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Correlation between Antibiotic Concentrations and Antibiotic Resistance Genes Contamination at Mafisa Wastewater Treatment Plant in Morogoro Municipality, Tanzania

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Abstract

Various antibiotics have been used for treating infectious diseases in humans, animals and aquaculture. Also antibiotics have been used as growth promoters in livestock production. After administration part of antibiotics is excreted as original compounds or metabolites through urine and faeces in environment, hence creating selection pressure which can lead to development of antibiotic resistance genes (ARGs). In this study the correlations of sulfonamides and tetracycline concentrations with their corresponding selected antibiotic resistant genes sul1, sul2 and tetM respectively was investigated at Mafisa wastewater treatment plant in Morogoro municipality.

The mean concentrations of tetracycline was 48.761 ± 7.9343,sulfonamides 18.7492 ± 5.4906 and quinolones 27.2753 ± 2.83878 in influents while effluent mean concentrations was 34.8635 ± 5.17469 tetracycline, 3.3136 ± 1.35145 sulfonamides and 24.1841 ± 2.14841. Mafisa wastewater treatment plant was contaminated with corresponding antibiotic resistance genes sul1, sul2 and tetM, but there was no correlations between antibiotic concentrations and corresponding antibiotic resistance genes (ARGs), suggesting that targeted ARGs are spread in a wide area without connection to the selection pressure.

Keywords: Antibiotics; Antibiotic-resistance genes; Sul1; Sul2 and tetM; Mafisa wastewater treatment plants; Tanzania

Introduction

Antibiotics have played an important role in treatment and preventing diseases in medical industries since penicillin was introduced by Fleming. Antibiotics are also used as growth promoters in livestock production. Antibiotics have been overused in preventing and treating infectious diseases in humans and animals. For example, approximately 210 million kilograms of antibiotics were used annually in the USA for human or agriculture use [1]. Antibiotics are only partially absorbed after administration [2]. Eventually, original compounds or metabolites are excreted into environment systems through urine and faeces.

These released residues generate selective pressure to the bacteria in the environment, thus contributing to the proliferation of antibiotic resistance genes (ARGs) and antibiotic-resistant microorganisms. The occurrence of antibiotic resistance changes the composition and structure of microbial community and increases the potential risks to human health and the environment [3,4]. Once microorganisms acquired resistance can compromise the effectiveness of antibiotic therapy.

Tetracycline and sulfonamides are the most widely used in treating infectious diseases. They have been used to treat human diseases for longer history. The same classes of drugs have shown cross-resistance characteristics. Currently more than 40 different tetracycline resistance genes have been observed in various environment systems [5]. Tetracycline resistance genes are divided as efflux pump genes (tetA, tetB, tetC, tetD, tetE and tetL, ribosomal protection protein genes (tetO, tetW, tetM and tetQ) and modification enzyme gene (tetX). The sulfonamides resistance genes include mainly sul1, sul2, sul3, and sulA.

The occurrence and spread of ARGs is closely associated with diverse resistance mechanisms and dissemination mechanisms. The plasmids, transposons and integrons

contributed to the increase of type and concentration of ARGs in various environments. The relationship between integrons and different ARGs were previously studied by Belinda and Zang et al. [6,7]. Wastewater treatment plants are considered as hotspots of antibiotic residues as well as normal human microbial and pathogenic bacteria that are introduced into ecological systems. These environmental factors inevitably create an advantageous condition for the enrichment and dissemination of antibiotic resistant microorganisms and ARGs. Thus wastewater treatment plants have high potential to be a threat to human and animal health because of its infectious and toxic characteristics.

Antibiotics and antibiotic resistant microorganisms are released to the environment from hospitals, livestock facilities and sewage treatment plants (STP) [8]. Although antibiotics are decomposed and diluted in the aquatic environment such as wastewater and rivers, but even at low concentrations they may act as signaling molecules in microbes for antibiotic resistance. Selection of ARGs mutation by very low concentrations of antibiotics was reported. It is, therefore critical to understand the fate of excreted antibiotics and ARGs in the environment, because antibiotic residual and ARGs in the environment pose a risk to humans and animals.

