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Distribution and prevalence of antibiotic resistant bacteria in fish farms in East Malaysia

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ABSTRACT

Aims: Aquaculture has grown tremendously in Malaysia over the past decades. However, guaranteeing aquaculture sustainability is a big challenge in terms of maintaining continuous output with a safe environment. Furthermore, the cultured species should be free from antibiotic resistance bacterial and antibiotic residue. This study aimed to monitor the existence and prevalence of antibiotic resistant bacteria associated with aquaculture farms in Sarawak.
Methodology and results: Samples of water, sediment and fish were collected from five aquaculture farms within Sarawak. The samples were plated on trypticase soy agar and incubated at 28 °C for 24 h. A total of 204 bacterial isolates were isolated and analysed by $(GTG)_5$-fingerprinting to determine genetic similarity among the bacterial isolates, so that representatives could be selected from similar clonal isolates. Based on the $(GTG)_5$ profiles, 50 representative isolates were chosen for species identification using 16S rRNA sequencing. The identified bacteria were tested against 25 antibiotics using standard disk diffusion method. The 16S rRNA analysis revealed that the isolates constitute of 14 genera of bacteria including *Bacillus* (38%), *Exiguobacterium* (16%), *Enterobacter* (14%), *Aeromonas* (6%), *Acinetobacter* (4%), *Citrobacter* (4%), *Staphylococcus* (4%), *Achromobacter* (2%), *Chitinophaga* (2%), *Fictibacillus* (2%), *Plesiomonas* (2%), *Pseudomonas* (2%), *Pseudoxanthomonas* (2%) and *Stenotrophomonas* (2%). The antibiotic resistance analysis revealed that the highest percentage of resistance was recorded against streptomycin (75.0%), followed by ampicillin (66.0%), ceftriaxone (50.0%), rifampin (43.3%), aztreonam (36.8%) and ceftazidime (31.6%). Resistance to more than two antibiotics was observed in 40.0% of isolates with an overall multiple antibiotic resistant (MAR) index ranging from 0 to 0.79.
Conclusion, significant and impact of study: The variability of antibiotic resistance patterns exhibited by different bacterial species suggests a dependence on selective pressures exhibited in different geographical locations. Our results show that the occurrence of MAR bacteria in an aquaculture environment with unknown history of antibiotics usage in the aquaculture system is possible, indicating a need to continuously monitor the presence of antibiotic resistant bacteria in the aquaculture system.

Keywords: Aquaculture, bacteria, 16S rRNA, antibiotic resistant

INTRODUCTION

Since the first antibiotic discovery by Alexander Fleming in 1928, many different classes of antibiotics have been developed and concurrently bacteria have slowly developed resistance towards those antimicrobial agents (Paulson *et al.*, 2016). Now, the phenomenon of antibiotic resistance is a global problem in every sector including aquaculture and agriculture (Radhouani *et al.*, 2014; Done *et al.*, 2015). In Malaysia, multiple resistance bacteria and resistance genes have been reported to be present in the aquaculture and its surrounding environments (Samuel *et al.*, 2011; Abdullahi *et al.*, 2013; Kui Soon *et al.*, 2014; Ng *et al.*, 2014; Seng Chiew *et al.*, 2018).

There have been continuous efforts in searching for

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potential sources of new antimicrobial agents by testing compounds from local natural resources (Samuel *et al.*, 2014a; Samuel *et al.*, 2014b; Farith *et al.*, 2015). However, very few of the antimicrobial agents have been successfully developed because of difficulties in identifying and isolating the exact compounds with effective activity against bacteria (Singh, 2014; Swamy and Rudramurthy, 2016).

The reliance on antibiotics to combat bacterial diseases in the rapidly growing aquaculture industry is unavoidable (Bostock *et al.*, 2010; Cabello *et al.*, 2013). The use of antibiotics in the aquaculture industry is mainly for the purpose of treatment, control and prevention of diseases as well as for promoting the growth of cultured species (Phillips *et al.*, 2004; Bush *et al.*, 2011; Romero *et al.*, 2012).

