Research Article

BASRAH JOURNAL OF VETERINARY RESEARCH, 2023, 22(1):56-68
https://bjvr.uobasrah.edu.iq/

Quantification of Oxytetracycline Residuei in Farmed Common Carp (*Cyprinus Carpio*) Meat in Sulaymaniyah Province/Iraq Using Hplc

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DOI: 10.23975/bjvetr.2023.178296

Received: 8 March 2023 Accepted: 23 March 2023.

Abstract

In Iraq, due to the government's lack of control over antibiotic use by the aquaculture farmers, random use of antibiotic is prominent, especially Oxytetracycline (OTC). Oxytetracycline is a potent antibiotic used in aquaculture industries because it is easily obtained, inexpensive, and effective against bacterial diseases. The present study aimed at quantifying and comparing the amount of OTC residue in the two most consumed muscles, dorsal and caudal skeletal muscles, of farmed common carp fish '*Cyprinus carpio*' by the Sulaymaniyah population. Samples of caudal and dorsal skeletal muscles of 54 (for each muscle type 27) carp fish were taken randomly from 9 large aquaculture ponds, which were composed of one or more earthen ponds, in Qaladze and Taqtaq areas near Sulaymaniyah Province/Iraq. The OTC residue was quantified using high-performance liquid chromatography (HPLC). Two-tailed, unpaired t test analysis of the results was performed by GraphPad Prism software to compare between the residue levels in the two regions of the skeletal muscles. Twenty-eight samples had OTC residue detected in them, while residues in twenty-six samples were not detectable, only two of the caudal skeletal muscle samples had residues that exceeded the Maximum Residual Limit (MRL), while all the dorsal muscles with OTC residues revealed lower levels than the set MRL. The present study revealed that there was OTC residues in common carp, and different muscles have OTC residues regardless of their location in the common carp's body. In addition, a specific MRL level and a legal regulation by the Iraqi government must be set to keep OTC use for the farms and residue levels in the fish meat under control.

Key words: Common carp, HPLC, antibiotic residue, oxytetracycline, OTC.

Introduction

One of the most critical issues that the world is continuously confronting is environmental pollution. Improper use of antibiotics contributes to one of the leading causes of this pollution (1). In the Kurdistan Region of Iraq (North of Iraq), the use of antibiotics is very high compared with the global rate of drug consumption, in addition to sub-optimal prescription that results in unnecessary use of antibiotics (2). In many ways, this results in an ascending rate of antibiotics occurrence in the natural environment of both human and animals equally, because after antibiotic administration, some are excreted through urine and feces into the environment in their active form, along with a common disposal route of remaining unused antibiotics by some people is flushing down drains (3). Additionally, if improper disposal methods are used by manufacturing industries of antibiotics and related products, it may lead to environmental pollution (3). Another reason that may contribute to raising the presence of antibiotics in the environment is giving antibiotics to animals (3). Using antibiotics in such various ways will lead to inevitable presence of residue around humans, such as in soil, water and animals (4). Throughout the world, due to the increased awareness of people about the sources of healthy food, over the past 50 years, seafood consumption, especially fish, is reaching the highest rates ever than before (5). Fish is one of the essential sources of food for people and considered as a rich source of animal protein (6). All the demands for fisheries have caused aquaculture to become the fastest growing sector of food production (7). In Sulaymaniyah Province/Iraq the rearing season of carp fish in artificial earthen ponds starts between February and May, while harvesting season starts from October to January. According to Omar (8) and Qasim (9) the most common type of fish species cultured in the fish farms in Kurdistan Region of Iraq is common carp (*Cyprinus carpio*) because its rearing is cheap, easy, and efficient with its ability to withstand diseases, and adapt to low water quality. Additionally, similar to other animals, fish is also prone to diseases of bacterial origin (10) and for treatment, one of the most used antibiotic drugs used in aquaculture is oxytetracycline, due to their wide spectrum activity against various bacteria, inexpensive price, and availability (11). Administration of oxytetracycline to fish is prescribed in cases of ulcer, furunculosis, red mouth ulcer, etc. (12). In recent years, due to the high demand on fish meat, the number of farms that rear common carp has increased, and in order to maximize the productivity, intensive cultivation system is used, thus upraising the probability of diseases and infection occurrences. For this reason, to treat the bacterial infections, oxytetracycline is administered to the fish (11).

