DETERMINATION OF ANTIMICROBIAL RESIDUES IN COMMERCIAL CULTIVABLE FISHES AND ITS IMPACT ON PUBLIC HEALTH

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Roll No.: 0120/01

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A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Pharmacology

DEPARTMENT OF PHYSIOLOGY, BIOCHEMISTRY AND PHARMACOLOGY

FACULTY OF VETERINARY MEDICINE

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CHATTOGRAM

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made.

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DEDICATED

To my

Beloved

Friends, Family and

Honorable Teachers

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The Author

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
%	Percent
µg	Microgram
ADI	Acceptable daily intake
AHPND	Acute hepato-pancreatic necrosis disease
AMR	Antimicrobial Resistance
CMA	Chattogram Metropolitan Area
CVASU	Chattogram Veterinary and Animal Sciences University
EDI	Estimated daily intake
EEZ	Exclusive economic zone
EMS	Early mortality syndrome
FAO	Food and Agriculture Organization
GC	Gas Chromatography
GIFT	Genetically improved farmed tilapia
gm	Gram
HPLC	High Performance Liquid Chromatography
HQ	Hazard quotient
Ig	Immunoglobulin
ITLOS	International Tribunal for the Law of the Seas
Kg	Kilogram
LC-MS	Liquid Chromatography and Mass Spectrometry
MRL	Maximum residue limit
NOAEL	No Observable Adverse Effect Level
NOEL	No Observable Effect Level
TLC	Thin Layer Chromatography
UHPLC	Ultra High Performance Liquid Chromatography

ABSTRACT

Antimicrobials are used as therapeutics and prophylaxis in commercial fish farms of Bangladesh to improve profitability. The Thin Layer Chromatography (TLC) method was performed to detect the presence of antimicrobial residues in commercial fishes. Moreover, the Ultra High Performance Liquid Chromatography (UHPLC) method was performed to determine the concentration of antimicrobial residues. Ciprofloxacin residues were detected in Tilapia (*Oreochromis aureus*), Stinging catfish (*Heteropneustes fossilis*), Climbing perch (*Anabas testudineus*), and Pabda (*Ompok pabda*) fish samples of 42%, 31%, 20%, and 10%, respectively. Enrofloxacin residues were detected 31% in Pabda, 22% in Tilapia, 22% in Stinging catfish, and 17% in Climbing perch fish samples. Levofloxacin residues were detected 27% in Pabda, 17% in Stinging catfish, 17% in Climbing perch, and 15% in Tilapia fish. Oxytetracycline residues were detected 41% in Pabda, 27% in Stinging catfish, 24% in Tilapia, and 23% in Climbing perch fish samples. Chlortetracycline residues were detected 49% in Tilapia, 43% in Pabda, 35% in Stinging catfish, and 21% in Climbing perch fish samples. Doxycycline residues were detected at 38% in Pabda, 32% in Tilapia, 28% in Stinging catfish, and 16% in Climbing perch fish. Furthermore, the average concentration of Ciprofloxacin residues in Stinging catfish was 45.85 µg/Kg, whereas in Pabda, Tilapia, and Climbing perch were 24.08 µg/Kg, 19.97 µg/Kg, and 18.29 µg/Kg, respectively. The average concentration of Enrofloxacin residues in Climbing perch was 69.32 µg/Kg, whereas in Pabda, Stinging catfish, and Tilapia were 66.21 µg/Kg, 61.13 µg/Kg, and 56.53 µg/Kg, respectively. The average concentration of Levofloxacin residues in Climbing perch was 37.59 µg/Kg, whereas in Pabda, Tilapia, and Stinging catfish were 24.74 µg/Kg, 22.28 µg/Kg, and 9.94 µg/Kg, respectively. The average concentration of Oxytetracycline residues in Pabda was 88.73 µg/Kg, whereas in Tilapia, Climbing perch, and Stinging catfish were 87.40 µg/Kg, 73.32 µg/Kg, and 68.44 µg/Kg, respectively. The highest Hazard Quotient (HQ) was found for Enrofloxacin in Climbing perch (0.480) followed by Pabda (0.460), Stinging catfish (0.420), and in Tilapia (0.387), which might be considered as potential HQ. Ciprofloxacin, and Levofloxacin residues were also potential HQ, but the values were less (<1.0). Although the residues might not show the toxicological effect as the HQ value was less than 1.0, they still have public health hazards due to antimicrobial resistance (AMR).

Key words: Antimicrobials, antimicrobial residues, commercial cultivated fishes, Thin Layer Chromatography, Ultra High Performance Liquid Chromatography, Hazard Quotient, antimicrobial resistance.

Chapter- I: INTRODUCTION

An antimicrobial is an agent that kills microorganisms or stops their growth (Strohl and bioprospecting, 2003). Antimicrobials have been used globally for many years in human and veterinary medicine practices. However, random use of antimicrobial drugs may lead to antimicrobial resistance (AMR). Antimicrobial residues and resistance are a burning issue worldwide nowadays (Patel et al., 2021). The maximum residue limit (MRL) of an antimicrobial has been set by the Food and Agricultural Organization (FAO) of the United Nations for determining the safety of animal-originated food sources (Hamilton, 2015). Several researches have been conducted related to this issue. People, especially from developing countries, are using antimicrobials randomly (Hart and Kariuki, 1998). That's why consumers intake antimicrobial residues with animal-originated food if harvested before completion of the withdrawal periods. The presence of antimicrobials in foods and their potential effects on human health is an area of growing concern. There is a risk that microorganisms get exposure to those antimicrobial drugs. Thus the AMR being developed could potentially transfer into human food sources (Frère and Rigali, 2016). Therefore, different methods have been developed to detect the presence of antimicrobial residues. Antimicrobial residues can be identified from foods in both qualitative and quantitative methods: Thin Layer Chromatography (TLC) is for qualitative detection and Ultra High Performance Liquid Chromatography (UHPLC) for quantitative detection (Tazrin, 2014).

Asia, "the home of aquaculture", contributed about 89% of global production (Ahmed and Lorica, 2002). In many Southeast Asian countries, including Bangladesh, aquaculture production has contributed significantly to their national economies. So aquaculture industry is now recognized as a potential sector for increasing economic growth. However, one of the most critical threats to the aquaculture industry is a bacterial infection (Defoirdt et al., 2011). A heavy amount of money is lost from the aquaculture industry due to diseases, especially bacterial infections. Due to the increase of both extensive and intensive aquaculture farming, waterborne pathogens are transmitting in higher numbers (Meyer, 1991). For Example, acute hepato-pancreatic necrosis disease (AHPND) is caused by *Vibrio parahaemolyticus* bacteria, formerly known as early mortality syndrome (EMS), causes devastating loss in aquaculture (He et al., 2019).

Bangladesh is an agricultural country with a huge population (Streatfield et al., 2008). To meet the protein requirement, extensive poultry, dairy, and fish farms produce meat, milk, egg, and fish commercially. Among these sectors, aquaculture plays an essential role as a source of animal protein in Bangladesh (Belton et al., 2011). Fish is the primary protein source in Bangladesh as it contributes about 60% of total animal protein (Mohan Dey et al., 2005). In terms of total aquaculture production, Bangladesh is ranked 10th, and in the case of inland fish production, Bangladesh is ranked 3rd in the world (Khan et al., 2021). Commercial fish and prawn farming are being popular in Bangladesh nowadays. Gracefully, Bangladesh has won the maritime boundary by the International Tribunal for the Law of the Seas (ITLOS) that provides an opportunity in fishing more (Shamsuzzaman et al., 2020).

In order to meet the demand of the world's growing population and achieve sustainable food production and security, aquaculture production has to be increased. Bangladesh is blessed with diversified fisheries resources, generally categorized into inland fisheries and marine fisheries (Shamsuzzaman et al., 2016; Uddin, 2019). Inland fisheries are distributed in 47.60 lakh hectares, divided into two sub-sectors, i.e., inland capture and inland culture. The inland capture includes the river, estuary, beel, haor, baor, and Kaptai lake (Shamsuzzaman et al., 2020). They occupy an area of 39.27 lakh hectares, where inland culture includes ponds, ditches, cage cultures, shrimp/prawn farms, and seasonal cultured water bodies, which occupy an area of 8.33 lakh hectares. In contrast, marine capture fisheries provide a vast amount of support for the growing economy of Bangladesh. It covers an area of about 118,813 km^2 along with 200 nautical miles of an exclusive economic zone (EEZ) from the baseline (Shamsuzzaman et al., 2020). Per capita, fish consumption in Bangladesh is about 62.58 gm, which is higher than their daily protein demand (60 gm) (Shamsuzzaman et al., 2020). Bangladesh is now considered self-sufficient in fish production and has already got global recognition as one of the biggest fish producers.

The fisheries sector can also contribute directly by employing fishers and other related trades, thereby sources of livelihood (Béné, 2006). More than 18 million people of Bangladesh are dependent on the entire fisheries sector to support their livelihoods directly and indirectly (Hossain and Fisheries, 2014). In addition, about 1.4 million women are dependent on the fisheries sector for their livelihoods through fishing, farming, fish handling and processing (Shamsuzzaman et al., 2020).

Fish farms are using antimicrobials as feed additives and medical and veterinary effluents, which have AMR bacteria, can contaminate the environment of aquaculture (Hassan et al., 2021a). However, people intake foods from this source. There is a chance of developing resistance against some specific antimicrobials. This leads to the transfer of AMR genes or bacteria into the human food chain (Ben et al., 2019).

Antimicrobials are administered in different route or purpose. As antimicrobials are distributed throughout the body with circulation, it can accumulate as residues in tissues, before they are completely metabolized or excreted from the body (O'Donnell et al., 2019). The occurrence of residues in fish or other animal tissues is observed when animals are harvested for human consumption before the withdrawal period while still on medication or shortly after medication. Various public health issues may arise when these foods are consumed (Bacanlı et al., 2019). The primary health concern is the development of AMR in the human body. Antimicrobial residues can occur in commercial fishes when the drugs are administered higher dose or without recommendations from registered veterinarian (Hassan et al., 2021b; Kalam et al., 2021). Random usage of antimicrobials in aquaculture is practiced everywhere in Bangladesh. It should be supervised by veterinarians but the actual scenario is quite different. The feed and drug dealers inspire the farmers to use antimicrobials (Sullivan, 2005).

'Maximum Residue Limit (MRL) is the maximum concentration of residue resulting from the use of a veterinary drug' (FAO, 2021). MRLs of veterinary drugs in foods are set with permitted quantities of drugs or metabolites in foods originating from animals that are safe for consumers (Passantino and Russo, 2008). Although steps have been taken to maintain the MRLs worldwide, MRLs are not the same from one place to another. Even MRLs in an animal product may vary from one country to another depending on their local food safety regulatory agencies and drug usage patterns. Most developing countries have not developed their own MRLs yet (Gaudin et al., 2010). 'Acceptable daily intake (ADI) is an estimate of the amount of a veterinary drug on a body weight basis' (FAO, 2021). ADI is also a critical standard that is set from the toxicological studies based on the No Observable Effect Level (NOEL) and safety factors (Nouws et al., 1994). ADI is the amount of residue that can be consumed daily over a lifetime without any health risk to the consumer. In fish farms, withdrawal periods of drugs are not maintained as the feed contains antimicrobials and even antimicrobials are used to prevent and cure different diseases in aquaculture just before harvesting. But these drugs must be elapsed from fishes before harvesting, then the edible products are considered safe for human consumption (Salte and Liestøl, 1983). This study has been performed to investigate the current state of the level of antimicrobial residues in cultivable fishes from commercial farming in Bangladesh.

