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Erythromycin residues in flesh of Nile Tilapia

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Abstract

The use of antibiotics in food production has generated considerable interest because of the widespread administration which may lead to the development of resistant human pathogens. So, the present study was planned to estimate the antibiotic residues in fish flesh either directly through chemical analysis by LC-MS/MS. Or indirectly by using sensitivity test for the isolated bacterial strains which were identified microscopically, biochemically, and were confirmed using PCR.

A total of 75 samples of Nile tilapia were gathered from different retail markets in Kafrelsheikh governorate and were classified into three groups, according to their weight, group (A) weighted 40-100 gm, group (B) weighted 100-200 gm and group (C) weighted 200-300 gm. The results of bacterial examination revealed that the means of aerobic plate count (APC) were $2.3\times10^5\pm 0.3\times10^5$ CFU/g, $4.3\times10^5\pm0.5\times10^5$CFU/g and $3.4\times10^5\pm 0.3\times10^5$CFU/g for group A, B, and C, respectively. *Aeromonas* spp. were isolated by 16% from all samples. Sensitivity test for the isolated strains revealed that *Aeromonas* spp. were susceptible to Norfloxacin, Ciprofloxacin, Cefotaxime, and Gentamycine but resistant to Erythromycin and Amoxiclave. Also, the isolated strains had antibiotic resistance gene for Erythromycin which was detected by PCR. Finally, the detection of antibiotic residues by using LC–MS/MS analysis showed that the concentration of erythromycin were 1.15±0.16 µg/g, 0.56±0.01 µg/g and 0.48±0.05 µg/g in group (A), (B) and (C). Results showed that all samples had erythromycin residue higher than maximum residue limit (MRL) according to European Union legislation, 1990 (EU).

Keywords: *Antibiotic residue, LC-MS/MS, Aeromonas spp., Nile tilapia fish.*

Introduction

Fish is a source of high quality animal protein, containing considerable quantities of valuable lipids, minerals and vitamins and it is assumed that freshwater fish are healthy food for human nutrition **(Steffen and Wirth. 2005).**

Aquaculture is an important system of fish production, which is growing worldwide faster than any other animal food-producing sectors **(FAO, 2014 and Romero, *et al.,* 2012**). The contribution of aquaculture to total fish production has grown from 13.4% in 1990 to 42.2% in 2012 **(FAO, 2014).** And the total amount of fish produced for human consumption ranged from 5% in 1962, to 37% in 2002 and to 49% in 2012 **(FAO, 2014 & Santos and Ramos, 2016).**

Although aquaculture has many advantages, the fast growth of this production system affected fish quality and safety, in which high densities of fish in small spaces can occur, subsequently increasing the risk of spreading diseases and high mortality rates among fish **(Aaresstrup and Nørrung, 2008; Quesada, *et al.,* 2013 & Santos and Ramos, 2016).** More over dissemination of disease can occur in it due to inadequate management and poor environmental conditions, including feeding levels, removal and restocking, and inadequate nutrition **(Quesada *et al*., 2013).**

The family *Aeromonas* associated with fish illnesses **(Hayes, 2003)**. They are widely distributed in freshwater, estuarine and marine environments worldwide **(Holmes *et al*., 1996).** *Aeromonas spp.* are pathogens that cause foodborne gastroenteritis in human and extraintestinal symptoms such as septicemia, meningitis, endocarditis and osteomyelitis with

