ASSESSMENT OF ANTIBIOTIC RESIDUE IN SHRIMP (*Penaeus monodon*) MUSCLE OF SOUTH-WEST REGION IN BANGLADESH

S.M. SHAHIN HOSSAIN

Examination Roll: MS-170616

Session: 2016-2017

Fisheries and Marine Resource Technology Discipline

Khulna University

Khulna

September, 2018

ASSESSMENT OF ANTIBIOTIC RESIDUE IN SHRIMP (*Penaeus monodon*) MUSCLE OF SOUTH-WEST REGION IN BANGLADESH

A Thesis

By

S.M. Shahin Hossain

Student No: MS-170616

Session: 2016-2017

A thesis submitted to Fisheries and Marine Resource Technology Discipline in partial fulfillment of the requirements for the Degree

of

Masters of Science in Fish Processing and Quality Control

September, 2018

ASSESSMENT OF ANTIBIOTIC RESIDUE IN SHRIMP (*Penaeus monodon*) MUSCLE OF SOUTH-WEST REGION IN BANGLADESH

Approved as to style and content

By

G. R. Banu 18.12.18

Dr. Ghausiatur Reza Banu

Professor and Head

Fisheries and Marine Resource Technology Discipline

Khulna University,

Khulna.

DECLARATION

ASSESSMENT OF ANTIBIOTIC RESIDUE IN SHRIMP (*Penaeus monodon*) MUSCLE OF SOUTH-WEST REGION IN BANGLADESH

The results presented in this thesis are entirely the candidate's own investigations and no part of the results has been accepted for any degree, not it is being concurrently submitted for any degree.

Candidate

S. M. SHAHIN HOSSAIN

S.M. Shahin Hossain
Examination Roll No: MS-170616
Session: 2016-2017

Co-Supervisor

02.12.18

Shamima Sultana
Assistant Professor
Fisheries and Marine Resource Technology Discipline
Khulna University, Khulna, Bangladesh.

Supervisor

G. R. Banu 02.12.18

Dr. Ghausiatur Reza Banu
Professor and Head
Fisheries and Marine Resource Technology Discipline
Khulna University, Khulna, Bangladesh.

DEDICATED TO

MY BELOVED PARENTS

Acknowledgement

At first I want to praise the Almighty Allah, the supreme creator and ruler of the universe from the deepest corner of my heart for His blessing that keeps me alive and enable me to accomplish this work successfully.

I am greatly indebted to my supervisor Professor **Dr. Ghausiatur Reza Banu**, Head, Fisheries and Marine Resource Technology Discipline, Khulna University, for her continuous guidance, valuable advice and constant encouragement in preparing this thesis.

I wish to express my sincere gratitude to my honorable co-supervisor **Shamima Sultana**, Assistant professor, Fisheries and Marine Resource Technology Discipline, Khulna University, for her valuable appreciation, comments and suggestions.

I would like to express my profound gratitude to **Md. Zillur Rahman**, Fish Inspection and Quality Control Officer (Quality Manager), Quality Control Laboratory, Department of Fisheries, Boyra, Khulna for his valuable suggestions and providence of all necessary facilities.

I would like to express my profound gratitude to Professor **Dr. Ghausiatur Reza Banu**, Head, Fisheries and Marine Resource Technology Discipline, Khulna University, for his valuable suggestions and providence of all necessary facilities.

Finally, I express my deepest gratitude to my parents and to my senior brothers and Joy Shome and other friends and specially thank all of my well-wishers for their continuous support and inspiration in the completion of this task.

S.M. Shahin Hossain

Khulna University

September, 2018

Abstract

The objectives of this investigation were to seek out the commonly used antibiotic residue level of nitrofurans and chloramphenicol in cultured shrimp in south west region in Bangladesh. 90 samples were collected randomly from various fish farm of different Upazillas of Khulna, Satkhira and Bagerhat districts during August-December, 2017, whereas each district was contained thirty samples. Antibiotic residues level present in the shrimp muscles were detected using ELISA by the Fish Inspection and Quality Control Wing of the Department of Fisheries of Khulna. To collect information about type of feed and fertilization status questionnaire interviews were performed during sample collection from the respective farmers. Antibiotic residue data showed all nitrofuran metabolites like SEM, AOZ, AMOZ, AHD and CAP present in all samples in which SEM was highest in Shatkhira district while lowest in Bagerhat district. In case of AHD highest residue were found in Bagerhat and lowest residue were detected in Sathkhira district. Additionally, AOZ and AMOZ and CAP were highest found in Khulna, Satkhira and Bagerhat district while lowest observed in Khulna, Khulna and Bagerhat district. The overall results revealed that nitrofuran metaboties and CAP residue present in all samples within the MRL with the exception of 6.67%, 3.33% and 10% samples of Khulna, Bagerhat and Sathkhira distrct respectively. Our study result also revealed that contamination of nitrofuran metabolites and chloramphenicol were more found in in shrimp farms where commercial feed was used rather than those with home-made feed.

Contents

List of Tables

List of Figures

List of abbreviations and non-English words

Abbreviation	Elaboration
cm	Centimeter
DoF	Department of Fisheries
et al.	et alli; Latin word meaning "and others "
FAO	Food and Agriculture Organization
FMRT	Fisheries and Marine Resource Technology
g	Gram
ha	Hector
Kg	Kilogram
Ltd.	Limited
MRL	Maximum Residue Level
ng	Nano-gram
pp.	Page
ppb	Parts per billion
ppm	Parts per million
PRA	Participatory Rural Appraisal
µg	Micro-gram
Viz.	Namely

Chapter One

INTRODUCTION

Chapter 1

Introduction

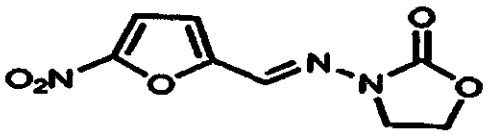
1.1 Background of the study

With the developing of aquaculture, it is now depending on the chemicals which have been used in various methods for centuries (Faruk *et al.*, 2008). Aqua drugs and chemicals have many uses in aquaculture, the types of drugs or chemicals used depending of the nature of the culture system and the species being cultured. Aqua drugs and chemicals are significant components in health management of aquatic animals, pond and tank construction, soil and water management, improve aquatic productivity, transportation of live organisms, feed formulation, manipulation of reproduction, growth promotion and processing and value addition of the final product. The benefits of chemical usage are many. Chemicals enhance production efficiency and decrease the waste of other resources. They help increasing hatchery production and feeding efficiency, and improve survival of fry and fingerlings to marketable size. They are used to reduce transport stress and to control pathogens, among many other applications (Rohana *et al.*, 1996).

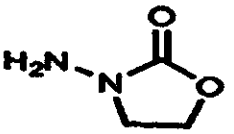
Aquaculture sector has showed a rapid growth in the last 30 years. The main aim of aquaculture is to maintain fish health as well as to improve fish performance. Antibiotics are commonly used to control fish disease, enhance the immune systems and as a growth promoter. However, the usage of antibiotics has been restricted in many countries due to the resistance inducted to bacteria and the residues that they could release in aquatic products (Citarasu, 2010).

Freshwater prawn (*Macrobrachium rosenbergii*) and marine shrimp (*Penaeus monodon*) together represent the second largest exportable iteam in Bangladesh. Targeting mainly for export, prawn and shrimp aquaculture has been expanded very fast over the last decades. Presently, 384700 ha water areas have been brought under prawn and shrimp aquaculture, out of which *M. rosenbergii* has been cultivated in 67,063 ha ponds and *P. monodon* is cultivated within 205,654 ha areas (DoF, 2017). In the past, to maximize production various antibiotics and chemicals like nitrofuran, chloramphenicol, malachite green, crystal violet, etc. were used in prawn/shrimp aquaculture, both prophylactically and therapeutically (Nowsad, 2007; Shamsuzzaman and Biswas, 2012; Hossain *et al.*, 2013).

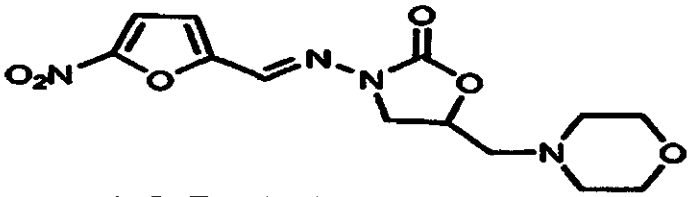
The nitrofurans are a group of broad-spectrum antimicrobials active against *Salmonella* sp., *coliforms*, *Mycoplasma* sp., *Coccidia* sp. and certain protozoa (Delatour *et al.*, 2003). Furazolidone, furaltadone, nitrofurantoin and nitrofurazone are the four main members of this group (Kennedy *et al.*, 2003). They are rapidly metabolized in the body within hours after administration (Leitner *et al.*, 2001). However, their metabolites reside in animal body for weeks, possibly months, as protein bound residues (Conneely *et al.*, 2002) (Fig. 1). Theuse of nitrofurans was banned in the European Union (EU) member states in 1993, with the exception of furazolidone due to the carcinogenicity of the parent drugs and their metabolites (EC., 1993). The ban was extended to cover furazolidone in 1995 due to the carcinogenicity of the parent drug and the unavailability of data concerning the safety of its metabolites (EC., 1995). Thereafter, it is prohibited to use nitrofurans on any animal in the EU and any animal or animal food products intended for export to the EU.



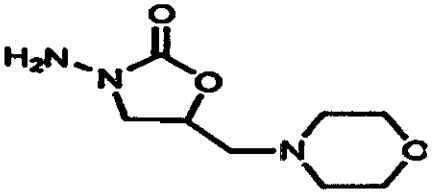
1-A. Furazolidone



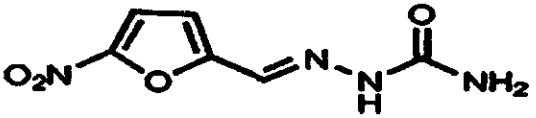
1-B. AOZ



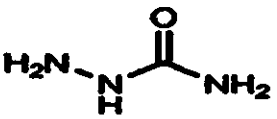
1-C. Furaltadone



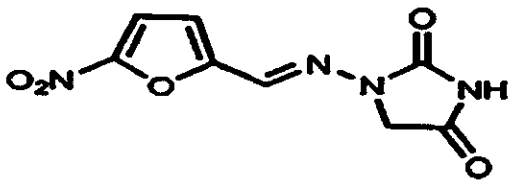
1-D. AMOZ



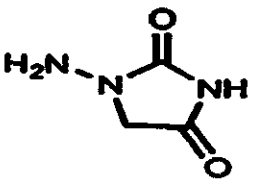
1-E. Nitrofurazone



1-F. SEM



1-G. Nitrofurantoin



1-H. AHD

Fig. 1. A-H. Structures of four nitrofuran antibiotics (on the left side) and their respective free metabolites (on the right side).

