RESEARCH ARTICLE

Environmental pollution

Screening and quantitative analysis of antibiotic resistance genes in hospital and aquaculture effluent in Sri Lanka as an emerging environmental contaminant

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Abstract: Hospital and aquaculture wastewaters play an important role in the evolution and spread of antibiotic resistance genes. In the present study, nine Antibiotic Resistance Genes (ARGs) were screened, which belong to two common groups of antibiotics: penicillin – *OPR D, bla TEM, bla OXA, amp a,* and *amp b,* and tetracycline – *tet A, tet M, tet B,* and *tet S*. The highest number of positive hospital wastewater sample locations were for *bla TEM* (51%) gene, followed in descending order by *amp a* (15%), *bla OXA* (14%), *OPR D* (5%) and *amp b* (1%). The highest number of positive sample locations for *tet (M)* genes was detected in aquaculture sampling sites (82%), followed by *tet (A), tet (S),* and *tet (B)* were 53%, 35%, and 18% respectively. A significant positive correlation ($p = 0.001$) between the concentrations of penicillin (0.001–0.006 µg/mL) and *bla TEM* gene ($7.56 \times 10^5 – 9.8 \times 10^5$ copies/mL) was found. The average concentrations of the *OPR D* and *amp a* was in the range $1.2 \times 10^2 – 1.56 \times 10^2$ copies/mL, $1.2 \times 10^5 – 6.56 \times 10^5$ copies/mL in hospital wastewater, whereas *tet M* and *tet A* in aquaculture effluent water was in the range $1.1 \times 10^5 – 9.23 \times 10^5$ copies/mL and $1.3 \times 10^4 – 4.56 \times 10^4$ copies/mL, respectively. The penicillin group (AMX, AMP, CLOX) in hospital wastewater effluent and tetracycline (TET, OTC) in aquaculture wastewater effluent were found to be important point sources of antibiotic pollution in their respective environments.

Keywords: Antibiotic-resistant genes, tetracycline, penicillin, *bla TEM*, *tet A*, *tet M*.

INTRODUCTION

The unregulated use of antibiotics in health care, livestock farming, and agriculture, has resulted in large amounts of such compounds being discharged directly into the natural ecosystem (Hernandes *et al*., 2013). Wastewater effluent from hospitals, intensive livestock, and aquaculture are the major sources of Antibiotic Resistance Genes (ARGs). According to the reports of World Health Organization (WHO) ARGs are one of the most critical human health challenges in the next century. ARGs serve as pathways of carrying out genetic manipulation and generate pressure for the development of antibiotic resistance in a susceptible microbial strain through horizontal gene transfer (Rodríguez-Mozaz *et al*., 2014; Liyanage & Manage, 2016a). The spread of antibiotic resistance is frequently associated with the high adaptive capacity of microorganisms.

Tetracycline and penicillin are widely used in livestock farming and healthcare. The major mechanisms for tetracycline resistance is prevention of drug uptake (efflux) into the cells, ribosomal protection, and enzyme inactivation (Ansari *et al*., 2009). Efflux genes are *tet A, tet B, tet C, tet D, tet K,* and *tet L* (Ansari *et*

al., 2009). The *tet M* and *tet O* are common genes for ribosomal protection proteins (Huddleston, 2014; Ansari *et al.*, 2009) while only *tet S*, *tetX, tet* 34, and *tet* 37 are coding for a protein capable of enzymatic inactivation of tetracycline (Ansari *et al.*, 2009). Penicillin is one of the common antibiotics, and major penicillin resistance mechanisms are antibiotic hydrolysis mediated by the bacterial enzyme *beta-lactamase* (*bla TEM)*, changes in Penicillin Binding Proteins (PBP) (*amp a)*, and decreasing of porin channel formation (*OPR D*). Among the penicillin resistance genes, *bla TEM* gene is one of the most frequently detected plasmid-borne antimicrobial resistance genes, which confers resistance to penicillin and extended-spectrum cephalosporin (Mroczkowska & Barlow, 2008).

Current EU legislation does not include specific regulations, neither the potential presence of antibiotic-resistant bacteria and ARGs in these waters nor their concentration thresholds. The evaluation of ARGs in clinical and environmental settings would therefore be the first step in tackling the rapidly growing resistance to antibiotics (Huddleston, 2014). Although the knowledge of antibiotic resistance in hospital wastewaters has largely depended on data provided by culture-based methods, the analysis of ARGs is limited in developing countries (Duong *et al*., 2008; Finley *et al*., 2013; Fekadu *et al*., 2015; Hocquet *et al*., 2016).