Materials and Methodology

Study area

Mafisa wastewater treatment plant is located in Morogoro municipality, Tanzania. It has eight ponds which are connected from entry (first) to the exit (eight). Morogoro is a town with approximately 2,218,492 inhabitants according to 2012 census. Mafisa is located next to the Morogoro river in northern part of the city, in an area with housing and farming, it receives wastewater from the city of Morogoro. The mafisa wastewater treatment system consists of two receiving ponds (gravity and track receiving pond) and six sedimentation ponds. While pond one is anaerobic pond, second pond is aerobic stabilization, ponds three to six are stabilization maturation ponds. The ponds have different functions as well as different dimensions. The dimension, flow rate and pH are summarized in **Table 1**. Sewage water is guided through Mafisa wastewater treatment plant; finally it joins the Morogoro river. During dry season, the water in the river is low; hence water from Mafisa wastewater treatment plant is used for irrigation of fields, mainly rice surrounding Mafisa and river. In rainy season the water joins the river immediately after outlet.

Table 1 The dimensions, dynamics, flow rate (Q) and pH of Mafisa wastewater treatment plant.

	1	2	3	4	5	6
Width (m)	48	59	59	59	59	59
Length (m)	72.2	133	133	133	133	133
Depth (m)	1.62	1.534	1.064	1.132	1.156	1.188
Q (m^3/sec	0.034	0.031	0.031	0.038	0.039	0.027
Volume (m^3)	5614	12037	8349	8883	9071	9322
Q (m^3/24 h)	2938	2678	2678	3283	3370	2333
Q/V	0.5232	0.2225	0.3208	0.3696	0.3715	0.2502
pH	7.4	7.2	7.6	7.5	7.8	7.3

Sample collection

Water samples were collected from eight ponds at identified sampling sites. Sampling sites included the raw influent from gravity and track receiving ponds, maturation ponds and effluent or exit pond. All samples were collected in triplicate, as grab samples in amber 2.5 L bottle. They were stored in cool box in which thermometer was included to monitor temperature, they were delivered to the laboratory within half an hour. They were processed within five hours.

Antibiotic detection from wastewater samples

Three antibiotics, quinolones, tetracycline and sulfonamides were screened. But only tetracycline and sulfonamides selected resistance genes were investigated. pH of samples was adjusted to 3.0 by using sulphuric acid. In the laboratory each sample was filtered twice, by filter paper from Munktell Company, Denmark. After filtration samples were divided into 1000 ml in amber bottle.

Solid phase extraction

Before extraction, the oasis hydrophilic Lipophilic balance (HLB) cartridges were preconditioned with 2 ml methanol, 2 ml ultrapure water, 4 ml 2 g/L Na2EDTA with pH 3.0 in sequence. 1000 ml water sample, was loaded into oasis HLB cartridges from waters (Milford, MA, USA) using a vacuum manifold and pump. The vacuum manifold was Vac Master from IST (Sweden) and pump was from Scan Vac (Denmark). After loading and running the samples, cartridges were air dried using vacuum and stored at -18°C before analysis. The analytes were eluted with 4 ml methanol. The elutes were concentrated under gentle stream of nitrogen and were dissolved in 1 ml methanol containing 0.1% formic acid. The detection of antibiotics was done by using Enzyme-Linked Immunosorbent Assay (ELISA) technique.

Reagents and standards

Standard salts of sulfonamides, tetracycline and quinolones were bought from Sigma-Aldrich (Augsburg, Germany), Ridascreen kits were bought from R. Biopharm AG (Darmstadt, Germany), methanol was bought from Sigma-Aldrich (Augsburg, Germany) ultrapure grade water was purchased from Sigma-Aldrich (Augsburg, Germany).

The basis of Enzyme- Linked Immunosorbent Assay

The basis of the test was the antigen-antibody reaction. The micro-titter wells were coated with capture antibodies directed against anti-sulfonamides antibodies, anti-tetracycline antibodies and anti-quinolones antibodies. Sample, antibiotic conjugate and anti-antibiotic antibodies were added. Free antibodies and antibiotic conjugate compete for the antibiotics antibody binding sites (competitive enzyme immunoassay). At the same time, the anti-antibiotics antibodies were also bound by the immobilized capture antibodies. Any unbound conjugate was removed in washing step. Substrate/chromogen was added to the wells and incubated.Bound conjugate converts the chromogen into a blue product. The addition of the stop solution changed color from blue to yellow. The measurement was made photo-metrically at 450 nm using ELISA reader. The absorption was inversely proportional to the sulfonamides, quinolones and tetracycline concentration in the sample.

