JOURNAL OF TROPICAL LIFE SCIENCE
2022, Vol. 12, No. 2, 207 – 218
http://dx.doi.org/10.11594/jtls.12.02.07

Research Article

Prevalence of Antibiotic Resistance Bacteria in Aquaculture Sources in Johor, Malaysia

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Article history:
Submission July 2021
Revised August 2021
Accepted October 2021

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ABSTRACT

The intensive use of antibiotics in aquaculture results in the proliferation of antibiotic. In this study, antibiotic resistant bacteria from six different aquaculture sources (pond of Fisheries Research Institute (FRI), and rivers of Kukup, Pulai, Pendas Laut, Sungai Melayu and Kong Kong) were isolated. These isolates were tested for antibiotic resistance against seven antibiotics via the disc diffusion method. Finally, phenotypic and genotypic identification via 16S rRNA sequencing and phylogenetic analysis were carried out. The results show that 58 out of 118 bacterial isolates are resistant to multiple antibiotics. The highest isolate resistance was observed towards rifampicin (89.66%), followed by ampicillin (79.31%) and sulfafurazole (67.24%). The isolates with multiple antibiotic resistant (MAR) index values with more than 20% were subjected to 16S rRNA gene sequencing. The majority of the bacterial strains exhibit multiple antibiotic resistance, indicating that they were isolated from highly contaminated sources based on the tested water qualities profiles, which showed the high level of turbidity and total dissolved solid (TDS) in most sampling sites with the high number of MAR bacteria obtained.

Keywords: Antibiotic resistance, Multiple antibiotic resistance, Aquaculture sources, MAR index value, 16S rRNA gene sequencing

Introduction

Antibiotic resistance (ABR) refers to the intrinsic resistance of bacterial species towards certain antibiotics [1]. The presence of antibiotic resistance genes will transform bacteria to tolerate antibiotics, thereby reducing the efficacy of such antibiotics in treating diseases or infections [2]. Propagation of such traits between different bacterial strains will pose a threat to the human population as antibiotics will no longer be effective in combatting bacterial infections that sometimes could be fatal [3].

Aquaculture is a large and fast-growing sector that supplies protein sources, in which diverse types of bacterial species and diseases could be found in the aquaculture stocks, including finfish and shellfish [4, 5]. This sector is also a reservoir of antibiotics residues. Thus a suitable environment for antibiotic resistance propagation since antibiotics commonly used as human medicine are also used in aquaculture [6]. Antibiotics are widely used in various industries, including aquaculture and agriculture, as prophylactics, growth promoters, and therapeutics [7]. Besides, antibiotic runoff from agriculture and the discharge from the farming industry into the river can lead to the sedimentation of antibiotic residues and accelerated antibiotic resistance in the environment [8].

Antibiotics used to treat bacterial infections in the aquaculture have resulted in selective pressure of antibiotic resistance amongst bacteria [9]. In China, the excessive use of antibiotics in the aquaculture environment to prevent and treat bacterial

How to cite:
Kam KY, Jaafar MF, Sani MH, Suhaimi MS, Zain NAM (2022) Prevalence of Antibiotic Resistance Bacteria in Aquaculture Sources in Johor, Malaysia. Journal of Tropical Life Science 12 (2): 207 – 218. doi: 10.11594/jtls.12.02.07.

infections speeds up the emergence of ABR [10]. In Thailand, approximately 500-600 metric tons of antibiotics are used in the shrimp farming, such as norfloxacin, oxytetracycline, enrofloxacin, and sulphonamides [11, 12]. In Malaysia, chloramphenicol has been used in aquaculture through the feed at 3-5 gm/kg [13]. The residues of these antibiotics can be dispersed to the surrounding aquatic environments via agriculture runoff or discharge from animal farms and wastewater treatment plant [14]. Selective pressure and longer exposure to the antibiotic residues might contribute to the development of multidrug-resistant bacteria [15]. This is because these antibiotic residues tend to remain in the sediment and alter the microflora composition and selection for antibiotic-resistance bacteria [16, 17, 18].

Studies have found a wide variety of antibiotic resistance bacteria surrounding the aquaculture site. Studies revealed that the aquaculture environments in most countries, especially Africa and South East Asia present a high level of antimicrobial resistance (AMR), and there is a strong correlation between multiple antibiotic resistance (MAR) from aquaculture and human clinical bacteria [19]. These bacteria possessed new and uncharacterized resistance determinants, which are able to emerge in the aquatic environment and could be transferred to other bacteria such as human and animal pathogens via horizontal gene transfer [20, 21, 22]. This could lead to the selection, emergence and spread of drug-resistant pathogens, one of the biggest threats to global health, food security, and development today [23, 24].

