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Article

Antibiotic residues in farmed raised tilapia (*Oreochromis niloticus*) of southwest region of Bangladesh

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Abstract: The objectives of this investigation were to seek out the residue level of nitrofurans and chloramphenicol in cultured tilapia muscle in south west region of Bangladesh. Ninety samples were collected randomly from various fish farm of different Upazillas of Khulna, Satkhira and Bagerhat districts during August-December, 2017, where each district was contained 30 samples. Antibiotic residues level present in the animal muscles were detected using ELISA in the Quality Control Laboratory of the Department of Fisheries, Khulna, Bangladesh. To collect information about the status of feed type questionnaire interviews were performed during sample collection from the respective farmers. The overall results revealed that nitrofuran metabolites and CAP residue existed in all the samples within the MRL (Maximum Residue Level) with the exception of 3.33%, 6.66% and 3.33% samples of Khulna, Bagerhat and Satkhira district respectively. Antibiotic residue data showed all nitrofuran metabolites like SEM, AOZ, AMOZ, AHD and CAP were present in all samples in which SEM was highest in Shatkhira district and lowest in Khulna district. In case of AHD, highest residue was found in Khulna and lowest was Satkhira district. Additionally, AOZ was highest in Khulna district and lowest in Bagerhat district and AMOZ was highest in Bagerhat district and lowest Satkhira district. CAP was found highest in Satkhira district while that was lowest in Bagerhat district. The results of the also revealed that contamination of nitrofuran metabolites and chloramphenicol were more in tilapia farms where home-made feed was used compared to commercial feed.

Keyword: nitrofuran; chloramphenicol; antibiotic; tilapia; feed

1. Introduction

Bangladesh is one of the world's leading fish producing countries. According to FAO statistics 2018, Bangladesh is ranked fifth in world aquaculture production. The role of fisheries sector to national economy has always been significant and it is considered as the main source of animal protein, employment opportunities, food security, foreign incomes and socio-economic improvement (Siddiq *et al.*, 2013). Bangladesh exports fish and fisheries products to more than 50 countries, including Belgium, United Kingdom, Netherlands, Germany, United States of America, China, France, Russian Federation, Japan and Saudi Arabia. However, some technical barriers to trade, such as international standards and regional technical regulations in the importing countries aimed at protecting consumers from the presence of chemical residues and contaminants in traded aquaculture products associated with intensive farming, may have significant impact on the efforts in these developing countries. Different drugs and chemicals have been widely used to improve the aquaculture production (Hossain *et al.*, 2018). A regrettable fact that, nitrofuran and chloramphenicol occupy an important place as aqua chemicals in the market of Bangladesh and the distribution as well as application of these drugs is most often by unqualified people. Most alarming point is the improper and uncontrolled use of these products without

observing the required withdrawal period can result in high residue levels in fisheries and the environment (Barani and Fallah, 2015), which ultimately can contribute to the development of antibiotic resistance, is a major concern for human and animal health worldwide (WHO, 2014) as well as hamper export sector. The world health organization (WHO), European Union (EU) and government of Bangladesh have a policy of zero tolerance toward the use of nitrofurans and chloramphenicol in food-producing aquatic animals.

Considering the above facts, the present study was conducted to determine the residue level of nitrofurans and chloramphenicol in cultured tilapia in south west region of Bangladesh as well as to trace out the actual sources this banned products.

2. Materials and Methods

2.1. Sample collection and questionnaire interview

Ninety samples were collected randomly from different Upazillas of Khulna, Satkhira and Bagerhat districts where 30 samples were collected from each district. The samples were directly collected from various fish farms during August-December, 2017. The weight of each fish sample was 130-150g. The samples were collected in polyethylene bags and kept in ice. Then the samples were brought to the Quality Control laboratory of Department of Fisheries in Boyra, Khulna and stored at -20^{o}C. While, to collect information about the status of feed type questionnaire interviews were performed during sample collection from the respective farmers. For that purpose, a structural questionnaire was developed to assemble information.

