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IDENTIFICATION, CHARACTERIZATION AND DETERMINATION OF MULTIPLE ANTIBIOTIC RESISTANCE (MAR) BACTERIA IN NILE TILAPIA (*O. NILOTICUS*)

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ABSTRACT

Concurrent resistance to antibiotics of different structural classes has arisen in a multitude of bacterial species which causes complicating the therapeutic management for both human and animal pathogenic diseases. The objectives of the study were to isolate and characterize antibiotic-resistant bacteria from Nile Tialapia (*O. niloticus*) and to determine Multiple Antibiotic Resistance (MAR) index of against; Tetracycline (TET), Ampicillin (AMP), Amoxicillin (AM.X), Sulfamethaxasol (SMX), Erythromycin (ERM), Cloxacillin (CLOX), Azythromycin (AZY) and Ciprofloxacin (CIP). The total of 165 antibiotic resistance bacteria strains from the intestine (75) and the gills (90) of Nile Tialapia was isolated. The highest antibiotic resistance of the bacteria was found against TET (44%) where the least resistance was recorded against CIP (2%), CLOX (1%), and AZY(1%) respectively. The *Bacillus* sp. was the dominant intestinal bacteria representing 40% and *E. coli* (20%) were recorded as the dominant and co-dominant bacteria in the gills. More than 50% of bacteria isolates exceeded the 420 ppm of MIC of the tested antibiotics except CIP, CLOX and AZY. The calculated MAR index ranged from 0.11 – 0.58, suggesting that the Nile Tilapia (*O. niloticus*) plays as a reservoir for antibiotic-resistant bacteria and may create health risk in human.

KEY WORDS : Nile Tilapia (*O. niloticus*), bacteria, antibiotics resistance, Minimum Inhibition Concentration (MIC), Multiple Antibiotic Resistance (MAR)

INTRODUCTION

FAO (2010) asserted that fish contributes about 60% of the world supply of protein demand and about 60% of fish production is from the developing world. In FAO report 2010, stated that 30% of protein to the world is supplied by the fishery industry. Though fish are generally regarded as safe nutritious protein food, fish, from marine and the freshwater environment was recorded potential sources of medically important zoonotic diseases. The continuous pollution in the aquatic environment has caused potential increases in infectious and non-infectious diseases (Raaijimakers and Mazzola, 2012; Manage, 2018). Recent studies have revealed that usage of a wide variety of non-degradable antibiotics in large amounts, has led to a growing health problem for humans and animals (Manage and Liyanage, 2019).

The total antibiotic consumption in the world has increased by more than 40% (approximately 50 billion to 70 billion Standard Units) from 2000 to 2012 (WHO, 2015), and the total human consumption of antibiotics in Asian countries especially in China and India have increased by nearly 30% compared to European countries, USA and UK during 2010 to 2014 (WHO, 2015; Manage and Liyanage, 2019). The same antibiotics used to treat infectious diseases in animal farms, in poultry, swine, and cattle farms to prevent diseases (FAO,

2010), and as growth promoters have also been recorded. It has been estimated that the usage of antibiotics for farm animal therapeutic purposes was greater than the number of antibiotics used for the entire world human population to prevent and treat infectious diseases in the world (FAO, 2010).

Most of antibiotics persist in the sediment of the aquatic environment for long time allowing administration in fish and aquatic organisms in aquaculture and animal farm industry (Liyanage and Manage, 2016; Manage and Liyanage, 2019). Thus, the uncontrollable uses of antibiotics have posed in the emergence of antibiotics resistant bacteria in aquatic environments that will pose the increase of antibiotics resistance in fish against bacteria pathogens (Liyanage and Manage, 2015).