Existing literature does not thoroughly address the use of antibiotics in aquaculture and the emergence of the multiple antibiotic resistance (MAR) bacteria in Malaysia. The use of antibiotics should be monitored from time to time so that antibiotics use could be traced and the limited data could be expanded from time to time. The correlation between antibiotic resistance in aquaculture sources and water samples is poorly understood. The lack of literature on antibiotic contamination and MAR in the natural environment could hinder the full understanding of the emergence of ABR in aquaculture in Malaysia. Therefore, aquaculture sources and water samples were collected and the water quality in each site was evaluated in this study. The bacterial strains from each water sample were subjected to isolation on nutrient agar as well as preliminary identification test. This study focuses on identifying antibiotic resistance bacteria (ARB) and the multiple antibiotic resistance bacteria (MAR). This study also includes the 16S rRNA sequencing and phylogenetic analysis to compare the resistance strains obtained with strains from the National Center for Biotechnology Information (NCBI) database.

Material and Methods

Sampling and sample preparation

Aquaculture sources (fish) and water samples were collected from selected districts in Johor, including Pulai, Kukup, Kong Kong, Pendas, Sungai Melayu rivers and Fisheries Research Institute Malaysia (FRI) pond located in Gelang Patah, where the samples collected from the FRI pond were used as control. Freshwater aquaculture in Pulai, Kukup, Kong Kong, Pendas and Sungai Melayu rivers were selected as these locations are the major aquaculture farms in Johore. FRI acts as an integrated fish farm and has served as a control for comparing antibiotic resistance bacteria between integrated fish farming and freshwater aquaculture. Water samples were collected from three points in each location by using dip sampling method and three fish samples were collected in each location. The collected fish samples are *Lates Calcarifer* (in FRI, Kukup, Pulai, and Kong Kong), *Lutianus Malabaricus* (in Pendas) and *Chanos Chanos* (in Sungai Melayu). According to the standard operating protocol, fish samples were transported at 4 °C to the laboratory and dissected within 4 h after collection [25]. The sample processing was performed on the fish samples where 10 g of internal guts and digestive tracts were grinded with 10 ml sterile distilled water and 1 ml aliquot volume was measured and homogenized in 9 ml of sterile distilled water to give a 1:10 dilution [26].

Water quality measurement

Water quality measurements was performed by using HORIIBA U-50 Multiparameter Water Quality Meter checker as described [27]. The measured parameters are temperature, pH, pHmV, ORPmV, conductivity, turbidity, dissolved oxygen (DO), total dissolved solid (TDS), salinity and seawater gravity (SG).

Primary isolation of bacteria

Serial dilution was performed for both fish and water samples to 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilution.

1 mL of water sample was added into 9 mL of sterile distilled water, giving a 10^{-1} dilution. A volume of 1 mL aliquot from the 10^{-1} dilution was added into another 9 mL of sterile distilled water to give a serial dilution of 10^{-2}, followed by 10^{-3} and 10^{-4}. Ten grams of fish samples including internal guts and digestive tracts were ground with 10 ml sterile distilled water and 1 ml aliquot volume was measured and homogenized in 9 ml of sterile distilled water, giving a 1:10 dilution, followed by serial dilution method as described [28]. The spread plate method was used for plate count while 0.1 mL of each water sample was collected and the fish aliquots were spread on nutrient agar, followed by overnight incubation at 37 °C for 24 h according to the standard method of American Public Health Association [29].

Phenotypic identification

The colony morphology of each bacteria strain was assessed based on the colour, shape, elevation, margin and texture after 24 h incubation. Gram staining was performed for each isolate as described [30] and examined under oil immersion at 1000× magnification.

Antibiotic Susceptibility Test (AST)

The Kirby-Bauer assay was carried out on the isolates. Disc diffusion method on Mueller-Hinton agar (MHA) was used to access the isolates susceptibility towards different antibiotics according to the Clinical and Laboratory Standard Institute [31, 32]. The bacterial suspension was prepared by suspending the colonies into 3 mL of sterile distilled water. Each MHA plate was divided into four sections and the bacterial suspension was swabbed onto the agar surface in three directions using sterile cotton buds. Agar diffusion method was employed by embedding the commercial antibiotic discs (Oxoid, UK) onto the MHA and gently pressing on the antibiotics disc. The inoculated agar plates were incubated at 37 °C for 18 to 24 h. The list of antibiotics tested for the isolates and the resistance of bacteria were tabulated in Table 2. Following incubation, the inhibition zones (zone size) formation was observed and measured to determine the degree of susceptibility of the bacterial isolates to the tested antibiotics according to the standard interpretative table [31, 33].

