ISOLATION AND IDENTIFICATION OF BACTERIAL PATHOGEN FROM TILAPIA FISH AND THEIR ANTIBIOGRAM STUDY

A THESIS

BY

SUME BEGUM

REGISTRATION NO. 1605130

SEMESTER: JULY-DECEMBER, 2017

SESSION: 2016

MASTER OF SCIENCE (M.S.)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY
UNIVERSITY, DINAJPUR-5200

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Hajee Mohammad Danesh Science and Technology University, Dinajpur

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HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY

UNIVERSITY, DINAJPUR-5200

DECEMBER, 2017

Dedicated

To

My Beloved Parents, Brother and Sister

ACKNOWLEDGEMENTS

ABSTRACT

This study was conducted on isolation and identification of bacterial pathogens from Tilapia fish (*Oreochromis niloticus*) that collected from local market of the Dinajpur city, Bangladesh and their antibiogram study. Sections of the skin, gills and intestine of twenty randomly selected fishes were aseptically removed by means of a sterile scalpel and pair of sterile scissors. A serial dilution was prepared and from last dilution 0.1ml was plated on nutrient agar plates. The purified isolates were identified according to Gram staining reaction, cell morphology, cultural characteristics, and biochemical characteristics. The sensitivity of bacteria isolated from Tilapia fishes to the following antibiotics Amoxicilin, Cefixime, Azithromicin, Chloramphenicol, Ciprofloxacin, Penicillin G, Erythromicin, Vancomycin, Gentamycin and Neomycin was performed. Total four different species of bacteria were isolated and identified. Gram negative include *Pseudomonas spp. Salmonella spp.* and *Escherichia coli*, while gram positive species include *Staphylococcus spp*. Some of these pathogens have tendancy to transmit to man (who eat fish meat or deal with fish and fish products). All collected samples showed bacterial growth and yielded 77 isolates. The isolated bacteria were 13(16.88%) *Pseudomonas spp,* 31 (40.26%) *Escherichia coli*, 10 (12.99%) *Salmonella spp*. and 23 (29.87%) *Staphylococcus spp*. The frequency of occurrences of the isolated Bacteria indicated that had the highest frequency of occurrence *Escherichia coli (*40.26), while *Salmonella spp*. had the least occurrence (12.99%). The result of sensitivity test was variable. Most species of bacteria isolated were sensitive to gentamycin, chloramphenicol, ciprofloxacin and Azithromycin but resistant to Amoxicilin, Penicilin G, Vancomycin and Erythromycin. The isolates were found to be of medical importance. Hence it is considered that a variety of bacterial species can be associated with fresh Tilapia fish related pathogen to humans.

Keywords: Bacteria, Gills, Intestine, skin and Tilapia fish.

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LIST OF ABBREVIATIONS

-	:	Negative
%	:	Percentage
+	:	Positive
µg	:	Microgram
^{0}C	:	Degree of celcius
Assist	:	Assistant
CIP	:	Ciprofloxacin
CEF	:	Cefixime
E	:	Erythromycin
E. coli	:	*Escherichia Coli*
e.g	:	Example
EMB	:	Eosin Methylene Blue
et al.	:	Associated
etc	:	Etcetera
EUCAST	:	European Committee on Antimicrobial Susceptibility Testing
GEN	:	Gentamycin
Gm	:	Gram
H_2O_2	:	Hydrogen peroxide
H_2S	:	Hydrogen sulphide
hrs	:	Hours
HSTU	:	Hajee Mohammad Danesh Science and Technology University
i.e.	:	That is
Ib	:	Pound
Kg	:	Kilogram
KOH	:	Potassium hydroxide
M.S	:	Master of Science
MC	:	MacConkey Agar
MDR	:	Multidrug resistant
MI	:	Milliliter
min	:	Minute
MIU	:	Motility Indole Urease
MR	:	Methyl Red
MSA	:	Mannitol Salt Agar
NA	:	Nutrient Agar
NB	:	Nutrient Broth
No.	:	Number
SL	:	Serial number
Spp.	:	Species
SSA	:	Salmonella Shigella Agar
TSI	:	Triple Sugar Iron
v/v	:	Volume by volume
VP	:	Voges-Proskauer

CHAPTER 1

INTRODUCTION

Fish has been one of the main source of foods for humans and still constitute an important part of the diet in many countries (Leisner, *et al.,* 2001). The shortage of human dietary protein can be provided by fish protein, particularly in developing countries, where the protein shortage is serious. The advantage of fish is its easy digestibility and high nutritional value. As a result, there is a considerable increase in the demand for fish being the cheapest source of animal protein (Ladipo, *et al.,* 1981). An estimated 1 billion people rely on fish as their main source of animal protein (FAO, 2007). Fish contributes about 60% of the world supply of protein, and 60% of the developing world derives more than 30% of their animal protein from fish (Emikpe, *et al.,* 2011). Fishes are generally regarded as safe, nutritious and beneficial but aquaculture products have sometimes been associated with certain food safety issues (WHO, 2007).

Fish play an important role in the Bangladeshi diet, contributing 60% of national animal protein consumption, representing a crucial source of micro-nutrients (Bolton, *et al.,* 2011). The total annual fish production in Bangladesh was estimated at 3.06 million tonnes in 2010–11 fiscal year, of which 1.46 million tonnes (48%) were obtained from inland aquaculture, 1.05 million tonnes (34%) from inland capture fisheries and 0.55 million tonnes (18%) from marine fisheries. The total annual fish production has gradually increased from 1.78 million tonnes in 2000-01 to 3.06 million tonnes in 2010-11, an average annual growth rate of 7% during the last decade (FRSS, 2012). Aquaculture contributes to the livelihoods and employment of millions of rural and urban poor in Bangladesh.

Tilapia is a tropical fish belonging to the family Cichlidae, genus tilapia native to Africa (Shirak, *et al.,* 2009). Due to its rapid growth and palatability, this species has been widely introduced in countries with a tropical climate around the world and has become globally important aquatic species produced in nearly 100 countries worldwide (Romana, *et al.,* 2004). Tilapia and fish in general can be contaminated with pathogenic microorganisms because of the texture of their flesh, their living habits and also from the microbe laden habitat they inhabit.

Isolation and identification of microbial food contaminants help to understand how infectious agents enter and spread through the food chain and therefore come up with procedures to prevent or minimize exposure of the consumer to such agents. Bacteria can enter the fish body through the gills or skin or it can stay on the surface of the body (Douglas, 2007). Fishes skin surface, intestine and gills, however, carries high microbial load (Mhango, *et al.,* 2010). Shell fish such as Tilapia have a particular large pool of nitrogenous extractives and are even more prone to raid spoilage, a factor which accounts for the common practice of keeping them alive until immediately prior to consumption (Adams, *et al.,* 1999). The speed with which a product spoils is also related to the initial microbial load on the product: the higher the count, the sooner spoilage occurs (Adams, *et al.,* 1999). Enterobacteria, Gram-positive cocci, Pseudomonads, and Vibrios have been recovered from the gills of healthy juvenile trout (Nieto, *et al.* 1984).