Acquisitions of Multiple Antibiotic Resistances (MARs) in the cultivated fish (Verraes *et al.,* 2013; Zhang *et al.,* 2015), MARs in bacteria isolated from fish, fish products and cropped from tropical water environments (Efuntoye *et al*., 2012) showed the antibiotic resistant bacteria could enter the humans body via unsafe handling of fish (Knapp *et al.*, 2012) and consumption of poorly cooked wild fish (Knapp *et al.*, 2012). The antibiotic resistance, increased frequency with treatment failure and severity of infection, lead to having a prolonged duration of illness, increased frequency of bloodstream infections and increased number of hospitalization has also been recorded in any parts of the world (Tiamiyu *et al.*, 2011).

In Sri Lanka, contamination status of antibiotics and ARGs are poorly understood and the recent study has been shown that TET (0.056 ± 0.001µg/ml) and OTC (0.234 ± 0.014 µg/ml) concentrations in shrimp hatcheries were greater than that in food fish farms (TET, 0.008 ± 0.012 µg/ml, OTC, 0.221± 0.012 µg/ml) and ornamental fish farms (TET, 0.009± 0.011 µg/ml, OTC, 0.031± 0.005 µg/ml) (Liyanage and Manage, 2016). Limited studies on the Antibiotic-Resistant Bacteria (ARB) in the wild fishes have been recorded in the world as well (Shah *et al.*, 2012; Torrissen *et al.*, 2013).

Thus, the aims of the present study were to screen and identify ARB following to determine the Multiple Antibiotic Resistance (MAR) index of the bacteria isolated from gills and intestinal tract of the Nile Thilapia (*O. niloticus*). According to the authors' knowledge, this is the first report on the isolation and characterization of ARB from Nile Tilapia and the screening of MDR for some selected therapeutic antibiotics in Sri Lanka.

MATERIALS AND METHODS

Sampling

Nile Tilapia (*O. niloticus*) samples in three different size classes were directly purchased from 15 fishing landing sites in Anuradhapura, Polonnaruwa and Hambanthota districts in Sri Lanka from February to April in 2017. Sizes were selected as large (> 20 cm), medium (15 cm-20 cm) and small (10-15 cm) according to the guidelines given by FAO (2010).

Processing of samples

Fishes were washed with sterile 0.9% saline water. Gills and intestine of each fish were aseptically removed and placed on sterile labeled Petri dishes as individual basis respectively.

Isolation of antibiotic resistance bacteria

Gills and intestine samples were treated with antibiotic; TET, AMP, AMX, SUF, SDI, TMP, CLOX, CIP, GEN, AZY, ERM at the final concentration of 60 ppm in 10 ml of sterilized 0.9% saline water Erlenmeyer flasks (Manage *et al.,* 2009; Liyanage m Manage, 2014). The flasks were incubated in 100 rpm at $28^0C \pm 1^0$ for 14 days. Standard modified pour plate method was performed to isolate and enumerate ARB using the Lauryl-Bertani (LB) medium (Liyanage and Manage, 2016). To enumerate ARB, filter-sterilized (0.2 mm) antibiotics; TET, AMP, AMX, SUF, SDI, TMP, CLOX, CIP, GEN, AZY, ERM at the final concentration of 60 µg/ml was spiked to each molting agar media (Liyanage and Manage, 2015). The colony appeared on agar plates were counted after 3-5 days of incubation at 28 ^0C as ARB antibiotic-resistant bacteria (CFU/ml) for the respective antibiotics.

Antibiotic susceptibility test

The cell density of the bacterial suspensions was equalized using McFarland No 0.5 (Liyanage and Manage, 2014; Liyanage and Manage, 2016). The MIC was determined by using an agar dilution method following CLSI guidelines (CLSI. 2015).

DNA extraction from isolated bacteria

Exponentially growing cells were harvested and then, the genomic DNA was extracted following Kim *et al*., (2004). Purified DNA was suspended in 30 mL of TE buffer and stored at -20 ^0C.

Identification of antibiotic resistance bacteria

A total volume of 200 µL of gDNA product was sent

to Macrogen, Korea for sequencing and DNA sequences were analyzed by the Basic Local Alignment Search Tool at the National Center for Biotechnology Information website (NCBI, http://www.ncbi.nlm.nih.gov/).