Multiple Antibiotic Resistance (MAR)

Multiple antibiotic resistance (MAR) index is defined by formula

$$MAR = {}^{a}/_{b}$$

where "a" represents the number of antibiotics that the bacteria are resistant to, while "b" represents the total number of antibiotics used for the evaluation of susceptibility [34].

Genotypic identification of bacterial isolates via 16S rRNA sequencing

The bacterial DNA was extracted from overnight culture by using the simple boiling method [35, 36]. The extracted DNA was used to perform PCR amplification with GOTAQ® Promega Green Master Mix and 0.5 µM forward primer (fD1, 5'-AGAGTTTGATCATGGCTCAG-'3) and reverse primer (rP1, 5'-ACGGTTACCTTGTTACGACTT-'3). The PCR mixtures (25 µL) contained bacterial DNA were amplified in the thermocycler, starting with the activation of *Taq* polymerase at 95°C for 3 mins, followed by 30 cycles of denaturation at 95°C for 40 sec, annealing process at 55°C for 30 sec and extension at 72°C for 1.5 min, with a final extension at 72°C for 5 min [37]. The PCR amplicons were electrophoresed in 1% w/v agarose gels with a molecular size marker (1kb GeneRuler) at 85 V for 45 min. The gel was stained with ethidium bromide for 5 minutes, rinsed, and viewed under ultraviolet light illumination. The resulting band size of the amplicons was ~1500bp.

DNA Sequencing and Phylogenetic Analysis

The unpurified PCR products were outsourced to Apical Scientific Sdn. Bhd. (Selangor, Malaysia) for sequencing. The resulting DNA sequences were analyzed by using the Bioedit software to obtain the complementary sequences. The sequences of the PCR products obtained were analyzed with nucleotide Basic Local Alignment Search Tool (BLASTn) by multiple sequence alignment using ClustalW program provided by the National Center of Biology Information (NCBI) website [38, 39]. The phylogenetic analysis was carried out by using MEGA 7 software to generate the phylogenetic tree and the relationship between the isolates of the most abundant bacterial species in each site. The multiple sequence alignments were performed with ClustalW [40] and the phylogenetic tree for each site was constructed by MEGA 7 with 1000 bootstraps [41].

Results and Discussions

Sampling sites' profile

In this study, the quality measurements of the water samples were performed using HORIBA U-50 Multiparameter Water Quality Meter checker except for samples from FRI site, where the data were provided by the Research Institute (Table 1). The dissolved oxygen (DO) in Kukup is the highest (11.26 mg/L), while Pulai River has the lowest concentration of DO (3.99 mg/L). Study has revealed that the concentration of DO of more than 5.00 mg/L is favorable for the growth of flora and fauna. Hence, for Pulai river, the DO concentration is slightly lower than the lower limit of concentration required for the good growth of the organisms in that site [42]. This finding suggests that the river is heavily polluted with low ability to sustain the aquatic biota. The data collected from all sites indicate that the TDS is higher than that of the standard level. High TDS levels could be due to the over-fertilization on all sites or from industrial effluents discharged to the surrounding aquatic environments [43].

As for the turbidity of the water, World Health Organization (WHO) stated that a standard level of 5 Nephelometric Turbidity Unit (NTU) is favorable for the survival of aquatic life [24]. Three samples show turbidity exceeding the standard level; FRI pond shows the highest turbidity value (85 NTU), followed by Kukup and Pulai Rivers. This could be contributed by industrial and human activities in surrounding areas resulting in antibiotic runoff and sedimentation in the sand, silt, and clay [44]. Samples from ponds tend to show a higher level of turbidity as in FRI samples due to lack of natural water flow and drainage, resulting in accumulation of suspended particles giving the water its characteristic light brown colour. These sampling sites were located near the agriculture sector and the domestic residential area. The water qualities profiles suggested that these sampling sites were possibly contaminated by agricultural activities, domestic, industrial waste water, and mass bathing occasions that could lead to increased pollutants in the water.

Assessment of antibiotic susceptibility of the bacterial isolates

There are 118 bacterial isolates from the six sites, composed of 24 isolates from FRI, 17 isolates from Kukup, 22 isolates from Pulai, 21 isolates from Pendas Laut, 20 isolates from Sungai Melayu and 14 isolates from Kong Kong. The antibiotic resistance profile for the bacterial isolates was interpreted based on the inhibition zone (zone size). The unique breakpoint zone size indicates susceptibility to that antimicrobial compound and is used to determine the degree of susceptibility of the bacterial isolates to the tested antibiotics according to the standard interpretative table [31]. Based on Figure 1, antibiotics including ampicillin, rifampicin and sulfafurazole were observed to have high percentage of bacterial resistance in all the freshwater locations. Resistance to these three antibiotics can be observed in different fish samples, as these antibiotics are widely used in fish farming for therapeutic, prophylactic or other purposes and feed efficiency [13]. The isolates obtained from different fishes (*Lates calcarifer* and *Chanos chanos*) showed similarity in the antibiotic resistance profile. Among all the seven antibiotics, the isolates obtained in *L. calcarifer* and *C.*