a high mortality rate in immuno-compromised persons **(Gold and Salit, 1993).**
Aeromonas species can grow and produce toxins in refrigerated conditions **(Eley *et al*., 1993).** This indicates that refrigeration cannot be effective enough to control the pathogen **(Kirov, 1993).** In spite of the fact that contaminations due to *Aeromonas* might be self-limiting, treatment with antibiotics is commonly important to control the progressing and persistence of the disease **(Albert *et al*., 2000** and **Palu *et al*., 2006).**
Therefore, antimicrobials are widely administered to food-producing animals for purposes of treatment and prevention of diseases and also for growth promotion purposes **(Quesada *et al*., 2013** and **Romero *et al*., 2012).**The extensive use of such antimicrobials can result in residues in aquatic products which are widely consumed all over the world **(Stolker and Brinkman, 2005)**. Moreover, these doses of such antimicrobials in foodstuffs may be consumed for long periods and may lead to an increase in resistant bacterial strains. The presence of such antimicrobial residues in food can be responsible for toxic effects, allergic reactions in individuals with hypersensitivity and can also result in the development of resistant strains of bacteria **(Freitas *et al.,* 2013).**
In recent years, bacterial resistance has become a worldwide concern and food-producing animals are a potential source of antibiotic-resistant bacteria in humans. As a result, there is an increasing pressure on laboratories responsible for ensuring the safety of food for human consumption **(Cháfer-Pericás, *et al.,* 2010).** To ensure human wellbeing, the European Union **(EU)** and the United States (**US)** Food and Drug Administration **(FDA)** have established safe maximum residue limits **(MRLs)** for these drugs and the use of veterinary drugs is regulated through **EU** Council Regulation 2377/90/EC that describes the procedure for establishing **MRLs** for veterinary medicinal products in foodstuffs of animal origin to ensure the quality and safety of consumer products **(CODEX, 2015; EC, 2010; Quesada *et al.*, 2013** and **Rezk *et al.,* 2015).** So, the aim of this study is to throw light on *Aeromonas* pathogen infection in freshwater fish and its antibiotic resistance genes, in addition to monitoring the presence of antibiotic residues in fish in order to allow international trade and to protect consumers from health hazards.

Materials and methods

Samples collection: A total number of 75 cultured tilapia fishes (*Oreochromis niloticus*) samples (25 samples weighting 40-100 gm, 25 samples weighting 100-200 gm and 25 samples weighting 200-300 gm) were collected from 5 different markets at different localities in Kafrelsheikh governorate. Fish were transported into sterile polythene bag from markets to Animal Health Research Institute Kafrelsheikh branch and subjected to bacteriological and biochemical examination.

1- Bacteriological examination:

1–1-Total bacterial count according to **(USDA, 1998):** Colonies were enumerated on Standard plate count agar after incubation at 37ºC for 24hrs.

1-2- Isolation of *Aeromonas species*: According to **Ashiru *et al.*, (2011)**: Five grams from the muscle of tilapia fish of each sample were weighted and macerated in sterile mortar and pestle then transferred into separate conical flasks. 45ml of alkaline peptone water were added into each sample and then incubated at 37ºC for 24hrs, then streaking on *Aeromonas* agar media to which *Aeromonas* selective supplement (**AES80004**) was added. The plates were incubated at 37ºC for 24hrs, green colonies with opaque center were selected.

1-3- Identification of isolated bacteria:

1-3-1- Microscopically: The bacteria were Gram negative rods.

1-3-2- Biochemically: The bacteria were catalase positive, urease negative, hydrogen sulphide negative and VP negative. Thus, the samples were confirmed to be *Aeromonas* spp., according to **Jatau and Yalubu, (2004).**

1–4- Confirmation of *Aeromonas* spp. by using PCR: As indicated by **Gordon *et al*., (2007).** The results of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE cradle at room temperature utilizing slopes of 5V/cm. For gel investigation, 15µl of the items were stacked in each gel opening. A gene ruler 100 bp stepping stool (Fermentas, Germany) was utilized to decide the part siz-

es. The gel was captured by a gel documentation framework (**Alpha Innotech, Biometra**) and the information was analyzed through computer software.

Table (A). Primers sequences, target genes, amplicon sizes and cycling conditions

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
16S rRNA	CTACTTTT-GCCGGCGAGCGG TGAT-TCCCGAAGGCACTCCC	953	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 50 sec.	72˚C 10 min.	Gordon *et al.*, (2007)

1.5- Antibiotic sensitivity test for isolated strains of *Aeromonas* species according to (NCCLS, 2002): Antibiotic sensitivity test was performed using disk diffusion method on Mueller-Hinton Agar (Oxoid) The antibiotics discs that applied were Norfloxacin Nor (10), Ciprofloxacin CIP (15), Gentamycine CN (10), Erythromycin E (10), Cefotaxime CTX (30) and Amoxiclave AMC (30) were dispensed on the surface of the medium and incubated aerobically at 37ºC for 18hrs. The results were recorded as resistant or susceptible by the measurement of the inhibition zone diameter.

1.6-Antibiotic resistant genes for *Aeromonas* spp.:

DNA extraction: DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200µl of the sample suspension was incubated with 10µl of proteinase K and 200µl of lysis buffer at 56°C for 10 min. After incubation, 200µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100µl of elution buffer provided in the kit.

