World Journal of Fish and Marine Sciences 5 (3): 302-309, 2013
ISSN 2078-4589

DOI: 10.5829/idosi.wjfms.2013.05.03.71214

Antibiotic Usage and Oxytetracycline Residue in African Catfish (*Clarias gariepinus* in Ibadan, Nigeria)

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Abstract: The practice of antimicrobial usage in African catfish (*Clarias gariepenius*) production and the presence of antimicrobial residue in catfish in Oyo State were investigated by cross sectional study. Twenty commercial fish farmers were interviewed using interviewers administered semi-structured questionnaire on the pattern of antibiotic usage, knowledge of occurrence and risks of antimicrobial residues in catfish produced for public consumption. In addition, 165 samples of *Clarias gariepenius* were obtained from ready to eat fish restaurants and farms for screening for antimicrobial residues using Premi®Test kit while oxytetracycline residue was determined by high performance liquid chromatography (HPLC). The results showed that majority (95%) of the respondents frequently administered drugs (in feed and water) to their fish without veterinary prescription and not observing withdrawal period. Oxytetracycline is the commonly used, extra-label drug usage were frequently practiced. Ninety eight (61.3%) liver and 84 (52.5%) muscle of catfish screened contained detectable antimicrobial residues, while 62 (38.8%) liver and 48 (30.0%) muscle had oxytetracycline residue by HPLC method. Oxytetracycline concentrations in the positive samples ranged between 234.3 and 987.5 μg kg^{-1} (mean = 875.32±45μg kg^{-1}) in the liver and 22.5 and 553.2 μg kg^{-1} (mean = 257.2±133μg kg^{-1}) in the fillets. Forty three (26.9%) liver and 30 (18.8%) fillets contained oxytetracycline residues above the Codex Alimentarius Commission established maximum residue limit of 600 and 200 μg kg^{-1} respectively. This implied that appreciable quantity of catfish consumed in Ibadan, Nigeria posed antibiotic residue risks and food safety consequences. Misuse of antibiotics in aquaculture production without veterinary prescription and control coupled with lack of awareness of the food safety consequences were the contributing factors for the high level of residue violation. Therefore, there is need for veterinary supervision of drug use in fish, residue monitoring and food safety education of producers and consumers.

Key words: Oxytetracycline Antibiotic · Residue · *Clarias gariepinus* (Catfish) · Ibadan

INTRODUCTION

The challenges of increasing world population and the need to cater for their nutritional needs through high food demand are driving force behind intensive agriculture and cardinal to the millennium development goals (MDGs). Fish is reported by the Food and Agriculture Organization [1] to contribute about 60% of the world's supply of protein and that 60% of the developing world derives more than 30% of their annual protein from fish. Aquaculture is fast gaining increasing relevance in the country to reduce the present gap, between fish demand of 2.66 million metric tons and local production of 0.62million metric tons with catfish production contributing greatest proportion in terms of geographic distribution, climatic suitability and acceptability. There has been recent increase attention on aquaculture in Nigeria to boost animal protein through the small, medium and large scale production. Though there are over hundred species of catfish in the world, *Clarias gariepinus* represents the most dominant of these fish species [2].

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In Nigeria, fish production is from both domestic or internal and imported sources with aquaculture as a major important local source. Fish in a small quantity is an important dietary supplement for the generality of the low income Nigerian who cannot easily afford animal protein and rely mainly on starch [3]. A study of household fish consumption in Ibadan reported that 91% of the surveyed population regularly consumed fish, out of which 68% consumed up to 3 kg of catfish monthly [4]. Catfish are relished as a delicacy among Nigerian for home consumption or at fast food joints and restaurants. The growing demand for catfish for home consumption and at restaurants due to high quality dietary protein and low fat content has led to intensification and private investment in aquaculture production in Nigeria to optimize yield and profit in Nigeria [5].

This current trend in fish production created the challenges of fish health conditions. Infectious and non-infectious diseases constitute major constraints to aquaculture productivity [6]. Antimicrobials regimens are being employed prophylactically and therapeutically to combat these challenges as well as for growth promotion. To reduce diseases outbreak in aquaculture, a wide range of chemotherapeutics has been used. Oxytetracycline (OTC) has been widely used in aquaculture as a therapeutic and prophylactic agent because of its broad-spectrum activity. It was the first antibacterial approved by USFDA for use in finfish aquaculture [7]. The difficulty of calculating the appropriate dose of these antibiotics, drug misuse has posed a threat to human health and environmental safety. In aquaculture, antibiotics are generally administered in feeds, having been either added during feed manufacture or surface-coated on to pellets by the manufacturer or the farmer. During outbreaks of disease, farmers may apply antibiotics using other routes. Antibiotics have not always been used in a responsible manner in aquaculture and control of their use has not provided a proper assurance of the prevention of risks to humans [8]. Clear instructions are required for the feed manufacturers, antibiotic dealers and farmers who are responsible for the use of antibiotics through the supervision of veterinary authorities and veterinarians. The withdrawal period for oxytetracycline in red Tilapia cultured in fresh water has been established [9].