In Iraq, there is no specific regulation of antibiotic administration. Thus, as a side effect of over- and improper administration of oxytetracycline, residue in edible tissues is inevitable (13) because of its hydrophilicity in nature and low volatility, their presence in aquatic environment will last for longer periods of time (14).

According to Food and Agriculture Organization (FAO), Food and Drug Administration (FDA) and Environmental Protection Agency (EPA), the maximum residue limit (MRL) of oxytetracycline in fish muscle is 200 ppb (200µg/kg) (15, 16). And excessive bioaccumulation of oxytetracycline in edible tissues that are consumed by humans as a source of food, may lead to undesirable effects on human body, because ingestion of oxytetracycline can cause tooth discoloration (17), allergy (12), and in pregnant women, teratogenicity in fetus may occur (18). Furthermore, when fish meat with OTC residue is eaten for a long period of time consistently, even presence of a small amount in the meat may lead to development of antibiotic resistance over time (12, 14, 19). And nowadays, one of the biggest concerns of the agencies regulating food safety is antibiotic resistance, and aquaculture is a potential source of this phenomenon (13). If there is no control over antibiotic use in fish ponds, the MRL in the meat may be reached easily and pose a threat to both the fish and the consumers altogether. To provide safe food, tolerance levels of authorized veterinary drugs has been established by national and international agencies, that includes oxytetracycline (which is 200 µg/kg or 30 µg/kg daily intake is the tolerance level of oxytetracycline according to (20). For that reason, many methods of different sensitivity and specificity have been developed for the quantification of tetracycline compounds. The aim of the present study is to detect and quantify the presence of oxytetracycline in common carp fish '*Cyprinus carpio'* in Sulaymaniyah Province/Iraq by Using high-performance liquid chromatography (HPLC), which was selected because of its efficiency, sensitivity, and specificity advantages.

Material and Method

Equipment, chemicals, and reagents: The HPLC used for the present study was a Shimadzu 10AV-LC system, which was equipped with a Binary Delivery Pump model: LC 10A Shimadzu, the eluted peaks were monitored by UV –Vis 10 A- SPD spectrophotometer. The column used for analysis was an HCMA-BIO 1000 C-18 ,3µm particle size (50 x 4.6 mm I.D) column. The mobile phase used for separation was optimized with a mixture of 0.03 M oxalic acid, acetonitrile and methanol (70:20:10 V/V/V), which was filtered through a 0.45 mm sized pores of MS® Nylon membrane filter and sonicated for 15 minutes before use. 1 ml/min was the flow rate, and detection UV was set at 254 nm. Pure standard oxytetracycline (99%) was obtained from Sigma-Aldrich company Ltd., St. Louis/USA. Deionized water was prepared in the lab by using a milli Q water purification system and was filtered through a 0.22 mm Millipore membrane filter. Acetonitrile and methanol were purchased from Sigma- Aldrich. Trifluoroacetic acid, ethylene diamine tetra-acetic acid (EDTA), Oxalic acid di-hydrate, Citric acid and tri-sodium citrate 2-hydrate were purchased from BDH Company, UK.

Study Area: This study was conducted in Sulaimanyah Province/Iraq, the two richest towns were selected in terms of fresh water and aquaculture, Qaladze and Taqtaq. Qaladze is a town in the north of Sulaymaniyah City, rich in fresh natural spring water, with average climate temperature of 8.5℃ during autumn season. Taqtaq is a town located in the northeast of Sulaymaniyah city and is dominated by Lesser Zab River. Many aquaculture ponds have been created in these two towns.

Stock Solution: The oxytetracycline stock solution was prepared by weighing 0.01g oxytetracycline standard and then dissolved in 10 ml of methanol to acquire a concentration of 1000µg/ml. The stock solution was kept in an environment protected from light, stored at -18℃, and prepared monthly. Working solution was acquired by combining 1 ml of the stock solution in a 10 ml of methanol and provided us with a concentration of 100µg/ml, the working solution was kept away from light, stored at 4℃, and was prepared weekly (11).