Because of the complexity of the processes and the relative scarcity of studies done, knowledge regarding ARGs and their role in the environment is still poor, and therefore environmental studies are given priority.

Documented evidence related to ARG levels and resistant bacteria in hospitals and aquaculture wastewater effluent is limited in Sri Lanka (Liyanage *et al.*, 2015; Liyanage & Manage, 2016a). This is the first report on screening and quantification of ARGs in hospital and aquaculture effluents in Sri Lanka. As an outcome, the study aimed to quantify ARGs for penicillin and tetracycline, as well as the level of antibiotic contamination in some selected hospital and aquaculture effluent water.

MATERIALS AND METHODS

Sampling sites and sample collection

Effluent water samples were collected in triplicate from 80 hospitals including the National Hospital Sri Lanka, 11 Teaching Hospitals, 15 General Hospitals, 30 Base Hospitals, 18 Divisional Hospitals, 2 Regional hospitals and 3 Private Hospitals (Table 1, Figure 1) and 16 aquaculture farms (food fish farms, ornamental fish farms, and shrimp farms) in different districts in Sri Lanka. Water samples were filtered through 150 µm plankton nets to remove debris and collected to sterile amber colour glass bottles (2 L). Then the samples were stored in ice boxes at 4ºC during transportation and kept in refrigerated conditions for subsequent analysis.

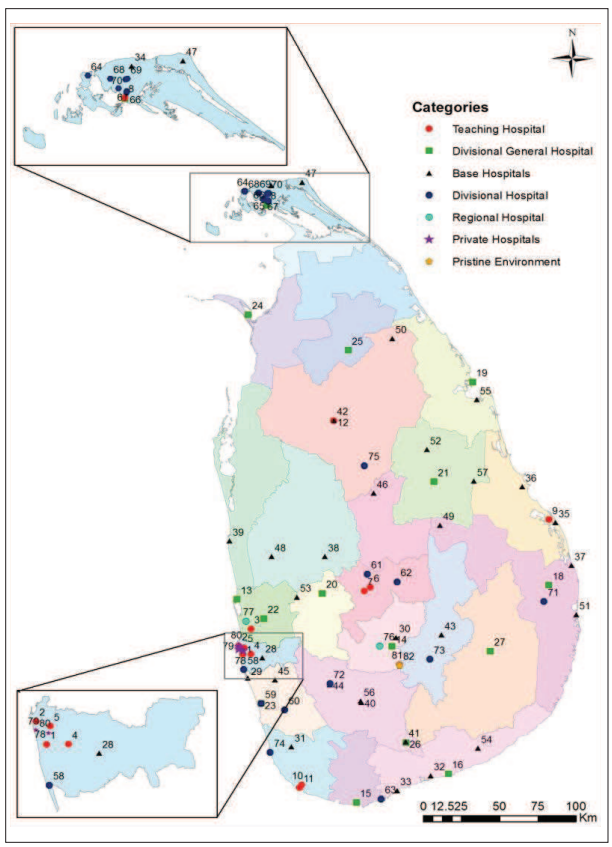

Figure 1: Hospital wastewater effluent sampling locations in the study

Quantification of tetracycline and penicillin in water samples

A 1L sample of wastewater was adjusted to pH 3 and filtered through a 0.22 µm Millipore filter. The filtered samples were spiked with antibiotic at a final concentration of 100 ppm and loaded onto a Sep-Pak Plus C18 cartridge after conditioning with 5ml of 100% HPLC grade methanol and following with 5 mL of deionized water (Liyanage *et al*., 2015). Pre-prepared

samples were then passed through the C18 cartridges set up with SPE unit at a flow rate of approximately 1–2 mL/min and then rinsed with 5 mL of deionized water. The analytes were eluted with HPLC grade 80% methanol (Liyanage & Manage, 2014; 2016b). The target antibiotics were quantified by using an Agilent 1200 series HPLC equipped with a diode array and fluorescence detector (Fernandez-Torres *et al*., 2010; Liyanage & Manage, 2016b). The injected volume was 20 µL and chromatography was performed at 30 ^{o}C.