Objectives:

1. To detect the presence of residues of antimicrobials of quinolones and tetracycline groups in commercial cultivable fishes (Tilapia, Climbing perch, Pabda, and Stinging catfish) by TLC method.
2. To determine the level of residues of antimicrobials (Ciprofloxacin, Enrofloxacin, Levofloxacin, and Oxytetracycline) in commercial cultivable fishes (Tilapia, Climbing perch, Pabda, and Stinging catfish) by UHPLC method.
3. To understand the public health significance of antimicrobial residues in commercial cultivable fishes and increase public awareness about AMU and AMR.

Chapter- II: REVIEW OF LITERATURE

2.1 Antimicrobial Drugs

A large number of drugs and formulations are available to manage infections. An antimicrobial is an agent that kills microorganisms or stops their growth (Strohl and bioprospecting, 2003). Antimicrobials have been used globally for many years in human and veterinary medicine practices (Prescott, 2017). Antimicrobials are generally classified according to their molecular structure and their antimicrobial mechanisms (Béahdy, 1974). Ideally, these mechanisms of action either interrupt the synthesis of structural components or alter specific metabolic functions that are unique to microbial cells. Antimicrobials kill microorganisms or hamper normal growth by inhibiting microbial metabolisms either by inhibiting cell wall synthesis or nucleic acid synthesis, or protein synthesis (Neu and Gootz, 1996). β-lactam antibiotics (such as Penicillin, Ampicillin, Amoxicillin, Cephalosporins, Carbapenems, and Monobactams), Vancomycin, and Bacitracin inhibit bacterial cell wall synthesis (Cho et al., 2014). Polymyxins inhibit bacterial cell membrane synthesis (Teuber, 1974). Sulfonamides and Trimethoprim hamper folic acid synthesis of bacteria (Huang et al., 2004). DNA-gyrase is the target of Quinolones (such as Ciprofloxacin, Enrofloxacin, and Levofloxacin) (Evans-Roberts et al., 2016). Rifampicin acts against bacterial nucleic acid synthesis by binding to the exit channel of the RNA polymerase (Alifano et al., 2015). Aminoglycosides and Tetracyclines are believed to act by binding to the 30S ribosomal subunit to impair bacterial protein synthesis (CP et al., 2019).

2.2 Antimicrobials in Aquaculture Industry

Antimicrobials are used in humans, food animals, and aquaculture, which can be categorized as therapeutic use, prophylactic use, or metaphylactic use (Romero et al., 2012). Therapeutic use means the treatment of established infectious diseases (Casadevall et al., 2004). Metaphylactic use means group-medication procedures to treat sick animals while also medicating others in that group to prevent disease. Prophylactic use means the preventative use of antimicrobials in individuals or groups to prevent infectious diseases (Baptiste and Pokludová, 2020). In aquaculture, antimicrobials are frequently administered at therapeutic levels for a particular period via oral route to the whole groups of fish that share the same tanks or cages (Okocha et al., 2018). All antimicrobial drugs should be used legally, which should be prescribed under the supervision of a registered veterinarian and these drugs must be approved by the government agencies responsible for veterinary medicine. These agencies may set the rules for antibiotic use, including routes of administration, dose forms, withdrawal periods, side effects, and use by species, including dose and drug limitations. But the actual scenario is different in Bangladesh (Hossain et al., 2017). The most common administration route for the fishes is the oral route, especially by mixing the antimicrobials with formulated feed from different companies. However, antimicrobials are not metabolized effectively in fish bodies and may remain in bodies or pass unused into the environment through feces. It has been estimated that about 75% of the antimicrobials are excreted into the water from fishes (Kümmerer, 2009). There is the fact that several bacterial diseases are increasing in intensive aquaculture, which leads to the use of more antimicrobials. The same levels of using antimicrobials in aquaculture cannot be determined easily because different companies have different feed formulation systems and the amount of antimicrobials used in aquaculture without any consultation from registered veterinarians.

2.3 Developing Antimicrobial Residues

The term antibiotic residue is the small amount of an antibiotic or its breakdown product that remains in or on an agricultural product following treatment with that antibiotic. Residues of veterinary drugs mean all pharmacologically active substances, whether active principles, recipients, or degradation products and their metabolites which remain in foodstuffs obtained from animals to which the veterinary medicinal product in question has been administered (Beyene, 2016). Nowadays, the use of antibiotics is frequent. Every living being is receiving antibiotics in direct or indirect ways. Antibiotics are used not only for treatment purposes but also for preventive and growth promoters (Serrano, 2005).

2.3.1 Antimicrobial residues in Aquaculture Industry

Antimicrobials are used indiscriminately in aquaculture in many countries, especially in Bangladesh. Though antimicrobials are used to get higher benefit commercially by improving productivity, there is a risk with their residues in the tissues of treated fishes or their derived products, which have health hazards to the consumers (Alderman et al., 1998). There are many reports which have shown evidence of antimicrobial residues in aquaculture worldwide. The leading cause behind the residual presence is using of antimicrobials without any consultancy from a registered veterinarian. Moreover, feeds from different companies contain antimicrobials to prevent diseases (Brunton et al., 2019). There is a lack of knowledge about withdrawal periods and the harmful effect of antimicrobial residues among farmers, drug, and feed dealers. Many farmers do not even think about it before harvesting (Okocha et al., 2018). They harvest fish a few days after medication or even on medication, sometimes to reduce loss from diseases and mortality.

2.4 Public health importance of antimicrobial residues

Antimicrobial residues in foods of animal origin may cause problems for several reasons. In addition to toxic effects, effects on intestinal microbiota, and the immune system are important. Microbiological effects are more critical and sensitive in the safety evaluation of antimicrobial residues in production animals than standard toxicological effects (Waltner-Toews and McEwen, 1994).

2.4.1 Growing Resistance Pathogen

The microbiota in the human gastrointestinal tract is from a highly compelled yet relatively stable ecological community, containing more than 400 bacterial species (Brewster et al., 2019). Administration of antimicrobial agents may cause disturbances in these normal bacterial functions. Disturbances in the ecological balance between host and microorganisms occur when antimicrobials are introduced. It depends on the spectrum of the antimicrobial agent, the dose, pharmacokinetic, and pharmacodynamics properties and in-vivo inactivation of the agent (Giguère, 2013). The major microbiological effects have been identified that could be of public health concern: modification of the metabolic activity of microbiota, changes in bacterial populations, and selection of resistant bacteria (Langdon et al., 2016).

In cases of reduced colonization resistance, not only are the minimal infectious or colonization doses of pathogenic or resistant bacteria considerably lower, but animals also excrete these bacteria in higher numbers and over a more extended period compared to animals with an intact colonization resistance. Some data have been reported on antimicrobial susceptibility and the emergence of resistant bacteria with low doses of antimicrobials (Kang et al., 2019).

2.4.2 Hypersensitivity reaction

Drug hypersensitivity is defined as an immune-mediated response to a drug agent in a sensitized patient and drug allergy is restricted to a reaction mediated by Ig E (Schnyder and Pichler, 2009). Drugs are foreign molecules for the body, but their molecular weight is usually too small to be immunogenic. For drugs to be immunogenic, they must act as haptens, which must combine with carrier proteins to be immunogenic and elicit antibody formation. Immunologic reactions may manifest from life-threatening anaphylactic reactions to milder reactions, such as rashes. Drug-induced allergic reactions may occur acutely, sub-acutely, or as latent responses (Schnyder and Pichler, 2009). The acute and some sub-acute disorders are often due to Type 1 (Ig-E)-mediated reactions and more rarely, due to Ig G antibodies (Type II). Immune complex disorders (Type III) are much rarer in this context. Type IV responses develop more slowly (Schnyder and Pichler, 2009). The principal types of disorder are Type 1: anaphylactic shock asthma and angioneurotic edema; type II: hemolytic anemia and agranulocytosis; type III: serum sickness and allergic vasculitis; and type IV: allergic dermatitis (Schnyder and Pichler, 2009).

Antimicrobial drug residues in animal tissues may cause hypersensitivity reactions in humans. Antimicrobial residues may trigger an allergic reaction in a previously sensitized individual (Schnyder and Pichler, 2009). Residues can not contribute to the overall response of primary sensitization because of its low concentrations. The duration of exposure is also short. Notwithstanding their non-toxic nature, β-lactams appear to be responsible for most reported human allergic reactions to antimicrobials. Aminoglycosides, sulphonamides, and tetracyclines may also cause an allergic reaction. Certain macrolides may, in exceptional cases, be responsible for liver injuries caused by a specific allergic response to macrolides metabolite modified hepatic cells (Schnyder and Pichler, 2009). However, only a few cases of hypersensitivity have been reported due to exposure to residues in fish. In one case, anaphylaxis was possibly caused by streptomycin residues and angioneurotic edema and chest tightness by penicillin residues in fish (Dayan, 1993).

2.5 Commercial cultivable fishes

Bangladesh is blessed with a perfect environment and many water bodies. Many fish species grow successfully in ponds, pools, and tanks, but only a few species are usually cultivated commercially (Thilsted and Wahab, 2014). The reason for this choice is to make more profit. The main goal of commercial aquaculture is achieving the maximum possible amount of fish production and profit through the perfect use of natural food and supplementary feed from different companies, which limits the choice of fish species for cultivation.

There are some standard criteria for the selection of suitable fish species for commercial cultivation. They must have the adaptability to the pond environment with a faster growth rate and should utilize natural food resources efficiently of the pond and convert supplementary feed efficiently (Meade, 2012). They should be compatible with other cultivable species of fish. Generally, the chosen species of fish should be palatable with high nutritive value. That is why they have higher market demand and price. Different types of carps meet these criteria; hence, the most widely cultivated commercial fishes are from this group in Bangladesh (E-Jahan et al., 2010; Shepon et al., 2020). Nowadays, Tilapia, Climbing perch, different species of catfish are getting popular as commercial cultivable fishes for their palatability and higher market demands.

2.5.1 Tilapia

Tilapia (*Oreochromis aureus*) is now a better choice as a commercial cultivable fish species in Bangladesh (Rahman et al., 2021a). It helps to meet protein demand for the increasing population in Bangladesh at a lower cost. Due to reducing yields from inland fisheries, fish consumption gets decreased at a point. Then the prospects for tilapia farming are improved in Bangladesh. Because of the introduction of new strains such as “genetically improved farmed tilapia” (GIFT), revolutionary change is observed among the farmers (Yáñez et al., 2020). Different hatcheries ensure the availability of different strains of Tilapia. That is why it is now widespread in ponds, markets, and diets (Boyd, 2004). Tilapia is cultivated in ponds as a poly-culture with carp or coastal shrimp production systems.

Tilapia respond relatively well to get better production and can be produced more cost-effectively than other species of carps and catfishes (Rahman et al., 2021a). Bangladesh is a growing producer of fish for the local market and the demand for tilapia is getting higher for its lower price. Rapidly growing demand and low prices in local markets make the farmers interested in Tilapia farming (Rahman et al., 2021a).

Most farmers and consumers now favor tilapia. It may be the most important fish in dry and least developed northwestern Bangladesh (Rahman et al., 2021a). Tilapia has contributed to protein demand for several decades in this particular area as there is a lack of marine fish. More than half of the households with ponds raised tilapia in drier areas, often together with carp.

