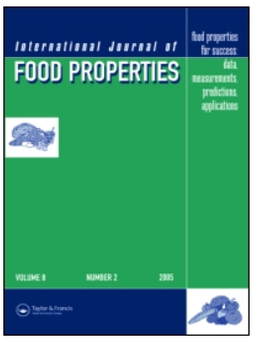

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Antibiotics and malachite green residues in farmed rainbow trout (*Oncorhynchus mykiss*) from the Iranian markets: A risk assessment

Milad Adel[a], Maryam Dadar[b], and Gea Oliveri Conti[c]

[a]Department of Aquatic Animal Health and Diseases, Iranian Fisheries Science Research Institute (IFSRI), Agricultural Research Education and Extension Organization (AREEO), Tehran, Iran; [b]Center of Biotechnology and Biology Research, Shahid Chamran University, Ahvaz, Iran; [c]Department of Medical Sciences, Surgical and Advanced Technologies "G.F. Ingrassia"-Hygiene and Public Health, Laboratory of Environmental and Food Hygiene (LIAA), University of Catania, Catania, Italy

ABSTRACT

Antibiotic and malachite green residues in farmed rainbow trout muscles were determined by high-performance liquid chromatography for a food risk assessment. The surveillance was carried out on total of 120 rainbow trout fillets, all fishes were randomly sampled from 20 fish markets of Iran. All antibiotics were detected in the range of 0.42–1.20 µg/g for Oxytetracycline, 0.02–0.34 µg/g for Enrofloxacin, 0.21–2.61 µg/g for Florfenicol, and finally 0.02–0.89 µg/g for Malachite green. Our results showed that 99 (82.5%), 36 (30%), 56 (46.6%), and 70 (58.4%) samples contained detectable residues of Oxytetracycline, Enrofloxacin, and Florfenicol antibiotics, and Malachite green, respectively. Our results showed that fish farmers use these drugs in large scale. Further investigations are needed to prevent: the foodborne risk to consumers, the possible environmental contamination, and the antimicrobial resistances.

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Oxytetracycline; Enrofloxacin; Florfenicol; Malachite green; Rainbow trout; Food hygiene

Introduction

Aquaculture is currently a worldwide growing food-producing industry (~10% per year). One of the most vulnerable points of aquaculture is the fish's mortality related to infectious diseases (10–20% of total mortality).[1] Infectious diseases are often consequence of stress conditions as an excessive density of fish in tank or basin, hypoxia, high nitrite, ammonia concentrations, etc. Therefore, antibiotics and antifungals are very used in modern aquaculture to prevent or treat the bacterial diseases in farmed fish.[2]

Oxytetracycline (OTC), Enrofloxacin (EFX), Florfenicol (FF) antibiotics, and Malachite Green (MG) are a group of compounds widely used in Iranian farms for prophylaxis and treatment of diseases in farmed fish. However, the extensive application of these drugs may cause certain serious including the presence of their residues in edible tissues of fish.[3,4] OTC is the most commonly used drug for farmed fish and is normally administered in medicated feed at a dose of 75 mg/kg fish /day for 7–10 days with a 21-day withdrawal before fish slaughter. After 21 days, OTC concentrations must be below the tolerance limit of 2 ppm (µg/g).[5] EFX is a fluoroquinolone extensively used to treat systemic bacterial infections in several farmed fish species.[6–8] Since the EFX is dangerous for consumers, the European Union (EU) set a maximum residue limit (MRL) of 100 µg/kg for EFX in the meat of fish (fillets) and of other farmed animals.[8]

CONTACT Dr. Maryam Dadar dadar.m77@gmail.com Center of Biotechnology and Biology Research, Shahid Chamran University, Ahvaz, Iran, 6135783151.

FF, a fenicol drug, is a synthetic and broad-spectrum antibiotic widely used in aquaculture to treat several infections.[9] FF, a fenicol drug, is a synthetic and broad-spectrum antibiotic widely used in aquaculture to treat several infections.[9] FF proved highly effective against a large number of fish pathogens including *Aeromonas salmonicida, Vibrio anguillarum, Vibrio salmonicida, Photobacterium damselae subsp. piscicida, and Edwardsiella tarda*.[10–12] MG is a cationic triphenylmethane, used both as a dyestuff which as antimicrobial in aquaculture, and in spite of its prohibition, is still illegally used in aquaculture industry to control the ectoparasites and fungal infections on fish eggs, fingerlings, and adult fish. MG is still used for its low cost, high efficacy and, unfortunately for lack of viable alternatives.[13–15]