Preparation of matrix-matched calibration standards: By spiking different concentrations of oxytetracycline in a number of antibiotic-free samples, a series of calibration standards were prepared. 6 different concentrations, between 0.15 to 4µg/ml were selected. Spiked samples were then treated as the other samples and under the same condition.

Sample collection and preparation

27 common carp for each dorsal and caudal skeletal muscle (total of 54) were randomly collected from 9 different aquaculture ponds (each composed of one or more earthen ponds) during autumn season, between November and December, 2021. The day of selling of the fish was chosen to collect the samples as the fish are directly delivered to the consumers. A questionnaire was asked about the history of the pond regarding diseases and dates of administration of OTC. Unfortunately, some farmers used OTC randomly without any observed diseases and some farms even were during withdrawal period and yet was sold to be delivered to consumers during the current study. 3 fish as samples (for each muscle type, total 6 fish) were taken from each pond. The mean weight of the samples was 1.5kg ± 100g. Each sample was properly packed and put in a cold box, transferred to the laboratory, in the preparation phase, samples from each fish were collected, from the two different muscles of the fish (caudal and dorsal muscles) and kept at -18℃ until analysis time, March, 2022. Performing the tests on the samples in less than 6 months was mandatory as OTC in freezing state reduces only 2.05% in up to 6 months, but more than 32% in a year (21). Two different muscles were chosen as to determine whether different locations of the skeletal muscle have any effect on the residue level in the same type of fish sample and to make a comparison between them. First, 10 grams of each sample was weighed, using a Sartorius digital scale, blended then put into a propylene tube. Then, 4 ml of 20% trifluoroacetic acid (TFA acid) and 2 ml of 0.01 M EDTA were added (22), and the

mixture was vortexed for 3 minutes. Next, a mixture of methanol: 0.01 M citrate and PH4 (80:20) was added to attain a total volume of 20ml, and the mixture was vortexed for 3 minutes and sonicated for 10 minutes at room temperature. After that, for 20 minutes it was centrifuged at 4,000 RPM. Finally, the supernatant was filtered through a 0.22 nylon filter, (this is done according to Alanazi, Almugbel (11) method), and as a modification to the methods used, the final volume was concentrated by a stream of liquid nitrogen pass through the propylene tube (bubbling) to reach 1ml to enhance the low detection limit, and 50µl was injected into a specific HPLC tube for analysis. And to analyze the final results, GraphPad Prism software was used to execute unpaired t-test and a group analysis test

Linearity

By injecting tube blank samples by standard solution of oxytetracycline hydrochloride at concentration rates of (0.015, 0.07, 0.17, 0.22, 0.3, 0.4 µg/ml) which were populated with the area under the curve, the calibration curve was prepared. The derived line equation was (y = 174153x + 3795.3). The correlation coefficient was (R2 = 0.9998) in which this equation manifests the linearity of the equation as all are shown in figure 1.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

By using signal-to-noise ratio, the limits of detection and quantification were measured. A concentration yielding 3:1 signal-to-noise ratio was defined as LOD, a concentration yielding 10:1 signal-to-noise ratio was defined as LOQ, which calculated as 0.015 µg/g and 0.05 µg/g, respectively.

Method Validation

Based on the guidelines of International Council of Harmonization (ICH), the evaluation and measurement of analytical methods and parameters were done in order to confirm the validity of the method (23).

Accuracy and precision

To demonstrate the accuracy and precision of the used method, recovery percentage, error percentage, and relative standard deviation, three blank samples were injected with three different concentration of the OTC standard (0.4, 0.6, 0.8 µg/g)) and spiked, to attain intra-day variation, this process was executed two times. In addition, to attain inter-day variation, the mentioned process was executed for three consecutive days. Due to the Relative Standard Deviation (RSD) result of less than 5%, the optimized method was considered to be precise and dependable.