Determination of Multi-Antibiotic Resistance (MAR) bacteria

According to the CLSI (2013) guidelines, multidrug resistance was defined as resistance to two or more classes of antibiotics. Liquid bacteria cultures were prepared and equalized using McFarland No 0.5. MAR against TET, AMP, AMX, SUF, SDI, TMP, CLOX, CIP, GEN, AZY and ERM at the final concentration of 60 ppm was determined using 96 well plate method and absorbance were taken at 0, 12, 24 and 48 hours interval at 595nm. Accordingly, the MAR index was calculated using the following formula (CLSI, 2013).

MAR index = Number of antibiotics which are the isolate was resistant

Number of antibiotics which are the isolate was exposed

RESULTS AND DISCUSSION

Antibiotics have lower bioaccumulation and higher mobility potential in soil and water than pesticides (Jorgensen and Turnidge, 2015) and lead to the development of antibiotic resistance in bacteria (Verraes *et al.*, 2013). The results of the present study showed that antibiotic resistance in the bacterial strains isolated from fish gills and intestine is different in the different size classes of fish. Bacteria isolated from gills and intestines of large size classes fish (>20 cm) showed greater antibiotic resistance than the other two sizes classes studied (15-20 cm, 10-15 cm) (Fig 1 A and B).

The results of the study showed that viable count of bacteria (CFU ml^{-1}) was greater in non-antibiotic supplemented control plate compare to the antibiotic supplemented plates in all the gills and intestine samples (Figure 1).

Among the three size classes, viable resistance bacteria counts (CFU ml^{-1}) from gills in the large size class of fish was ranged from 0.3×10^{4}- 3.7×10^{4} where the medium size class was from 0.2×10^{4} to 2.1×10^{4} respectively. The lowest number of resistant bacteria was recorded in a small size class of fish (0.3×10^{4} to 1.8×10^{4} CFU ml^{-1}) (Figure 1 A). The viable counts of resistant bacteria in gills (0.2×10^{4} - 3.7×10^{4} CFU ml^{-1}) were greater than those bacteria recorded from intestinal content (0.1×10^{4}- 2.1×10^{4} CFU ml^{-1}). In the intestinal, the highest number of bacteria was detected from the large size class fish (0.1×10^{4}- 2.1×10^{4} CFU ml^{-1}) where the lowest was in small size class (0.1×10^{4} -0.9×10^{4} CFU ml^{-1}) of fish

