Quantitation of Oxytetracycline residue in three common cultured fishes of Bangladesh by analytical High Performance Liquid Chromatography (HPLC)

A dissertation submitted to the Department of Pharmacy, East West University in partial fulfillment of the requirement for the Degree of Master of Pharmacy

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Dedication

I dedicated my dissertation to my parents, in-laws and my husband Tanvir Ahmed.

With love and appreciation

Declaration by the research candidate

I, Amna Rasul, hereby declare that the dissertation entitled **"**Quantitation of Oxytetracycline residue in three common cultured fishes of Bangladesh by analytical High Performance Liquid Chromatography (HPLC)**"**, submitted by me to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the award of the degree of Masters of Pharmacy is a record of original research work carried out by me during Fall-2011-Spring 2012 under the supervision and guidance of Dr. Chowdhury Faiz Hossain, Professor, Department of Pharmacy, East West University and it has not formed the basis for the award of any other Degree/Diploma/Fellowship or other similar title to any candidate of any University.

Dhaka

Date: 17/06/12 Signature of the candidate

(Amna Rasul)

Thesis Certificate

This is to certify that the thesis entitled **"**Quantitation of Oxytetracycline residue in three common cultured fishes of Bangladesh by analytical High Performance Liquid Chromatography (HPLC) **"**, submitted to the Department of Pharmacy , East West University, in partial fulfillment of the requirements for the award of the degree of Masters in Pharmacy is a record of original Research work carried out by Amna Rasul during the period Fall 2011-Spring 2012 of her research in the Department of Pharmacy at East West University, under my supervision and guidance and the thesis has not formed the basis for the award of any Degree/Diploma/Fellowship or other similar title to any candidate of any University.

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Acknowledgement

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ABSTRACT

Labeo rohita (Rui), *Pangasius pangasius* (Pangas) and *Oreochromis niloticus* (Tilapia) are popular in the Bangladesh because of reasonable market prices and consumer demand. These fishes from five local markets (Dhanmondi, Mohammadpur, Shukrabad, Banani and Gazipur) were purchased randomly.

The objective of this experiment was to do quantitation of oxytetracycline (OTC) residue in three common cultured fishes of Bangladesh by analytical High Performance Liquid Chromatography (HPLC). Oxytetracycline is one of two available FDA-approved antibiotics and regularly used in aquaculture farms of Bangladesh. This antibiotic was chosen for this experiment because it is excreted primarily unchanged through the urine and very minute amount gets deposits in fish muscle. It was necessary to find out whether this minute amount of antibiotic deposition in fish fulfills the requirement i.e. the range of oxytetracycline concentration in fish muscle according to regulatory body (FDA) should be within 55-83mg/Kg. Therefore, through this experiment fish extraction was done and oxytetracycline concentrations were determined by using High Performance Liquid Chromatography (HPLC). In this experiment, presence of residual of oxytetracycline in boiled fish is also included as a parameter to be observed. Two different fishes with confirmed oxytetracycline of two market areas were selected and individually each sample was boiled for 30 minutes. After boiling, the fish extraction process was carried once again and then finally the samples were run in HPLC.

From the experiment it was found that concentration of oxytetracycline in Rui fish of Mohammadpur Townhall market, Shukrabad Kachabazar, Banani Kachabazar and Gazipur Kachabazar were 3237 mg/Kg, 1000 mg/Kg, 923.6 mg/Kg and 1287.5 mg/Kg resceptively. Similarly, Pangas fish of Dhanmondi (Meena bazar), Mohammadpur Townhall market, Shukrabad Kachabazar, Banani Kachabazar and Gazipur Kachabazar contained 1500 mg/Kg, 1061.8 mg/Kg, 1125 mg/Kg, 6250 mg/Kg, 1675 mg/Kg of oxytetracycline respectively. Concentration of oxytetracycline in Tilapia fish of Mohammadpur Townhall market, Shukrabad Kachabazar, Banani Kachabazar and Gazipur Kachabazar were 874 mg/Kg, 1237.6 mg/Kg, 9123.3 mg/Kg, 1625 mg/Kg respectively. Rui and Tilapia fish of Dhanmondi (Meena bazar) showed no trace of oxytetracycline. Finally results of the experiment revealed that residual of oxytetracycline in all three different fishes of different local market areas- *Labeo rohita* (Rui), *Pangasius pangasius* (Pangas) and *Oreochromis niloticus* (Tilapia) were within the range of 874-9123 mg/Kg- which is far too much in amount than the range being fixed by the regulatory body. It indicates whichever fish farms supplying Rui, Pangas and Tilapia in those five areas (Dhanmondi, Mohammadpur, Shukrabad, Banani and Gazipur) are using antibiotics at a very high concentration. Result of boiled fish states- two fish samples (*Labeo rohita* and *Pangasius pangasius*) had no trace of oxytetracycline after boiling. There is a possibility that during the boiling or cooking the oxytetracycline gets released from the fish and dissolves in the boiled water or in the fish gravy.

Apart from this extreme concentrations level and the chronic presence of oxytetracycline inside the fish, sediments at the bottom of the pond should also be considered seriously because oxytetracycline excretes primarily unchanged through the waste materials of the fish. In spite of the potential risks related to the use of antibiotics and micro-organisms getting resistant to antibiotics, the concentrations found in the studied fishes are significantly higher than the regulatory body.

Key words: Oxytetracycline, *Labeo rohita, Pangasius pangasius, Oreochromis niloticus,* HPLC, Antibiotic resistant microorganisms.

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1.0. Introduction:

By virtue of its geographical location to the great Himalayan ranges in the north and the Bay of Bangal in the south, Bangladesh has the unique advantage of possessing the world's largest river delta the Ganga delta, which offers a vast and varied fisheries potential in its fresh, brackish and marine waters. The country is also blessed with the network of innumerable rivers, tributaries, canals, creeks, large lakes, ox-bowlakes (baors), land depression (haors) and huge floodplain during the monsoon. There are 0.114 million hectors of haors, 68,800 hectors of lakes and 2.83 million hectors of floodplains in the country making a total of 4.047 million hectors of open water inland capture fisheries resource. In addition to these resources, there are about 0.27 million hectors of closed water fishery resources, comprising of about 0.146 million hectors of ponds, 5500 hectors of oxbowlakes and over 0.14 million hectors of brackish water shrimp culture enclosures. These make the total inland fisheries resources 4.34 million hectors during the monsoon month. These inland water of Bangladesh are inhabited by 260 indigenous and 11 exotic species of fish[1] and 25 species of prawn.[2]

1.1. Aquaculture Production of Bangladesh:

Over the last decade aquaculture in Bangladesh has expanded, diversified, intensified and technologically advanced. Aquaculture falls under food production sectors, where one of the major ingredient required for successful fish production is chemical. Chemicals are essential ingredient to successful aquaculture, which has been used in various forms for a long time.[3]

For health management of aquatic animals as well as for pond construction- soil and water management, improve aquatic productivity, transportation of live fish, feed formulation, manipulation of reproduction, growth promotion and processing and value addition of the final product chemicals and antibiotics play vital roles.[3]

Bangladesh is a densely populated country of 147 570 km2 with a population of 130 million people. It is fortunate in having an extensive water resource in the form of ponds, natural depressions (haors and beels), lakes, canals, rivers and estuaries covering an area of 4.56 million hactors.[4]

Bangladesh is one of the world's leading inland fisheries producer with a production of 1 646 819 tonnes during 2003–4, with marine catch total of 455 601 tonnes and a total production from aquaculture of 914 752 tonnes during 2003–4. Bangladesh's total fish production for the year totaled above 2.1 million tones.[4] FAO (2005) ranked Bangladesh as sixth largest aquaculture producing country with its estimated production of 856 956 tonnes in 2003.[5] According to the Department of Fisheries, aquaculture accounted for about 43.5 percent of the total fish production during 2003–4, with inland open water fisheries contributed 34.8 percent.[4]

The Ministry of Fisheries and Livestock (MoFL), Department of Fisheries (DoF), Bangladesh Fisheries Development Corporation (BFDC) and the Bangladesh Fisheries Research Institute (BFRI) are the main organizations responsible for aquaculture and its development. Universities, organizations within other ministries and local and international NGOs are also involved in this area.

Fish play an important role among the population in Bangladesh as indicated by the proverb *machte bhate Bangali* (fish and rice make a Bengali). Situated in the delta of the Brahmaputra, Meghna, and Ganges rivers, the climate, water, and soil conditions of Bangladesh are favorable for inland fisheries and aquaculture. At the height of the rainy season, more than a third of the total land area (147,570 square kilometers) of the country is submerged.[6] According to the Bangladesh Bureau of Statistics (BBS), the fisheries sector, including aquaculture and capture fisheries, has had an annual growth exceeding 7% since 1995 and contributed 6% to the country's GDP in 2000.[7] Freshwater aquaculture led this with an annual growth exceeding 10% over the last decade. With annual fish consumption of about 14 kilograms (kg)/person in 2000,[8] fish account for 60–80% of the animal protein consumed by the population, and also provide essential vitamins, minerals, and fatty acids. Inland fisheries and freshwater aquaculture are the main source of these nutrients for most of the rural and urban poor.[9]

In Bangladesh, aquaculture production systems are mainly extensive and extended extensive, with some semi-intensive and in very few cases intensive systems. Nevertheless, over last ten years, yield from closed water aquaculture has been increasing steadily.

Indigenous freshwater carps (22 percent) and exotic carps (10 percent) from both the farming and capture sectors are the primary contributors to total production;[10] other freshwater fish include catfish, snakeheads and small indigenous species. However, carp polyculture in ponds is more productive, capital intensive and is a more profitable activity when compared to the other culture systems.

Feed and labor comprise the two most important components of the total operating cost for most culture systems in Bangladesh, each accounting for approximately 20 percent and 17 percent, respectively of the total operating costs.

In general fish markets in Bangladesh are situated in both rural and urban areas, they tend to be unhygienic, unscientific, dirty and operate using weak management systems. Approximately 97 percent of the inland fish production is marketed internally for domestic consumption while the remaining 3 percent is exported.[11]

A large number of people are involved in the fish marketing chain and include farmers, processors, traders, intermediaries, day laborers and transporters. Four categories of markets are involved in the distribution of fish, these are: primary markets, secondary markets (assembly markets), higher secondary markets (wholesale markets) and central markets. Locally these steps in the chain are known as: Fisherman Nikary (collector), Chalani (transporter), Aratdars (wholesaler), Paiker (retailer) and consumer.[12]

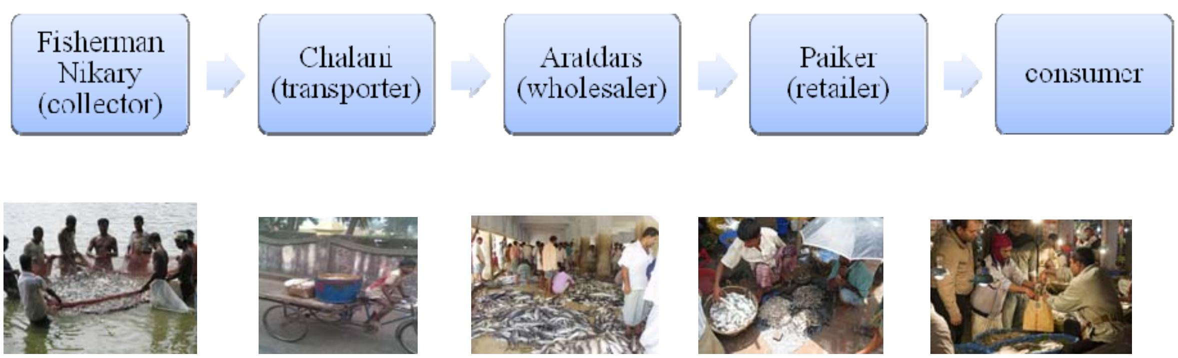


Figure 1: Flow chart showing distribution of fish from fisherman to consumer

According to Alam (2002), a fish farmer receives 56 percent of the price paid by the final consumer, in other words 44 percent of the retail price is taken by the various intermediaries.[12] The country's main exportable product is frozen shrimp, other exported products include frozen fish, frozen frog, dry fish, salted fish, turtles, crabs, shark fins and fish maws (dried fish swim bladders).[13]

Both fisheries and aquaculture in Bangladesh play a major role in alleviating protein deficiency and malnutrition, in generating employment and foreign exchange earnings. Fish and fishery products are the country's third largest export commodity contributing 5.10 percent of its exchange earnings, in 2002–2003 Bangladesh earned US$ 324 million of which shrimp alone contributed 72 percent of the total by quantity and 89 percent by value.[13]

There is no doubt that the growth of aquaculture in Bangladesh is significantly high. However, due to excess population growth, reduce access of common water resources, environmental changes related to rice production, urban construction, use of irrigation, pesticides and fertilizers made fisheries of Bangladesh to be under great threat. Because of intense agriculture and aquaculture processes various small fishes are a extinction as a result of big fishes, especially culture fishes of which edible parts are mainly muscles are replacing the small fishes which finally has a reducing or declining effect on micronutrient intake of the population of Bangladesh.[14] Therefore it is important to follow sustainable aquaculture rather than only considering aquaculture as a profitable business. Aquaculture is defined according to the definition currently used by FAO i.e: - “Aquaculture is the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. Farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated. Therefore, sustainable aquaculture means the management and conservation of the natural resource base and the orientation of technological and institutional change in such a manner as to ensure the attainment and continued satisfaction of human needs for present and future generations. It means, while performing aquaculture, owner as well as people who are directly and indirectly involved with fish farming must consider the effect of aquaculture on the environment. It is necessary for them to consider the consequence of using various chemicals and antibiotics and their on the food-chain that is being naturally builded around the water and water related species.

Below given a diagram from which it is easily understand about the factors involved in a sustainable aquaculture: [15]

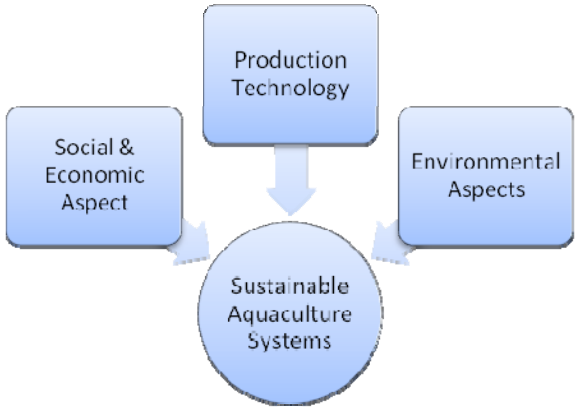


Figure 2: Flow chart showing factors involved in sustainable aquaculture

1.2. Water Quality in Aquaculture:

Normal water quality condition always plays important role for keeping good health state of fish. Any alteration in the environment disturbs this equilibrium causing stress to the fish and become susceptible to disease. There is a relationship of environment, pathogen and fish. The presence of the fish pathogens will result in epizootics only if unfavorable environmental conditions exist. If the relationship is balanced, good health and growth of fish will occur. If it is unsatisfactory, poor growth and over disease will occur. Therefore water quality parameters of fish culture facilities are of utmost importance for raising a healthy crop.

Thus the significant water quality parameters fish health of tropical water bodies are discussed below :

1. Oxygen:

Oxygen is the most important factor influencing the health condition of fish in the water body as an environment. For optimum normal growth and reproduction in tropical waters the dissolved oxygen value for fish health management is 5.0 mgl^-1. Lethal condition arises when the dissolve oxygen concentration become 0.3-0.8 mgl^{-1}

Factors influence dissolved oxygen

a. Temperature: Water holds less oxygen at higher temperature.
b. Salinity: Water holds less oxygen at high salinity.
c. Atmospheric pressure: Water holds less oxygen at low atmospheric pressure.
d. Phytoplankton: As a result of photosynthesis during bloom period the dissolved oxygen values fluctuates during the day.
e. Respiration: Dissolved oxygen in water is utilized for respiration of fish and other aquatic vertebrate and invertebrate fauna.