Matrix Effect and Recovery Study

Matrix effect was evaluated in order to measure the effect of the matrix components on the detector's response. By comparison between the peak areas of calibration standards, which are matrix matching (added standards to blank samples) and non-matrix matching (standard solutions), the quantitation was attained. The evaluation can be done by comparing the peak areas or concentration of the matrix matched solutions with the analytical standard signals at the same concentration (Table 1). This

equation (demonstrated below) can be used at any chosen concentration. MEs can be calculated as follows: ME (%) = (found concentration of the standard in matrix – concentration of the standard in pure solvent)/concentration of the standard in pure solvent) × 100. In addition, to assess the recovery rate, an extracted muscle that was blank sample that did not have any oxytetracycline response when tested, was used and was prepared with 5 different OTC concentration levels (0.05, 0.15, 0.35, 0.6, and 0.8 µg/g). After the extraction procedure and test, the obtained signals of the samples were compared with that of the standard solution. The attained recoveries percentage ranged from 94 to 102% showing an acceptable recovery range according to ICH (23) suggesting the effectiveness of the procedure used to extract oxytetracycline in fish meat samples as shown in Table 1.

Table 1: Recovery study and matrix effect.

	Retention Time	Added Concentration (µg/g)	Found Concentration (µg/g)	Recovery %	ME %
OTC	3.0 to 3.2 minutes.	0.8	0.80495	100.621	0.618
		0.6	0.61413	102.35	2.355
		0.35	0.3565	101.86	1.857
		0.15	0.14267	95.11	-4.886
		0.05	0.04721	94.44	-5.58

Result

Figure 1 shows the linearity graph of the calibration curve that has been conducted to show the efficacy of the equipment used in the current study. While figure 2 shows the peak area (224386µv) and retention time (3.053 min) of the OTC standard used in the current study. Figure 3 is demonstrating the blank sample of caudal skeletal muscle that has been used with no response at the retention time, and figure 4 is representing the dorsal skeletal muscle blank sample. In addition, figure 5&6 are of a random sample of caudal and dorsal skeletal muscles, respectively. The results of the caudal and dorsal muscles' samples tested for any OTC residue are shown in Table 2. OTC residue was found in 28 samples (52% of the samples), either in dorsal muscle or in caudal muscles, and OTC residue was not

detected in 26 samples (48% of the samples). In most of the samples where residue was present, the rate was lower than the MRL set by FAO, WHO, FDA and EPA. In two caudal skeletal muscle samples the level was slightly higher than the MRL (sample 8, pond 3 & sample 14, pond 5) while all the residues detected in dorsal muscles were lower than the MRL level. To analyze the results, GraphPad Prism Statistical Analysis Software was used, the results of the samples that had residues in them were compared by a two-tailed, unpaired t test (as shown in Graph 1) that clarified there is no significance difference between the muscle locations to bear residues with a p value of (0.5338). In addition, a group test analysis was performed (Graph 2) in between the ponds' samples to show the comparison between the residues in the dorsal and caudal muscles, and according to the result, there is no relation between muscle location and amount of residue.

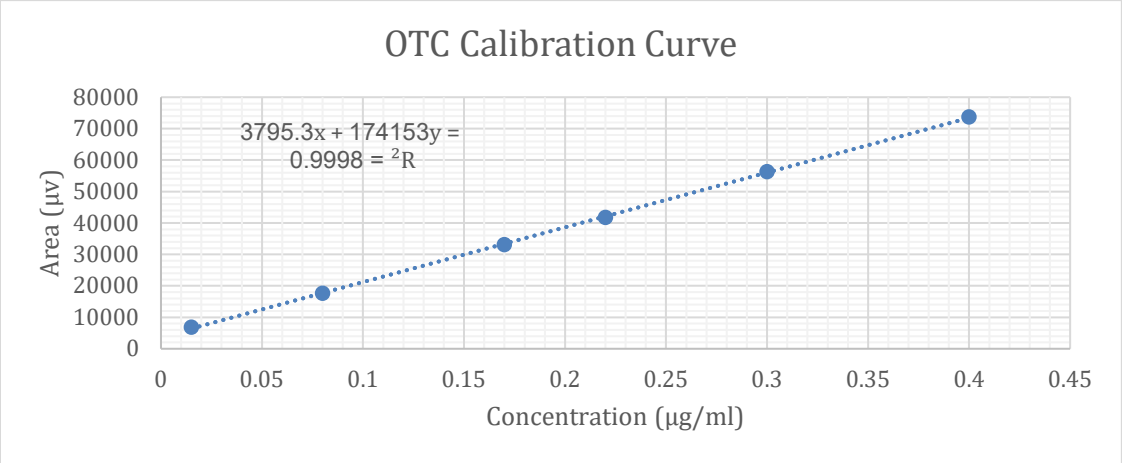


Figure 1: Calibration curve of OTC, Y-axis implies area of sample in µv, X-axis implies concentration of sample in µg/ml, R2 implies correlation coefficient.