However, it is highly controversial that either the emergence of resistance bacteria is attributed to anthropogenic activities or as a result of intrinsic resistance and gene transfer among environmental bacteria (Bhullar *et al.*, 2012). There has been growing evidence that antibiotic resistant bacteria (ARB) carries antibiotic resistance genes (ARGs) by mobile genetic elements such as, integrons and plasmids that have shown to be shared between aquatic bacteria and terrestrial animals and human pathogen (Cabello *et al.*, 2013; Cantas *et al.*, 2013). These genes movement allows a bacterium to build on existing adaptations to better adapt to the changing environment (Perry and Wright, 2013).

Malaysian brackish water aquaculture displayed a considerable growth in production by 6.7% (324.3 thousand tonnes) in 2017 and a slight decline in freshwater aquaculture by 0.8% (102.5 thousand tonnes) against the preceding year (Department of Statistic Malaysia, 2018). The existing aquaculture industry is, however, associated with an increasing number of large farms, high density of fish and poor sanitary conditions which could only lead to greater levels of resistance in the human commensal microbiota (Schmidt *et al.*, 2000; Barton and Fløysand, 2010; Deng *et al.*, 2016). This has become a global problem because the misuse of antibiotics in aquaculture has been identified to drives the emergence of antibiotic resistant bacteria (ARB) and led to an unwelcoming implication to the public health (Schmidt *et al.*, 2000; Heuer *et al.*, 2009; Deng *et al.*, 2016; Patil *et al.*, 2016; Paulson *et al.*, 2016).

Thus, there is a need for surveillance on the use of antibiotics in aquaculture with a comprehensive regulatory framework for the registration of antibiotics drug, as current standards vary widely from one country to another (Cabello *et al.*, 2013; Watts *et al.*, 2017). An active enforcement by the health sector is of vast importance to ensure its safety and effectiveness. It is important that people working in the aquaculture industry learn how to use antibiotics in such a way that maximize their efficacy while minimizing the increased frequencies of resistant variants as the consequence of the antibiotics usage (Smith, 2008).

A clear view on the development and spread of resistance in aquaculture to protect the humans, animals, and ecosystem can be achieved by a better understanding of antibiotic resistance (AR) ecology through characterisation of antibiotic resistant bacteria and the ecology of antibiotic resistance prevalence in an aquaculture environment. Therefore, the objectives of this study are to isolate bacteria from the aquaculture and its environment and to determine the extent to which they are resistant to the commonly used antibiotics.

MATERIALS AND METHODS

Sampling sites

Five aquaculture farms located within the southern part of Sarawak, East Malaysia, were selected for the sampling in this study. The location of the farms on the map is shown in Figure 1. The global positioning system (GPS) coordinates of the farms were; Farm 1 (1°26'57.80" N 110°24'21.30"E), Farm 2 (1°32'53.43" N 110°32'53.99"E), Farm 3 (1°24'02.40" N 110°19'50.60"E), Farm 4 (1°26'59.5"N 110°10'12.0"E), and Farm 5 (1°26'09.6"N 110°10'10.0"E).

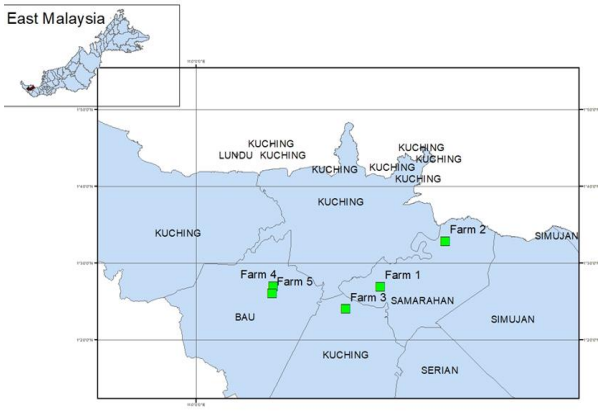

Figure 1: Sampling locations of five different farms in northern part of East Malaysia.

Sample processing and bacterial isolation

Samples of sediment, water and fish collected from the farms were processed according to the method described by Huys (2003). After the sample processing, the samples were plated on duplicate tryptone soy agar (Oxoid, USA) and incubated at 28 °C for 18 to 24 h. Bacterial colonies grown on the agar were randomly picked, purified and stocked in glycerol. Two hundred and four bacteria were isolated and kept in glycerol stock in – 20 °C freezer.