The use of antimicrobial has created the concerns for antibiotic resistant pathogens and residues in food chain. The result of this study also confirms the general concerns that such misuse of antimicrobials occur in aquaculture. However, these compounds can come into contact with humans by means of the food chain [10]. It is important to pay attention to this contamination because of the potential hazards associated to the presence of these products in edible tissues. So, there is a global concern about the consumption of aquatic food containing low levels of antibiotics.

Several classes of antibiotics are commonly used in large quantity in fish industry, especially in developing countries where their uses are not regulated. Some of these antibiotics are often non-biodegradable and persist in the aquatic environment as residues. When antibiotic are mixed with fish feeds, residues may be deposited in the meat and consumers are inadvertently consuming the residues which may lead to changes in their microbial flora [11].

Indiscriminate use of drugs in could lead to undesirable deposition of their residues in edible tissues offered for human consumption which could pose public health risks to the consumers. Associated public health risks include acute or cumulative allergic, toxic, mutagenic, teratogenic or carcinogenic effects. Antibiotic residues transferred to humans through food can also alter the intestinal ecology thereby favouring the emergence of resistant microflora [12]. Other side effects of antimicrobial residue in human include aplastic anaemia with chloramphenicol [13], damage to urinary vestibular and auditory functions by aminoglycoside antibiotics [14], hypersensitivity reactions in human by penicillin [15]. Toxic and allergic reactions in humans and animals caused by oxytetracycline have only been observed at therapeutic dose [16]. Oxytetracycline has been reported to produce immunosuppression in some fish species [17].

About 70-80% of drugs used in aquaculture ends up in the environment, such contamination with antimicrobials lead to resistant environmental microflora and resistant genes which can be transferred to human beings through food of contact with the fish or water [18, 19].

In Nigeria, the administration of veterinary drugs in food animals including fish production is characterized by indiscriminate use without appropriate veterinary supervision, regulation and control to protect the consumers. This misuse of veterinary drugs as well as violative residues of antimicrobials in Nigerian livestock products have been reported by several authors [20-22]. However, there are paucity of data on quantitative risks assessment of antimicrobial residues and usage in fish in Nigeria. This present study therefore determine the patterns of antimicrobial usage in catfish from two major fish farms and determined the presence of residues especially oxytetracycline in table size catfish available for human consumption from the farms and public eateries.

This study was designed to determine the practice of antimicrobial usage in *Clarias gariepenius* production and the presence of antimicrobial residue in catfish produce for human consumption in Ibadan metropolis. Premi®Test developed and validated as a screening test based on the inhibition of growth of *Bacillus stearothermophilus*, a thermophilic bacterium sensitive to many antibiotics [23] employed in the detection of antibiotics residues in meat within 3 hours of slaughter was used in this study to determine the prevalence of antibacterial residues in catfish sold for human consumption in Ibadan metropolis.

MATERIALS AND METHODS

Study Area: This study was undertaken in Ibadan, the capital city of Oyo State, is in the south western part of Nigeria, on grid reference latitude 07°22' North and longitude 03°58' East. The choice of the city for this study is based on its unique cosmopolitan metropolis with broad socio-economic diversity with low, medium and high income individuals who are government employee, private sector workers, artisans, farmers, etc. of above 2 million population (2006, census). There are growing numbers of fish farms and hatcheries as well as a several retail outlets of fresh and ready-to-eat catfish markets and restaurants in the city. Small, medium and large scale catfish productions are engaged within the urban and peri-urban areas of the metropolis. Questionnaires administration and fish samples were collected from aquaculture farmers and retailers in Ibadan metropolis.

Questionnaire Survey and Sample Collection: Semi-structured questionnaire was randomly administered to twenty aquaculturists engaged in fish farming randomly selected across six Local Government Areas in the peri-urban of Oyo State. The overall objectives of the study; antibiotic efficacy and residue safety were explained to the respondents.