Table 1: Hospital wastewater effluent sampling locations screened in the study

No	Location	No	Location	No	Location	No	Location
1.	Colombo-NHSL	21.	Polonnaruwa-GH	41.	Ambilipitiya- BH	61.	Akurana- -DH
2.	Colombo South Teaching Hospital (CSTH)	22.	Gampaha-GH	42.	Anuradhapura- BH	62.	Madadumbara -DH
3.	Colombo Northern Teaching Hospital (CNTH)	23.	Kalutara-GH	43.	Badulla -BH	63.	Dickwella -DH
4.	J'pura hospital – TH	24.	Mannar-GH	44.	Awissawella –BH	64.	Karainagar -DH
5.	Castle Street Hospital for Women (CSHW)- TH	25.	Vauniya-GH	45.	Horana-BH	65.	Konadavil -DH
6.	Kandy- TH	26.	Embilipitiya –GH	46.	Dambulla-BH	66.	Kokuvil -DH
7.	Peradeniya- TH	27.	Monaragala-GH	47.	Point pedro-BH	67.	Manipay-DH
8.	Jaffna- TH	28.	Homagama -BH	48.	Kuliyapitiya-BH	68.	Vaddukkoddai -DH
9.	Batticaloa- TH	29.	Panadura- BH	49.	Dehiattakandiya-BH	69.	Chunnakam -DH
10.	Karapitya- TH	30.	Ambewala - BH	50.	Padaviya-BH	70.	Uduvil -DH
11.	Mahamodara- TH	31.	Alpitiya- BH	51.	Thirukkovil-BH	71.	Damana -DH
12.	Anuradhapura- TH	32.	Ambalanthota- BH	52.	Medirigiriya-BH	72.	Rathnapura-DH
13.	Negombo-GH	33.	Tangalle- BH	53.	Warakapola-BH	73.	Bandarawela-DH
14.	Nuwaraeliya-GH	34.	Tellippalai- BH	54.	Tissamaharama-BH	74.	Ambalangoda-DH
15.	Matara-GH	35.	Kattankudy- BH	55.	Muthur-BH	75.	Kekirawa-DH
16.	Hambanthota-GH	36.	Valaichchenai- BH	56.	Kahawatta-BH	76.	Lindula-RH
17.	Jaffna-GH	37.	Kalmunai- BH	57.	Welikanda-BH	77.	Jae la-RH
18.	Ampara-GH	38.	Kurunegala -BH	58.	Moratuwa -DH	78.	Asiri hospital-Colombo
19.	Trincomalee-GH	39.	Puttalam- BH	59.	Kalutara -DH	79.	Durdans Hospital-Colombo
20.	Kegalle-GH	40.	Kahawatta- BH	60.	Mathugama -DH	80.	Nawaloka hospital-Colombo

TH; Teaching Hospitals, GH; General Hospitals, BH; Base Hospitals, DH; Divisional Hospitals, RH; Regional Hospitals.

Extraction of environmental DNA from wastewater effluent

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

Detection of ARGs by PCR

PCR was performed to detect antibiotic resistance genes and the PCR mixtures contained 0.5 µL of target primer (10 µM), 5 µL of Go taq reaction buffer, 0.5 µL of dNTPs, 2.0 µL of 25 mM $MgCl_2$ and 0.1 µL of Gotaq DNA polymerase, adjusted to a total volume of 25 µL. Purified DNA (5 µL) was used as the PCR template. Optimized conditions used for the primers are shown in Table 1. PCR amplification was performed using the BIOLAB PCR system thermal cycler (BYQ6078E-757, China)

and utilized 35 cycles (denaturation at 95 °C for 30 s, annealing for 30 s, and extension at 72°C for 1 min). The final extension was performed at 72 °C for 5 min. The amplified products were analysed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

Quantification of Antibiotic Resistance Genes (ARGs)

Real-time PCR (qPCR) assays were employed to quantify five ARGs: *bla TEM, bla OXA, amp a, tet A,* and *tet M*, which confer resistance to the main antibiotic families' penicillin and tetracycline. Quantitative PCR (qPCR) assays were performed using the SYBR Green chemistry (Applied Bio systems) using an Applied Bio system 7500 real-time machine (Marti *et al*., 2013). Each gene was amplified using specific primer sets (Table 2) and the PCR conditions included an initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s and at the annealing temperatures are given in the Table 2 for 20 s. After each qPCR assay, a dissociation curve was constructed by increasing the temperature from 65 to 95 °C to confirm the specificity of the amplified products.

