
13	VaFrEBTeCaz	6	P6
14	CVaFrKfEBTe	7	P10,P11,C59
15	VaFrKfEBTeCaz	7	C54
16	CVaFrKfNorEBTe	8	C53
17	CVaFrKfBAkTeK	8	C56
18	CVaFrKfEBCipTe	8	C57
19	VaFrKfNorESBTe	8	P33
20	VaFrKfESBCipTe	8	P45
21	CFrKfSBCnAkCazK	9	P2
22	CFrNorSCipCnAkTeCazK	10	P1
23	FrKfSBCipCnAkTeCazK	10	P4
24	FrNorESBCipCnAkTeCazK	11	P14, P17
25	CNorESBCipCnAkTeCazK	11	P18
26	CKfNorSBCipCnAkTeCazK	11	P22
27	CFrESBCnIpmAkTeCazK	11	P32
28	CVaFrKfNorESBCipCnTeK	12	P44
29	VaFrKfNorSBCipCnAkTeCazK	12	P5
30	CFrKfNorESBCipCnAkCazK	12	P7
31	VaFrKfNorESBCipCnTeCazK	12	P8
32	FrNorESBCipCnIpmAkTeCazK	12	P12
33	CFrKfNorESBCnAkTeCazK	12	P24
34	VaKfESBCipCnIpmAkTeCazK	12	P29
35	FrKfNorSBCipCnIpmAkTeCazK	12	P30
36	CFrKfNorESBCipCnAkTeCazK	13	P3
37	CVaFrNorESBCipCnAkTeCazK	13	P9
38	CFrNorESBCipCnIpmAkTeCazK	13	P16
39	CVaFrKfNorSBCipCnAkTeCazK	13	P19
40	CVaFrKfESBCipCnAkTeCazK	13	P25
41	CVaFrKfNorESBCipCnAkTeCazK	14	P13

[a]Tested for Chloramphemicol (C), Vancomycin (Va), Furazolidone (Fr), Cephalothin (Kf), Norfloxacin (Nor), Erythromycin (E), Streptomycin (S), Bacitracin (B), Ciprofloxacin(Cip), Gentamicin (Cn), Imipenem (Ipm), Amikacin (Ak), Tetracycline (Te), Ceftazidime (Caz), Kanamycin (K).

should also be explained to the entire freshwater farm aquaculturist by the authority in charge. At the retail level, the cleanliness of the handlers and the good hygiene practices while handling the freshwater fish should also be considered in order to keep the fish from cross-contaminated with other pathogenic bacteria sources.

The results of this study had provide a useful information in the search for safe and efficient antibiotics. In addition it also gives us some insight into the problems and to create awareness to the consumers towards the antibiotic resistance level in freshwater aquaculture fish in Malaysia and indirectly to warn all the aquaculturist of the extensive used of antibiotic in their freshwater aquaculture farms and the effects to the future generations.

In conclusion, this study showed an extremely high level of multiresistant isolates of *V. cholera* and *V. parahaemolyticus* to as many as 15 antibiotics tested with the overall MAR index value of 0.13 to 0.93 respectively from both *Vibrio* spp. under study. In term of biosafety, the high resistant level of Furazididone, Bacitracin and Tetracycline were of concern as being the drug choice to treat *Vibrio* infection in future. It can also be the potential growing threat in our region. However, we discover in this study that *Vibrio* infection still can be treats as the antimicrobial under the group of β-lactams was found to be the best antibiotics against *V. cholera* and *V. parahaemolyticus* infections as shown with a high percentage of susceptibility toward Imipenem.

Other than that, the cluster analysis used based on the antibiotic profiles in this study, had provide us with a good analysis and information in a more simple and effective way to observed on the spreading of antibiotic resistance pathogens in particular geographic area, sources specific and patterns of resistance among the isolates under study. However, on-going controlled studies are needed to determine the current effects of antimicrobial therapy on the ecology of aquaculture ponds, particularly at the microorganisms level.

Acknowledgements

This study was supported by a Science fund (project No. 02-01-04-SF0390) from the Ministry of Science, Technology and Innovation, Malaysia and in part by a Grant-in-Aid for Scientific Research (KAKENHI 191010) from Japan Society for the Promotion of Sciences.

References

Anon. Food safety and the Antibiotic Resistance Crisis. Available at *www. Save antibiotics.org/resources/ FoodsafetyAMR.pdf*. Accessed on 7 June 2010.

Anon. Performance and Achievements of the Fisheries Sector. Available at *http://www.dof.my/18*. Accessed on 31 Jan 2011.

Bauer, A. W., Kirby, W. M. M., Sheris, J. C. and Turck, M. 1966. Antibiotics susceptibility testing by standardized single disk method. American Journal of Clinical Pathology 45: 493-496.

Chai, L. C., Fatimah, A. B., Ghazali, F. M., Lee, H. Y., Tunung, R., Shamsinar, A.T., Laila, R.A.S., Thahirahtul, A. Z., Malakae, P. M., Nakaguchi, Y., Nishibuchi, M. And Son, R. 2008. Biosafety of *Campylobacter jejuni* from Raw Vegetables Consumed as Ulam with Reference to their resistance to Antibiotics. International Food Research Journal 15(2): 125-134.

Chinabut, S., Somsiri, T., Bartie, K. L., Huys, G., Oanh, D.T.H., Giacomini, M., Bertone, S., Shariff, M., Yusoff, F. M., Swings, J. and Teale, A. The use of antimicrobials in asian aquaculture : Aims of the EU-asiaresist project. Available at *http://www.medinfo.dist. unige.it/Asiaresist/Publications/WAS%20Abstract. pdf*. Accessed on 21 May 2011.