Some of these pathogens could transmit to man who eat fish meat or deal with fish and fish products (Goncalves, *et al.* 1992; Weinstein, *et al.* 1997; Zlotkin, *et al.* 2003). *Aeromonas sp*. And *Pseudomonas sp*. isolated from *O. niloticus* by 35.96% and 16.88% respectively (Abou El-Atta, 2003).). Pseudomonas was widely distributed in ecosystem and was recognized as one of the primary cause of bacterial hemorrhagic septicemia in fish, pseudomonas septicemia, usually is associated with environmentally stressful conditions such as overcrowding, low temperature, injuries (Aly, 1994; Allen, *et al.* 1983). It may be a secondary invader of damaged fish tissue (Roberts and Home, 1978). *P. fluorescens* considered the causative agent of red spot disease attack all kinds of cultured fishes where the disease raised in running water ponds, stagnant water ponds as well as in cages (Angka and Lioe,1982), the disease favored by stressor as low temperature, injuries and recorded that the incidence of pseudomonas septicemia was 11% (Eissa, *et al.* 1996).

Current knowledge of the health and environmental impact of antibiotics used in aquaculture is poor particularly in developing countries. Drug residues may remain in fish used for human consumption and consequently the antibiotics released into the environment can lead to the development of antibiotic resistant bacteria in the food chain (Cabello, 2006).

By monitoring the bacteria contents of fish organs, the quality of fish can be measured since these will affect the storage life and quality of the fishery products (Kaneko, 1971).

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In order to provide a predictive capability for possible disease outbreaks and provide an opportunity to design preventive management actions, detailed information of the bacterial load and types of bacteria associated with the organs of apparently healthy Tilapia fish is needed.

The present study aimed to isolate and identify bacterial pathogens in locally available Tilapia fish species, to demonstrate the drug-resistance traits of the isolates with a view to provide potential approaches for improving the quality assurance and create awareness among the consumers. An attempt is made in this study to investigate the bacterial micro flora associated with fresh Tilapia fish, sold at the local market of dinajpur city, Bangladesh.

Considering the above points the present study was undertaken with the following objectives-

i) To isolate and identify the bacterial pathogens from Tilapia fish samples collected from local fish market of Dinajpur city.
ii) To detect the antibiotic sensitivity pattern of identified isolates.

CHAPTER 2

REVIEW OF LITERATURE

The review of literatures related to the present study is briefly presented under the following headings:

Huicab-Pech *et al.,* **(2017)** Determined the diversity of pathogenic bacteria at the genus level in the species *O. niloticus var.* Styrling during growing stage in the fattening and pre-fattening phases. Tilapia samples were collected and analyzed; each sample were evaluated by microbiological tests and conventional biochemical tests aimed at the production of glucose, sucrose, lactose, oxidase, catalase, indole and Gram staining. Microbiological and biochemical analysis showed the presence of eleven bacterial genera known as *Arthrobacter sp., Enterococcus sp., Staphylococcus sp., Micrococcus sp., Streptococcus sp., Aeromonas sp., Pseudomonas sp., Edwardsiella sp., Flexibacter sp. and Flavobacterium sp.,* with a predominance of 55% Gram-negative bacilli in tilapia crops. According to the results, it is necessary to take preventive and corrective measures in order to avoid possible risks during production cycles, mainly when handling organisms**.**

Henrietta *et al.,* **(2017)** Isolated aerobic heterotrophic bacteria from Clarias gariepinus and Tilapia zillii in Unwana River, Ebonyi State, Nigeria by using cluster analysis.Both aerobic heterotrophic Gram positive and Gram negative bacteria were isolated. The Gram positive bacteria isolated include *Staphylococcus lugdunensis, S. hominis, S. cohnii, Streptococcus pyogenes, S. pneumoniae,* while the Gram negative bacteria include *Klebsiella pneumoniae, Aeromonas hydrophila, A. veronii, Proteus vulgaris, Serratia fonticola, and Enterobacter gergoriae.* Cluster analysis showed some degree of similarity among the different clusters of isolated bacteria. The result of this study presumptively shows that the water sample is polluted; and this in turn affects fresh water fishes in the river. Therefore the microbiological examination of the water at this study site is necessary for monitoring and controlling the quality and safety of the water for usage by the locals.

Meidong *et al.,* **(2017)** Isolated 119 bacterial strains from various samples such as healthy tilapia fish (*Oreochromis niloticus*). These bacterial isolates were screened for antibacterial activities against bacterial fish pathogens i.e. *Aeromonas hydrophila, A.*

caviae and *Streptococcus agalactiae* using an agar well diffusion assay. It was identified as *Lactobacillus plantarum* by using both conventional and molecular methods. The other probiotic properties were evaluated in vitro which revealed that strain CR1T5 tolerated the simulated gastrointestinal conditions well, showed high capacity to adhere intestinal mucosa and did not lyse red blood cells. The efficiency of *L. plantarum* CR1T5 was also examined in vivo. *O. niloticus* were employed in the feed-trial experiments. Fish fed a diet containing strain CR1T5 displayed not only no mortality but also growth improvement. At the end of feed-trial, fish were challenged by intramuscularly injection of *A. hydrophila*) The *L. plantarum* CR1T5-fed fish survived (87.5%) better than the fish fed a control diet (12.5%) after a two week-challenge. This study clearly shows that *L. plantarum* strain CR1T5 is a promising probiotic candidate for farmed fish.

Younes *et al.,* (2016) Isolated the *Vibrio alginolyticus* and *Vibrio vulnificus* from Nile tilapia fishes (*Oreochromis niloticus*) without fish morbidity or mortality documented. The water parameters showed rise in ammonia and water salinity. Isolates were biochemically and molecularly identified using primers for 16srRNA.*V.alginolyticus* and *Vibrio vulnificus* were isolated from 87.5% and 12.5% of the examined fishes respectively. The *V. Alginolyticus* isolates were pathogenic to Nile tilapia with LD50.The isolated strains were sensitive to cefotaxime, streptomycin and chloramphenicol.

Saqr *et al.,* (2016) Isolated forty *E. coli* (29 from gills and 11 from muscles) from tilapia fish. Further, the isolates were studied for their antimicrobial susceptibility patterns using 9 antibiotics commonly used in the veterinary and medical fields. The highest resistances were by *E. coli* isolates from the gills against ampicillin, streptomycin, sulfamethoxazole-trimethobrim and tetracycline with 97.5%, 65%, 57% and 50%, respectively. While sensitivities were detected to Amikacin (97.5%), Ciprofloxacin (90%), Cefotaxime (67.5%) and Gentamycin (60%). Multiple antibiotic resistances were detected in 95% (38/40) of the isolates.. These results collectively indicate that Nile tilapia in market can harbor pathogenic *E. coli* and act as a reservoir for multi-resistance *E. coli* and facilitate its transmission and dissemination

Kato *et al.,* (2016) Isolated and Identified potential probiotics bacteria from the gut of *Oreochromis niloticus* and *Clarias gariepinus* in uganda **.**Bacterial infections remain a hindrance to aquaculture expansion globally. Increased fish mortality and poor performance resulting from ill health has forced farmers to resort to the use of antibiotics

globally. However, prolonged use of these drugs in aquaculture is becoming restrained as pathogens develop resistance to drugs and unpredicted long term effect on public health. Alternative approaches to control disease are proposed of which probiotics have come forward.