Symptoms of low dissolved oxygen in ponds:

Fishes surfacing in the water body in the early morning hours gulping air with mouth wide open and gills looking pale. This is a condition of depleted dissolved oxygen level causing stress to fish.

Maintenance of dissolved oxygen concentration:

Rectification of depleted oxygen concentration can be done in the following methods:

- Manual- In this method during oxygen depletion the water surface is splashed with bamboo sticks. This helps in dissolving atmospheric oxygen in water.
- Mechanical- In this method a diesel water pump is operated. The water is pumped out and simultaneously sprayed into the water body again. This helps to dissolve the atmospheric oxygen.
- Aerators- Aerators are the mechanical floating devices in the water bodies. Their rotating blades churn the water helping in dissolution of atmospheric oxygen in water.

2. Ammonia

The total ammonia concentration in water comprises two forms, *viz.*,

i. NH_3 - unionized ammonia
ii. NH_4^+ – ionized ammonia

They maintain the equilibrium as per the equation

$NH_3 + H_2O$ --------------- $NH_4^+ + OH^-$

Unionized ammonia fraction is more toxic to fish and the amount of the total ammonia in this form depends on pH and temperature of water. As a rule, higher the pH and temperature, higher the percentage of total ammonia is present in the toxic unionized form. Safe unionized ammonia level in tropical fish health management is 0.02-0.05 mgl^{-1}. Lethal unionized ammonia level in tropical fish health management is 0.4-2.5 mgl^{-1}.

Factors of ammonia concentration in water:

a. Excess of organic matter is generated from fertilization with organic manure, ammonia based fertilizers and artificial feeds.
b. Industrial and domestic wastes released in water.
c. Death of phytoplankton bloom.
d. Denitrification of nitrates in deoxygenated waters.

Symptoms due to ammonia stress:

a. Gill hyperplasia.
b. Reduced growth and activities.
c. Liver, kidney and brain damage.

Maintenance of safe ammonia concentration in water:

Generally high salinity, dissolved oxygen and carbon dioxide concentration reduce toxicity.

- Aeration will increase the oxygen concentration.
- Healthy phytoplankton population removes ammonia from water.
- Sodium chloride is used to reduce the toxicity of ammonia in water.
- Biological filter maybe used.

3. Nitrite:

Nitrite is an intermediate product in biological oxidation of ammonia to nitrite called the nitrification process. Nitrite concentration is low in natural and well maintained water bodies. Sublethal level of nitrite value for fish health management is 0.02-1.0 mgl^{-1}. Lethal level of nitrite value for fish health management is 1.0-10.0 mgl^{-1}.

Factors of nitrite concentration in water:

a. High organic pollution.
b. Low oxygen concentration.

Symptoms due to nitrite stress in fish:

a. Toxic to fish
b. Reacts with haemoglobin to form methaemoglobin when absorbed.
c. Thus gives brick red color to fish gill.

Maintenance of safe nitrite concentration in water:

Correct stocking, feeding, fertilization and biofiltration are the parameters needed to be maintained for safe nitrite concentration in water.

4. pH

The pH is a measure of concentration of hydrogen ions. It is defined on a scale of 1 to 14. A pH less than 7 is acidic and a pH greater than 7 is alkaline. The optimum pH for most fishes is 6 to 9.

Factors responsible for acidic or alkaline waters:

Acidic waters originated from draining swamps, acidic rocks or acidic sulphate soils. Pollution from industrial discharges many also be acidic.

Alkaline waters originated from calcium and silica rich area. Pollutants from certain industries may also be alkaline.

Rectification of alkaline or acidic water bodies:

Alkaline waters:

- Ensuring good water management
- By application of acid forming fertilizers
- Addition of acid to water supply.

Acid waters:

- $CaCO_3$, $Ca(OH)_3$, CaO or dolomite maybe used.
- Salt water like sea water maybe flushed to water bodies.

5. Carbon dioxide:

The concentration of carbon dioxide in most water bodies is low. Normally natural water contains free CO_2 (< 6 mg l^{-1}). It occurs in water in three closely related forms – a) free carbon dioxide, b) bicarbonate ions (HCO_3) and c) carbonate ions (CO_3). Its amount depends on the pH of the water. Carbon dioxide value of 12-50 mg l^{-1} in fish health management is sublethal. Its effects maybe respiratory stress and development of kidney stones. Carbon dioxide value of 50-60 mg l^{-1} in fish health management is lethal on prolong exposure.

Factors influence carbon dioxide concentration in water:

a. For acidic ground water.
b. Due to high phytoplankton population carbon dioxide may reach high level due to a) death of phytoplankton bloom b) at night due to phytoplankton respiration c) during cloudy weather.
c. If water area heavily loaded with water manure or feed.
d. During transportation of fish the excrete carbon dioxide and high concentration may build up.
e. High concentration may occur after herbicide treatment.

Control measures of high CO_2 concentration:

- Aeration of water repeatedly
- Increasing pH by adding hydrated lime (calcium hydroxide)
- The phytoplankton population and the organic loading in a water body should be regulated by correct stocking, feeding and fertilization.

In addition to these parameters suspended solids, metals, pesticides cause several problems to fish health. Some environment mediated diseases like- Gas bubble disease, algal toxicosis are also recorded. In gas bubble disease fish lose balance due to accumulation of large gas bubbles in the intestine. The disease occurs in water area where load of organic fertilizers are high at the pond bottom. On the other hand gill clogging occur by the algae causing respiratory distress. Convulsion occurs of severe cases in fishes. Some blue-green algae- *Mycrocystis* and *Anabaena* spp. are often encountered for algal toxicosis. Use of copper sulphate and cow dung are partial covering the water surface by water hyacinth for blocking the sunlight penetration.[16]

1.3. Chemicals widely used in freshwater aquaculture for health management:

Different other chemicals are also used for health management of fish apart from antibiotics. Some common chemicals include sodium chloride, formalin, malachite green, methylene blue, potassium permanganate, hydrogen per oxide, copper compounds, glutaraldehyde and trifluralin.[17]

1.4 Prophylaxis and Treatment of Fish Disease:

Fish disease is the interaction between a susceptible fish, the pathogen and the environment. A clean environment always helps in a good growth of fish whereas a bad environment favors multiplication of pathogens. This relationship is a very important factor in the outbreak of infectious diseases of fishes. Any occurrence of fish health problem in a water body is generally recognized by fish becoming restless as exhibited by splashing, surfacing, whirling, rubbing its body against ponds bank and non-acceptance of food etc. Essential features of the strategy for prevention of diseases are good husbandry and proper monitoring of fish health. It’s essential and therefore to be taken care off to prevent entry of pathogens into fish and to treat the diseased fish.

a) Prophylaxis

Prophylaxis or action taken to prevent the development of disease is one of the most critical factors in determining the success or otherwise of a fish farming enterprise.

Water source of fish for rearing farms is an important component for disease prevention. Direct rainwater fill up in ponds is ideal for culture, because it is pathogen free. The rearing water mainly collected from irrigation through shallow machine which should be treated to prevent entry of pathogens. Stocking density is probably the most important factor and problems usually arise from overstocking. High stocking density of fish often seen to act as predisposing factor for disease out-break. This factor will also lead to decrease oxygen level in water and also to build-up of toxic waste products. At the same time if growth is to be obtained, the amount of food added to a particular volume of water will also be dramatically increased and any excess uneaten food will further complicate matters through its decomposition.

When pH of water changes an adverse conditions have a direct effect on the epithelium of gills and skin and cause increased mucus production, hyper plastic responses in the gills and often erosion and ulceration of the skin. Normally saprophytic bacteria and parasites readily invade these lesions.

Balanced nutrition is another factor in preventing the occurrence of disease. Lack of food often causes nutritional deficiency diseases and increase susceptibility to many infectious diseases.

Sterilization of pond-bottoms in between batches of fish is important in cutting down the number of parasites. This should be carried out with calcium hydroxide. The quantity used will be varied with soil type.

b) Treatment of fish:

It must be given attention that majority of infectious diseases arise directly as a result of some husbandry problems and there is no point of carrying out specific parasitic or bacterial treatment without improving the background husbandry quality. Nutritional or non-infectious diseases should obviously treated by identifying the particular husbandry or dietary defects.

The types of treatment method available are limited and will depend on the size of the fish to be treated and type of diseases. At its simplest, therapy can be divided into three categories:

i) **Immersion treatment** (addition of chemicals to water) - This involves the application of chemical treatment to the water to control external disease agents affecting skin and gills. The type of immersion will vary from the “dip” treatment i.e. fishes are placed in a high concentration for a short time, to bath treatment which involves much lower concentrations of chemicals for extended period of time.

ii) **Systemic treatment** by the oral routes (addition of chemicals to feed) – It involves the incorporation of chemicals into the food to treat internal infections- This involves the incorporation of chemicals into food to treat internal infections. Its practical use is in the treatment of systemic bacterial disease with antibiotics although this route may also treat helminthes infections.

iii) **Injection** (application of chemicals directly to the fish) - This usually involves antibiotic therapy and is seldom financially worthwhile except in the case of valuable brood stock fish. Injection is rarely used as it is very labor intensive and requires the fish to be handled that can be harmful in its self. For this reason it tends to be restricted to valuable fish such as brood stock. Only antibiotics are usually injected. It is an efficient means of getting large concentration of antibiotic into a fish quickly.

c) Treatment Compounds:

Several factors need to be considered when choosing a method and/or chemicals for treatment. First of all it may not be worth attempting treatment if mortalities are low and cost of treatment is high. The alternatives are –

a. to either let the disease continue and accept a certain percentage of loss removing heavily infected fish to percentage build-up of pathogens or,
b. if fish are near market size, it may be possible to harvest the fish and avoid losses, or
c. treatment may be necessary.

A number of compounds at present in use for fish farming presented in the following table. The permanent bath method, treating the whole pond by spraying the compound over the surface as evenly as possible and the use of oral therapy are available. In early stage, dip treatment are more readily used and expense becomes less of a problem.

Repeat treatments are also occasionally necessary but care should be taken to avoid accumulated toxicity. Repeated application of many of the chemicals used e.g. Formalin, Malachite green causes irritation to the gills and may reduce the respiratory efficiency.[16]

Table1. Important Fish Disease and Their Treatment Method[17]

Disease or Agent to be treated	Compounds/Chemicals	Treated method and dose rate
External protozoa and monogenic trematodes	Formaline (40% formaldehyde)	Bath: 20-45 min, 100-250ppm Bath: Permanent,25-50ppm
External protozoa, Leeches, Crustacea	Salt	Bath: Indefinite,0.1-0.2% Bath:20-30min, 3.0%
Leeches, Crustacea, For persistent monogenean infections	Trichlorphon	Bath: Parmanent, 0.25ppm
External protozoa *Saprolegnia*	Malachite green	Bath: Parmanent, 0.1ppm
External protozoa	Copper sulphate	Bath: Parmanent, 0.2-2ppm dependent on hardness Do not use in soft water
External protozoa and monogenic trematodes	Potassium Permanganate ($KmnO_4$)	Bath: Parmanent, 2ppm Repeat treatment may be necessary
Mycobacterial skin and gill infections. Skin ulceration caused by bacteria.	Quaternary ammonium compounds e.g. Roccal, Hyamine	Bath: 1hr, 1-4 ppm depending on hardness
External protozoa especially *Ichthyophthirius* and some monogenic trematodes	Malachite green/ Formalin mixture	Bath: Indefinite, 0.1ppm Malachite, 25ppm
Myxobacterial infections	Nifurpirinol	Food: 7g/100kg fish/day for 10days Bath: Indefinite, 0.1ppm
Intestinal helminths	Magnesium sulphate	Food: 3% in ration
Systemic bacterial infections	Nitrofurans	Food: 10g/100kg fish/day for 10days Bath: 4ppm
Systemic bacterial infections	Oxytetracycline	Food: 7g/100kg fish/day for 10days Injection 50mg/kg
Systemic bacterial infections	Sulphonamides	Food: 15g/100kg fish/day for 10days
Systemic bacterial infections	Potentiated Sulphonamides (Tribrissen)	Food: 5g/100kg fish/day for 10days

1.5. Selected Fish Sample for the Experiment:

Fish production is an important source of livelihoods among the world's poor, and fish consumption has long been known to have nutritional benefits. Protein, fatty acid, essential vitamins and minerals are found in fish in enormous amount. All these indicate fish to be a rich source of nutrition. Nowadays, people are becoming more and more health conscious than the previous generations. As a result, in case of animal protein people are focusing more toward the fish protein rather than animal protein such as red meat or white meat. Rising incomes and high consumer preferences for fish, especially in Asia, have caused global fish consumption to double in the past 30 years to 15 kilograms per person per year, according to the Food and Agriculture Organization of the United Nations (FAO).[14] The reasons for choosing *Labeo rohita*, *Pangasius pangasius* and *Orechromis niloticus* as samples of this experiment are their popularity among the population due to low cost, taste, availability and their extensive culture in the industry of aquaculture of Bangladesh.

Sample 1:

Figure 3: Fish sample *Labeo rohita*

Family: Cyprinidae

Genus: Labeo

Scientific Name: Labeo rohita

English Name: Rohu, Rohu Carp

Local Name: Rui, Rohita, Rohu, Rau

Morphology: Body of the fish is moderately elongated. This fish has small eyes which are not visible from the underside of the head. Mouth is small with thick lips.

Habitats and Geographical Distribution: Naturally, Rui fish feed on plant matters including decaying vegetations. In culture condition, the species also feed on supplementary fish food, such as rice bran and oil cake. Normally, it is a riverine fish and one of the member of Indian Major Carp. Rui is extensively stocked in ponds and other closed water bodies such as beels, deep pools, clearly sluggish streams, ponds and tanks, paddy field and floodplains.

This fish is geographically distributed in North and Central India, Pakistan, Bangladesh, Nepal and Myanmar. It has been introduced in many countries including Bhutan, Mainland China, Japan, Malaysia, Philippines, Sri Lanka, Thailand, Vietnam and Zimbabwe.

Economic importance: Rohu is the abundant among the species of genus Labeo, a commercially important species, and preferred as food fish throughout Bangladesh and other countries. *L.*rohita along with other major carps namely *Catla catla* contributes some 4,75,000 mt or about 22.6% of total production of fish in Bangladesh. A maximum quantity is produced from culture fishery.

The species is also extensively bred in hatcheries artificially to meet the demand of seed for aquaculture. The fish fetches a better market price than any other fish because of its exquisite taste. This fish is basically caught by drag nets because *L.rohita* is an excellent game fish and puts up a big fight when caught by anglers with a hook and bait.

Ecological role: *L.rohita* feeds mainly on plant matters and decaying vegetation from the bottom of the pond and does not compete with other carps for food and space.

<u>Sample 2:</u>

Figure 4: Fish sample *Pangasius pangasius*

Family: Pangasiidae

Genus: Pangasius Valenciennes

Scientific Name: *Pangasius hypophthalamus, Pangasius pangasius*

English Name: Pungas, Yellowtail Catfish, Pungas Catfish

Local Name: Thai Pangas, Pangwash

Morphology: *Pangasius pangasius* is indigenous and *P.hypophthalamus* has been introduced into Bangladesh. Distributed in South and Southeast Asia. The fish body is compressed, elongated and has rounded abdomen. Nostrils are widely separated and lips are thin.

Habitats and Geographical Distribution: The fish is omnivorous. Feeds on crusteceans, fishes and vegetable debris. Inhabits large river. This fish is mainly found in Southeast Asia: Thailand, Cambodia and Vietnam. Introduced in many country's for aquaculture.

Economic importance: *P.hypophthalmus* was first introduced in Bangladesh from Thailand in the 1990s. The pangas has great market acceptability, sells at a reasonable price and is considered as one of the important species for aquaculture in the country. This species is fast growing and intensively cultured by Bangladeshi farmers. Farmers can sell fresh fish in market. Usual price is Taka 70-80 per kg.