CAP is well known as a broad spectrum antibiotic. The use of CAP in shrimp farms and hatcheries has been reported (Graslund and Bengtsson 2001). Although the use of antibiotics in aquaculture production can promote growth and improve the production of aquatic products, excessive use of these compounds will lead to concentration of antibiotics in aquatic products that is higher than national and international food safety standards. Because of the well-known risk of anemia and carcinogenic properties of CAP, the presence of CAP in food is considered illegal and unacceptable in the European Community (EC) since 1994. Furthermore, according to the European Commission Decision 2001/699/EC, 2001/705/EC, 2002/249/ EC, 2002/250/EC, and 2002/251/EC, certain fishery and aquaculture products, imported from China, Vietnam, Indonesia, Thailand, and Myanmar, intended for human consumption, must be subjected to testing to ensure the absence of CAP residues (Impens *et al.*, 2003). CAP is one of nine types of food additives that are banned in Indonesia (Permenkes No. 722/Menkes/Per/IX/88). Nevertheless, the use of CAP in fishery commodities (shrimp and fish) has spread in local, regional, and international levels that it has hampered exports, especially shrimp from Indonesia to various countries in the world. Peak export failure the implementation of zero tolerance occurs when the content of CAP by EU countries for commodities imported shrimp (Islamulhayati *et al.*, 2005).

Health problems have become very familiar in fish and shrimp culture, especially in intensive systems. Protozoan and crustacean ectoparasites, as well as bacterial diseases caused by *Aeromonas hydrophila* and *Vibrio* spp. account for huge mortality in freshwater fish culture and brackish water shrimp culture. To reduce these problems, various antibiotics and other chemicals have been widely used for treatment and prevention of infectious diseases in fish and shrimp farms. The application of chemicals by immersion and the addition of antibiotics to fish feed are the methods that are normally practiced by the farmers (Supriyadi *et al.*, 1996).

Shamsuzzaman and Biswas (2013) mentioned that the active ingredients of antibiotics are mainly oxytetracyclin, chloro tetracycline amoxicillin, co-trimoxa zole, sulphadiazine and sulphamethaoxazole. Antibiotics are effective against bacterial disease. Monsur (2012) found that Oxysentin 20%, Aquamycine, Captor and Acimox powder as antibiotics. Most of the antibiotics are effective against bacterial disease.

The shrimp farming industry has been one of the most important economic activities in the aquaculture sector of many developing countries due to the high demand in the EU member states, Japan and USA. However, due to fundamental deficits in disease control, management and biosecurity practices the disease prevalence is high in shrimp farms (Munasinghe *et al.*, 2010). High disease prevalence, lack of resources and facilities to test food of animal origin for such banned antibiotics and inadequate legislative frame work may increase the risk of abusing nitrofurans by the shrimp farmers. Therefore, it is essential to screen shrimp (and other food of animal origin) for banned antibiotics compounds including nitrofurans to safeguard the consumers and export industry (Fernando *et al.*, 2017).

Considering the above facts, the present study was conducted to identify different types of antibiotics used in aquaculture activities in south west region as well as to evaluate whether those items present in appropriate limit or not.

1.2 Objectives

To determine the residue level of nitrofurans and chloramphenicol in cultured shrimp in South-West region in Bangladesh.

Chapter Two

LITERATURE REVIEW

Chapter 2

Literature Review

2. Literature Review

Degroodt *et al.*, (1992) researched on chloramphenicol and nitrofuran residue analysis by HPLC and photodiode array detection in meat and fish and described a chromatographic method for the analysis of residues of chloramphenicol and nitrofurans in meat and fish. Chloramphenicol, furazolidone, furaltadone and nitrofurazone were extracted from the tissues with ethyl acetate. The extract was purified with petroleum ether and n-pentane and then analyzed with a liquid chromatograph and a photodiode array detector using a Chromspher C-8 column. Chloramphenicol and nitrofurans were detected at 280 nm and 360 nm respectively. Blank samples fortified with chloramphenicol and nitrofurans at levels of 5, 10 and 20 pgkg gave recoveries in the range of 69 to 88%. A quantitation limit of 1 pgkg for nitrofurazone and fwazolidone and of 2 pgkg for chloramphenicol and furaltadone in meat and fish has been reached.

Holmström *et al.*, (2003) researched on Antibiotic use in shrimp farming and implications for environmental impacts and human health and showed that a large proportion of shrimp farmers along the Thai coast used antibiotics in their farms. Of the seventy-six farmers interviewed, 74% used antibiotics in shrimp pond management. Most farmers used them prophylactically, some on a daily basis, and at least thirteen different antibiotics were used. Many farmers were not well informed about efficient and safe application practices. A more restrictive use of antibiotics could have positive effects for the individual farmer and, simultaneously, decrease impacts on regional human medicine and adjacent coastal ecosystems. It was likely that dissemination of information could contribute to a decreased use of antibiotics, without decreasing the level of shrimp production.

Ramos *et al.*, (2003) conducted on determination of chloramphenicol residues in shrimps by liquid chromatography–mass spectrometry and showed that a liquid chromatographic method with mass spectrometric detection and identification (LC–MS) is presented for the determination of chloramphenicol (CAP) in shrimp tissues. Homogenized shrimp samples were extracted with phosphate buffer (pH 7.0). Clean-up was carried out on a C

SPE cartridge. Chloramphenicol was determined by LC–MS-ESI in 18 negative modes. The column used was a Symmetry Shield with a mixture of acetonitrile–water (25:75) as mobile phase. 21 Shrimp samples were fortified at CAP levels between 0.2 and 50 ng/g with 5D-CAP as internal standard. At these levels, accuracies lay between 101 and 110% and between-day reproducibility were lower than 7.1%, expressed as the variation coefficient of the mean. Limit of decision (CCa) was 0.02 ng/g. Limit of quantitation (LOQ) was 0.2 ng/g.

Le and Munekage, (2004) conducted on Residues of selected antibiotics in water and mud from shrimp ponds in mangrove areas in Viet Nam and showed that these antibiotics are found in all samples in both shrimp ponds and surrounding canals. The highest concentrations of TMP, SMX, NFXC and OXLA are 1.04, 2.39, 6.06, and 2.50 ppm in water samples; and 734.61, 820.49, 2615.96, 426.31 ppm (based on wet mud weight), respectively. The comparison of antibiotics residues between study sites and types of shrimp ponds will be discussed in this paper. The results also suggest that antibiotics residues may cause harmful effect on ecosystems in the study sites.

Samanidou and Evaggelopoulou, (2007) conducted on Analytical strategies to determine antibiotic residues in fish and resulted in the emergence of antibiotic-resistant bacteria in aquaculture environments, in the increase in antibiotic resistance in fish pathogens as well as in the transfer of these resistance determinants to human pathogens. Moreover, the use of large amounts of antibiotics may lead to the presence of residual antibiotics in fish tissue and fish products. Fluoroquinolones, tetracyclines, penicillins, sulphonamides and other antibiotics, exhibiting activity against both Gram-positive and Gram-negative bacteria, are widely used for the treatment and prevention of diseases in fish. An extended and comprehensive review on the recent analytical methodologies concerning antibiotic residues in fish reported in the literature is provided in the present article. Emphasis is given on sample preparation regarding isolation and purification, chromatographic conditions and method validation according to legislation. Results of published assays are comparatively presented and criticised.

Vass *et al.*, (2008) conducted on Nitrofuran antibiotics: a review on the application, prohibition and residual analysis and provided an overview of nitrofuran toxicity, metabolism, and also specific aspects of legislation surrounding their prohibition. Special

attention is devoted to semicarbazide – a nitrofuran metabolite and food contaminant. Analytical procedures for nitrofuran analysis in various matrices and validation requirements for screening and confirmation methods with respect to EU regulations are also reviewed.

Chico *et al.*, (2008) experimented on High-throughput multiclass method for antibiotic residue analysis by liquid chromatography–tandem mass spectrometry and found that a simple and rapid method has been developed for the residue analysis of 39 antibiotics (tetracyclines, quinolones, penicillins, sulfonamides and macrolides) in foodstuffs of animal origin. The method combines an effective extraction technique, which uses water–methanol as extracting solvent, with ultra-high-pressure liquid chromatography–tandem mass spectrometry, allowing both confirmation and quantification in a single chromatographic run. The multiresidue method has been validated in chicken muscle matrix according to European Union Decision 2002/657/EC. It has been implemented as a routinemethod in a Public Health Laboratory, instead of the five plates test and LC methods previously used.

Tsai *et al.*, (2010) researched on Quantitative Determination of Four Nitrofurans and Corresponding Metabolites in the Fish Muscle by Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry and found that this developed method carried limits of quantification lower than 10 μg/kg for the nitrofurans and 1.0 μg/kg for the metabolites. It was observed that the decision limit ($CC\alpha$) ranged from 2.93 to 5.01 μg/kg for the nitrofurans and 0.19 to 0.43 μg/kg for the metabolites. The detection capability (CC_β) was between 3.62 and 6.20 μg/kg for the nitrofurans and between 0.23 and 0.54 μg/kg for the metabolites. The linear calibration curve parameters in the fortified fish muscle were between 1.0-100.0 μg/kg and 0.1-10.0 μg/kg for the nitrofurans and metabolites, respectively.

Cháfer-Pericás *et al.*, (2011) researched on Multiresidue determination of antibiotics in feed and fish samples for food safety evaluation. Comparison of immunoassay vs LC-MS-MS and found that 20 fish and 4 feed samples were analysed in order to confirm the feasibility of the immunoassays for detecting antibiotic residues. Sulfonamide residues were not detected in any fish sample. Tetracycline residues were detected in some fish samples from marine farms, with total concentrations between 2.1 and 152 ng/g. In all

cases, the obtained results correlated well with those achieved by LCMS- MS. Therefore, the new methodology allows for food safety of the medicated fish.