Table 1. Measurement of water quality in each location

Water Quality Parameter	Point A FRI	Point B Kukup	Point C Pulai	Point D Pendas Laut	Point E Sungai Melayu	Point F Kong Kong
Temp (°C)	31	31.18	30.44	30.57	31.28	29.99
pH	N.A.	8.31	7.62	8.63	8.77	7.41
pHmV	N.A.	-62.67	-28	-89	-98	-16
ORPmV	N.A.	166.5	125	70	77	165.5
Conductivity (mS/cm)	N.A.	44.1	45.8	45.8	42.7	39.7
Turbidity (NTU)	85	69.4	9	1.9	1.3	3.6
DO (mg/L)	6.24	11.26	3.99	6.98	10.16	6.46
TDS (g/L)	N.A.	26.8	27.9	27.9	26	24.3
Salinity (ppt)	15	28.5	29.7	29.6	27.4	25.3
Seawater gravity (SG)	N.A.	16.5	17.8	17.7	15.8	14.7

† N.A.: Not Applicable.

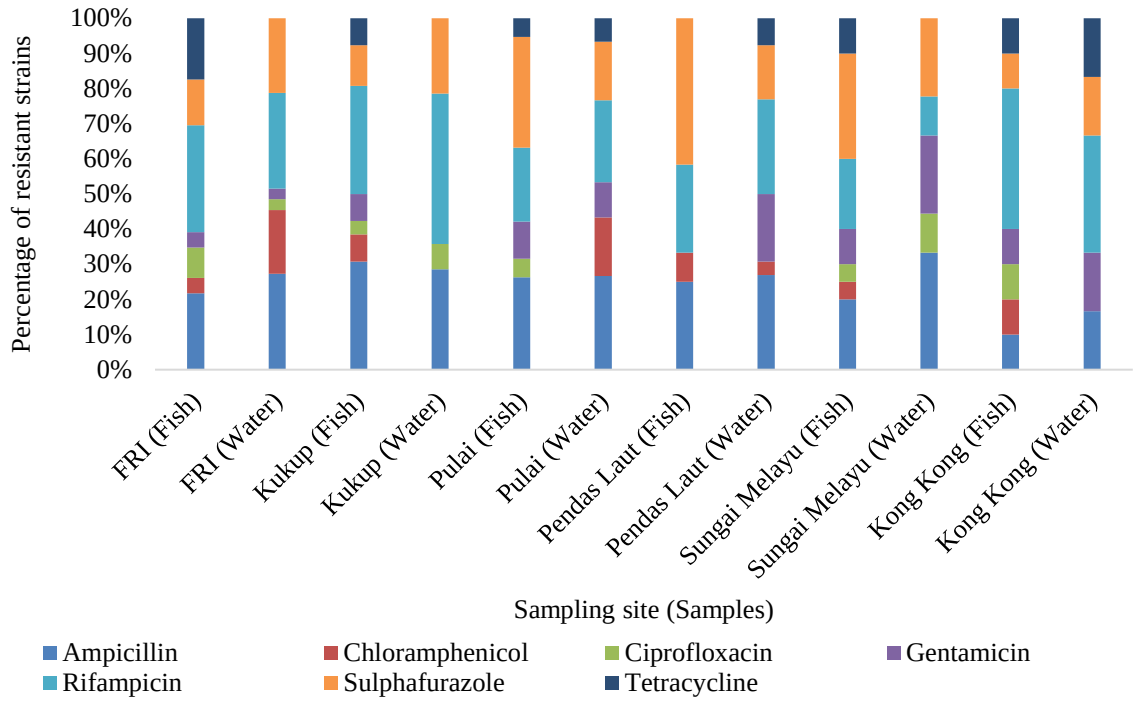

Figure 1. The percentage of bacterial strains resistant towards different antibiotics in each location

Table 2. Resistance of bacteria towards different antibiotics

Antibiotics used (amount)	Zone Size Interpretation (mm)			Number of resistant isolates	Isolates with resistance (%)
	Resistance	Intermediate	Susceptible		
Ampicillin (10 µg)	13	14-16	17	58	49.15
Chloramphenicol (30 µg)	12	13-17	18	18	15.25
Ciprofloxacin (5 µg)	15	16-20	21	9	7.63
Gentamicin (10 µg)	12	13-14	15	20	16.95
Rifampicin (5 µg)	16	17-19	20	62	52.54
Sulphafurozole (300 µg)	12	13-16	17	46	38.98
Tetracycline (30 µg)	11	12-14.	15	15	12.71

chanos have shown resistance to all antibiotics tested. The majority of the bacterial isolates from different freshwaters and fish samples have shown the similarity in the antibiotic resistance profile, in which all of the isolates are resistant to at least one antibiotic tested.