Oligonucleotide Primer: Primers used were supplied from *Metabion* (Germany) are listed in the table (A).

PCR amplification: Primers were utilized in a 25µl reaction containing 12.5µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1µl of each primer of 20pmol concentrations, 4.5µl of water, and 6µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

Analysis of the PCR Products: The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A gene ruler 100bp ladder (Fermentas, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

2-Detection of antibiotic residues by using LC–MS/MS analysis:

2.1- Sample preparation according to Sun *et al*., (2015): Two grams homogenized muscle sample was weighed into a 35mL centrifuge tube, added 6mL 50% Acetonitrile (ACN) (ACN: H_2O=1:1, v/v), and vortexed 30s. The mixture was shaken by a multitube vortexer for 10min and centrifuged at 8000 r/min for 5min. After centrifugation, the remaining supernatant was removed to a 10mL centrifuge tube, and the residue was extracted by 4mL ACN in the same steps. Then the supernatant was combined and loaded onto a MEGA BE-NH2 cartridge previously conditional with 6 mL ACN. The extraction passed through the cartridge was evaporated under a stream of

nitrogen and redissolved with 1 mL of a mixture of ACN/ H_2O (50:50 v/v). Finally, 5 µL of the sample extract that filtered through a 0.2 µm Pall GHP Acrodisc filter (Pall Corporation, East Hill,NY, USA) were injected into the UPLC system.

2.2- Mobile phase preparation

Mobile phase A: Add 2ml of 1M ammonium formate solution and 2ml of formic acid to 996ml of HPLC-grade water.

Mobile phase B: Add 2ml of 1M ammonium formate solution and 2ml of formic acid to 996ml of HPLC-grade acetonitrile.

2.3- LC–MS/MS analysis

The analyses were carried out in the LC–MS/MS 4000 QTRAP (Applied Bioscience): Advanced Linear Ion Trap liquid chromatography was used for quantitative analysis. Liquid nitrogen and ultra-high purity (99%) argon gases used in the LC/MS/MS interface were supplied by TIG (**Bangplee, Samutprakarn, Thailand**).

Results and Discussion

The Nile tilapia is one of the most important fish species in the fisheries of tropical Africa and also from one of the most important species for 21stcentury aquaculture and is produced in more than100 countries. Tilapias are now the second most popular farmed fishes after carps in the world **(El-Sayed, 2006; Eknath *et al*., 2007; Yitayew, 2012 and Moawad *et al.,* 2017).** The global production of farmed tilapia exceeded 2,002,087 metric ton **(Yitayew, 2012).**

Results in table (1) showed the APC in groups A, B, and C ($2.3x10^5 \pm 0.3x10^5$, $4.3x10^5 \pm 0.5x10^5$ and $3.4x10^5 \pm 0.3x10^5$). The Higher finding were reported by **Ahmed and Uddin (2005)** and **Mohammed, and Hamid (2011),** while lower results recorded by **Alagoa *et al.*, (2018) and Saadia *et al., (*2017).** Our results meet the acceptable limit of an international commission of microbiological specification for food **(ICMSF, 1998)** which is ($5x10^5$-10^7).

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The presence of a high bacterial load in gills and intestine of fish might be due to the high metabolic activity of fish associated with increased feeding rates at higher temperatures.

APC is frequently used as criteria to assess the quality and safety of food and can be used as a useful indicator to predict the shelf life of raw fish **(Cahill M.M. 1990).** Fish nutrient composition and high moisture content allow the growth of a large range of microorganisms which affect the fish quality and safety rendering fish unacceptable for human consumption. The microbiological quality of raw fish results from a microbiological load of aquatic habitat, methods of capture, transportation, chilling and storage conditions **(Gram and Huss 2000).** The quality of fish degrades due to a complex process in which physical, chemical and microbiological forms of deterioration are implicated. Fish of good quality should have a bacterial count of less than 10^5cfu/g **(Mahendra *et al*., 2016).**

Table (2) showed that *Aeromonas* spp. were isolated from 12 samples with percent 16% from all samples. These results were lower than that recorded by **(Kingombe *et al*., 2004; Janda and Abbott, 2010 and Abd-El-Malek, 2017)** who isolated *Aeromonas* spp. by (20%; 31% and 36%) respectively, while these results were nearly similar to **Praveen *et al.* (2014)** who isolate it by (18.89%) and finally the present results were higher than that recorded by **Radu *et al. (*2003)** who found it by (13.13%) in this study the low percent of isolation of *Aeromonas* spp. might be due to the presence of antibiotics which control the progressing of the pathogenic organisms.