Analytical procedure

Standard solutions used to plot standard curves for sulfonamides, quinolones and tetracycline were included in ELISA kits. Standard solutions for sulfonamides and quinolones were diluted to final concentrations ready for use. Their concentrations were 0, 1, 3, 10, 30, 100 µg/l, sulfonamides and 0, 0.5, 1.5, 3, 6, 18 µg/l quinolones. Standard solutions for tetracycline were provided as concentrates. In order to produce tetracycline standards ready for use, 50 µl standard concentrate was diluted with 450 µl sample buffer, all were included in kits. Each 50 µl of the following standard concentrates 0, 0.5, 1.5, 3, 6, 18 µg/l were diluted with 450 µl sample buffer to make 0, 0.05, 0.15, 0.3, 0.6 and 1.8 µg/l final concentrations.

Enzyme-Linked Immunosorbent Assay Reaction

Fifty microliters of each standard or prepared sample was added to micro-plate wells of ELISA plate in duplicate. Fifty microliters of conjugate was added to each well. Then fifty microliters of antibody was added to each well mixed gently, incubated for one hour at room temperature. The solution in the wells was discarded and the micro-plate was tapped three times in blotting paper to ensure complete removal of solution from wells. The wells were filled with 250 µl of washing buffer. The liquid again was poured out; the wash step was repeated three times. One hundred microliters of substrate/chromogen was added to each well, mixed gently and incubated for 15 minutes at room temperature in the dark. One hundred microliters of stop solution was added. Absorbance was read at 450 nm using ELISA reader, thirty minutes after adding stop solution. Average absorbance of duplicate samples or standards were inserted in the below formula to calculate the percentage absorbance B/B0%. The B/B0% was interpolated in calibrated standard curve to obtain the concentrations of antibiotics.

$$\frac{B}{B0}\% = \frac{\text{Absorbance standard/sample}}{\text{Absorbance at Zero standard concentration}} \times 100$$

In order to obtain the sulfonamides, quinolones and tetracycline concentrations in the samples, the B/B0% values were interpolated on the calibration standard curve built with sulfonamides, quinolones and tetracycline. Results were multiplied by two as a dilution factor to obtain final concentration.

Limit of detection (LOD) of antibiotics in wastewater was 0.05 µg/l and Limit of quantification (LOQ) was 1.6 µg/l. Recoveries of tetracycline, quinolones and sulfonamides were 80.5 to 105%, 75.2 to 91.4% and 60.7 to 83.6% respectively (**Table 3**).

DNA extraction from wastewater samples

Wastewater samples 1000 ml were filtered twice, by filter paper from munktell company, Denmark. The filters were placed in the extraction tubes of the power water DNA isolation kit (MoBio Laboratories, Inc). DNA extraction was performed following the manufacturers protocol. The quality and concentration of the purified DNA were evaluated by spectrophotometry analysis (Nanodrop, MA, USA) and 2% agarose gel electrophoresis. The purified DNA was stored at -18°C prior to real-time qPCR analysis.

Quantitative Analysis of Antibiotic Resistance Genes (ARGs)

The sulfonamides resistance genes Sul1, Sul2 and tetracycline resistance genes tetM were quantified by quantitative PCR (qPCR) from assemblage using DNA trapped on 0.2 µm pore filter as shown in **Table 2**. DNA extraction from filter was previously reported by Suzuki et al. [9]. DNA from filter was obtained from triplicate samples. qPCR was performed using a (FX96 Real-Time system 9BioRa, Laboratories, Hercules, CA, USA) to detect an increase of double-stranded DNA with an increase in fluorescence according to [9]. PCR amplifications were performed in 20 µl reaction volume containing 1X Sso Fast Ever-Green Supermix (Bio-Rad), 500 nM of each primers and 1 µl of sample DNA. qPCR was performed using a previously designed primers; bacterial 16SrRNA genes [10], sul1 [11], sul2 [12], tetM [13]. 16SrRNA gene from E.Coli K12, Sul1 from plasmid R388, Sul2 from plasmid RSF1010 [11], and tetM from PFD310 fragments [14] were used as standard for quantification.