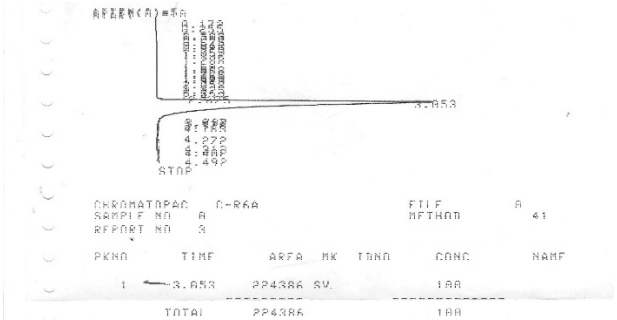


Figure 2:HPLC peak area result and retention time of OTC standard.

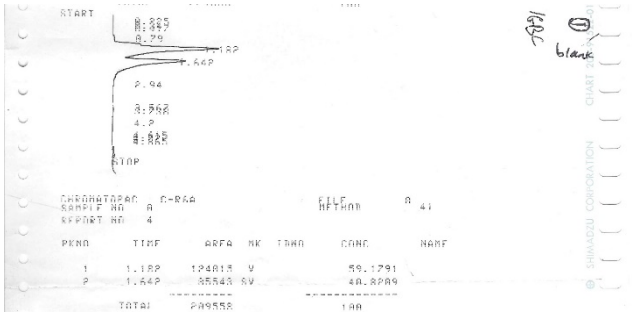


Figure 1:HPLC peak area result and retention time of caudal skeletal muscle of the blank sample.

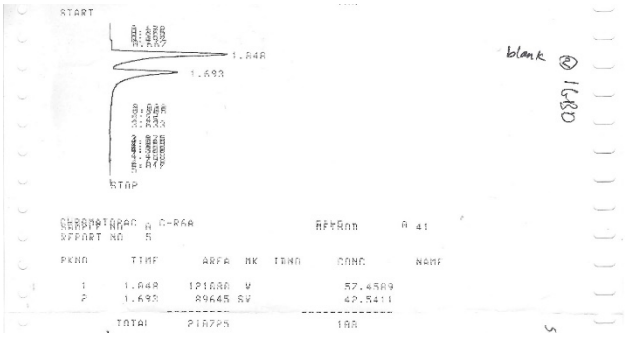


Figure 3: HPLC peak area result and retention time of dorsal skeletal muscle of the blank sample.

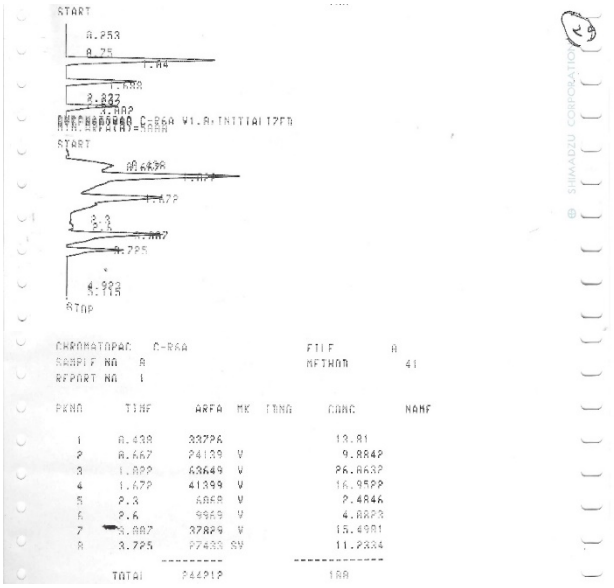
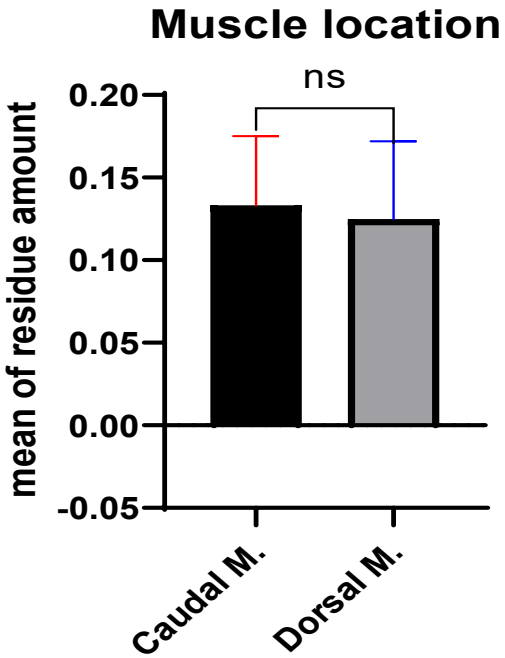