2.2. Laboratory procedure

Nitrofuran metabolites and Chloramphenicol residues present in the shrimp muscles were detected using Enzyme- Linked Immunosorbent Assay (ELISA) in the Quality Control Laboratory of the Department of Fisheries, Khulna, Bangladesh. TMSOP/C-02, TMSOP/C-07, TMSOP/C-08 and TMSOP/C-016 Test Methods were used for determination of nitrofuran metabolites (AHD, AMOZ, AOZ, SEM) in fish using ELISA while TMSOP/C-01 test method for Chloramphenicol (CAP) by Quality Control Laboratory of the Department of Fisheries, Khulna, Bangladesh.

2.2.1. Sample preparation

Firstly, frozen sample was thawed and kept in normal temperature. Then the sample was washed with reverse osmosis water. After removal of head, scale and body appendages as well as air drying, samples were chopped individually and taken into a blender to make paste.

2.2.1.1a. Extraction procedure for chloramphenicol (CAP)

At first 3g tissue was added with 6 ml ethyl acetate and homogenized in vortex for 1 minute. Then the mixture was centrifuged at 2000 rpm for 15 minute and two layers were appeared. Four ml of upper phase were removed and reduced to dryness at 70°C under N_2 atmosphere. The residue was dissolved in 2 ml of isooctane/chloroform (2:3) and again vortexed for 1 min then 0.5 ml of diluted Tissue Extraction Buffer was added and vortexed for 2 min. The mixture was centrifuged at 2000 rpm for 15 minute and the upper phase was ready for application to microtitre plate.

2.2.1.1b. Sample preparation procedure for ELISA

Before starting ELISA procedure all reagents were kept in room temperature (19-25^{o}C) in order to minimize edge effects. The assay was performed in duplicate following a layout prepared according to sample number.

Twenty-five microliter (25 µl) standards from lower to higher concentration were pipetted into the appropriate wells of the microtitre plate (8x12). Then 25 µl sample extract of the 2 tissue blank were pipetted in duplicate. One set of blank duplicates at the beginning and once at the end of the set were plated. Again 25 µl sample extract of the recoveries were pipetted in duplicate, then plated them once at the beginning and once at the end of the set. Then 25µl of extracted sample were pipetted in to separate wells. New pipette tip was used for each standards or sample. 100µl conjugate were pipetted to each plate. Then the microtitre plate was covered with adhesive film. After that the microtitre plate was tappped from side to side for a few seconds before incubating for half hour at room temperature (+19 to + 25°C) in the dark. The plate was inverted and liquid were tapped out. Microtitre plate were washed 6 times with diluted wash buffer over a 10-15-minute period. After final washing liquid were discarded and tapped onto tissue paper until completely dry. Immediately after washing, 125µl of the one shot substrate solution were pipetted into each well. Microtitre plate were tapped from side to side and incubated the microtitre plate for 20 minutes at room temperature (+19 to +25°C) in the dark. Colour reaction were stopped by the addition of 100 µl of Stop Solution per well. A colour change from blue to yellow

were evident. The optical density of each well at 450 nm and 630 nm filter as the reference wavelength were measured within 10 minutes of stopping the colour reaction.

2.2.1.2a. Extraction procedure for nitrofuran

At first each sample was homogenized after removing head, shell and body appendages. One gram of homogenized sample was weighed in a 50 ml falcon tube. Weigh four 1g of previously analyzed blank tissue were used as two positive and two negative controls. Fortify two positive controls with AOZ, AMOZ, AHD and SEM respectively at 0.5 µl/kg. After that, 1g of homogenized sample was added with 4ml distilled H_2O, 0.5ml 1M HCl and 50µl 10mM 4-Nitrobenzaldehyde.Vo rtex for 1 minute and incubated for 2 hours at 50^oC. After vortexed 5ml 0.1M K_2HPO_4, 0.4ml 1M NaOH and 6ml ethyl acetate were added and vortex for 1minute.Then the sample was centrifuged for 10 minutes at 4000 rpm at 25^oC and two layers were appeared. After 10 minutes 3ml of the upper ethyl acetate layer of each sample was transferred into test tube and dried at 60^oC under N_2 atmosphere. The residue was then dissolved in 1ml hexane and 1ml diluted Wash Buffer and vortex for 2 minutes. Then the sample was centrifuged for 10 minutes at 4000rpm at 25^oC.The lower phase was ready for application to microtitre plate.