Table 2: Primers, primer sequences and annealing temperatures used to amplify selected resistant genes of the two antibiotics, *i.e*., Tetracycline and Penicillin

Antibiotic group	Resistance gene	Primer pair	Nucleotide sequence 5' – 3'	Annealing temperature (°C)
Tetracycline	tet (A)	tet A-F	GCGCGATCTGGTTCACTCG	56
		tet A-R	AGTCGACAGYRGCGCCGGC	
	tet (M)	tet M - F	GTTAAATAGTGTTCTTGGAG	48
		tet M- R	CTAAGATATGGCTCTAACAA	
	ORP (D)	ORP- F	TTGGTTAGGGGCAAGTTTTG	64
		ORP- R	GTAATGGGCCAATAACACCG	
Penicillin	TEM-1	TEM-1- F	CATAGACAAGCCGTTGACC	57
		TEM-1- R	ATGTTTTTGGAACGACAGAG	
	amp a	amp a -F	CATAGACAAGCCGTTGACC	32
		amp a -R	ATGTTTTTGGAACGACAGAG	

Development of standard curves

Standard curves were generated by cloning the amplicon from positive controls into the PBR322 vector (Invitrogen, USA), and the corresponding copy number was calculated as follows: copy number $\mu L^{-1} = (A \times 6.022 \times 10^{23}) (660 \times B)^{-1}$, where A is the plasmid DNA concentration (g μL^{-1}), B is the plasmid length (bp) containing the cloned sequence, 6.022×10^{23} is Avogadro's number and 660 is the average molecular weight of one base pair (Perini *et al*., 2011). A ten-fold serial dilution was used to construct the standard curve for each ARG, which was run in parallel with the samples to obtain absolute quantification.

Statistical analysis

Comparisons of average antibiotic and ARG concentrations among different sampling points were carried out using ANOVA tests. Correlation between antibiotic and ARG values was made using Pearson's test (all variables satisfied the normality assumption).

Differences were considered significant at $p < 0.05$. All statistical analysis was performed using Minitab 17 software.

RESULTS AND DISCUSSION

At present great attention has been paid to the heavy use of antibiotics in hospitals and the aquaculture industry by national and international health agencies due to the development of antibiotic resistance (Aminov & Mackie, 2007; WHO, 2015).

The most abundantly use antibiotic classes in the worldwide are β-lactams, glycopeptides, sulphonamides, quinolones and trimethoprim (Kümmerer, 2001; Kimosop *et al*., 2016). β-Lactams and tetracycline are the most intensively used antibiotic classes for human therapies in Sri Lanka (Ministry of Health, 2015). However, the same antibiotics which are used for human infections are used for different diseases and as growth hormones in the aquaculture industry (NAQDA, 2015). Despite Sri Lanka's lengthy history of antibiotic use, information on antibiotic manufacturing and usage trends is scarce due to a lack of publicly available information, systematic monitoring, and documentation efforts.

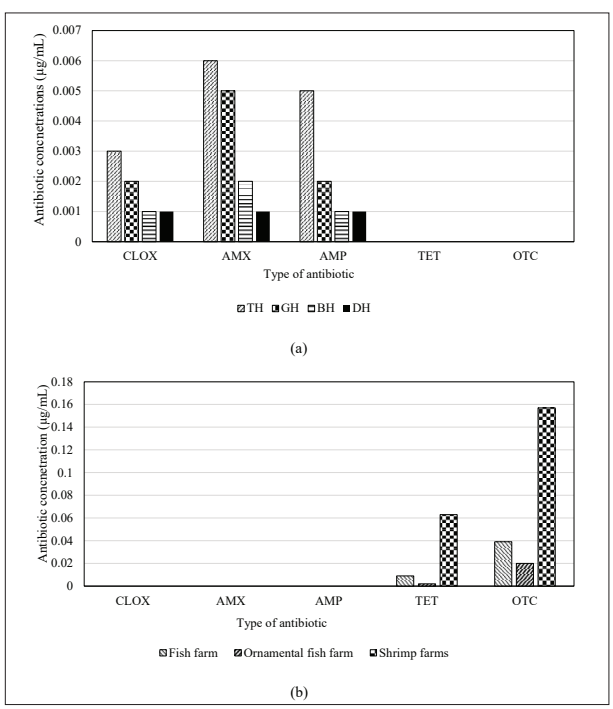

Figure 2: Mean antibiotic contamination in (a) Hospital effluent (TH: Teaching Hospitals, GH; General Hospitals, BH; Base Hospitals, DH; Divisional Hospitals) and (b) Aquaculture effluent (n = 3)

Antibiotic concentrations in hospital and aquaculture wastewater

The mean concentrations of antibiotics in the penicillin (AMX, AMP, CLOX) group ranged from 0.001 µg/mL to 0.006 µg/mL for hospital effluent water. However, OTC and TET were not recorded in hospital effluent during the study period (Figure 2 a).

