DETECTION OF TETRACYCLINE AND OXYTETRACYCLINE RESISTANT BACTERIA AND GENES IN EFFLUENT WATER OF ZOOLOGICAL GARDEN, SRI LANKA

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Abstract- The treatment of bacterial infections is increasingly complicated due to development of resistance to antimicrobial agents. The present study records, occurrence of tetracycline (TET) and oxytetracycline (OTC) resistance bacteria in the effluent waste water of Zoological garden, Sri Lanka. Moreover, corresponding antibiotics resistance genes of the isolated bacteria were screened.Four bacterial strains which were resistant to both TET and OTC were isolated by enrichment culture method following standard pour plate. Different concentrations of OTC and TET (0 - 900 ppm) were used to detect Minimum Inhibition Concentration (MIC) of isolated bacteria using standard pour plate and 96 well plate methods. Chromosomal DNA of the bacterial isolates was extracted and the presence of resistance genes (*tetA*, *tet M*, *tet S*) were identified by Polymerase Chain Reaction (PCR). Isolated bacterial strains were identified as *Acinetobacter junii, Acinetobacter calcoaceticus, Staphylococcus aureus* and *Staphylococcus arlettae* by 16S rRNA sequencing. The MICs of the *A. junii* (OTC=480ppm, TET= 540ppm), *A. calcoaceticus* (OTC= 540ppm, TET= 660ppm), *S. aureus* (OTC =780ppm, TET= 840ppm), and *S. arlettae* (OTC= 600ppm, TET= 540ppm) were detected respectively. It was identified that *S. aureus* bear *tetA*, *tet S* and *tet M* genes where *tet A* gene was detected from*A. junii*, *A. calcoaceticus*. The resistant genes of *tet M* and *tet S* were detected in *S. arlettae*. *Acinetobacter* strains were commonly known for transmitting antibiotic resistance genes which are associated with urinary tract infections, pneumonia and wound infections. Thus, the finding of the present study shows the contamination status of antibiotics in the environment and it would lead to develop antibiotic resistance in most of the pathogenic bacteria.

Keywords- *A. junii*, *A. calcoaceticus, S. arlettae, S. aureus,* Oxytetracycline, Tetracycline, antibiotic resistance, Minimum Inhibition Concentration (MIC)

I. INTRODUCTION

The tetracyclines (tetracycline, doxycycline, minocycline, oxtetracyclin) which were discovered in 1940s, are a family of antibiotics that inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site [1]. Tetracyclines are broad-spectrum agents, exhibiting activity against a wide range of gram-positive and gram-negative bacteria, atypical organisms such as chlamydiae, mycoplasmas, and rickettsiae, and protozoan parasites [2].

The tetracycline have widely been used for the past forty years as therapeutic agent in human and veterinary medicine but also as growth promoter in animal husbandry. The favorable antimicrobial properties of these agents and the absence of major adverse side effects have led to their extensive use for human therapy and animal infectious diseases [3].

Undoubtedly the use of tetracyclines in clinical practice has been responsible for the selection of antibiotic resistant organisms. Nevertheless the use of tetracyclines and other antibiotics as animal growth promoters is becoming increasingly controversial because of concerns that this practice may be contributing to the emergence of resistance in human pathogens[4]. The increasing incidence of bacterial resistance to tetracyclines has in turn resulted in efforts to establish the mechanisms by which genetic determinants of resistance are transferred between bacteria and the molecular basis of the resistance mechanisms themselves.

The increase of TC resistance bacteria is a serious issue in recent years, not only in human clinics but also in other fields such as animal husbandry, aquaculture and etc [4]. Chronic, low-dose application of TC for the promotion of growth of farm animals, particularly cultured for food, causes an increase in the presence of TC resistant bacteria, not only in pathogenic bacteria but in commensal environmental bacteria as well [5].Three different specific mechanisms of tetracycline resistance have been identified so far: tetracycline efflux, ribosome protection and tetracycline modification.