Ecological role: By feeding on foul and decaying animals and vegetable matter the species keeps the bottom of the ecosystem clean.

Sample 3:

Figure 5: Fish sample *Oreochromis niloticus*

Family: Cichlidae

Genus: Tilapia

Scientific Name: *Orechromis niloticus*

English Name: Nile Tilapia

Local Name: Tilapia, Nilotica

Morphology: Tilapia's body is elongated, fairly deep and compressed; mouth is large. The most distinguishing characteristic of the species is the presence of regular vertical stripes along the sides.

Habitats and Geographical Distribution: Tilapia is omnivorous and feed on almost anything from algae to insects. Juveniles are carnivorous and adults tend to be herbivorous. This species is fast growing and can tolerate a wide range of salinity ranging from 34-48ppt. It is a fresh water fish, inhibiting estuarine areas, pools of sluggish streams, canals, lakes and ponds.The natural habitats of this fish are the rivers and lakes in West, North and South Africa but it has been introduced to many countries of the world and is cultured in Indonesia, Malaysia, the Philippines, Thailand, China, Southeast and far East Asia, including Bangladesh.

Economic Importance: Nilotica was introduced into Bangladesh from Thailand in 1974 with the assistance of UNICEF. Now the fish is one of the most widely cultured species in the world.

Ecological Role: Generally the adult fish performs the role of the first consumer and the juveniles as the higher level of consumers in the aquatic food chain. This species competes with other small indigenous fish and gradually occupies. The fish has proved to be harmful to the other fish species, because of its ability to mature early and to breed more frequently resulting in its overpopulation in the ponds.[18]

Below given the table which shows the nutritional values of these three fishes:

Table2. Nutritional content of cultured fish in Bangladesh[14]

Name of cultured fish species	Vitamin A	Calcium	Iron	Fat
Labeo rohita(rui)	<30g	86g	NA	NA
Pangasius pangasius(pangas)	NA	NA	2g	4g
Orechromis niloticus(tilapia)	<30g	NA	5g	NA

1.6. Antibacterial Agents

1.6.1. Definition of Antibiotics:

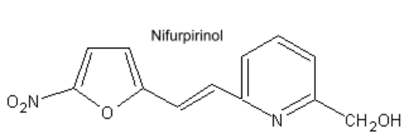
Antibiotics are drugs of natural or synthetic origin that have the capacity to kill or to inhibit the growth of micro-organisms. Antibiotics that are sufficiently non-toxic to the host are used as chemotherapeutic agents in the treatment of infectious diseases of humans, animals and plants. Such chemical agents have been present in the environment for a long time, and have played a role in the battle between man and microbes. In the last century, the discovery of new antibiotics revolutionized the treatment of infectious diseases, leading to a dramatic reduction in morbidity and mortality, and contributing significantly to improvements in the health of the general population.

1.6.2. Fish Disease and Antibacterial Agents:

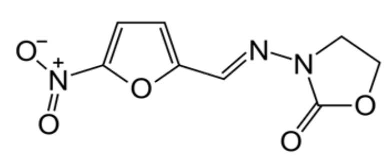
Table3. Name and Doses of various Antibiotics used to treat different fish diseases [17]

Myxobacterial infections	Nifurpirinol	Food: 7g/100kg fish/day for 10days Bath: Indefinite, 0.1ppm
Systemic bacterial infections	Nitrofurans	Food: 10g/100kg fish/day for 10days Bath: 4ppm
Systemic bacterial infections	Oxytetracycline	Food: 7g/100kg fish/day for 10days Injection 50mg/kg
Systemic bacterial infections	Sulphonamides	Food: 15g/100kg fish/day for 10days
Systemic bacterial infections	Potentiated Sulphonamides (Tribrissen)	Food: 5g/100kg fish/day for 10days

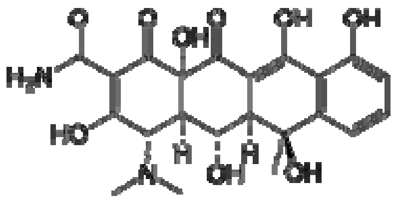
Below given chemical structures of the antibiotics used in the table 3:

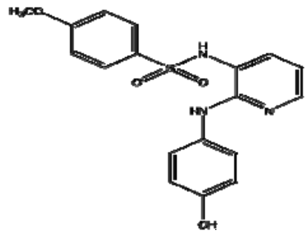


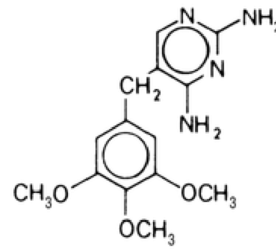
Nifurpirinol



Nitrofurans







Oxytetracycline Sulphonamides Potentiated Sulphonamides (Tribrissen)

1.6.3. Use of Antibacterial Agents in Aquaculture:

Nowadays, the use of antibiotic substances has become a controversial topic. Bacterial diseases of fish can be successfully treated with antibiotics. It is widely recognized that excessive use of antibiotics contributes the development of resistant strains of bacteria. Of the antibiotics four antibiotics like Oxysentin 20%, Renamox, Renamycin and Orgamycine15% were used widely by the farmers (Table 4) for the treatment of fish diseases.

Table4. Names of Chemicals widely used by the Farmers of Bangladesh to treat fish diseases[17]

Chemicals	Number of farmers (n = 50)	% of farmer
Oxytetracycline HCl BP	17	34
Amoxicilline Trihydrate	6	12
Oxytetracycline	14	28
Oxytracycline HCl BP (WSP)	11	22

According to M.A.R.Faruk et al, about 14 branded antibiotics with different trade names were seen in the market as well as used by the fish farmers which are shown in Table 4. The active ingredients of such antibiotics are mainly Oxytetracycline, Chlorotetracyclin, Amoxicillin, Co-trimoxazole, Sulphadiazine and Sulphamethoxazole. The price seems quite affordable by the commercial aqua farmers. As a result, all the people who are involved with aquaculture in Bangladesh use antibiotic in an unethical level and without considering regulations about the use of antibiotic.

Table5: 14 branded antibiotics with different trade names found in the market which are used by the fish farmers[17]

Trade name	Active ingredient	Dose	Source	Price (Tk.)
Oxysentin 20%	Oxytetracline HCl BP	100-200 g/100 kg feed,5-7 days (For treatment)	Novartis Pharmaceuticals	700/kg
Chlorsteclin	Chlortetracycline	200-300 g/100 Kg feed (5-7days)	Novartis Pharmaceuticals	300/kg
Ranamox	Amoxicillin Trihydrate	28-40 g/100 bd of fish, 10 days continuously.	Renata Pharmaceuticals Ltd	140/100 g
Renamycin	Oxytetracycline	28-42 g/100 kg feed, 10 days.	Renata Pharmaceuticals	82/100 g
Fish cure	Chloro-tetracyclin HCl	500 g/100 kg feed (3-5 days)	Rals Agro Ltd.	275/kg
Orgamycin 15 %	Oxytracycline HCl BP (WSP)	In case of prevention 60 gm/100 kg feed 10days In treatment 120-240 g/100 kg feed, 5-7 days	Organic Pharmaceuticals Ltd.	70/100 g
Orgacycline-15%	Chlorotetracycline	200-300 g/100 kg feed (5-7days)	Organic Pharmaceuticals Ltd	-
Acimox(vet) Powder	Amoxiciline (Trihydrate)	1 g/1 kg feed	ACI Animal Health	75/100 g
Bactitab	Oxytetracyclin 20%	50 g/kg body weight , 5-7 days	ACI Animal Health	70-80/100 g
Contrim vet Bolus	Co-Trimoxa zole	Mixed with feed; 1bolus/ 10-12 kg body wt	Square Pharmaceuticals	64 /100 g
Otetra vet power 50	Oxytetracycline	Mixed with feed; 11-16 g/ 100 kg body wt	Square Pharmaceuticals	156/100 g
Sulfatrim	Sulphadiazine & Trimethoprim	50 g/kg body weight , 5-7 days	Square Pharmaceuticals	70-80/100 g
Oxin WS	Oxytetracycline 20%	50 mg/kg body weight	Navana	70-80/100 g
Cotrim-Vet	Sulphamethoxazole + Trimethoprime	0.50mg/kg body weight	Square Pharmaceuticals	70-80/100 g

1.6.4. Antibiotic Tetracycline

The tetracyclines were the first major group of antimicrobial who exhibit activity against both gram-positive and gram-negative bacteria agents for which the term broad-spectrum was used for tetracyclines. Most natural tetracyclines have a common structure with the β-diketone system in rings B and C. *Streptomyces rimosus* is used for the production of natural tetracyclines by commercial fermentation. In *S. rimosus*, a mixture of tetracycline and oxytetracycline is produced, but as the 5-hydroxylase enzyme is extremely active, therefore the equilibrum is far in favor (>95%) of oxytetracycline production. Oxytetracycline (OTC) is a broad-spectrum antibiotic produced by *S. rimosus*. Oxytetracycline (OTC) is a member of the "polyketide" class of secondary metabolites biosynthesized by condensation of coenzyme A derivatives of metabolic precursors. Polyketides are a large group of secondary metabolites of varied structure. They exhibit many physiological actions on organisms. Some of them are useful drugs for humans, including antibacterials (e.g. erythromycin, tetracycline), anticancer agents (e.g. daunomycin), antifungal agents (e.g. amphotericin), cholesterol-lowering agents (e.g. lovastatin), immunosuppressants (e.g. rapamycin) and veterinary products (e.g. the antiparasitic, avermectin; the feed additive, monensin), others can be allergenic, toxic, and in some cases carcinogenic. The backbone of oxytetracycline is consisted of 19 carbon atoms.[19] In comparison to other antibiotics, tetracyclines rank among the antimicrobial substances most frequently used in the animal food production.[20] Tetracyclines display a wide spectrum of antimicrobial action; apart from a stronger action on the gram-positive bacteria and a weaker one on the gram-negative ones, they exercise action also on mycoplasmas, chlamydiae, rickettsias, spirochetes, actinomycetes, and some protozoa.[21] The sum of tetracycline action is bacteriostatic. The main goal of the antibacterial action of tetracyclines is proteosynthesis inhibition. They bind to the bacterial 30S ribosomal subunit and prevent attachment of aminoacyl tRNA to the ribosomal receptor site.[22, 23]

1.6.5. Oxytetracycline:

Oxytetracycline (460.40 MW) is a natural tetracycline compound that is derived from the fungus, *Streptomyces rimosus*. Tetracycline compounds are broad-spectrum antibiotics that are bacteriostatic by inhibiting bacterial protein synthesis at the 30S ribosome. Tetracyclines are 4 ring amphoteric compounds with side chain substitutions made for multiple drug compounds and activity (Figure 6).

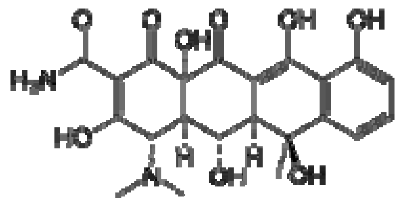


Figure 6: Structure of Oxytetracycline

Oxytetracycline was the second of the broad-spectrum tetracycline group of antibiotics to be discovered. Tetracyclines generally act as bacteriostatic antibiotics and inhibit protein synthesis by reversibly binding to 30S ribosomal subunits of susceptible organisms, thereby preventing binding to those ribosomes of aminoacyl transfer-RNA. Tetracyclines also are believed to reversibly bind to 50S ribosomes and additionally alter cytoplasmic membrane permeability in susceptible organisms. In high concentrations, tetracyclines can also inhibit protein synthesis by mammalian cells. Against gram positive bacteria, the tetracyclines have activity against some strains of *staphylococcus* and *streptococci*, but resistance of these organisms is increasing. Gram positive bacteria that are usually covered by tetracyclines, include *Actinomyces sp., Bacillus anthracis*, *Clostridium perfringens* and *tetani*, *Listeria monocytogenes,* and *Nocardia*. Among gram negative bacteria that tetracyclines usually have *in vitro* and *in vivo* activity against include *Bordetella sp., Brucella, Bartonella, Haemophilus sp., Pasturella multocida, Shigella,* and *Yersinia pestis*. Many or most strains of *E. coli, Klebsiella, Bacteroides, Enterobacter, Proteus* and *Pseudomonas aeruginosa* are resistant to the tetracyclines. Oxytetracycline works by interfering with the ability of bacteria to produce proteins that are essential to them. Without these proteins the bacteria cannot grow, multiply and increase in numbers. Oxytetracycline therefore stops the spread of the infection and the remaining bacteria are killed by the immune system or eventually die. Tetracyclines are highly lipophilic, which allows them to widely distribute in the body. In mammals, tetracyclines are typically well absorbed from the gastrointestinal tract of animals with systemic bioavailability of OTC ranging between 60 - 80%[24]; however, absorption rates are variable between species, drug formulations and chelation status. Protein binding affinity varies among the different tetracycline drugs but generally protein binding is moderate (20 - 40%). Bioavailability of OTC in fish, both fresh and saltwater fish, ranges from 0.6 - 80%.[25, 26, 27, 28, 29, 30, 31, 32] These drugs are renally excreted (60%) primarily unchanged: indicative of minimal metabolism/biotransformation. Renal excretion occurs primarily by glomerular filtration, but may occur by tubular secretion. [33, 34, 24] Oxytetracycline has a low risk of toxicosis and diffuses into most body fluids and tissues.[26]

Despite of many therapeutic qualities of OTC, the use of oxytetracycline in fish also has several disadvantages. One primary problem that has developed with the widespread use of this drug is bacterial resistance. Veterinarians should be aware that when treating diseased animals or populations, drug distribution and or elimination may be altered such that residues may persist past officially recommended withdrawal times.[35] Awareness should also be grown among those who are directly and indirectly involved with aquaculture industry. Drug residues in products entering the human food chain may lead to bacterial resistance and other potential consumer health threats, such as allergic reactions.[36, 37] In most farmed fish species, the primary edible portions are muscle and skin; so residue studies are typically limited to these tissues.[36] Oxytetracycline is commonly used in human medicine. Indiscriminate exposure through contaminated meat products or water could lead to increased OTC resistance in human and animal pathogens.[38] Through R-plasmid mediated bacterial resistance, many fish pathogens, such as *Aeromonas* sp., are now resistant to OTC therapy.[34, 37]

Bacterial resistance to OTC may occur by three mechanisms which are: 1) decreased intracellular OTC concentrations because of plasmid-borne transporters pumping drug out and decreased cellular permeability, 2) production of proteins that interfere with the binding to ribosomes and 3) enzyme inactivation.[39] Another concern is the low bioavailability of OTC in fish species.[34] One cause of the limited bioavailability of OTC in fish is its affinity to bind to plasma proteins. The binding of drug to plasma proteins influences the concentration of active drug in the plasma and its distribution in the tissues.[28]

However, OTC is one of only two FDA-approved chemotherapeutics available to fish farmers and the only FDA-approved route of therapeutic antibiotic drug exposure in fish is through medicated feeds.

Chemistry:

Among the most important broad-spectrum antibiotics are members of the tetracycline family. Nine such compounds – tetracycline, rolitetracycline, oxytetracycline, chlorotetracycline, demeclocyline, meclocycline, methacycline, doxycycline and minocycline

– have been introduced into medical use. The tetracyclines are obtained by fermentation procedures from *Streptomyces* spp. or by chemical transformation of the natural products. Their chemical identities have been established by degradation studies and confirmed by the synthesis of three members of the groups, Oxytetracycline, 6-demethyle-6-deoxytetracycline, and anhydrochlortetracycline in their a-forms. The antibiotic spectra and the chemical properties of these compounds are very similar but not identical. The stereochemistry of the tetracyclines are very complex. Carbon atoms 4, 4a, 5, 5a, 6 and 12a are potentially chiral depending on substitution. Oxytetracycline and doxytetracycline each with a 5-a-hydroxyl substitute have six asymmetric centers. [40]

Structure of Tetracyclines:

The tertacyclines are amphoteric compounds, forming salt with either acids or bases. In neutral solution, these substances exist mainly as zwitterions. The acid salt, which are formed through protonation of the enol group on C-2, exist as crystalline compounds that are very soluble in water. These amphoteric antibiotics will crystallize out in aqueous solution of their salts, however, unless stabilize in an excess of acid. The hydrochloride salts are most commonly for oral administration and usually are encapsulated because they are bitter. Water-soluble salts are obtained from bases, such as sodium or potassium hydroxides, but they are not stable in aqueous solutions. Water-insoluble salts are formed with divalent and polyvalent metals.