Dangwetngam *et al.,* (2016) Conducted a study reports the serotype distribution and antimicrobial susceptibilities of *Streptococcus agalactiae* isolated from infected tilapia cultured in Thailand. One hundred and forty-four GBS isolates were identified by analyses. Of these 144 GBS isolates, 126 were serotype Ia and 18 were serotype III. Antimicrobial susceptibilities of the 144 GBS isolates were determined by the disc diffusion method. Most isolates were susceptible to lincomycin, norfloxacin, oxytetracycline, ampicillin, erythromycin and chloramphenicol, but resistant to oxolinic acid, gentamicin, sulfamethoxazole and trimethoprim. Oxytetracyline-resistant isolates were found to be susceptible to ampicillin, lincomycin, norfloxacin, erythromycin and chloramphenicol, respectively. Moreover, all 17 oxytetracycline-resistant isolates demonstrated resistance to trimethoprim, oxolinic acid, gentamicin, sulfamethoxazole and oxytetracycline, respectively. These findings are useful information for antibiotic usage in fish aquaculture.

Etyemez and Balcazar (2016) Isolated and characterized the bacteria with antibacterial properties from Nile tilapia (*Oreochromis niloticus*).One hundred and twenty bacterial isolates were obtained from the intestinal mucus of Nile tilapia (*Oreochromis niloticus*) and screened for antagonistic activity and adherence abilities. Based on *in vitro* antagonism against two pathogens (*Streptococcus iniae* and *Edwardsiella piscicida*), five isolates were selected and identified by 16S rRNA gene sequence analysis. All antagonistic isolates were affiliated to the genus *Bacillus*, which showed inhibitory activity against *S. iniae*. Only the isolate B191 (closely related to *Bacillus mojavensis*) inhibited the growth of both pathogens. Moreover, isolate B191 adhered significantly better to fish intestinal mucus than other antagonistic isolates. According to our results, these bacterial isolates, particularly isolate B191, should be further studied to explore their probiotic effects under *in vivo* conditions.

Tiamiyu *et al.,* (2015) Determined bacteriological quality of both wild and cultured Nile Tilapia, *Oreochromis niloticus*, sourced in some area of Ibadan, Southwest, Nigeria. Bacteria flora of the skin and stomach of fish samples were examined and compared

statistically. The resistance of bacterial isolates to commonly used antibiotics was also studied. The bacteria in the genera *Staphylococcus*, *Escherichia*, *Proteus*, *Bacillus*, *Klebsiella*, *Micrococcus*, *Serratia*, *Pseudomonas*, *Salmonella and Streptococcus* were isolated and had been proven to be of great public health significance. About 87.5% of the tested isolates were resistant to augmentin, 83.33% to cotrimoxazole and nalidixic acid. In addition, 79.17% of the isolates were resistant to tetracycline, 75.0% to amoxicillin, 54.17% to nitrofuratoin, 50.00% to ofloxacin and 29.17% to gentamicin. Among the eight antibiotics tested, six patterns of drug resistance were obtained and all of them were multiple drug resistance with three to eight. The public health implications of these observations are discussed**.**

Budiati *et al.,* **(2015)** Determine the microbiological quality in catfish (*Clarias gariepinus*) and tilapia (*Tilapia mossambica*) obtained from wet markets and ponds in Malaysia. A total of 108 samples (32 catfish, 32 tilapia, and 44 water samples) were obtained from nine wet markets and eight ponds in Penang, Malaysia. The feed in fish ponds were chicken offal, spoiled eggs and commercial fish feed. Using standard procedures, aerobic plate counts (APC), coliform, fecal coliform including *E. coli* were performed. A total 31/32 of catfish and 31/32 of tilapia exceeded the recommended microbiological standard for the APC. *E. coli* was less than 3 MPN/g for all catfish and tilapia samples. Homemade feed using chicken offal and spoiled egg may contribute to the microbiological quality in fish. This highlights the importance of feed in aquaculture system.

Bibi *et al.,* **(2015**) Dertermined the occrence of salmonella in freshwater fish.Smoked fishes serves as a vehicle for the transmission of *Salmonella* that exists on skin, in gills and intestine. The absence of a suitable hygiene programme to overcome the transmission of *Salmonella* in the fisheries production sector may be the main reason for the spread of the diseases. The impact of this pathogen in human, particularly in the very young or older, includes gastroenteritis, abdominal cramps, enteric fever and bacteremia resulting from ingestion of uncooked fishes. In spite of these unhealthy impacts, their epidemiology is still poorly understood. Studies are, therefore, needed to summarize the impact of *Salmonella* infected fishes, with particular attention to freshwater fishes in human.

Grema *et al.,* (2015) Isolated Multi-drug resistant bacteria from fresh fish. The bacteria isolated include *Staphylococcus aureus*, *Streptococcus sp*, *E. coli*, *Klebsiella sp*, *Proteus sp*. and *Brucella sp*. These bacterial isolates were subjected to antibiotic susceptibility testing using disc diffusion technique against ten antimicrobial agents. *S. aureus* isolates showed resistance to gentamycin, tetracycline, oxacillin, ciprofloxacin and cefoxitin while *Streptococcus sp* were resistant to tetracycline, chloramphenicol and clindamycin. All the bacterial isolates were resistant to tetracycline while susceptible to cefoxitin, cephazolin, erythromycin and clindamycin. The multi-drug resistance pattern of *Staphylococcus aureus* isolates showed resistance to three and more antimicrobial agents while none was resistant to 10 antimicrobial agents. All other isolates were resistant to four and more different antimicrobial agents while no isolates was resistant to one and ten antimicrobial agents. Therefore the continuous monitoring and surveillance of multi-drug resistant bacteria in fish and fish handlers will not only reduce the risk of disease to the fishes but public health hazard to fish handlers and consumers in general.

Fatima *et al.,* (2014) Analyzed the bacterial habitat and content of Tilapia fish. Total seven fishes were taken and eighteen different species of bacteria were isolated and identified. Gram negative include *Pseudomonas sp. and Enterobacter sp.,* while gram positive species include *Staphylococcus aureus and Streptococcus sp.* Some of these pathogens have tendancy to transmit to man (who eat fish meat or deal with fish and fish products). Streptococcus infection was detected in high prevalence among cultured fresh water fishes, especially during summer seasons. Hence it is considered that a variety of bacterial species can be associated with fresh Tilapia fish related pathogen to humans.

Edris *et al.,* (2014) Examined one hundred random samples of fresh *Tilapia nilotica* samples were purchased from different fish markets in Cairo, Kalyobia and Beheira governorates. The obtained results revealed that 7 of *L. monocytogenes* were isolated from fresh *Tilapia nilotica* fish samples in Egyptian fish market. The highest prevalence of *Listeria monocytogenes* (3%) was observed in fresh *Tilapia nilotica* from marketed fish while lower prevalence of *L. monocytogenes* (1%) was seen in fresh *Tilapia nilotica* from farm fish. Concerning the other *Listeria* species, 27 isolates, eight isolates (8%) were *L. ivanovii*, five isolates (5%) were *L. innocua*, five isolates (5%) were *L. seeligeri*, six (6%) isolates were *L. welshimeri*, and three (3%) isolates were *L. grayi*. The public health significance of the isolated microorganisms and the probable sources of Tilapia

nilotica contamination as well as the suspected recommendations to prevent them to gain access to such food items were discussed.