Figure (1) illustrated the positive amplification of 953 bp fragment of positive *Aeromonas* spp. by using PCR test which confirmed the biochemical identification of isolated strains of *Aeromonas* spp. From the specific media.

The genus *Aeromonas* consists of ubiquitous Gram-negative rods that are widely distributed in freshwater, estuarine and marine environments worldwide **(Holmes *et al.,* 1996).** It is also widespread in freshwater fishes and has been associated with diseases of fish

(**Gonzalez *et al.,* 1999; Wang and Silva, 1999 and Hayes, 2003).** Moreover, it is an emerging pathogen and is recognized to cause a variety of diseases in humans. This pathogen is associated with food poisoning and some human diseases as gastroenteritis and extraintestinal symptoms such as soft-tissue, muscle infections, septicemia, and skin diseases in humans **(Batra *et al.,* 2016).**

Table (3) and Figure (3) showed that the positive samples of *Aeromonas* were susceptible to norfloxacin, ciprofloxacin, cefotaxime, and gentamycine but resistant to erythromycin and amoxiclav. These results indicate the presence of erythromycin and amoxiclav resistant genes in isolated *Aeromonas* spp. **(Kirkan *et al*., 2006 and Saikot *et al*., 2013)** were found the same results of Ciprofloxacin, Erythromycin, and Amoxiclav on isolated *Aeromonas* spp., while **Sharma *et al.* (2009) and Alperi *et al.* (2008)** were found similar results of *Gentamycin* on *Aeromonas* spp. On the other hand, **Guz and Kozinska (2004)** found the same results of *Norfloxacin* on it.

The use of antibiotics is one of the most important factors influencing the emergence of resistance in bacterial pathogens. Multi-resistant *Aeromonas* spp. were isolated from different parts of the world and are reported to be resistant to penicillin and ampicillin, but sensitive to aminoglycosides, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, quinolones, and second-and third-generation cephalosporins **(Vivekanandhan *et al*., 2002 and Igbinosa *et al.,* 2012).** However, the increase in *Aeromonas* spp. resistance to antibiotics is a public health concern therefore, there should be a continuous and concerted effort to monitor the existence of this opportunistic pathogen globally **(Kaskhedikar, and Chhabra, 2010).** Antibiotic resistance frequencies and profiles varied according to the source of the strains **(Ko *et al*., 1996).**

Intensive fish farming is accompanied by a number of bacterial diseases, resulting in increased use of antibiotics. The prevention and treatment of fish diseases through extensive application of antibiotics contributes to the development of antibiotic-resistant bacterial strains **(Rhodes *et al.,* 2000),** moreover, frequent use of antibacterial drugs in aquaculture could lead to increased antimicrobial resistance and unacceptable levels of drug residues in aquaculture products and the environment **(Rahman *et al.,* 2009).**

Figure (2) illustrated the positive gene resistance for antibiotic erythromycin by using a PCR test that technique confirms the presence of the erythromycin-resistant gene in *Aeromonas* spp. As reported by **Adebayo *et al*., (2012)** the spread of resistance to antimicrobial drugs in foodborne pathogens had increased during the last decades, likely as a result of the use of these drugs in livestock raised for human consumption. In addition, **Daood, (2012)** reported that the development of antimicrobial resistance in aquatic microbial pathogens will significantly decrease the therapeutic efficiency of drugs. Furthermore, increased antibiotic resistance confers additional virulence properties to bacterial pathogens.

Aeromonas spp. carries stable plasmids that playing an important role in microbial resistance and virulence. Those plasmids are mobile genetic elements carrying microbial resistance genes and they are transmitted among bacteria of various species via horizontal gene transfer. Plasmids transfer these genes in the bacterial chromosome or may gather together multiple genes conferring resistance resulting in the emergence of additional bacterial strains resistant to several antimicrobial drugs, i.e multi-resistance **(Del Castillo *et al.*, 2013).**

In recent years, numerous bioanalytical methods have been developed to improve the sensitivity and specificity for quantification of antibiotic residue particularly as LC-MS/MS which is now routinely used in pharmacokinetic experimental and clinical laboratories **(Jin *et al*., 2014).**