The questionnaire comprised of sections on of socio-demographic characteristics, fish farming operations, their knowledge and practices of antibiotic usage and withdrawal period in their fishes as well as attitude to antibiotic resistance and residues. A pre-test of the questionnaire exercise was carried out with four catfish producers for a better understanding and clarity of the questions. The practice of antibiotic usage involving, indications, prescription, sources and administration were assessed. Also the farmers' knowledge and attitudes towards antibiotic residues and resistance were investigated through practices of observance or non-observance of withdrawal period, sales and consumption of fish under recent medications.

Also, approximately 150 g of 160 liver and muscle samples were randomly obtained from ready to eat fish restaurants and catfish farms in Ibadan. The samples were aseptically collected in Nasco Whirl-Pak® sample bags (Sigma Aldrich, St. Louis, MO USA) and transported in cool icebox to the laboratory of the Department of Veterinary Public Health and Preventive Medicine stored at -20°C until the time for subsequent extraction and residue analysis.

Screening for Antimicrobial Residues: Approximately $2cm^3$ of each liver and fillets were cut into the meat press to extract the meat juice into a Petri-dish. Using a micropipette, 100µl was carefully drawn onto each ampoule of the pre-seeded agar. The agar with the extract were allowed to stand for 20 minutes for pre-diffusion at room temperature and then flushed carefully twice with distilled water. The agar was drained of the extract after which the ampoules were closed with foil. These were incubated in the heating block for 3 hours at 64°C. The results were observed through colour change indicator in the agar.

HPLC Detection and Analysis of Oxytetracycline Residue in Fish Samples: Equipment: HPLC apparatus (Agilent, 1200 series, made in Germany, product no GI3104) connected to UV detector with C18 (4.5 x 150 mm, 5µm) as the solid phase column. The flow rate was set at 1.3 ml/min while the mobile phase was methanol: acetonitrile: 0.01M oxalic acid in the ratio of 1:1.5:2.5 v/v.

Chemicals: Laboratory grade oxytetracycline hydrochloride (reference standard) purchased from Sigma chemical Co, St Louis, USA, acetonitrile (HPLC grade), methanol (HPLC grade), oxalic acid, hydrochloric acid, methylene chloride, petroleum ether and deionised water. All chemicals were analytical grade and were properly degassed by sonication.

Preparation of Standard Curve: Calibration curve (Figure 1) was prepared from the peak areas obtained by injecting corresponding concentration of oxytetracycline standard solutions of 10.0, 5.0, 2.5, 1.0, 0.5 and 0.25ppm made in 10 ml volumetric flasks. The linear equation obtained $y= 19.598x + 6.6594$ was obtained where y= peak area and x= concentration of the oxytetracycline (ppm) and the correlation coefficient (r^2) = 0.9955 showing the

linearity (Figure 1). The detection limit for oxytetracycline was 0.01ppm while the mean retention time of the oxytetracycline was approximately 2.8 to 3.5 minutes.

Sample Preparation: Liquid-liquid partitioning extraction as well as clean up procedures were employed to obtain the analyte. 25g of each sample was homogenized and deproteinized with 3 volumes of 1N hydrochloric acid after which 8 ml of the homogenate was thoroughly swirled with 32mls acetonitrile and allowed to stand for 5 minutes. The supernatant was decanted through a glass wool on the stem of a glass funnel and 20 ml of the filtrate was vigorously shaken with 20 ml petroleum ether and 20 ml methylene chloride in a separating funnel. The aqueous layer containing the analyte was collected for HPLC analysis.

HPLC Analysis of Oxytetracycline Residue: HPLC detection and quantitation according to Muriuki *et al.* [24] was used. The oxytetracycline was quantified for oxytetracycline residue using a HPLC apparatus equipped with a constant flow pump and a variable wavelength U.V detector set at 280 nm and flow rate of 2 ml/min. Elution of oxytetracycline from the analyte was done on a nucleosil C18 (4.5x150 mm, 5µm ID) column with Methanol-Acetonitriel-0.01M aqueous Oxalic acid solution (1: 1.5: 2.5) at pH 2.0 as the mobile phase. 20 µl injection volume of the analytes from each sample was injected in duplicate to obtain average peak area of positive sample corresponding to the retention time of 2.8 to 3.5 minutes of the reference standard. The concentrations of oxytetracycline residue in the samples were calculated from the linear equation obtained from the standard curve.