Rocha *et al.,* (2014) Conducted a study to isolate *E. coli* strains from the gills, muscle, and body surface of farmed Nile tilapias (*Oreochromis niloticus*) fresh-marketed in supermarkets in Fortaleza (Cear´a, Brazil), to determine their susceptibility to antibiotics of different families (amikacin, gentamicin, imipenem, cephalothin, cefotaxime, ciprofloxacin, aztreonam, ampicillin, nalidixic acid, tetracycline, and sulfametoxazol-trimetoprim).Forty-four strains (body surface = 25, gills = 15, muscle = 4) were isolated, all of which were susceptible to amikacin, aztreonam, cefotaxime, ciprofloxacin, gentamicin, and imipenem.The multiple antibiotic resistance index was higher for strains isolated from body surface than from gills. The overall high antibiotic susceptibility of *E. coli* strains isolated from fresh-marketed tilapia was satisfactory, although the occasional finding of plasmidial resistance points to the need for close microbiological surveillance of the farming, handling, and marketing conditions of aquaculture products.

Lara-Flores *et al.,* (2014) Identified the Mycobacterium Agent Isolated from Tissues of Nile tilapia (*Oreochromis niloticus*). Nile tilapia (*Oreochromis niloticus*) samples (60%) of spleen, liver and stomach display multiples granulomas with a thick capsule, walled off by epithelioid cells and robust acid-fast rods on the necrotic center. A great number of acid-fast bacilli where detected with Ziehl-Neelsen stain protocol. The bacteriological analysis of kidney displayed a 90% (26 organisms) of positive development in Lowenstein-Jensen medium with yellow or orange colonies when exposed to light and acid-fast rods. A 562-bp fragment confirms the positive result of the presence of the *Mycobacterium* spp.by PCR analysis; where phylogenetic analysis suggests that *Mycobacterium fortuitum* and *M. marinum* were the principal mycobacterial species associated with tilapia mortality in Campeche, Mexico.

Jimoh *et al.,* (2013) Analysed the microbial load and diversity in the gastro-intestinal tract of Nile Tilapia Oreochromis niloticus caught in River Dandaru, Ibadan. The isolated bacteria were *Proteus mirabilis, Bacillus licheniformis, Pseudomonas aeruginosa, Bacillus megaterium, Staphylococcus aureus, Flavobacterium aquatile, Pseudomonas cepacia, Lactobacillus brevis*. The microbial count of Nile Tilapia caught from river Dandaru Ibadan were; the total plate count was 3.3×10^6 CFUs/g, total

coliform count was 2.4×10^4 CFUs/g, total anaerobic count was 1.0×10^3 CFUs/g, total faecal coliform count was 1.1×10^3 CFU/ml, total fungi count was 7.5×10^3 spore/g.

Wang *et al.,* (2013) Isolated and characterized *Streptococcus agalactiae* from Nile Tilapia *Oreochromis niloticus* in China. In 2009, eight Gram-positive, chain-forming coccus strains were isolated from the outbreak epidemical diseased tilapias from Hainan Province, China. Characteristics based on morphological, physiological and biochemical tests show that the eight strains were suggested to *Streptococcus agalactiae*. All results indicated that the eight isolated strains were *S. agalactiae*. All the isolates were sensitive to cefoxitin, and had quite different sensitiveness to other antibiotics. This research hinted that *S. agalactiae* had become an important potential bacterial agent of tilapias in the south of China.

Karki *et al.,* (2013) Conducted to investigate the presence of antibiotic resistant bacteria in hatchery-reared fish that are important in commercial aquaculture. Two fish species, tilapia (*Oreochromi sniloticus*) and cohosalmon (*Oncorhynchu skisutch)* were obtained from certified hatcheries to investigate if antibiotic resistant bacteria are present in their guts. The antibiotic resistant bacteria were isolated on the basis of their resistance to ampicillin. These results indicated prevalence of antibiotic resistant bacteria in guts of both fish species. Five bacterial isolates were taken from each of these nine fish that yielded ampicillin resistant bacteria (45 bacterial isolates in total) to test antibiotic sensitivity using six additional antibiotics: penicillin, vancomycin, chloramphenicol, tetracycline, streptomycin, and gentamicin. In addition to ampicillin, all isolates showed resistance to penicillin and sensitivity to gentamicin. Overall, ten different resistance phenotypes were observed and many isolates displayed multidrug resistance

Zapata and Lara-Flores (2013) Conducted a study to isolate and identify lactic acid bacteria as new probiotic from the intestinal microflora of Nile tilapia (*Oreochromis niloticus*) fish. A total of five lactic acid bacteria were isolated from intestine to evaluate with antibacterial properties. These strains were identified by conventional and molecular techniques as: *Enterococcus faecium, Leuconostoc mesenteroides, Lactobacillus fermentum, Lactobacillus plantarum, Enterococcus durans. Leuc. mesenteroides* present more ability to inhibit growth of fish pathogens bacteria and selected as possible probiotic bacteria to use in aquaculture.

Sichewo *et al.,* (2013) Conducted a study aiming at the isolation of human pathogenic bacteria in gills, intestines, mouth and the skin of apparently healthy fish, Tilapia rendali and Oreochromic mossambicus, from the Fletcher dam. The following human pathogenic bacteria were isolated *Salmonella typhi, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Vibrio cholerae, Shigella dysenteriae and Enterococcus faecalis*. All the bacterial species which were isolated from the fish were also present in the initial water samples collected. The isolation of enteric bacteria in fish serves as indicator organisms of faecal contamination and or water pollution. Their presence also represents a potential hazard to humans

Shayo *et al.,* (2012) Determined the virulence of Aeromonas hydrophila (4), A. veronii (2) and Pseudomonas aeruginosa (1) recovered from normal and ulcerative affected 200 healthy Oreochromis niloticus (50–100gm) were used in which 20 fish were stocked in each aquarium. The infected fish were observed for 14 days. The injected bacteria were then recovered from the experimental fish and subjected to morphological, biochemical and antibiotic susceptibility tests. Results showed that; 112 out of 180 infected fish developed clinical abnormalities such as skin darkness, scales detachment, blindness and large irregular hemorrhages on the body surface, fin necrosis, exophthalmia and eye cataract/trachoma within four days and mortality rate of 95%. The recovered strains were motile, gram- negative, and were resistant to Ampicillin, Streptomycin, Amoxyllin and Novobiocin. This study concluded that Aeromonas and Pseudomonas species are responsible for the ulcerative disease outbreaks in Mtera Dam. However, the study is not conclusive as to whether the same bacteria are responsible for development of eye cataract and blindness to the infected fish.

