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RESEARCH ARTICLE

Occurrence and distribution of tetracycline resistance determinants and their pollution profile in selected aquaculture environments in Sri Lanka

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Abstract: Tetracycline (TET) has been extensively used in aquaculture for chemotherapy against various fish diseases. The contamination levels in 16 aquaculture farms in Sri Lanka were analyzed using high performance liquid chromatography (HPLC). The presence of antibiotic-resistant bacteria was identified using the 16SrRNA gene sequencing and the corresponding antibiotic resistant genes (ARGs) were screened at each location using the PCR method. The concentration of TET in the aquaculture farm samples ranged between 0.001± 0.001 - 0.112± 0.017 ppm. *Bacillus* and *Staphylococcus* were recorded as the most dominant resistant bacterial genera against TET. *Acinetobacter* sp., *Achromabacter* sp., *Staphylococcus* sp., *Micrococcus* sp. were also identified as resistant bacteria. In the present study, *tet* (M) and *tet* (A) were the abundant resistance genes (11/16 isolates), followed by *tet* (S) (5/16) and *tet* (B) (4/16). It was found that *tet* (M) is one of the most widely distributed tetracycline resistance determinants in the aquaculture environment in Sri Lanka.

Keywords: Antibiotic resistant genes (ARGs), aquaculture, tetracycline (TET)

INTRODUCTION

In recent years, there has been an increasing interest in the study of the occurrence and distribution of veterinary antibiotics in the environment, due to their potential adverse effects on the ecosystem and human health (Moges *et al*., 2014). Among the management of drugs approved for agriculture, antibiotics were widely administered for animal health, especially in aquaculture applications (Allen *et al*., 2010).

At present, aquaculture production is increasing worldwide as a source for human consumption. It is known that antibiotics are used extensively in aquaculture to treat infectious diseases caused by a variety of bacterial pathogens in aquatic life and as a sub-therapeutic dose to improve production (Pruden *et al*., 2013; Liyanage & Manage, 2016b). Antibiotics can be metabolised after administration; however up to 80 % of the antibiotics administrated are excreted through urine or feces without complete decomposition (Muziasari *et al*., 2014). Therefore, it is possible that antibiotics can find their way into the aquatic environment from a variety of sources such as the excretion of animals and discharge from sewage waste. Thus, the prophylactic and therapeutic use of antibiotics result in the occurrence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in the aquaculture environment (Ritter *et al*., 2008; Manage & Liyanage, 2019).

ARGs in the aquaculture environment can be transferred horizontally among microbes and ultimately be transferred to fish as pathogens (Shah *et al*., 2012). In many Asian countries including Sri Lanka, fish is the major diet of the local people (82 %) because of the consumption habit, health and its nutritional benefits (MFARD, 2016). Therefore, humans have an exposure

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risk to ARB and ARGs *via* ingestion of contaminated aquaculture food and water use. Tetracycline (TET) has been extensively used in aquaculture for chemotherapy against fish diseases such as fin rot and skin ulcers (Mortazavi, 2014), and TET resistant (TETr) bacteria were reported in fish pathogens and environmental bacteria (Ajayi *et al*., 2013). *Aeromonas, Proteus* (Vincent *et al*., 2015) and *Pseudomonas* (Nikokar *et al*., 2013) have been reported to carry the resistance genes to TET.

Resistance to TET occurs *via* two primary mechanisms; one is the energy dependent efflux of TET and the other is production of ribosomal protection proteins (RPPs) (Kim *et al*., 2004; Zhu *et al*., 2013).

