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ORIGINAL ARTICLE

Determination of Florfenicol Residues in the Muscle and Liver of Cultured Rainbow Trout in Iran by ELISA

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KEYWORDS

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ABSTRACT: Florfenicol is a broad-spectrum antibiotic, widely used in veterinary medicine. The aim of this study was to determine florfenicol residues in the muscle and liver of cultured rainbow trout in Iran by ELISA. The samples were collected from three areas (Kiar, Ardal, and Koohrang), considered as areas with high production of trout in the western part of Iran. Sampling was completed during the spring and summer of 2011. All of the samples were categorized into three weight groups (below 50g, 50 to150g and over 150g) and five individuals were randomly selected from fish belonged to each weigh group, and then collected samples sent for determination of antibiotic residues. The highest and the lowest antibiotic residues were 31.42±53.52 ng g^{-1} (>150 g fish) and 10.35±2.33 ng g^{-1} (<50 g fish) for liver samples and 48.84±50.36 ng g^{-1} (50-150 g fish) and 18.20±15.41 ng g^{-1} (> 150 g fish) for muscle samples, respectively. In different areas, the highest antibiotic levels were found in Koohrang and Ardal with mean of 37.00±63.61 and 15.33±10.45 ng g^{-1} for liver samples and 40.74±40.80 and 28.24±45.91ng g^{-1} for muscle samples. The results indicated that florfenicol residues are lower than the maximum permissible level has been announced by the European Union.

INTRODUCTION

Antibiotics often used in aquaculture, as feed additives, and as prophylactic and therapeutic agents represent a potential hazard for human health. These compounds are used in food-producing animals both for treatment of

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disease, and to sub-therapeutically maintain health and promote growth except for fish [1]. Amphenicols (e.g. thiamphenicol (TAP), florfenicol (FF) and chloramphenicol) are synthetic antibiotics with similar broad spectrum of activities. FF is a fluorinated synthetic analog of thiamphenicol and a primarily bacteriostatic antibiotic with a range of activities similar to that of chloramphenicol including many gram-negative and gram-positive organisms [2]. FF has a fluorine atom instead of the hydroxyl group located at C-3 in the structure of chloramphenicol and thiamphenicol [3].

Because of the broad-spectrum and absorbed rapidity, FF has been used in veterinary medicine to treat infectious diseases [4]. FF was first approved for use in freshwater-reared salmonids for the control of mortality due to cold-water disease associated with *Flavobacterium psychrophilum* in 2007. In addition, it was effective in controlling mortality in *Tilapia oreochromis* sp. due to *Streptococcus iniae* in laboratory and field efficacy trials when fed at a dose rate of 15 mg/kg BW/d for 10 consecutive days [5]. The European Union (EU) has been set the tolerance level for these compounds as the maximum residues limit (MRLs) to ensure safety for the food production industry. The value of MRLs was calculated by the sum of FF and its metabolites. The total residues of FF and FF amine were not exceeding 1000 µg/kg in fish [6]. FF and FF amine were determined in muscle and liver tissues of rainbow trout [7]. Alsoa competitive ELISA has been developed for detecting FF and thiamphenicol in swine feed [8]. Their results obtained from HPLC-tandem mass spectrometry indicated that ELISA procedures could be used as a convenient method for rapid screening of FF and thiamphenicol in swine feed.

In Iran, there has been an increase in the production and consumption of freshwater fish and shrimp reared in aquaculture systems in recent years, mainly the rainbow trout (*Oncorhynchus mykiss*). Coldwater fish productionin Iran reached to near 100000 tons in 2012, which 18.5 percent of it will be allocated to the Chaharmahal and Bakhtiari Province as the largest producer of trout in Iran [9]. According to information taken from farm owners FF is mostly used in the control of infectious diseases.

The aim of the present study was to determine FF residues in major trout farms of this area.

MATERIALS AND METHODS

Study area

The study was done in the trout farms of western part of Iran (Ardal, Koohrang and Kyar) from spring to summer of 2011. The use of antibiotics usually increases in these seasons.