Thillaimaharani *et al.,* (2012) Analysed the intestinal bacterial flora of the fish *Oreochromis mossambicus* and optimization of alkaline protease production .The isolated strains were identified as *Virgibacillus pantothenticus, Bacillus cereus, Bacillus licheniformis, Enterococcus faecalis* and *Virgibacillus alginolyticus*. Among the isolated bacteria, *V. pantothenticus* gave the maximum yield of alkaline proteas.The studies on fish gut microflora are very much needed for the management both in aquaculture and public health. The result of alkaline protease production by *V. pantothenticus* is encouraging and it can be applied for bioconversion of fish waste and sea weed from *aquafeed* formulation and subsequent reduction in feed related waste outputs.

Kapute *et al.,* (2012) Assessed the quality of Lake Malawi Tilapia (local name: Chambo) from local and super markets in Malawi. Most isolated bacteria species were Corynebacterium, Micrococcus, Pseudomonas, Bacillus, Flavobacterium and Escherichia coli.Fish collected from local markets had significantly higher levels of TVB-N (P<0.05) compared to those from super markets. Fish from both local and super markets were contaminated generally due to poor handling by sellers but not necessarily at the selling points. The study nevertheless observed that despite being microbiologically contaminated, fish were not wholly spoiled. This suggests that product declared unfit for consumption through sensory evaluation may still be nutritionally good hence need for validating such results with other methods.

Ashiru *et al.,* (2011) Analyzed for the presence of *Aeromonas* species and their susceptibility to antibiotics from the surface and the intestinal tract of catfish and tilapia fish purchased from Makoko market, Lagos metropolis in Nigeria. The surface and intestinal tract of the fishes were found to be contaminated with *Aeromonas* species and this is a potential risk for public health. *Aeromonas caviae* was predominant in tilapia fish. The *Aeromonas* species exhibited different level of antibiotics susceptibility based on the zone of inhibition observed around the antibiotics disc. *Aeromonas caviae*, *Aeromonas sobria* and *Aeromonas hydrophila* were all resistant to tetracycline, nitrofurantoin and augmentin but highly susceptible to pefloxacin, ofloxacin and ciprofloxacin while they were randomly susceptible to ceftriazone, gentamycin, cotrimozazole and amoxycillin. Hence, pefloxacin, ofloxacin and ciprofloxacin are suitable drugs that can be use in the treatment of *Aeromonas* associated infections. There is need for antibiotic susceptibility test before treatment of *Aeromonas* associated infection since some strains of the *Aeromonas* species were randomly susceptible to the some of the antibiotics.

Donkeng *et al.,* (2011) Investigated that the presence of *Aeromonas spp.*, *Vibrio* spp. and *Plesiomonas shigelloides* in Cameroonian tilapia.120 samples (gills, intestine and skin of individually fish) of tilapia were collected and inoculated in culture media. In addition, the antimicrobial pattern of the identified strains to ten different antibiotics was studied using the agar disk diffusion method. The results obtained from this study show the presence of bacterial species belonging to *Aeromonas spp*. (n=39), *Vibrio* spp. (n=41) and *P. shigelloides* (n=14). The results of antimicrobial susceptibility show that the highest rates of resistance was found in ampicillin (100% of isolates), nitrofurantoin

(more than 68% of isolates), association of trimethoprim and sulfamethoxazole (51% of isolates) and sulfamide (46 % of isolates). This work therefore highlights an important incidence of *Aeromonas spp.*, *Vibrio* spp. and *P. shigelloides* with potential antimicrobial resistance, isolated from tilapia in four localities of Cameroon**.**

Musefiu *et al.*, (2011) Isolated and identified the bacteria flora of wild and cultured *Clarias gariepinus* (African catfish) and *Orepchromis niloticus* (Nile Tilapia). 210 tissue samples harvested from skin ($1cm^2$) and stomach (1g) were aseptically analysed. The bacteria species isolated are those in genera *Bacillus, Proteus, Pseudomonas, Klebsiella, Streptococcus, Salmonella, Staphylococcus, Micrococcus, Serratia and Escherichia*. The highest number of different bacteria count was recorded in tilapia species captured from natural river. Escherichia coli were most frequently encountered (13.06%). The bacteria isolated were of public health significance and the implications of these observations are discussed.

Mhango *et al.*, (2010) Analyzed the Tilapia from the supermarkets and tilapia and catfish from street vendors for detected microbial load, presence of indicator microorganisms, opportunistic and pathogenic bacteria. Though coliforms were found in 84% of fish from the street vendors, only 16% of the fish had coliforms above the acceptable limits.No *Escherichia coli* were isolated. *Staphylococcus* species were isolated frequently in all fish analyzed. *Salmonella arizonae, Salmonella paratyphi* (22-40%) was only isolated in street vended fish. Other bacterial isolates from fish included *Enterobacter sakazakii, Enterobacter cloacae, Vibrio cholorae, Proteus mirabilis, Proteus vulgaris, Klebsiella pneumoniae* and *Aeromonas hydrophila*. The presence of diverse enteric bacteria indicated the degree of cross contamination from the handlers. Stringent regulations on registration of fishermen, traders, education and mandatory observance of sanitation at trading points and use of ice have to be enforced to ensure safety of fish consumption.

Shinkafi and Ukwaja (2010) Conducted on bacteria micro flora associated with fresh Tilapia fish (*Oreochromis niloticus*) sold at Sokoto central market, Sokoto. Nigeria. Sections of the skin, gills and intestine of ten randomly selected fishes were taken. A total of nine (9) bacterial species were isolated and identified. Eight bacteria were identified to specie level and one to genus level. Six (6) were grampositive namely: *Bacillus megatanium, Listeria monocytogenes, Bacillus Pumilus, Bacillus alvei, Bacillus*

Licheniformis and Staphylococcus saprophyticus and three gram negative bacteria namely: *Serratia mercescens, Providentia stuartii and Salmonella spp*. The frequency of occurrences of the isolated Bacteria indicated that *Bacillus pumilius* had the highest frequency of occurrence *(*19.35%), while *Salmonella spp.* had the least occurrence (3.2%). The isolates were found to be of medical importance.

Gisain *et al.,* (2010) Iisolated bacteria from the brain, eye and kidney of red tilapia, as well as water and debris samples. In Kenyir Lake, bacterial isolates that predominated in the fish were *Micrococcus* spp. and *Aeromonas hydrophila* at 13.64 %, in water samples it was *Staphylococcus xylosus* at 40% .In the Semantan River, the predominant bacteria in fish and debris samples were *Aeromonas hydrophila* at 23.53 % and 90 % respectively. In the water samples, *Staphylococcus lentus* and *Staphylococcus xylosus* were the predominant bacteria with 30 and 20%, respectively. The ammonia, sulphide, iron and nitrite-nitrogen levels in the Semantan River were over the acceptable limits and this may lead to high fish mortality. This study concluded that *Aeromonas hydrophila* and *Staphylococcus spp.* were the most predominant bacteria in red tilapia and poor water quality played a major role in red tilapia succumbing to infections by pathogenic bacteria.