Tetracycline efflux is achieved by an export protein from the Major Facilitator Superfamily (MFS). The export protein was shown to function as an electroneutralantiport system which catalyzes the exchange of tetracycline-divalent-metal-cation complex for a proton [6]. In Gram-negative bacteria the export protein contains 12 TMS (transmembrane fragments) whereas in Gram-positive bacteria has 14 TMS. Ribosome protection is mediated by a soluble protein which shares homology with the GTPases participating in protein synthesis, namely EF-Tu and EF-G. The third mechanism involves a cytoplasmic protein that chemically modifies tetracycline. This

reaction takes place only with the presence of oxygen and NADPH and does not function in the natural host (*Bacteroides*) [6].

The two first mechanisms are the most widespread and most of their genes are normally acquired via transferable plasmids and/or transposons. These two mechanisms were observed both in aerobic and anaerobic gram-negative or gram-positive bacteria demonstrating their wide distribution among the bacterial kingdom [7].

OTC and TET are the most common analogues of tetracycline (TC), widely used in Sri Lanka for the culture of farm animals and fish.Thus, the aim of the present study was to examine the occurrence of resistant bacteria for both OTC and TET and identify the tet (M), tet (S). tet (A) genes which are responsible to develop tetracycline resistant in effluent water from national zoological garden, Sri Lanka.

II. MATERIALS AND METHODS

2.1. Chemicals and reagents

OTC and TET standards, HPLC and Bacteriological grade chemicals were purchased from Sigma Aldrich, USA.

2.2. Sampling

The triplicate effluent water samples were collected from three sites in National Zoological garden. Water samples were filtered through 150µm plankton nets to remove debrises and stored in sterilized bottles and transported to the laboratory in ice box. Samples were kept in freezer (-20^{0}C) until further analysis[8].

2.3. Enrichment and isolation of antibiotic resistance isolates

50ml of water from each sampling sites in 100 ml erlenmeyer flasks were enriched with inoculating, TET and OTC at final concentration of 60ppm. Then the final volume was topped up to 100ml with sterile water and then flasks were incubated at 25^{0}C ± 1^0 in 100rpm for 14 days in the shaking incubator.

After 14 days of enrichment, 1ml of sample aliquots was taken from the flask for isolation and enumeration of bacteria following standard pour plate method. LB medium containing 60ppm of TET and OTC (Lauryl-Bertani (LB) medium; Tryptone, 9.1g; 4.6g, Sodium chloride, 4.6g; Yeast extract, 4.6g; agar ,13.1; per liter) were used to isolate TET and OTC resistant bacteria[8].

After three days of incubationat 25^{0}C, bacterial colonies with different morphological characters were picked up and re-suspended in liquid LB medium. Subsequently pure bacterial cultures were sub cultured and stored in agar slants at -20 ^{0}C in LB-glycerol media for further studies and identification purposes.

2.4. Determination of Minimum Inhibition Concentration (MIC)

The LB broth cultures were prepared and a loop of isolated bacterial strains were inoculated and incubated at 28^{0}C, 100 rpm overnight. The turbidity of the bacterial suspensions was equalized using McFarland.

The 96 well plates were used to determine the MIC of antibiotic for each isolate. A well in the plate contained optimal density adjusted bacterial suspension (10 µl), LB broth and antibiotic (60ppm-900ppm). Each bacterial strain tested against TET and OTC in triplicates.

The plate was incubated at 25 ^{0}C and absorbance was taken at 0 and 24 hours incubation using an ELISA plate reader (Thermo Scientific, USA) at 595nm[9]. The positive control wells in the plate contained bacterial isolates and LB brothwhere as negative control contained antibiotic, LB medium and sterilize saline water.

2.5. DNA extraction from isolates and PCR amplification

Genomic DNA was extracted following the modified method of Kim et al (2004) [10]. A PCR mixture contained 0.5µl of each primer(10µm), 5 µl Go taq reaction buffer, 0.5 µldNTPs, 2.0 µl from 25 mM MgCl2 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. PCRamplification was performed with BIOLAB PCR system (BYQ6078E-757, China). An optimized conditions used for the primers were shown in table 1.

