



RESEARCH ARTICLE

Investigation of tetracycline residues in fish caught from surrounding fish farms in Muğla district

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Muğla bölgesindeki balık çiftlikleri çevresinden avlanan balıklarda tetrasiklin aranması

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Öz

Amaç: Su ürünlerindeki ilaç ve metabolitlerinin kalıntı analizleri Ulusal Kalıntı İzleme Planı kapsamında düzenli olarak yapılmaktadır. Fakat Türkiye’de bu kalıntıların sucul ortama etkilerine yönelik araştırmalar sınırlıdır. Bu çalışmada, Muğla bölgesindeki balık çiftliklerinin etrafından avlanan balıklarda tetrasiklin grubu antibiyotiklerin (oksitetrasiklin, tetrasiklin, klortetrasiklin, doksisisiklin) kalıntılarının belirlenmesi amaçlandı.

Gereç ve Yöntem: Analiz için 70 ayrı çiftlik etrafından toplanan 70 adet balık örneği kullanıldı. Balıklarda tetrasiklin kalıntıları LC MS/MS yöntemi ile analiz edildi. Seçicilik, doğruluk, geri kazanım, duyarlılık ve kesinlik ölçütlerinde metod validasyonu yapıldı.

Bulgular: Analizin geri kazanımları oksitetrasiklin için %100.5, tetrasiklin için %101.3, klortetrasiklin için %99 ve doksisisiklin için %100.5 olarak belirlendi. Analizlerin sonucunda balık örneklerinin hiçbirinde tetrasiklin grubu antibiyotikler tespit edilemedi.

Öneri: Yapılan analizlerde balık örneklerinde hesaplanabilir limitlerde tetrasiklin kalıntısı bulunmaması çevre ve halk sağlığı açısından memnuniyet verici olarak kabul edilebilir.

Anahtar kelimeler: Balık, tetrasiklin, kalıntı, LC MS/MS

Abstract

Aim: Residue analyses of drugs and metabolites in the aquaculture are regularly conducted under the National Residue Monitoring Plan in Turkey. However, the research on the impact of these residues on the aquatic environment is limited. In this study, it was aimed to analyze the residues of tetracycline group antibiotics in fishes caught around fish farms in the Muğla district.

Materials and Methods: Seventy fish samples collected from the vicinity of 70 different fish farms were used for analysis. Tetracycline residues in the fish were analyzed with the LC MS/MS method. Validation was performed with the criteria of specificity, linearity, recovery, precision and sensitivity.

Results: Recovery values were determined as follows: Oxytetracycline 100.5%, tetracycline 101.3%, chlortetracycline 99%, and doxycycline 100.5%. At the end of the analyses, no tetracycline antibiotic residues were found.

Conclusion: It can be expressed that no tetracycline residues were detected in the samples at the detectable limits is a satisfactory result in terms of public health and environment.

Keywords: Fish, residue, tetracycline, LC MS/MS



Introduction

In addition to having important nutrient value for the nutrition of an increasing world population, aquaculture products are also important for national economies. Industrial aquaculture production is rapidly increasing in developed and developing countries (Cabello 2006). According to FAO data from 2012, 158 million tons of aquaculture products were obtained, 91 million tons of which were obtained by catching and 67 million tons of which were obtained by production. World fish consumption per capita is estimated to be 19.2 kg, while the world fish market value is estimated to be around \$ 129.5 billions (FAO 2015). Fisheries are an important field of activity in Turkey due to fish being a significant source of nutrition and being one of the sources of income in the coastal areas. According to TSI data from 2013, total aquaculture production in Turkey was 607 thousand tons, 374 thousand tons of which were obtained by catching, 233 thousand tons of which were obtained by production (TSI 2014). Turkey is ranked 38th worldwide and 6th EU-wide at catching; while it is ranked 22nd worldwide and 3rd EU-wide at production (FAO 2015).