Stable chelate complexes are formed by the tetracyclines with many metals, including calcium, magnesium and iron. Such chelates are very insoluble in water causing impaired absorption of most tetracyclines.[40]

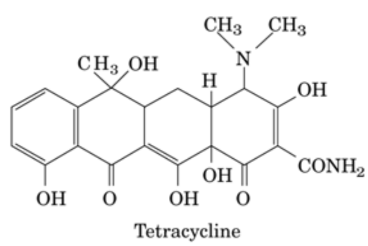


Figure 7: Structure of Tetracycline

Spectrum of Activity:

The tetracyclines have the broadest spectrum of activity of any known antibacterial agents. They are active against a wide-range of Gram-positive and Gram-negative bacteria, spirochetes, myoplasm, rickettsiae and chlamydiae. Their potential indications are, therefore, numerous. Their bacteriostatic action, however is a disadvantage in the treatment of life-threatening infections such as septicemia, endocarditis, and meningitis; ame aminoglycosides and/or cephalosporins is preferred for Gram-negative and Penicillins for Gram-positive infections. Because of incomplete absorption and their effectiveness against the natural bacterial flora of the intestine, tetracycline may induce superinfections caused by pathogenic yeast *Candida albicans*. Resistance to tetracycline among both Gram-positive and Gram-negative bacteria is relatively common. Parenteral tetracyclines may cause severe liver damage, especially when given in excessive dosage to pregnant women or to patients with impaired renal function. [40]

Mechanism of Action:

Tetracyclins are specific inhibitors of bacterial protein synthesis. They bind to 30S ribosomal sub-unit and there by prevent the binding of aminoacyl tRNA to the m-RNA and the binding of tetracyclines at the ribosomal binding site requires magnesium ions**.** Tetracyclines also bind to mammalian ribosomes but with lower affinities and they apparently do not achieves sufficient intracellular concentrations to interfere with protein synthesis. The selective toxicity to tetracycline toward bacteria depends strongly on self-destructive capacity of bacterial cells to concentrate these agents in the cell. Tetracycline enters bacterial cells by two processes: Passive diffusion and active transport. The active up-take of tetracycline by bacterial cells is an energy-dependent process and requires adenosine triphosphate (ATP) and magnesium ions. [41]

Mechanism of Resistance:

Tetracycline has been a widely used antibiotic because of its low toxicity and broad spectrum of activity. However, its clinical usefulness has been declining because of the appearance of an increasing number of tetracycline-resistant isolates of clinically important bacteria. Three biochemically distinct mechanisms of resistance to tetracyclines have been described in bacteria (a) efflux mediated by transmembrane spanning, active transport proteins that reduces the intracellular tetracycline concentrartion; Recently it was reported that at least one of the ribosome protection genes is regulated by attenuation. Tetracycline resistance genes are often found on transmissible elements. Efflux resistance genes are generally found on plasmids, whereas genes involved in ribosome protection have been found on both plasmids and self-transmissible chromosomal elements (conjugative transposons). One class of conjugative transposon, originally found in streptococci, can transfer itself from streptococci to a variety of recipients, including other gram-positive bacteria, gram-negative bacteria, and mycoplasmas. Another class of conjugative transposons has been found in the Bacteroides group. An unusual feature of the Bacteroides elements is that their transfer is enhanced by preexposure to tetracycline.[40] Thus, tetracycline has the double effect of selecting for recipients that acquire a resistance gene and stimulating transfer of the gene[42] (b) ribosomal protection, in which bacterial protein synthesis apparatus is rendered resistant to the action of tetracyclines by an inducible cytoplasmic protein; and (c) enzymetic oxidation be regulated by an attenuation mechanism.

Pharmacology of Oxytetracycline:

Broad-spectrum bacteriostatic activity of Oxytetracycline, made it useful for systemic therapy as well as locally for gastric or intestinal infections. Due to rapid development in science and technology, it is currently discovered that additional mechanism of Oxytetracycline includes antioxidant, anti-inflammatory and immunosuppressive activity of Oxytetracycline HCl. Presently, this antibiotic is considered as therapy of choice in papulopustulous acne, rosacea and perioral dermatitis and also for primary and secondary skin infection. Gastrointestinal irritative effects such as stomach upset, epigastric burning, nausea and vomiting made Oxytetracycline a less favorite antibiotic nowadays in human therapy. [43]

1.6.6. Antibiotic and its Growth promoting factor:-

Antibiotics act by eliminating the subclinical population of pathogenic microorganisms. Antibiotics alter the non-pathogenic intestinal flora, producing beneficial effects on digestive processes and more efficient utilization of nutrients in feeds. The feeding of antibiotics is associated with decreases in animal gut mass, increased intestinal absorption of nutrients and energy sparing. This causes reduction in the nutrient cost for maintenance, so that a larger portion of consumed nutrients can be used for growth and production, thereby improving the efficiency of nutrient use. Antibiotics may prevent irritation of the intestinal lining and may enhance the uptake of nutrients from the intestine by thinning of the mucosal layer. Intestinal bacteria inactivate pancreatic enzymes and metabolize dietary protein with the production of ammonia and biogenic amines. Antibiotics inhibit these activities and increase the digestibility of dietary protein.[44]

Oxytetracycline as animal growth promoter: Antibiotics represent one of the few classes of drugs that can be used in food animals both therapeutically to treat disease and subtherapeutically, usually over long periods, to improve their rate of growth and feed conversion efficiency. The practice of adding low concentrations of antibiotics, defined in the United States as ,< 200 g/ton of feed [44, 45], to animals to improve growth and feed efficiency is referred to as growth promotion or growth enhancement [46, 44, 45, 47, 48]**.** An obvious outcome of this practice is that animals need less food to reach marketable weight. The mechanisms responsible for growth promotion have not been fully understood yet but appear to include enhancement of vitamin production by gastrointestinal microorganisms, elimination of subclinical populations of pathogenic organisms, and increased intestinal absorption of nutrients[50].The growth-promoting properties of tetracyclines were discovered in 1949, when it was observed that low levels of chlortetracycline in livestock rations beneficially affected the rate of growth and feed utilization by young chickens[49]. In the United States these antibiotics were approved by Food and Drug Administration as feed additives in 1951 (chlortetracycline) and 1953 (Oxytetracycline). The sub-therapeutic application of tetracyclines and other antibiotics to farm animals might contribute to the development of resistant human isolates, caused ban of the use of tetracyclines for growth promotion in Europe in the early 1970s [50].

1.7. Worldwide Antibiotic Treatment Vs Bangladeshi Treatment

1.7.1. Use of Antibiotics in Fish Culture in the United States

To maximize production, fish culture facilities often maintain fish at high stocking densities and their aim remain to maximize growth by high feed intake. High stocking densities and the resulting poor water quality typically lead to compromised fish health, disease and mortality.

Infectious diseases are one of the most common causes of population and economic losses in commercial aquaculture.[51, 52] Therefore, chemotherapeutics are frequently involved in the treatment of bacterial diseases in cultured fishes.[53]

Currently in the United States, there are only two antibiotics available and approved by the Food and Drug Administration (FDA) for use in foodfish: oxytetracycline (OTC, Terramycin for Fishr) and Rometr. The FDA-approved dose of OTC in feed is 55 – 83 mg OTC/kg of body weight for 10 d. Recommended doses for injectable formulations of OTC are 25 – 50 mg OTC/ kg of body weight.[54] According to FDA the tolerance limit of 2 ppm OTC in the raw edible portions of fish. In addition, veterinarians should be aware that when treating diseased animals or populations, because later on the cowdung or the excretory compound is used as food for the fishes in aquaculture. Therefore, veterinarians should be aware of alteration of drug distribution and or elimination provided residues remaining are within FDA range [34]. Drug residues in products entering the human food chain may lead to bacterial resistance and other potential consumer health threats, such as allergic reactions [36, 55]. In most farmed fish species, the primary edible portions are muscle and skin; so residue studies are typically limited to these tissues [36]. According to author, Du *et al* common cooking procedures of OTC-treated channel catfish (*Ictalurus punctatus*) fillets did not completely degrade the drug. Oxytetracycline is commonly used in human medicine such that indiscriminate exposure through contaminated meat products or water could lead to increased OTC resistance in human and animal pathogens [38].

Total prevention of disease in aquacultural systems is likely to be unattainable in practice. Disease management, therefore, depends upon good culture practice in combination with chemotherapeutic agents. Some agents may be administered (often in feed) on a prophylactic basis, although in many countries, the US for example, this is forbidden. Parasiticides, anaesthetics, spawning hormones, oxidants, disinfectants and herbicides are all routinely used. In the case of antibiotics, the development of vaccines has led to a sharp reduction in use in many of the finfish cultures in Europe and North America.

In Europe, fish oral drugs, as well as external antibacterial and antiparasitic compounds, are considered as Veterinary Medicinal Products (VMP) and the Salmonidae are classed as major animal food producing species. The availability of antimicrobial agents for aquaculture use is affected by the setting of maximum residue limits (MRLs), which was adopted by a Regulation in 1990 (EC, 1990). However, the setting of an MRL is only a preliminary step towards achievement of full marketing authorisation, although the European Medicine Agency (EMEA) recently extrapolated the MRLs of twelve antibiotics to all food producing animal species. Nevertheless, the list of fully authorised licensed pharmaceuticals for aquaculture is still quite small (Table 1). In addition, although Europe has taken steps to harmonise the availability of antimicrobial agents there is still large variation between individual European countries. The use of antimicrobial agents in food animal species, including fish, is controlled by regulations, particularly in Europe and the USA. Strict safety and efficacy standards, including residue testing, are required before approval of any such agent. This rigorous approval process is very costly and time consuming though and the sales potential for the aquaculture market in global terms is limited, which in some cases has meant a certain lack of interest on behalf of pharmaceutical companies for developing new antimicrobials and registering them.[56]

A number of antibiotics have been banned, either because they have been reserved for use in humans or because of their toxic effects. In the case of the USA, the following drugs are specifically banned for use in raising animals [56]

(i) Chloramphenicol;
(ii) Clenbuterol;
(iii) Diethylstilbestrol (DES);
(iv) Dimetridazole;
(v) Ipronidazole;
(vi) Other nitroimidazoles;
(vii) Furazolidone, nitrofurazone, other nitrofurans;
(viii) Sulphonamide drugs in lactating dairy cattle (except approved use of sulphadimethoxine, sulphabromomethazine, and sulphaethoxypyridazine);
(ix) Fluoroquinolones; and
(x) Glycopeptides.

The release of large quantities of antibiotics into the environment due to animal production (including aquaculture) and human use has produced the phenomena of microbial resistance, although different bacteria can acquire resistance to antibiotics and the development of antibiotic resistance by pathogenic bacteria is considered to be one of the most serious risks to human health at the global level.[57]

1.7.2 Use of Antibiotics in Fish Culture in Bangladesh:

With the expansion of aquaculture in Bangladesh, there has been increasing trend in using chemicals in aquatic animal health management. Commonly used chemicals in Bangladesh aquaculture are lime, rotenone, various forms of inorganic and organic fertilizers, phostoxin, salt, dipterex, antimicrobials agents, potassium permanganate, copper sulphate, formalin, sumithion, melathion etc. Table 4 has 14 branded antibiotics used in Bangladeshi market by the farmers to treat fish diseases.

Unfortunately, little or no attention has been paid on the documentation of chemicals and antibiotics used in aquaculture industry in the country. As a result, there is a lack of information regarding the present status and consequences of chemicals and antibiotics using in aquaculture sector especially in aquatic animal health management and needs examination.[57]

1.8 Health Hazards of Antibacterial Agents:

In the case of food hazards, adverse health effects refer to human beings. The residues of veterinary drugs and chemicals utilized in aquaculture form part of the large group of so-called "chemical hazards". Hazards coming from the use of chemical and veterinary drugs in aquaculture are not the only hazards that could affect aquacultured fish. Perhaps hazards due to post-harvest handling (e.g. microbiological) continue, as in wild fish, to be the most important from the point of view of public health, in situations where a responsible use of chemicals and veterinary drugs and good aquaculture practices are implemented.[58] During the last decade, some environmental hazards, like the accumulation of polychlorinated biphenyls (PCBs) and dioxins in farmed fish, have gained the attention of food risk managers.[59] The probability of a hazard actually being present in fish and fish products from aquaculture, either at the time of slaughter or at the time the final product is consumed, depends, in turn, on a number of factors. For instance, the relevance of the hazard (risk) could vary for the same type of cultured fish due, for instance, on the one hand to the environment and the rearing system, and on the other hand to cooking and consumption patterns and the susceptibility of the consumer. The current tendency is towards a risk analysis approach, which means an approach where the risk assessment, risk management and risk communication are clearly separated and could play a role in the mitigation of the actual identified risk.[60] Residues of chemicals and veterinary drugs that could constitute a hazard to consumers of fish and fish products from aquaculture comprise a number of substances.

They are usually sub-divided into two groups:
(i) Antibacterial substances (including sulphonamides).
(ii) Veterinary drugs other than antibiotics.

These groups include substances that in some cases could have a maximum regulatory limit (MRL) –those approved for use– whereas others must be absent from fish and fish products. Nevertheless, at the same time, a drug could be accepted in one country and not in another.[60] Furthermore, the presence of chemical and drug residues in fish could be due not only to the intentional use of the specific chemical or drug, but as a result of an interaction between the cultured fish and the environment. It is well known that some drugs and chemicals biodegrade relatively soon, or do not form toxic products in the process of biodegradation, whereas others remain for a long time in the environment or produce toxic degradation products, which in turn could remain for a long time in the environment.[61, 62] The half-life for OTC in sediments under fish farms ranged between 9 and 419 d depending on temperature, oxygen and water currents [38], [63, 64, 65]. Contamination of sediment with OTC is likely associated with the accumulation of uneaten medicated feed particles and fish feces. Because OTC is poorly absorbed from the intestinal tracts of fish, fecal material deposited under pens or cages may contain high levels of the drug.[66, 67, 68, 69] Oxytetracycline medicated feed is not highly palatable to fish, decreased feed intake may result in more medicated feed remaining in the environment and also may lead to subtherapeutic levels in fish populations.[70] These instances of low level drug exposure increase the potential for bacterial resistance in the targeted pathogen and in other organisms in the animal and in the environment.

Human use (and misuse) of medicines (chemicals and antibiotics) seems to be mainly responsible for residues in aquatic environments.[71, 72] Antibacterial substances (antibiotics and sulphonamides) are utilized in aquaculture production with the purpose of prevention (prophylactic) and treatment (therapeutic use) of bacterial diseases.[73] Antibiotics have also been utilized as growth factors, even if this is not now considered to be a good practice. Antibiotics utilized in aquaculture are of the same type utilized to treat bacterial diseases in humans.

Hazards of antibacterial substances utilized in aquaculture production to public health could be classified as follows:

(i) Hazards due to the residues of approved antibiotics in fish and fish products.

(ii) Hazards due to the residues of unapproved or banned antibiotics.