2.5.2 Pabda

Commercial Pabda fish (also known as Butter Catfish or *Ompok pabda*) farming is gradually becoming popular in Bangladesh (Shamsuzzaman et al., 2020). Pabda fish is an Indian freshwater catfish species with excellent market demand in this country.

Pabda fish farming is getting popular for its higher market value, mainly for its better palatability due to delicate flesh with soft meat texture and it has very high nutritional value and relatively low bones. The Pabda fish is generally cultivated in the ponds along with some other carp fish species.

Commercial Pabda fish farming does not have much attention due to the non-availability of information regarding its breeding and rearing technique (Ngasotter et al., 2020). But it is very profitable and it is now a commercially viable business for the rural unemployed youth. Though Pabda fish farming is not very widely distributed, it has excellent potential for growing market value and demand. Researchers are trying to change this situation by improving the breeding and rearing methods of Pabda.

2.5.3 Climbing Perch

The climbing perch (*Anabas testudineus*), locally called Koi, is an indigenous fish species in Bangladesh (Shamsuzzaman et al., 2020). It is a native air-breathing fish, generally found in swamps, rivers, streams, lakes, canals, and estuaries (Izmaniar et al., 2018). It can survive in several adverse environmental conditions, such as low oxygen for its air-breathing ability and a wide range of temperatures (Izmaniar et al., 2018). It is a prevalent fish in south-east Asian countries like Bangladesh, Malaysia, Indonesia, Vietnam, Laos, Cambodia, Thailand, the Philippines, and India (Edwards et al., 1997). It plays a significant role in the aquaculture sector in Bangladesh due to its high nutritious value with better taste and flavor. It contains a higher amount of iron and copper, which support hemoglobin synthesis and it also contains high-quality poly-unsaturated fatty acids and many essential amino acids (Ahmadi). So the demand for Climbing perch species is very high. Though this species is found in natural habitats, they are also important in commercial farming due to their market value and demand. The breeding technologies of this species have been developed for commercial rearing in ponds or cages with different culture strategies (Slamat et al., 2019).

2.5.4 Stinging Catfish

The stinging catfish (*Heteropneustes fossilis*), locally called Shing, is an indigenous fish species in Bangladesh (Kohinoor et al., 2012). The species is famous for its delicious taste, market value, and its nutritional and medicinal properties. It contains a high amount of iron and calcium compared to many other fishes (Zafar and Khan, 2019). It is considered a nutritious fish species and recommended for sick, convalescent patients, pregnant women, and breastfeeding mothers. It is a lean fish, so it is also recommended for those avoiding fatty diet for health purposes (Farhat, 2013). But the fish gets endangered gradually due to over-harvesting and destruction of the natural habitats and breeding grounds for application of pesticides and fertilizers in rice cultivation, the release of chemical effluents from industries and reduction of water bodies (Sultana et al., 2017). So stinging catfish cultivation is getting famous due to higher market price and demand (Kohinoor et al., 2012). Scientists are developing breeding and farming strategies for commercial farming to achieve optimum benefit.

2.6 Control of Antimicrobial residues

Antimicrobials are used in aquaculture by farmers without any consultation from a registered veterinarian (Hassan et al., 2021a). Generally, they got influenced by the drug and feed dealers (Kalam et al., 2021). Even many fish feed companies use antimicrobials to formulate their feed. At the period of antimicrobial use, farmers may harvest fish when mortality starts. Withdrawal periods are not appropriately maintained all the time. So this malpractice should be under control. Law enforcement agencies should get involved to restrict random use of antimicrobials not only in aquaculture but also in all veterinary aspects (Costello et al., 2001). Foods of animal origin should be tested regularly to determine certain substances and residues to identify the situation (Hassan et al., 2021a).

2.7 Safety evaluation of antimicrobial residues

To assess the safety of ingested antimicrobial residues, national and international committees evaluate data on chemical, pharmacological, toxicological, and antimicrobial properties of the drugs derived from studies of experimental animals and observations in humans. To demonstrate the safety of the veterinary drugs, tests are performed in order to determine a NOAEL (no observed adverse effect level) (Dorato et al., 2005). This level is the basis for the development of microbiological methods for detecting and identifying antimicrobial residues in fish, calculating an ADI.

2.7.1 Acceptable Daily Intake

The ADI estimates the residue, expressed on a body weight basis, that can be ingested daily over a lifetime without any appreciable health risk (Mojsak et al., 2018). The ADI approach was initially developed to take account of effects based on classical toxicology and it was applied to the results of standard toxicity studies. These studies were used to derive a NOAEL. The ADI was calculated by dividing this by a suitable safety factor, usually 100, which assumes that humans are 10 times more sensitive than animals and that there is a 10 fold range of sensitivity (Woodward and therapeutics, 1998). Standard toxicological studies are inadequate in evaluating the adverse effects of antimicrobials. Thus the determination of microbiological endpoints is necessary.

2.7.2 Maximum Residue Limits (MRLs)

'Maximum residue limit means the maximum concentration of residue resulting from the use of a veterinary medicinal product which may be legally permitted or recognized as the development of microbiological methods for the detection and identification of antimicrobial residues in fish acceptable in food, allocated to individual food commodities' (Mitchell et al., 1998). It is based on the type and amount of residue without any toxicological hazard for human health as expressed by the ADI or based on a temporary ADI that utilizes an additional safety factor. In calculating an MRL, the ADI, the residue depletion patterns of a compound in the edible tissues of a particular food-producing animal and the theoretical food intakes are taken into account (Mitchell et al., 1998). Once the process of safety evaluation is complete and MRLs have been derived for a particular substance, considerations are given to the likely level of residue which may be expected to remain after the use of the substance following good practice and to the availability of analytical detection methods suitable for use for routine monitoring purposes.

2.7.3 Withdrawal period

'This is the period of time between the last administration of a drug and the collection of edible tissue or products from a treated animal that ensures the contents of residues in food comply with the maximum residue limit for the veterinary drug' (FAO, 2021). To ensure drug residues have declined safe concentration following the use in animals, specific period for withdrawal must observed prior to harvest for human consumption (Takele and Berihun, 2014). The time passes between the antimicrobial given the animal and when the concentration residues in the tissues: muscle, liver, kidney get lower than or equal to the MRL. Typically it could be 7-15 days for different antimicrobials (Khatun et al., 2018). Harvesting should be kept off till completing withdrawal periods for specific antimicrobial drugs (Takele and Berihun, 2014).

2.8 Detection of Antimicrobial residues

Antimicrobial residues are a global concern now. AMR can emerge from this. So foods of animal origin should be checked if there is any antimicrobial residue. Qualitative detection of antimicrobial residues can be performed by TLC and quantitative determination is performed by UHPLC (Sattar et al., 2014; Hassan et al., 2021a).

2.8.1 Chromatographic detection of antimicrobial residues

TLC is a sensitive and reliable method for monitoring low amounts of different chemicals. Illumination of antibiotics against UV light helps to detect their presence. Determination of drug residues in foods from animal origin is an essential application of TLC (Stead and Applications, 2000). Penicillin can be determined by using hydrocarbon impregnated TLC plates. Post chromatographic derivatization using fluorescamine was performed and quantification of the bright pale blue fluorescent zone was achieved with fluorimetric scanning. Qualitative detection of antimicrobial residues can be performed in foods originated from animals by TLC (Stead and Applications, 2000). The fluorescent zone achieved by residues in foods are compared with respective antimicrobials and its presence can be detected.

2.8.2 Confirmatory analytical detection of antimicrobial residues

Commonly used procedures for the detection of veterinary drug residues include high-performance liquid chromatography (HPLC), gas chromatography (GC), liquid chromatography and mass spectrometry (LC-MS) (Masiá et al., 2016). Chemical methods are usually performed with a preliminary extraction to isolate the drugs of interest from the biological matrix. The main objectives of sample treatment are removing macromolecules and other matrix constituents that may adversely affect the chromatographic system with the detection and enrichment of the analytes to achieve the required low limits of detection. The low solubility of some antimicrobials in organic solvents has made it challenging to develop procedures to extract and concentrate their residues from biological matrices. Other antimicrobials are either insufficiently volatile or too thermally unstable to permit their GC analysis (Masiá et al., 2016). Liquid chromatography has emerged as the method of choice to determine antimicrobials, which are relatively polar, non-volatile and sometimes heat sensitive. The development of coupled liquid chromatography-mass spectrometry (LC-MS) has increased the range of antimicrobials for which assays based on molecular spectrometry can be developed (Masiá et al., 2016).

The aim of chromatography, in general, is the resolution or separation of different molecular species. As the analytes in the mobile phase pass over the stationary phase, those with polarity closer to that of the stationary phase are retained selectively for a time on the column. Passing through the instrument monitor sequentially, these groups of molecules give rise to peaks on the chromatogram (Masiá et al., 2016).

Chapter- III: MATERIALS AND METHODS

Antimicrobial residues were determined from commercial cultivated fishes collected from wet markets of Chattogram, Bangladesh. Qualitative and quantitative detection of antimicrobial residues were done by performing TLC and UHPLC methods.

3.1 Study Design and Selection of the Study Markets

A repeated cross-sectional and an intervention study were conducted in different wet markets in the Chattogram Metropolitan Area (CMA) of Chattogram district from October 2020 – March 2021. A census of wet markets offering different types of fishes for sale in CMA was performed within the context of another research project (Food safety issues: antimicrobial uses and their residues in foods of animal origin - SD2019967 - supported by the Ministry of Education, Bangladesh). Five wet markets (Bahaddarhat, Chawkbazar, Jhawtala, Pahartali, and Reazuddin Bazar) were selected for sampling.

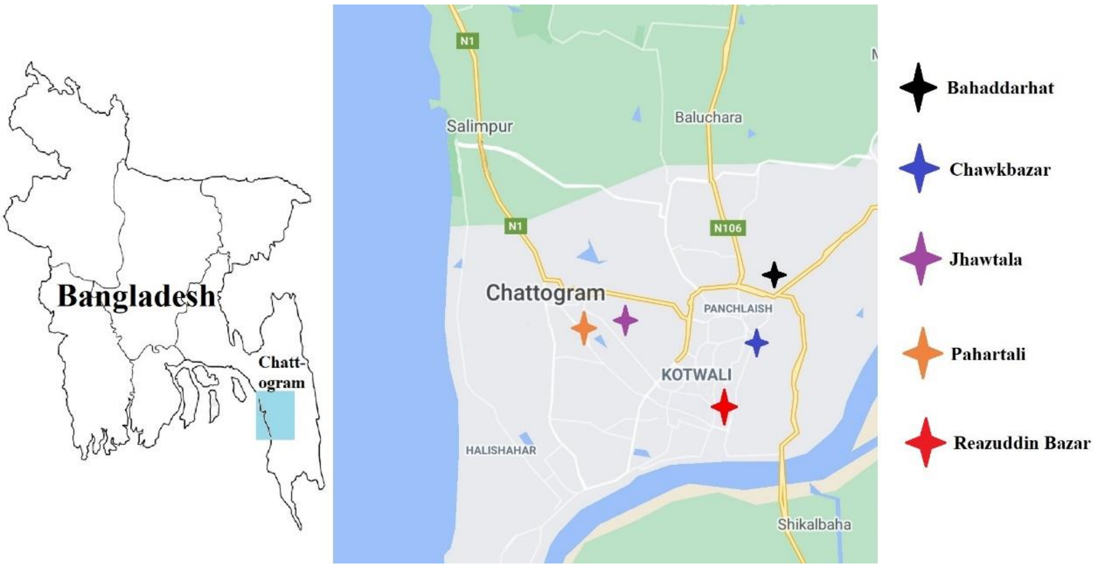

Figure 1: Locations of selected wet markets of Chattogram Metropolitan Area

3.2 Sampling

In total, 400 fish samples were collected for the study from five markets (80 from each market). Four types of commercial cultivable fish (Tilapia, Climbing perch, Pabda, and Stinging catfish) were selected for this study. Twenty samples were collected from each type of fish as total were taken to determine antimicrobial residues. All 400 fish samples were tested with the TLC method, whereas 100 samples (25 from each type) were chosen randomly with the same sample size for UHPLC.