Trout production in Muğla province began in the 1970's while marine aquaculture production began in 1985. Thirty per cent the aquaculture products produced in Turkey in 2013 was produced in the fish farms in Muğla province (MFAL 2015a). There are 81 trout farms in the Muğla province as of 2014. These establishments produced 15.5 thousand tons of trout in 2014. Sixty-five thousand tons of bream and bass, 2.500 kilograms of meager and corb were produced in 100 net cage facilities and 163 earth pond farms. The aquaculture production of the province in 2014 was 83.000 tons (MFAL 2015a).

One of the most important factors causing environmental pollution is antibacterials used for public health and animal health purposes and also used in the aquatic environment. Antibiotics are used to protect fish from bacterial diseases and to treat sick animals (Avsever et al 2010). Antibacterial drugs have been used for a long time in aquaculture production. Sulfanilamide and oxytetracycline are the primary antibacterials used in aquaculture production (Erdogdu 2012). Drugs used in aquaculture are administered either inside the feed or as bath solutions. As of April 15, 2015 there were 41 antibacterials certificated for use in fish in Turkey. Seventeen of them contain florfenicol, 11 of them contain oxytetracycline, nine of them contain sulfadiazine + trimethoprim, two of them contain amoxicillin, and two of them contain enrofloxacin (MFAL 2015b). In aquaculture products, metabolites or decomposition products of antibiotics accumulate in the body as residues. The elimination period of the drugs in fish is 500 °C/day (MFAL 2015c). This period was confirmed during the studies with tetracycline (25 days at 20°C; Ozak and Cengizler 2015). In the Turkish Food Codex Communiqué No.

2011/20, the maximum residue limit (MRL) of the sum of oxytetracycline and its 4'-epimer is 100 µg/kg (ppb) (Official Gazette 2011).

Tetracyclines, which were discovered in the 1940s, are effective against gram-positive and gram-negative bacteria, chlamydia, mycoplasmas, rickettsia, and single-cell parasites, and they are a group of broad-spectrum antibiotics. Bi- or trivalent metal compounds (eg. Ca, Fe, Zn, Al, Mg) form chelates in the gastrointestinal tract with tetracyclines and inactivate them, and since these chelates do not dissolve in water, they are not absorbed in the intestines (Chopra and Roberts 2001). Tetracyclines are absorbed by the liver from blood circulation and then transported to the intestine via bile. In the intestines, they are re-absorbed into the blood circulation and excreted from the body by the kidneys. They have a bacteriostatic effect in bacteria ribosomes by inhibiting protein synthesis, while at higher doses they have a bactericidal effect (Chopra and Roberts 2001, Yazar et al 2009). Tetracyclines are photosensitive; when used as bath solutions for treatment, they decompose and turn brown. This form could be toxic for humans and animals (Samanidou and Evaggelou 2007).

It was reported that the accumulation of antibacterials on sediments below fish farms were sufficient to prevent bacterial growth (Kummerer 2008). Even the low level of antibacterial presence reduces microbial activity. Therefore, antimicrobials in the environment affect the improvement of waste waters, which require microbial activity unfavorably. This could result in eutrophication (Bakal et al 2001, Serrano 2005). Olatoye and Basiru (2013) investigated oxytetracycline residues in the muscle and liver samples of a total of 160 fishes collected from fish farms and restaurants. Forty-three liver samples (26.9%) exceeded the MRL laid down in the Nigerian Codex with 600 µg/kg and 30 fillet samples (18.8%) exceeded the MRL with 200 µg/kg oxytetracycline. Barani and Fallah (2014) investigated the oxytetracycline residues in 138 trout samples. Tetracycline was found in 87 of the samples between 1.43-101.4 µg/kg. Antibiotic residues in the trout samples did not exceed the MRL levels laid down in Iranian legislation (200 µg/kg). Baydan et al (2015) investigated the oxytetracycline residues in samples of fish (*Oblada melanura*, *Mullus barbatus*) in the natural habitat, caught in the vicinity of fish farms around Aegean Sea, Bodrum, and Salihli Island, sea water, and sediment using the HPLC method. No oxytetracycline residues exceeding the detection limit were detected in these samples.