Eissa *et al.,* (2010)　Charecterised the presence of pseudomonas species isolated from tilapia *(Oreochromis niloticus*) that collected from Qaroun and El Rayan lakes. The organisms were found in 30.83% of the 480 examined fish. Culture from liver, spleen, kidneys and gills on pseudomonas agar media yielded different species of pseudomonas. The prevalence of infection revealed significant difference among four batches, it was 43.33% (April 2008), 24.44% (August 2008), 21.11% (November 2008) and 17.77% (January 2009); and the organisms were mainly isolated from liver and kidney (35 and 30%, respectively). Challenge tests revealed that *P. angulliseptica* was the highest pathogenic one and induced 96.66% mortality, while *P. fluroscens* injected groups showed 2% mortality. Antibiogram sensitivity revealed high sensitivity to Avatryl and Amikicin and sensitivity to Gentamicin, Erythromycin, Novobiocin and Sulfa-trimethoprime. It was concluded that different pseudomonas diseases were considered Qaroun and El-Rayan lakes causes septicemia in *O. niloticus* and the pathogenic species include *P. anguilliseptica, P. putida* and *P. aureginosa*. Avatryl and Amikicin are the best drug for treatment of septicemia caused by the above mentioned species in *O. niloticus*.

Soliman *et al*., (2010) Conducted a study to isolate different species of *E. coli* and identifying it by different methods and. The higher isolates of *E. coli* obtained from intestine and liver. The results obtained during the course of *E. coli* incidence cleared that the high organic matter and un-ionized ammonia (NH3) can affect the incidence and pathogenicity of *E. coli*. The Antibiogram test indicated that, the sensitivity of isolated *E. coli* to different antibiotics that, the antibiotics of high effect on *E. coli* were Enrofloxacine, Oxanilic acid and spectinomycine and the least effect include Erythromycin, Chloramphenicol. Also, our results indicated that, the most important *E. coli* isolates, that isolated from +ve fish samples to *E. coli* were O55, O148, O157 and O125.

Mandal *et al.,* (2009) Investigated the abundance of coliform bacteria in Nile tilapia sampled from different sources. Densities of total aerobic bacteria, total coliform (TC), faecal coliform, (FC) and *Escherichia coli* were measured from different organs of Nile tilapia sampled from pond, *gher* and market using serial dilution and spread plate techniques. Significant differences were observed in various parameters of bacterial density between and within different organs of Nile tilapia (p<0.05). Significantly higher density of faecal coliform was detected in the muscle, gill and intestine in Nile tilapia sampled from pond than that of market. Findings of the present study suggest that Nile tilapia may be faecally contaminated during culture period, storage and transportation and unhygienic marketing.

Yagoub (2009) Isolated *Enterobacteriaceae* from gills, skin, muscles and the intestine of 83 out of 150 (55%) randomly collected fishes, the most dominants isolates were *E. coli*, *Citrobacter spp*, *Enteriobacter spp* and *Klebsiella spp*. This together with the highly pathogenic *Enterobacteriaceae* including *Salmonella spp* and *Shigella spp. Proteus spp*, and *Alklegens* spp. Potential pathogenic organisms were also among the isolates. On the other hand *Pseudomonas spp* was isolated from 62% of randomly collected fishes. The number and percentages of the isolated bacteria were compared according to seasons. The total bacterial count, coliform count and *E. coli* count were estimated from all parts of collected samples. The negative impacts of the presence of *Enterobacteriaceae* and *Pseudomonas spp* in fisheswere discussed based on their potential pathogenic effect toward public health and their role to enhance rapid spoilage of fishes.

Boari *et al.,* (2008) Investigated the bacterial ecology of tilapia (*Oreochromis niloticus*) fresh fillets and some factors that can influence its microbial quality. Samples of fish cultivation water (n = 20), tilapia tegument and gut (n = 20) and fresh fillets (n = 20) were collected in an experimental tilapia. *Staphylococcus* spp.*, Aeromonas spp.,* *Enterococcus* spp. and Enterobacteriaceae were quantified using selective plating. For the enumeration of *Pseudomonas spp.*, the most probable number technique (MPN) was utilized. Bacterial colonies (n = 198) were identified by Gram strain and biochemical tests. *Aeromonas spp., Pseudomonas spp., Enterococcus* spp. and Enterobacteriaceae were found in the cultivation water (water from a fishpond cultivation), tegument, gut, and fresh fillets. *Staphylococcus* spp. was not isolated in the cultivation water. *Salmonella* spp. was not detecte. Associated to freshwater tilapia fillet processing, there is a large variety of microorganisms related to foodborne illnesses and fish products deterioration.

Newaj-Fyzul *et al., (*2008) Conducted a study was on local tilapia industry to determine the microbial quality of pond water, prevalence of bacterial pathogens and their anti-microbial resistance using the disk diffusion method the predominant bacteria from fish slurry were *Pseudomonas spp.* (60.0%), *Aeromonas spp.* (44.0%), Plesiomonas (41.3%) and Chromobacterium (36.0%) compared with isolates from pond water where Bacillus spp. (80.0%), Staphylococcus spp., *Alcaligenes spp.* and *Aeromonas spp.* (60.0%) were most prevalent. Using eight anti-microbial agents, to test bacteria from five genera (Aeromonas, Chromobacterium, Enterobacter, Plesiomonas and Pseudomonas), 168 (97.1%) of 173 bacterial isolates from fish slurry exhibited resistance to one or more anti-microbial agents compared with 47 (90.4%) of 52 from water. Resistance was high to ampicillin, 90.2% (158 of 173), erythromycin, 66.5% (115 of 173) and oxytetracycline, 52.6%, (91 of 173) but relatively low to chloramphenicol, 9.8% (17 of 173) and sulphamethoxazole/trimethoprim, 6.4% (11 of 173). Resistance was generally high to ampicillin, 78.8% (41 of 52), erythromycin, 51.9% (27 of 52) and oxytetracycline, 34.5% (18 of 52) but low to sulphamethoxazole/trimethoprim, 7.7% (four of 52) and norfloxacin, 3.8% (two of 52). It was concluded that the rather high prevalence of bacterial pathogens in tilapia along with their high prevalence of resistance to anti-microbial agents might pose therapeutic problems as well as health risk to consumers. The microbial presence and their anti-microbial resistance in the tilapia industry are being reported for the first time in the country.

Al-Harbi and Uddin (2005) Observed the Bacterial diversity of tilapia (Oreochromis niloticus) cultured in brackish pond water in Saudi Arabia. In total, 19 bacterial species were identified. The bacteria were predominantly Gram-negative rods (87%). Pond water and sediment bacteria influenced the bacterial composition of gills and intestine of tilapia. In contrast to gill bacteria, more diversification was observed in intestinal bacteria. The predominant (prevalence >10%) bacterial species were *Vibrio parahaemolyticus, Vibrio carchariae, Vibrio alginolyticus, Chryseomonas sp., Vibrio vulnificus, and Streptococcus sp.* in all the populations with the exception of the sediment population where *Streptococcus sp.* was replaced by *Shewanella putrefaciens. Vibrio spp.* (58% of the total isolates) dominated the total bacterial population.