Recovery Experiment: Validation of the protocol by recovery experiment and precision were determined using relative standard deviation (RSD) and percentage recovery. Four replicate oxytetracycline free fish spiked with different concentrations oxytetracycline standard were taken through the extraction, clean-up and HPLC procedures to validate our methods and results.

Statistical Analysis: One way analysis of variance (ANOVA) was used to compare the levels of oxytetracycline residue in the samples from different locations at confidence level of 95% ($p<0.05$).

RESULTS

Catfish Farmers Characteristics and Operations: Out of the 20 catfish farmers interviewed, majority (75.0%) were male, while 25.0% were female with mean age and years of fish production experience of 48 and 6.5 years respectively. Their education levels ranged from primary school certificate to university degree among who are two veterinarians. All the respondents produced the fish for commercial purposes and domestic consumption. The average stocking density was 15,000 fishes comprising of 10% large-scale producers using modern re-circulatory water system and the others on earthen ponds.

Drug Use in Aquaculture Production: Majority (90%) of the fish farmers interviewed engaged in routine administration of antibiotics to prevent fish diseases. Extra-label use of human preparations of antibiotics was also were routinely done. About 65% of the farmers claimed to administer drugs routinely to prevent disease outbreak or mortality losses without engaging the services of veterinarians for disease diagnosis and treatment. Majority of the respondents complained about lack or unavailability of fish veterinary specialist. All the respondents (100%) claimed they routinely administered several antibiotics ranging from oxytetracycline, procaine penicillin G, Malachite Green and enrofloxacin to their fish stocks for disease prevention, treatment and productivity performance. Oxytetracycline was the most frequently administered antibiotics both to the fish stocks and fish feed by 73% of the respondents. About 85.5% of the respondents were neither aware of the withdrawal period for antibiotics nor the potential hazards of antibiotic residue in human.

Oxytetracycline Standard Curve and Recovery Results: The calibration curve obtained from the standard oxytetracycline is shown in Figure 1 with the linear

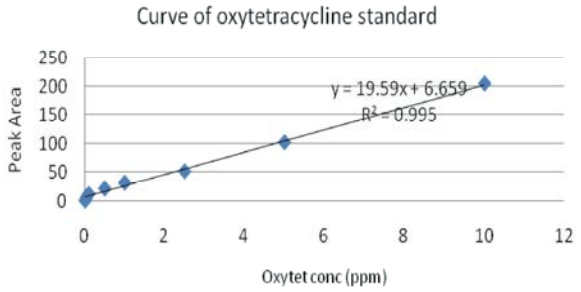

Fig. 1: Calibration curve of oxytetracycline standard solution

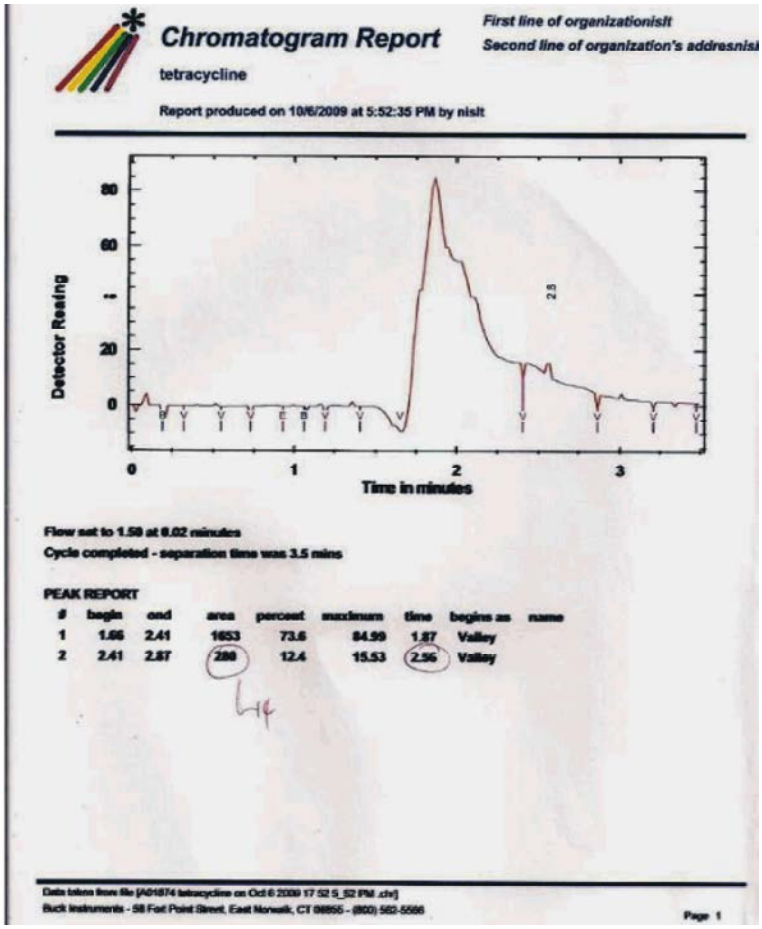

Fig. 2: Chromatogram of oxytetracycline residue in catfish liver sample

Table 1: Antimicrobial and oxytetracycline residues in restaurants and aquaculture farms in Ibadan