Table (4) and Figure (4) revealed that in group A (fish weighted from 40 to 100 gm) the concentration of erythromycin ranged from 0.899 to 1.787µg/g with mean value of 1.15±0.16µg/g, while in group B (fish weighted from 100 to 200 gm) the concentration of erythromycin ranged from 0.515 to 0.6µg/g with mean value of 0.56±0.01µg/g and finally in group C (fish weighted from 200 to 300 gm) the concentration of erythromycin ranged from 0.269 to 0.593µg/g with a mean value of 0.48±0.05µg/

g. From the previous result, the LC-MS/MS detected only one antibiotic which was erythromycin and the means of it in all groups higher than MRL (maximum residue limit) which was 200 µg/Kg according to **European Union legislation, (2377/1990/EC). Park *et al.* (2008)** detected the MRL of erythromycin in fish as 576.2 µg/Kg, that result was nearly similar to our results in group B, lower than the results in group A and higher than that in group C. Also, **Jo *et al*. (2011)** detected the MRL of erythromycin in fish as 734.5 µg/Kg, that result was higher than that recorded in group B and C while it was lower than that recorded in group A. So the high residue of erythromycin in group (A) may be attributed to high initial dose of antibiotic for fish as prophylactic dose to decrease the general bacterial load, stop the progress of pathogenic organisms and improve the general status of fish, while that concentration of antibiotic residue began to decrease gradually by passing the time in group (B) and (C) due their metabolism and degradation in fish bodies. However, this concentration of antibiotic residue (MRL) still high at the age of marketing according to **EU, (1990)** which constitute high public health on the fish consumer.

Erythromycin is one member of macrolides which are the most effective medicine against diseases produced by many micro-organisms and have been widely used in the rearing of food-producing animals, including fish to prevent and treat diseases **(Horie *et al*., 2003).** A large increase in the demand for seafood products has occurred in the last century which led to heavy use of formulated feeds containing antibiotics, among other substances. Therefore, accurate and sensitive determination of antibiotic residues is now a necessity, in order to protect human health **(De La Pena and Espinosa-Mansilla, 2009).**

The antibiotic residues may include the non-altered parent compound and metabolites **(Cháfer-Pericás *et al*., 2010).** Because of the misuse of it, the antimicrobial residues in products of animal origin brought a concern to consumers and the residue of this kind of drugs can be directly toxic or even cause allergic reactions in some hypersensitive individuals. In addition, low-level doses of antimicrobial in foodstuff consumed for long periods can lead to the spread of drug-resistant micro-organisms **(Lopes, 2012).**

Conclusion

From this study, it was found that samples were considerably contaminated with *Aeromonas* species and this causes risks for public health, especially for an immuno-compromised person, children and aged. Hence, there is a need for public enlighten and general education to assist in controlling the outbreak of diseases in human through ingestion of the bacteria along with fish. Also, the correct identification of the infectious agent is essential for the rapid selection of antibiotic therapy. Ciprofloxacin, norfloxacin, and gentamycin are suitable antibiotics that can be used in the treatment of *Aeromonas* associated infections, in addition, using LC-MS/MS detection method for determination of different antimicrobial classes in Nile tilapia's muscle is characterized by its high trueness, precision, and sensitivity. In fact, it allows the identification and quantification of target compounds in the range of low parts-per-billion in fish farms and was a reliable tool which could be applied to analyze antimicrobials residues in fish for surveillance programs, safety monitoring research and quality control. In addition, it generated less hazardous waste and it was friendly to the environment.

Table (1). Statistical analytical results of the aeropic bacterial count (APC) of examined 3 groups (A, B, C) of raw fish samples (each of 25).

Type of groups	Total bacterial count (CFU/g)		
	Min.	Max.	Mean ±SE.
A	6×10^4	7×10^5	$2.3\times10^5\pm 0.3\times10^5$
B	1.1×10^5	8.3×10^5	$4.3\times10^5\pm 0.5\times10^5$
C	8×10^4	6.5×10^5	$3.4\times10^5\pm 0.3\times10^5$

Values as mean ±SE. Group (A) (fish weight from 40-100 gm), group B (fish weight from 100-200gm) and group C (fish weight from 200-300gm).