Hassan *et al.,* (2013) researched on monitoring the presence of chloramphenicol and nitrofuran metabolites in cultured prawn shrimp and feed in the Southwest coastal region of Bangladesh and found that antibiotic residues present in the animal muscles, feeds and feed ingredients were detected using LC–MS–MS by the Fish Inspection and Quality Control Wing of the Department of Fisheries. The study revealed that farmers did not deliberately use those banned antibiotics, but these chemicals were detected in many *M. Rosenbergii* and *P. monodon* samples in 2008, 2009 and 2010, in both fresh muscles, pre-export and post-export consignments. The percentage of contamination with this banned antibiotics in *M. rosenbergii* samples were 17.74%, 22.89%, 13.60% and in *P. monodon* samples were 12.65%, 15.79%, 11.85% in 2008, 2009 and 2010 respectively.

Nowsad *et al.*, (2013) experimented on studies on nitrofuran contamination in exportable shrimp and prawn products and found that A series of antibiotics sold in local veterinary drug shops of Cox's Bazar and Khulna were found to be used in shrimp hatcheries. While conducting stakeholder based dialogue in Khulna and Cox's Bazar, hatchery operators were directly blamed by shrimp/prawn farmers, depot holders and processors for using nitrofuran drug. Many hatchery technicians mentioned that they used nitrofuran drugs in the hatcheries 5-6 years back, much before than it was declared banned on shrimp products, but at present they were not using it. While asking about the unlabelled drugs using in their hatcheries, they did not respond but mentioned the efficacy of nitrofuran in the survival of shrimp/prawn PL and promotion of their growth. From the participatory stakeholder based study it was understood that both the prawn/shrimp hatchery and poultry feed/fish feed used as shrimp/prawn feed in grow out ponds might be the possible sources of contamination of nitrofuran drugs in exportable shrimp/prawn products.

Suseno *et al.,* (2016) performed an experiment of elimination simulation of CAP in tiger shrimp (*Penaeus monodon*) and white shrimp (*Litopenaeus vannamei*). After 5 days of depuration process, the concentration of CAP in *P. monodon* decreased to 94.85% (muscle), 97.98% (cephalothoraxes), and 90.30% (exoskeleton). The elimination half-life of CAP in *P. monodon* was 0.596 day in the muscle, 0.716 day in cephalothorax, and 0.437 day in exoskeleton. On the other hand, concentrations of CAP in *L. vannamei*

decreased to 97.74% (muscle), 90.30% (cephalothoraxes), and 97.63% (exoskeleton). The elimination halflife of CAP in *L. vannamei* was 0.6624 day (muscle), 0.859 day (cephalothorax), and 0.796 day (exoskeleton). CAP was retained better by *P. monodon* compared to *L. vannamei.*

Fernando *et al.*, (2017) experimented on Determination of nitrofuran metabolites in shrimp muscle tissue by liquid chromatography-photo diode array detection and described a High Performance Liquid Chromatography Diode Array Detection (HPLC-DAD) method to detect tissue bound nitrofuran metabolites, namely 3-amino-2-oxazolidinone (AOZ), 3- amino-5-morpholino-methyl-1,3-oxazolidinone (AMOZ), semicarbazide (SEM) and 1-aminohydantoin (AHD). The bound residues were hydrolysed and derivatized into the corresponding nitrophenyl derivatives (NPAOZ, NPAMOZ, NPSEM and NPAHD) with 2-nitrobenzaldehyde and analysed by HPLC-UV at 275 nm. The linearity of the photometric detector response to the four derivatized nitrofuran metabolites was verified using matrix matched calibration standards in the range of 1-20 mg/kg and the average correlation coefficients for NPAOZ, NPAMOZ, NPSEM and NPAHD were 0.9960, 0.9951, 0.9984 and 0.9993, respectively. The decision limit of the method was below the Minimum Required Performance Limit (MRPL) of 1 mg/kg, for all four nitrofuran metabolites, which makes it compatible with the EU requirements. The average recoveries for (fortified samples between 1 and 5 mg/kg) AOZ, AMOZ, SEM and AHD were 107%, 107%, 115% and 114%, respectively.

Chapter Three

MATERIALS AND METHODS

Chapter 3

Materials and Methods

3. Materials and Method

3.1 Sample Collection and Period

A total 90 shrimp samples were collected randomly from different Upazillas of Khulna, Satkhira and Bagerhat districts whereas each district was contained thirty samples. The samples were directly collected from various fish farm during August-December, 2017. The weight of each sample was 20-25g.The samples were collected in polyethylene bag and iced. Then the samples were brought to the Quality Control laboratory, DOF, Boyra, Khulna and kept in -20°C.

3.2 Questionnaire Interview

To collect information about type of status questionnaire interviews were performed during sample collection from the respective farmers. For that purpose, a detailed questionnaire was developed to assemble information about that. Questionnaire form is attached in appendix section.

3.3 Sample Preparation

Frozen sample was kept in air to bring it normal temperature. Then the sample was washed with reverse osmosis water. Head, scale and body appendages were removed from the body. After air drying samples were chopped individually using a knife and then the sample was taken into a blender to make paste and from the paste 3.0g was taken for extraction procedure by using an electric weighing machine. Then the samples were taken for further preparation.

3.4 Laboratory Procedure

Nitrofuran metabolites and Chloramphenicol residues presented in the shrimp muscles were detected using ELISA by Quality Control of Department of Fisheries, Khulna, Bangladesh.

3.4.1 Required Materials for Chloramphenicol (CAP)

Diluent/Wash buffer (Conc.), conjugate (Conc.), tissue extraction buffer (Conc.), one shot substrate, standards, stop solution, 100 ng/ml chloramphenicol spiking material.

3.4.1.1 Apparatus and Instrument

CAP microtitre plate, micro plate reader (Biotek, Model ELx 800), lint free tissue, water bath/N_2 evaporator, eppendorf tube, 10 ml test tube, 50 ml falcon tube, pipettes: 10 µl to 1000 µl, plate sealer, analytical balance, centrifuge, vortex, blender, personal computer with printer.

3.4.1.2 Extraction Procedure

At first 3g tissue was added with 6 ml ethyl acetate and homogenised in vortex for 1 minute. After centrifuging at 2000 rpm for 15 minute two layers were appeared. 4 ml of upper phase were removed and reduced dryness at 70°C under N_2 atmosphere. The residue was dissolved in 2 ml of isooctane and chloroform (2:3). Again vortex for 1 min as well as added 0.5 ml of diluted tissue extraction buffer and vortex for 2 min. Then centrifuged at 2000 rpm for 15 minute. Upper phase was ready for application to microtitre plate.

3.4.1.3 Sample preparation procedure for ELISA

Before starting ELISA procedure all reagents were kept in room temperature (19-25°C) in order to minimize edge effects.

The assay was performed in duplicate following a layout prepared according to sample number.

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

Solution per well. A colour change from blue to yellow were evident. Measured the optical density of each well a 450 nm and 630 nm filter as the reference wave length within 10 minutes of stopping the colour reaction.

3.4.2 Required Materials for Nitrofuran

Microtitre plate, Conjugate diluent, Diluent/Wash buffer (Conc.), One shot substrate, Stop solution, Standards, Conjugate (Concentrate), 10 mM 4-Nitrobenzaldehyde.

3.4.2.1 Apparatus and Instrument

Respective Microtitre Plate, Micro plate reader equipped with a 450 nm filter with a recommended reference filter of 630 nm, Lint free tissue,Water bath/Incubator, N_2 Evaporator, Eppendorf tube, 10 ml test tube, 50 ml falcon tube, Pipettes : 10 µl to 1000 µl and 1ml to 10 ml, Plate sealer, Analytical balance, Blender, Centrifuge, Vortex, Personal computer with printer.

3.4.2.2 Extraction Procedure

At first homogenized the sample after remove head, shell and body appendages. Weight 1g of homogenized sample in a 50 ml falcon tube. Weigh four 1g portions of previously analyzed blank tissue were used as two positive and two negative controls. Fortify two positive controls with AOZ/AMOZ/AHD and SEM respectively at 0.5 µl/kg.

After that, 1g of homogenized sample was added with 4ml double distilled H_2O, 0.5ml 1M HCl and 50 µl 10mM 4-Nitrobenzaldehyde.Vortex for 1 minute and incubated for 2 hours at 50°C. After vortex for 2 hours 5ml 0.1M K_2HPO_4, 0.4ml 1M NaOH and 6ml ethyl acetate were added and vortex for 1minute. Then centrifuged the sample for 10 minutes at 4000 rpm at 25°C and two layers were appeared. After 10 minutes 3ml of the upper ethyl acetate layer were transferred into a test tube and dry down at 60°C under N2 atmosphere. Dissolved the residue in 1ml hexane and 1ml diluted Diluent/Wash Buffer were added and vortex sample for 2 minutes. Then centrifuged the sample for 10 minutes at 4000 rpm at 25°C. Lower phase was ready for application to microtitre plate.

3.4.2.3 Sample preparation procedure for ELISA

Before starting ELISA procedure all reagents were kept in room temperature (19-25°C) in order to minimize edge effects.

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

3.5 Analysis of data

The collected data were scrutinized and summarized carefully and then they were tabulated. Normality test was done to check the data after input the data in Microsoft Excel 2010. As the data were parametric, One Way Variance was conducted.

Chapter Four

RESULTS

Chapter 4

Result

4. Result

4.1 Types of nitrofuran and cholarmphenicol metabolites

About four the types metabolites namely furazolidone (AOZ), nitrofurazone (SEM), furaltadone (AMOZ) and nitrofurantoin (AHD) and chloramphenicol (CAP) were found to be bind with the protein in the body muscle of shrimp in present study.

4.2 Level of nitrofuran metabolites and chloramphenicol (ppb) in shrimp muscle

Out of 90 *P. monodon* samples tested, a total of 90 samples were detected with banned antibiotics though below maximum residual limit (MRL) (Table 1).

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

Types of antibiotic/metabolites	No. of contaminated sample	Range in ppb	MRL in ppb
SEM	90	0.017-0.405	0.351
AHD	90	0.001-0.081	0.364
AOZ	90	0.001-0.031	0.365
AMOZ	90	0.001-0.046	0.356
CAP	90	0.003-0.024	0.108

4.3 Area wise level of different metabolites in shrimp muscle

The percent of contamination with banned antibiotics in Khulna, Bagerhat and Satkhira were 100%, 100% and 100% respectively. Different types of nitrofuran metabolites were found in different shrimp samples in different levels. In most cases more than one metabolite was found in one or more samples. Area wise the range of the detected levels of metabolites is given in Table 2.