Taking into consideration the literature and theses available, it could be suggested that the studies on tetracycline residues in fish except the "National Residue Monitoring Plan" are limited. The aim of the study was to detect the tetracycline residues in fish caught in the vicinity of 70 farms in the region due to the following factors:





- Muğla province comprises 30% of the production and aquaculture in Turkey and the products from this region is exported to EU Member States;
- Tetracyclines are broad-spectrum and cheap antibiotics, therefore they are very widely used in fish production and they are important antibiotics for human health; and
- Fish living in the natural environment in the vicinity of fish farms are considered as bio-indicators of environmental pollution.

Materials and Methods

Approval of the ethics committee

This project was carried out with the approval of Selcuk University Faculty of Veterinary Medicine Ethics Committee dated 08.10.2013 and numbered 2013/048.

Devices and materials

Routine laboratory materials in the toxicology laboratory were used.

LC MS/MS device

ZORBAX SB-C 18 4.6x100 mm, 3.5 μ m column, liquid chromatography (Agilent 1200), degasser (Agilent 1200), LC pump (Agilent 1200), autosampler (Agilent 1100), and mass detector (Agilent 6460).

Chemicals and stock solutions and standards

Reference standards: Chemical properties of oxytetracycline hydrochloride (Riedel De Haen 46598), tetracycline hydrochloride (Fluka 31741), chlortetracycline hydrochloride (Riedel De Haen 46133), doxycycline hyclate (Fluka 33429), and demeclocycline hydrochloride (Sigma Aldrich d6140) are analytical grade (99.9%). Acetonitrile (Sigma Aldrich, 34851), formic acid (Merck 2635), oxalic acid dehydrate (Merck), methanol (Sigma Aldrich 34885), titriplex (Na_2EDTA Sigma) are chemically analytical grade. Stock solutions of oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), and doxycycline (DC) and demeclocycline, which is used internally, were prepared in methanol at a proportion of 1 mg/mL. Stock solutions were diluted with 2-100 μ g/mL methanol in order to simulate working standards of tetracycline mix and demeclocycline.

Two g tissue samples whose calibration standards did not contain TC mix (OTC, TC, CTC, and DC) were administered at 50, 100, 150, 200, and 400 μ g/kg to simulate working standards. Fifty, 100, and 150 μ g/kg of the calibration standard levels were selected as quality control samples. These samples were used in the recovery and precision description of the method.

LC MS/MS device conditions

Pump conditions: T-flow: 0.8 mL/min, B Con 10%, Pressure limit: P. max: 300 bar, column oven conditions: oven temp; 35°C. Autosampler conditions: Sampler temperature; 10°C, injection volume: 20 μ L. Source parameters: Gas temp; 350°C, gas flow; 9 L/min, nebulizer; 40 PSI, sheath gas temp; 400°C, sheath gas flow; 10 L/min.

Study area and collection of samples

Fish samples used in the study were collected using fishing rods in the vicinity of 70 poultry farms spanning from Göl-türkbükü, Torba, and Güvercinlik Bay around Güllük Bay of Muğla province to Kuşadası in the north. Seventy samples of the caught gilt-head bream (*Sparus aurata*), common sea bream (*Sparus pagrus*), and smaris (*Maena smaris*) were used for analysis. Fish were caught between 01 Sept 2013 and 01 Dec 2013 on five different days at a depth of 25-35 meters, taking into account the availability of the sea for sampling (wave height: 2-4 m) and the species were identified. Fish were kept at -20°C until analysis.