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The qPCR program consisted of an initial denaturation of 30s at 95°C and 40 cycles of 5s at 95°C and 10s at 50°C for 16SrRNA gene and 10s at 51°C for Sul1 and Sul2 and 20s at 57°C for tetM respectively. Melting curves for the amplicons were measured by raising temperature slowly from 60°C and 65°C to 95°C for 16SrRNA gene, Sul1, Sul2, tetM respectively, while monitoring fluorescence. Each sample was measured in triplicate. The copy number of Sul1, Sul2 and tetM were normalized by dividing by the 16SrRNA gene copy number at the respective points. The results were analyzed using a Big dye terminator Kit on a 3130ABI Prism sequencer, Applied Bio systems, Foster City, CA, USA. PCR products were sequenced to confirm they were not non-specific product (**Table 2**).

Statistical analysis

Statistical analysis was performed by using SPSS 16.0 version. Correlation was used to calculate Pearson bivariate correlations and p values One-Way ANOVA was used to assess the homogeneity of variance with a significance level of 5% ($p<0.05$).

Results and Discussion

The distribution of antibiotic concentrations in mafisa wastewater treatment plant showed that tetracycline was a major contaminant along with quinolones. This suggests that these two antibiotics are frequently used and highly excreted in the study area. Also this may reflect the increased use of antibiotics by community without prescription by healthcare professional and veterinarians; hence most of metabolized and un-metabolized drugs are released through urine and faeces, ending up in wastewater treatment plant. The concentration of sulfonamides was low compare to tetracycline and quinolones, but its concentration ranged to µg/l the same way like tetracycline and quinolones. It has been reported that sulfonamides is frequently used in African countries to control bacteria and protozoan in HIV patients [15]. Some reports in Ghana, Mozambique, Kenya and South Africa showed sulfonamides is in highest concentration among screened antibiotics [16], but it was not the case in this study. Despite the use of sulfonamides being restricted in recent years for resistance and dissemination among treated patients, sulfonamides resistance genes Sul1 and Sul2 has been detected in various aquatic environments including hospital wastewater [17], livestock lagoons [18] and even fresh water rivers [19]. Sulfonamides antibiotic resistance was acquired by mutations in enzyme dihydropteroate synthase (DHPS) in the folic acid pathway Sul1, Sul2 and Sul3 were alternative DHPS genes (**Table 4**). The contamination levels of Sul1 and Sul2 were comparable to those in wastewater treatment systems of swine farms [20]. The concentrations of Sul1 and Sul2 in this study were at the same level as shown in **Figures 1 and 2**. The detection frequency of selected tetracycline resistance gene was 100% by conventional PCR analysis, and this result demonstrated the prevalence of tetracycline in mafisa wastewater treatment plant. One ribosomal protection protein tetM was selected as an object in this study. The ribosomal protection genes have been identified in various bacterial species, including gram-positive and gram negative, thus contributing to their high concentrations in different environmental systems according to previous report [21,22]. There were no correlations between the concentrations of antibiotics and level of their resistant genes in mafisa wastewater treatment plant (**Table 5**), since the resistant genes were detected in all ponds at the same level of contamination, this suggest that the selection pressure of increasing ARGs was not only from the corresponding classes of antibiotics, but from non-corresponding classes of antibiotics. The environmental behaviour and propagation mechanisms of ARGs and antibiotics in the environment probably contributed to weak correlation between them. Along with effect of microorganisms and other factors, antibiotics would gradually be degraded. However, ARGs can persistently be disseminated and migrated within the microbial community. A study done by Aktan et al. [23] found that different classes of antibiotics and heavy metals ions in the wastewater may affect the correlations between ARGs and antibiotics concentrations. Intergrons and other mobile genetic elements could play an important role in developing resistance among microorganisms by transferring various resistance genes simultaneously. Concentrations of tetracycline, quinolones and sulfonamides detected at mafisa wastewater treatment plant are summarized in **Table 2**. The three antibiotics were detected in the influent and effluent samples, but only resistance genes for tetracycline and sulfonamides were investigated. The reason why antibiotics resistance is universal concerned is that it has been increasingly difficult to treat and cure pathogen infectious diseases by antibiotic prescriptions present in the market.

Table 2 PCR primers of the ARGs used in this study.