In present study, a total of 90 *P. monodon* samples from three different districts were tested for all the nitrofuran and CAP residues. As we know that maximum residual limit (MRL) of SEM, AHD, AOZ, AMOZ and CAP in shrimp muscle were 0.351, 0.364, 0.365, 0.356 and 0.108 ppb Out of 90 samples, SEM, AHD, AOZ, AMOZ and CAP were detected in all samples. The result of the present study also revealed that more than 93% samples contained those antibiotic residues though exist in FIQC recommended acceptable limit. Only 6 samples contained SEM which was detected above FIQC recommended MRL (Table 2).

Table 2. Area wise percentage of nitrofuran metabolites contamination above MRL

District	No. of sample tested	No. of contaminated sample (Above MRL)	% of contamination	Metabolite
Khulna	30	2	6.67	SEM
Bagerhat	30	1	3.33	SEM
Satkhira	30	3	10	SEM

Area wise mean value and range of nitrofuran metabolites and chloramphenicol (ppb) in shrimp muscles present in Table 8.

Here we can see that mean and range of nitrofuran metabolite SEM in ppb of Khulna, Bagerhat and Sathkhira district were 0.2094±0.1094, 0.1798±0.1155, 0.2534±0.1639 and 0.018-0.404, 0.017-0.401, 0.017-0.405 respectively. From the above finding it could be revealed that SEM was highest in Shatkhira district while lowest in Bagerhat district (Table 3) (Fig 2 a). There was no significance difference in SEM level among Khulna, Bagerhat and Satkhira at 5% level of significance. As mentioned before only 6 samples contained SEM which was detected above FIQC recommended MRL in which 3 samples were collected from Satkhira district and 2 were collected from Khulna district and 1 from Bagerhat district.

Table 3. District wise mean and range of nitrofuran metabolite SEM in shrimp muscle

Districts	Range (ppb)	Mean±Stdv (ppb)	MRL (ppb)
Khulna	0.018-0.404	0.2094±0.1094[a]	0.351
Bagerhat	0.017-0.401	0.1798±0.1155[a]	
Satkhira	0.017-0.405	0.2534±0.1639[a]	

Difference in superscript represents significantly different at 5% level of significance.

In case of AHD, that mean and range in ppb of Khulna, Bagerhat and Sathkhira district were 0.0279±0.0177, 0.0313±0.0245, 0.0145±0.0111 and 0.001-0.066, 0.001-0.081, 0.001-0.047 respectively. From table 4 it could be said that highest residues of AHD were found in Bagerhat and lowest residues were detected in Sathkhira district (Fig 2 b). There was significance difference in AHD level between Khulna, Bagerhat and Satkhira at 5% level of significance while no difference in AHD level between Khulna and Bagerhat (Table 4).

Table 4. District wise mean and range of nitrofuran metabolite AHD in shrimp muscle

Districts	Range (ppb)	Mean±Stdv (ppb)	MRL (ppb)
Khulna	0.001-0.066	0.0279±0.0177[a]	0.364
Bagerhat	0.001-0.081	0.0313±0.0245[a]	
Satkhira	0.001-0.047	0.0145±0.0111[b]	

Difference in superscript represents significantly different at 5% level of significance.

Mean and range in ppb of Khulna, Bagerhat and Sathkhira district were 0.0202±0.0105, 0.0247±0.0106, 0.0283±0.0094 and 0.001-0.039, 0.007-0.043, 0.005-0.046 respectively. From Table 5 it could be said that highest residues of AMOZ were found in Sathkhira and lowest

residues were detected in Khulna district (Fig 2 c). There was significance difference in AMOZ level found between Khulna and Satkhira at 5% level of significance.

Table 5. District wise mean and range of nitrofuran metabolite AMOZ in shrimp muscle

Districts	Range (ppb)	Mean±Stdv (ppb)	MRL (ppb)
Khulna	0.001-0.039	0.0202 ± 0.0105^{a}	0.356
Bagerhat	0.007-0.043	0.0247 ± 0.0106^{ab}	
Satkhira	0.005-0.046	0.0283 ± 0.0094^{b}	

Difference in superscript represents significantly different at 5% level of significance.

From Table 6 we can see that the mean and range in ppb of Khulna, Bagerhat and Sathkhira district were 0.0127±0.0073, 0.0088±0.0049, 0.0113±0.0037 and 0.001-0.031, 0.003-0.021, 0.003-0.023 respectively. Above findings revealed that highest residues of AOZ were found in Khulna and lowest residues were detected in Bagerhat district (Fig 2 d). There was significance difference in AOZ level found between Khulna and Bagerhat district at 5% level of significance.

Table 6. District wise mean and range of nitrofuran metabolite AOZ in shrimp muscle

Districts	Range (ppb)	Mean±Stdv (ppb)	MRL (ppb)
Khulna	0.001-0.031	0.0127 ± 0.0073^{a}	0.365
Bagerhat	0.003-0.021	0.0088 ± 0.0049^{b}	
Satkhira	0.003-0.023	0.0113 ± 0.0037^{ab}	

Difference in superscript represents significantly different at 5% level of significance.

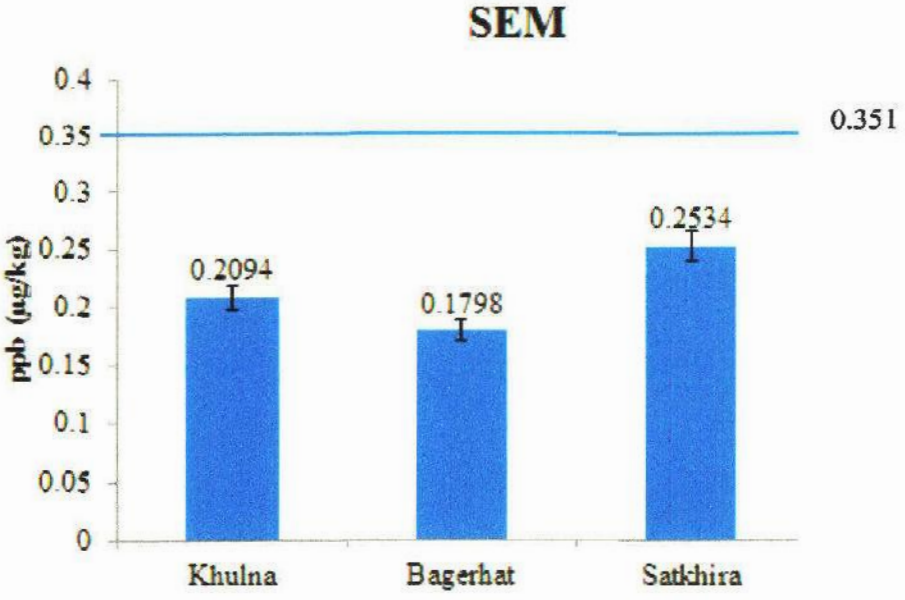
Table 7 showed that the mean and range in ppb of Khulna, Bagerhat and Sathkhira district were 0.0091±0.0045, 0.0089±0.0038 and 0.0123±0.0036 and 0.003-0.019, 0.003-0.017, 0.007-0.024 respectively. Above findings revealed that highest residues of CAP were found in Satkhira

awhile lowest residues were detected in Bagerhat district (Fig 2 e). There was significance difference in CAP level found between Khulna, Bagerhat and Satkhira district at 5% level of significance while no significance difference was found between Khulna and Bagerhat district.

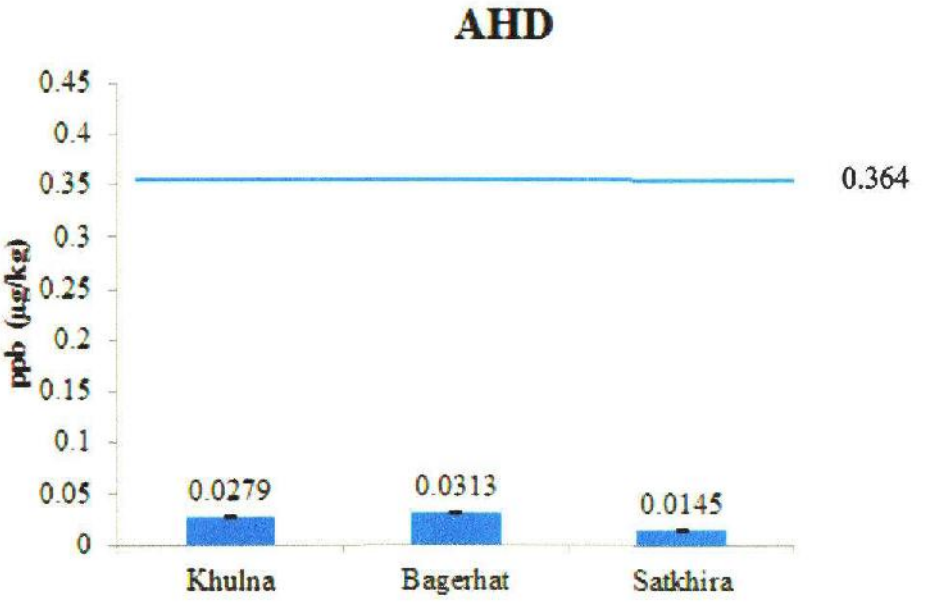
Table 7. District wise mean and range of nitrofuran metabolite CAP in shrimp muscle

Districts	Range (ppb)	Mean±Stdv (ppb)	MRL (ppb)
Khulna	0.003-0.019	0.0091 ± 0.0045^{a}	0.108
Bagerhat	0.003-0.017	0.0089 ± 0.0038^{a}	
Satkhira	0.007-0.024	0.0123 ± 0.0036^{b}	

Difference in superscript represents significantly different at 5% level of significance.