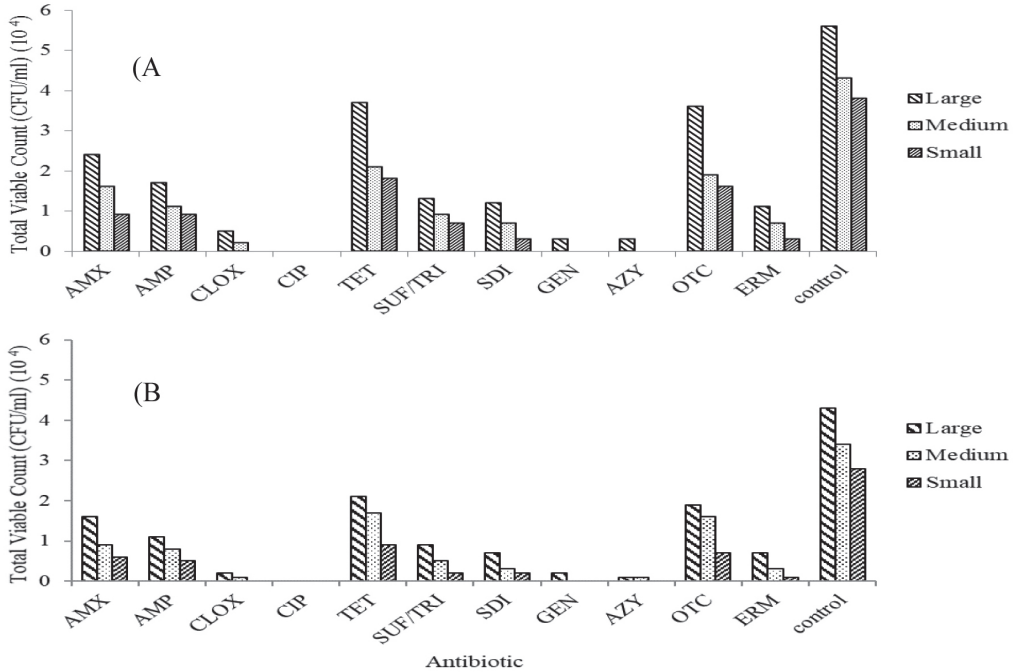

Fig. 1. Abundance of Total Viable Counts (TVC) of antibiotic-resistant bacteria in different size class of fish (A) TVC of bacteria in Gills (B) TVC of bacteria in intestine samples. When error bars are not shown, the standard deviation was less than the width of the symbol

(Figure 1B). The detection of a lower number of resistant bacteria in intestinal content may due to some selective action of acids secreted by the gastrointestinal tract of fish (Miranda and Zemelman, 2001). Several studies have suggested that intestinal bacteria may nutritionally beneficial to fish (Ray, 2016; Guardiola *et al.,* 2017) or that they participate in preventing colonization of fish pathogenic bacteria in the intestine. Therefore, the selective pressure exerted by antibiotics in water might provoke changes in the intestinal microflora with unexpected consequences on fish health.

The number of tetracycline (TET) and oxytetracycline (OTC) resistance bacteria from gill (1.8×10^{4}- 3.7×10^{4} CFU ml^{-1}) and intestine (0.9×10^{4}- 2.1×10^{4} CFU ml^{-1}) was high, whereas Cloxacillin (CLOX), Ciprofloxacillin (CIP) and Azithromycin (AZM) from the same samples were found to be significantly low ($p < 0.05$) (Figure 1). A moderate proportion (30-40%) of bacteria isolated from the gills and intestine of the fishes were resistant to Ampicillin (AMP), Amoxicillin (AMX), Sulfanomide (SUF), Sulfadiazine (SDI) and Erythromycin (ERM) (Figure 1).

Out of 165, antibiotic resistant bacteria isolates, 75 were recorded from intestines and 90 from gills of three different size classes of Nile Tilapia. Bacillus sp. was the most abundant intestinal bacteria representing 40% from the total following *E.coli* (23%), *Lactobacillus* sp. (12%), *Enterobacter* sp. (10%), *Streptococcus* sp. (8%), *Vibrio* sp. (6%) and *Klebsiella* sp. (1%) respectively (Figure 2a).

Aeromonas was found to be the dominant bacteria genera in gills of the fish representing 30% to the total following of *E. coli* (20%), *Streptococcus* sp. (18%), *Pasteurella* sp. (10%), *Lactobacillus* sp. (6%), and *Moraxella* sp. (3%), respectively (Figure 2b).

Plasmids-mediated resistance to antibiotics by fish pathogens; *Aeromonas salmonicidia, Aeromonas hydrophila, Vibrio anguillarium, Pseudomonas fluorescences, Pasteurella piscicidia*, *Edwardsilla tarda* (Brown *et al.,* 2012) and *Yersinia ruckeri* (Robert *et al.,* 2015) revealed that the exposure of the aquatic animals to low doses of antibiotics for chronic exposure or transfer will enhance the resistance genes leading the development of antibiotic resistance (Brown *et al.,* 2012; Robert *et al.,* 2015; Liyanage and Manage, 2016). Thus, the determinants of antibiotic resistance that have emerged and selected in the aquatic environment