2.2.1.2b Sample preparation procedure for ELISA

Before starting ELISA procedure all reagents were kept in room temperature (19-25^oC) in order to minimize edge effects. The assay was performed in duplicate following a layout prepared according to sample number. 100 µl standards from lower to higher concentration were pipetted into the appropriate wells of the microtitre plate supplied for AMOZ/AOZ/AHD/SEM. Then pipetted 100 µl sample extract of the two tissue blank in duplicate. One set of duplicates at the beginning and the once at the end of the set were plated. Again 100 µl sample extracts of recoveries were pipetted in duplicate and plated them once at the beginning and once at the end of the set. Then 100 µl of extracted sample were pipetted into separate wells. A new pipette tip for each standards or sample was used. 50 µl of conjugate were added in each well and mixed well by gently rocking the plate for 1 minute. Then the plate was incubated for 30 minutes at room temperature (20-25^oC). After 30 minutes the plate were washed 6 times with the wash buffer. After the last wash the plate was inverted and gently tapped the plate dry on paper towels. After drying 100 µl of one shot substrate were added and mixed the solution by gently rocking the plate for 1 minute. After that the plate was incubated for 20 minutes at room temperature (20-25^oC) in dark. Colour reactions were stopped by the addition of 100 µl of stop solution per well. A colour change from blue to yellow was evident. The optical density of each well at 450 nm and 630nm filter as the reference wavelength was measured within 10 minutes of stopping the colour reaction.

2.3. Analysis of data

The collected data were scrutinized, summarized carefully and tabulated. Normality test was done to check the data after input the data in Microsoft Excel 2013. As the data were parametric, One Way Variance was conducted.

3. Results

3.1. Types of nitrofuran metabolites and chloramphenicol

Chloramphenicol (CAP) and four types of nitofuran metabolites namely furazolidone (AOZ), nitrofurazone (SEM), furaltadone (AMOZ) and nitrofurantoin (AHD) were found to be bind with the protein in the body muscle of tilapia.

3.2. Level of different metabolites in tilapia muscle

All the 90 samples of *O. niloticus* were detected with banned antibiotics below the level of maximum residual limit (MRL) (Table 1).

3.3. Area wise level of different metabolites in tilapia muscle

The contamination of sample with banned antibiotics in Khulna, Bagerhat and Satkhira were 100, 100 and 100% respectively. Different types of nitrofuran metabolites were found in different animal samples at different levels. In most cases more than one metabolite was found in one or more samples. Area wise percentage contaminated samples above MRL are given in Table 2.

In the present study, a total of 90 tilapia samples from three different districts were tested for all the nitrofuran and CAP residues, only 4 samples were found to be contaminated with banned antibiotics above maximum residue level. Among them 3 samples were positive with AHD metabolite and 1 sample was positive with SEM

metabolite. SEM, AHD, AOZ, AMOZ and CAP were detected in all the samples and no samples were completely free from antibiotic residue. The study also revealed that more than 95% samples does not exceed the FIQC recommended acceptable limit.

Mean value of nitrofuran metabolites and chloramphenicol (ppb) in tilapia muscles in different areas are presented in Table 3. It was evident that all nitrofuran metabolites like SEM, AOZ, AMOZ, AHD and CAP existed in all samples in which SEM was highest in Shatkhira district that was lowest in Khulna district. In case of AHD, highest residue were found in Khulna and lowest in Satkhira district. Additionally, AOZ was highest in Khulna district and lowest in Bagerhat district and AMOZ was highest in Bagerhat district and lowest in Satkhira district. CAP was found highest in Satkhira district and that was lowest in Bagerhat district.