Ibrahim, A. B., Mohd khan, A., Ayob, M. Y. and Norrakiah, A.S. 2010. Pesticide and antibiotic residues in freshwater aquaculture fish : Chemical risk assessment from farm to table. Asian Journal and Agro-Industry 3 (3) : 328-334.

Imziln, B. and Hassani, L. 1994. Antimicrobial susceptibility on non-01 *Vibrio cholera* isolated from wastewater stabilization ponds in Marrakesh, Morocco. World

Journal of Microbiology and Biotechnology 10 : 230-231.

Krumperman, P. H. 1983. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. Applied and Environmental Microbiology 46: 165-170.

Kumar, P. A., Patterson, J. and Karpagam, P. 2009. Multiple Antibiotic Resistance Profiles of *Vibrio cholera* non-01 and non-0139. Japan Journal of Infectious Diseases 62 : 230-232.

Kummerer, K. 2009. Antibiotics in the Aquatic environment – A review – Part 1. Chemosphere 75(4): 417-434.

Li, J., Yie, J., Foo, R.W.T., Ling, J.M.L., Xu, H. And Woo, N.Y.S. 1999. Antibiotic Resistance and Plasmid Profiles of *Vibrio* Isolates from Cultured Silver Sea Beam, *Sparus sarba*. Marine Pollution Bulletin 39 (1-12): 245-249.

Lim, V. K. E., Cheong, Y. M and Suleiman, A. B. 1993. Pattern of antibiotic usage in hospitals in Malaysia. Singapore Medical Journal 34: 525-528.

Majusha, S., sarita, G. B., Elyas, K.K. and Chandrasekaran, M. 2005. Multiple Antibiotic Resistances of *Vibrio* Isolates from Coastal and Brackish Water Areas. American Journal of Biochemistry and Biotechnology 1 (4): 201-206.

Matyar, F., Kaya, A. And Dincer, S. 2008. Antibacterial agents and heavy metal resistance in Gram-negative bacteria isolated from seawater, shrimp and sediment in Iskenderun Bay, Turkey. Science of the Total Environment 407: 279-285.

Micky, V. 2001. Reported Cholera in Miri, Sarawak: A Case Study by Molecular Technique for Use in Public Health Surveillance. Master of Science dissertation. Universiti Putra Malaysia.

National Committee for Clinical Laboratory Standards. 2004. Performance Standards for Antimicrobacterial Susceptibility Testing, Fourteenth Informational Supplement Document Number: NCCLS/CLSI M100-S14 Clinical and Laboratory Standards Institute, Villanova, P.A., USA.

Noorlis, A., Ghazali, F. M., Cheah, Y. K., Tuan Zainazor, T. C., Ponniah, J., Tunung, R., Tang, J. Y. H., Nishibuchi, M., Nakaguchi, Y. and Son, R. 2011. Prevalence and Quantification of *Vibrio* species and *Vibrio parahaemolyticus* in Freshwater Fish at Hypermarket Level. International Food Research Journal 18: 673-679.

Ottaviani, D., Bacchiocchi, I., Masini, L., Leoni, F., Carraturo, A., Giammoarioli, M. And Sbaraglia, G. 2001. Antimicrobial susceptibility of potential pathogenic halophilic vibrios isolated from seafood. International Journal of Antimicrobial Agents 18: 135-140.

Papadopoulou, C., Economou, V., Sakkas, H., Gousia, P., Giannakopoulos, X., Dontorou, C., Filioussis, G., Gessouli, H., Karanis, P. And Leveidiotou, S. 2008. Microbiological quality of indoor and outdoor swimming pools in Greece: Investigation of the antibiotic resistance of the bacterial isolates. International Journal of Hygiene and Environmental Health 211: 385-397.

Rafi, S., Qureshi, A. H., Saeed, W., Ali, A., Ahmadani, M. And Khawaja, S. A. 2004. Changing Epidemiology and Sensitivity Pattern of *Vibrio cholera* at Rawalpindi. Pakistan Journal of Medical Sciences 20(4) : 357-360.

Roque, A., Molina-Aja, A., Bolan-Mejia, C., Gomez-Gil, B. 2001. *In vitro* susceptibility to 15 antibiotics of *vibrios* isolated from panaeid shrimps in Northwestern Mexico. International Journal Of Antimicrobial Agents 17: 383-387.

Sarter, S., Nguyen, H.N.K., Hung, L. H., Lazard, J and Montet, D. 2007. Antibiotic resistance in Gram-negative bacteria isolated from farmed catfish. Food Control 18: 1391-1396.

Spanggaard, B., Jorgensen, F., Gram, L. And Huss, H.H. 1993. Antibiotic esistance in bacteria isolated from three freshwater fish farms and an unpolluted stream in Denmark. Aquaculture 115: 195-207.

Tuan Zainazor, T. C. 2006. Prevalence and Molecular Characterization of *Vibrio parahaemolyticus* Isolated from Cultured Tiger Prawns (*Penaeus Monodon*) from Malacca. Master of Science dissertation. University Putra Malaysia.

Vaseeharan, B., Ramasamy, P., Murigan, T. and Chen, J. C. 2005. *In vitro* susceptibility of antibiotics against *Vibrio* spp. and *Aeromonas* spp. isolated from *Penaeus monodon* hatcheries and ponds. International Journal of Antimicrobial Agents 26: 285-291.

Zulkifli, Y., Alitheen, N. B., Raha, A. R., Yeap, S. K., Marlina, Son, R. And Nishibuchi, M. 2009. Antibiotic resistance and plasmid profiling of *Vibrio parahaemolticus* isolated from cockles in Padang, Indonesia. International Food Research Journal 16: 53-58.