The detected mean OTC concentration (0.157 ± 0.001 µg/mL) in shrimp hatcheries was comparatively higher than OTC concentrations (0.039 ± 0.012 µg/mL) recorded in food fish farms and ornamental fish farms (0.020 ± 0.011µg/mL). Similarly, a high mean TET concentration was detected in shrimp hatcheries (0.063 ± 0.019 µg/mL) compared to ornamental (0.002 ± 0.001 µg/mL) and food fish farms (0.009 ± 0.001 µg/mL) (Figure 2b). Antibiotics in the penicillin group were not detected in any aquaculture wastewater effluent during the period.

Screening of Antibiotic Resistance Genes (ARGs)

Many researchers have reported the potential presence of ARGs in environmental samples worldwide (Hsieh *et al*., 2011; Pruden *et al*., 2013), including in the pristine environment (Hsieh *et al*., 2011; Pruden *et al*., 2013; Liyanage & Manage, 2016a). According to the authors' knowledge, so far, no records are available regarding ARGs (*tet M, tet S, tet A, amp a, amp b, amp c, bla TEM,* and *bla OXA*) in environmental samples in Sri Lanka (Liyanage & Manage, 2015). Thus, the present study is the first report of screening at more than 90 sampling locations including hospital effluents and aquaculture farms for the potential of ARGs in Sri Lanka.

The percentage of positive sampling locations for penicillin resistance genes (*bla TEM, bla OXA, OPR D, amp a, amp b*) detected ranged from 5% to 51%, which is higher than the percentage of positive sampling locations of tetracycline resistance genes (*tet A, tet M, tet S* and *tet B*) in hospital wastewater samples (Figure 3). The highest number of positive hospital wastewater sample locations were for *bla TEM* (51%) gene, followed in descending order by *amp a* (15%), *bla OXA* (14%), *OPR D* (5%) and *amp b* (1%) [Figure 3(a)].

By contrast, the resistance genes *tet A, tet M, tet B,* and *tet S* belonging to the tetracycline group (OTC and TET) was detected in aquaculture sites with high frequency, ranging from 17% to 82%, compared with hospital wastewater effluents (0.5% to 11%), suggesting that the usage of specific antibiotics in

particular applications cause ARGs development in the environment [Figure 3(b)].

It should be noted that in the present study, a pristine reference sample was collected from the Horton plains, which is situated 2100 m above sea level and was declared a World Heritage Site (WHS) by UNESCO (UNESCO, 2010). The investigators of the study assumed that the Horton plains are free of antibiotic contamination due to minimum anthropological impact and strict regulations for human entry into the Horton plains. Interestingly, none of the antibiotics was detected in the Horton plains, supporting the assumption that it is a pristine environment for the study. However, AMX and TET resistance bacteria were detected in Horton plains water samples and one sample was even positive for the *bla TEM* gene. The presence of ARGs in such environments may be the result of Horizontal Gene Transfer (Hsieh *et al*., 2011; Liyanage & Manage, 2017), which can facilitate the development of new resistance bacteria. Thus, further studies are needed to investigate how the level of antibiotic resistance has developed and spread, and also how these levels vary in different environments with different bacterial communities.