Experiments

In the each area three farms were randomly selected and each farm was divided into three weight groups (50 g, 50 to150 g and >150 g) of 5 fish per group. A total of 45 rainbow trout were obtained from fish farms in all groups, in all weight groups, liver and muscle tissue samples of fishes were taken and sent for laboratory procedures under standard conditions. Samples were stored at -18 ºC until ELISA testing, and water quality conditions of farms were recorded in different fish farms.

Sample preparation and ELISA test

To determine the antibiotic residues florfenicol ELISA test kit (Shenzhen Lvshiyuan Biotechnology Co. China) was used. This test kit was based on the competitive enzyme immunoassay for the detection of FF in the fish. Sensitivity, detection limit, and recovery rate of kit for fish was 0.05 ppb, 0.05 ppb and 80 ±10%, respectively. Each immunoassay kit contained sufficient material for 96 measurements. Each microtiter consisted of 96 wells, coated with capture antibodies against antiflorfenicol

antibodies. There were six concentration FF level (0, 0.5, 1.5, 4.5, 13.5 and 40.5 ppb) in aqueous solution standards of FF. The microtitre plate spectrophotometer (Stat Fax 2100) was set at 450 nm. Frozen trout were thawed at 4 °C overnight. The tissue samples (muscle and liver) were homogenized by using Ultra Turrax T25. Three grams of tissue homogenate was taken for centrifuge and 6ml ethyl acetate was added to it. Sample homogenate was centrifuged (Eppendorf Centrifuge 5810 R) at above 4000 rpm at the room temperature for 10 min. An amount of 2 ml of the supernatant was taken and dried at 50-60 °C by blowing of Nitrogen (N-EVAP111 Orgnization Associates Inc., Berlin, MA, USA). The dry residues was dissolved in 1 ml N-hexane, then 1 ml of the diluted redissolving solution was added to it, and then shaken vigorously for 30 seconds then centrifuged at above 4000r/min at room temperature for 15 min. Finally, 50 µl of the lower were placed in each well of microtitre plate, the procedure was continued based on kit instructions and finally the optical density of samples at 450 nm was measured and the results were recorded.

STATISTICAL ANALYSIS

All data were expressed as mean ± SD and means between different areas and weights compared with statistical tests (One Way ANOVA) analysis and Duncan's multiple at 95% confidence interval. Moreover, SPSS software, version 19 (Chicago, IL, USA) was used for statistical analysis.

RESULTS

We determined the amount of FF residues in liver and muscle of rainbow trout cultured in western part of Iran. Table 1 shows the comparison between antibiotic residues in the muscle and liver of fish in different weights. The highest and the lowest antibiotic residues were 31.42±53.52 ng g^{-1} (>150 g fish) and 10.35±2.33 ng g^{-1} (<50 g fish) for liver samples and 48.84±50.36 ng g^{-1} (50-150 g fish) and 18.20±15.41 ng g^{-1} (> 150 g fish) for muscle samples, respectively. No significant statistical differences were observed between compared groups (P<0.05). The highest antibiotic levels were found in fish in Koohrang and Ardal areas with mean of 37.00±63.61 and 15.33±10.45 ng g^{-1} for liver samples and 40.74±40.80 and 28.24±45.91 ng $g^{-1}$for muscle samples (Table 2). There were no significant relation between florfenicol levels and locations (P<0.05).

Table1. Antibiotic residues (mean ±SD) in the liver and muscle of fish in different weights

Weight group	Muscle(ng g^{-1})	Liver (ng g^{-1})
<50g	29.63±36.50	10.35±2.33
50-150g	48.84±50.36	28.82±44.52
>150g	18.20±15.41	31.42±53.52

Table 2. Antibiotic residues (mean ±SD) in the liver and muscle of fish in different area

Study area	Muscle(ng g^{-1})	Liver(ng g^{-1})
Ardal	28.24±45.91	15.33±10.45
Kyar	40.71±44.74	30.95±49.03
Koohrang	40.74±40.80	37.00±63.61

DISCUSSION

In aquaculture, FF demonstrates potent activity against a wide range of fish pathogens *in vitro* and *in vivo* [10]. The drug has been authorized in many countries for use in aquaculture including Iran, in which antibiotic is being used to control and treatment of infectious diseases in the farmed trout. The aim of this study was to determine FF residues in the muscle and liver of farmed rainbow trout regarding to the over use of this antibiotic in fish farms.