Preparation of muscle samples for analysis

The analyses of fish samples used in the study were performed at the Toxicology Laboratory of Ministry of Food and Agriculture and Livestock, Bornova Veterinary Control and Research Institute (İzmir). After the frozen fish were thawed in the refrigerator, the fillet samples were taken from the dorso-lateral area between the dorsal fin and lateral line just behind the left operculum using scissors and a knife and homogenized using a laboratory blender. A 2 ± 0.02 g muscle sample was placed in a 50 mL polypropylene centrifuge tube. One hundred μ L of 2 ppm internal standard (demeclocycline) was added and vortexed for five seconds. Two hundred μ L 0.1 M Na_2EDTA and 10 mL 70% methanol was added into the mixture and vortexed for 15 minutes. Then the mixture was centrifuged at 3°C and 4.000 rpm for 18 minutes. One thousand eight hundred μ L distilled water was added to 200 μ L supernatant and mixed for a few seconds. The diluted extract was obtained using a 2 mL injector and filtered to a vial. Twenty μ L were injected into the LC MS/MS system.

Method validation

Criteria which determine the characteristic performance of a method are specificity, linearity, recovery, precision and sensitivity (EC 2002, Aydın and Oguz 2012). The validation of the method used in the detection of tetracycline residues in the fish samples and the analysis of the samples were carried out using the method accredited by the EU and developed by Bornova Veterinary Control and Research Institute, benefiting from the methods developed by Oka et al. (1998), Lykkeberg (2004), and Granelli et al (2009). For the validity of this



method of analysis, Commission Decision 2002/657/EC and the Procedure for Trial Methods and Validation of Trial Methods were considered as guidance and method performance criteria were established (EC 2002).

Results

Method validation

Specificity: The chromatogram obtained following the addition of internal standard into the sample not containing TC mix, the chromatogram obtained as a result of the analysis of fish samples and the chromatogram obtained from fish samples.

Linearity: The correlation coefficient was $r^2 \geq 0.999$.

Recovery: The recovery of analysis was $100.5 \pm 3.51\%$ for OTC, $101.3 \pm 4.65\%$ for TC, $99 \pm 2.47\%$ for CTC, and $100.5 \pm 3.21\%$ for DC.

Precision: Intra- and inter-day variation values were below the VK% (15%) as determined by Commission Decision 2002/657/EC (EC, 2002). Intraday variation values were: OTC 1.8, TC 2.0, CTC 1.8, and DC 1.7; while interday variation values were: OTC 4.4, TC 5.6, CTC 3.4, and DC 4.4.

Sensitivity: The limits of detection (LOD) were: 11 $\mu\text{g}/\text{kg}$ for oxytetracycline, 13 $\mu\text{g}/\text{kg}$ for tetracycline, 7.4 $\mu\text{g}/\text{kg}$ for chlortetracycline, and 9.4 $\mu\text{g}/\text{kg}$ for doxycycline. Limits of quantitation (LOQ) were: 22.2 $\mu\text{g}/\text{kg}$ for oxytetracycline, 26 $\mu\text{g}/\text{kg}$ for tetracycline, 14.9 $\mu\text{g}/\text{kg}$ for chlortetracycline and 18.7 $\mu\text{g}/\text{kg}$ for doxycycline.

Results obtained from fish samples: The residues of tetracyclines (oxytetracycline, tetracycline, chlortetracycline, and doxycycline) were investigated in 70 fish samples (40 gilt-head breams, 16 smarid, and 14 common sea breams) from 70 different fish farms around Muğla province. At the end of the study, no residues of tetracyclines were detected in the analyzed fish samples.

Discussion

Aquaculture production plays a significant role in the environmental spread of drug residues. Each year, tons of antibiotics are released into the aquatic environment worldwide. The aim of this study was to detect the presence of any tetracycline residues in fish living in the vicinity of farms, which are considered bio-indicators of antibiotic contamination in aquatic environment. In this study, the presence of tetracycline (OTC, TC, CTC, DC) residues were analyzed in samples from 70 fish caught in the vicinity of approximately 90 fish farms operating in the region. No tetracycline residues were detected as a result of the study. Tetracyclines are sources

of contamination for the ecosystem since they are cheap, and they are used to treat diseases in humans and animals and as feed additives in production, and are resistant to environmental conditions (Sorensen 2000). A study with oxytetracycline has shown that this substance can remain intact for almost six months on sea sediments (Samuelsen 1989). In antibiotics, complex formation or binding to particles may cause losses in antimicrobial activity and difficulties in detection at analyses (Kummerer 2009). Antibiotic residues in fish may cause toxic and allergic reactions in living beings and resistance in bacteria. Antibiotic residues around fish farms also cause resistance to bacteria and eutrophication in the environment (Serrano 2005). Studies on antibiotic residues in fish living in their natural habitat and sediments are limited in Turkey.