Rainbow trout (*Oncorhynchus mykiss*), a member of Pacific salmonids, is the most farmed freshwater fish species in Iran. Its farming has started in 1959 in Iran, and production increased from 599 tons in 1978 to 62,630 tons in 2009 and with a production of 112,396 tons in 2014.[3] The rainbow trout's intensive farming, however, caused the onset of some bacterial diseases (streptococcosis, lactococcosis, ecto-parasies, and fungal infections) giving rise to many economic losses in Iranian aquaculture.[3] For the management of infectious diseases, the fish farmers, in recent years, have increased the use of antibiotics also giving rise to the increase of antimicrobial resistance against many common antibiotics.[16,17] For the importance of this issue and of related public health risks of exposure to these drugs, several control measures against the disinfectants (MG) and antibiotics (e.g., sulfonamides and tetracyclines) have been set in several countries. Few studies were carried out in Iran on this topic, and no report of drug residues in Iranian farmed fish is today available. So, we studied the hygienic quality of Iranian farmed fish, relatively to content of these drugs residues, with the aim of improve the management of risk of consumers in relation to farmed fish consumption.

Materials and Methods

Sampling

During the year 2015, 24 trout markets were selected randomly in central, northern, western, and north-west of Iran. The mentioned areas produce more than 95% of rainbow trout in Iran market. From each market, five market-size (450 ± 50 g) rainbow trout were obtained and transported to the laboratory inside an insulated ice chest. During the year 2015, 24 trout markets were selected randomly in central, northern, western, and north-west parts of Iran. The mentioned areas produce more than 95% of rainbow trout in Iran market. From each market, five market-size (450 ± 50 g) rainbow trouts were sampled and transported at a controlled temperature (4°C) to the laboratory. Then, the fish were eviscerated, beheaded, deboned, and filleted. The fillets were homogenized with a IKA-WERKE ULTRA-TURRAX homogenizer and placed into labeled plastic bags. The specific protocols of homogenization of samples were described in dedicated subsections. The homogenate samples (n = 120) were stored at −20°C until analyses.

Methods of Drugs Detection

OTC, EFX, FF, and MG residues in fish samples were determined by high-performance liquid chromatography (HPLC) analysis, the gold standard standard technique for drugs analysis. For all drugs the limit of detection (LOD) and of quantification (LOQ) for muscle were calculated (see Table 1) according to the International Conference on Harmonization requirements.[17]

FF Analysis

For FF detection, 3 g of homogenized fillet was prepared and the extract injected manually into the HPLC system (Varian 9012, USA) coupled with florescence detector Varian 9070. The separation was carried out on a C18 column (250 × 4.6 mm i.d., 5 μm; Merck). The analysis was carried out

Table 1. Occurrence and levels of antibiotic and MG residues in farmed rainbow trout in Iran.

Compound residues	Region	Sample	Positive sample(%)	Mean ± SD µg/g	Range µg/g	>LOD^ (%)	Legal limit µg/g
OTC	Central	30	23 (76.6%)	0.61 ± 0.09 (a)	0.48–0.78	7 (23.4%)	
	Northern	30	29 (96.6%)	0.62 ± 0.13(a)	0.42–0.78	1 (3.6%)	
	Western	30	17 (56.6%)	1.01 ± 0.11(b)	0.86–1.20	13 (43.4%)	
	North-west	30	30 (100%)	0.95 ± 0.14(b)	0.72–1.20	0 (0%)	
	Total	120	99 (82.5%)	0.79 ± 0.22	0.42–1.20	21 (17.5%)	0.1
EFX	Central	30	12 (40%)	0.03 ± 0.02(b)	0.02–0.08	18 (60%)	
	Northern	30	8 (26.6%)	0.21 ± 0.05(a)	0.13–0.28	22 (73.4%)	
	Western	30	11 (36.6%)	0.18 ± 0.04(a)	0.13–0.26	19 (63.4%)	
	North-west	30	5 (16.6%)	0.28 ± 0.04(c)	0.21–0.34	25 (83.4%)	
	Total	120	36 (30%)	0.18 ± 0.11	0.02–0.34	84 (70%)	0.1
FF	Central	30	14 (46.6%)	0.6 ± 0.25(a)	0.21–0.96	16 (53.4%)	
	Northern	30	18 (60%)	1.3 ± 0.24(a)	0.92–1.74	12 (40%)	
	Western	30	16 (53.3%)	0.43 ± 0.4(b)	0.22–1.15	14 (46.7%)	
	North-west	30	8 (26.6%)	1.8 ± 0.56(c)	1.1–2.61	22 (73.4%)	
	Total	120	56 (46.6%)	1.04 ± 0.68	0.21–2.61	64 (53.4%)	1
MG	Central	30	17 (56.7%)	0.12 ± 0.01(a)	0.07–0.13	13 (43.3%)	
	Northern	30	21 (70%)	0.13 ± 0.04(a)	0.12–0.26	9 (30%)	
	Western	30	13 (43.4%)	0.15 ± 0.25(a)	0.02–0.86	17 (56.7%)	
	North-west	30	19 (63.4%)	0.53 ± 0.35(b)	0.02–0.89	11 (36.6%)	
	Total	120	70 (58.4%)	0.22–0.26	0.02–0.89	50 (41.6%)	0.002