Molinari *et al.*, (2003) Measured the total bacterial numbers in the gastrointestinal tract of tilapia, *Oreochromis niloticus* cultured cultured in a semi-intensive system. *Aeromonas hydrophila, Aeromonas veronii, Burkholderia cepacia, Chromobacterium violaceum, Citrobacter freundii, Escherichia coli, Flavimonas oryzihabitans* and *Plesiomonas shigelloides* were the most frequently isolated Gram negative bacilli. From these samples, only *Plesiomonas shigelloides* was present in both gastrointestinal regions, with higher number in the posterior gut (76%), when compared to anterior gut (4.8%) and stomach (0.6%). *Aeromonas hydrophila* (0.6%), *Escherichia coli* (7.4%), and *Flavimonas oryzihabitans* were isolated only in the stomach, and *Citrobacter freundii* and *Burkholderia cepacia* were found only in the posterior gut. *Chromobacterium violaceum* was the dominant bacteria isolated from stomach and anterior gut, with 90% and 55%, respectively. Unidentified organisms comprised 0 - 39.3% of the gastrointestinal microflora.

Al-Harbi and Uddin (2003) Documented Quantitative and qualitative studies on bacterial flora of hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) cultured in earthen ponds in Saudi Arabia. In total, 15 bacterial genera and 18 species were identified. Pond water and sediment bacteria reflected the bacterial composition in the gills and intestine of tilapia. In contrast to gill bacteria, more diversification was observed in intestinal bacteria. Corynebacterium urealyticum, Shewanella putrefaciens and Aeromonas hydrophila predominated in all samples. In pond water, C. urealyticurn, S. putrefaciens, A. hydrophila, Flavobacterium sp. and Pseudomonas sp. were the most predominant bacterial species (prevalence > 10%), whereas A. hydrophila, C. urealyticum, S. putrefaciens and Escherichia coli were predominant in pond sediment, and C.

urealyticum, S. putrefaciens and A. hydrophila were predominant in both the gills and intestine of tilapia.

Son *et al.,* **(1997)** Observed Antibiotic resistance and plasmid profile of *Aeromonas hydrophila* isolates from cultured fish, Telapia (*Telapia mossambica*). Strains of Aeromonas hydrophila isolates from skin lesions of the common freshwater fish, Telapia mossambica, were screened for the presence of plasmid DNA by agarose gel electrophoresis and tested for susceptibility to 10 antimicrobial agents. Of the 21 fish isolates examined, all were resistant to ampicillin and sensitive to gentamycin. Most isolates were resistant to streptomycin (57%), tetracycline (48%) and erythromycin (43%). While seven of 21 isolates harboured plasmids, with sizes ranging from 3 to 63.4 kilobase pair (kb), it was only possible to associate the presence of a plasmid with antibiotic resistance (ampicillin and tetracycline) in strain AH11. Both the plasmid and the associated antimicrobial resistance could be transferred to an Escherichia coli recipient by single-step conjugation at a frequency of 4.3 x 10(-3) transconjugants per donor cell.

Al-Harbi (1994) Documented epizootic of streptococcicosis has been recorded among cultured hybrid tilapia (*Oreochromis niloticus × O. aureus*) in Saudi Arabia. Affected fish showed erratic swimming, melanosis, exophthalmia, haemorrhaging around the jaws and at the base of the pectoral and dorsal fins and tail, and the presence of ascitic fluid in the adbominal cavity. The causal agent was characterized biochemically, and identified as an α-haemolytic *Streptococcus* sp.

Kamei *et al.,* **(1985)** Isolated the anaerobic bacteria from the intestinal tracts of the freshwater fish Tilapia and identified as the genus Bacteroides were separated into two groups at the species level (Bacteroides ATS and BTS) according to morphological and physiological properties. The two groups are significantly different in NaCI requirement, optimal growth temperature, pH, metabolic products, and antibiotic susceptibility. They have little ability to degrade macromolecular compounds, except for some strains which hydrolyzed the starch or chitin. Bacteroides ATS was predominant in the intestines of all species of Tilapia throughout their lives, but Bacteroides BTS tended to increase in accordance with fish growth to a level similar to that of Bacteroides ATS.

Sugita *et al.,* **(1985)** Investigated Microflora in the contents of intestinal tract of carp, grass carp and tilapia by using 7 different agar media. The intestinal microflora varied

with the species of fish. The predominant bacteria in the intestinal tract of carp were *Aeromonas hydrophila, Bacteroides* type A, *Citrobacter freundii, Pseudomonas* and *Micrococcus.* The intestinal microflora of grass carp mainly consisted of A. hydrophila and *Bacteroides* type A. The intestinal microflora of tilapia was mainly composed of *Bacteroides* type A, *Bacteroides* type B, *Plesiomonas shigelloides* and *A. hydrophila.* The ratio of the obligate anaerobes to the facultative anaerobes plus aerobes of carp, grass carp and tilapia was 0.05 to 0.95, 0 to 0.49 and 32.4 to 111.1, respectively. It is suggested that the predominance of obligate anaerobes in the intestine of tilapia was due to their long intestine.

Silva and Widanapathirana (1984) Observed the bacteriological examination of the gastrointestinal microfiora of two fresh water cichlid fish species *(Sarotherodon mossambicus* and *Tilapia nilotica) that* was performed, resulting in the becteria enumeration of total viable counts of 1.06×10^7 /g and 7.75×10^7 /g of gastro- bacteria intestina tract plus contents (wet weight) respectively. The majority (78 %) of the total gut isolates from both fish species was Gram positive mesophilic which is characteristic of the higher ambient temperature in the tropics these isolates were fastidious in their nutritional requirements and together with the rest are isogenous to *bacteria autochthonous to* soil and water. The occurrence of such organisms is attributed to the feeding habits of these fish. The gastrointestinal bacteria isolated in this study are transient residents but not indigenous' in these cichlid fish.

CHAPTER 3

MATERIALS AND METHODS

This study was conducted in the Microbiology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh during the period from July to December, 2017. The details outline of Materials and Methods are given below:

3.1 Materials

3.1.1 Study Site

20 Fresh Tilapia fishes were collected randomly from respective local market of the Dinajpur city {Bahadur bazaar (10) and Gopalgong bazaar (10)}, Bangladesh. Then the samples were brought to the laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh for isolation and identification of bacterial pathogens with antibiogram study.

3.1.2 Collection of fish samples

A total number of 60 samples {gill (20), skin (20) and intestine (20)} of Tilapia (*Oreochromis niloticus*) fishes of different sizes 200 g -300 g and length 17 cm–23 cm were collected aseptically for the isolation and identification of bacteria.

Gills:

One to 2 cm^2 pieces of gills from Tilapia (*Oreochromis niloticus*) fishes were cut with sterile scissor and taken by sterile forceps for inoculation onto appropriate culture medium.

Intestines:

One to 2 cm proximal segments of intestines from Tilapia (*Oreochromis niloticus*) fishes were cut with sterile scissor and taken by sterile forceps for inoculation onto appropriate culture medium.

Skin:

Skin sample collected from Tilapia (*Oreochromis niloticus)* fishes with the help of cotton swab for inoculation onto appropriate culture medium.