As shown in Table 2, the bacterial isolates are mostly resistant to rifampicin (52.54%), followed by the ampicillin (49.15%), and sulfafurazole (38.98%). To a lesser extent, the bacterial isolates are also resistant to other antibiotics including gentamicin (16.95%), chloramphenicol (15.25%), tetracycline (12.71%) and ciprofloxacin (7.63%). High ampicillin resistance and low gentamicin resistance were also observed in Mandal's study on the bacterial strains including *E. coli*, *Proteus mirabilis*, *Proteus vulgaris*, *Citrobacter freuundii*, *Morganella morganii*, *Pseudomonas aeruginosa* and *Serratia marcescens* [45]. Meanwhile, the study reported that most isolates from the water samples are generally 100% resistant to ampicillin. At the same time, none of them is resistant to gentamicin [46], which is confirmed with the finding that, majority of the isolates obtained from the

different freshwater locations have shown resistance to ampicillin and less resistant to gentamicin. Antibiotics use could be one of the causes of contamination, and it may propagate to the coastal environment from rivers, sewage treatment plants and industrial effluent [47]. The antibiotic resistance in water samples is correlated with the antibiotic resistance in fish samples as the majority of the resistance patterns towards the antibiotics tested in the bacterial isolates from fish and water samples are similar.

Multiple Antibiotic Resistance (MAR)

In this study, the determined MAR index values ranged from 0 to 0.8517, where the isolates with MAR index value of more than 0.2 indicate that the bacteria were resistant to more than two antibiotics. Isolates with MAR index value higher than 0.2 showed that the bacterial isolates were isolated from high-risk antibiotic-contaminated sources including municipal, commercial poultry farms, swine, and dairy cattle where antibiotics are often used [34].

Analysis on overall isolates revealed that 49.15% (n=58) isolates belong to the group of Multiple Antibiotic Resistance (MAR) index value greater than 0.2. In comparison, 50.85% (n=60) isolates belong to the group of MAR index value less than 0.2. As shown in Figure 2, bacterial isolates with MAR index value greater than 0.2 were mainly isolated from the FRI, Kukup and Pulai, while only a few of MAR strain were isolated from Pendas Laut and Sungai Melayu. FRI has served as a control due to integrated fish farming activities with little exchange of water. Hence, the overfeeding and water currents in FRI have indicated that the accumulation of antibiotic residual has potential risk towards the development of multidrug-resistant bacteria, compared to the freshwater aquaculture [48].

The high number of MAR isolates obtained in the FRI might be due to the lack of natural water flow and the accumulation of sediment in the pond, which contributed to the high level of turbidity (85 NTU) in FRI, suggesting that the water quality profile is correlated with antibiotic resistance properties of the isolated bacteria. This correlation can be observed in the Kukup and Pulai that consist of high number of MAR isolates obtained, in which these freshwater locations have higher turbidity and TDS compared to the other freshwater locations, suggesting that this high level of TDS and turbidity in water quality profile could be due to the industrial and human activities in surrounding areas, resulting in antibiotic runoff and sedimentation in the sand, silt, and clay and cause the occurrence of this antibiotic resistance properties.

Among the MAR isolates, bacteria show the highest percentage of resistance to rifampicin (89.66%), followed by the ampicillin (79.31%), sulfafurazole (67.24%), gentamicin (29.31%), chloramphenicol (27.59%), tetracycline (22.41%) and ciprofloxacin (12.07%). In this study, samples from FRI show the highest percentage of antibiotic resistant bacteria while Kong Kong has the lowest percentage. The lowest frequency of antibiotic resistant bacteria in Kong Kong might be due to less