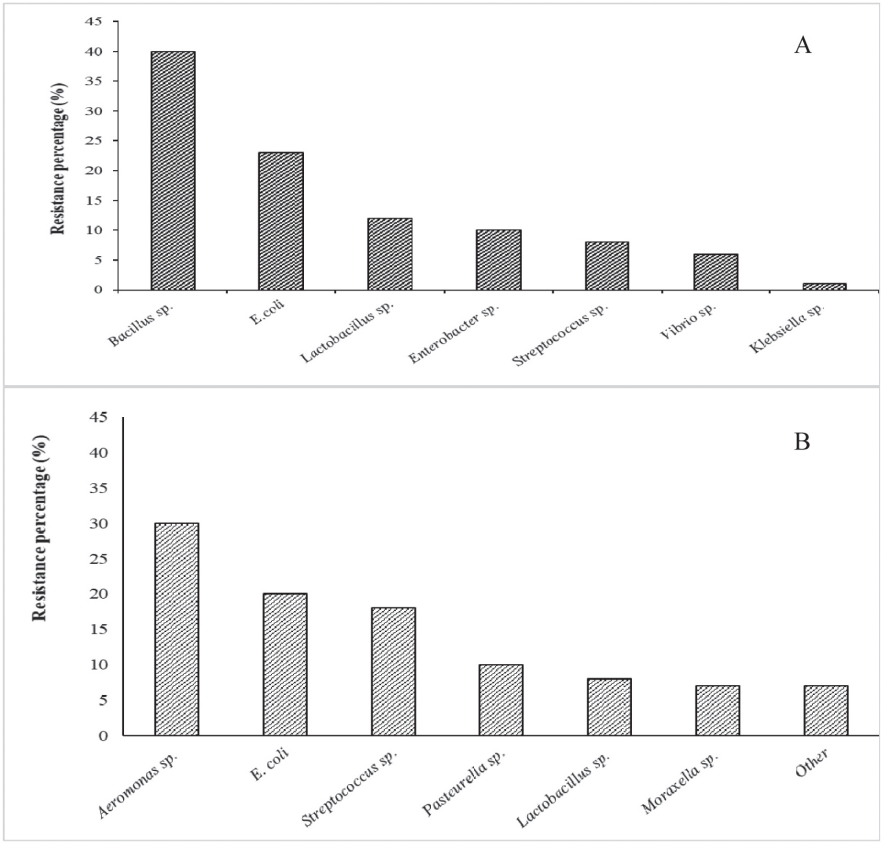

Fig. 2. Percentage of different bacteria isolated in; (a) Intestine samples (b) Gill samples

have the potential of being transmitted horizontally to pathogenic bacteria in the terrestrial environment to become human and animal pathogens (Pruden *et al.*, 2013; Manage, 2018; Manage and Liyanage, 2019).

The horizontal gene transfer mechanisms involved in exchanging resistance determinants between aquatic and terrestrial bacteria via conjugation and conjugative transposition for the development of antibiotic-resistant (Pruden *et al.*, 2013; Manage, 2018). Among the isolated resistant bacteria, 46% showed MIC range between 420-540 µg/ml where 4%, 5%, 6%, 15% and 24% of isolates having the MIC ranges from 60 to 180 µg/ml, 180-300 µg/ml, 660-780 µg/ml, 540-660 µg/ml and 300-420 µg/ml respectively (Fig. 3). The lowest MIC range (60-180 µg/ml) was found to be against the CIP, CLOX, AZY, SDI, GEN and AZY antibiotics (Fig. 2). Moges *et al.*, (2014) recorded that the *E. coli* has developed greater than 82% resistance against, ampicillin, chloramphenicol, oxytetracycline and the MIC ranged between 500-600 µg/ml. Knap *et al.*, (2012) found that the antibiotic-resistant bacteria isolated from the intestine and gills of Nile tilapia