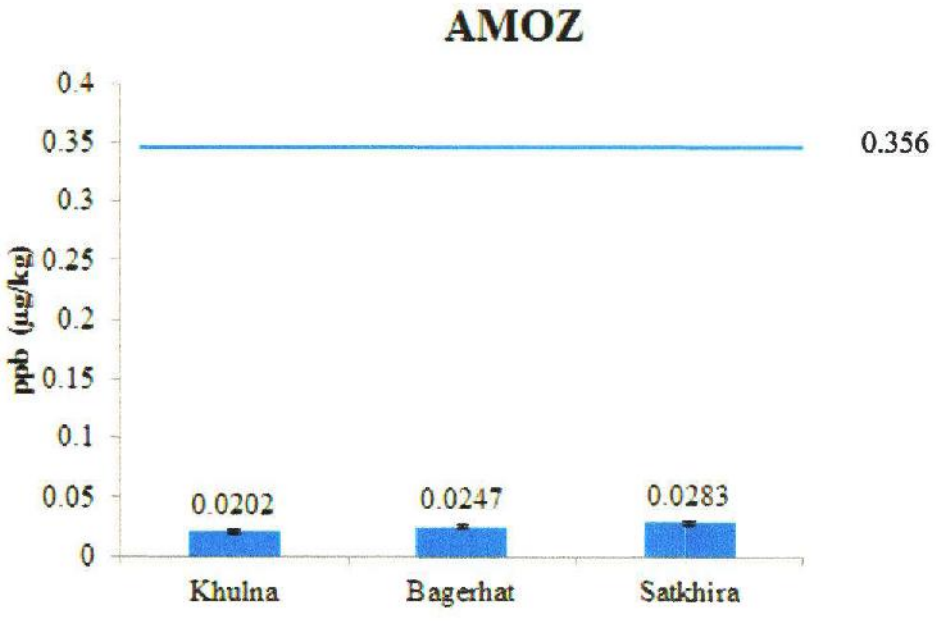
a)


AHD
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
ppb (µg/kg)
0.364
0.0279
0.0313
0.0145
Khulna
Bagerhat
Satkhira

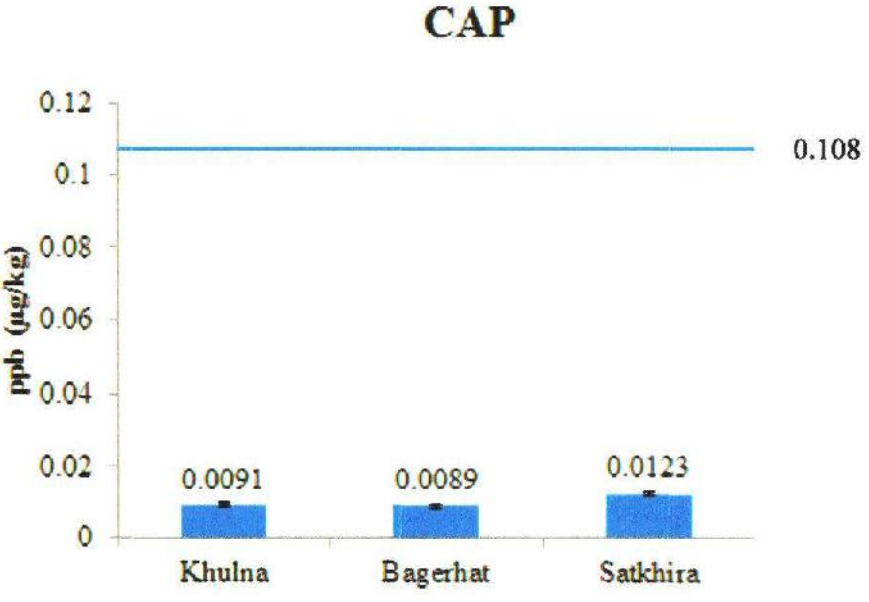
b)


AOZ
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
ppb (µg/kg)
0.365
0.0127
0.0088
0.0113
Khulna
Bagerhat
Satkhira

c)



d)



e)

Fig 2: Mean±SD value of CAP and nitrofuran metabotites residues in shrimp muscle of three different districts; a) SEM, b) AHD, c) AOZ, d) AMOZ and e) CAP

Table 8. Area wise nitrofuran metabolites and chloramphenicol (ppb) in shrimp muscles.

District	SEM			AHD			AOZ			AMOZ			CAP		
	Range	Mean±Stdv	MRL	Range	Mean±Stdv	MRL	Range	Mean±Stdv	MRL	Range	Mean±Stdv	MRL	Range	Mean±Stdv	MRL
Khulna	0.018-0.404	0.2094±0.1094^{a}		0.001-0.066	0.0279±0.0177^{a}		0.001-0.031	0.0127±0.0073^{a}		0.001-0.039	0.0202±0.0105^{a}		0.003-0.019	0.0091±0.0045^{a}	
Bagerhat	0.017-0.401	0.1798±0.1155^{a}	0.351	0.001-0.081	0.0313±0.0245^{a}	0.364	0.003-0.021	0.0088±0.0049^{b}	0.365	0.007-0.043	0.0247±0.0106^{ab}	0.356	0.003-0.017	0.0089±0.0038^{a}	0.108
Satkhira	0.017-0.405	0.2534±0.1639^{a}		0.001-0.047	0.0145±0.0111^{b}		0.003-0.023	0.0113±0.0037^{ab}		0.005-0.046	0.0283±0.0094^{b}		0.007-0.024	0.0123±0.0036^{b}	

Difference in superscript represents significantly different at 5% level of significance.

4.4 Factors responsible behind antibiotic residue

There were several factors played important role behind this. Some major factors which might responsible were, i) direct application of antibiotics in supplementary feed, ii) use of commercial poultry feed, iii) use of homemade feed with organic like cowdung and inorganic fertilizers and so on. Mainly four types of management practice observed in those farms. Some farmers used fed with commercial feed, with homemade feed and used organic and inorganic fertilizer, some only fed with organic and inorganic fertilizer, and rest used no feed or fertilizer. The percentage of different types of feed and fertilizers used in tested shrimp farms was present in Table 9 and list of different commercial feed was presented in Table 10.

Table 9. Area wise percentage of different type of feeds and fertilizers applied for tested shrimp

Types of farm	**% of farm applied**		
	Khulna	**Bagerhat**	**Satkhira**
Fed with commercial feed	50	43.33	50
Fed with homemade feed with organic and inorganic fertilizer	16.67	26.67	23.33
Only fed with organic and inorganic fertilizer	13.33	13.33	16.67
Without feed or fertilizer	20	16.67	10

Table 10. List of commercial feed that were used in the various fish farm in Khulna, Bagerhat and Satkhira district

Feed Name	Company	Ingredients	Dose	Status
Shrimp Grower	Aman Feed	Animal protein, Soyabean cake, Rape seed cake, Wheat flour, Rice polish, Maize, Vitamin –Mineral premix, Feed additives.		Commercial feed
Tiger Brand Eon Nursery Feed	Eon Animal Health Product Ltd.	Fish meal, Soyabean meal, Flour, Betain, Vitamin, Mineral, Euca extract, Enzyme and Natural attractant.		Commercial feed
Shrimp Pre Nursery Feed	Paragon Group	Soyabean meal, Wheat flour, Maize, Rice polish, Enzyme , Vitamin and Mineral.		Commercial feed
CP Prawn Feed	CP Aquaculture Private Ltd.	Fish meal, Wheat flour, Soyabean meal, Fish oil, Phospholipid, Vitamin and Mineral.	4 times per day.	Commercial feed

LA One	Unipresident, Vietnam		4 times per day	Commercial feed
Biomar	All Well Marketing Ltd.		4 times per day	Commercial feed
Excell-Nutri	Saudi-Bangla Fish Feed Ltd.	Fish meal, Fish oil, Soyabean meal, Gluten, Yeast meal, Wheat flour, Growth Promoter.		Commercial feed
Supplementary feed		Rice bran, Wheat bran, Oil cake and occasionally Incorporating Soybean meal and Fish meal.	Once or twice in a week	Home made feed
Farm made feed		Rice bran, Wheat bran, Oilcake, Fishmeal, Flour, Maize, Oyster shell, Salt, Antibiotics, Vitamin premix and Additives.	Once or twice in a week	Farm made feed

Chapter Five

DISCUSSION

Chapter 5

Discussion

5. Discussion

The analyzed results showed that antibiotic residues were detected in all sample but below MRL. It can be seen that type of antibiotic concentrations varied area wise widely. Antibiotic residue monitoring data when compared with the questionnaire survey results of the major observed findings were: (i) among the 4 nitrofuran metabolites, SEM was more abundantly found (ii) shrimps raised in farms fed with commercial feed were contaminated with nitrofuran metabolites more than prawns raised in farms fed with home-made feed with organic and inorganic fertilizer only iii) CAP also widely distributed among samples. Many researchers in Asian countries have also detected SEM in many crustacean muscles and they mentioned that two types of metabolites sometimes naturally occur in samples in low concentration. Antibiotic residue present in muscle below shell occurs due to nature while present in muscle near stomach due to different foods. The most frequent administration route for antibiotics in shrimp is oral, in which the antibiotic is incorporated in the feed with subsequent exposure to the extremely aggressive aquatic environment (Pereira *et al.*, 2004; Saari and Peltonen, 2004).

Nouws and Laurensen (1990) and McCracken *et al.* (2005) mentioned that these parent compounds metabolise rapidly after ingestion to form corresponding tissue bound metabolites. Due to this instability, effective monitoring of their illegal use has been difficult. Additionally, Nouws and Laurensen (1990) stated that the short in vivo half-life of the parent drugs (7 to 63 minutes) results in rapid depletion of nitrofurans in blood and tissue. However, several researchers reported that the formed metabolites (AOZ, AMOZ, AHD and SEM) bind to tissue proteins in the body for many weeks after treatment, making them more practical for monitoring public compliance of the EU ban (Hoogenboom *et al.*, 1991; Horne *et al.*, 1996; McCracken and Kennedy, 1997; Cooper *et al.*, 2005). Although according to Leitner *et al.* (2001) the metabolism of nitrofurans is not well documented, a suggested mechanism is through cleavage of the nitrofuran ring, leaving the specific tail group covalently bound to tissue. Furthermore, Hoogenboom *et al.* (1992) found in In vivo test, these metabolites can be released by natural stomach acids; this fact is taken into consideration in the isolation of metabolites for residue analysis. According to Cooper and Kennedy (2007), the stability of metabolites during the storage and cooking of meat was demonstrated recently. Eight months storage did not

have any significant effect on the residual concentration of nitrofurans in incurred muscle and liver pig samples.