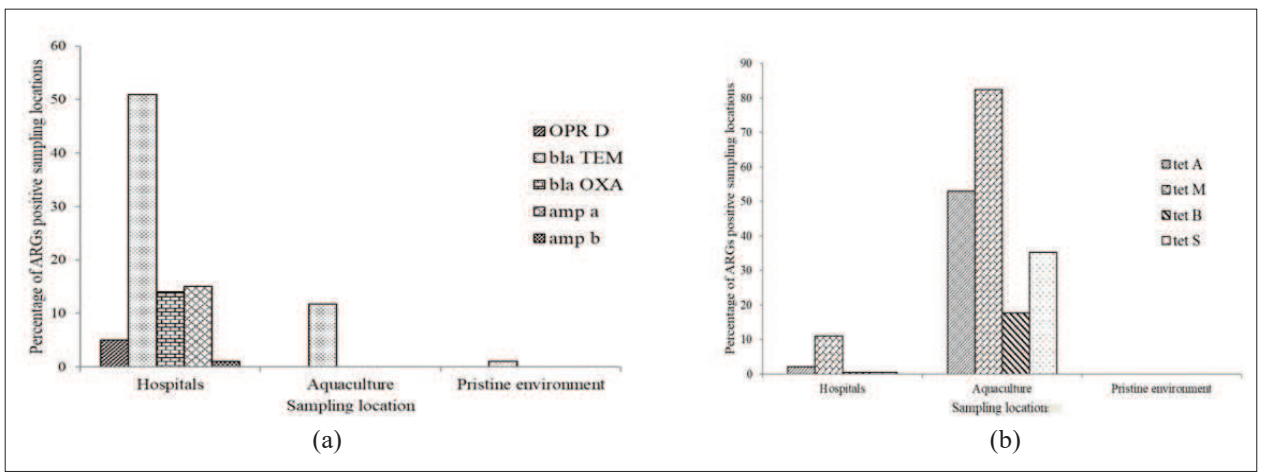

Figure 3: - Penicillin (a) (*bla* TEM, *bla* OXA, *OPR* D, *amp* a, *amp* b) and tetracycline (b) (*tet M*, *tet S*, *tet A*, *tet B*) resistance gene percentages at the wastewater effluent in hospitals, aquaculture farms and pristine environment

Quantification of Antibiotic Resistance Genes (ARGs)

In the present study, culture-independent approaches were used to determine the occurrence and abundance of ARGs. Out of five 5 genes which were initially investigated during the pre-screening process in the wastewater of 80 hospitals, three genes were selected from each resistance mechanism (changes in penicillin-binding protein, secretion of b-lactamases, and decreasing porin channel formation) with high abundance in hospital wastewater samples. In aquaculture wastewater samples two genes were selected for further analysis based on detection frequency and responsible mechanism. Subsequently, these selected genes were quantified using qPCR assays. All qPCR assays showed high R^2 values (> 0.99) and high efficiencies from 96.84% to 107.71%. ARG quantification overestimates the actual gene-carrying bacteria in a sample as an indicator of environmental impact and the possibility of further dissemination of ARGs, and it may be a superior indicator compared to an accurate measure of the number of resistant bacteria (Mao *et al*., 2015).

Among the detected ARGs, the most ARGs, ranging from 7.68×10^5 copy/mL to 1.572×10^2 copy/mL, were recorded in teaching hospitals, where 7.26×10^5–1.24×10^2 copy/mL, 7.0057×10^4– 8.00 copy/mL and 37.2–3.4 copy/mL were detected in General Hospitals, Base Hospitals, and Divisional hospitals respectively [Figure 4(a)].

A higher absolute copy number of b-lactam resistance genes ($p < 0.05$) was recorded in Teaching Hospital (TH) effluent than in effluent water from other types of hospitals [Figure 4(b)]. According to previously published literature, the *bla TEM* gene is one of the most frequently detected plasmid-borne antimicrobial resistance genes, which confers resistance to penicillin

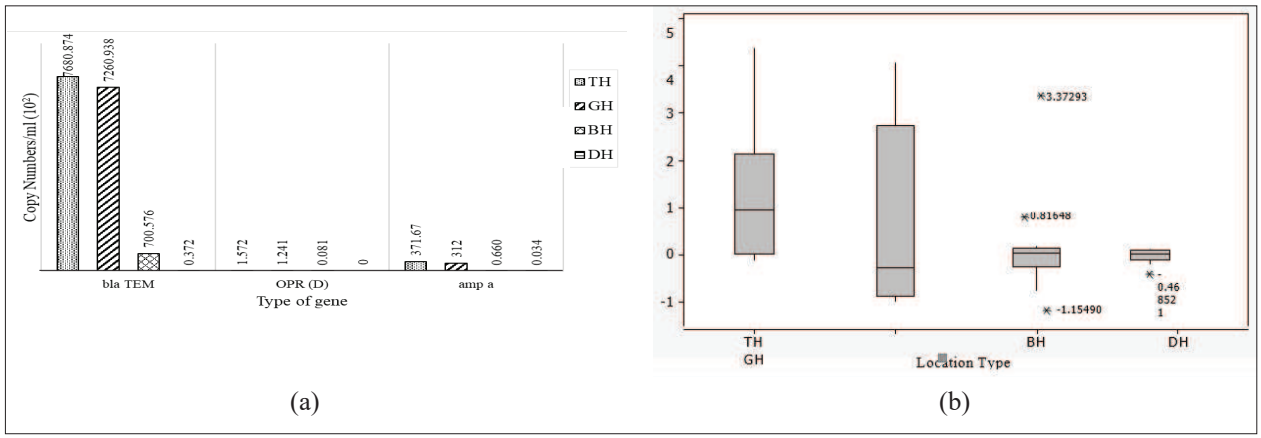

Figure 4: (a) Variations of total copy numbers of antibiotic resistance genes with hospital categories; (b) Absolute concentration of b-lactam resistance genes in the hospital effluent water samples. Within the box plot chart, the crosspieces of each box plot represent (from top to bottom) maximum, upper-quartile, median (black bar), lower quartile and minimum values, when error bars are not shown, standard deviation was less than the width of symbol.