Bacterial characterization and identification

Pure bacterial colonies were analysed for their genetic differences using $(GTG)_5$-PCR. Based on the $(GTG)_5$-PCR profile as shown in Figure 2, a representative of fifty isolates was selected and identified using 16S rRNA gene

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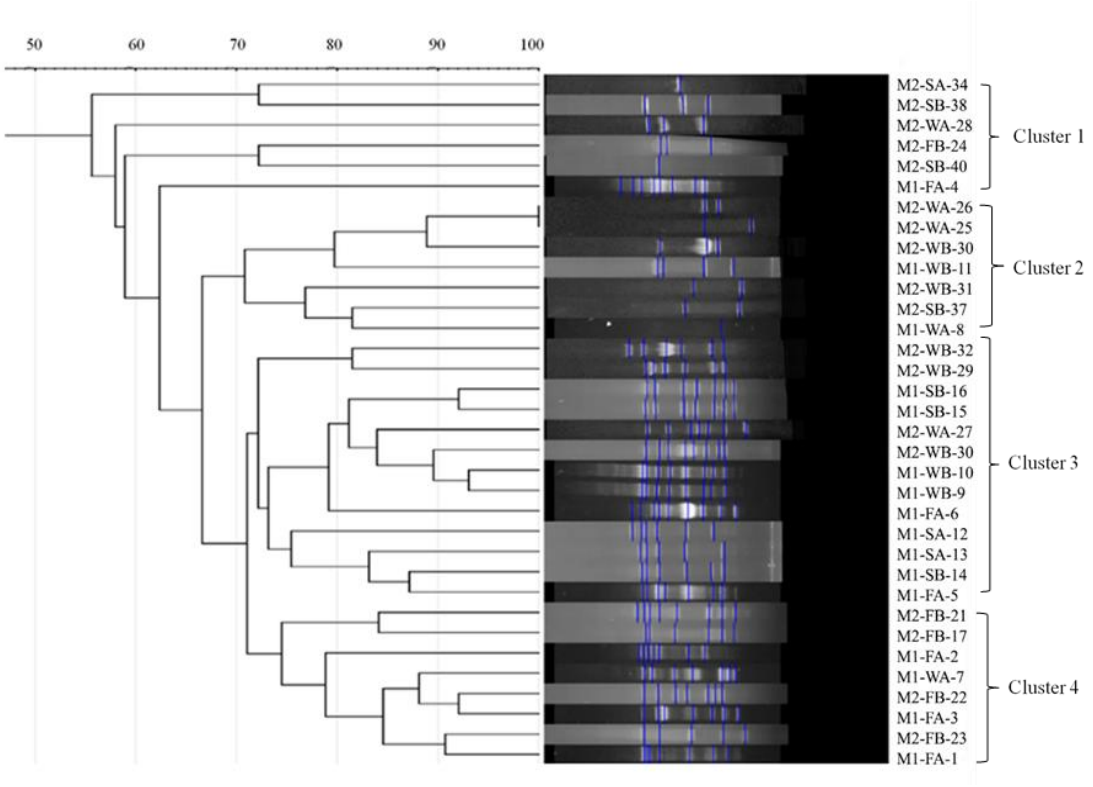

Figure 2: Representative profile and dendrogram of $(GTG)_5$-PCR of the bacterial isolated from Farm 1. Dendrogram based on Dice similarity method and UPGMA linkage of $(GTG)_5$-PCR fingerprints obtained from bacterial isolates. Cluster 1: M2-SA-34, M2-SB-38, M2-WA-28, M2-FB-24, M2-SB-40, M1-FA-4; Cluster 2: M2-WA-26, M2-WA-25, M2-WB-30, M1-WB-11, M2-WB-31, M2-SB-37, M1-WA-8; Cluster 3: M2-WB-32, M2-WB-29, M1-SB-16, M1-SB-15, M2-WA-27, M2-WB-30, M1-WB-10, M1-WB-9, M1-FA-6, M1-SA-12, M1-SA-13, M1-SB-14, M1-FA-5; Cluster 4: M2-FB-21, M2-FB-17, M1-FA-2, M1-WA-7, M2-FB-22, M1-FA-3, M2-FB-23, M1-FA-1.

sequencing with 27F (5'-CAGGCCTAACACATGCAAGTC- 3') and 519R (5'-GWATTACCGCGGCKGCTG - 3') primers in accordance with Iñiguez-Palomares *et al.* (2007). Sequencing of the PCR product was carried out by First Base Laboratories (Selangor, Malaysia). The 16S rRNA sequence data were compared with available sequence data in the GenBank using BLAST.