Table 1: Optimized PCR conditions for *tet (A)*, *tet (M)* and *tet (S)* genes

Primer pair	Sequence	PCR annealing temperature (^{0}C)	Amplicon size (bp)
Tet A-FP *Tet A*-RP	GCGCGATCTGGTTCACTCG AGTCGACAGYRGCGCCGGC	58	164
Tet M - FP *Tet M*- RP	GTTAAATAGTGTTCTTGGAG CTAAGATATGGCTCTAACAA	48	657
Tet S- FP *Tet S*- RP	CATAGACAAGCCGTTGACC ATGTTTTTGGAACGACAGAG	46	667

2.6. Identification of OTC and TET resistance bacteria

The bacteria isolates were subjected to gram stain and biochemical tests for tentative identification. Confirmations of the bacterial strains were done by 16S rRNA gene sequence analysis.

III. RESULTS AND DISCUSSION

Human and animal pathogenic and potentially pathogenic bacteria are constantly released with wastewater into the aquatic environment. Many of bacteria harbor antibiotic-resistance genes, eventually inserted into genetic mobile platforms (plasmids, transposons, integrons) able to spread among water and soil bacterial communities as well.

Several studies have suggested that increasing use of antibiotics has promoted the dissemination of antibiotic resistant bacteria and resistance genes in the localized natural environment (Popowska et al., 2012).Chee-Sanford et al., (2001) observed that fecal waste from animals could be a major source of resistant bacteria to various environments.

Presentstudy examined the occurrence of TET and OTC resistance bacteria in effluent water taken from national zoological garden in Sri Lanka and their MIC for tested antibiotics.

The sampling sites in present study do not directly receive antibiotics, but contaminated via waste water which discharged from animal cages and fecal matters. The OTC and TET contamination levels of effluent water were recorded as 0.007 ±0.043ppm and 0.003 ±0.002ppm respectively [8]. Thus, there is a possibility to occurrence of OTC and TET resistance genes in environment.

The isolated OTC and TET resistance bacteria were identified as *Acinetobacter junii, Acinetobacter calcoaceticus, Staphylococcus aureus* and *Staphylococcus arlettae.*

Acinetobacter spp. are aerobic gram-negative coccobacilli commonly present in soil and water as free-living saprophytes. Infections associated with *Acinetobacter* spp. include ventilator-associated pneumonia, skin and soft-tissue infections, wound infections, urinary tract infections, peritonitis, secondary meningitis and bloodstream infections [13]. Their optimism was shaken by the emergence of resistance to multiple antibiotics among such pathogens as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis* [14].

3.1 Minimum Inhibition Concentrations (MIC)

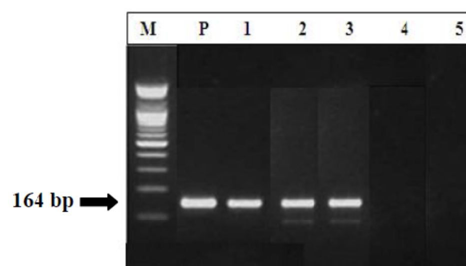
Table 2:MIC of OTC and TET resistance bacteria

Name of isolate	MIC	
	OTC	TET
A.junii	480ppm	540ppm
A. calcoaceticus	540ppm	660ppm
S. aureus	780ppm	840ppm
S. arlettae	600ppm	540ppm

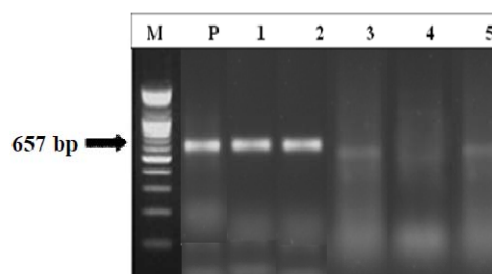
The MIC values for the bacteria isolates were shown in table 2. Each isolate had MIC greater than 420ppm for both TET and OTC. Among 4 isolates from TET and OTC resistance, *S. aureus* had MIC greater than 700ppm for both TET and OTC.