(iii) Hazards due to the development of resistance to antibiotics in microbial pathogens in the environment due to the use of antibiotics (approved or not).[74]

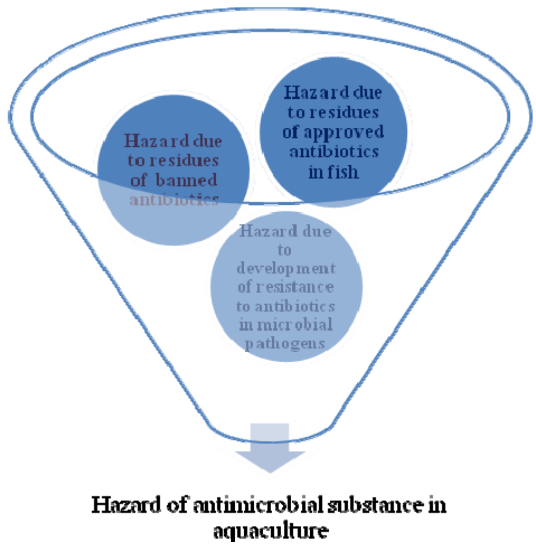


Figure 8: Funnel chart shows how various hazards together merge to bring negative impact on the aquaculture

When specific pathogenic bacteria have developed resistance against a specific antibiotic, microbial illnesses that could be treated are no longer treatable with that antibiotic. Many pathogenic bacteria already show multiple resistances, which means resistance to several antibiotics. Attention in this case focuses on pathogenic bacteria that are clearly already relevant hazards according to epidemiological records[75] and stresses the need for continued surveillance of food-borne zoonotic bacterial pathogens from imported foods entering the United States".

Bacteria from the aquaculture ecosystem may also be transmitted directly to humans through handling of fish. Recently, the fish pathogen *Streptococcus iniae* has caused invasive infections in persons who handled store-bought aquacultured tilapia; *S.iniae* was isolated from the aquaculture ecosystem and on fish in grocery stores.[76] Similarly, a new biotype of *Vibrio vulnifcus* caused hundreds of serious infections among persons handling live tilapia produced by aquaculture in Israe.[77] Bacteria on fish may also be transmitted to humans when the aquacultured products are eaten, or when other foods are eaten that have been cross-contaminated by bacteria from fish. For example, *Vibrio parahaemolyticus* is a common food-borne disease in Japan, where infections have been linked to the consumption of aquacultured finfish.[78] Antibiotic-resistant bacteria have been isolated from the carcasses of catfish from the retail market.[79] These bacteria can be transferred during food preparation at home or by handling in the market.

1.9 Risk Management:

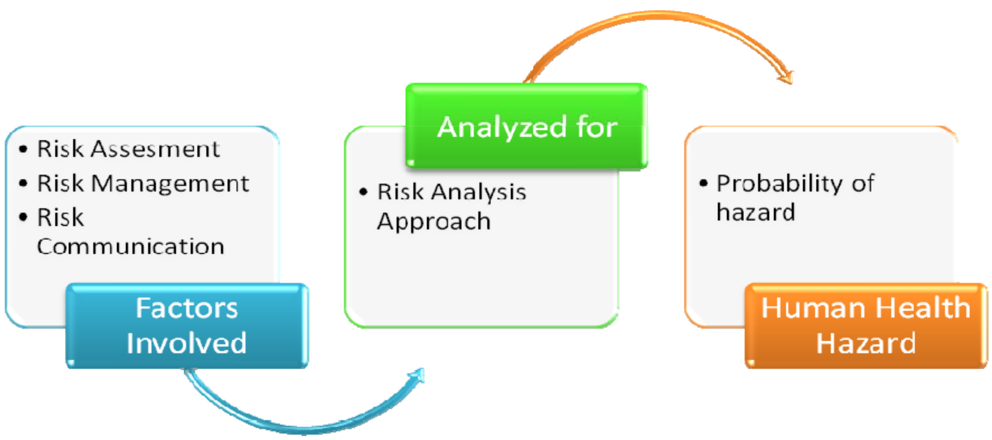


Figure 9: Flow chart shows risk approach analysis can measure possible human health hazard along with three factors which are important for risk approach analysis.

Figure 9 shows how risk assessment, risk management and risk communication all together needed for risk analysis approach. This risk analysis approach is needed to measure probability of human health hazard. By measuring the intensity of human health hazard it is possible to make a strategy toward risk management options. Below given several risk management options which can be practiced by the farmers of Bangladesh, so that they are not completely depending on the antibiotic therapy to treat their fishes.

Hygienic procedures

It is important to note that good aquaculture management practices are essential to maintain a healthy environment for farmed fish. Priority should be given to good hygiene and other preventive efforts in containment of resistant infections. The most common diseases in aquaculture are infectious diseases, with various causative organisms. The use of antibiotics as prophylactic agents could be reduced, stressing hygiene measures, with proper handling practices. These practices will help prevent infectious diseases, including those of viral origin, which cannot be treated with antibiotics established a series of hygiene measures available for aquaculture farmers. These measures are considered below. [80]

Disinfecting of fish eggs with iodine

In the case of eggs that have to be transported, the packaging has to be disinfected as well, or destroyed in a manner that will not pose a contamination or health risk to water or other living organisms at the end destination. Disinfecting eggs with iodine can be carried out for various fish species, but it is most commonly used for Salmonidae. For other species, preliminary tests should be conducted to determine safer concentrations.

Disinfecting fish farms

The choice of substances and disinfecting procedures depends upon size, type and nature of the materials and sites to be disinfected. Chlorine and iodine can be used according to the procedures mentioned by FDA.

Removal of aquaculture therapeutants by carbon adsorption

Activated carbon filtration has been used extensively for water treatment after ozone or chlorine treatment, and for organic color removal. Organic chemical therapeutants, including antibiotics, can be effectively removed from the water column by adsorption onto activated carbon[81]. Because therapeutants could be in competition with other aquaculture components for the available adsorption sites on the carbon, some attempt have been made to design appropriate carbon filters.[82]

Alternative therapy:

Low immune system and responses of fish may result in very high mortalities due to specific pathogens that antibiotics are helpless against. For example, White Spot Syndrome Virus (WSSV) is one of the most devastating viruses in the shrimp industry. Different medicinal plants and herbs and/ or combinations of them known to have properties such as anti-bacteria, anti-fungal, physiological systems (immune system, digestive system,) supporting, hormonal balancing and many other properties. Strategies for prophylaxis and control of various virus include improvement of environmental conditions, stocking of specific pathogen free (SPF) post-larvae and enhancement of disease resistance by using immuno-stimulants. Immuno stimulants are substances, which enhance the non-specific defense mechanism and provide resistance against pathogenic organisms. Many herbs and plants have been used as home remedies in many cultures around the world for both human and animals. Some of these remedies have potent anti-viral as well as anti-bacterial and anti-fungal properties. These natural plant products have been reported to have various properties such as anti-stress, growth promoters, appetizers, tonic and immuno-stimulants. Moreover, these substances also possess other valuable properties; they are nontoxic, biodegradable and biocompatible. No herbal-resistance immunity has been found by any pathogen to date. Herbals can be used not only as remedies but even more so, as growth promoters, stress resistance boosters and preventatives of infections. Therefore, the use of herbal extract as feed additives can significantly benefit any organism cultured. The development of drug-resistant pathogens has been reported from all areas of aquaculture. Treating microbial infections in fish and crustaceans involves dissolving high quantities of broad-spectrum chemotherapeutic agents in the culture medium or supplying it in the food. Most of these antibiotics and drugs are now banned for use in the EU, USA and many other countries. Natural plant products present a viable alternative to antibiotics and other banned drugs being safer for the reared organism and humans, as well as, the environment.[83]

1.10 High Performance Liquid Chromatography (HPLC) used to detect the Residue of Antibacterial Agents:

Figure 10: Shimadzu HPLC used in East West University

Analytical method suited for this experiment is high-performance liquid chromatography (HPLC). This a reliable analytical method required for monitoring residues of any compound in the aquatic environment. HPLC is an efficient method for determination of trace of tetracycline antibiotics.

HPLC developed in late 1960s early 1970s. The technique is also known by other synonyms such as *high speed chromatography*, *high resolution chromatography* and *high efficiency chromatography* and is considered as the most sensitive method with continuous major developments. Today it is the most versatile and widely used technique for the separation, qualitative identification and quantitative determination of species in a variety of organic, inorganic, biological and other complex materials. Modern HPLC has emerged from the confluence of need, the human desire to minimize work, technological capability and the theory to guide development along rational lines. In some cases, HPLC may detect nanogram or even picogram quantities. HPLC separations are based on specific interactions between sample molecules with both the stationary and mobile phases. It is a type of elution chromatography where the sample, a mixture of solutes, is in a liquid solvent or mobile phase. HPLC is accomplished by injection of a small volume of liquid sample into a moving stream of liquid that pass through a column packed with particles of stationary phase. Separation of mixtures into its components depends on different degrees of retention. The extent to which a component is retained in a column is determined by its partitioning between the liquid mobile phase and stationary phase. Retention time is the time between injection and detection. Since the compound has different motilities', the exit the column at different time i.e. they have different retention time, t_R. HPLC apparatus is consist of a mobile phase reservoir, which is just a clean solvent jug, a solvent delivery system consisting of a pump for delivering precise and constant amount of mobile phase, a simple inlet, a column, the detectors with associate electronics and an interface to the outside. The pump which is used to deliver the mobile phase often ranging from 500-5000 psi.

2. Literature Review:

Whereas it is true that pathogen resistance to antibiotics is due to different sources (human and pet use, and animal production other than fish), and that caught fish can harbour resistant bacteria as well as aquaculture fish, a number of authors, as already mentioned, have already reported a correlation between findings of increased bacterial resistance levels on and around fish farms and the antimicrobial agents used at the farms. Consequently, as is happening, for instance, with the use of antibiotics in human medicine, a full strategy to achieve responsible use of antibiotics in aquaculture seems necessary.

Author Hossain, M.G. mentioned Major groups like Tilapia, Catfish, Climbing Perch and Shrimp are being cultured in suitable fresh and brackish waterbodies. Because of the high profits in scientific aquaculture, the number of ponds and the rate of adoption of technology-based aquaculture are both increasing. However, the reliable growth of this sub-sector is constrained by the following limitations: i) basic inputs like fish eggs, fish feeds and other on-farm inputs are not available to most pond owners. More specifically, non-availability of quality seeds is the single largest limitation in expansion of aquaculture; ii) poor quality fish stocks due to inbreeding depression at private hatcheries which results in poor fish growth; and, iii) disease epidemics and loss of fish crop due to water quality deterioration resulting from inappropriate management.[84] According to author M.A.R. Faruk et. al, chemicals and antibiotics are important components in health management of aquatic animals, pond construction, soil and water management, improve aquatic productivity, transportation of live fish, feed formulation, manipulation of reproduction, growth promotion and processing and value addition of the final product . A variety of other chemicals are also used in aquaculture for health management of fish apart from antibiotics. Some common chemicals include sodium chloride, formalin, malachite green, methylene blue, potassium permanganate, hydrogen per oxide, copper compounds, glutaraldehyde and trifluralin.[17]

According to author Cabello, a disadvantage of aquaculture is the increasing risk of fish disease outbreaks. An increased infection pressure results from: the high density at which fish are kept; accumulation of left-over fish feed and faeces at the bottom of basins. This sediment layer provides ideal circumstances for the growth and reproduction of bacteria; decreased genetic variation as a result of selective breeding of fish; stress as a result of the manipulations that the fish undergo when they are being raised in captivity. This stress leads to a decreased effectiveness of the fishes' immune system to clear up bacterial colonization and infection.[85] Therefore, author Serrano mentioned, in order to compensate for these fish health issues, antibiotic agents are commonly used, either as an additional compound in fish feed, directly added to the waters, or occasionally by injections. Antibiotics are being used: on a therapeutic basis: to fight bacterial infections in diseased fish; on a prophylactic basis: to reduce the number of potentially pathogenic bacteria in the environment and in the fish's gastro-intestinal tract, in order to prevent disease outbreaks; as a growth promoter: the feeding of antibiotics is associated with decreases in animal gut mass, increased intestinal absorption of nutrients and energy sparing. Depletion of naturally occurring intestinal bacteria leads to more efficient uptake from nutrients in consumed food. According to the author, the classic concept antibiotic concerns compounds of natural or synthetic origin, that have the capacity to inhibit the growth or multiplication of, or kill, bacteria or other microorganisms. Antibiotics are relatively small organic molecules that are able to penetrate bacterial cells and block the replication process via various pathways, for instance by interfering with the processes of DNA replication, synthesis of the cell wall or synthesis of relevant proteins.[60]

Author Ibrahim, A.B et. al mentioned, the uses of pesticides and antibiotics in fish farming are common practices to avoid the overgrowth of herbal plants and fish diseases beside promoting the fast growth of the fish. Generally chemical residues used at farm level can be accumulated in fish and could cause chronic health effects to consumers. They have the potential to gradually accumulate in the body and cause certain organ or system malfunction. There have been many studies about the effect of chemical residues in fish on consumers. Among the health problems caused by chemical residues are cancer, nerve problems and immunological problems.[86] Author M.A.R. Faruk et. al have also indentified some problems associated with the use of chemicals and antibiotics which included i) lack of technical knowledge of fish farmers on fish health management ii) lack of knowledge about use of chemicals iii) unavailability of appropriates therapeutic iv) lack of knowledge of application of chemicals and antibiotics v)indiscriminate use of chemicals vi) pressure on farmers from pharmaceutical companies and pesticide sellers vii) poor quality chemicals viii) lack of assistance from GO and NGOs ix) lack of awareness about the safety issues in using hazardous chemicals x) lack of information on the label of chemical about possible hazard xi) lack of knowledge about residual effect and expiry date xii) lack of diagnostic facilities for proper disease diagnosis and xiii) lack of trained manpower to prescribe fish medicine.[17]

According to author Choo, P.S. Oxytetracycline is widely used antibiotic in aqauculture and is also added to prawn and fishes as a growth promoter. In the USA, oxytetracycline is one of the two antibiotic registered by the US Food and Drug Administration for the treatment of bacterial disease in cultured catfish. In many countries, especially those is the tropics there is no legislation governing use of antibiotics for aquaculture, although in the more developed countries legislation to control their use is available. With expention of aquaculture worldwide, there is increasing concern about antibiotic abuse.[87]

Author Shaikh, B. and Moats, W.A. mentioned significant progress on development of HPLC methods for determination of the tetracycline group of antibiotics in food substrates including honey, egg, milk, tissues.[88] Author Navratilova, P et.al developed a high-performance liquid chromatography (HPLC) method was developed for determination of Oxytetracycline. They developed HPLC method, using ODS-C18 column at UV detector (365 nm). The measurement took place in isocratic mode. Mobile phase (flow rate of 0.700ml/min) was a mixture of acetonitrile, methanol and 0.05mol/l of oxalic acid in the ratio of 13:13:74. The sample injection volume was 20 µl and the column temperature was 30^{o}C. Detection limit was 50µg/L and 100µg/L in standard solution.[89]

3 Method and Materials

3.1. Chemicals, Equipment & Conditions:

(a). Reagents:

HPLC grade water

HPLC grade Methanol

HPLC grade Acetonitrile

GR grade Ethyl Acetate

Hydrochloric acid solution 6M

Sodium Hydroxide

Oxalic acid

(b). Equipments:

Test Tubes of 20ml

Volumetric Flasks of 50ml and 250ml

Pipette of 5ml and 10ml

Conical flask 250ml and 500ml

Measuring cylinder of 50ml, 100ml

Beakers of 250ml, 500ml and 1000ml

Round Bottom Flasks of 250 ml

Blender

Water Bath Machine (Model YCM-010, Gemmyco)

Shimadzu HPLC

Ultrasonic bath (Power Sonic 520)

Degassing pump

(c). HPLC Conditions:

Mobile Phase: Methanol: Acetonitrile: Oxalic Acid (13:13:74)

Flow Rate: 0.700 mL/min

Column Temperature: 30^{o} C

Injection Volume: 20 uL

Wavelength : 365nm

Run time: 15min

Column Type: ODS-2 C18

3.2.1. Preparation of Stock Solution:

To prepare the stock solution, 6mg of Oxytetracycline was dissolved in 3ml of HPLC grade methanol to make the concentration of 2mg/ml of Oxytetracycline.