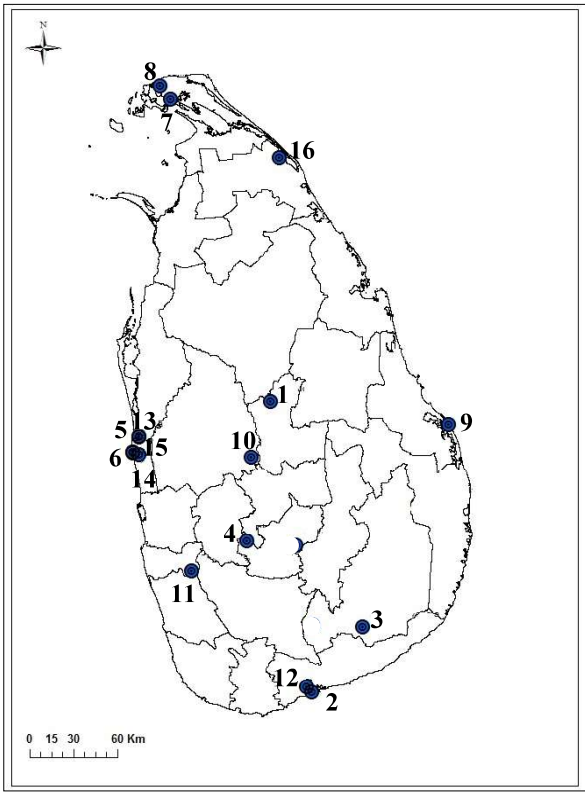
To regulate antibiotic usage, some monitoring programmes for antimicrobial resistance have been established in Europe, North America and Latin America (Harbarth *et al*., 2015). However, little information about the status of antibiotic contamination and ARGs is available in Sri Lanka. The annual aquaculture production in Sri Lanka has increased from 44,300 Mt to 334,890 Mt from 1999 to 2014 (NAQDA, 2015). Therefore, monitoring studies of antibiotics, ARB and ARGs is necessary to develop a safe aquaculture industry in Sri Lanka. Contamination of antibiotics, ARB and ARGs in Vietnam, Thailand (Suzuki & Hoa, 2012) and South Africa (Suzuki *et al*., 2015) have recently been reported. As for aquaculture environments in Sri Lanka, frequent use of TET is likely in fish farms.

Thus, the present study aims to determine the status of antibiotic contamination, ARB and ARGs in 16 aquaculture farms (freshwater fish farms, brackish water and shrimp farms) in Sri Lanka. The selected genes in the present study include both efflux pump genes (*tet* A, *tet* B) and RPP genes (*tet* M, *tet* S), which are widely detected among the TET resistance genes.

METHODOLOGY

Chemicals and standards

Tetracycline, HPLC grade chemicals and bacteriological grade chemicals were purchased from Sigma-Aldrich.



Site	No
Fish farms	
Dambulla (Da)	1
Ranna (Ra)	2
Lunugamwehera (Lu)	3
Ginigathhena (Gi)	4
Muthupanthiya (Mt)	5
Udappuwa (Ud)	6
Jaffna 01 (Jf 1)	7
Jaffna 02 (Jf 2)	8
Batticalo 01 (Bt 1)	9
Ornamental fish farm	
Rambodagalle (Rm)	10
Orna fish farm- Colombo (Or)	11
Shrimp hatcheries	
Kahandamodara (Ka)	12
Ambakandawila 1 (Am 1)	13
Ambakandawila 2 (Am 2)	14
Ambakandawila 3 (Am 3)	15
Puthukudirippu (Pt)	16

Figure 1: Location Map of the sampling sites in the study

Sampling sites and sample collection

Triplicate effluent water samples were collected from 16 aquaculture farms: including 9 freshwater food fish farms (Dambulla, Ranna, Lunugamwehera, Ginigathhena, Muthupanthiya, Udappuwa, Jaffna 01, Jaffna 02, Batticaloa 01), 2 ornamental freshwater fish farms (Rambodagalle, Orna-fish farm) and 5 brackish water shrimp farms (Kahandamodara, Ambakandawila 1, Ambakandawila 2, Ambankandawila 3, Puthukudirippu) (Figure 1). Water from Horton Plains were collected as samples from a pristine environment. Water samples were filtered through 150 µm plankton nets to remove debris and collected into sterile amber coloured glass bottles. The samples were stored in the dark in an ice box during transportation and then stored at 4 ºC until analysis.

Total viable counts (TVC) of bacteria in environment samples

The total viable counts were measured using the standard pour plate method with Lauryl-Bertani (LB) medium; Tryptone, 9.1 g; Sodium chloride, 4.6 g; Yeast extract, 4.6 g; agar ,13.1 g; per liter) (Manage *et al*., 2009). The colony numbers (CFU/mL) were counted after incubation at 28 ºC for 3 – 5 days. For counting of TETr bacteria, TET at a final concentration of 60 µg/mL were added to each medium (Kim *et al*., 2012; Liyanage & Manage, 2015; 2018). Bacteria growing on TET treated media were defined as TETr bacteria and enumeration was done after 3 d of incubation in the dark.