3.3 Thin Layer Chromatography

3.3.1 Sample processing and preparation

Fish samples were transported via the insulated cool box to Chattogram Veterinary and Animal Sciences University (CVASU) laboratory. Fishes were processed and washed with tap water and kept at -20°C until analysis at the CVASU laboratory. The washed samples were placed in an individual plastic bag. All the fish samples were prepared in the same procedure. The fish sample was separated from fish bone and cut into small pieces and then it was grinded with a grinder.

A four-gram sample was weighted with a digital weighing balance and taken into a falcon tube. Then 10ml phosphate-buffered saline (PBS) (pH-6.5) was added and mixed by vortexing. Then 2ml 30% trichloroacetic acid was added and mixed by vortexing. Then it was centrifuged at 3500 rpm for 20 minutes. The supernatant was collected 5 ml and filtered by filter paper. Then it was mixed with Di-ethyl-ether properly by vortexing. Then it was left to settle down to separate fatty portion of the sample. The extracted sample was then collected carefully with a dropper to a cryovial (Touchstone, 1992).

3.3.2 Preparation of standard and mobile phase

Ciprofloxacin, Enrofloxacin, Doxycycline, Levofloxacin, Oxytetracycline, and Chlortetracycline were selected to perform TLC to detect antimicrobial residue from a commercial fish sample. Commercially available products were collected as a standard. If the product was in powder form, then 0.1 gm of powder was mixed with 4 ml Methanol.

To perform TLC a mobile phase or solvent system was prepared. Methanol (50 ml) was mixed with 50 ml of Acetone to a TLC tank and used as mobile phase.

3.3.3 Pointing and running of TLC

TLC plate was cut according to the size of TLC tank. A line was drawn above the level of solvent and there were three spots are made on the line about 2 cm distances from one another. First one is used for standard and other two for two different samples from the cryovial tube with the help of capillary tube. Then the plate was dried for a minute. Pointed TLC plate was placed on TLC tank containing mobile phase carefully. Then it was left for about 15 minutes until the solvent rise upto the top of the TLC plate. Then it was removed carefully and dried.

That TLC plate is placed in UV chamber to examine under the ultraviolet light at 256 nm. Both standard and samples were checked if the distances had traveled from the start line in cm. The outline of the top spot was marked with a pencil (Touchstone, 1992).

3.3.4 Determination of Retardation factor

To define the relative migration rate of substances under various conditions retardation factor is determined. It is the ratio distance moved by the substance and distance moved by solvent. For this, the distance that each spot had travelled from the start line was measured in centimeter (Touchstone, 1992). The calculation of R_f values:

$$R_f = \frac{\text{Distance moved by the substance}}{\text{Distance moved by the solvent}} \text{ .... (1)}$$

The relative migration rate of substances was considered to identify the unknown chemicals as a particular chemical substance.

3.4 Ultra High Performance Liquid Chromatography (UHPLC)

To determine the concentration of antimicrobial residues UHPLC was performed in the laboratory of Centre for Advanced Research in Sciences (CARS), University of Dhaka.

3.4.1 Chromatographic equipment and conditions

UHPLC was performed on SIL 20 series Prominence UHPLC (Shimadzu, Japan) equipped with an auto sampler (Model SIL-20 AC), dual pumps (Model 20 AD), column oven (Model CTO-20A), vacuum degasser (Model DGU-20A), UV-visible detector (Model SPD-20A), and LC solution software was used. Analytical reversed phase C-18, Luna 5µ, 250 x 4.6 mm, Phenomenex, Inc., Japan was used (Xu, 2013).

Mobile phase: Acetic acid (10%): Acetonitrile (90: 10)

UV detection: 280 nm

Run time: 15 minutes

Flow rate: 1.0 mL/min

Column temperature: Room temperature

Injection volume: 20 µL Elution: Isocratic.

3.4.2 Preparation of reagents and standard

The chemicals and techniques used for extraction, detection, and quantification of residual concentration of Ciprofloxacin, Enrofloxacin, Levofloxacin, and Oxytetracycline are-

Methanol: UHPLC grade, DUKSAN, Korea; Acetonitrile: UHPLC grade, DUKSAN, Korea; Deionized water, Phosphate Buffer Saline (PH 6.5); Trichloroacetic acid AR., 98% analytical grade, Loba Chemie, and Research-Lab Fine Chem Industries. Purity of all standard chemicals and reagents were at least 98%. To prepare standard solution, 10 mg of Ciprofloxacin, Oxytetracycline, Levofloxacin, and Enrofloxacin were weighed and diluted with 10ml 5% TCA in a volumetric flask containing a concentration of 1000ppm. Then this solution was mixed by vortexing for 5 minutes and was filtrated by 0.45 μm nylon filter. Serial dilution was done to get concentration 1250ppb-15,000ppb i.e., 1.25-15.00 μg/mL with TCA solution. The supernatant was filtrated by 0.45 μm nylon filter and then sample was injected into UHPLC (Xu, 2013).

3.4.3 Sample preparation and extraction

Samples (2 gm) were weighted with digital weighing machine and taken into a falcon tube. Then 8ml of 30% trichloroacetic acid was added and mixed properly by vortexing. Then it was centrifuged at 3000 rpm for 20 minutes. Supernatant was collected and filtered by syringe filter. Then it was collected carefully to a vial.

3.4.4 Running of UHPLC

Sample containing vials were placed to input section of UHPLC machine for auto-sampling. After completion of UHPLC run, chromatographic peaks were found as output. Then these peaks were calibrated through software to determine the residue concentration in samples which were found by comparison with chromatographic peaks of standards.

3.5 Statistical analysis

All the data from TLC and UHPLC results were sorted (according to fish type and wet market) in Microsoft excel 2013 for statistical analysis. Then the data was analyzed in R software (R studio version1.4.1717). Data organized and tidied with the library tidyverse. Descriptive statistics was performed to identify the TLC and UHPLC positive samples. Univariate analysis were performed for particular antimicrobials tested both in TLC and UHPLC methods to get the prevalence and 95% confidence interval. Prevalence of particular antimicrobials along with P value of Chi square test was analyzed for TLC method. Mean concentration with standard error along with P value of One-way ANOVA was analyzed for UHPLC method. P value less than 0.05 considered as a significant difference. Different values of prevalence and concentration were arranged in tables according to fish type, different wet markets, and different antimicrobials.

3.6 Estimation of Hazard Quotient (HQ) and risk assessment

HQ model was used to assess the risk of consuming residues with fish.

Acceptable Daily Intake (ADI) is an estimated amount of residue allowed for ingested daily over a lifetime without any appreciable health risk expressed on a bodyweight basis. ADI value of Oxytetracycline is 30 µg/Kg/day (APVMA, 2021) and Quinolones (Ciprofloxacin, Enrofloxacin, and Levofloxacin) are 0.15 µg/Kg/day (Budiati and Technology, 2010).

The mean level of antimicrobial residue concentrations in fish was calculated. Then the value of the mean concentration and average daily fish consumption based on 60 kg body weight were taken into consideration. Per capita fish consumption in Bangladesh is about 62.58 gm/ day (Shamsuzzaman et al., 2020).

The estimated daily intake (EDI) was calculated (Eq. 2) by the following given equation (Rahman et al., 2021b).

$$\text{EDI} = \frac{(\text{concentration of residue as } \mu g/kg) \text{ x } (\text{daily intake of food in kg/person})}{\text{Adult body weight (60 kg)}} \quad \text{.....………… (2)}$$

The HQ (Eq. 3) is the ratio of the potential exposure to a substance and the level at which no adverse effects are expected (Rahman et al., 2021b).

$$\text{HQ} = \frac{\text{Estimated daily intake (EDI)}}{\text{Accepted daily intake (ADI)}} \quad \text{…………….. (3)}$$

A HQ less than or equal to one indicates negligible hazard, while greater than one states the toxicological effects on the health of consumers (Rahman et al., 2021b).

Chapter- IV: RESULTS

4.1 Descriptive analysis:

Ciprofloxacin, Enrofloxacin, Doxycycline, Oxytetracycline, Chlortetracycline, and Levofloxacin residues were tested through TLC from 400 commercial fish samples (Pabda, Stinging catfish, Tilapia, and Climbing perch: 100 from each sample type), collected from five different wet markets (80 from each market). Based on descriptive analysis, highest prevalence was found in Tilapia fishes 86% with 95% CI: 77.62-92.12 containing at least one residue from above mentioned antimicrobial. 77% Pabda fish with 95% CI: 67.51-84.82, 75% Stinging catfish with 95% CI: 65.34-83.12, and 62% Climbing perch fish samples with 95% CI: 51.74-71.52 contain antimicrobial residues. Among all five different wet markets, fish samples from Jhawtala contains highest prevalence 81.25% (95% CI: 70.96-89.11) of fishes containing antimicrobial residues followed by Chawkbazar 77.50% (95% CI: 66.79-86.08), Pahartali 77.50% (95% CI: 66.79-86.08), Reazuddin Bazar 71.25% (95% CI: 60.04-80.82), and Bahaddarhat 67.50% (95% CI: 56.1-77.55) (Table 1).

Table 1: Prevalence of TLC positive fish samples based on samples types and wet market

Factor	Category	N	TLC positive (%)	95% CI
Sample type	Stinging catfish	100	75 (75)	65.34-83.12
	Climbing perch	100	62 (62)	51.74-71.52
	Pabda	100	77 (77)	67.51-84.82
	Tilapia	100	86 (86)	77.62-92.12
Wet market	Bahaddarhat	80	54 (67.50)	56.1-77.55
	Chawkbazar	80	62 (77.50)	66.79-86.08
	Jhawtala	80	65 (81.25)	70.96-89.11
	Pahartali	80	62 (77.50)	66.79-86.08
	Reazuddin Bazar	80	57 (71.25)	60.04-80.82

Ciprofloxacin, Enrofloxacin, Oxytetracycline, and Levofloxacin residues were tested through UHPLC from 100 commercial fish samples randomly taken from TLC positive samples (Pabda, Stinging catfish, Tilapia, and Climbing perch: 25 from each sample type), collected from five different wet markets (20 from each market). Based on descriptive analysis, Pabda fishes were found with the highest prevalence (96%; 95% CI: 79.65-99.90) containing at least one residue from above mentioned antimicrobial. 84% Climbing perch fish with 95% CI: 63.91-95.46, 80% Tilapia with 95% CI: 56.29-93.17, and 24% Stinging catfish samples with 95% CI: 9.35-45.13 contain antimicrobial residues. Among all five different wet markets, fish samples from Jhawtala, Bahaddarhat, and Chawkbazar contains highest prevalence (75%; 95% CI: 50.89-91.34) of fishes containing antimicrobial residues followed by Pahartali, and Reazuddin Bazar (65%; 95% CI: 40.78-84.61) (Table 2).