	Samples positive (Premi®test screening)		Samples with detectable otc by HPLC		Sample with otc>MRL	
Sample Fish location	Liver n=40	Muscle n=40	Liver n=40	Muscle n=40	Liver n=40	Muscle n=40
Samonda Restaurant	26 (65.0)	20 (50.0)	14 (35.0)	15 (37.5)	10 (25.0)	10 (25.0)
Adamasingba Restaurant	19 (47.5)	16 (40.0)	11 (27.5)	8 (20.0)	8 (20.0)	6 (15.0)
Iwo Road Restaurant	23 (57.5)	23 (57.5)	15 (37.5)	10 (25.0)	8 (20.0)	4 (10.0)
AquaLink Farm	16 (40.0)	13 (32.5)	12 (30.0)	9 (22.5)	9 (22.5)	6 (15.0)
Oredegbe Fish Farm	14 (35.0)	12 (30.0)	11 (27.5)	6 (15.0)	8 (20.0)	4 (10.0)
Total	98 (61.3)	84 (52.5)	63 (39.4)	48 (30.0)	43 (26.9)	30 (18.8)

Table 2: Mean recovery values at different oxytetracycline levels

Spike (µg kg^{-1})	Mean Recovery Rate (%)	RSD %	Coefficients of Variation (CVs)
0.5	85.5	2.38	2.4
1.0	86.0	2.12	2.1
2.5	87.8	1.43	2.2
10	88.5	2.14	1.2

equation y= 19.598x + 6.6594 where y= peak area (cm^2) and x= concentration of the oxytetracycline (ppm) and the correlation coefficient (r^2) = 0.9955 showing the linearity. The detection limit for oxytetracycline was 0.01ppm while the mean retention time of the oxytetracycline was approximately 2.3 to 3.2 minutes. Sample of the chromatographs is shown in Figure 2. The mean recovery values for the oxytetracycline spiked to fish varied

between 85.2% and 88.5% while relative standard deviation (RSD) ranged from 1.43 and 2.38% (Table 2). These results showed good linearity and reproducibility (R^2 = 0.993). LOD and LOQ were 10.0 and 32.3 μg kg^{-1} respectively while the coefficients of variation (CVs) of the spiked standard solutions ranged between 1.2% and 2.4% and for samples between 0.02% and 0.4%.

Antibiotic Residue Prevalence in Fish: The overall prevalence of antibiotic residue in catfish using the Premi®Test was 61.3% of liver and 52.5% of fillet samples with fishes from the restaurants and farms containing variable proportion of unclassified antimicrobial residues (Table 1). HPLC analysis indicated that 62 fish samples (39.4%) contained detectable oxytetracycline. Oxytetracycline concentrations in the liver samples ranged between 234.3 and 987.5μg kg^{-1} (mean = 875.32±45SD μg kg^{-1}) while the range in fillets was between 22.5 and 553.2 μg kg^{-1} (mean = 257.2±133μg kg^{-1}). Forty three (26.9%) and 30 (18.8) liver and fillets samples respectively was detected to contain oxytetracycline residues above codex maximum residue limit (MRL) of 600 and 200 μg kg^{-1} respectively. The distributions of prevalence of antimicrobial and oxytetracycline residues across the surveyed fish restaurants and farms are shown in Table 1. Highest prevalence of residues was obtained in restaurants. There was no significant difference in the prevalence of antimicrobial residues in all the different locations. However, the mean concentration of oxyteracycline in liver was significantly higher ($p<0.05$) than in the fillets.