Enrichment and isolation of antibiotic resistant isolates

From each sampling site 50mL of water was enriched by inoculating TET at final concentration of 60 µg/mL in 100 mL Erlenmeyer flasks. The final volume was topped up to 100 mL with sterile water and the flasks were incubated at 28 ºC ± 1º in 100 rpm for 14 ds in the shaking incubator.

After 14 ds of enrichment, 1 mL sample aliquots were taken from the flask for isolation and enumeration of bacteria following the modified pour plate method (Manage *et al*., 2010). LB medium which contained 60 µg/mL of TET was used to isolate TET resistant bacteria (Kim *et al*., 2012; Liyanage & Manage, 2014).

After 3 d of incubation at 28 ºC, bacterial colonies with different morphological characters were picked and re-suspended in liquid LB medium. Subsequently pure bacterial cultures were sub cultured and stored in agar slants at -20 ºC in LB-glycerol media for further analysis and identification.

Antibiotic susceptibility test

The LB broth cultures were prepared, and a loop of isolated bacterial strains was inoculated and incubated in shaking incubator in 28 ºC at 100 rpm overnight. The cell density of the bacterial suspension was equalised using McFarland No 0.5 (Liyanage & Manage, 2014; Jorgensen & Turnidge, 2015). The minimum inhibition concentration (MIC) was determined by agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2015).

Extraction of environmental DNA from water samples

For DNA extraction, 250 mL water sample was collected and filtered through a 47 mm diameter polycarbonate filter (0.22 µm pore size, Millipore). Each filter paper was soaked in 10 mL of 70 % methanol and stored at -20 ºC until use. Extraction of DNA from the filter papers was carried out following the modified version of Kim *et al*., (2012). Purified DNA was subjected to PCR analysis.

DNA extraction from isolated bacteria culture

Isolated bacteria were cultured in 5 mL of LB liquid medium at 28 ºC, 100 rpm for 24 h. Cells were harvested by centrifugation at 12,000 rpm for 2 min, and the genomic DNA was extracted following the method described by Kim *et al*., (2004). Purified DNA was re-suspended in 30 µL of TE buffer and stored at -20 ºC until PCR analysis.

Detection of ARGs by PCR

PCR was performed to detect antibiotic resistant genes and the PCR mixture contained 0.5 µL of each primer (10µm), 5 µL Go taq reaction buffer, 0.5 µLdNTPs, 2.0 µL of 25 mM $MgCl_2$ and 0.1 µL of Go taq DNA polymerase, adjusted to a total volume of 25 µL. Purified DNA (5 µL) was used as the PCR template. The optimised conditions used for the primers are shown in Table 1. PCR amplification was conducted in a BIOLAB PCR system (BYQ6078E-757, China) thermal cycler and utilised 35 cycles [denaturation at 95 ºC for 30 s, annealing for 30 s, and extension at 72 ºC for 1 min]. A final extension was performed at 72 ºC for 5 min. The amplified products were analysed by electrophoresis on a 1.5 % agarose gel and stained with ethidium bromide.

Table 1: Optimised conditions for tet primers (Call *et al*., 2003)

Primer pair	Sequence	PCR annealing temp (°C)	Amplicon size (bp)
tet A-FP	5′-TTGGCATTCTGCATTCACTC-3′		
tet A-RP	5′-GTATAGCTTGCCGGAAGTCG-3′	60	494
tet M - FP	5′-ACACGCCAGGACATATGGAT-3′		
tet M- RP	5′-ATTTCCGCAAAGTTCAGACG-3′	55	536
tet B - FP	5′-CAGTGCTGTTGTTGTCATTAA-3′		
tet B- RP	5′-GCTTGGAATACTGAGTGTAA-3′	60	571
tet S - FP	5′-CGCTACATTTGCGAGACTCA-3′		
tet S- RP	5′-GAATGCCACTACCCAAAGGA-3′	46	555

Identification of tetracycline resistant bacteria

A total volume of 200 µL of gDNA product was sent to Macrogen, Korea for sequencing. For identification of bacteria, DNA sequences were analysed by the basic local alignment search tool at the National Center for Biotechnology Information (NCBI) website (*http://www.ncbi.nlm.nih.gov/*).