3.4. Factors responsible behind antibiotic residue

Several factors might play an important role behind this and among them direct application of antibiotics in supplementary feed, use of commercial poultry feed, use of homemade feed with organic fertilizer like cow dung and inorganic fertilizers.

The questionnaire survey revealed that, in Khulna district, 40% farms used commercial feed, 23.33% farm used homemade feed with organic and inorganic fertilizers such as cow dung, poultry litter, urea, triple super phosphate and murate of potash, 16.67% farms used only organic and inorganic fertilizer and 20% farms operated without feed or fertilizer. In Bagerhat, 26.67% farms used commercial feed, 36.67% farms used homemade feed with organic and inorganic, 23.33% farms used only organic and inorganic fertilizer and 13.33% farms operated without feed or fertilizer. In Satkhira, 23.33% farm used commercial feed, 33.33% farms used homemade feed with organic and inorganic fertilizers, 16.67% farms used only organic and inorganic fertilizer and 26.67% farms operated without feed or fertilizer (Table 4) and the feeds those are frequently used in farms are presented in Table 5.

Table 1. Nitrofuran metabolites and chloramphenicol (ppb) in tilapia muscles.

Types of antibiotic/metabolites	No. of contaminated sample	Range in ppb	MRL
SEM	90	0.003-0.364	0.346
AHD	90	0.007-0.403	0.374
AOZ	90	0.005-0.054	0.355
AMOZ	90	0.002-0.037	0.348
CAP	90	0.007-0.029	0.108

Table 2. Area wise percentage contaminated samples above MRL.

District	No. of sample tested	No. of contaminated sample (Above MRL)	% contamination	Metabolite
Khulna	30	1	3.33	AHD
Bagerhat	30	2	6.67	AHD
Satkhira	30	1	3.33	SEM

Table 3. Area wise nitrofuran metabolites and chloramphenicol (ppb) in tilapia muscles.

District	SEM			AHD			AOZ			AMOZ			CAP		
	Range	Mean±Stdv	MRL	Range	Mean±Stdv	MRL	Range	Mean±Stdv	MRL	Range	Mean±Stdv	MRL	Range	Mean±Stdv	MRL
Khulna	0.008-0.291	0.097±0.079[a]		0.067-0.403	0.187±0.072[a]		0.005-0.054	0.024±0.012[b]		0.002-0.037	0.011±0.007[ab]		0.005-0.029	0.014±0.005[a]	
Bagerhat	0.005-0.339	0.092±0.099[a]	0.346	0.027-0.389	0.181±0.086[a]	0.374	0.005-0.022	0.012±0.005[a]	0.355	0.002-0.037	0.012±0.008[b]	0.348	0.007-0.025	0.013±0.004[a]	0.108
Satkhira	0.003-0.364	0.121±0.085[a]		0.054-0.249	0.182±0.058[a]		0.005-0.036	0.015±0.008[a]		0.002-0.019	0.007±0.004[a]		0.007-0.028	0.015±0.005[a]	

Different superscripts indicate significant difference at 5% level of significance

Table 4. Area wise percentage of different types of feed used in different farms.

Types of farm	% of farm applied		
	Khulna	Bagerhat	Satkhira
Commercial feed	40	26.67	23.33
Homemade feed with organic and inorganic fertilizer	23.33	36.67	33.33
Only organic and inorganic fertilizers	16.67	23.33	16.67
Without feed or fertilizer	20	13.33	26.67

Table 5. Name of the feeds that frequently used in different farms in three districts.