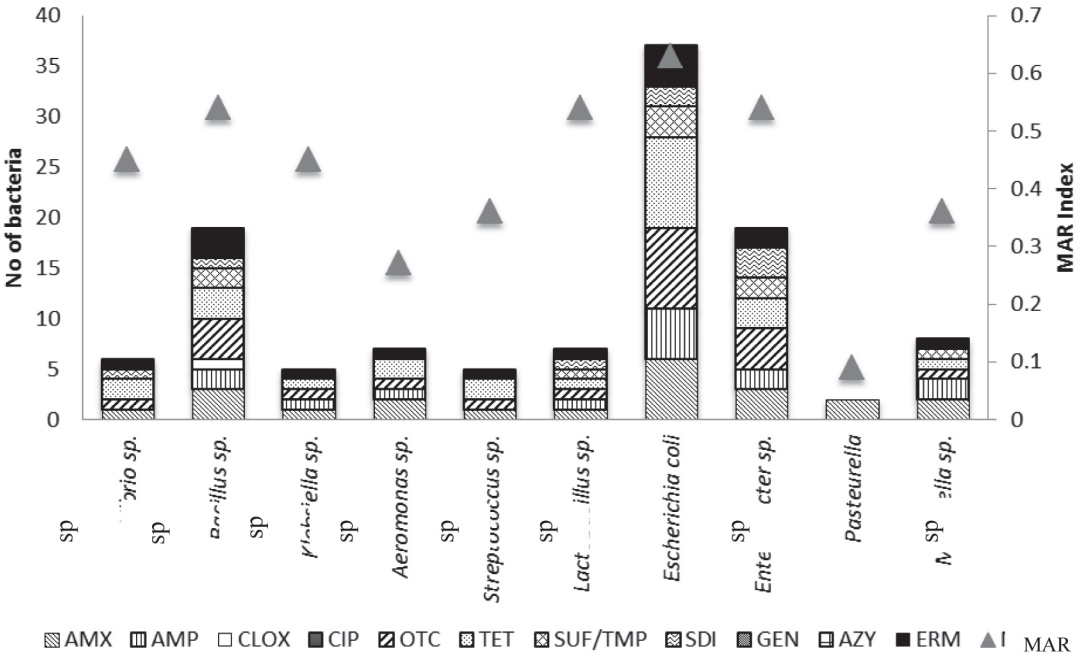

Fig. 4. Multiple antibiotic resistance of isolated bacteria against selected antibiotics and their Multiple Antibiotic Resistance (MAR) index indicated the MAR)

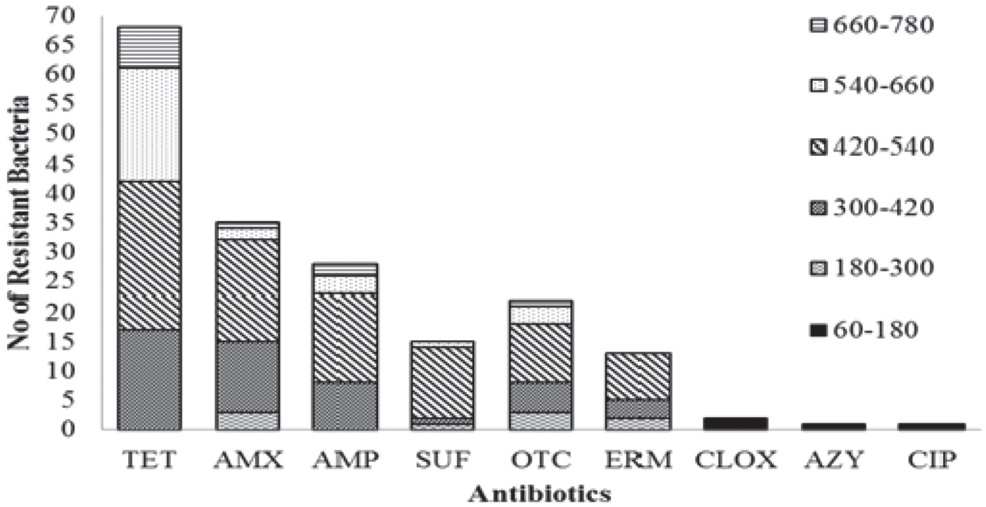

Fig. 3. MIC variations of isolated bacteria

resist against OTC and TET (480-660 µg/ml) compared to CIP (< 60 µg/ml).