Table 2: Prevalence of UHPLC positive fish samples based on samples type and wet market

Factor	Category	N	UHPLC positive (%)	95% CI
Sample type	Stinging catfish	25	6 (24)	9.35-45.13
	Climbing perch	25	21 (84)	63.91-95.46
	Pabda	25	24 (96)	79.65-99.90
	Tilapia	25	20 (80)	56.29-93.17
Wet Market	Bahaddarhat	20	15 (75)	50.89-91.34
	Chawkbazar	20	15 (75)	50.89-91.34
	Jhawtala	20	15 (75)	50.89-91.34
	Pahartali	20	13 (65)	40.78-84.61
	Reazuddin Bazar	20	13 (65)	40.78-84.61

4.2 Univariate analysis:

Ciprofloxacin residues were detected through TLC from 400 commercial fish samples (Pabda, Stinging catfish, Tilapia, and Climbing perch: 100 from each sample type), collected from five different wet markets (80 from each market). Tilapia possesses the highest prevalence (42%) among all other fishes followed by Stinging catfish (31%), Climbing perch (20%), and Pabda fish (10%). According to wet markets, fish samples from Jhawtala were found with high prevalence (33.75%) of Ciprofloxacin residues followed by Pahartali (26.25%), Bahaddarhat (25%), Chawkbazar (23.25%), and Reazuddin Bazar (20%) (Table 3).

Table 3: Prevalence of Ciprofloxacin residues in fish samples by TLC

Factor	Category	N	Ciprofloxacin residue	
			Yes (%)	**P-value**
Sample type	Stinging catfish	100	31 (31)	<0.001
	Climbing perch	100	20 (20)	
	Pabda	100	10 (10)	
	Tilapia	100	42 (42)	
Wet market	Bahaddarhat	80	20 (25)	0.372
	Chawkbazar	80	19 (23.75)	
	Jhawtala	80	27 (33.75)	
	Pahartali	80	21 (26.25)	
	Reazuddin Bazar	80	16 (20)	

Enrofloxacin residues were detected through TLC from 400 commercial fish samples (Pabda, Stinging catfish, Tilapia, and Climbing perch: 100 from each sample type), collected from five different wet markets (80 from each market). Pabda fish possesses the highest prevalence (31%) among all other fishes followed by Stinging catfish (22%), Tilapia (20%), and Climbing perch (17%). According to wet markets, fish samples from Pahartali were found with high prevalence (28.75%) of Enrofloxacin residues followed by Jhawtala (23.75%), Chawkbazar (22.50%), Bahaddarhat (21.25%), and Reazuddin Bazar (18.75%) (Table 4).

Table 4: Prevalence of Enrofloxacin residues in fish samples by TLC

Factor	Category	N	Enrofloxacin residue	
			Yes (%)	P-value
Sample type	Stinging catfish	100	22 (22)	0.124
	Climbing perch	100	17 (17)	
	Pabda	100	31 (31)	
	Tilapia	100	22 (22)	
Wet market	Bahaddarhat	80	17 (21.25)	0.647
	Chawkbazar	80	18 (22.50)	
	Jhawtala	80	19 (23.75)	
	Pahartali	80	23 (28.75)	
	Reazuddin Bazar	80	15 (18.75)	

Levofloxacin residues were detected through TLC from 400 commercial fish samples (Pabda, Stinging catfish, Tilapia, and Climbing perch: 100 from each sample type), collected from five different wet markets (80 from each market). Pabda fish possesses the highest prevalence (27%) among all other fishes followed by Stinging catfish (17%), Climbing perch (17%), and Tilapia fish (15%). According to wet markets, fish samples from Jhawtala were found with high prevalence (27.50%) of Levofloxacin residues followed by Chawkbazar (26.25%), Bahaddarhat (17.50%), Pahartali (15%), and Reazuddin Bazar (8.75%) (Table 5).

Table 5: Prevalence of Levofloxacin residues in fish samples by TLC

Factor	Category	N	Levofloxacin residue	
			Yes (%)	**P-value**
Sample type	Stinging catfish	100	17 (17)	0.126
	Climbing perch	100	17 (17)	
	Pabda	100	27 (27)	
	Tilapia	100	15 (15)	
Wet market	Bahaddarhat	80	14 (17.50)	0.012
	Chawkbazar	80	21 (26.25)	
	Jhawtala	80	22 (27.50)	
	Pahartali	80	12 (15.00)	
	Reazuddin Bazar	80	7 (8.75)	

Oxytetracycline residues were detected through TLC from 400 commercial fish samples (Pabda, Stinging catfish, Tilapia, and Climbing perch: 100 from each sample type), collected from five different wet markets (80 from each market). Pabda fish possesses the highest prevalence (41%) among all other fishes followed by Stinging catfish (27%), Tilapia (24%), and Climbing perch (23%). According to wet markets, fish samples from Chawkbazar were found with high prevalence (37.50%) of Oxytetracycline residues followed by Pahartali (30%), Jhawtala (28.75%), Reazuddin Bazar (25%), and Bahaddarhat (22.50%) (Table 6).

Table 6: Prevalence of Oxytetracycline residues in fish samples by TLC

Factor	**Category**	**N**	**Oxytetracycline residue**	
			Yes (%)	**P-value**
Sample type	Stinging catfish	100	27 (27)	0.017
	Climbing perch	100	23 (23)	
	Pabda	100	41 (41)	
	Tilapia	100	24 (24)	
Wet market	Bahaddarhat	80	18 (22.50)	0.275
	Chawkbazar	80	30 (37.50)	
	Jhawtala	80	23 (28.75)	
	Pahartali	80	24 (30)	
	Reazuddin Bazar	80	20 (25)	

Chlortetracycline residues were detected through TLC from 400 commercial fish samples (Pabda, Stinging catfish, Tilapia, and Climbing perch: 100 from each sample type), collected from five different wet markets (80 from each market). Tilapia possesses the highest prevalence (49%) among all other fishes followed by Pabda (43%), Stinging catfish (35%), and Climbing perch (21%). According to wet markets, fish samples from Jhawtala were found with high prevalence (53.75%) of Chlortetracycline residues followed by Bahaddarhat (38.75%), Chawkbazar (35%), Pahartali (30%), Reazuddin Bazar (31.25%), and Pahartali (26.25%) (Table 7).

Table 7: Prevalence of Chlortetracycline residues in fish samples by TLC

Factor	Category	N	Chlortetracycline residue	
			Yes (%)	**P-value**
Sample type	Stinging catfish	100	35 (35)	<0.001
	Climbing perch	100	21 (21)	
	Pabda	100	43 (43)	
	Tilapia	100	49 (49)	
Wet market	Bahaddarhat	80	31 (38.75)	0.005
	Chawkbazar	80	28 (35)	
	Jhawtala	80	43 (53.75)	
	Pahartali	80	21 (26.25)	
	Reazuddin Bazar	80	25 (31.25)	

Doxycycline residues were detected through TLC from 400 commercial fish samples (Pabda, Stinging catfish, Tilapia, and Climbing perch: 100 from each sample type), collected from five different wet markets (80 from each market). Pabda fish possesses the highest prevalence (38%) among all other fishes followed by Tilapia (32%), Stinging catfish (28%), and Climbing perch (16%). According to wet markets, fish samples from Jhawtala were found with high prevalence (35%) of Doxycycline residues followed by Pahartali (32.50%), Reazuddin Bazar (28.75%), Bahaddarhat (23.75%), and Chawkbazar (22.50%) (Table 8).

Table 8: Prevalence of Doxycycline residues in fish samples by TLC

Factor	**Category**	**N**	**Doxycycline residue**	
			Yes (%)	**P-value**
Sample type	Stinging catfish	100	28 (28)	0.005
	Climbing perch	100	16 (16)	
	Pabda	100	38 (38)	
	Tilapia	100	32 (32)	
Wet market	Bahaddarhat	80	19 (23.75)	0.332
	Chawkbazar	80	18 (22.50)	
	Jhawtala	80	28 (35)	
	Pahartali	80	26 (32.50)	
	Reazuddin Bazar	80	23 (28.75)	

The concentration of Ciprofloxacin residues were determined by UHPLC from 100 commercial fish samples (Pabda, Stinging catfish, Tilapia, and Climbing perch: 25 from each sample type), collected from five different wet markets (20 from each market). Among them 1 Stinging catfish sample was found positive with highest concentration (45.85 µg/Kg) followed by 2 Pabda fish samples (24.08 µg/Kg ± 9.18), 3 Tilapia samples (19.97 µg/Kg ± 6.64), and 13 Climbing perch samples (18.29 µg/Kg ± 2.37). According to wet markets, 3 fish samples from Reazuddin Bazar were found for Ciprofloxacin residues containing highest concentration (30.81 µg/Kg ± 9.46) followed by 3 samples from Chawkbazar (20.50 µg/Kg ± 6.40), 3 samples from Pahartali (19.97 µg/Kg ± 6.64), 4 samples from Bahaddarhat (18.70 µg/Kg ± 4.86), and 6 samples from Jhawtala (17.17 µg/Kg ± 3.23) (Table 9).

Table 9: Mean Concentration of Ciprofloxacin residues in fish samples by UHPLC

Factor	**Category**	**Positive**	**Ciprofloxacin (µg/Kg)**	
			Mean ± SE	**P-value**
Sample type	Stinging catfish	1	45.85±NA	0.076
	Climbing perch	13	18.29 ± 2.37	
	Pabda	2	24.08 ± 9.18	
	Tilapia	3	19.97 ± 6.64	
Wet market	Bahaddarhat	4	18.70 ± 4.86	0.518
	Chawkbazar	3	20.50 ± 6.40	
	Jhawtala	6	17.17 ± 3.23	
	Pahartali	3	19.97 ± 6.64	
	Reazuddin Bazar	3	30.81 ± 9.46	