Antibiotic susceptibility test

Fifty environmental isolates from 14 different genera were tested for antibiotic susceptibility using the disk diffusion method according to the recommendations of the CLSI (2017). All isolates were grown in Mueller-Hinton Broth (HiMedia, India) and then swabbed evenly onto the surface of Mueller-Hinton agar (HiMedia, India) plates. The plates were dried for 2-5 min before an antibiotic disk was placed on the agar surface using sterile forceps. The plates were then incubated at 28 °C for 18-20 h. Twenty-five antibiotics selected for the test were amikacin (AK, 30 µg), gentamicin (CN, 10 µg), kanamycin (KA, 30 µg), streptomycin (S, 10 µg), doxycycline (DO, 30 µg), tetracycline (TE, 30 µg), penicillin (P, 10 µg), piperacillin (PRL, 100 µg), ampicillin (AMP, 10 µg), chloramphenicol (C, 30 µg), levofloxacin (LEV, 5 µg), norfloxacin (NOR, 10 µg), ciprofloxacin (CIP, 5 µg), nalidixic acid (NA, 30 µg), rifampin (RD, 5 µg), erythromycin (E, 15 µg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg), cefepime (FEP, 30 µg), ceftazidime (CAZ, 30 µg), meropenem (MEM, 10 µg), ertapenem (ETP, 10 µg), imipenem (IMP, 10 µg), aztreonam (ATM, 30 µg), ceftriaxone (CRO, 30 µg) and cephalothin (KF, 30 µg). Cultures of *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were included as controls in the susceptibility testing. The assessment of the bacterial susceptibility to the twenty-five different antibiotics is shown in Table 1.

MAR index assessment

The multiple antibiotic resistance (MAR) index analysis was employed in accordance to Krumperman (1983) formula, where the number of antibiotics to which the bacterium was resistant to was divided by the number of antibiotics to which the isolates were tested upon.

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Table 1: Antibiogram of aquatic bacteria from the fish farms and their MAR index.

Bacterial codes	Bacterial species	Antibiotics																									MAR index
		AK	TE	DO	CN	P	C	LEV	RD	NOR	E	CIP	PRL	SXT	FEP	MEM	CAZ	KA	NA	S	AMP	ATM	ETP	IMP	KF	CRO	
PM 249	*Bacillus* sp.	S	S	S	S	R	S	S	R	S	R	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.27
LT 26	*Bacillus cereus*	S	S	S	S	S	S	S	I	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.09
LT 17	*Bacillus pumilus*	S	S	S	S	S	S	S	I	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.09
J 200	*Bacillus cereus*	S	R	S	I	R	S	S	R	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.36
J 187	*Bacillus* sp.	S	S	S	S	S	S	S	I	S	I	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.18
LT 18	*Bacillus pumilus*	S	S	S	S	S	S	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0
PM 207	*Bacillus* sp.	S	S	S	S	R	S	S	I	S	I	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.27
LT 21	*Bacillus aquimaris*	S	S	S	S	S	S	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0
LT 40	*Exiguobacterum profundum*	S	S	S	S	S	S	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0
PM 185	*Bacillus indicus*	S	S	S	S	S	S	S	I	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.09
SM 103	*Staphylococcus* sp.	S	I	S	S	S	S	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.09
SM 94	*Bacillus zhangzhouensis*	S	S	S	S	S	S	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0
LT 63	*Exiguobacterium profundum*	S	S	S	S	S	S	S	S	S	R	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.09
LT 64	*Exiguobacterium aurantiacum*	S	S	S	S	S	S	S	S	R	R	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.18
SM 91	*Bacillus cereus*	S	S	S	S	R	S	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.09