MAR of bacteria in fish and fish products (canned fish etc.) were recorded from the tropical water environments (Efuntoye *et al.*, 2012). The results of the present study recorded that the MAR index was ranged from 0.09 to 0.63 where the highest MAR index was recorded for *E.coli* (0.63) following the lowest for *Pasteurella* sp. (0.08) (Figure 4). It was found that the isolated antibiotic-resistant bacteria in the present study was belonging to the different genera of the bacteria with the varying number of species; *Vibrio* sp., *Bacillus* sp. (2), *Klebsiella* sp. (3), *Aeromonas* sp. (8), *Streptococcus* sp. (1), *Lactobacillus* sp. (7), *E.coli* (7), *Enterobacter* sp. (9), *Pasteurell*a sp., *Moraxella* sp. against to the tetracycline group of antibiotics (TET and OTC). *P. aeruginosa* resisted against ampicillin (63.6%), amoxicillin (54.5%), nalidixic acid (63.6%), and oxytetracycline (72.7%) were found where the *Salmonella* sp. resist to Erythromycin (85.7%). More or less similar results were recorded by Musefiu and Olasunkanmi (2015) recording the *Aeromonas hydrophila* and *Edwardsiella* sp. were to be important fish pathogens in the warmer countries (Musefiu and Olasunkanmi, 2015). In a recent study has documented that some bacterial genera: *Bacillus, Proteus, Pseudomonas, Klebsiella, Streptococcus, Salmonella, Staphylococcus, Micrococcus, Serratia* and *Escherichia* isolated from fish and fish products cropped from feral and cultured ponds were resistance for antibiotics TET and OTC used in aquaculture (Tiamiyu *et al.*, 2011)

The number of bacterial isolate from gills of fish has high multidrug-resistance than those isolated from the intestinal tract was found. Bacteria isolated from gills (90) of Nile Tilapia showed resistance against 10 antibiotics (AMX, CLOX, CIP, SDI, GEN, OXY, TET, ERM, SUF, and AMX), where isolates from the intestine (75) remained resistance against only for five antibiotics (OXY, TET, ERM, SUF, and AMX) in the study. Approximately 40% of *E. coli* strains isolated from the intestinal tract of the Tilapia captured from reservoirs were resistant to one or more antibiotics and 56% strains isolated from gills were resistant against three to four antibacterial drugs also were recorded (Paniagua *et al.*, 2017). Moreover, seepage of the residue of the antibiotics from the surrounding environment into an aquatic environment might have resulted in the emergence of antibiotics resistance (Efuntoye *et al.*, 2012). Therefore, if food fish contaminated with antibiotic-resistant bacteria, which cause disturb the microflora of the human intestinal tract and increased the risk for certain infectious diseases (Pham *et al.*, 2015). Thus, the increased the number of treatment failure will lead to the severity of infection as a result of antibiotic resistance. This may result in the prolonged duration of illness, increased the frequency of infections (WHO, 2015) which may lead to hospitalization of high numbers of patients. Therefore, further studies need to be conducted and course of action needs to be worked out.

Therefore, the results of the present study emphasize the importance of research, especially concerning the genes which encoding resistance in different bacterial species and the possibility of the returning of resistance genes to the human through fish consumption.

CONCLUSION

The results of the present study suggest that the contamination of antibiotics residues and enteric antibiotic-resistant bacteria in the aquatic environment might influence on increasing numbers of resistant bacteria in commercial fish which facilitate the transfer resistance determinants to human pathogenic bacteria which may create ecological and public health implications. As a result of antibiotic resistance, increased the number of treatment failure will lead to the severity of infection.

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