^LODs: 1.3, 4, 0.3, and 0.1 µg/kg for tetracyclines, sulfonamides, fluoroquinolones, and florfenicol, respectively.
The limits of quantification (LOQ) for OTC, EFX, FF and MG in fish are 13, 1.4, 30, and 0.3 µg/kg, respectively.
The values for each antibiotic and Malachite green with different letters are significantly different among the regions ($p < 0.05$).

according to Pouliquen and Morvan[18] using acetonitrile, 0.01 M sodium dihydrogen phosphate containing 0.005 M sodium dodecyl sulfate, and 0.1% triethylamine, adjusted to pH 4.8 with 85% phosphoric acid (A/B, 35:65, v/v) as the mobile phase, at a flow rate of 1 mL/min. The samples were filtered using 0.45 µm disposable syringe filter and the filtrate was directly injected into the HPLC. The chromatographic system was calibrated using FF external standard by Sigma Aldrich with purity of 93.2% and with a linear regression near to 0.99. The mean recovery rate was 95%, reagent blank processing failed to disclose any trace of FF.

EFX Analysis

For EFX, optimum separation occurred using a mixture of acetonitrile and 0.01 M phosphate at a flow rate of 1 mL/min as described by Soltani.[17] Briefly, 5 ± 0.01 g of muscle samples were mixed with homogenizer and then added in a polypropylene container containing 15 mL of sterile phosphate buffered solution (PBS; 0.02, mol, pH = 9.1). After 15 min, a volume of 5 mL acetonitrile (Merck) was added, mixed well, and the sample was extracted through an ultrasonic bath for 10 min. The supernatant of the extracts was separated and concentrated almost to dryness (by nitrogen flow at 40°C). The final extracts were purified through the activated SPE (Bond Elut Agilent) cartridge C18 (4 mL of methanol and 4 mL of PBS) and finally were eluted with 5 mL methanol containing 2% hydrochloric acid (Merck) and dried by nitrogen flow at 40°C.[17] Finally, samples were filtered using 0.45 µm disposable syringe filter and the filtrates were directly injected into the HPLC. The chromatographic system was calibrated using external EFX standard by Sigma Aldrich with purity of 98% and with a linear regression near to 0.99. The mean recovery rate was 98%. Reagent blank processing failed to disclose any trace of EFX.

OTC Analysis

Detection of OTC in samples was carried out according to Nordlander and Soltani.[2,17] Briefly, 5 ± 0.01 g of homogenized samples were mixed in sterile polypropylene containers containing 15 mL of sterile PBS (0.02

M, pH = 2.25) with acetic acid 50% (W/V; Merck) and 2 mL of trichloroacetic acid 50% (TCA) prepared in pure distilled water. The mixing process was repeated three times (30 s for turn) with homogenizer before centrifugation at 6000 × *g* for 10 min. The extracts were collected into sterile containers. The supernatants of extracts were purified through the activated solid phase extinction (SPE) (Bond Elut Agilent) cartridge C18 (4 mL of methanol and 4 mL of buffer) and dried by nitrogen flow at 40°C. Finally, the extracts were filtered using 0.45 μm disposable syringe filters and the filtrates were directly injected into the HPLC. The chromatographic system was calibrated using OTC external standards by Sigma Aldrich with purity of 99.5% and with a linear regression near to 0.99. The mean recovery rate was 94%. Reagent blank processing failed to disclose any trace of OTC.