The use of drugs in the treatment of disease in finfish and shrimp is a common practice. In order to ensure that tissue residues are within tolerance when antibiotics are used in the treatment of disease in aquatic species, established minimum drug withdrawal times must be strictly adhered to prior to harvesting. The ideal aquaculture drug would be rapidly and highly absorbed. It would distribute to desired tissues and have a relatively short half-life (Guarino *et al.,* 1988). Antibiotic therapy should always be used wisely to minimize the development of drug resistance in target pathogens (Frappaolo and Guest 1986), a phenomenon well documented for pathogens of aquatic species. Development of drug resistance in bacteria is not only a human safety concern but may also contribute to the increased incidence and prevalence of chronic and recurrent forms of bacterial disease in aquatic species (Bullock 1984; DePaola 1988; Ganzhorn 1987; Hastings and McKay 1987; MacMillan 1985; McPhearson *et al.* 1991; Taylor 1987).

Honculada Primavera, *et al.*, (1993) mentioned that the daily feeding of antibiotics to shrimp encourages antibiotic resistance in the ponds. On average, shrimp eat only 20 percent of their feed. That means the other 80 percent, including the antibiotics it contains, end up in the water and on the muddy pond bottom. Many antibiotics are not biodegradable and persist in the surrounding environment, where they fight against bacteria that continue to develop resistance. Furthermore, Cabello (2006) reported that studies of shrimp ponds in Thailand, Vietnam, the Philippines and Mexico had found relatively high levels of bacteria that were resistant to antibiotics, especially *Vibrio* bacteria. Any time you handle or eat raw or undercooked shrimp, you run the risk of getting food poisoning. However, when the shrimp you eat were grown with large quantities of antibiotics, you take on the additional risk of getting food poisoning from antibiotic-resistant bacteria, which by definition is much more difficult to treat.

Chloramphenicol is a drug of last resort to treat typhoid fever and meningitis in humans. It is generally not used when less toxic drugs are available. Unfortunately, the drug also is used in industrial shrimp production. Although many countries restrict the direct application of chloramphenicol, it is still often applied illegally or indirectly by mixing it with the shrimp feed. According to analysis of FDA data Food and Water Watch obtained by submitting a Freedom of Information Act request, 39 shipments of shrimp failed

import inspections due to the presence of chloramphenicol between 2003 and 2006. Wallerstein *et al.*, (1969) reported that the drug was used sparingly in human medicine because it can cause aplastic anemia, a condition in which bone marrow stops producing red and white blood cells and platelets, which are essential for carrying oxygen and for a healthy immune system. Aplastic anemia is often irreversible and fatal, and onset may occur three weeks to 12 months after exposure. Shakila *et al.*, (2006) reported that Chloramphenicol was only partially deactivated by cooking. In one study, shrimp cooked for 30 minutes at 212° F still retained 71 percent of the antibiotic. Even less chloramphenicol was destroyed when the shrimp was cooked for a shorter, more typical length of time.

Chemical agents should only be applied if there is an appropriate diagnosis of the situation and always under previously established control protocols. According to Páez-Osuna *et al.*, (2003) and Nakata *et al.*, (2005), to evaluate the impact of antibiotic administration, the usage standards, the hydrology of the area, and the physical-chemical properties of the water should be known. Chávez- Sánchez 2003 stated that best practices in aquaculture management should be prioritized to avoid the entrance of pathogens into the shrimp cultivation systems, and antibiotics should only be administered as a last resort. The most frequent administration route for antibiotics in shrimp is oral, in which the antibiotic is incorporated in the feed with subsequent exposure to the extremely aggressive aquatic environment. Cabello (2004) opinion on this matter was for this reason, it is important that the antibiotic be contained within a pellet to maintain its stability and protect it from factors such as leaching and binding to trivalent and divalent cations. It must be certain that the shrimp will eat the food when the antibiotic therapy is applied, because the disease will otherwise not be treated, the environment will be contaminated, and the emergence of bacterial resistant strains will be favored. Cuzon *et al.*, (2004) mentioned that the consumption of food by the farmed organisms may decrease during the molting period, due to environmental factors, or factors related to the infections, reducing the quantity of the antibiotic ingested.

Additionally, Chávez and Montoya (2004) and Montoya (2002) reported that the water temperature of the farm ponds is a critical point to consider, because parameters such as the maximum concentration, distribution volume, and rate of elimination of the antibiotic may be affected. The pH, oxygenation, salinity, stage of disease, climatic changes, and presence of natural food in the ponds are other factors that affect antibiotic therapies

among aquatic organisms. The use of pharmacological agents, antibiotics, and other chemical agents should be considered as methods of last resort in shrimp farming and aquaculture in general. None of the antibiotics is approved for use in shrimp in the United States. Futrher more accordance with FDA (2001), the medicated feed is used in an extralabel manner only for treatment of minor species as defined in the Code of Federal Regulations (21 CFR 514.1(d)(1)(ii). In an aquatic species, the extralabel use of medications added to feed in limited to products approved for use in other aquatic species.

The use of antibiotics in aquaculture is associated with environmental and human health problems, including bacterial resistance, persistence of the disease in the aquatic environment, and effects on the biogeochemical composition of the sediment. The accumulation of antibiotic residues in the edible tissues of shrimp may also alter human intestinal flora and cause food poisoning or allergy problems (Ma *et al.*, 2006).

Antibiotics must not be used as a preventative measure, since bacteria very rapidly develop resistance to them, leading to their ineffectiveness. Chemical agents should only be applied if there is an appropriate diagnosis of the situation and always under previously established control protocols

Chapter Six

CONCLUSION and RECOMMENDATION

Chapter 6

Conclusion and Recommendation

6. Conclusion and Recommendation

Overall, the occurrence of nitrofuran metabolites and CAP contamination in farm raised shrimps under acceptable limit except 6.67%, 3.33%, and 10% sample of Khulna, Bagerhat and Satkhira district respectively in recent days. Though all the residues (CAP, AOZ, AMOZ, AHD, and SEM) were present in all the samples, SEM, AOZ, AMOZ and CAP were highest in Shatkhira district while SEM was lowest in Bagerhat whereas AOZ and AMOZ were lowest in Khulna district. Additionally, CAP was lowest in Bagerhat district. However, in case of AHD the scenario was different, highest AHD level were found in Bagerhat and lowest residue were detected in Sathkhira district. Moreover, contamination of nitrofuran metabolites and chloramphenicol were much more in shrimp fed with commercial feed than those with home-made feed as well as homemade feed with organic and inorganic fertilizer than organic and inorganic fertilizer. Present European legislation does not permit any confirmed concentration of nitrofuran residues in food commodities, although an MRL of 1 μg/kg has been laid down by the European Commission for nitrofuran metabolites in edible tissues of animal origin. Detection of a parent nitrofuran or its metabolite below the concentration of 1 μg/kg requires enforcement action to be initiated. Regulatory authorities and producers are required to identify and eliminate the contamination source to ensure the chemical safety of foods available to the consumer. However, National Residue Monitoring Plan should be intensified and farmers, feed processors and sellers should be aware and involved in NRMP programmes through appropriate traceability network linkage in Bangladesh. All commercial feeds should be strictly screened and any chance of deliberative, accidental or incidental leakage should be checked. Because the effects and fates of these residues in cultured organisms, within the aquaculture system and in the aquatic environment in general is still quite unknown. There are some important concerns about this matter such as human health concerns, product quality concerns, environmental concerns etc. therefore this practice should be taken under scrutiny.

Chapter Seven

REFERENCES

Chapter 7

References

7. Reference

Bullock, G.L. and Snieszko, S.F. 1979. *Enteric redmouth disease of salmonids* (No. 57). US Fish and Wildlife Service.

Cabello, F.C. 2004. Antibióticos y acuicultura en Chile: consecuencias para la salud humana y animal. *Revista médica de Chile*, *132*(8), pp.1001-1006.

Cabello, F.C. 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental microbiology*, *8*(7), pp.1137-1144.

Cháfer-Pericás, C., Maquieira, Á., Puchades, R., Miralles, J. and Moreno, A. 2011. Multiresidue determination of antibiotics in feed and fish samples for food safety evaluation. Comparison of immunoassay vs LC-MS-MS. *Food Control*, *22*(6), pp.993-999.

Chávez, S. M. C. and Montoya, R. L. 2004. Medidas de bioseguridad para evitar la introducción y dispersión de enfermedades virales en granjas camaronícolas, VII *Simposium Internacional de Nutrición Acuícola.*

Chávez-Sánchez, M. C. and Higuera-Ciapara, I. 2003. Manual de Buenas Prácticas de Producción Acuícola de Camarón para la Inocuidad Alimentaria. Servicio Nacional de Sanidad Inocuidad y Calidad Agroalimentaria (SENASICA), ISBN 968-5384-04-5, Mazatlán, Sinaloa, México.

Chico, J., Rúbies, A., Centrich, F., Companyó, R., Prat, M.D. and Granados, M. 2008. High-throughput multiclass method for antibiotic residue analysis by liquid chromatography–tandem mass spectrometry. *Journal of Chromatography* , *1213*(2), pp.189-199.

Citarasu, T. 2010. Herbal biomedicines: a new opportunity for aquaculture industry. *Aquaculture International*, *18*(3), pp.403-414.

Conneely, A., Nugent, A., and O'keeffe, M. 2002. Use of solid phase extraction for the isolation and clean-up of a derivatised furazolidone metabolite from animal tissues. Analyst, 127(6), pp.705-709.

Cooper, K.M. and Kennedy, D.G. 2007. Stability studies of the metabolites of nitrofuran antibiotics during storage and cooking. *Food additives and contaminants, 24*(9), pp.935-942.

Cooper, K.M., McCracken R.J. and Kennedy D.G. 2005. Nitrofurazone accumulates in avian eyes – a replacement for semicarbazide as a marker of abuse. Analyst, 130, pp.824–827.

Cuzon, G., Lawrence, A., Gaxiola, G., Rosas, C. and Guillaume, J. 2004. Nutrition of Litopenaeus vannamei reared in tanks or in ponds. *Aquaculture, 235*(1-4), pp.513-551.

Degroodt, J.M., De Bukanski, B.W., De Groof, J., Beernaert, H. and Srebrnk, S. 1992. Chloramphenicol and nitrofuran residue analysis by HPLC and photodiode array detection in meat and fish. *Journal of Liquid Chromatography and Related Technologies, 15*(13), pp.2355-2371.