Table (2). Incidence of *Aeromonas* spp. in examined fish samples no. (75)

Isolated bacteria	Positive samples	
	No.	%
***Aeromonas* spp.**	12	16

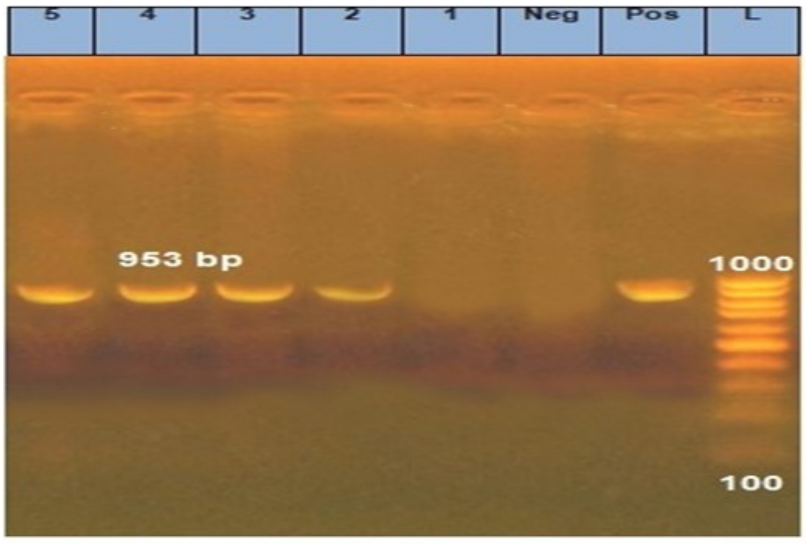

Fig. (1): Agarose gel electrophoresis of PCR amplification products of *Aeromonas* spp. isolated from fish samples. Lane L: 100bp ladder as molecular size DNA marker. Lane Pos: Control positive *Aeromonas* spp. genes. Lane Neg: Control negative. Lanes1: negative to *Aeromonas* spp. Lanes 2, 3, 4 and 5: Positive to *Aeromonas* spp. at 953 bp.

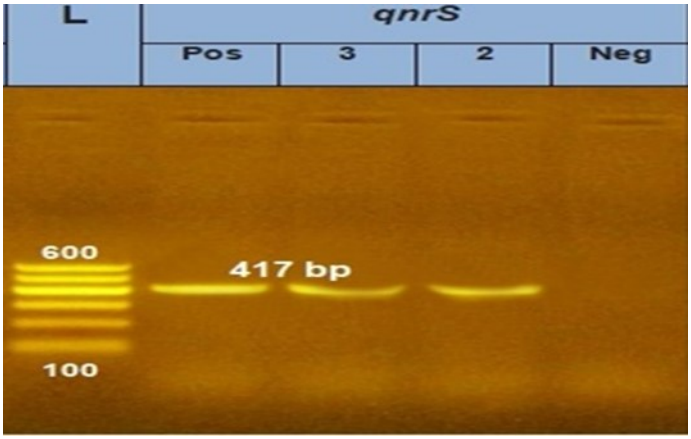

Fig. (2): Agarose gel electrophoresis of PCR amplification for gene resistance for antibiotic (erythromycin) of *Aeromonas* spp. isolated from fish samples.
Lane L: 100bp ladder as molecular size DNA marker. Lane Pos: Control positive erythromycin resistance genes. Lane Neg: Control negative. Lanes 2and3: positive erythromycin resistance genes at 417 bp

Fig. (3): Antibiotic sensitivity test results of Erythromycin against *Aeromonas* spp.

Table-3. Antibiotic sensitivity test results of selected antibiotics against *Aeromonas* spp.

Antibiotic	Zone inhibition(mm)	Sensitivity
Norfloxacin, NOR (10)	4.0	Susceptible
Ciprofloxacin, CIP (5)	3.6	Susceptible
Cefotaxime, CTX (30)	3.5	Susceptible
Gentamycin, CN (10)	2.0	Susceptible
Erythromycin, E (15)	0	Resistant
Amoxiclav, AMC (30)	0	Resistant

Table-4. Concentration levels of antibiotic (erythromycin) in examined 3 groups (n=25)

Groups	Min	Max	Mean + SE (µg/g)	*MRL (µg/g)
A	0.899	1.787	1.15±0.16 [a]	0.2
B	0.515	0.6	0.56±0.01 [b]	
C	0.269	0.593	0.48±0.05 [b]	

Means with deferent small litters in the same column are significantly different at ($p>0.05$).
*MRL (maximum residue limit) according to European Union legislation, (2377/1990/EC).