MG Analysis

The analysis of residues of MG in muscles of rainbow trout, was carried out according to Fallah and Van de Riet[19,20] Briefly, the samples were extracted according to modified Van de Riet method.[20] The modification has been to use the 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 98% purity) solution to oxidize colorless leucomalachite green (LMG) to its chromic analog, MG. A total of 5 ± 0.01 g of the homogenized fish sample mixed with 16 mL of acidified acetonitrile in a Falcon tube. The homogenate was diluted to 25 mL with pure dichloromethane, shaken for 10 min, then centrifuged at 1500 rpm for 10 min. Subsequently, the supernatant (10 mL) was transferred to a tube and 3 mL of DDQ solution (0.001 M) were added and, the oxidation reaction occurred after 30 min of incubation.

Then, the oxidized sample was concentrated to 3 mL under a gentle stream of nitrogen at 40°C and purified through a SPE column previously activated with 2.5 mL of acetonitrile. The first eluate was collected. The SPE (Bond Elut Agilent) column was also rinsed with 2 mL of acetonitrile. This second eluate was collected and combined with the first. Afterwards, the mixed eluate was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 1 mL of mobile phase containing acetonitrile, acetate buffer (0.05 M, pH 4.5), and ascorbic acid (1 mg/mL; 47.5:47.5:5, v/v/v). The sample, finally, was filtered through a 0.45 μm disposable syringe filter and directly injected into the HPLC. The recovery rate of MG was in the range of 99.6–104%. Reagent blank processing failed to disclose any trace of MG.

Statistical Analysis

The one-way analysis of variance (ANOVA) was carried out using the SPSS software version no. 18 (SPSS Inc., Chicago, IL, USA) for the evaluation of differences in antibiotic and MG concentrations among the various geographical areas of sampling. All data have been reported as mean values ± standard deviation (SD). ANOVA analysis, along with Duncan's method, was carried out to verify mean differences among the various geographical areas of sampling. *p*-Values of ≤0.05 were used to determine significant differences.

Results

The detected (LOD) and measured (LOQ) levels of OTC, EFX, FF antibiotics, and MG are presented in Table 1. From the 120 samples obtained from Iranian trout markets, 99 (82.5%), 36 (30%), 56 (46.6%), and 70 (58.4%) samples showed detectable residues of OTC, EFX, FF antibiotics, and MG, respectively. Also, the residues of the antibiotics and MG in trout samples did exceeded the maximum residue levels (MRLs) recommended by the Institute of Standards and Industrial Research of Iran (ISIRI). The range of detected concentrations in positive samples was 0.42–1.20 μg/g for OTC, 0.02–0.34 μg/g for EFX, 0.21–2.61 μg/g for FF, and 0.02–0.89 μg/g for MG.

OTC was detected in 76.6, 96.6, 56.6, and 100% of the samples obtained from fish markets in central, northern, western, and north-west Iranian territories, respectively. There were statistically

significant difference between the concentrations of OTC in central and northern areas of Iran with western and north-west areas ($p < 0.05$). According to the obtained results, 46.6% in Central, 60% in Northern, 53.3% in western, and 26% in North-west of Iranian markets of rainbow trout showed FF presence.

Residues of FF ranged from 0.21–2.61 μg g^{-1} with a mean of 1.04 ± 0.68 μg g^{-1}. There were statistically significant difference between the concentrations of this antibiotic and geographical areas of sampling. In our study, the occurrence of EFX in trout samples of the central (40%) and western (36.6%) areas was significantly higher ($p < 0.05$) than those from northern (26.6%) and north-west (5%) areas. Most of the positive samples showed an EFX content ranging by 0.02 to 0.34 (Table 1). Our results shown that 56.7% of samples (in central, 70% in northern, 43.4% in western, and 63.3% in north-west) of Iranian markets of rainbow trout contained with the residue of MG. The measured concentrations of MG ranged to 0.02–0.89 μg g^{-1} with a mean of 0.22–0.26 μg g^{-1} (Table 1). There were statistically significant differences between the concentrations of MG in geographical regions of sampling.