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SM 130	*Bacillus pumilus*	S	S	S	S	S	S	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0
SM 139	*Staphylococcus haemolyticus*	S	S	S	S	S	S	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0
J 162	*Bacillus* sp.	S	S	S	S	S	S	S	I	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.09
J 149	*Bacillus megaterium*	S	S	S	S	R	S	S	R	S	R	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.27
M 4	*Bacillus* sp.	S	S	S	S	S	S	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0
M 9	*Bacillus* sp.	S	S	S	S	S	S	S	I	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.09
M 1	*Bacillus altitudinis*	S	S	S	S	S	S	S	I	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.09
SM 142	*Bacillus pumilus*	S	S	S	S	S	S	S	I	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.09
LT 54	*Exiguobacterium profundum*	S	S	S	S	S	S	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0
M 21	*Fictibacillus macauensis*	R	S	S	S	S	I	S	R	R	I	I	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.55
LT 52	*Exiguobacterium* sp.	S	S	S	S	R	S	S	S	S	R	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0.18
LT 31	*Bacillus* sp.	S	S	S	S	S	S	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0
LT 47	*Exiguobacterium* sp.	S	S	S	S	S	S	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0
M 11	*Acinetobacter junii*	S	S	S	S	NT	NT	NT	S	NT	NT	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0
LT 43	*Acinetobacter* sp.	S	S	S	S	NT	NT	NT	S	NT	NT	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0
M 14	*Enterobacter asburiae*	S	NT	NT	S	NT	S	S	NT	S	NT	NT	S	NT	NT	S	S	S	S	I	S	S	S	NT	NT	NT	0.07
M 16	*Enterobacter asburiae*	S	NT	NT	S	NT	S	S	NT	S	NT	NT	S	NT	NT	S	S	S	S	I	S	S	S	NT	NT	NT	0.07
J 171	*Citrobacter* sp.	S	NT	NT	S	NT	S	S	NT	S	NT	NT	S	NT	NT	S	S	S	S	R	S	S	S	NT	NT	NT	0.07
J 184	*Enterobacter cloacae*	S	NT	NT	S	NT	S	S	NT	S	NT	NT	S	NT	NT	S	S	S	S	I	R	S	S	NT	NT	NT	0.14

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PM 205	*Enterobacter* sp.	S	NT	NT	S	NT	S	S	NT	S	NT	NT	S	NT	NT	I	S	S	S	I	R	S	I	NT	NT	NT	0.29
LT 23	*Enterobacter* sp.	S	NT	NT	S	NT	S	S	NT	S	NT	NT	S	NT	NT	S	I	S	S	S	R	S	S	NT	NT	NT	0.14
J2 176	*Citrobacter* sp.	S	NT	NT	S	NT	S	S	NT	S	NT	NT	S	NT	NT	S	S	S	S	S	R	S	S	NT	NT	NT	0.07
SM 101	*Chitinophaga* sp.	R	NT	NT	R	NT	R	S	NT	I	NT	NT	R	NT	NT	S	R	R	S	R	R	R	R	NT	NT	NT	0.79
J 151	*Enterobacter amnigenus*	S	NT	NT	S	NT	S	S	NT	S	NT	NT	S	NT	NT	S	S	S	S	I	R	S	S	NT	NT	NT	0.14
PM 246	*Enterobacter* sp.	S	NT	NT	S	NT	S	S	NT	S	NT	NT	I	NT	NT	S	R	S	S	S	S	R	S	NT	NT	NT	0.21
PM 216	*Enterobacter* sp.	S	NT	NT	S	NT	S	S	NT	S	NT	NT	S	NT	NT	S	S	I	S	I	R	S	I	NT	NT	NT	0.29
LT 78	*Enterobacter* sp.	S	NT	NT	S	NT	R	S	NT	S	NT	NT	S	NT	NT	S	S	S	S	R	R	S	S	NT	NT	NT	0.21
SM 79	*Pseudoxanthomonas mexicana*	NT	NT	NT	R	NT	NT	S	NT	NT	NT	S	I	NT	R	R	R	NT	NT	NT	NT	R	NT	R	NT	NT	0.78
J 152	*Achromobacter* sp.	NT	NT	NT	S	NT	NT	S	NT	NT	NT	S	S	NT	S	S	S	NT	NT	NT	NT	R	NT	S	NT	NT	0.11
PM 199	*Pseudomonas sp.*	NT	NT	NT	S	NT	NT	S	NT	NT	NT	S	S	NT	S	S	S	NT	NT	NT	NT	S	NT	S	NT	NT	0
LT 16	*Stenotrophomonas* sp.	NT	NT	NT	NT	NT	NT	S	NT	NT	NT	NT	NT	S	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	0
M 40	*Aeromonas veronii*	S	S	NT	S	NT	S	NT	NT	NT	NT	S	NT	NT	NT	S	R	NT	NT	NT	NT	R	NT	S	S	I	0.27
PM 257	*Plesiomonas shigelloides*	I	I	NT	I	NT	S	NT	NT	NT	NT	S	NT	NT	NT	S	S	NT	NT	NT	NT	S	NT	S	R	S	0.36
PM 183	*Aeromonas veronii*	S	S	NT	S	NT	S	NT	NT	NT	NT	S	NT	NT	NT	S	S	NT	NT	NT	NT	R	NT	S	S	S	0.09
J 181	*Aeromonas jandaei*	R	S	NT	S	NT	S	NT	NT	NT	NT	S	NT	NT	NT	S	R	NT	NT	NT	NT	R	NT	S	S	R	0.36