Feed Name	Company	Ingredients	Rate of feeding	Status
Tongway Tilapia Grower	Tongway Feed Mill company Ltd.	Fish meal, Soyabean meal, Meat and bone meal, Flour, Rice bran, Vitamin	2-3% of body weight.	Commercial feed
Tilapia Finisher	Spectra Hexa Feeds Ltd.	Fish meal, Wheat, Rice polish, Masterd oil cake, Soya meal, Meat and bone meal, Flour, Salt, D.C.P. Vitamin and Mineral premix.	2 times per day	Commercial feed
Carp-Tilapia Pre Nursery powder Feed	SMS Feeds Ltd.	Fish meal, Soyabean meal, Flour, Fish oil, Rice bran, Amino acid, Vitamin and Mineral premix, Enzyme, Natural growth promoter.		Commercial feed
Floating Tilapia Feed	Paragon Group	Fish meal, Soyabean meal, Rice polish, Flour, Vitamin and Mineral premix.		Commercial feed
Floating Tilapia Grower	Unique Hatchery and Feeds Ltd.	Corn, Soya meal, Full fat soya, Vegetable protein, Fish meal, Flour, Rice polish, Deoiled rice bran, Amino acid, Vitamin, Mineral and Feed additives.		Commercial feed
Floating Carp Fish Feed	Paragon Group	Fish meal, Soyabean meal, Rice polish, Flour, Vitamin and Mineral premix.		Commercial feed
Saudi-Bangla feed	Saudi-Bangla Fish Feed Company Ltd.	Fish meal, Fish oil, Soyabean meal, Gluten meal, Yeast meal, Wheat flour, Growth promoter.		Commercial feed
Supplementary feed		Rice bran, Wheat bran, Oil cake and occasionally incorporating Soybean meal and Fish meal.	Once or twice in a week	Homemade feed
Farm made feed		Rice bran, Wheat bran, Oilcake, Fishmeal, Flour, Maize, Oyster shell, Salt, Vitamin premix and Additives.	Once or twice in a week	Farm made feed

4. Discussion

The analyzed results showed antibiotic residues in the all sample but below MRL. It was also found that antibiotic concentrations widely varied in different area. Compared antibiotic residue monitoring data with the questionnaire survey, major observed findings were: (i) 3.33%, 6.67% and 3.33% sample of Khulna, Bagerhat and Satkhira district contaminated with AHD, AHD and SEM, ii) among the 4 nitrofuran metabolites, AHD was abundantly found (iii) tilapia raised in farms fed with homemade feed with organic and inorganic fertilizers were more contaminated with nitrofuran metabolites than the farms fed with commercial feed iv) CAP also widely distributed among samples. Many researchers in Asian countries have detected SEM in many crustacean muscles (Pereira *et al.*, 2004; Saari and Peltonen, 2004). Researchers reported that these type of metabolites sometimes naturally occur in samples in low concentration. Antibiotic residue presents in muscle below shell occur due to nature while present in muscle near stomach due to feeding antibiotic. The most frequent administration route for antibiotics in white fish is oral, in which the antibiotic is incorporated into the feed with subsequent exposure to the extremely aggressive aquatic environment.

It has been found that, the residue of AHD was highest among 4 nitrofuran metabolites of three districts and 6.33% and 3.33% contaminated samples were detected in Bagerhat and Khulna districts with AHD metabolite above maximum residue level. But in Satkhira, 3.33% contaminated samples were detected with SEM metabolite.

In aquaculture a variety of feeds are used in Bangladesh including supplementary feed, farm-made feeds and industrially manufactured pelleted feeds. In general, extensive farmers mainly use supplementary feed (Mamun-Ur-Rashida and Belton, 2014). Homemade feed was usually prepared by rice bran, wheat bran, oil cake and occasionally by soybean meal and fish meal. It has been showed that, farms those used homemade feed with organic and inorganic fertilizers mostly used antibiotic intentionally or unintentionally in Bagerhat and Khulna district. In Bagerhat district, 36.67% farms were found which use homemade feed with organic and inorganic fertilizer and in Khulna district 23.33% farms were found to use homemade feed. In two cases, 6.66% and 3.33% contaminated fish were found in Bagerhat and Khulna district respectively. But in Satkhira district, 3.33% contaminated fish were identified which fed with commercial feed.