Discussion

Regular fish consumption is recommended for a healthy diet; however, the poor fish quality (both farmed and wild) can negatively affect the human health.[21,22] Aquaculture is currently a growing industry. One of the most vulnerable points of aquaculture are diseases, has been estimated that 10 to 20% of farmed fish mortality occurs due to infectious diseases.[1,3] An important aspect in aquaculture business is the need of more accurate controls and monitoring for better fish and consumers health management. In fact the disease has become a primary constraint to the growth of same aquaculture. Intensive culture practices, with poorly controlled use of feed and production of waste, have adversely affected the environments as the increase of eutrophication and related problems.[23–25] The lack of effective treatments for the control of certain infectious diseases such as the *Piscirickettsia salmonis, Acinetobacter spp*., gram-negative bacteria, and *Streptococcus agalactiae* and *Streptococcus iniae* gram-positive bacteria have emphasized the need to develop techniques for disease prevention. The occurrence of these diseases have promoted an extensive research on the health of aquatic species to reduce losses in production. These research include the evaluations of microbial dietary supplements and prebiotics in breeding of finfish and shellfish, the scientific community has also focused their interest in approaches to these issues in aquaculture.[1,26] It was recognized that a widespread use of antibiotics and MG could cause negative health effects on consumers if exposed to their residue but also, some environmental problems since antibiotics are released into the surrounding water during therapeutic treatment of fish stocks. In fact, the use of illegal compound or misuse of approved compound in farmed fish lowers the quality of commercial product and causes several health risks because, these drugs may be toxic, allergenic, or carcinogenic for consumers.[22]

The WHO has long recognized that antibiotic use in industrial livestock farms may contribute to the growing public health problem of antibacterial resistance in human and veterinary medicine.[22,27] Our study showed that most of the positive samples contained OTCs, EFX, FF antibiotics, and MG residues. Residues detected in our samples (from four parts of Iran) exceed the MRLs recommended by the ISIRI. Also, our data suggests that the antibiotic and MG concentrations found in the fish fillet sample from all over the markets of Iran were higher than the standard limits set by various agencies, thus indicating that an important health risk is associated with the consumption of fish contaminated by drugs residues.[27,28]

Barani and colleague,[3] in a recent study, showed that from a total of 138 samples obtained from Iranian trout farms, 87 (63.1%), 24 (17.4%), 27 (19.6%), and 56 (40.6%) samples contained measurable residues of tetracyclines, sulfonamides, fluoroquinolones, and FF, respectively, but never exceeding the MRLs recommended by the ISIRI.[3] In another study conducted in Nigeria,[29] 30% of the catfish fillet samples contained OTC, ranging between 22.5 and 553.2 μg/kg, and 18.8% of

samples exceeded the limit of 200 μg/kg.[22] In Croatia, 87.6% of the analyzed fish products contained tetracyclines, with a maximum value of 9.4 μg/kg.[30]

Our results showed that the peak concentrations of OTCs in farmed fish fillet were comparable to other studies carried out in other countries. Bjorklund and Bylund[31] found peak OTCs concentrations of 0.6–1.5 μg/g in farmed rainbow trout and salmon. Monteiro and colleagues found both OTC and FF concentrations in muscle samples of Nile tilapia ranging from 15.6 to 1231.8 μg/kg and from 521.2 to 528.0 μg/kg, respectively.[32] The high antimicrobial concentrations showed in our study and reported by the cited studies from other countries suggest that further investigation on antimicrobials residues in farmed fish samples is necessary worldwide to prevent foodborne risk to consumers and to prevent possible environmental contamination and increase of emergence of resistant bacteria. However, these results can be used to provide a database for the risk assessment associated with consumption of Iranian farmed fish. Furthermore, constant monitoring of the seafood markets in different locations is recommended in view of the increased use of antibiotics and MG on the farmed fish of this regions that disturb the nature cycle of microbial organisms.

Conclusion

The antibiotics and MG residues in farmed rainbow trout from the Iranian markets must be considered like a severe risk for consumers. This study provides an accurate assessment of residues of certain drugs in farmed fish of market of Iran. The rainbow trout specie in Iran can serve as sentinel for the total quality of aquaculture monitoring, and make contributions to the environmental authorities, who should also monitor the health of people and make or suggest good solutions to reduce the antimicrobial and forbidden compounds in aquaculture. This report is the first step for an integrated evaluation of the antimicrobial and MG drugs accumulation in tissues of farmed fish in markets of Iran. Further studies are needed to measure residues of other drugs and in different time of year and, to establish the food risk for the consumers.

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