3.2.2. Sample Collection:

15 samples of fish (5 Tilapia, 5 Pangas and 5 Rui fish) were randomly purchased from the local-markets situated in and around Dhaka city. The areas which are being focused for this experiment were Dhanmondi, Mohammadpur, Shukrabad, Banani and Gazipur. Tilapia fishes collected were within the range of weight 300-375g; Pangas and Rui fishes collected were of weight 500-600g.

3.2.2. Sample Preparation:

Head and the intestinal parts of the fish were excluded and only the muscle of the fish was collected. It was done by cutting the fish into fillet at first and then peeling of the skin from the fish. Then the muscles were homogenized in a blender and kept at -80^{o} C for 24 hours.

3.2.3. Extraction of Fish:

From the homogenized fish muscle accurately 2g was measured and dipped into hydrochloric acid (6mol/ml) of 5 ml for 20 minutes for pre-digestion purpose. During this time, it had been observed that the texture of the muscle becomes rubbery and whitish in color. Then it was transferred in a round bottom flask and heated in a water-bath for 30 minutes at 99.9^{o}C.

Initially the muscle of the fish appeared minced whitish and gradually the sample became partially dissolved and finally it appeared completely dissolved and cloudy in color.

After 30 minutes, sample was removed from the water-bath heat and then adjusted the pH 8-9 with sodium hydroxide. Then the sample volume were measured and transferred into a test tube and equal volume of ethyl acetate was added into the sample and vigorously shaken for 10 minutes. Then the sample was allowed to settle down until two clear layers form. With the help of a pipette of only the upper clear layer was collected carefully and then evaporated the ethyl acetate with an air pump until dry final compound is obtained.

Finally the volume of the collected sample was made up to 50 ml with methanol. This fish extract was stored in a refrigerator and was used within 5days to obtain an accurate result. If the sample extraction were kept for two to three weeks or even for a longer period of time, then HPLC result will not be accurate.

3.2.4. Calculations:

After running the standard solution of several concentration of oxytetracycline calibration curve was obtained. From the calibration curve it was found that the absorbance of the oxytetracycline has a linear relationship with various concentraion and had the following equation:

$y = ax + b$

where,

y = absorbance

x = concentration of oxytetracycline in mg/ml

b = 0 at y-intersect (the absorbance value when concentration of oxytetracycline is 0)

Thus, using the above equation it was possible to calculate the unknown concentrations of oxytetracycline present in the fish samples.

Let us consider the sample *Labeo rohita* of Mohammadpur Townhall market. As the peak area of this particular sample is 5554, therefore the concentration of oxytetracycline derived from that peak area is 5554/2138357 = 0.00259 mg/ml.

As the volume injection was 20 uL, which is equal to 0.02ml.

0.02ml of sample fish contains 0.00259mg of Oxytetracycline

Therefore, 50ml of sample fish contains (.00259*50)/0.02 of Oxytetracycline

= 6.475mg of Oxytetracycline

The total weight of the fish was 560g and only 2g from 27g of fish muscle for the extraction, so we can consider:

2g of fish sample has 6.475mg of Oxytetracycline

27g of fish muscle has (6.475*27)/2 mg of Oxytetracycline

= 87.4 mg of Oxytetracycline

Therefore, 560g fish contains (87.4*560)/27 mg of Oxytetracycline

= 1,812.7 mg of Oxytetracycline.

According to FAO, WHO and EC, the unit for maximum residual unit is mg/kg

As, 560g of fish sample contains 1,812.7mg of Oxytetracycline

Therefore, 1000g of fish may contains (1,812.7*1,000)/560 mg of Oxytetracycline

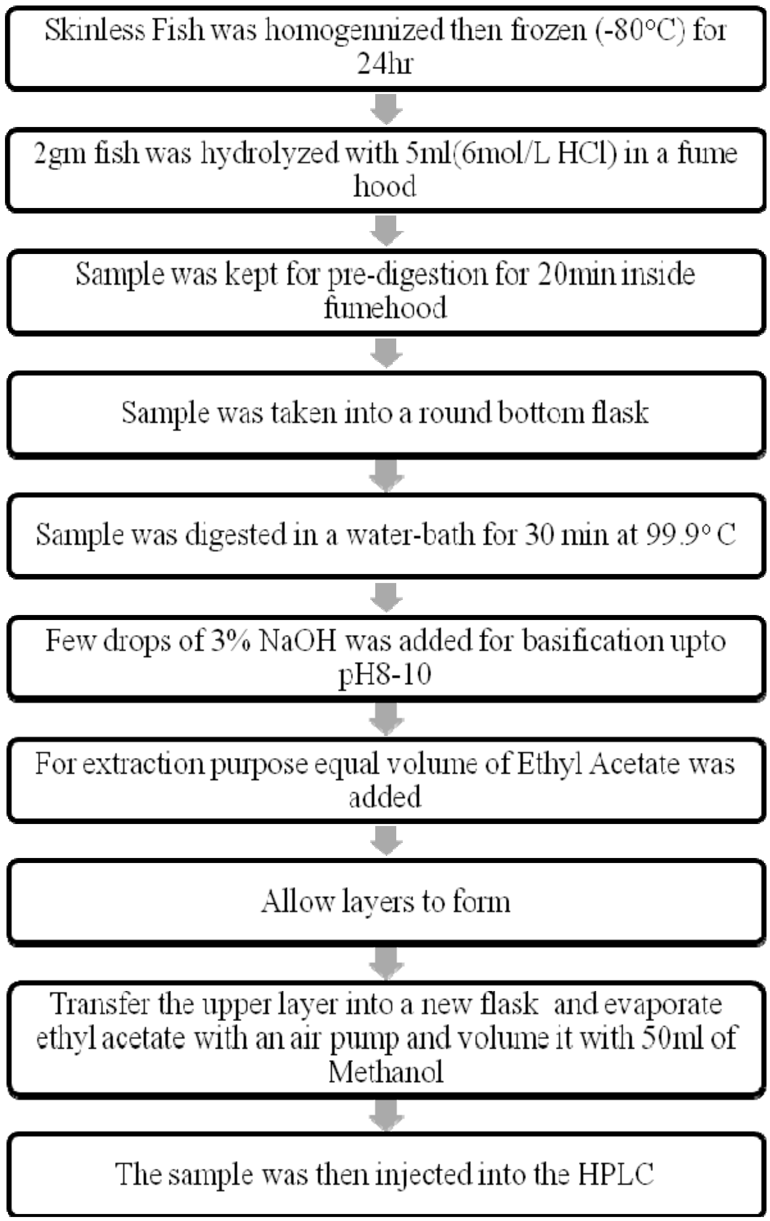
= 3,237 mg of Oxytetracycline.

Hence we can assume, residual of oxytetracycline in specific *Labeo rohita* of Mohammadpur area is 3237mg/kg, which is far too much in amount than the range being fixed by FDA i.e. 55-83mg/kg.

Similarly we can calculate the residual of oxytetracycline found in other 14 samples and compare the values with the range fixed by the regulatory bodies.

4. Result and Discussion:

4.1. Summary for the process of Fish Sample Extraction:



Below given the pictures which show how the fish sample is processed before and during the fish extraction:

Figure 11(a): One whole Tilapia fish

Figure 11(b): Fillet and only muscle of Tilapia fish

Figure 11(c): One piece of Pangas fish

Figure 11(d): One piece of Rui fish

Figure 11(e): One piece of Pangas fish muscle

Figure 11(f): Fish muscles are being homogenized without skin (in the same way skin of Rui fish has also been peeled off, which is not shown here)

Figure 12(a) 2g of fish paste

Figure 12(b)Fish hydrolyzed in HCl for 20 min

Figure 12(c) Sample digested in water bath for 30 min

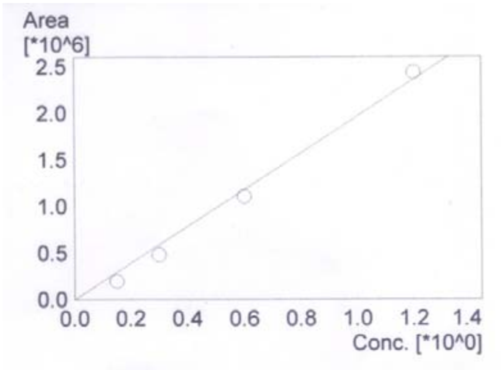
Figure 12(d): Whitish minced sample in conc^{n} HCl At the beginning of water-bath process .

Figure 12(e): At the middle of water-bath process sample appears to be partially dissolved

Figure 12(f): Dissolved cloudy colored sample

Figure 11(g) Evaporation of ethyl acetate with an air pump

4.2. Construction of Calibration Curve:



#	Conc (Ratio)	Area	Area
2	1.200	2429464.6	2429465
3	0.600	1108406.6	1108407
4	0.300	478609.0	478609
5	0.150	197488.9	197489

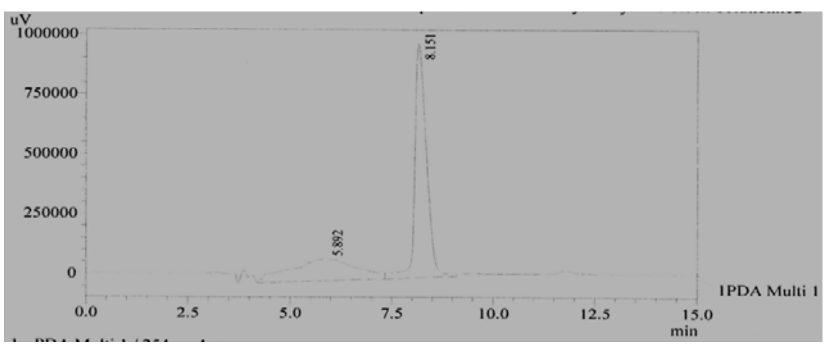
$$y = 1.96267e{+}006x - 0$$
$$R^2 = 0.999$$

Figure 13: Calibration Curve for Oxytetracycline

Concentration	Area
0	0
0.15	197489
0.3	478609
0.6	1108407
1.2	2429465

Table 5: Concentrations and respective absorbance of Oxytetracycline

4.3. Result with the stock solution of Oxytetracycline:

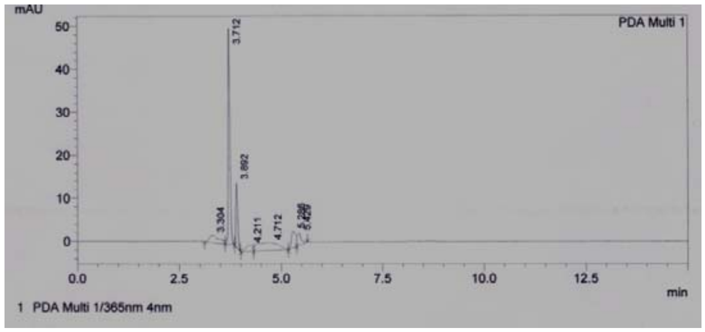


PDA Multi 1/365nm 4nm

Name	Ret.Time	Area	Height
Oxytetracycline	8.151	19774993	1067118

4.3.1. Result with *Labeo rohita* fish of five different markets:

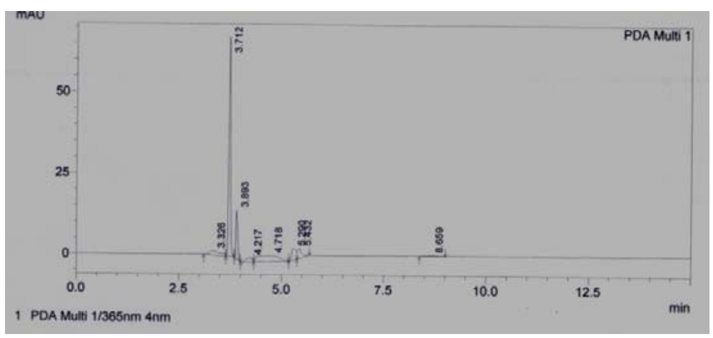
Dhanmondi (Meena Bazar):



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	Not Found	Not Found	Not Found

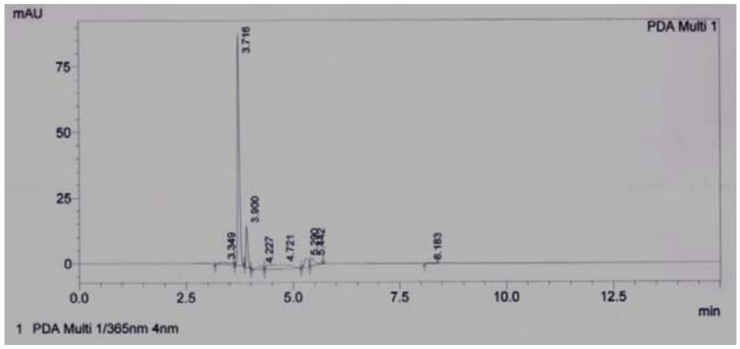
Mohammadpur (Townhall Market):



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.659	5554	276

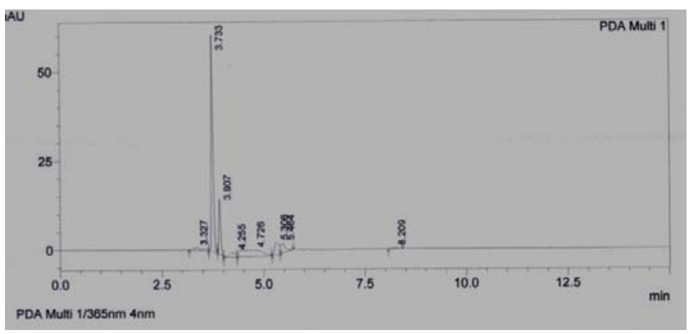
Shukrabad Kacha Bazar:



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.183	1802	240

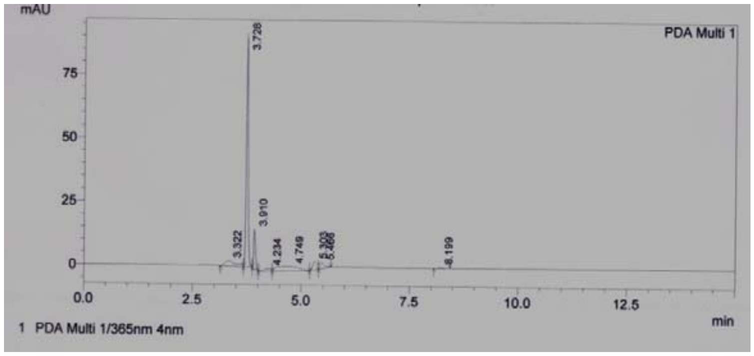
Banani Kacha Bazar:



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.209	1593	211

Gazipur Kacha Bazar:

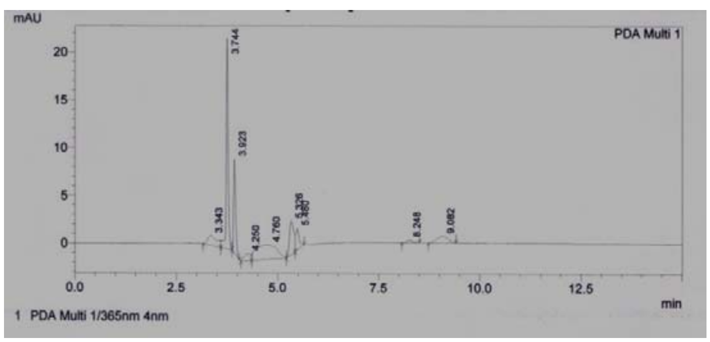


Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.199	2203	279

4.3.2. Result with *Pangasius pangasius* fish of five different markets:

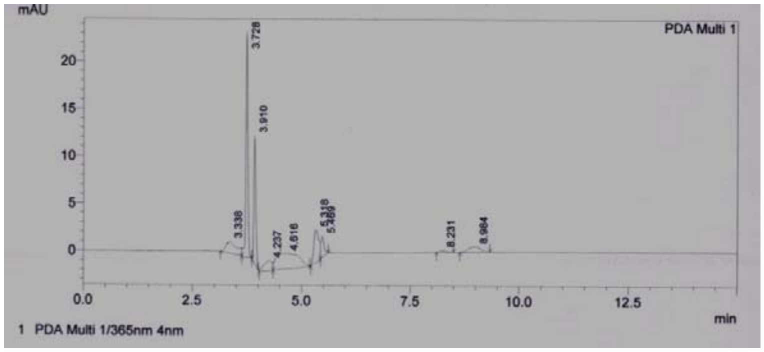
Dhanmondi (Meena Bazar):



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.248	2587	263

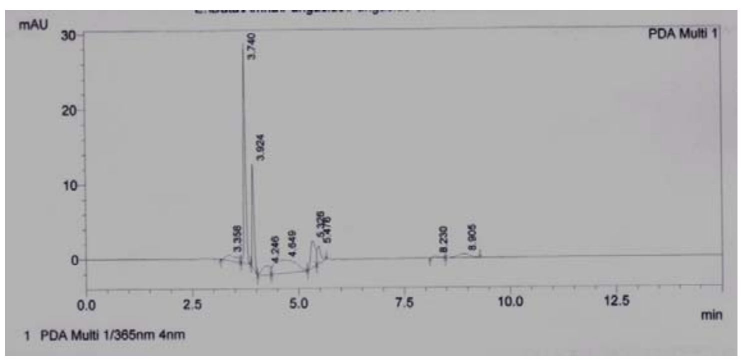
Mohammadpur (Townhall Market):



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.231	1830	212

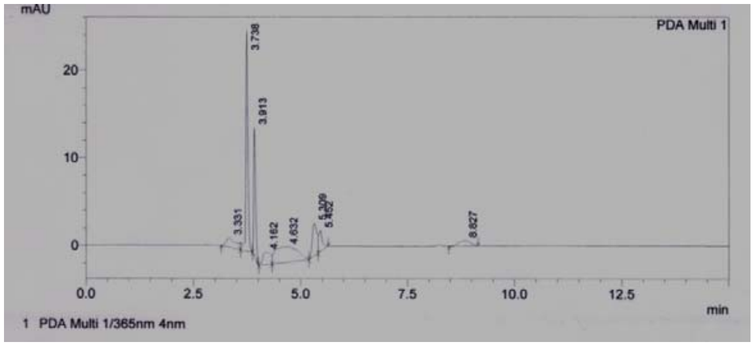
Shukrabad Kacha Bazar:



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.230	2019	251

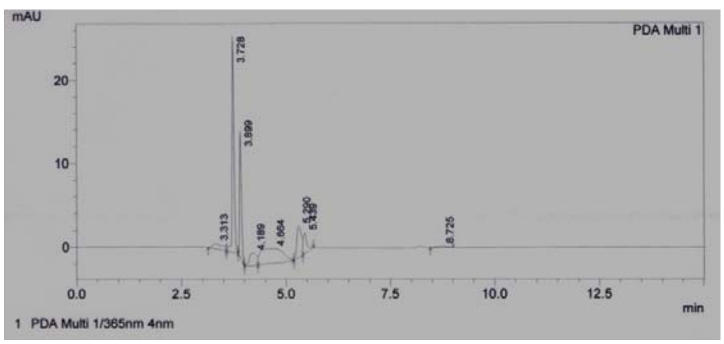
Banani Kacha Bazar:



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.827	10721	589

Gazipur Kacha Bazar:

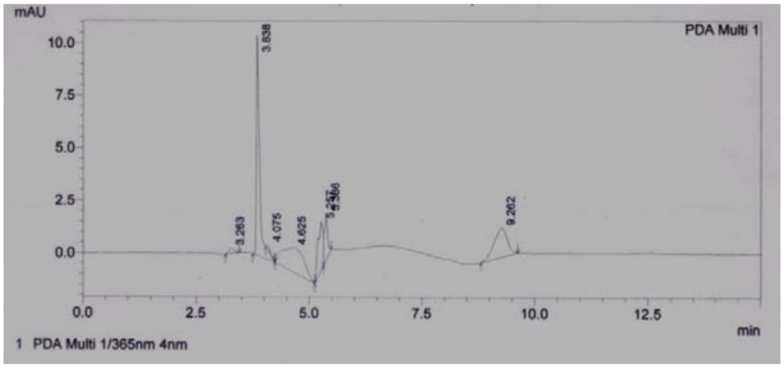


Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.725	2873	165

4.3.3. Result with *Oreochromis niloticus* fish of five different markets:

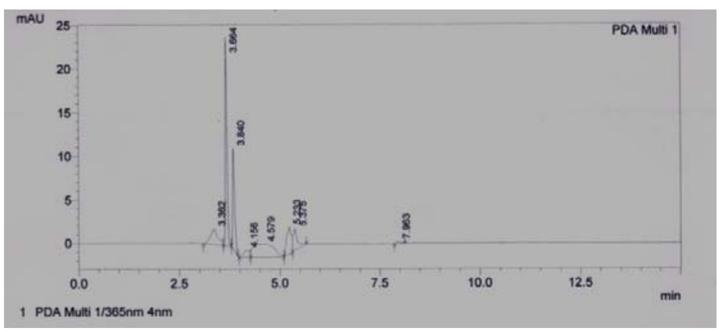
Dhanmondi (Meena Bazar):



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	Not Found	Not Found	Not Found

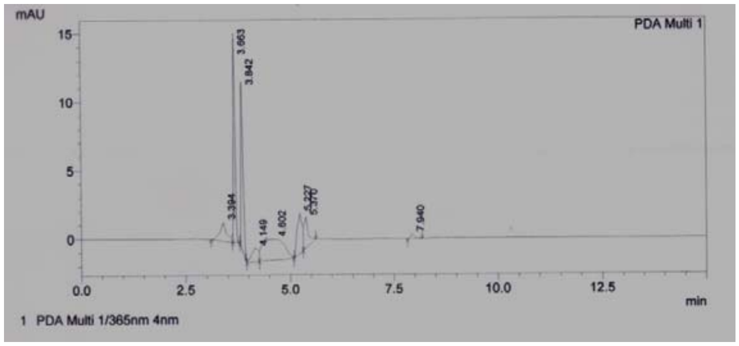
Mohammadpur (Townhall Market) :



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	Not Found	Not Found	Not Found

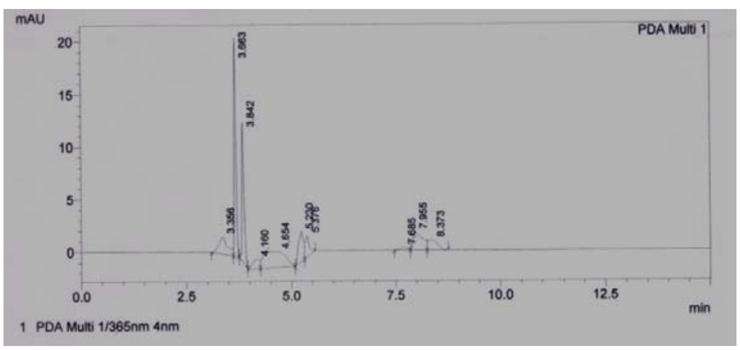
Shukrabad Kacha Bazar:



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	Not Found	Not Found	Not Found

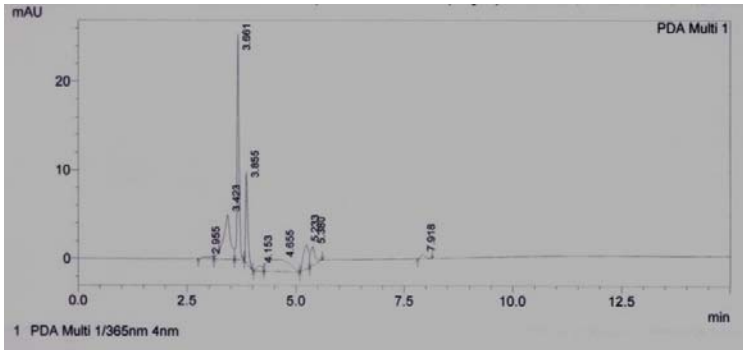
Banani Kacha Bazar:



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.373	15590	970

Gazipur Kacha Bazar:

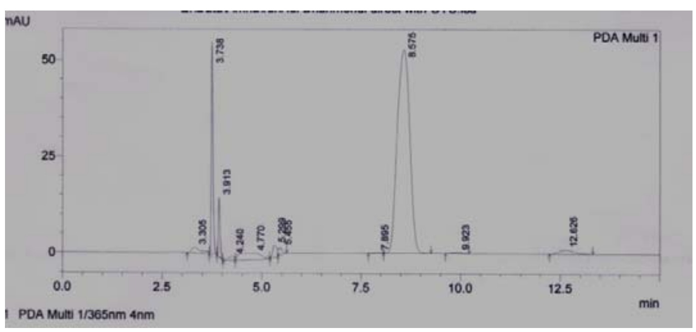


Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	Not Found	Not Found	Not Found

4.3.4. Result with spiked sample of *Labeo rohita* fish of five different markets:

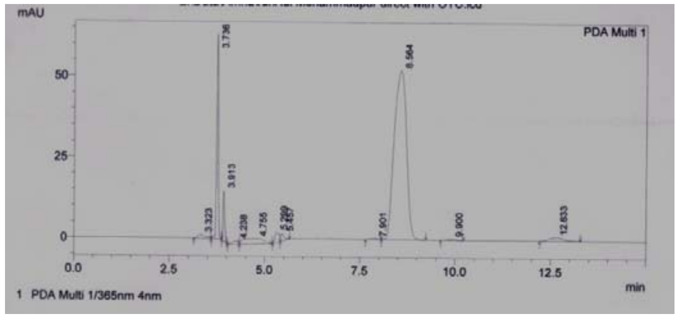
Dhanmondi (Meena Bazar):



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.575	1155937	52874

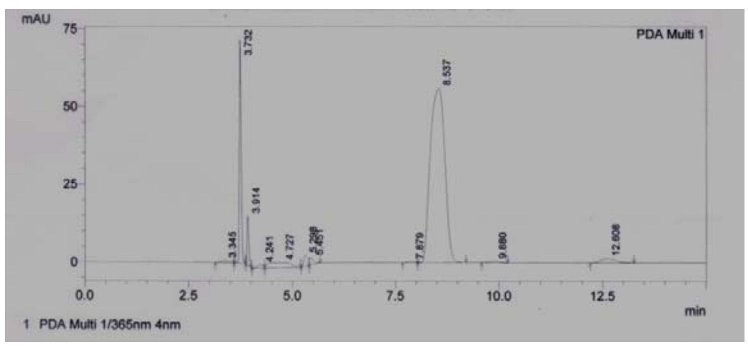
Mohammadpur (Townhall Market):



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.564	1177647	52163

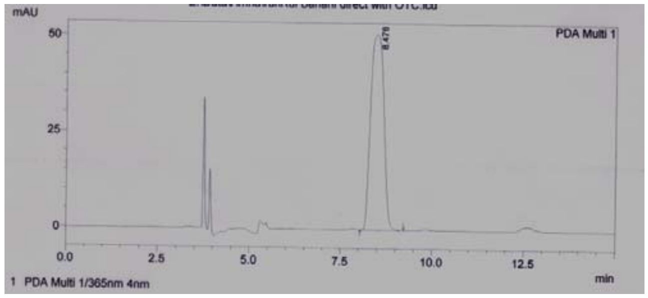
Shukrabad Kacha Bazar:



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.537	1363177	55868

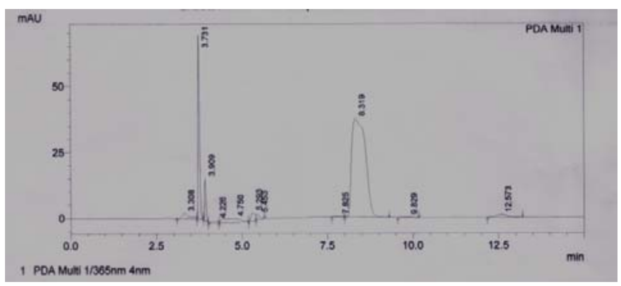
Banani Kacha Bazar:



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.476	1276517	50582

Gazipur Kacha Bazar:

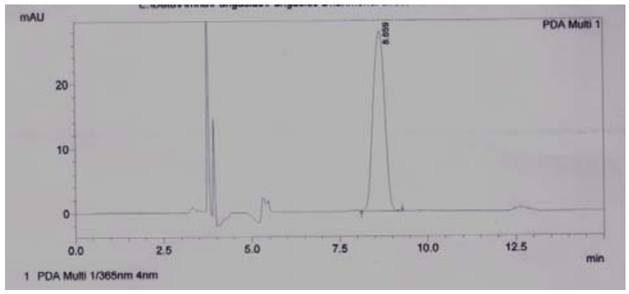


Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.319	15269	38672

4.3.5. Result with spiked sample of *Pangasius pangasius* fish of five different markets:

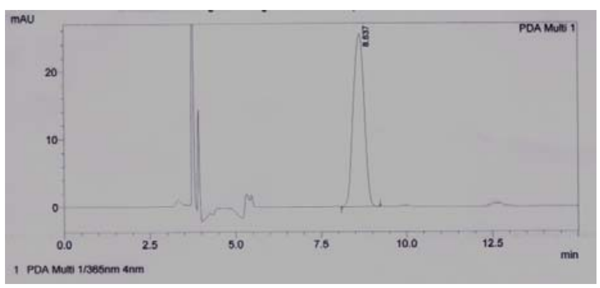
Dhanmondi (Meena Bazar):



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.659	639880	27850

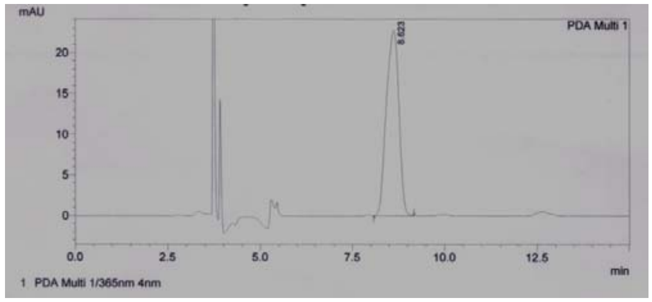
Mohammadpur (Townhall Market):



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.637	568877	25478

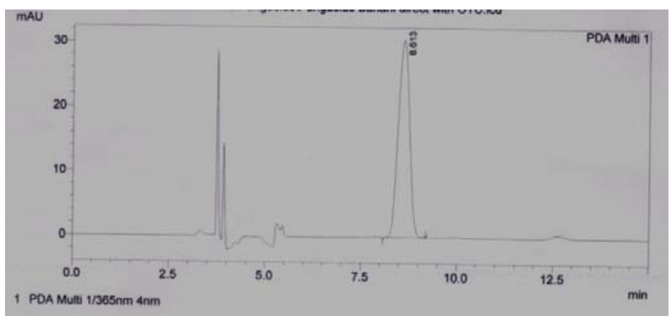
Shukrabad Kacha Bazar:



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.623	536736	22717

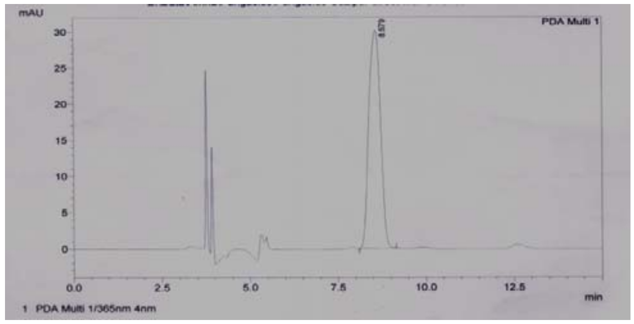
Banani Kacha Bazar:



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.613	620235	30524

Gazipur Kacha Bazar:

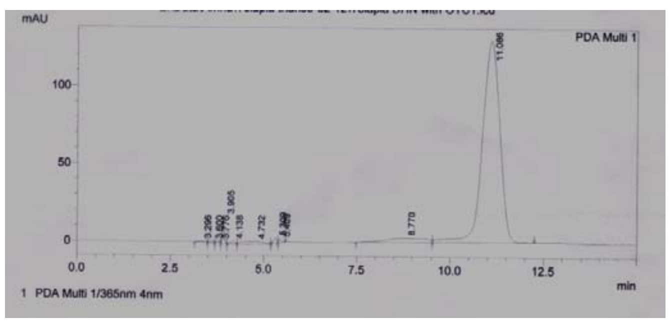


Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.579	698346	30163

4.3.6. Result with spiked sample of *Oreochromis niloticus* fish of five different markets:

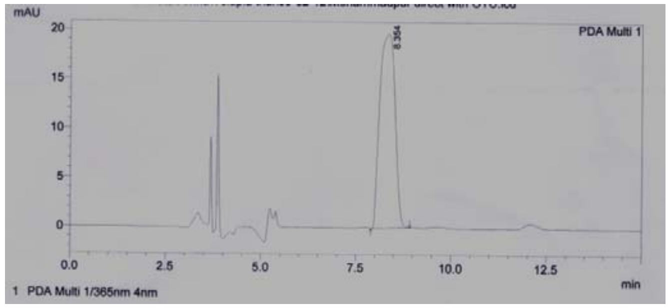
Dhanmondi (Meena Bazar):



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.770	199115	2686

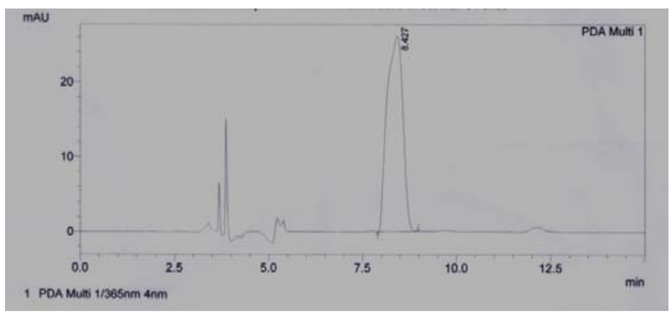
Mohammadpur (Townhall Market):



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.354	553929	19631

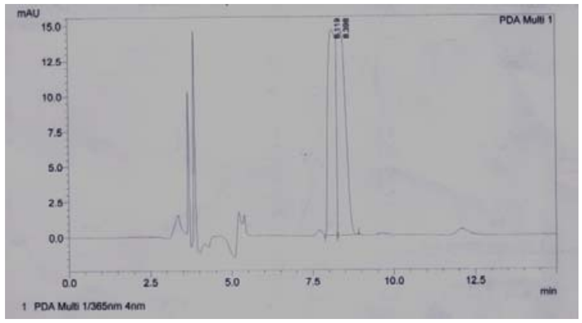
Shukrabad Kacha Bazar:



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.427	772255	26095

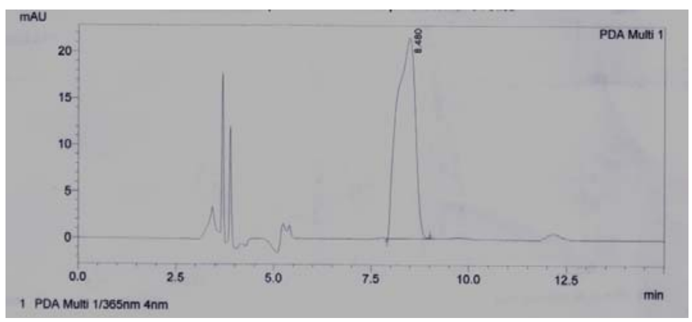
Banani Kacha Bazar:



Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.258	256470	14587

Gazipur Kacha Bazar:



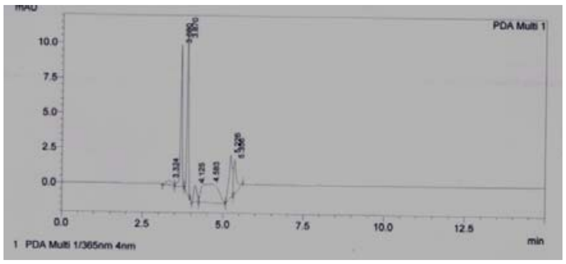
Quantitative Result

Name	Ret.Time	Area	Height
Oxytetracycline	8.480	698779	21484

Spiked sample was prepared by adding 2-3drops of Oxytetracycline intentionally in the sample which had been prepared from the fish extract and then ran through HPLC. Purpose of spiked sample is to become more assured about the peak of Oxytetracycline. There can be many other compounds in fish sample which also give peak. Therefore, due to the intentional addition of Oxytetracycline if the same peak becomes sharper and peak height increases then it can be confirmed that Oxytetracycline is present in the sample.

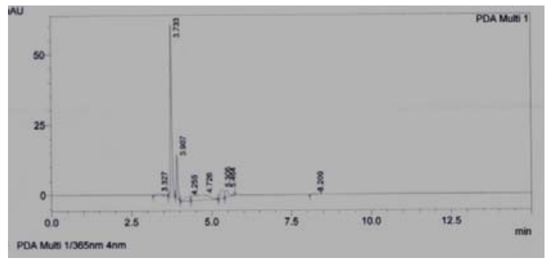
4.3.7. Result with boiled fish in comparison to raw fish:

Boiled *Labeo rohita* of Banani Market:



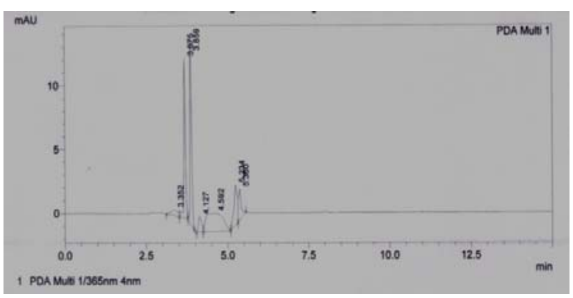
Name	Ret.Time	Area	Height
Oxytetracycline	Not Found	Not Found	Not Found

Raw *Labeo rohita* of Banani Market



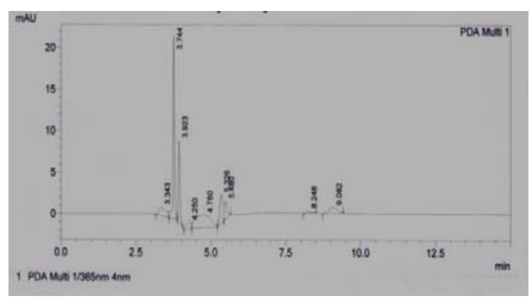
Name	Ret.Time	Area	Height
Oxytetracycline	8.209	1593	211

Boiled *Pangasius pangasius* of Dhanmondi Market:



Name	Ret.Time	Area	Height
Oxytetracycline	Not Found	Not Found	Not Found

Raw *Pangasius pangasius* of Dhanmondi Market:



Name	Ret.Time	Area	Height
Oxytetracycline	8.248	2587	263

Around the world, in many countries people do eat raw fish but in most of the places fish has been cooked with various spices or simply boiled with salt and served with black pepper, lemon juice and coriander leaves. In terms of Bangladesh, fish is really cooked in different ways with light to heavy spices. In whichever way the fish is cooked, it's being boiled for at least 20-30 minutes. In this experiment, presence of residual of Oxytetracycline in boiled fish is also included as a parameter to be observed. Two fishes with confirmed Oxytetracycline of two different market areas were selected and individually each sample was boiled for 30minutes. After boiling, the fish extraction process was carried once again and then finally the samples were run in HPLC. It was observed that two fish samples (*Labeo rohita* and *Pangasius pangasius*) had no trace of Oxytetracycline after boiling. There is a possibility that during the boiling or cooking the Oxytetracycline gets released from the fish and dissolves in the boiled water or in the fish gravy.

Table 6: Presence of residual of Oxytetracycline before and after boiling of fish sample:

Name of the fish sample	Concentration of Oxytetracycline before boiling	Concentration of Oxytetracycline after boiling
Labeo rohita (Rui) of Banani	924 mg/kg	0
Pangasius pangasius (Pangas) of Dhanmondi	1500 mg/kg	0

Table 7: Concentration (mg/kg) of Oxytetracycline in a fish sample given by the regulatory body and concentration (mg/kg) of Oxytetracycline in a fish sample found from the experiment:

Area of Local Market	**Concentration (mg/Kg) of OTC in a fish sample**		
	Rui	Pangas	Tilapia
FDA (Min)	55	55	55
FDA (Max)	83	83	83
Dhanmondi	0	1500	0
Mohammadpur	3237	1061.8	874
Shukrabad	1000	1125	1237.6
Banani	923.6	6250	9123.3
Gazipur	1287.5	1675	1625

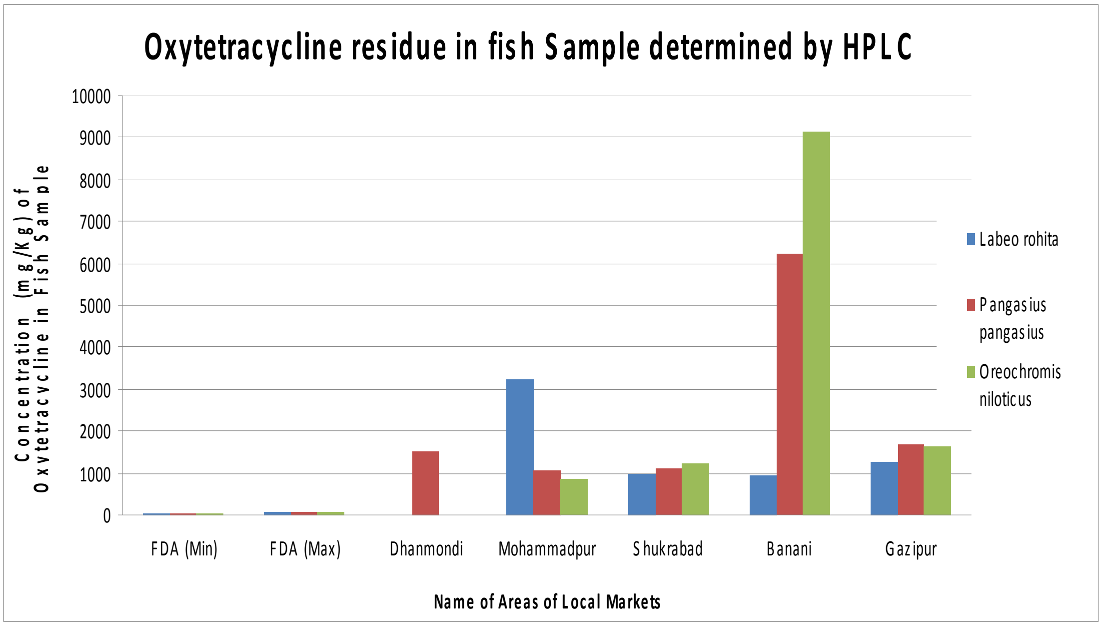


Figure 14: Bar graph showing result of Oxytetracycline residue in fish sample determined by HPLC

According to Food and Drug Administration (FDA), the maximum residual limit (MRL) of Oxytetracycline should remain within the range of 55-83mg/kg in case of fish farming. However, the data of this experiment which were derived from HPLC and the bar graph which was constructed from those values represented a different point of view, i.e. mg/kg of Oxytetracycline used in aquaculture of Bangladesh are exceeding the range given by the regulatory body.

5. Conclusion:

Aquaculture is still the fastest growing food-production sector in the world. It provides a significant supplement to, and substitutes for, and creates employment, generates income and provides opportunities for human development. Aquaculture in Bangladesh is expanding rapidly. Over the last decade it has expanded, diversified, intensified and technologically advanced. In aquaculture, one of the inputs required for successful fish production is chemicals and antibiotics.

The objective of the experiment was to study the presence of residual of antibacterial residue of Oxytetracycline in three common cultured fishes of Bangladesh by analytical HPLC method. End result obtained from this experiment was not very satisfactory and pretty much threatening to human health because whichever fish farms supply *Labeo rohita*, *Pangasius pangasius* and *Oreochromis niloticus* in those five areas (Dhanmondi, Mohammadpur, Shukrabad, Banani and Gazipur) are not following the rule for using antibiotic (oxytetracycline) given by the regulatory body. This study also focused on current status of antibiotics used in aquatic animal health management and pointed out some problems of use of these antibacterial agents by the farmers. Most of the farmers are not well-educated regarding the use of antibiotics. Pharmaceutical companies which manufacture antibiotics as veterinary drug and for aquaculture must instruct the medical officers to go to various fish farms and train up the farmers about using antibiotics. Even those who construct small ponds around the house and willingly do fish farming must also know about the pharmacology, toxicology, adverse effect, dose and dosage form, rout of administration of a specific antibiotic. There remains opportunity for further developments to be carried out by the future research students in this experiment such as experiment can be repeated with several antibiotics and also with other compounds, negative control should be added, as well as instead of collecting single sample from each area they can collect minimum 10-15 samples from each market. Summation and mean values of large number of samples definitely give significant result which helps to obtain a clear picture of current use of antibiotics in aquaculture of Bangladesh.

Unfortunately, little attention has been paid on the documentation of chemicals and antibiotics used in aquaculture industry in Bangladesh. As a result, there is a lack of information regarding the present status and consequences of chemicals and antibiotics using in aquaculture sector especially in aquatic animal health management. Simply less use of chemicals and antibiotics is the best alternative to minimize the adverse effects in aquaculture and on human health. Other alternatives could be used as bioremediation and use of probiotics, immunostimulants, vaccination and alternative therapeutic can also be considered. However, policy makers, researchers, and scientists should work together in addressing the issues of antibiotic as a therapeutic agent and a growth promoter in aquaculture with the view to reduce the negative impacts.

Bacteria, the major group of pathogens, pose one of the most significant threats to successful fish production throughout the world. Bacterial diseases are responsible for heavy mortalities in cultured fishes and most of the causative microorganisms are naturally occurring opportunist pathogens which invade the tissue of a fish host rendered susceptible to infection. Bacteria are able to develop antibiotic resistance when exposed to low doses of drugs over long periods of time. To promote growth and weight gain, entire flocks of farm fish are routinely fed antibiotics at low levels in their feed or water—a practice that has been identified as a contributor to antibiotic resistance. When a person or animal/fish is infected with an antibiotic-resistant bacterium, treatment becomes more difficult because standard antibiotic therapies become less effective or may not work at all. These impacts on human health can result in both higher frequency and longer duration of hospitalizations, raising the cost of health care. The overuse of antibiotics in food animals and aquaculture is leading to increased risk of human illness and increased health care costs.

Government of Bangladesh along with non-governmental organizations (NGOs) must make several laws and regulations regarding the use of antibiotics in aquaculture and should keep updated information whether the aquaculture farms are actually following the rules or not. Actions must be taken against the particular farm which is not following the regulation properly. It is government's responsibility to aware public about unethical and misuses of antibiotics and also about the dangerous consequences of antibiotics resistant pathogens which are directly related to human health. Since it is a matter of public health, pharmaceutical companies should focus on educating the farmers regarding the use of antibiotics in aquaculture. Researchers and scientists must concentrate on various experiments with fish and factors related with aquaculture of Bangladesh. In the near future, lots of other similar work as well as experiments with different indicators will be possible in the field of aquaculture.

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