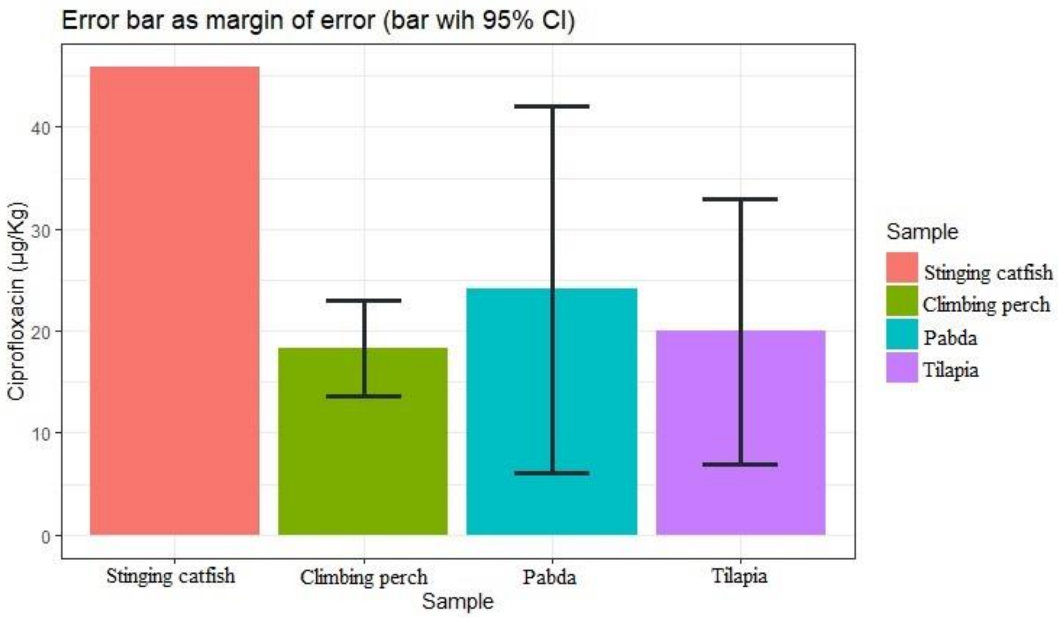

Figure 2: Concentration level of Ciprofloxacin residues in fish samples

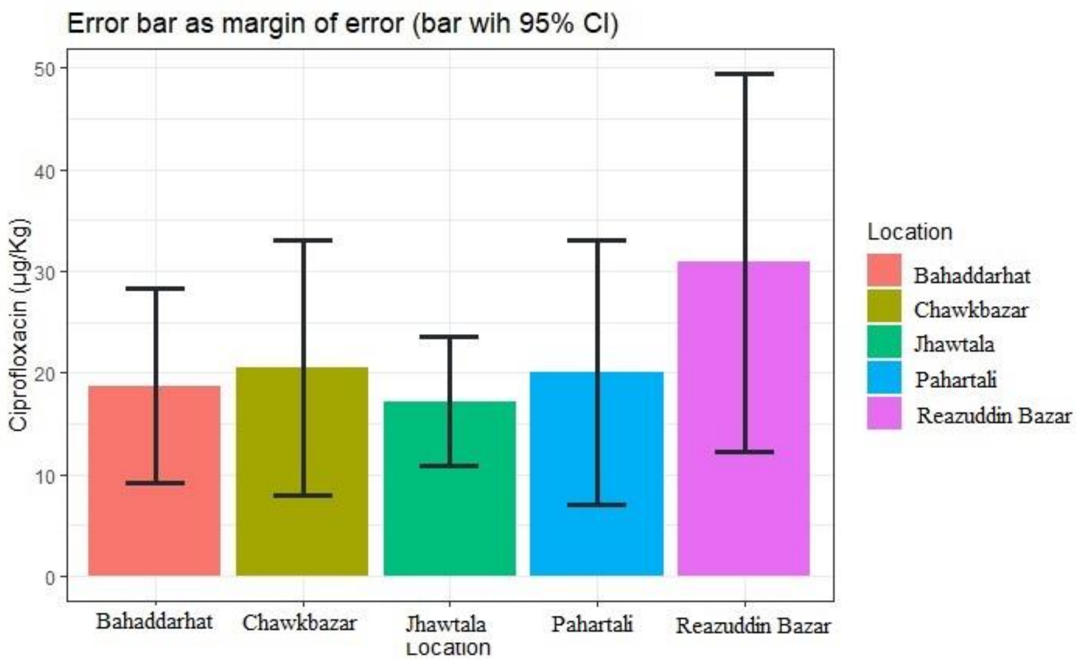

Figure 3: Concentration level of Ciprofloxacin residues in fishes from different wet market

The concentration of Enrofloxacin residues were determined by UHPLC from 100 commercial fish samples (Pabda, Stinging catfish, Tilapia, and Climbing perch: 25 from each sample type), collected from five different wet markets (20 from each market). Among them 9 Climbing perch samples were found positive with highest mean concentration (69.32 µg/Kg ± 8.05) followed by 18 Pabda fish samples (66.21 µg/Kg ± 5.25), 2 Stinging catfish samples (51.13 µg/Kg ± 13.32), and 6 Tilapia samples (56.53 µg/Kg ± 5.96). According to wet markets, 5 fish samples from Jhawtala were found for Enrofloxacin residues containing highest concentration (71.62 µg/Kg ± 5.62) followed by 4 samples from Chawkbazar (68.95 µg/Kg ± 3.43), 10 samples from Pahartali (66.70 µg/Kg ± 6.46), 10 samples from Reazuddin Bazar (65.37 µg/Kg ± 6.75), and 6 samples from Bahaddarhat (53.76 µg/Kg ± 13.50) (Table 10).

Table 10: Mean Concentration of Enrofloxacin residues in fish samples by UHPLC

Factor	**Category**	**Positive**	**Enrofloxacin (µg/Kg)**	
			Mean ± SE	**P-value**
Sample type	Stinging catfish	2	61.13 ± 13.32	
	Climbing perch	9	69.32 ± 8.05	0.708
	Pabda	18	66.21 ± 5.25	
	Tilapia	6	56.53 ± 5.96	
Wet market	Bahaddarhat	6	53.76 ± 13.50	
	Chawkbazar	4	68.95 ± 3.43	
	Jhawtala	5	71.62 ± 5.62	0.687
	Pahartali	10	66.70 ± 6.46	
	Reazuddin Bazar	10	65.37 ± 6.75	

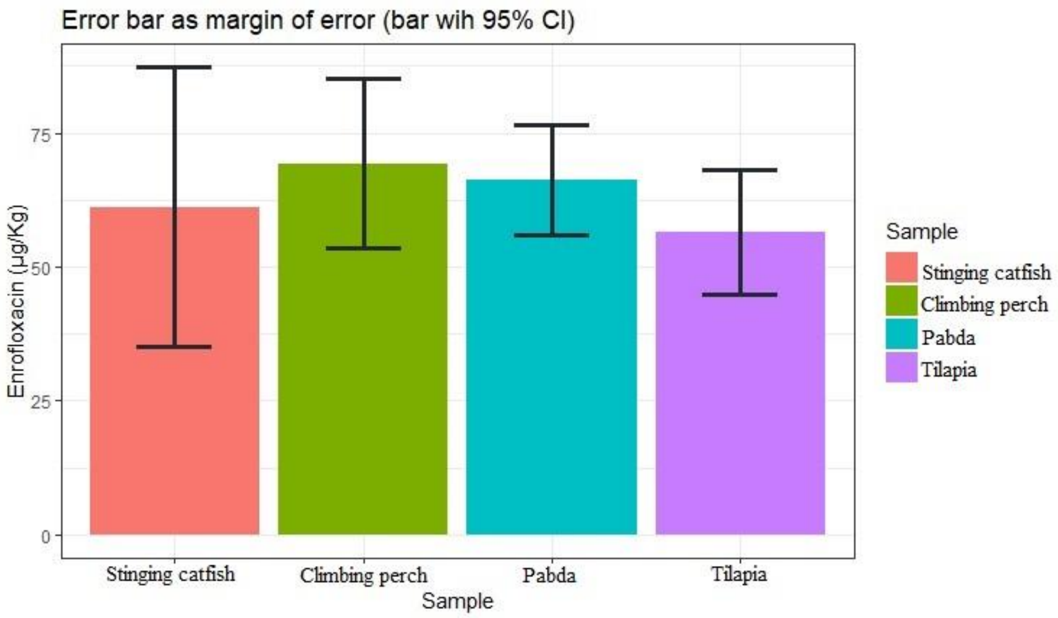

Figure 4: Concentration level of Enrofloxacin residues in different fish samples

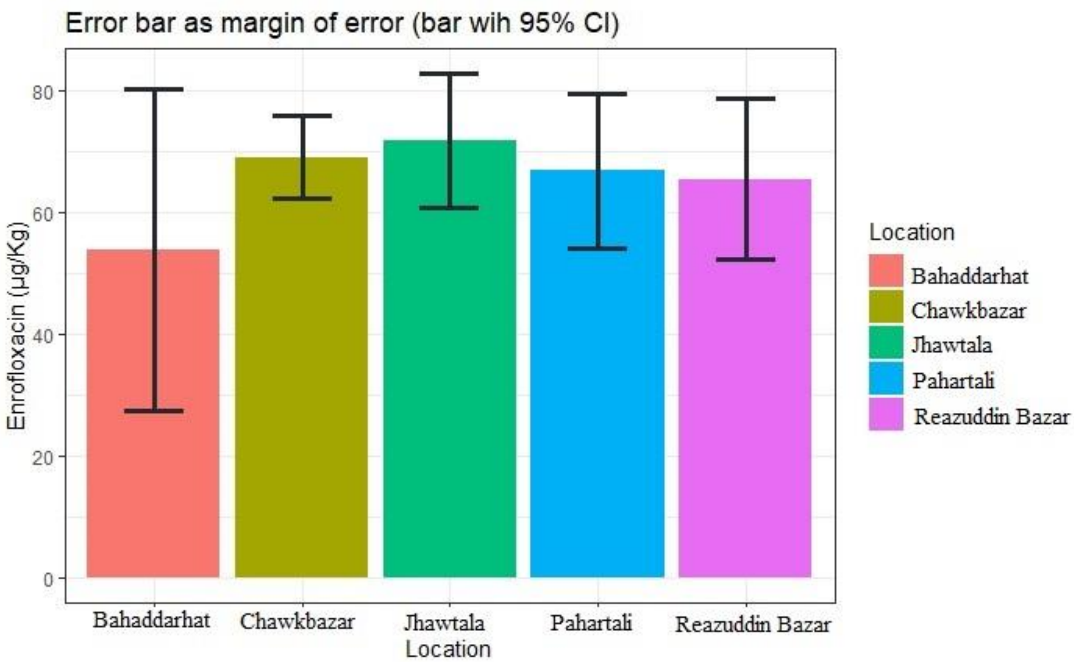

Figure 5: Concentration level of Enrofloxacin residues in fishes from different wet market

The concentration of Levofloxacin residues were determined by UHPLC from 100 commercial fish samples (Pabda, Stinging catfish, Tilapia, and Climbing perch: 25 from each sample type), collected from five different wet markets (20 from each market). Among them 6 Climbing perch samples were found positive with highest mean concentration (37.59 µg/Kg ± 12.93) followed by 16 Pabda fish samples (24.74 µg/Kg ± 6.14), 5 Tilapia samples (22.28 µg/Kg ± 9.66), and 1 Stinging catfish sample (9.94 µg/Kg). According to wet markets, 9 fish samples from Jhawtala were found for Levofloxacin residues containing highest concentration (38.28 µg/Kg ± 8.37) followed by 8 samples from Bahaddarhat (25.31 µg/Kg ± 9.34), 9 samples from Chawkbazar (19.87 µg/Kg ± 8.22), 1 sample from Pahartali (9.71 µg/Kg), and 1 sample from Reazuddin Bazar (7.13 µg/Kg) (Table 11).