Figure 4: HPLC peak area result and retention time of caudal skeletal muscle of a sample.



Graph 1: Two-tailed, unpaired t test analysis of the residue results to compare the amount of OTC residue in each type of muscle, and shows that the location of the muscle does not change the amount of residue level.

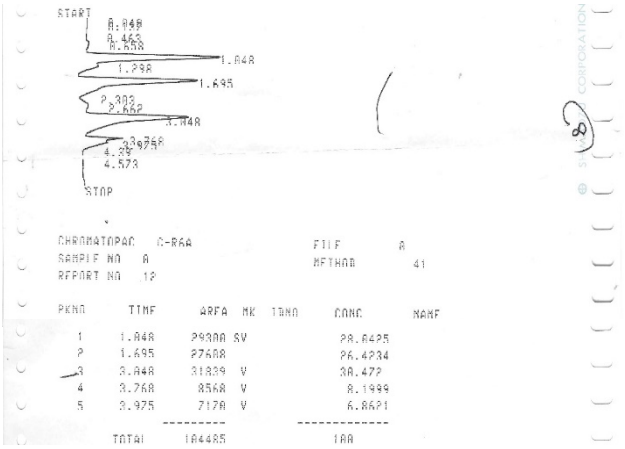
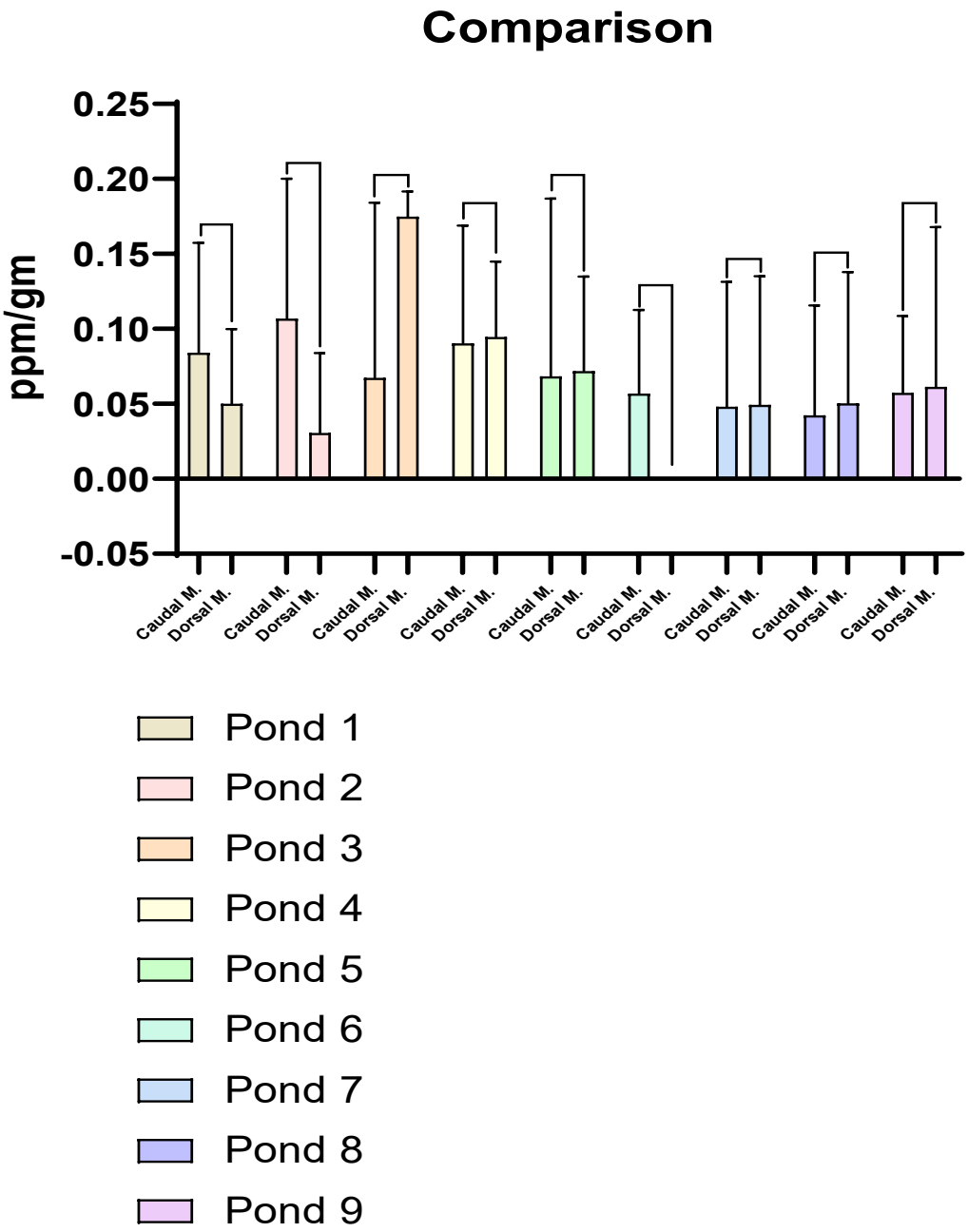