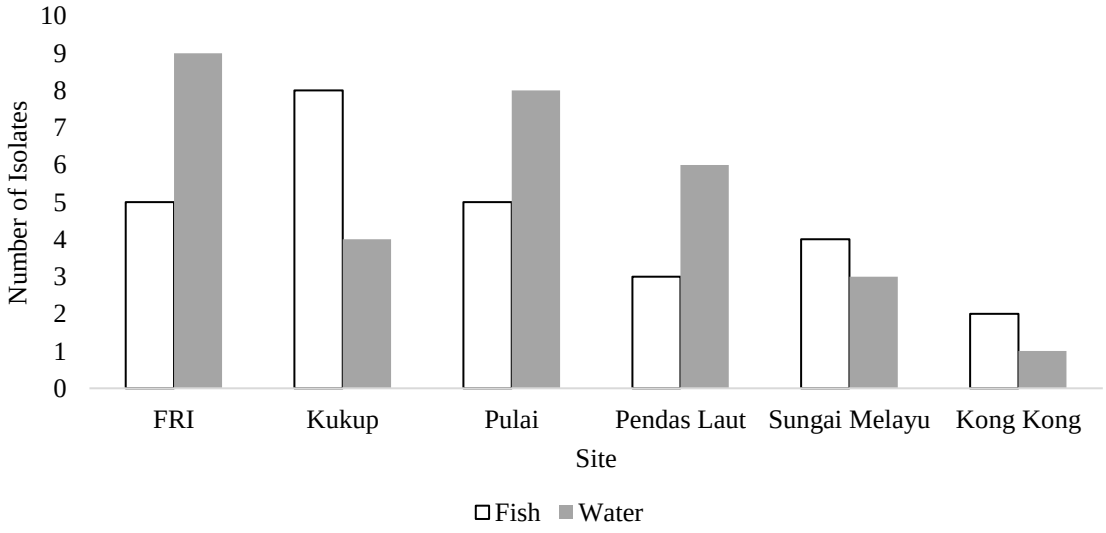

Figure 2. Number of isolates with MAR index value > 0.2

activity that may contribute to the contamination of antibiotics in that area [49]. As shown in Figure 2, FRI shows the highest number of isolates with a MAR index value of more than 0.2. The aquaculture farm is consisted of individual ponds that are not connected to streams. There is possibility that antibiotics residues were absorbed into the sediment. Water sampling in FRI was also carried out in a pond, in which the water sample collected from FRI fish ponds might contain the sediments including accumulates of fish feeding and feces, and eventually causing the bacterial selection, which is the selection of antibiotic resistant bacteria in the place where an antibiotic is present at a

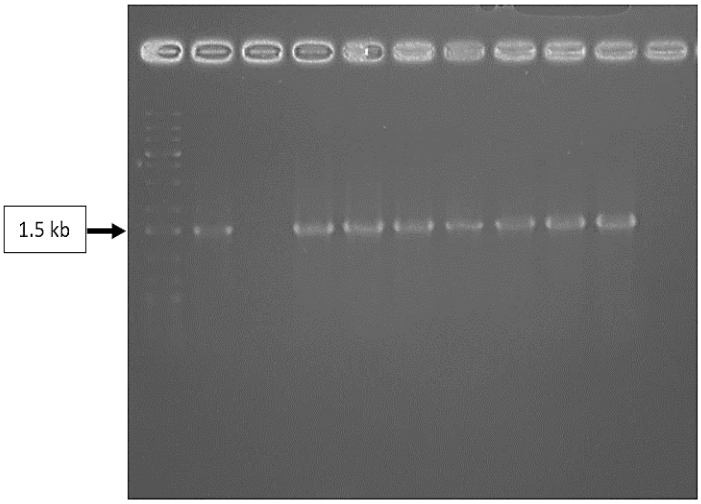

Figure 3. Gel analysis of 16s amplicons of the bacterial isolates of FRI water sample from PCR that yielded band size of 1.5 kb. Lane 1, GeneRuler 1kb DNA ladder; lane 2, E. coli as positive control; lane 3, negative control; lane 4, Isolate R2W1; lane 5, Isolate R2W2; lane 6, Isolate R2W3; lane 7, Isolate R2W4; lane 8, Isolate R2W5; lane 9, Isolate R2W6; lane 10, Isolate R2W7.