In particular, over use of antibiotics in human therapy as well as in veterinary therapy is probably a major source of antibiotic-resistant organisms and antibiotic-resistancegenes released into the environment.

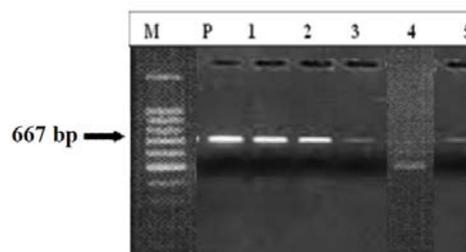
3.2 Detection of tet (A), tet (M) and tet (S) genes in isolated bacteria



(a). Presence of *tet(A)* gene; 1: *A.junii*, 2: *A. calcoaceticus,* 3: *S. aureus*4: *S. arlettae* 5: Negative control



(b). Presence of *tet(M)* gene; 1:*S. aureus,* 2: *S. arlettae,* 3:*A.junii* 4:*A. calcoaceticus* 5: Negative control



(c). Presence of *tet (S)* gene; 1: *S. aureus*, 2: *S. arlettae*, 3: *A.junii* 4: *A. calcoaceticus* 5: Negative control

Figure 1: Presence of *tet (A), tet (M)* and *tet (S)* genes in resistance genes. (a) *tet (A)*-167bp; (b) *tet (M)*-657bp ; (c) *tet (S)* – 667bp (M-100bp ladder, P-positive control)

It was detected that *S. aureus* bear *tet A*, *tet S* and *tet M* genes.One of the resistant gene (*tet A*) was detected in the isolates of *A. junii*, *A. calcoaceticus* where two resistant genes (*tet M* and *tet S)* were detected in *S. arlettae*.

The previous studies showed that 21-96% of TC bacteria possessed the *tet (M)* and *tet (A)*genes, suggesting that *tet (M)* and *tet (A*) are a significant contribution to the TC mechanism in environmental bacteria. The possessing rate of tet(M) and tet (A) were almost the same between isolates from culture plates with high and low concentrations of TC, suggesting that *tet (M)* and *tet (A)*possessing rate was not depend on TC level [15].

The bacterium *S. aureus* bear all three genes and show highest MIC values (OTC=780ppm, TET = 840ppm) for both TET and OTC respectively. The present study showed that among the 4 isolates *S. aureus* showed more resistance for TET and OTC respectively.

The long-term dispersion of liquid manure on fields may result in serious contamination, especially when certain antibiotics, such as tetracycline, accumulate in the environment. Previous studies reported, that a broader range of bacteria including *Vibrio* sp., *L. garvieae* and *P. damsela* and subspecies in *Staphylococccus*with *tet (M)* and *tet (A)* in diseased fish [16]. Widespread presence of tetracycline resistance genes is thought to be due to broad-host-range conjugative plasmids and transposons that may play a significant role in dissemination of resistance among clinically important species [7].

The impact of antimicrobial drugs administered to animals in terrestrial and aquatic environments depends not only on the used amount and the type of administration, but also on animal husbandry practices, metabolism within the animal, manure handling and storage and degradation rates in it. Also the long-term dispersion of liquid manure in environment may result in serious contamination, especially when certain antibiotics, such as tetracycline, accumulate in the environment [4].

Antimicrobials released into the environment can enhance the formation of single, cross- and even multiple resistances in pathogens, commensal and environmental bacteria [17, 18]. The role of antimicrobials in the development and maintenance of single or multiple antibiotic-resistant bacterial populations, especially pathogenic bacteria, is still a subject for further studies.

Therefore, further studies are necessary to fully understand of the distribution mechanisms of tetracycline resistance genesin environment.

ACKNOWLEDGEMENT

The study was financially supported by the University of Sri Jayewardenepura, Sri Lanka under the research grant no. ASP/06/RE/SCI/2013/20.

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