Table 11: Mean Concentration of Levofloxacin residues in fish samples by UHPLC

Factor	**Category**	**Positive**	**Levofloxacin (µg/Kg)**	
			Mean ± SE	**P-value**
Sample type	Stinging catfish	1	9.94 ± NA	0.636
	Climbing perch	6	37.59 ± 12.93	
	Pabda	16	24.74 ± 6.14	
	Tilapia	5	22.28 ± 9.66	
Wet market	Bahaddarhat	8	25.31 ±9.34	0.481
	Chawkbazar	9	19.87 ± 8.22	
	Jhawtala	9	38.28 ± 8.37	
	Pahartali	1	9.71±NA	
	Reazuddin Bazar	1	7.13 ± NA	

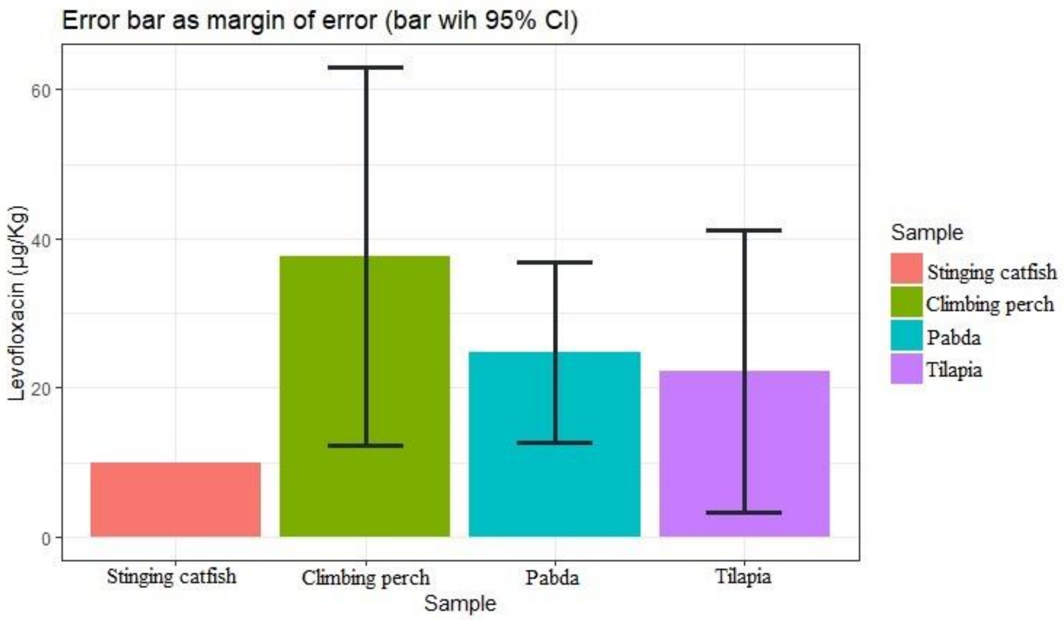

Figure 6: Concentration level of Levofloxacin residues in different fish samples

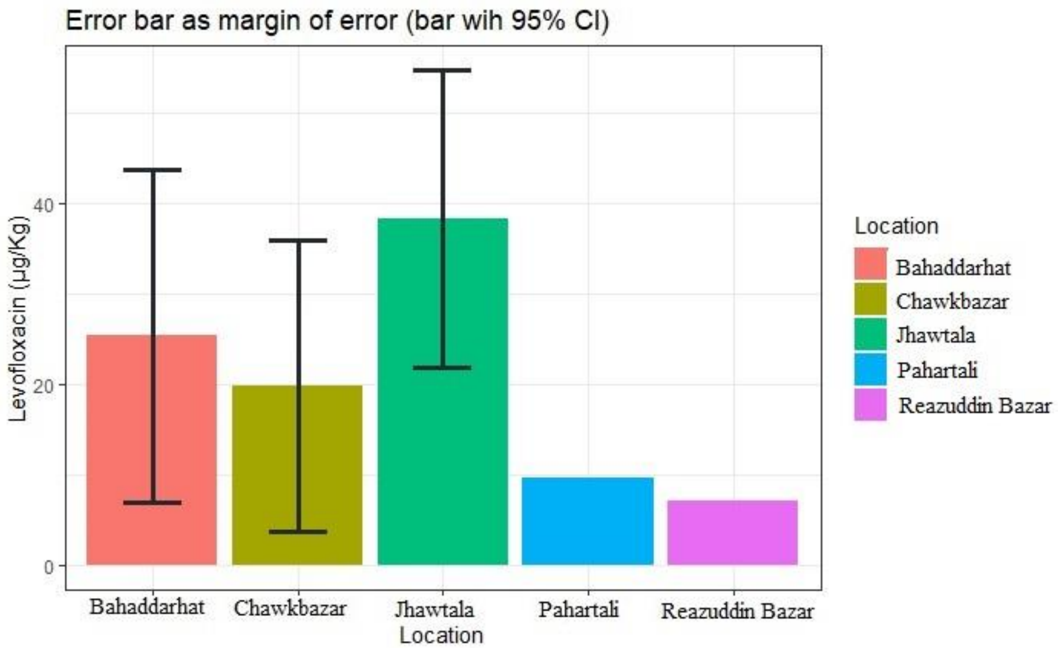

Figure 7: Concentration level of Levofloxacin residues in fishes from different wet market

The concentration of Oxytetracycline residues were determined by UHPLC from 100 commercial fish samples (Pabda, Stinging catfish, Tilapia, and Climbing perch: 25 from each sample type), collected from five different wet markets (20 from each market). Among them 18 Pabda fish samples were found positive with highest mean concentration (88.73 µg/Kg ± 3.92) followed by 15 Tilapia samples (87.40 µg/Kg ± 3.87), 9 Climbing perch samples (73.32 µg/Kg ± 4.60), and 5 Stinging catfish samples (68.44 µg/Kg ± 3.79). According to wet markets, 3 fish samples from Reazuddin Bazar were found for Oxytetracycline residues containing highest concentration (100.65 µg/Kg ± 17.14) followed by 14 samples from Jhawtala (89.58 µg/Kg ± 10.80), 13 samples from Bahaddarhat (79.84 µg/Kg ± 11.72), 3 samples from Pahartali (78.76 µg/Kg ± 23.81), and 14 samples from Chawkbazar (77.15 µg/Kg ± 11.16) (Table 12).

Table 12: Mean Concentration of Oxytetracycline residues in fish samples by UHPLC

Factor	**Category**	**Positive**	**Oxytetracycline (µg/Kg)**	
			Mean ± SE	**P-value**
Sample type	Stinging catfish	5	68.44 ± 3.79	0.638
	Climbing perch	9	73.32 ± 4.60	
	Pabda	18	88.73 ± 3.92	
	Tilapia	15	87.40 ± 3.87	
Wet market	Bahaddarhat	13	79.84 ± 11.72	0.858
	Chawkbazar	14	77.15 ± 11.16	
	Jhawtala	14	89.58 ± 10.80	
	Pahartali	3	78.76 ± 23.81	
	Reazuddin Bazar	3	100.65 ± 17.14	

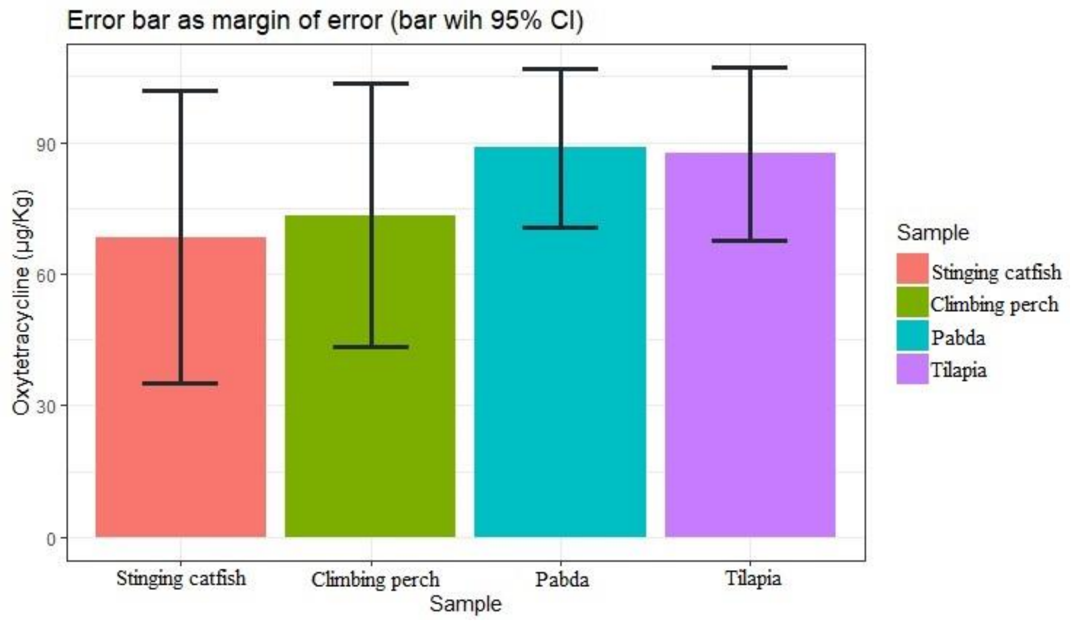

Figure 8: Concentration level of Oxytetracycline residues in different fish samples

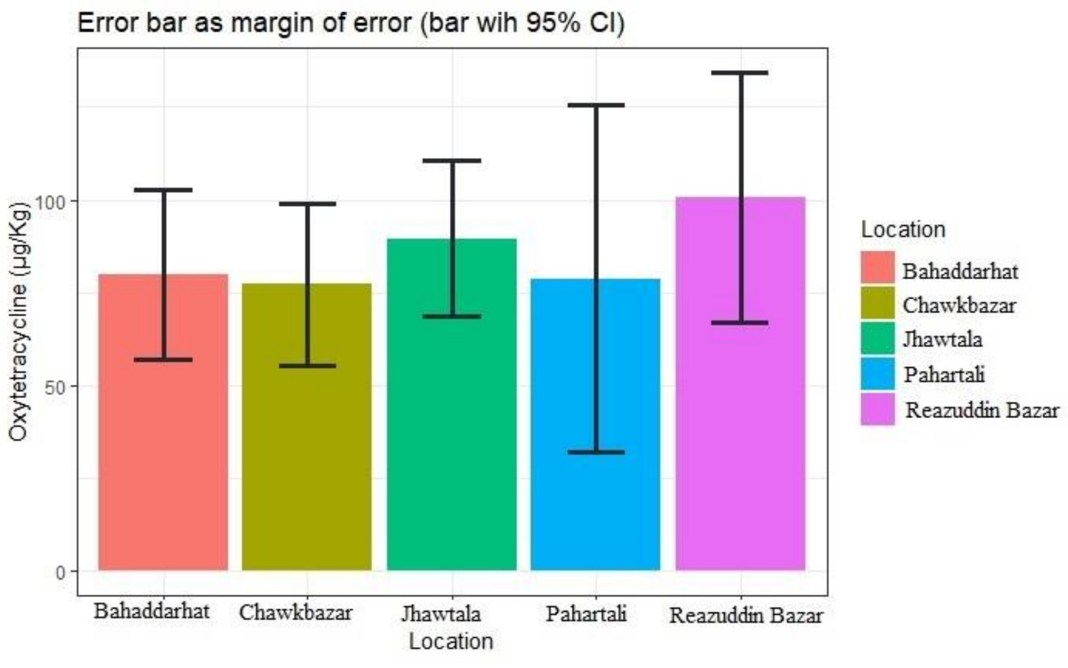

Figure 9: Concentration level of Ciprofloxacin residues in fishes from different wet market

Based on the mean value of antimicrobial residues in different fishes, the HQ was calculated to characterize the risk of dietary exposure to Ciprofloxacin, Enrofloxacin, Levofloxacin, and Oxytetracycline. Highest HQ was found for Enrofloxacin in Climbing perch (0.480) followed by Pabda (0.460), Stinging catfish (0.420), and in Tilapia (0.387). For Ciprofloxacin, highest HQ was found in Stinging catfish (0.320) followed by Pabda (0.167), Tilapia (0.140), and Climbing perch (0.127). For Levofloxacin, highest HQ was found in Climbing perch (0.260) followed by Pabda (0.173), Tilapia (0.153), and Stinging catfish (0.067). For Oxytetracycline, highest HQ was found in Pabda (0.0031) followed by Tilapia (0.003033), Climbing perch (0.002567), and Stinging catfish (0.002367) (Table 13).