Figure 5: HPLC peak result and retention time of dorsal muscle of a sample.



Graph 2: Group test analysis to compare the residue amount between dorsal and caudal muscle of the samples of the same pond.

Table 2: Residue levels of the 54 random samples (27 for each muscle type) taken from 9 aquaculture ponds. MRL=200ppb, BDL=Below Detection Limit, 1ppb=0.001µg/g.

Ponds	Samples	Caudal Residue (ppb)	Dorsal Residue (ppb)
	1	BDL	48
	2	119	100
Pond 1	3	133	BDL
	4	BDL	92
	5	148	BDL
Pond 2	6	172	BDL
	7	BDL	193
	8	202	170
Pond 3	9	BDL	160
	10	137	82
	11	BDL	51
Pond 4	12	133	149
	13	BDL	118
	14	205	96
Pond 5	15	BDL	BDL
	16	59	BDL
	17	BDL	BDL
Pond 6	18	111	BDL
	19	BDL	BDL
	20	144	BDL
Pond 7	21	BDL	148
	22	126	BDL
	23	BDL	BDL
Pond 8	24	BDL	151
	25	99	BDL
	26	BDL	BDL
Pond 9	27	72	184

Discussion

In advanced countries, major concerns about antibiotic resistance have been raised due to antibiotic use in animals that are considered food product origins (1, 4, 19). In the recent years, in Kurdistan Region of Iraq (North of Iraq), the demand on fish meat, especially common carp, for consumption has increased significantly, thus prompting farming of the common carp. In this process, the use of antibiotic for treating the diseases that affect the farms has increased simultaneously. The use of oxytetracycline antibiotic in aquaculture by veterinarians are drug of choice due to its wide range of antibacterial activity (11). Since this study is the first research done on quantifying oxytetracycline residue in aquatic culture using HPLC in north Iraq and on the most

commonly consumed type of fish meat, and the fact that Iraqi veterinary organization does not keep track accurately and has not officially set a maximum dose for this drug, thus the results are dependent on either the European commission or the surrounding areas that geographically and veterinary medicine-wise resemble Iraq's. HPLC testing system was selected to conduct our research due to its high sensitivity and accuracy, in addition to its specificity level (24, 25) as it was used in a research that was conducted in a geographically near area by Rafati, Ehrampoush (12) that lead to the conclusion of having residue in all their fillet samples, which the mean was 236.42 ± 26.37 ppb, and most exceeded the MRL. In this research, 48% of the samples were clear of any detectable residue, while 52% contained residues. Out of the 28 fish samples that contained residues, 2 of them (7%) exceeded the MRL while the others bearing residues (93%) didn't exceed the MRL.