Note: S, Susceptible; I, Intermediate; R, Resistant; NT, Not tested. Antibiotics: AK, amikacin; CN, gentamicin; KA, kanamycin; S, streptomycin; DO, doxycycline; TE, tetracycline; P, penicillin; PRL, piperacillin; AMP, ampicillin; C, chloramphenicol; LEV, levofloxacin; NOR, norfloxacin; CIP, ciprofloxacin; NA, nalidixic acid; RD, rifampin; E, erythromycin; SXT, trimethoprim-sulfamethoxazole; FEP, cefepime; CAZ, ceftazidime; MEM, meropenem; ETP, ertapenem; IMP, imipenem; ATM, aztreonam; CRO, ceftriaxone; KF, cephalothin.

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RESULTS

Antibiotics selection was dependent on the bacterial genera, as different bacterial groups may be intrinsically resistant to certain antibiotics which explained the unnecessary need to be tested against certain antibiotics classes. The list of bacterial isolates consisting 14 different genera of bacteria tested in this study is shown in Table 1.

The highest percentage of resistance recorded was against streptomycin (75.0%), followed by ampicillin (66.7%), ceftriaxone (50.0%), rifampin (43.3%), aztreonam (36.8%) and ceftazidime (31.6%) as shown in Table 2. In addition, the bacterial isolates showed susceptibility towards four antibiotics; doxycycline, levofloxacin, trimethoprim-sulfamethoxazole and nalidixic acid. A representative profile together with the dendrogram constructed out of $(GTG)_5$-PCR of the bacteria isolated from Farm 1 is shown in Figure 2. The dendrogram was constructed based on the Dice similarity method and UPGMA linkage of $(GTG)_5$-PCR fingerprints obtained from bacterial isolates.

Twenty-four different resistance patterns were found in this study as shown in Table 3. Resistance to only one antibiotic was seen in 32.0% (16/50) of isolates. Out of all the isolates, 40.0% (20/50) were found to be multiple resistances. There were four resistance patterns shared by at least two or more bacteria. The variability of antibiotic resistance patterns exhibited by different bacterial species suggests selective pressures exhibit in the aqueous habitats which is in agreement with studies conducted by Lesley *et al.* (2011) and Kathleen *et al.* (2016).

Fisher's exact test was applied on *Bacillus* sp. which accounts for the majority of the isolates (38%, n = 19/50) to determine if there was any significant difference in the proportion of *Bacillus* between the sample sources (sediment, water, and fish) and the risk level from antibiotic contaminated areas (high ≥ 0.2 or low ≤ 0.2). Group sizes of *Bacillus* from the three-sample sources were unequal, where 10 *Bacillus* sp. were identified from the sediment, 5 isolates from water and 4 isolates from fish. Based on this study, there was no significant difference in proportions of *Bacillus* sp. from low or high-risk level from antibiotic contaminated areas in these three sources (p= 0.373). This finding is in agreement with Schmidt *et al*. (2000), where the resistance level between water and fish isolates showed no significant difference.

Table 2: Percentage of bacterial resistance to different antibiotics in the fish farms.