Delatour, T., Gremaud, E., Mottier, P., Richoz, J., Arce Vera, F. and Stadler, R.H. 2003. Preparation of stable isotope-labeled 2-nitrobenzaldehyde derivatives of four metabolites of nitrofuran antibiotics and their comprehensive characterization by UV, MS, and NMR techniques. *Journal of agricultural and food chemistry, 51*(22), pp.6371-6379.

DePaola, A., Flynn, P.A., McPhearson, R.M. and Levy, S.B. 1988. Phenotypic and genotypic characterization of tetracycline-and oxytetracycline-resistant Aeromonas hydrophila from cultured channel catfish (Ictalurus punctatus) and their environments. *Applied and environmental microbiology, 54*(7), pp.1861-1863.

Department of Fisheries. 2011. National Fisheries Week. Department of Fisheries, Bangladesh, Souvenir, 20–26 July.

European Community. 1993. Council regulation 2901/93 of 18 October 1993 amending Annexes I, II, III and IV to regulation (EEC) No. 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Official Journal of the European Communities*, 264, pp.1-4.

European Community. 1995. Commission regulation 1442/95/EC of 26 June 1995 amending Annexes I, II, III and IV of Council regulation (EEC) No. 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Official Journal of the European Communities*, 143, pp.26-30.

Faruk, M.A.R., Ali, M.M. and Patwary, Z.P. 2008. Evaluation of the status of use of chemicals and antibiotics in freshwater aquaculture activities with special emphasis to fish health management. *Journal of Bangladesh Agricultural University*, *6*(2), pp.381-390.

Fernando, R.U.C.H.I.K.A., Munasinghe, D.M.S., Gunasena, A.R.C. and Abeynayake, P. 2017. Determination of nitrofuran metabolites in shrimp muscle by liquid chromatography-photo diode array detection. *Food Control*, *72*, pp.300-305.

Food and Drug Administration. 2001. United States Food and Drug Administration. Aquaculture drugs (a chemical hazard). In: Fish and fishery products hazards and controls guidance. 3rd edition, Washington, DC. Centre for Food Safety and Applied Nutrition, Office on Seafood, pp.127-144.

Frappaolo, P.J. and Guest, G.B. 1986. Regulatory status of tetracyclines, penicillin, and other antibacterial drugs in animal feeds. *Journal of Animal Science*, *62*(Supplement_3), pp.86-91.

Ganzhorn, J. 1987. Aeromonas salmonicida, apparent drug resistance to ormetoprim-potentiated sulfadimethoxine. *American Fisheries Society, Fish Health Section Newsletter*, *15*(4).

Gräslund, S. and Bengtsson, B.E. 2001. Chemicals and biological products used in south-east Asian shrimp farming, and their potential impact on the environment—a review. *Science of the Total Environment*, *280*(1-3), pp.93-131.

Guarino, A.M., Plakas, S.M., Dickey, R.W. and Zeeman, M. 1988. Principles of drug absorption and recent studies of bioavailability in aquatic species. *Veterinary and human toxicology*, *30*, pp.41-44.

Hassan, M.N., Rahman, M., Hossain, M.B., Hossain, M.M., Mendes, R. and Nowsad, A.A.K.M. 2013. Monitoring the presence of chloramphenicol and nitrofuran

metabolites in cultured prawn, shrimp and feed in the Southwest coastal region of Bangladesh. *The Egyptian Journal of Aquatic Research, 39*(1), pp.51-58.

Hastings, T.S. and McKay, A. 1987. Resistance of Aeromonas salmonicida to oxolinic acid. *Aquaculture, 61*(3-4), pp.165-171.

Holmström, K., Gräslund, S., Wahlström, A., Poungshompoo, S., Bengtsson, B.E. and Kautsky, N. 2003. Antibiotic use in shrimp farming and implications for environmental impacts and human health. *International journal of food science and technology, 38*(3), pp.255-266.

Honculada, P. J. 1993. A Survey of Chemical and Biological Products used in Intensive Prawn Farms in the Philippines. *Marine Pollution Bulletin*, 26(1), pp.35-40.

Hoogenboom, L.A., Berghmans, M.C., Polman, T.H., Parker, R. and Shaw, I.C. 1992. Depletion of protein-bound furazolidone metabolites containing the 3-amino-2-oxazolidinone side-chain from liver, kidney and muscle tissues from pigs. *Food Additives and Contaminants, 9*(6), pp.623-630.

Hoogenboom, L.A.P., Tomassini, O., Oorsprong, M.B.M. and Kuiper, H.A. 1991. Use of pig hepatocytes to study the inhibition of monoamine oxidase by furazolidone. *Food and Chemical Toxicology, 29*(3), pp.185-191.

Horne, E., Cadogan, A., OKeeffe, M. and Hoogenboom, L.A.P. 1996. Analysis of protein-bound metabolites of furazolidone and furaltadone in pig liver by high-performance liquid chromatography and liquid chromatography mass spectrometry. Analyst, 121, pp.1463–1468.

Impens, S., Reybroeck, W., Vercammen, J., Courtheyn, D., Ooghe, S., De Wasch, K., Smedts, W. and De Brabander, H. 2003. Screening and confirmation of chloramphenicol in shrimp tissue using ELISA in combination with GC–MS2 and LC–MS2. *Analytica Chimica Acta, 483*(1-2), pp.153-163.

Islamulhayati, I., Keman, S. and Yudahstuti, R. 2005. Pengaruh Residu Khloramfenikol Dalam Udang Windu Terhadap Kejadian Anemia Aplastik Pada Mencit. *Jurnal Kesehatan Lingkungan, 1*(2).

Kennedy, D. G., Young, P. B., and McCracken, R. J. 2003. Analysis of veterinary drug residues in food: The nitrofuran issue. *Mitteilungen aus Lebensmitteluntersuchung und Hygiene*, *94*(6), pp.510-526.

Le, T.X. and Munekage, Y. 2004. Residues of selected antibiotics in water and mud from shrimp ponds in mangrove areas in Viet Nam. *Marine pollution bulletin*, *49*(11-12), pp.922-929.

Leitner, A., Zöllner, P. and Lindner, W. 2001. Determination of the metabolites of nitrofuran antibiotics in animal tissue by high-performance liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A*, *939*(1-2), pp.49-58.

Ma, D., Hu, Y., Wang, J., Ye, S. and Li, A. 2006. Effects of antibacterials use in aquaculture on biogeochemical processes in marine sediment. *Science of the total environment*, *367*(1), pp.273-277.

MacMillan, J. 1985. Infectious diseases. In: Tucker CS channel catfish culture. *Elsevier Science Publishers*, Amsterdam, Netherlands, pp.405-496.

McCracken, R.J., Rhijn, J.A., Kennedy, D.G. 2005. The occurrence of nitrofuran metabolites in the tissues of chickens exposed to very low dietary concentrations of the nitrofurans. *Food additives and contaminants*, 22, pp.567–572.

McPhearson, R.M., DePaola, A., Zywno, S.R., Motes, M.L. and Guarino, A.M. 1991. Antibiotic resistance in gram-negative bacteria from cultured catfish and aquaculture ponds. Aquaculture, pp.99-203.

Monsur, A. 2012. Use of Aqua Drugs and Chemicals of Aquaculture in Jamalpur and Sherpur region. MS Thesis. Department of Aquaculture, Bangladesh Agricultural University, Mymensingh, Bangladesh.

Montoya, V.N. 2002. Residuos de antibióticos en camarones: límites residuales y detección de anfenicoles, In. *Centro nacional de acuicultura e investigaciones marinas edgar arellano*,54.

Munasinghe, M.N., Stephen, C., Abeynayake, P. and Abeygunawardena, I. S. 2010. Shrimp farming practices in the Puttallam district of Sri Lanka: implications for

disease control, industry sustainability, and rural development. *Veterinary Medicine International.*

Nakata, H., Kannan, K., Jones, D.P. and Giesy, P.J. 2005. Determination of fluoroquinolones antibiotics in wastewater effluents by liquid chromatography-mass spectrometry and fluorescente detection. *Chemosphere*, 58, pp.759-766.

Nouws, J.F.M. and Laurensen, J. 1990. Postmortal degradation of furazolidone and furaltadone in edible tissues of calves. *Veterinary quarterly*, 12, pp.56–59.

Nowsad, A.K.M., Islam, M.R. and Faruk, M.A.R. 2013. Studies on nitrofuran contamination in exportable shrimp and prawn products. *Progressive Agriculture*, *20*(1-2), pp.143-151.

Nowsad, A.K.M.A. 2007. Final report on studies on nitrofuran contamination in exportable shrimp and prawn products. Bangladesh fisheries research forum, Dhaka.

Páez-Osuna, F., Gracia, A., Flores-Verdugo, F., Lyle-Fritch, L.P., Alonso-Rodriguez, R., Roque, A. and Ruiz-Fernandez, A.C. 2003. Shrimp aquaculture development and the environment in the Gulf of California ecoregion. *Marine pollution bulletin,* 46(7), pp.806-815.

Pereira, A.S., Donato, J.L. and De Nucci, G. 2004. Implications of the use of semicarbazide as a metabolic target of nitrofurazone contamination in coated products. Food Addit. Contam. 21, pp.63–69.

Ramos, M., Munoz, P., Aranda, A., Rodriguez, I., Diaz, R. and Blanca, J. 2003. Determination of chloramphenicol residues in shrimps by liquid chromatography–mass spectrometry. *Journal of chromatography bulletin, 791*(1-2), pp.31-38.

Rohana, P., Subasinghe, Barg, U. and Tacon, A. 1996. Chemicals in Asian aquaculture: need, usage, issues and challenges. *Southeast Asian Fisheries.*

Saari, L., Peltonen, K. 2004. Novel source of semicarbazide: levels of semicarbazide in cooked crayfish samples determined by LC/MS/ MS. Food Addit. Contam. 21, pp.825–832.

Samanidou, V.F. and Evaggelopoulou, E.N. 2007. Analytical strategies to determine antibiotic residues in fish. *Journal of separation science*, *30*(16), pp.2549-2569.

Shakila, R.J. 2006. Stability of chloramphenicol residues in shrimp subjected to heat processing treatments. *Food Microbiology*, 23, pp.47-51.

Shamsuzzaman, M.M. and Biswas, T.K. 2012. Aqua chemicals in shrimp farm: A study from south-west coast of Bangladesh. *The Egyptian Journal of Aquatic Research.* 38 (4), pp.275–285.