DISCUSSION

The use of antimicrobial agents in food producing animals has become a great public health concern especially in the developing country where they are administered indiscriminately [25]. This study confirmed catfish farmers are also engaged in uncontrolled administration of antibiotics for aquaculture production in Ibadan, Nigeria. Most of the drugs used by the aquaculture farmers interviewed were not specifically indicated for fish, extra-label use of mostly poultry and human antibiotic preparations was observed to be commonly practiced. This practice coupled with inadequate numbers of veterinarians trained in fish medicine as well as lack of food safety (withdrawal periods and residue in fish) knowledge by the respondents is of public health risk. Also the ineffective regulations of veterinary drugs handling and use in Nigeria could account for such practices. Some countries or regions, such as European Countries, Canada and Norway, approve a limited number of antibiotics specifically for use in aquaculture. In Canada, the antibiotics approved for aquaculture use are: oxytetracycline, sulfadiazine (trimethoprim), sulfadimethoxine (ormetoprim) and florfenicol Also in USA, sulfonamides and tetracyclines are two classes of antibiotics broadly used in aquaculture to treat infections in fish [26].

The accelerated growth of aquaculture in the country as witnessed in Ibadan could result in pollution of the aquatic environment with large amounts of antibiotics and presence of residual antibiotics in fish tissue and fish products. This was confirmed by the high prevalence of residues obtained by the screening out of which fewer samples contained oxytetracycline. Most of the samples positive by Premi® Test would contain more than one antibiotic since the test is sensitive to several groups of antimicrobial agents. There is also, possibility of false positive results due to tissue matrices interference with the inhibition. However, appreciable prevalence (39.4% and 30.0% of liver and fillets respectively) of oxytetracycline residue in the sample confirmed the drugs as one of the frequently used antibiotics in catfish. Oxytetracycline being a broad spectrum and cheap antibiotic is widely used by farmers and confirmed by result of administered questionnaire, making it most misused antibiotic in aquaculture. Hence, the presence detectable oxytetracycline residue in 39.4% 0f the samples confirmed the level of misuse or abuse of antibiotics by the producers. This is consistent with other studies in food animal's products in Nigeria [20, 21, 27-29]. However, there has been little or no survey and quantitative analysis of antibiotics in fish consumed in Ibadan. The higher prevalence of antimicrobial presence and antibiotic residue obtained for the restaurant might be as result of use of locally formulated feed fortified with antibiotics just before the harvesting fish to boost growth to prevent or treat against disease as well as protecting the fish from transportation stress. Thus, the higher proportion of the samples (26.9% liver and 18.8 fillets) containing oxytetracycline above codex MRLs indicated that withdrawal period nor human hazards associated with antibiotic residue in fish were not of concern to the farmers. Cháfer-Pericás [30] showed that the withdrawal time needed to reduce oxytetracycline concentration to around MRL before marketing in Mediterranean fish was about 37 days.

Catfish farming is a rapidly growing business in Nigeria including Ibadan, attracting the attention of wide range of people with different educational background. This study revealed that most farmers in Ibadan have low level of education and have unrestricted access to antibiotics (over the counter), thereby engaging in self medication of their stocks as a routine practice without proper diagnosis and not observing of withdrawal period. High proportions (75%) of the respondents in this study were not knowledgeable on the deposition and the public health implication of the residues in fish meat. This could account for the non-observance of the withdrawal periods by most of these farmers and also for the high prevalence of antimicrobial residues in the present study. The unhygienic practices characterizing the studied farms with earthen pond could be a predisposing factor to disease outbreak and frequent use of prophylactic and therapeutic antimicrobials obtained from the result of administered questionnaires. These practices can influence the presence of antimicrobial residues in fish and the consequence of resistant bacteria.

CONCLUSION

This study revealed that appreciable quantity of catfish consumed in Ibadan, Nigeria posed antibiotic residue risks and food safety consequences. Misuse of antibiotics in aquaculture production without veterinary prescription and control coupled with lack of awareness of the food safety consequences were the contributing factors for the high level of residue violation. Antibiotics are necessary for specific and diagnosed need uses in aquaculture, (HACCP) as a risk-based management tool will ensure prudent antibiotic use in aquaculture. Also, regulation of their commercial availability is advocated. The proper use of approved antibiotics will continue to be necessary in animal production, including aquaculture, for consumers to be reassured that the use of approved antibiotics, in particular under "label use" conditions, does not imply a hazard. Vaccination options and the use of immunostimulants and probiotics for disease control in aquaculture will also help to reduce the use of antimicrobial agents. Therefore, government should enforce strict regulation of use of veterinary drugs in aquaculture, educate fish farmers, provide fish laboratory and train more professional in fish medicine in order to safeguard Nigerian populace from hazards associated with antibiotic misuse and residue in aquaculture.

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