Antibiotics	Abbreviation of antibiotics	Total of resistant isolates (total isolates tested)	Percentage of resistance (%)
Streptomycin	S	9 (12)	75.0
Ampicillin	AMP	8 (12)	66.7
Ceftriaxone	CRO	2 (4)	50.0
Rifampin	RD	13 (30)	43.3
Aztreonam	ATM	7 (19)	36.8
Ceftazidime	CAZ	6 (19)	31.6
Erythromycin	E	8 (28)	28.6
Ertapenem	ETP	3 (12)	25.0
Cephalothin	KF	1 (4)	25.0
Penicillin	P	6 (28)	21.4
Cefepime	FEP	1 (5)	20.0
Piperacillin	PRL	3 (17)	17.6
Kanamycin	KA	2 (12)	16.7
Imipenem	IMP	1 (7)	14.3
Meropenem	MEM	2 (21)	9.5
Tetracycline	TE	3 (34)	8.8
Chloramphenicol	C	3 (38)	8.8
Amikacin	AK	4 (46)	8.7
Gentamycin	CN	4 (49)	8.2
Norfloxacin	NOR	3 (40)	7.5
Ciprofloxacin	CIP	1 (37)	2.7
Doxycycline	DO	0 (30)	0.0
Levofloxacin	LEV	0 (42)	0.0
Trimethoprim-sulfamethoxazole	SXT	0 (3)	0.0
Nalidixic Acid	NA	0 (12)	0.0

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Table 3: Resistant patterns and MAR index of aquaculture bacteria.

MAR index	Resistant pattern	Isolates code	Percentage of isolate (%)
0.79	NOR-AK-CN-C-PRL-CAZ-KA-S-AMP-ATM-ETP	SM 101	2.0
0.78	PRL-CN-FEP-MEM-CAZ-ATM-IMP	SM 79	2.0
0.55	C-E-CIP-AK-RD-NOR	M 21	2.0
0.36	CN-TE-P-RD AK-TE-CN- KF AK-CAZ-ATM-KF	J 200 PM 257 J 181	6.0
0.29	MEM-S-ETP-AMP KA-S-ETP-AMP	PM 205 PM 216	4.0
0.27	P-RD-E CRO- CAZ-ATM	PM 249, PM 207, J 149 M40	8.0
0.21	PRL-CAZ-ATM C-PRL-AMP	PM 246 LT 78	4.0
0.18	RD-E NOR-E P-E	J 187 LT 64 LT 52	6.0
0.14	S-AMP CAZ-AMP	J 184, J 151 LT 23	6.0
0.11	ATM	J 152	2.0
0.09	RD TE E P ATM	LT 26, LT17, PM 185, J 162, M 9, M 1, SM 142 SM 103 LT 63 SM 91 PM 183	22.0
0.07	S AMP	M 14, M 16, J 171 J 176	8.0
0	-	LT 18, LT 21, LT 40, SM 94, SM 130, SM 139, M 4, LT 54, LT 31, LT 47, M 11, LT 43, PM 199, LT 16	28.0

Note: AK, amikacin; CN, gentamicin; KA, kanamycin; S, streptomycin; DO, doxycycline; TE, tetracycline; P, penicillin; PRL, piperacillin; AMP, ampicillin; C, chloramphenicol; LEV, levofloxacin; NOR, norfloxacin; CIP, ciprofloxacin; NA, nalidixic acid; RD, rifampin; E, erythromycin; SXT, trimethoprim-sulfamethoxazole; FEP, cefepime; CAZ, ceftazidime; MEM, meropenem; ETP, ertapenem; IMP, imipenem; ATM, aztreonam; CRO, ceftriaxone; KF, cephalothin.

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DISCUSSION

The direct use of antibiotics in human health and the use of the antimicrobial agents in animal husbandry for growth promoters and disease treatment have been responsible for the significant rise in multiple antibiotic resistance with the potential for transfer of the organisms or of genetic material coding for resistance to humans. The antimicrobial resistance of bacteria in animals including fish reared in the aquaculture sector should be monitored continuously.

In this study, it was found that 16 out of 50 (32%) representative environmental bacteria were resistant to only one of the antibiotics tested. Not surprisingly, antibiotic resistance in aquatic bacteria was found in the water sample as it is accepted as a mixing ground for gene exchange between environmental bacteria (Perry and Wright, 2013). It is, however, difficult to conclude that antibiotic resistance in the fish farms is attributed to anthropogenic activities only, as Chamosa *et al*. (2017) suggested that there could be a bidirectional flow of similar antimicrobial resistant bacteria and genes encountered in both the environment and human microbiota.