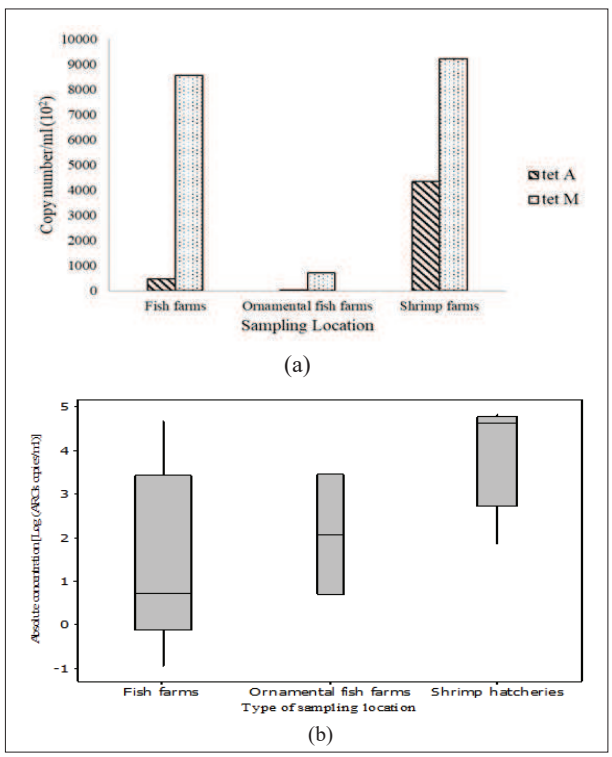

Figure 5: (a) Variations of total copy numbers of antibiotic resistance genes with farm categories (b) Absolute concentration of tetracycline resistance genes in the hospital effluent water samples. Within the box plot chart, the crosspieces of each box plot represent (from top to bottom) maximum, upper-quartile, median (black bar), lower quartile and minimum values, When error bars are not shown, the standard deviation was less than the width of the symbol

and extended-spectrum cephalosporin (Zhang & Li., 2011). The present results agree with previous studies suggesting that hospital discharges could contribute to the spread of ARGs into the aquatic environment. McCoy *et al.* (2011) observed that the abundance of *bla TEM* gene in hospitals was higher in populated urban areas than in rural areas. Czekalski *et al.* (2015) recently demonstrated that the abundance of *bla TEM* in hospital effluent was an indicator for high penicillin contamination in water. In the case of penicillin resistance genes (*bla TEM, OPR D, amp a),* detected concentrations in effluent water from hospitals may become severe, due to detecting the respective gene without detecting any of the selected penicillin (AMX, AMP, CLOX) in the water samples (Figure 4). Thus, it will be confirmed that the spreading of ARGs and ARB is not dependent on antibiotic concentration. Altogether, these observations undoubtedly demonstrate the contribution of hospital effluent water discharges to the spread of antibiotic resistance in the natural environment.

Among genes conferring resistance to tetracycline, both *tet M* and *tet A* genes were detected in shrimp farms ranging from 7.93×10^4 to 9.23×10^5 copy/mL and from 1.23×10^2 to 4.39×10^5 copy/mL respectively. The recorded concentrations were greater than the values recorded in fish farms (*tet M*- 1.1–8.560×10^5 copy/mL; *tet A*- 1.3–4.56×10^4 copy/mL) and ornamental fish farms (*tet M*- 2.2×10^2–7.23×10^4 copy/mL; *tet A*~ 3.2×10^3 copy/mL) [Figure 5 (a)].

A significant difference ($p < 0.05$) was also observed in the concentration of ARGs between resistance genes

detected in each hospital category [Figure 5 (b)]. Further, the genes; effect size estimate showed that 90–95% (χ^2) of the variability in rank scores is accounted for by location and it was not due to random events. Recorded values (1.1–9.23 × 10^5 copies/mL) were greater than the aquaculture farms (2.3–4.32 × 10^3 copies/mL) in the Philippines (Suzuki *et al*., 2013) and in Finnish sediment (3.0–2.35 × 10^3 copies/mL) (Muziasari *et al.*, 2014), but are comparable to values (~7.93 × 10^4 copies/mL) reported for aquaculture farms in Thailand (McKinney *et al*., 2010). Thus, the present results suggest that tetracycline resistance genes are persistent even in the absence of tetracycline contamination in the water.