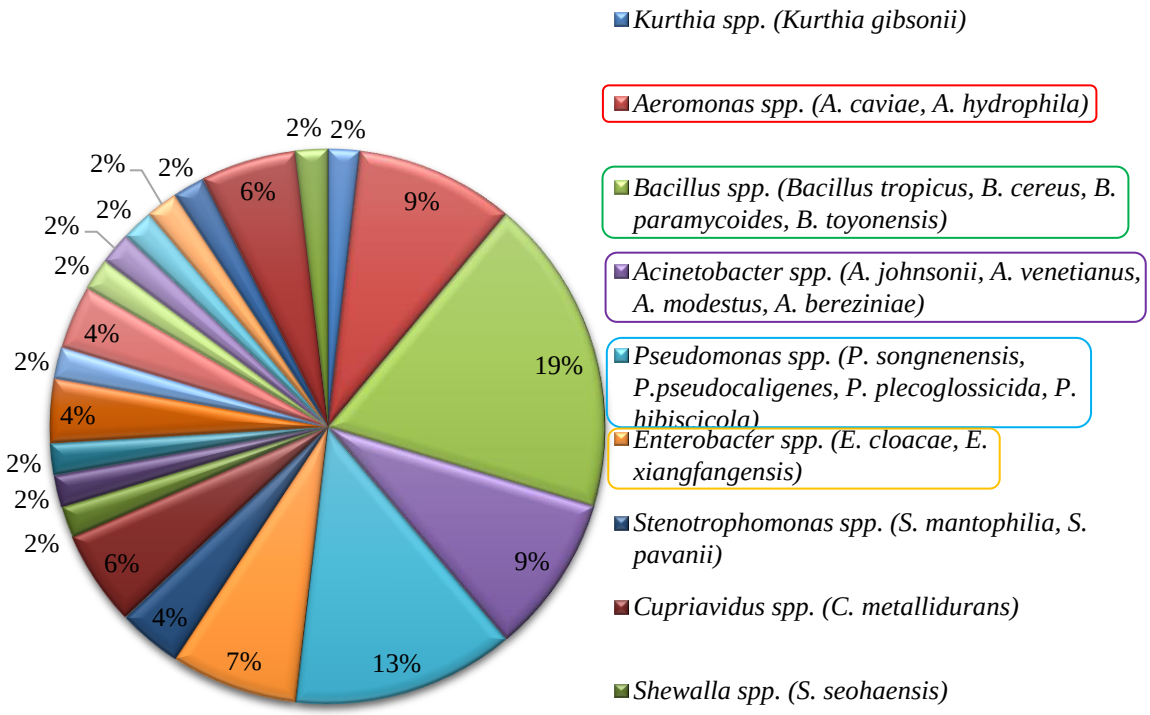

Figure 4. The percentage of bacterial species obtained after BLASTn analysis

selective concentration [50]. A high concentration of antibiotic residue in the pond will increase the rate of antibiotics bioaccumulation and raise additional concerns about the transmission of ARB, especially in the cross-species transmission of the pathogen [8, 51, 52, 53, 54]. Bioaccumulation of antibiotics will eventually lead to the acquisition of resistance by the bacteria in the ponds. The MAR index reflects the number of antibiotics used and the number of bacteria isolates [55].

PCR and 16S rRNA Sequencing

All of the isolates yielded amplicons with the expected band size of 1.5 kb with good intensity and brightness in gel analysis as shown in Figure 3. High identity percentages (92-100%) and low E-values were observed in the BLASTn analysis, which strongly suggest the accuracy and reliability of the identification results [56].

With MAR index value greater than 0.2, most of the bacterial species isolated are *Bacillus* spp. (19%), *Pseudomonas* spp. (13%), *Aeromonas* spp. (9%), *Acinetobacter* spp. (9%) and *Enterobacter* spp. (7%) as illustrated in Figure 4. All of these bacteria are commonly found in the aquaculture environment and can be isolated from fish species, including tilapia, catfish and shellfish [57, 58, 59, 60].

Bacillus spp. was mostly isolated from fish samples, in which all of them are resistant to ampicillin and rifampicin. This finding correlates with the report by Gazi *et al.* [61], which stated that *Bacillus* spp. is resistant to ampicillin. The *Bacillus* spp. occurs in the highest percentage in the fish samples as they are commonly used as probiotics in aquaculture [62]. The study revealed that high resistance (90%) to sulfamethoxazole and rifampicin was observed for the *Pseudomonas* spp. in the antibiotic susceptibility test [63], which agrees well with the finding of this study. Previous studies have reported that the antibiotics are widely used in food and aquaculture production for treatments, and eventually caused 50% to 90% of the antibiotics used to be released into the environment and promote the development of antibiotic resistance [64].

Phylogenetic analysis

The newly obtained 16S rRNA sequences was deposited in NCBI database (www.ncbi.nlm.nih.gov) under the accession number MK294292 for SeqO1. The neighbour-joining (NJ) phylogenetic tree presented in Figure 5 was constructed using MEGA7 software. The result of multiple alignments for the phylogenetic tree showed O1 strain was clustered with a bootstrap value of 1000 and a scale bar of 0.001 substitutions per site. This finding was observed to be 99% similar to *Shigella dysenteriae*. The bootstrapping values describe how confident and well-