Since the liver is the most important metabolizing organ, the highest amount of antibiotic aggregate in live. On the other hand, the larger fish have higher level of antibiotic. The results show that liver has the highest rate of FF residue in fish>150 g while the lowest residues has been observed in trout < 50 g. Differences between weight groups can be explained as an influential factor in receiving antibiotics. In the muscle samples, however, the highest rate of FF was seen in group 50-150g which is related to the duration of treatment (P<0.05), and is not correlated with weight gain. The high density of fish was found in Koohrang farms; therefore, muscle and liver samples of this area had the highest rate of antibiotic residue. In addition, the mean water temperature in koohrang was low (8°C) and metabolism of drugs occurred in a longer time. There is acorrelationbetween different liver andmusclegroups.

According to the obtained results, FF residues in muscle and liver were lower than the MRLs recommended by the EU (< 1000 ng g^{-1}). As a result, there is no concern for human, but fish pathogens which may have developed acquired resistance as a result of drug usage and the continuous presence of residual levels in the fish body, can act as a host for resistance genes that can be transferred to human pathogens [11]. Most studies have been done in FF determination using HP LC analysis. FF and FF amine were determined in muscle and liver tissues of rainbow trout by HPLC analysis, LOD values were determined 20 ng g^{-1} for muscle and 50 ng $g^{-1}$for liver [7]. FF amine was determined in catfish skinless fillet samples with LOD values 4 ng g^{-1}[12] and FF in catfish muscle with LOD 44 ng g^{-1}[13]. In addition, the FF was determined in plasma of catfish, lake trout, rainbow trout, and lake sturgeon, using an LC method [14]. Other antibiotics have also been investigated in fish. Enrofloxacin residues were assayed in muscle and liver of rainbow trout cultured in Chaharmahal-va-Bakhtiary Province using ELISA method. Drug residuals were lower than the maximum acceptance limit determined by the European Union that similar to our works [15]. Tetracycline with 80 mg/kg bw has been used in rainbow trout by oral administration and antibiotic residues was detected 10 µg/g in the kidney through TLC-B (Thin layer chromatography-bioautography) assay. No drug residue was detectable in liver and muscles of fish [16].

Molecular and epidemiological evidence has demonstrated that antibiotic resistance determinants of resistant *Salmonella enteric* serotype Typhimurium DT104, an emergent pathogen and the cause of several outbreaks of salmonellosis in humans and animals in Europe and the USA, probably originated in aquaculture settings of the Far East [17, 18]. This FF determinant, *floR*, was detected for the first time in the fish pathogen *Vibrio damsel* [19]. Aquaculture is a fast-growing sector of agriculture with tremendous growth of more than 30 percent worldwide during the last ten years. Culture of salmonids is one of the fastest growing industries in Iran in the recent years, and in this country, majority of water resources have been allocated to rainbow trout farming. One of the most economical regions in rainbow trout production is located in the western part of Iran. The rapid growth of the aquaculture industry is inhibited by the large number of diseases. Various methods have been used and developed to prevent and control these infections. Antibiotics are widely used in aquaculture for treatment and prevention of bacterial diseases due to

the infection of cultured aquatic species. Antibiotics are widely used in aquaculture and present certain problems, but this can result in developing resistance to the antibiotics

CONCLUSIONS

For these reasons, regulatory agencies have made decisions to keep these substances under control. To limit the spread of resistance, unnecessary dosing of antibiotics should be minimized. Control of usage in aquaculture requires monitoring antibiotic residues in different biological samples. In addition to limiting the spread of resistance, monitoring of residues, also, prevents the access of possible allergenic antibiotics to finished food products and ensures that the residues do not interfere with food production processes.

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