Farmers mainly use organic and inorganic fertilizers in three districts. The most widely used organic fertilizer was cow dung, which is relatively cheap and readily available in rural Bangladesh. The use of inorganic fertilizer is not widespread. It has been found that, in most cases, farmers often use inorganic fertilizers like urea, lime, murate of potash and triple super phosphate (TSP), which are usually used in combination with cow dung. But the questionnaire survey revealed that the use of poultry litter as organic fertilizer was common in three districts.

Antibiotic was frequently used in farms which reared fish with homemade feed containing organic and inorganic fertilizers. In this study, it was found that farms which used homemade feed with organic and inorganic fertilizer, 6.33% and 3.33% fish were found contaminated with AHD metabolite in Bagerhat and Khulna district. But in Satkhira district, 3.33% fish were found contaminated with SEM metabolite where commercial feed were used.

According to analysis of FDA (2001) data Food and Water Watch obtained by submitting a Freedom of Information Act request, 39 shipments of shrimp failed import inspections due to the presence of chloramphenicol between 2003 and 2006. However, nitrofurans and chloramphenicol concentration in finfishes was still not well documented.

The most frequent administration route for antibiotics in fish is oral, in which the antibiotic is incorporated in the feed with subsequent exposure to the extremely aggressive aquatic environment. Nitrofurans parent compounds metabolize rapidly after ingestion to form corresponding tissue bound metabolites. Due to this instability, effective monitoring of their illegal use has been difficult. The short *in vivo* half-life of the parent drugs (7 to 63 minutes) results in rapid depletion of nitrofurans in blood and tissue (Nouws and Laurensen, 1990). However, the formed metabolites (AOZ, AMOZ, AHD and SEM) bind to tissue proteins in the body for many weeks after treatment, making them more practical for monitoring public compliance of the EU ban (Cooper *et al.*, 2005). While chloramphenicol is only partially deactivated by cooking. In a study, fish cooked for 30 minutes at 212º F still retained 71 percent of the antibiotic. (Shakila *et al.*, 2006). Even less chloramphenicol was destroyed when the fish was cooked for a shorter, more typical length of time.

Studies of shrimp ponds in Thailand, Vietnam, the Philippines and Mexico have found relatively high levels of bacteria that are resistant to antibiotics, especially *Vibrio* bacteria (Cabello, 2006). Any time you handle or eat raw or undercooked shrimp/fin fish, you run the risk of getting food poisoning. However, when the muscle of fish you eat were grown with large quantities of antibiotics, you take on the additional risk of getting food poisoning from antibiotic-resistant bacteria, which by definition is much more difficult to treat.

In order to ensure that tissue residues are within tolerance when antibiotics are used in the treatment of disease in aquatic species, established minimum drug withdrawal times must be strictly adhered to prior to harvesting. The ideal aquaculture drug would be rapidly and highly absorbed. It would distribute to desired tissues and have a relatively short half-life (Guarino *et al.*, 1988). However, many antibiotics are not biodegradable and persist in the surrounding environment, where they fight against bacteria that continue to develop resistance. Development of drug resistance in bacteria is not only a human safety concern but may also contribute to the increased incidence and prevalence of chronic and recurrent forms of bacterial disease in aquatic species (Egidius and Anderson, 1979). The accumulation of antibiotic residues in the edible tissues may also alter human intestinal flora and cause food poisoning or allergy problems (Ma *et al*., 2006).

5. Conclusions

Overall, the occurrence of nitrofuran metabolites and CAP contamination in farm raised tilapia beyond acceptable limit except 3.33%, 6.67% and 3.33% samples of Khulna, Bagerhat and Satkhira district respectively. However National Residue Monitoring Plan should be intensified and farmers, feed processors and sellers should be aware and involved in NRMP programs through appropriate traceability network linkage. All commercial feeds should be strictly screened, any chance of deliberative, accidental or incidental leakage should be checked and public awareness should be created against illegal use of antibiotic. Regulatory authorities and producers are required to identify and eliminate the contamination source to ensure the chemical safety of foods available to the consumer.

Conflict of interest

None to declare.

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