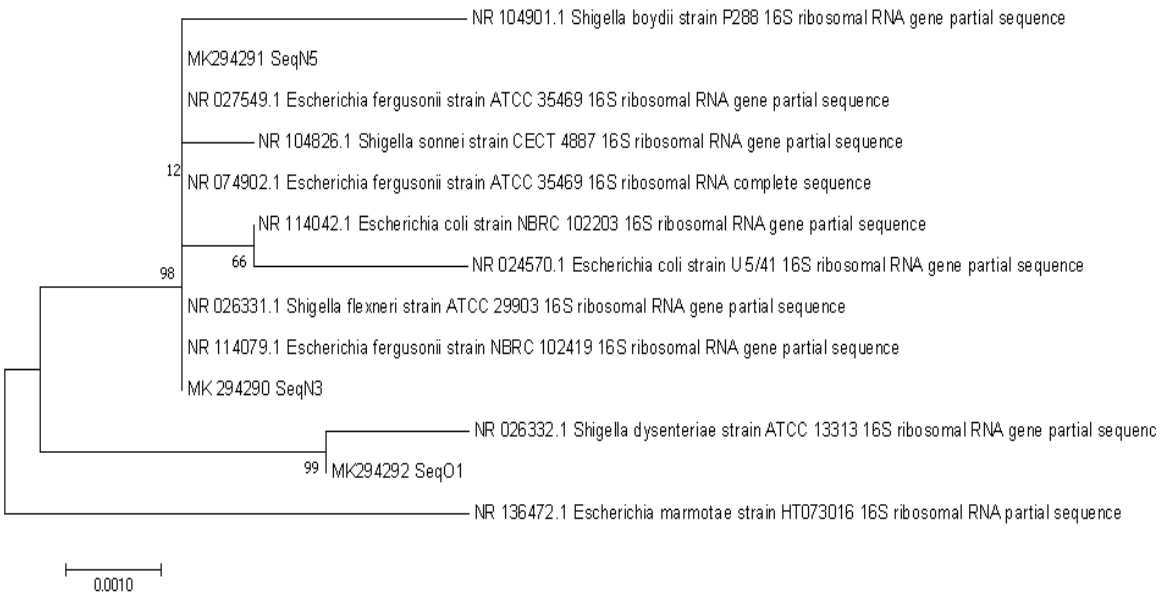

Figure 5. Neighbour-joining tree illustrates the phylogenetic positions of the three most abundant antibiotic resistance strains obtained from Sungai Melayu (highlighted in red bracket) and other strains of species of the *Shigella* spp. group based on 16s rRNA sequencing

supported the phylogenetic tree was measured and a value of 100 showed the constructed tree is well-supported [65]. Based on the phylogenetic analysis, only O1 strain showed a high bootstrapping value (99), while other strains demonstrated a close relationship with the bacteria strains that aligned in the NJ tree with low bootstrap values. For instance, the 16s rRNA gene obtained in FRI has suggested that the strains belong to the *Pseudomonas* group but are unable to assign it accurately to a certain species due to its low discrimination [66]. Therefore, there is a possibility that these strains might serve as potential novel species and further analysis may apply in order to demonstrate the potential of these strains.

Conclusion

This study reports the quality of the water samples and the presence of antibiotic resistant bacteria from the selected sites. High TDS in the water samples could result from over-fertilization and probably agriculture runoff nearby the sampling area. Bacteria isolates show the highest percentage of antibiotic resistant to rifampicin (52.54%) and ampicillin (49.15%), followed by sulfafurazole (38.98%), gentamicin (16.95%), chloramphenicol (15.25%), tetracycline (12.71%) and ciprofloxacin (7.63%). The MAR index has revealed that 58 out of 118 isolates were resistant towards multiple antibiotics. Sequencing results have shown that majority of the bacteria strains obtained are *Bacillus* spp. (19%), *Pseudomonas* spp. (13%), *Aeromonas* spp. (9%), *Acinetobacter* spp. (9%) and *Enterobacter* spp. (7%). Majority of the bacteria strains exhibited multiple antibiotic resistance that could result from over-supplied of antibiotic agents. Majority of the bacteria strains obtained are potentially pathogenic and clinically important strains. The multiple antibiotic resistance in these bacteria strains especially the clinically important strains are able to cause serious disease and threaten the effective prevention and treatment of infection. Therefore, the development of multiple antibiotic resistance bacteria should not be overlooked and should be investigated thoroughly since it has adverse risk towards human health and the environment in general.

Acknowledgement

This study was funded by the Cagayan State University (CSU) through the leadership of the University President Dr. Urdujah G. Alvarado and was supported by the Cagayan State University-Gonzaga Campus (CSU-G) through its Campus Executive Officer, Dr. Froilan A. Pacris, Jr., CSU-G Project Ariwat/Ayo Team (Dr. Calixto B. Alicay, Charlot L. Maramag, Gerlie U. Bayani, Marvin V. Baloloy, Lyle B. Sumer, Dr. Claribel A. Gaspar, and Jean S. Torres); Municipal Environment and Natural Resources Offices (MENROs) of northeastern Cagayan municipalities; Coast Guard Station Aparri; Coast Guard Sub-Station Santa Ana, Cagayan; CSU Lal-lo Campus; Central Analytical Laboratory of the CSU-Andrews Campus; and the Regional Soils Laboratory of the Cagayan Valley Integrated Agricultural Laboratory, Department of Agriculture Region 2, Tuguegarao City, Cagayan.

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