A high percentage of isolates (74.0%, *n* = 37/50) display MAR indices less than 0.2. According to Krumperman (1983), isolates originate from a lower antibiotic contaminated source showed MAR indices of 0.2 and below. This suggests that most isolates originate from a lower antibiotic contaminated source (Krumperman, 1983; Tanil *et al*., 2005), which in turn suggests a low or no history of usage of antibiotics in the aquaculture farms studied (Lesley *et al.*, 2018). Similar results were obtained from a study conducted by Kathleen *et al*. (2016), whereby most isolates (63.1%, *n* = 94) were found to be from lower antibiotic contaminated sources. The overall results indicated that MAR indices ranged from 0 to 0.79, with the highest resistance seen in *Chitinophaga* sp. (Table 1). This bacterial species showed resistance to 11 out of the 14 antibiotics tested.

Table 2 shows the percentage of bacterial resistance to different antibiotics in the fish farms. A high percentage of resistance to streptomycin (75%; 9/12) and ampicillin (66.7%; 8/12) was observed among the aquacultural bacterial isolates. This observation is consistent with a study by Chelossi *et al*. (2003), which also recorded a high resistance towards streptomycin and ampicillin in the benthic bacterial community of a marine aquaculture. The prevalence of streptomycin resistance has also been reported in numerous fish and shrimp farms (Dung *et al*., 2008; Kian Giap *et al*., 2012; Shah *et al.*, 2014). High incidence of ampicillin resistance has been reported in *Vibrio* sp. in tropical water (You *et al*., 2016), and in *Enterococci* in the recreational water in Malaysia (Dada *et al*., 2013). In another study conducted by Letchumanan *et al*. (2015), 82% of *Vibrio parahaemolyticus* in retail shrimps in Malaysia displayed a high resistance to ampicillin. This might suggest that high incidences of streptomycin and ampicillin resistance are not restricted to a particular water body, but they are indeed widely distributed in aquatic environments.

A high susceptibility was recorded towards doxycycline, levofloxacin, trimethoprim-sulfamethoxazole and nalidixic acid. From this finding, the high susceptibility of the bacterial isolates towards doxycycline might suggest that doxycycline was not commonly used among fish farmers (Pham *et al*., 2015). Similarly, high susceptibility of levofloxacin was observed in *Vibrio parahaemolyticus* isolated from retail shrimps by Letchumanan *et al*. (2015) and Saifedden *et al.* (2016). High susceptibility towards trimethoprim-sulfamethoxazole was also seen in *Aeromonas hydrophila* in catfish aquaculture (Paola *et al*., 1995) and bacteria isolated from sea bass (Bourouni *et al.*, 2000). *Esherichia coli* isolated from tilapia species in Brazil (Rocha *et al.*, 2014) and *Vibrio* species isolated from aquaculture water in Sabah (Ransangan *et al.*, 2013) also revealed a high sensitivity towards nalidixic acid. However, in a short period of time, these originally susceptible bacteria may become resistant through acquiring resistance genes via horizontal gene transfer or gene mutations (Allen *et al*., 2010).

Based on the results of this study, the bacteria were resistant towards a variety of antibiotics, which might be an offshoot of diverse genes that protects them against the therapeutic dose of antibiotics. These genes which are also known as the resistome have the potential to be transferred to pathogens and there has been evidence showing that some clinically relevant resistance genes originated from environmental bacteria (Cattoir *et al.*, 2008). Therefore, bacteria with antibiotic resistance genes get selective advantage over antibiotic-sensitive bacteria in presence of antibiotics, and evidently creates a plethora of resistance patterns exhibited by the aquatic bacteria. The fact that antibiotic resistant bacteria were seen in all five fish farms where no antibiotics were used Further confirmed that resistance genes exist naturally in the environment (Allen *et al.*, 2010).

CONCLUSION

Based on this study, a high percentage (74.0%; n= 37/50) of bacteria found in aquaculture in Sarawak possess MAR indices less than 0.2; suggesting that most isolates have been originated from low antibiotic contaminated areas. However, 40.0% (n= 20/50) of the isolates displayed multiple antibiotic resistance which should be of concern, as no known antibiotics were used in these farms. The frequency of bacterial resistance to different classes of antibiotics suggested that those bacteria could be reservoirs for antibiotic resistance genes. There is a need for public authorities to reinforce a systematic management of antibiotics in an aquaculture system. Routine screening of antibiotic resistant bacteria in aquaculture could contribute to a better understanding of the role of aquaculture environment and cultured species in the transmission of MAR among human pathogens.

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