Quantification of tetracycline (TET)

Extraction of TET from environmental samples using SPE

One liter of the effluent water sample was adjusted to pH 3 and filtered through 0.22 µm nuclearpore filter. Filtered samples were spiked with the standard antibiotic concentration of 10 µg/mL and loaded onto a Sep-Pak Plus C18 cartridge after conditioning with 100 % methanol by followed 5 mL deionised water (Baquero *et al*., 2008; Liyanage & Manage, 2014). Pre-prepared samples were then passed through the cartridges at a flow rate of approximately 1–2 mL/min and then rinsed with 5 mL of deionised water. The analytes were eluted with 3 mL of 100 % methanol (Liyanage & Manage, 2014).

HPLC quantification

TET was quantified using Agilent 1200 series HPLC equipped with a diode array and fluorescence detector (Fernandez-Torres *et al*., 2010; Liyanage & Manage, 2014). The injected volume was 20 µL and chromatography was performed at 30 ºC. The mobile phase consisted of a mixture of 0.1 % glacial acetic acid in water (Component A): 0.1 % glacial acetic acid in acetonitrile (Component B) 99:1 (v/v) was pumped in the beginning at a flow rate of 0.7 mL/min for TET. Then followed linear elution gradient from 99 % to 70 % in 25 min for TET at 230 nm (Liyanage & Manage, 2014). Retention time was 10.56 min for TET. The antibiotic concentrations of the environmental samples were determined by using a calibration curve prepared for TET using external standards from 0.05 to 100 µg/mL.

Calibration curves

The concentration of TET was determined using the following equation, which was derived from the calibration plot for TET.

$$C_{TET} = (A_{TET} - 7.345) / 0.149$$

where,
A_{TET} = peak area of TET
C_{TET} = concentration of TET

The concentration of TET in the environmental sample was obtained by subtracting the corresponding value obtained for a 10 µg/mL aqueous sample of the standard antibiotic treated in the same way as the spiked environmental sample from C_{TET}.

Recovery test

The recovery test was carried out for TET as follows. A sample of sterilised distilled water (1 L) was spiked with TET to a final concentration of 100 µg/mL. The prepared sample was subjected to solid phase extraction (SPE) by using Sep-Pak Plus C18 cartridge and analysed as described above. A recovery of 87 % was obtained.

Statistical analysis

Principal component analysis (PCA) was done for antibiotic concentration in environmental samples and biological parameters of collected water samples in order to see the cluster of sampling locations depending on the presence of antibiotic resistant bacterial strains.

The phylogeny was constructed by neighbour-joining method based on Mega 6/ Cluster W from alignment of antibiotic resistant genes.

All statistical analyses were performed using Minitab 17 software.

RESULTS AND DISCUSSION

The present study simultaneously investigated the concentrations of TET, TRB and occurrence of *tet* (M), *tet* (S), *tet* (A), *tet* (B) genes in aquaculture effluent water. It is not clear whether the antibiotics and ARGs from fish feeds and fish excrement remain in aquatic environment in Sri Lanka. In other countries, it is known that the selection for the resistant bacteria and ARGs may have occurred in medicated fish feeds (Fedorova *et al*., 2014) or inside the fish intestines and fish faeces that entered the water (Xiong *et al*., 2015), or selection through the antibiotics present in water (Xiong *et al*., 2015).

Table 2: TET concentrations in water samples collected from fish farms and shrimp hatcheries

Sampling site	TET (µg/mL)*
Fish farms	
Dambulla (Da)	N.D
Ranna (Ra)	0.002 ± 0.001
Lunugamwehera (Lu)	0.001 ± 0.001
Ginigathhena (Gi)	0.001 ± 0.001
Muthupanthiya (Mt)	N.D
Udappuwa (Ud)	N.D
Jaffna 01 (Jf 1)	0.076 ± 0.022
Jaffna 02 (Jf 2)	0.008 ± 0.002
Batticaloa 01 (Bt 1)	0.005 ± 0.001
Ornamental fish farm	
Rambodagalle (Rm)	0.002 ± 0.001
Orna fish farm- Colombo (Or)	0.001 ± 0.001
Shrimp hatcheries	
Kahandamodara (Ka)	0.012 ± 0.009
Ambakandawila 1 (Am 1)	0.112 ± 0.017
Ambakandawila 2 (Am 2)	0.092 ± 0.001
Ambakandawila 3 (Am 3)	0.076 ± 0.022
Puthukudirippu (Pt)	0.023 ± 0.008
Pristine environment	
Horton Plains	0.000

N.D: Not detected

* Average ± standard division (n = 3)