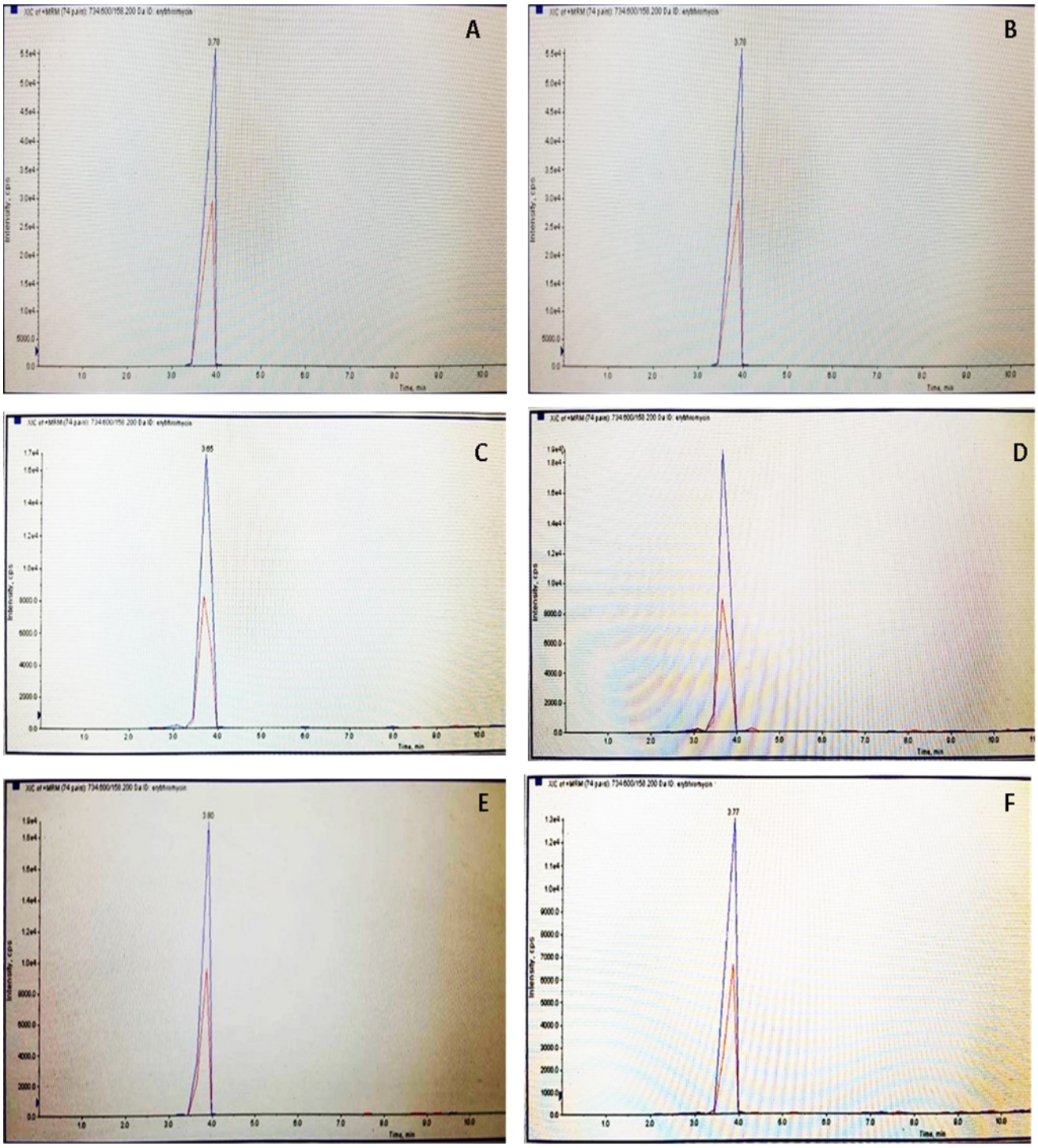

Fig. (4); A, B: illustrated the minimum and maximum concentration (0.899-1.787) µg/g of erythromycin in group A.
C, D: illustrated the minimum and maximum concentration (0.515µg -0.600) µg/g of erythromycin in group B.
E, F: illustrated the minimum and maximum concentration (0.269-0.593) µg/g of erythromycin in group C.

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