Conclusion

As the population increases in an area, the demand on food increases. Because aquatic food composes a large part of the food chain, the demand on it has increased as well. Due to this increased demand, creation of ponds and building farms of aquatic culture has increased in Kurdistan Region of Iraq (KRI). One of the most consumed and demanded aquatic food in KRI is common carp (*Cyprinus carpio*). Many ponds are built to rear carp, and it is susceptible to diseases and when infected with bacterial diseases, many veterinarians use oxytetracycline to cure the disease. Due to increased usage of oxytetracycline, the risk of residue has increased. The result was promising as well as threatening, because if the government does not regulate the use of oxytetracycline and limit its usage, the results of residue may increase in the upcoming years and expose great risks on the general health of the consumers.

Acknowledgment

The author is grateful to the University of Sulaimani /College of Veterinary Medicine for all the facilities to achieve this study. Many thanks to Mr. Brwa Abdulla and Dr. Aram Bagzada for their help in the sample collection. Best Regards to Miss Lara A. Othman for her help in the calculation and analysis process.

Conflict of Interest

There is no conflict of interest.

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القياس الكمي لبقايا أوكسي تتراسيكلين فى لحوم اسماك الكارب العادي المستزرعة فى محافظة السليمانية/ العراق

شيخ محمد على عثمان، نسرين محى الدين عبد الرحمن، نهلة محمد سعيد

كلية الطب البيطرى/ جامعة السليمانية/ السليمانية/ العراق.

الخلاصة

بسبب نقص سيطرة الحكومة في العراق على استخدام المضادات الحيوية من قبل مزارعي الأحياء المائية، يعد استخدام المضادات الحيوية بشكل عشوائي بارزًا، وخاصة المضاد الحيوي أوكسي تتراسيكلين . (OTC) يستخدم أوكسي تتراسيكلين كمضاد حيوي فعال في صناعات الأحياء المائية لأنه سهل الحصول عليه ورخيص التكلفة وفعال ضد الأمراض البكتيرية. هدفت الدراسة الحالية إلى قياس ومقارنة كمية بقايا OTC في أكثر عضلات الاسماك المزروعة استهلاكًا، وهي العضلات الظهرية والعضلات الذيلية، من قبل سكان محافظة السليمانية. تم أخذ عينات من العضلات الظهرية والعضلات الذيلية لـ 27 سمكة كارب (لكل نوع عضلة 27 عينة، 54 عينة في المجموع) بشكل عشوائي من تسع مزارع سمكية، التي تتألف من حوص ترابي واحة أو أكثر، في مناطق قلعةدزه و تەقتەق بالقرب من محافظة السليمانية في العراق. تم قياس بقايا OTC باستخدام الكروماتوغرافيا السائلة عالية الأداء (HPLC) . تم إجراء تحليل t-test غير المتوافق ذو الذيلين للنتائج باستخدام برنامج GraphPad Prismلمقارنة مستويات البقايا في منطقتي العضلات الظهرية والذيلية. كانت بقايا OTC موجودة في 44 عينة، في حين أن البقايا لم يتم الكشف عنها في 10 عينات، وكانت هناك عينتان فقط من عضلات الذيلية زادت عن الحد الأقصى المسموح (MRL) بينما كانت جميع عينات العضلات الظهرية اقل من .MRL كشفت الدراسة الحالية عن وجود بقايا OTC في اسماك الكارب العادي، وأن هناك بقايا في العضلات المختلفة بغض النظر عن موقعها في جسم اسماك الكارب العادي. بالإضافة إلى ذلك، يجب تحديد مستوى MRL ووضعها في التشريعات القانونية من قبل الحكومة العراقية للحفاظ على استخدام OTCفي المزارع السمكية ومستويات البقايا في لحوم الأسماك تحت السيطرة.

الكلمات المفتاحية: الكارب العادي، HPLC، البقايا التراكمية، أوكسي تتراسيكلين.