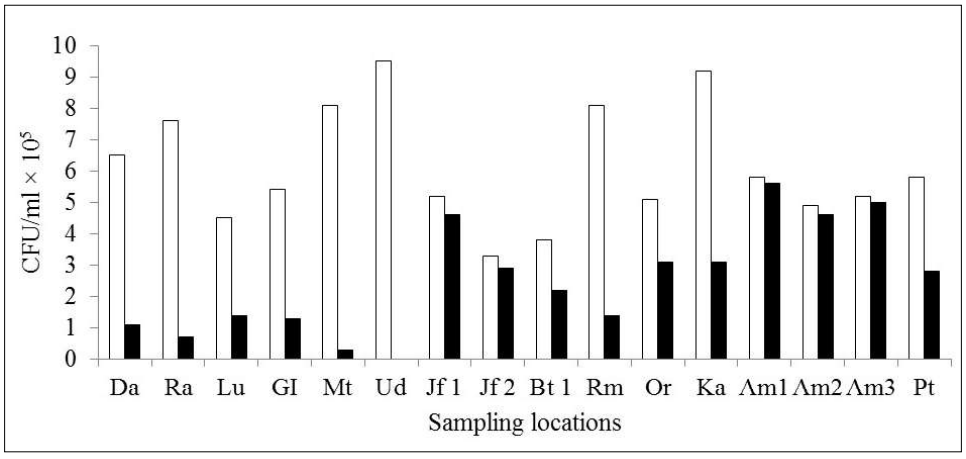


Figure 2: Total viable counts (TVC) (□) and TET resistant bacteria (■) at each sampling site [fish farm: Da; Dambulla, Ra; Ranna, Lu; Lunugamwehera, Gi; Ginigathhena, Mt; Muthupanthiya, Ud; Udappuwa, Jf 1; Jaffna 01, Jf 2; Jaffna 02, Bt1; Batticaloa 01, Ornamental fish farms: Rm; Rambodagalle, Or; Orna-fish farm, Shrimp hatcheries: Ka; Kahandamodara, Am1; Ambakandawila 1, Am 2; Ambakandawila 2, Am 3; Ambakandawila 3), Pt; Puthukudiirippu]

Occurrence of TET in water samples

The concentrations of TET in water samples are presented in Table 2. TET was detected at all sampling locations except Dambulla, Muthupanthiya and Udappuwa.

The concentrations of TET ranged from 0.001± 0.031 µg/mL to 0.112± 0.017 µg/mL where the highest concentration was recorded at Ambakandawila 1 and the lowest was at Ginigathhena, Lunugamwehera and Orna fish farm. In Muthupanthiya and Udappuwa aquaculture sites TET was not detected, because aquaculture practices were not being conducted at these sites during the sampling period. High TET concentrations were detected in shrimp hatcheries (0.012 ± 0.019 µg/mL – 0.112 ± 0.017 µg/mL) compared with ornamental (0.001 ± 0.002 µg/mL – 0.002± 0.031 µg/mL) and food fish farms (0.001 ± 0.031 µg/mL – 0.076 ± 0.022 µg/mL), respectively.

In the present study, TET concentration ranged from 0.001 to 0.112 µg/mL. The WHO recommended concentration is less than 0.001 ppm of antibiotic residues in the aquatic environment and less than 0.1 µg/mL in soil (Connor & Aga, 2007). Thus, the results of the present study showed that antibiotics detected in effluent water exceeded the recommended concentrations given by the WHO (WHO, 2015).

The high frequency of detection and concentration of TET are likely due to the large amount of TET used for feed additives and to control infectious diseases in aquaculture sites (Shah *et al*., 2014). The antibiotic concentration levels found in the present study are higher than those reported in some developed countries such as Victoria Harbor, China, Japan, Vietnam (Gao *et al*., 2012) and South Africa (Chenia & Vietze, 2012).

Water samples collected from Horton Plains, which was declared a World Heritage Site (WHS) by UNESCO (UNESCO, 2010) and situated 2100 m above sea level, were considered as reference samples from a 'pristine' environment. TET and ARGs were not detected in Horton Plains.

Total viable bacterial counts (TVC)

As shown in Figure 2, total viable counts (TVC) of cultivable bacteria ranged between $3.3 \times 10^5 – 9.5 \times 10^5$ CFU/mL in the sampling sites. The highest TVC of cultivable bacteria was recorded in Udappuwa (9.5×10^5 CFU/mL) whereas the lowest TVC was detected in Jaffna farm 2 (3.3×10^5 CFU/mL).