Within the scope of Residue Monitoring Plan for 2006-2012, the presence of tetracycline residues were analyzed in 613 samples of aquaculture products at Bornova Veterinary Control and Research Institute Toxicology Laboratory. In five samples, oxytetracycline levels exceeded the maximum residue limit (MRL; 100 $\mu\text{g}/\text{kg}$) (Erdogdu 2012). Segmenoglu (2014) analyzed the presence of tetracycline residues in 386 fish samples at Adana Veterinary Control and Research Institute Toxicology Laboratory between 2011 and 2013. Oxytetracycline residues were detected in three samples and the levels of these residues exceeded MRL limits laid down in the codex (100 $\mu\text{g}/\text{kg}$). Fortt et al (2007) analyzed the presence of oxytetracycline in 13 fish samples caught in the vicinity of salmon farms in Chile. Eighty-seven $\mu\text{g}/\text{kg}$ of oxytetracycline was detected in one of the fishes. Oxytetracycline levels of fish caught in the sea were below the levels laid down by Chilean fisheries authorities (tetracycline 100 $\mu\text{g}/\text{kg}$). Bjorklund et al (1990) detected oxytetracycline residues in fish caught in their natural habitat in the vicinity of farms where fish were administered tetracycline, up to 13 days after the antibiotic administration. Furthermore, they found that the bacteria isolated from the intestines of the caught fish were resistant to OTC. Kil-Bo et al (2010) carried out residue analyses in 108 samples of fish that were caught in their natural habitat in the Republic of Korea. No tetracycline residues were detected in the samples. Within the scope of this study, 0-60 $\mu\text{g}/\text{kg}$ of oxytetracycline and tetracycline residues were detected in some of the 111 farmed fish. They reported that these levels were below the MRL laid down in Korean Food Legislation. Furthermore Kim et al (2013) analyzed the presence of tetracycline residues in a study in trout in the Republic of Korea. Tetracycline residue was below 200 $\mu\text{g}/\text{kg}$ and it was found that this level was below the MRL laid down in Korean legislation. Olusola et al (2012) studied the tetracycline levels in fresh and frozen fish, while Chaleshtori et al (2013) studied the tetracycline levels pre- and post-frying. They reported that frying and long-term freezing relatively reduced the tetracycline levels in fish. Data from the current study are similar to the data from the studies of Kil-Bio et al (2010) and





Baydan et al (2015), which were carried out using fish caught in their natural environment. On the other hand, Bjorklund et al (1990) and Fortt et al (2007) who collected samples in the vicinity of farms after administering antibiotics, demonstrated that the antibiotics were not only detected in the animals within the cages but also spread to non-target species.

Conclusion

- In this study, tetracycline residues were not detected in the fish caught in the vicinity of fish farms around Muğla province.
- It was confirmed that a valid, accredited method for the detection of tetracycline residues in fish could be used in routine analyses.
- The fact that no tetracycline residues exceeding the limits of detection were found in the analyses is important for the public and environment health.
- The fact that no tetracycline residues were detected in the analyzed fish samples may be attributed to the exports from the region to the EU. Also the positive contribution of training and controls of Ministry of Food, Agriculture and Livestock regarding production and food safety targeting fish farmers.
- Antibiotics should only be used for treatment purposes in fishes. The elimination period of antibiotics used as feed additives or for treatment purposes should be taken into consideration.
- The sampling process should be extended to 12 months in upcoming studies.
- Sediments should also be taken into consideration regarding antibiotic presence, if the studies will concern the fish caught in the vicinity of farms.
- Bacteria can be isolated from the intestines of fish in their natural habitat. The isolated bacteria that are resistant are an indicator and a result of antibacterial contamination.

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