Supriyadi, H. and Rukyani, A. 1996. The use of chemicals in aquaculture in Indonesia. *Southeast Asian Fisheries.*

Suseno, H., Hudiyono, S. and Muslim, M. 2016. Elimination of chloramphenicol by tiger shrimp (Penaeus monodon) and white shrimp (Litopenaeus vannamei). *HAYATI Journal of Biosciences*, *23*(3), pp.117-120.

Taylor, P. 1987. Antibiotic resistance to Romet-30 in bacterial infections of catfish. American Fish Society, 15(4).

Tsai, C.W., Tang, C.H. and Wang, W.H. 2010. Quantitative determination of four nitrofurans and corresponding metabolites in the fish muscle by Liquid Chromatography-Electrospray Ionization-Tandem. *Journal of Food and Drug Analysis*, *18*(2).

Vass, M., Hruska, K. and Franek, M., 2008. Nitrofuran antibiotics: a review on the application, prohibition and residual analysis. *Veterinarni Medicina*, *53*(9), pp.469-500.

Wallerstein, R.O. 1969. Statewide study of chloramphenicol therapy and fatal aplastic anemia. *Journal of the American Medical Association*, pp.208-2045.

Chapter Eight

APPENDIX

Appendix 1: Questionnaire

Basic information

1. Name of District:
2. Name of Upazilla:

Farm/Hatchery Information:

1. Name of the farm/ghar/hatchery:
2. Name and Age of farmer :
3. Education level:
4. Contact:

No	Name of antibiotic	Stage	Types of feed	Method of application	Duration of application	Source	Fin fish/Shrimp
1							
2							
3							
4							
5							

Appendix 2: Multiple Comparisons

Dependent Variable		(I) Districts	(J) Districts	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
CAP	Tukey HSD	Khulna	Bagerhat	.000267	.001035	.964	-.00220	.00273
			Satkhira	-.003167*	.001035	.008	-.00563	-.00070
		Bagerhat	Khulna	-.000267	.001035	.964	-.00273	.00220
			Satkhira	-.003433*	.001035	.004	-.00590	-.00097
		Satkhira	Khulna	.003167*	.001035	.008	.00070	.00563
			Bagerhat	.003433*	.001035	.004	.00097	.00590
AOZ	Tukey HSD	Khulna	Bagerhat	.003867*	.001593	.045	.00007	.00767
			Satkhira	.001367	.001593	.668	-.00243	.00517
		Bagerhat	Khulna	-.003867*	.001593	.045	-.00767	-.00007
			Satkhira	-.002500	.001593	.264	-.00630	.00130
		Satkhira	Khulna	-.001367	.001593	.668	-.00517	.00243
			Bagerhat	.002500	.001593	.264	-.00130	.00630
AMOZ	Tukey HSD	Khulna	Bagerhat	-.004567	.002633	.198	-.01085	.00171
			Satkhira	-.008100*	.002633	.008	-.01438	-.00182
		Bagerhat	Khulna	.004567	.002633	.198	-.00171	.01085
			Satkhira	-.003533	.002633	.376	-.00981	.00275
		Satkhira	Khulna	.008100*	.002633	.008	.00182	.01438
			Bagerhat	.003533	.002633	.376	-.00275	.00981
AHD	Tukey HSD	Khulna	Bagerhat	-.003367	.004804	.764	-.01482	.00809
			Satkhira	.013433*	.004804	.017	.00198	.02489

	Bagerhat	Khulna	.003367	.004804	.764	-.00809	.01482
		Satkhira	.016800*	.004804	.002	.00534	.02826
	Satkhira	Khulna	-.013433*	.004804	.017	-.02489	-.00198
		Bagerhat	-.016800*	.004804	.002	-.02826	-.00534
SEM Tukey HSD	Khulna	Bagerhat	.029567	.034048	.662	-.05162	.11075
		Satkhira	-.044033	.034048	.403	-.12522	.03715
	Bagerhat	Khulna	-.029567	.034048	.662	-.11075	.05162
		Satkhira	-.073600	.034048	.084	-.15479	.00759
	Satkhira	Khulna	.044033	.034048	.403	-.03715	.12522
		Bagerhat	.073600	.034048	.084	-.00759	.15479

* The mean difference is significant at the 0.05 level.

CAP

	Districts	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	Bagerhat	30	.00887	
	Khulna	30	.00913	
	Satkhira	30		.01230
	Sig.		.964	1.000
Tukey B[a]	Bagerhat	30	.00887	
	Khulna	30	.00913	
	Satkhira	30		.01230

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

AOZ

	Districts	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	Bagerhat	30	.00880	
	Satkhira	30	.01130	.01130
	Khulna	30		.01267
	Sig.		.264	.668
Tukey B[a]	Bagerhat	30	.00880	
	Satkhira	30	.01130	.01130
	Khulna	30		.01267

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

AMOZ

	Districts	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	Khulna	30	.02017	
	Bagerhat	30	.02473	.02473
	Satkhira	30		.02827
	Sig.		.198	.376
Tukey B[a]	Khulna	30	.02017	
	Bagerhat	30	.02473	.02473
	Satkhira	30		.02827

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

KU. Lib-Acc. No-36955 20.2.19

AHD

	Districts	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	Satkhira	30	.01453	
	Khulna	30		.02797
	Bagerhat	30		.03133
	Sig.		1.000	.764
Tukey B[a]	Satkhira	30	.01453	
	Khulna	30		.02797
	Bagerhat	30		.03133

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

SEM

	Districts	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	Bagerhat	30	.17980
	Khulna	30	.20937
	Satkhira	30	.25340
	Sig.		.084
Tukey B[a]	Bagerhat	30	.17980
	Khulna	30	.20937
	Satkhira	30	.25340

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

Appendix 3: Photo Album

Plate 1: Data collection from pond in Satkhira district

Plate 2: Data collection from Khulna district through PRA

Plate 3: Data collection from pond in Bagerhat district

Plate 4: Sample in FIQC lab

Plate 5: Chopping and Vortex of sample in FIQC lab

Plate 6: Sample after Vortex and Centrifuge of sample in FIQC lab

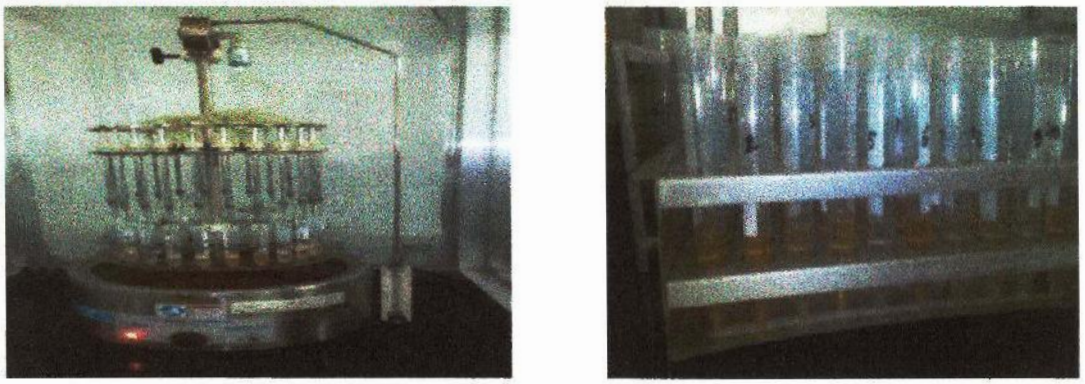


Plate 7: N_2 evaporator and sample after evaporating in FIQC lab

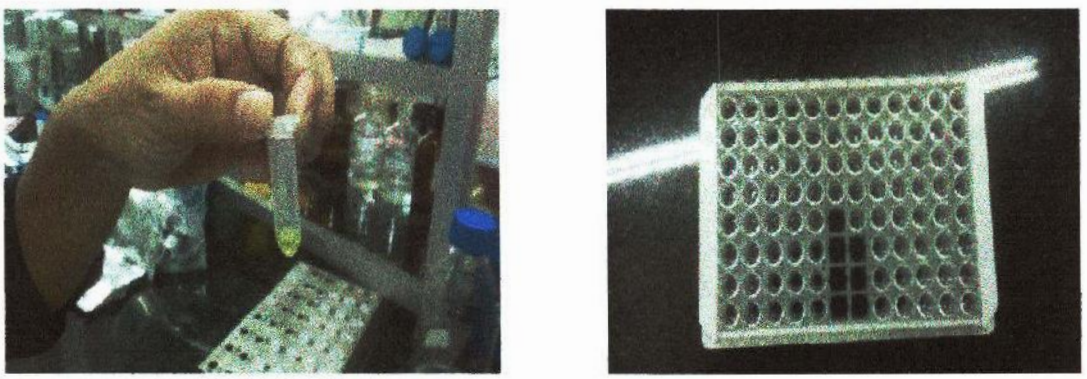


Plate 8: Sample before using in Kit and a Randox Kit in FIQC lab

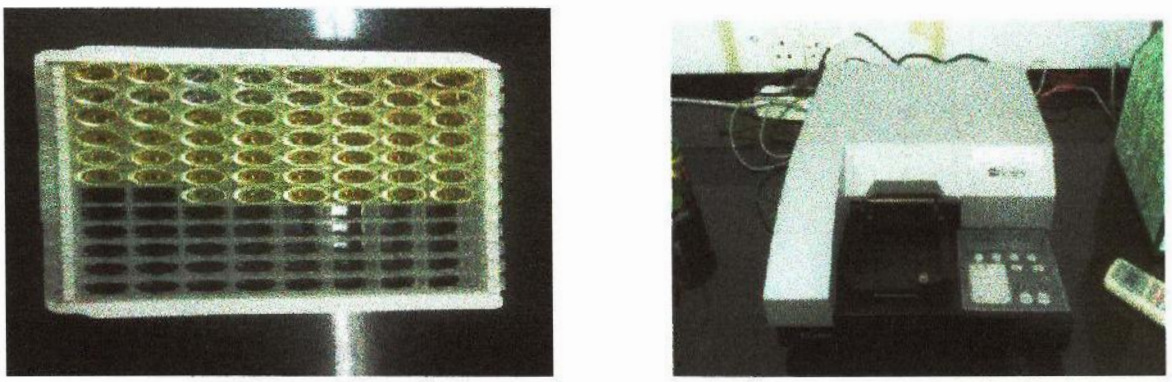


Plate 9: Sample after Kit operation and an ELAISA in FIQC lab