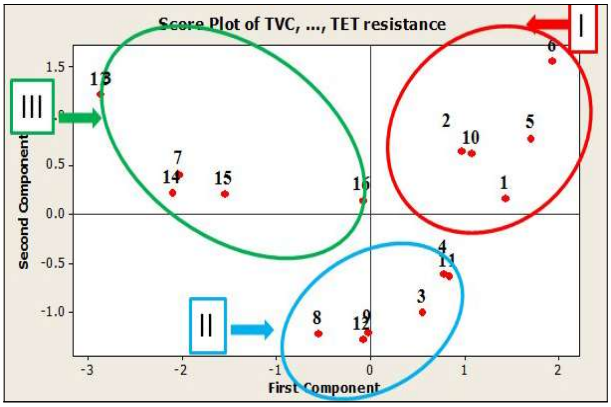


Figure 3: PCA analysis for total viable count, TET concentration CFU of TETr bacteria

The resistance percentages for TETr (defined as the number of bacteria that grew on antibiotic plates divided by the total number of bacteria that grew on plates containing no antibiotics) were calculated for effluent water samples from each sampling site.

The TETr bacterial percentage in fish farms varied between 2.1 to 72.91 %; in ornamental fish farms between 7.84 to 12.16 % and in shrimp hatcheries between 13.41% to 78.18 %, respectively. TETr bacteria were not detected in Dambulla and Muthupanthiya aquaculture sites. This may be due to the lack of prior exposure to TET at these sampling sites (Table 2).

The results obtained from PCA are shown in Figure 3. The clusters (I, II, III) correspond to TVC, CFU of TETr bacteria and TET concentration. TET concentrations and number of resistant bacteria were recorded to be high in cluster III compared to clusters I and II. In cluster I and II, detected antibiotic concentrations were very low, or not in the detectable level. Thus, antibiotic concentration and the number of resistant bacteria exhibited a strong relationship.

Gao *et al*., (2012) reported that the rate of occurrence of TETr bacteria was high in aquaculture farms in Northern China compared to other environments. In previous studies, the recorded percentage of tetracycline resistant bacteria in fish farms in Malaysia varied from 2.7 to 60.7 % (Shariff *et al*., 2000), whereas it varied between 4.7 to 64.8 % in shrimp hatcheries in Singapore (Shariff *et al*., 2000) and the percentage of occurrence of TETr bacteria against 60 µg/mL of TET was from 0.79 to 14.7 % in ornamental fish farms in Malaysis (Shariff *et al*., 2000). Compared to these, the results of the

present study were more or less similar to the recorded concentrations for ornamental and shrimp hatcheries.

Contrary to the present study, a higher incidence of bacterial resistance to TET at aquaculture farms was found in Australia, Denmark and the Netherlands by comparing resistance percentages in shrimp hatcheries (Akinbowale *et al*., 2006). The results of the present study showed high percentage of occurrence (2.1 – 78.1 %) of TETr bacteria compared to the other studies (Shariff *et al*., 2000; Akinbowale *et al*., 2006). The findings suggest that TETr bacteria is linked to various drug resistance mechanisms, but a specific trend of pattern would be present due to regional differences (Akinbowale *et al*., 2006). Additionally, the distribution of antibiotics also depends on the physical and chemical stability of antibiotics (Martinez, 2017).

Identification of antibiotic resistant bacteria

Forty strains of bacteria were randomly isolated from aquaculture effluent water. 16S rRNA analysis was carried out to identify the isolated TETr bacteria. Species composition of TET resistant bacteria, the resistance genes detected and their MIC values are summarised in Table 3.

Table 3: MIC of tetracycline and detection of *tet* (M), *tet* (S), *tet* (A) and *tet* (B) genes in tetracycline resistant bacteria isolated from aquaculture farms