Table 13: Estimation of risk assessment by Hazard Quotient for mean concentration of residues in fishes

Antimicrobial	**Fish**	**EDI (µg/Kg/day)**	**ADI (µg/Kg/day)**	**Hazard Quotient**
Ciprofloxacin	Stinging catfish	0.048	0.15	0.320
	Climbing perch	0.019	0.15	0.127
	Pabda	0.025	0.15	0.167
	Tilapia	0.021	0.15	0.140
Enrofloxacin	Stinging catfish	0.063	0.15	0.420
	Climbing perch	0.072	0.15	0.480
	Pabda	0.069	0.15	0.460
	Tilapia	0.058	0.15	0.387
Levofloxacin	Stinging catfish	0.010	0.15	0.067
	Climbing perch	0.039	0.15	0.260
	Pabda	0.026	0.15	0.173
	Tilapia	0.023	0.15	0.153
Oxytetracycline	Stinging catfish	0.071	30	0.002367
	Climbing perch	0.077	30	0.002567
	Pabda	0.093	30	0.0031
	Tilapia	0.091	30	0.003033

Chapter- V: DISCUSSION

In this study, a total of 400 fish samples (Tilapia, Climbing perch, Pabda, and Stinging catfish) were randomly collected from five different wet markets of Chattogram metropolitan area during October 2020 to March 2021. After collection, we performed antimicrobial residues test through TLC and UHPLC. The prevalence of antimicrobial residues were calculated along with its concentration in fish samples. A significant number of fish samples were detected containing different antimicrobial residues with various level of concentration.

In Bangladesh, antimicrobials are frequently being used for treating diseases in aquaculture industry. In addition, they are used as feed additives as growth promoter mixed with formulated feeds are the common feature in this industry since last couple of years (Hassan et al., 2021a; Kalam et al., 2021). The rapidly growing burden of antimicrobials in aquaculture industry for treatment, prophylaxis, and enhancing growth increase the risk of antimicrobial residue deposition. However, all those events usually exceed the MRLs of the particular antibiotic that set up by the FAO (Mog et al., 2020).

Generally, antimicrobials mixed with feeds as growth promoters help in improving the growth rate and play a vital role against chronic disease conditions (Najafian and Babji, 2012). Therefore, farmers are inclined to use antimicrobials as growth promoter in commercial fish farms. Without this approach, their farm will be infected through various infectious diseases and the farms profitability being hampered drastically (Sekkin and Kum, 2011). Inadequate treatment records, inadequate management, failure to notice drug withdrawal periods, accessibility of antimicrobials to commercial fish farm owners are considered as the principle causes of residues in commercial fishes. Moreover, consumers do not have enough awareness about the human health hazards associated with residues consumption. National legislative bodies should take proper steps to establish restrictive legislation to use antimicrobials and increase public awareness. The most common antibiotics that been utilized in aquaculture industry are Ciprofloxacin, Enrofloxacin, Levofloxacin, Oxytetracycline, Doxycycline, Colistin sulfate, and Neomycin (Petersen and Dalsgaard, 2003). In this study, Ciprofloxacin, Enrofloxacin, Levofloxacin, Oxytetracycline, Chlortetracycline, and Doxycycline residues were detected with high prevalence along with significant concentration.

A review article entitled 'Residual antimicrobial agents in food originating from animals' has shown the overall condition of antimicrobial residues in food animals in Bangladesh (Hassan et al., 2021a). There were few studies conducted on fish samples in Bangladesh (Bakar et al., 2013; Barman et al., 2018; Hossain et al., 2018; Ferdous et al., 2019). Qualitative detection was performed with TLC and tests results were analyzed to calculate the prevalence of antimicrobial residues present in commercial fishes which are getting popular nowadays. Ciprofloxacin, Enrofloxacin, Levofloxacin, Oxytetracycline, Chlortetracycline, and Doxycycline residues were detected in Tilapia, Stinging catfish, Climbing perch, and Pabda fish samples from wet markets of CMA. There was another study conducted also in CMA in 2015, where Amoxicillin and Oxytetracycline residues were detected in Climbing perch, Tilapia, Rui, Bombay duck, and Shrimp (Ferdous et al., 2019). Oxytetracycline residues were found 8.3% in Climbing perch and 3.03% in Tilapia fish samples (Ferdous et al., 2019), which were considered as very low in comparison of this study.

Furthermore, quantitative detection was performed with UHPLC where concentration of residues in fishes were determined with the unit µg/Kg. The average concentration of Ciprofloxacin, Enrofloxacin, Levofloxacin, and Oxytetracycline residues were determined in Tilapia, Stinging catfish, Climbing perch, and Pabda fish samples. A study in Sylhet district was conducted in 2016, where concentration of Oxytetracycline residuses in Tilapia fishes were conducted by HPLC method and mean concentration was determined 38.88 µg/Kg (Barman et al., 2018), which was very low in comparison of this study. Another study in Sylhet district was conducted in 2016, where concentration of Oxytetracycline residuses in Pungas fishes were conducted by HPLC method and mean concentration was determined 35.11 µg/Kg (Hossain et al., 2018). There was another study also conducted in Chattogram in 2013, where Chloramphenicol residues were detected in Climbing perch, Tilapia, Rui, Pungas, and Trout fish samples by LC-MS method which were determined as very high concentration (Bakar et al., 2013).

The HQ expresses the risk posed to human health by consuming fishes having residues and presents the intensity of the hazardous effect (Rahman et al., 2021b). With the help of calculated mean concentration of antimicrobial residues HQs were calculated for various antimicrobials in fishes. Highest HQ was found for Enrofloxacin residues in Climbing perch followed by Pabda, Stinging catfish, and Tilapia which might be considered as potential Hazard quotient. Ciprofloxacin residues were also considered as potential HQ. HQ for Ciprofloxacin and Levofloxacin residues in Stinging catfish, Pabda, Tilapia, and Climbing perch were also considered as potential HQ. Oxytetracycline residues in Pabda, Tilapia, Climbing perch, and Stinging catfish were found as negligible HQ. Results revealed that the HQ for Ciprofloxacin, Enrofloxacin, Levofloxacin, and Oxytetracycline residues were found below 1.0 which proved that detected levels of residues in fishes had no significant toxicological effects on the health of consumers in the study area as per capita fish consumption were taken into consideration. However, more consumption of these four fishes may show toxicological effects if the EDI gets higher than the acceptable daily intakes (ADI) especially for Enrofloxacin, Ciprofloxacin, and Levofloxacin residues. Although the residues might not show toxicological effect as HQ values were less than 1.0, they still have public health hazard due to AMR issue.

AMR in human pathogens is now a major public health issue globally (Roca et al., 2015). An alarming issue at the moment is the relation between use of antimicrobials in food producing animals and the development of resistance in human pathogens (Chantziaras et al., 2014). Resistance against few enteric bacteria originated from human has evolved due to transfer of resistant bacteria or resistance genes from animals to people via the food chain. Over-use and misuse of antimicrobials for therapeutic purpose is the principal cause of the resistance issue in human medicine (Komolafe, 2003), whereas prophylactic use and growth promoting use of antimicrobials in aquaculture have contributed most to the emergence of resistant bacteria in cultivable fishes (Cabello, 2006). Evidently, the use of antimicrobials in aquaculture sector has been associated with the development of human associated AMR. The fish feed containing low prophylactic level of antimicrobials might be responsible for developing bacterial resistance to those antimicrobials (Mog et al., 2020).

Development and persistence of resistance against the antimicrobials by the microorganisms are quite variable. Some microorganisms show resistance quite readily, whereas others do not. Tetracycline resistance persist long after the withdrawal of the drug, whereas Avoparcin resistance declines quite rapidly after withdrawal (Wegener, 2003). However, re-exposure to previously withdrawn antimicrobial drug will lead to re-emergence of resistance rapidly. Use of antimicrobials as feed additives, use of poultry offal and poultry litter in aquaculture might have low level of exposure of antimicrobials in fish (Hassan et al., 2021a). The presence of antimicrobial residues in fishes is the ultimate result of the use of those poultry and livestock wastage in aquaculture in Bangladesh. It was concluded that the probable public health hazard of AMR arises from consuming sub-therapeutic levels of antimicrobials acquired from food producing animals (Chowdhury et al., 2015). Relation between antimicrobial use in animals and the development of resistance in human pathogens is described in several reports. If any antimicrobial residue cross the MRL is an alarming concern for public health (Aytenfsu et al., 2016). Consumers should be aware of this issue, proper food processing might reduce the residue level (Ferdous et al., 2019).

This is the first report on risk assessment against antimicrobial residues in commercial cultivable fishes in Bangladesh. Previously this sort of report has been developed in milk samples of Chattogram (Rahman et al., 2021b). Therefore this study has tremendous significance to implement strategic control plan against AMR. However, our study findings suggest adequate nationwide policy making to mitigate the residual impact in aquaculture industry.

Chapter- VI: CONCLUSION

A significant number of samples were found with antimicrobial residues with some remarkable concentration. It was stated that probably in some cases commercial fish farm owners do not maintain withdrawal periods before harvesting. The use and sometimes misuse of antimicrobials in aquaculture have resulted in the emergence of resistant pathogens and resistance genes. Antimicrobial resistant bacteria in fishes can affect not only fish production, but also affect public health. Although the HQs were found less than one, some values are potential when fish consumption will increased. As some potential values of the HQ were found, there may exist some cases of toxicological effect of antimicrobial residues on health of consumers through more fish consumption. Although the residues might not show toxicological effect as HQ value was less than one, they still have public health hazard due to AMR issue. More restrictions in using of antimicrobials in food animals will reduce the selection of resistant bacteria and help to preserve these valuable antimicrobials for both human and veterinary medicine. To reduce the overuse and misuse of antimicrobials, prescriptions must be provided by registered veterinarian for antimicrobials for disease control in aquaculture. AMR is one of the most important public health issues now. National authorities should take proper steps to restrict antimicrobial use at random in aquaculture. Laboratory detection of antimicrobials should be adopted in every corner to ensure food quality for public.

Chapter- VII: RECOMMENDATIONS

To minimize the devastating consequences for human health few recommendations are suggested through this study. These are applicable for the public health authorities, commercial fish farm owners, veterinarians, and consumers.

The public health authorities should set up a permanent national fish quality control program (for antimicrobial residues and AMR). They should arrange seminars on public health hazard due to antimicrobial residues and resistance. Laboratories should be established to control the veterinary drug residues in foods from animal origin.

Commercial fish farm owners should not use antimicrobials without suggestions from registered veterinarians. They should follow the withdrawal periods before harvesting.

Veterinarians should prescribe the actual dose of antimicrobials and suggest about withdrawal periods.

Consumers should get aware of health hazard from antimicrobial residues. They should reject foods having antimicrobial residues that could harm their health.

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