Relationship between antibiotic concentration vs antibiotic resistance genes

The abundance of types of ARGs (Figure 3) showed a similar pattern as the corresponding classes of antibiotics, where b-lactams had the highest concentration in the hospital effluent water and tetracycline in the aquaculture wastewater.

A correlation analysis was done to determine a potential link between the absolute concentration of b-lactam resistance genes and the penicillin concentration (Figure 6).

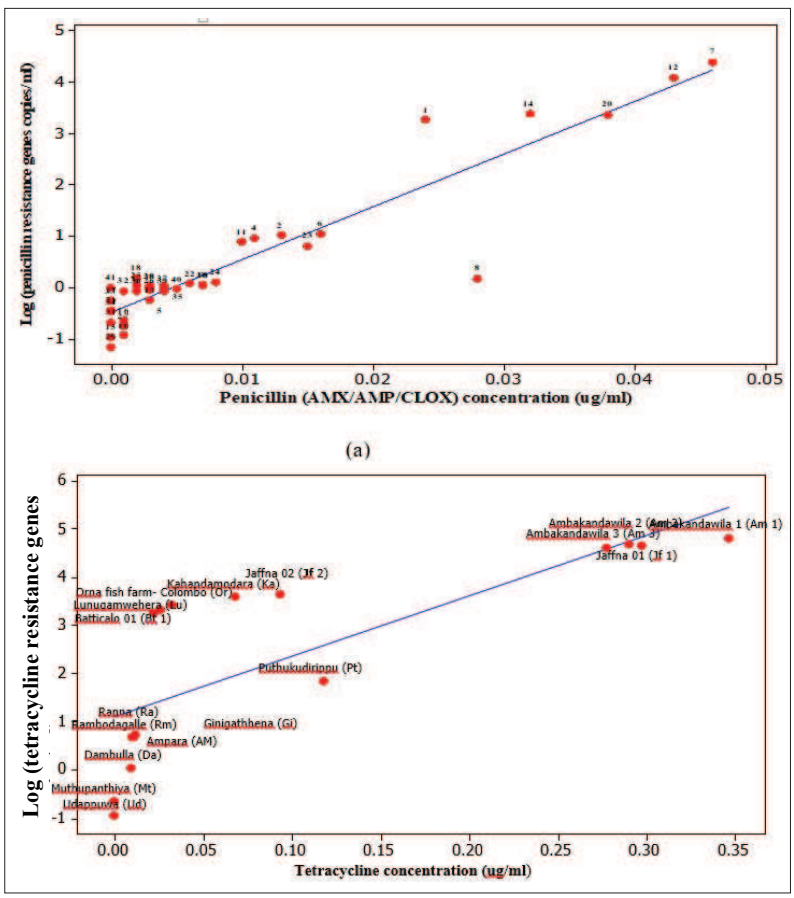

Figure 6: Correlation between the concentrations of antibiotics and antibiotic resistance genes (a) penicillin ($p < 0.05$, $R^2 = 0.889$); (b) tetracycline ($p > 0.05$, $R^2 = 0.776$). Sample locations were represented by red circles.

As a result, a significant positive correlation between penicillin concentrations and b-lactam resistance genes was found. In fact, there was a link between penicillin and b-lactamase resistance genes ($p = 0.001$, $R^2 = 0.889$). The correlations also revealed that ARGs increase with antibiotic exposure concentration (Fig. 6). However, no significant ($p = 0.052$, $R^2 = 0.776$) differences in *tet M* and *tet A* gene concentrations were found in aquaculture samples.

Furthermore, it was found that tetracycline resistance genes could be detected in the absence of tetracycline in water. These finding suggest that the increase in the prevalence of tetracycline resistance genes is caused by their persistence in the absence of selection pressure (Kim *et al*., 2012). However, the results discussed in this study are consistent with previous research indicating that antibiotic exposure may be a major factor leading to selective pressure for ARGs (Franje *et al*., 2010).

In the future, the author suggests that more exploration be conducted to determine at what concentrations antibiotic resistance is developed and disseminated, as well as how these concentrations vary in different environments with different bacterial communities and ARGs.

CONCLUSION

The present study is the first in Sri Lanka to look at antibiotics and ARGs in hospital and aquaculture effluents. The results reveal that hospital effluents, fish farms, and shrimp hatcheries are ARG reservoirs, as well as showing the presence of potential resistant strains. It is also suggested that pathogen-associated taxonomic groups in fish farms have implications for human health. The study results highlighted the significant impact of heavy and unregulated antibiotic use, as well as the discharge of untreated wastewater into the aquatic environment, which could lead to significant contamination by both antibiotics and ARGs.

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