Isolate	Group or species	MIC (µg/mL)	*tet* (M)	*tet* (S)	*tet* (A)	*tet* (B)
HF586507	*Achromobacter* sp.	760	+	-	-	-
DQ174269	*Achromobacter xylosoxidans*	550	+	-	+	+
NR042387	*Acinetobacter calcoacetius*	640	-	+	+	-
KL234156	*Aeromonas hydrophila*	680	+	+	+	-
FJ982654	*Bacillus cereus*	410	+	+	-	-
KN514297	*Bacillus thurengiensis*	420	+	+	+	-
D1186353	*Bacillus* sp.	360	+	-	-	-
HV492635	*Bacillus* sp.	780	-	-	+	-
AS238910	*Bacillus pumilus*	670	-	-	+	-
HF947328	*Staphylococcus* sp.	580	+	+	+	+
KU691844	*Flexibacter* sp.	540	+	-	+	+
AS678425	*Bacillus anthracis*	450	+	-	+	-
PR617299	*Pseudomonas aeruginosa*	840	+	-	+	-
LS345678	*Micrococcus luteus*	430	-	-	-	+
KS675433	*Staphylococcus epidermis*	620	+	-	-	-
KS678987	*Staphylococcus haemolyticus*	320	+	-	-	-

The genera *Achromobacter, Micrococcus, Pseudomonas* and *Acinetobacter* are recognised as proteobacteria, while *Bacillus and Staphylococcus* belong to the firmicutes group. *Bacillus* was found to be the most dominant resistant genus in the present study.

The isolated strains except *Bacillus thuringiensis* have been recorded as pathogenic bacteria that cause different diseases such as fin rot, mouth rot, skin ulcers and abdominal swelling in fish and shrimp (Vincent *et al*., 2015).

MIC values of TETr bacteria varied from 320 µg/mL to 840 µg/mL. The highest MIC was recorded for *Pseudomonas aeruginosa* and the lowest was for *Staphylococcus haemolyticus*.

The *tet* (M) and *tet* (S) are well studied ribosomal protection protein genes, which are known to be distributed widely in the aquatic environment, whereas *tet* (A) and *tet* (B) are considered as genes that efflux tetracycline from the cell (Vincent *et al*., 2015).

In the present study, 12 strains out of 16 isolates were positive for *tet* (M) and 10 out of 16 strains were positive for *tet* (A), suggesting that these genes were harboured in cultural bacteria in aquaculture farms effluent water. In contrast, *tet* (S) (5/16) and *tet* (B) (4/16) detected only in a few TETr isolates.

Either *tet* (M) or *tet* (A) gene was detected in most of the resistant bacteria and the detection frequency was 68.75 % for both genes whereas a detection frequency for *tet*(S) and *tet* (B) were recorded as 31.25 % and 25 %, respectively.

Bacillus sp., *Achromabacter* sp. and *Staphylococcus* sp. were found to be the most dominant resistant strain in TETr bacteria in the present study. These results are different from a study conducted in Vietnamese shrimp and fish ponds where *Acinetobacter* was the commonest tetracycline resistant bacterium (Hoa *et al*., 2011). Another study in Vietnamese shrimp ponds found that *Vibrio* sp. was a common tetracycline-resistant bacterium, followed by *Bacillus* (Bien *et al*., 2015). These results may reflect regional differences in species composition of the bacteria population. Bonnin *et al*., (2013) also pointed out that the presence of *Bacillus* strains producing antimicrobial compounds may contribute to the high incidence of bacterial resistance to the antibiotics studied.

In the present study, some species of *Pseudomonas, Staphylococcus, Achromobacter, Flexibacter, Aeromonas* and *Micrococcus* are considered as pathogenic microorganisms which cause fish diseases such as fin rot and ulcers (Ritter *et al*., 2008). Therefore, bacteria resistant to antibiotics in the intestinal tract are excreted into the environment and then ARG transfer may occur between indigenous bacteria and intestinal bacteria, which possibly spread ARGs to human pathogens, thereby posing a risk to human health. Thus, the results of the study suggest that ARB isolated from fish ponds which belong to diverse taxonomic bacterial groups may be an important reservoir of a diverse group of tetracycline resistance genes conferring resistance by different mechanisms.

tet (M) gene has been detected at high percentages in bacteria, which were isolated from aquaculture sites in Korea and Japan (Kim *et al*., 2004). Similarly, this study also detected *tet* (M) in many isolated resistant bacteria strains (Table 3).