Target	Primers	Sequence	qPCR annealing temperature	Amplicon size (bp)
etM	tetM-F	ACAGAAAGCTTATTATATAAC	55	171
	tetM-R	TGGCGTGTCTATGATGTTCAC		
Sul1	Sul1-F	CACCGGAAACATCGCTGCA	65	163
	Sul1-R	AAGTTCCGCCGCAAGGCT		
Sul2	Sul2-F	CTCCGATGGAGGCCGGTAT	57.5	191
	Sul2-R	GGGAATGCCATCTGCCTTGA		

F-Forward, R-Reverse			

Table 3 Mean concentrations of antibiotics and removal efficiency at Mafisa wastewater treatment plant. Cluster 1-Influent, anaerobic and facultative ponds, Cluster 2-maturation ponds and Cluster 3-Effluent and exit point.

Antibiotics	Cluster	Mean	Removal efficiency
Quinolones	Cluster 1	27.2753 ± 2.83878	11.33%
	Cluster 2	24.0660 ± 5.1280	
	Cluster 3	24.1841 ± 2.14841	
Tetracycline	Cluster 1	48.7615 ± 7.9343	28.50%
	Cluster 2	41.5620 ± 8.43186	
	Cluster 3	34.8635 ± 5.17469	
Sulfonamides	Cluster 1	18.7492 ± 5.4906	82.32%
	Cluster 2	10.3945 ± 1.77246	
	Cluster 3	3.3136 ± 1.35145	

Table 4 Detection of ARGs in different ponds by conventional PCR (+present, - absent, ARGs-Antibiotic resistance genes, PCR-Polymerase Chain Reaction).

ARGs			
Pond	**tetM**	**sul1**	**sul2**
P1	+	+	+
P2	+	+	+
P3	+	+	+
P4	+	+	+
P5	+	+	+
P6	+	+	+
P7	+	+	+
P8	+	+	+
Control	+	+	+

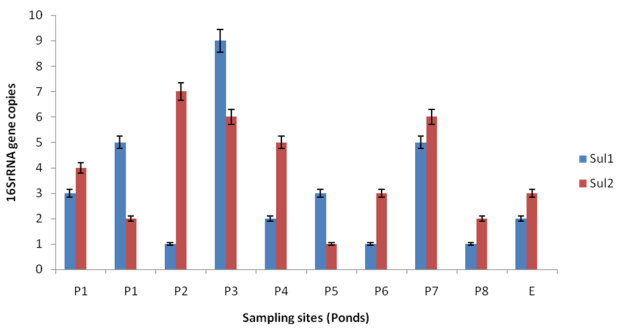

Figure 1 Relative concentrations of sulfonamides resistant genes in different ponds in mafisa wastewater treatment plant (p1-p8 means pond 1 to pond 8, E-Exit point).

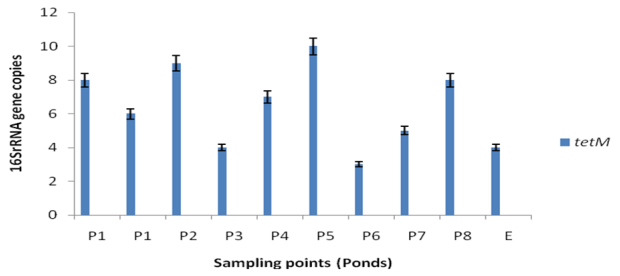

Figure 2 Relative concentrations of tetracycline resistant genes in different ponds in mafisa wastewater treatment plant (p1-p8 means ponds one to eigth, E-Exit point).

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Table 5 Correlations of antibiotics concentrations and ARG relative concentrations in different ponds at Mafisa wastewater treatment plant. *Correlation is significant at the 0.05 level. There were no correlations of antibiotics concentrations and their antibiotic resistance genes.

	Sul1	Sul2	tetM
Sulfonamides	0.638	0.611	0.114
Tetracyclines	0.503	0.471	0.356

Conclusion

In this study there was no correlation between antibiotic concentrations and their corresponding selected resistant genes in mafisa wastewater treatment plant. This suggests that antibiotic resistance genes are wide spread in the environment, hence do not depend on the concentrations level of the antibiotic to be present. This study provide basic information, more studies should be done in future by using different techniques.

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