PCR detection of ARGs in water samples

tet (M) gene was detected in 14 sampling sites out of 16, while *tet* (A), *tet* (B) and *tet* (S) were detected in 9, 4, and 6 sampling sites, respectively. From the target ARGs, *tet* (A), *tet* (M) and *tet* (S) genes were found at three sampling sites, namely, Rambodagalle, Jaffna farm 1 and Batticaloa 1 whereas *tet* (A) and *tet* (M) genes were recorded at seven sampling sites of Dambulla, Rambodagalle, Udappuwa, Jaffna farm 1, Batticaloa, Ambakandawila 3 and Puthukuduirippu. Interestingly, TETr bacteria were not detected in Udappuwa sampling site, although *tet* (A) and *tet* (M) genes were detected (Figure 5).

Accordingly, the present study recorded *tet* (M) at many sampling sites where *tet* (S), *tet* (A) and *tet* (B) were found in only limited sampling sites (Figure 5). Further, a high detection frequency of *tet* (M) was recorded in this study and similar results were reported in Vietnamese shrimp and fish ponds as well (Hoa *et al*., 2011; Novais *et al*., 2012).

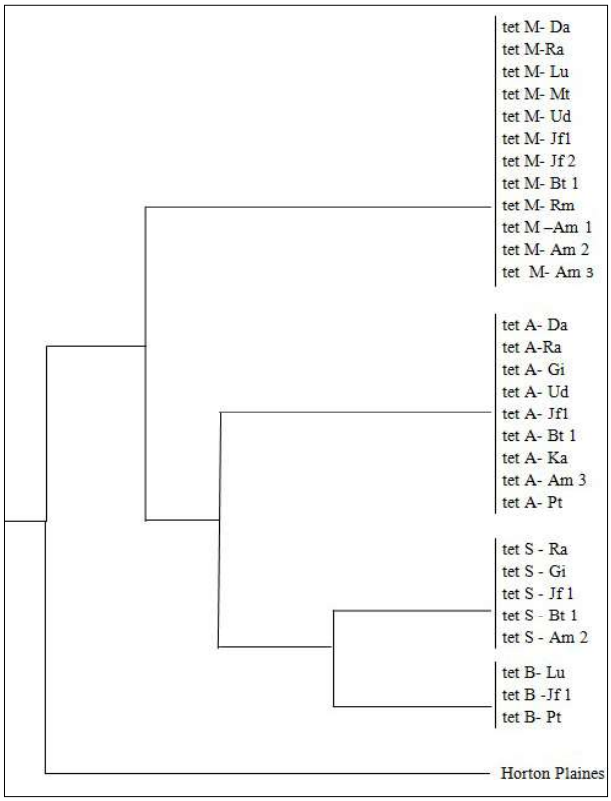


Figure 5: Phylogenetic tree of *tet (M), tet (A), tet (S*) and *tet (B)* from environmental DNA in water samples [fish farm: Da; Dambulla, Ra; Ranna, Lu; Lunugamwehera, Gi; Ginigathhena, Mt; Muthupanthiya, Ud; Udappuwa, Jf 1; Jaffna 01, Jf 2; Jaffna 02, Bt1; Batticaloa 01, Ornamental fish farms: Rm; Rambodagalle, Or; Orna-fish farm, Shrimp hatcheries: Ka; Kahandamodara, Am1; Ambakandawila 1, Am 2; Ambakandawila 2, Am 3; Ambakandawila 3), Pt; Puthukudiirippu]

Moreover, the presence of antibiotics in sub-inhibitory concentration may induce the horizontal gene transfer system in bacterial communities (Suzuki *et al*., 2008; Ryu *et al*., 2012; Manage, 2018), which further increases the prevalence of ARGs.

The results of the present study indicates that fish farms and shrimp hatcheries are reservoirs of ARGs and the presence of potential resistant and pathogen-associated taxonomic groups in fish farms have implications for human health. To the best of our knowledge, this is the first study on detecting the status of antibiotic contamination, ARGs, and composition of the antibiotic resistant bacterial community in selected aquaculture environments in Sri Lanka. The results highlight the negative impacts of overuse of antibiotics in aquaculture and the potential risk to human health.

CONCLUSION

In conclusion, the study demonstrates that aquaculture farms are a reservoir of TET, ARB and ARGs. The TETr bacteria, especially the possible opportunistic pathogens isolated from aquaculture environments and the presence of TETr genes, implies an urgent need for implementing a monitoring system for antibiotic usage in aquaculture, as ARGs and isolated ARB in farm water may lead to diseases outbreaks which would eventually lead to production losses at fish and shrimp farms. Further, the ARB and ARGs might have an impact on human society. Understanding the route and mechanisms of ARG proliferation is a step to mitigate the risks to human health.

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