3.2 Laboratory preparation

3.2.1 Sterilization

a. Flaming:

It was used to sterilize glass slides, cover slips, needles and scalpels.

b. Red heat:

It was used to sterile wire loop, points and searing spatulas by holding them over Bunsen burner flame until they became red-hot.

c. Hot air oven:

It was used to sterilize glass wares such as test tubes, graduated pipettes, flasks and forceps, and cotton swabs. The holding period was one hour and oven temperature was 160 °C.

d. Steaming at 100 °C

Repeated steaming (Tyndallization) was used for sterilization of sugars and media that could not be autoclaved without detriment effect to their constituents. It was carried out as described by Barrow and Feltham (1993).

e. Moist heat (autoclave):

Autoclaving at 121°C (15 Ib/inch^2) for 15 minutes was used for sterilization of media and plastic wares. Autoclaving at 115°C (10 Ib/inch^2) 40 for 10 minutes was used for sterilization of some media such as sugars.

3.3 Bacteriological Media used for culture

3.3.1 Liquid media

- Nutrient broth.

- 1% peptone water (Hi-media, India).

3.3.2 Solid media

- Nutrient agar base (Hi-media, India).
- MacConkey agar medium (Hi-media, India).
- Eosin methylene blue (EMB) agar (Hi-media, India).
- Salmonella-Shigella agar (Hi-media, India).
- Cetrimide agar (Hi-media, India).
- Mannitol Salt Agar (MSA) (Hi-media, India).
- Mueller Hinton Agar (Hi-media, India).

3.3.3 Media for biochemical test

- Triple sugar iron (TSI) agar slant (Hi-media, India).
- Motility, Indole, Urease (MIU) medium (Hi-media, India).
- Methyl Red (MR) media
- Voges-Proskauer (VP) media
- Simmons's Citrate agar media

3.3.4 Reagents

- Gram's staining reagent: Crystal violet, Gram's iodine, Acetone and Safranin.
- Alpha-naphthol solution.
- Kovac's reagent.
- Ethyl alcohol (70% and 95%).
- Phosphate Buffered Saline (PBS)
- Physiological Saline Solution (PSS)
- Methylene Blue stain
- Voges-Proskauer (VP) Solution
- Indol Solution
- Methyl Red (MR) Solution
- Potassium-di-hydrogen phosphate (0.2M, KH_2PO_4 $2H_2O$)
- Di-sodium hydrogen phosphate (0.2M, $Na_2HPO_4 12H_2O$)

3.4 Preparation of culture media

All the media, broth and reagents used in this experiment were prepared according to instruction of the manufacturer.

3.4.1 Liquid Media

3.4.1.1 Nutrient broth

The medium was prepared by adding 13 g of nutrient broth powder to one liter of distilled water and well mixed. The pH was adjusted to 7.4. The mixture was distributed in 5 ml volumes into clean bottles, and then sterilized by autoclaving at 121 °C (15 Ib/inch^2) for 15 minutes (Carter, 1979).

3.4.1.2 Peptone water:

This medium was prepared by dissolving 10 g of peptone water and 5g sodium chloride in 1litre of distilled water. The mixture was distributed in 5 ml volumes into clean bottles, and sterilized by autoclaving at 121°C (15lb/inch^2) for 15 minutes (Carter, 1979).

3.4.2 Solid media

3.4.2.1 Nutrient agar

28.0 grams of nutrient agar powder (Hi-media, India) was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile petridish and allowed to solidify. After solidification of the medium in the petridishes, these were incubated at 37°C for overnight to check their sterility and used for culture characterization (Carter, 1979).

3.4.2.2 MacConkey agar

51.50 grams of dehydrated Bacto-MacConkey agar (Difco) was suspended in 1000 ml of cold distilled water taken in a conical flask and was heated up to boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured sterile glass petridishes. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petridishes partially removed. The

sterility of the medium was judged and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.4.2.3 Eosin methylene blue agar

Eosin methylene blue (EMB) agar medium was used to observe the growth of *Escherichia coli* (Cheesebrough, 1985).

36 gram of EMB agar base (Hi-media, India) was added to 1000 ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured in to sterile glass. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petridishes partially removed. The sterility of the medium was judged and used or stored at 4°C in refrigerator for future use (Carter, 1979).

3.4.2.4 Salmonella Shigella Agar

Salmonella Shigella (SS) agar is the selective medium for the isolation of *Salmonella* and *Shigella*. 63.0 grams SS agar powder was dissolved in 1000 ml of distilled water. It was mixed well until a homogeneous suspension is obtained. It was heated with frequent agitation and boiled for one minute. It did not sterilized by autoclaved. It was cooled to 45ºC and 50°C and distributed in Petri plates and allow the medium to solidify partially uncovered. (HIMEDIA and Leifson *et al.,* 1935)

3.4.2.5 Mannitol salt agar

111.02 grams of Mannitol salt agar powder (Hi-media, India) was suspended in 1000 ml of distilled water in a flask and heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving. The precipitate was resuspended by gentle agitation to avoid bubbles and pour the plates while the medium is hot and allowed to solidify. Alternatively, the medium was cooled to 45-50°C. After solidification of the medium in the petridishes, these were incubated at 37°C for overnight to check their sterility and used for culture characterization (Carter, 1979).

3.4.2.6 Cetrimide agar

46.7 grams of Cetrimide agar powder (Hi-media, India) was suspended in 1000 ml of distilled water containing 10 ml glycerol in a flask and heated to boiling for dissolving

the medium completely. The medium was then sterilized by autoclaving. The precipitate was resuspended by gentle agitation to avoid bubbles and pour the plates while the medium is hot and allowed to solidify. Alternatively, the medium was cooled to 45-50°C. After solidification of the medium in the petridishes, these were incubated at 37°C for overnight to check their sterility and used for culture characterization (Carter, 1979).

3.4.2.7 Mueller Hinton Agar

Mueller Hinton Agar is used in antimicrobial susceptibility testing by the disk diffusion method. 38 grams of Mueller Hinton agar powder was suspended in 1000 ml of distilled water. It was mixed well. It was heated agitating frequently and boiled for about one minute. It was dispensed and sterilized in autoclave at 116 - 121°C (15 lbs. sp) for 15 minutes. It was cooled to 45° or 50°C (Carter, 1979).

3.4.2.8 MIU medium

18 grams of MIU agar (Difco) was suspended in 950 ml of cold distilled water taken in a conical flask and heated up to boiling to dissolve the medium completely. Ninety five ml was dispensed into flasks and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Then was Cooled to about 50-55°C and aseptically add 5ml was added of sterile 40% basal medium. After mixing were dispensed into sterile test tubes. Allow to cool in an upright position. The sterility of the medium was judged and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.5 Reagents preparation

3.5.1 Methyl Red-Voges Proskauer broth

A quantity of 3.4 grams of Bacto MR-VP medium was dissolved in 250 ml of distilled water dispensed in 2 ml amount in each test tube and then the test tubes were autoclaved. After autoclaving, the tubes containing medium were incubated at 37°C for overnight to check their sterility and used for biochemical characterization or stored at 4°C in refrigerator for future use (Cheesbrough, 1984).

3.5.2 Methyl red solution

The indicator methyl red (MR) solution was prepared by dissolving 0.1 gm of Bacto methyl red (Difco) in 300 ml of 95% alcohol and diluting this to 500 ml with the addition of 200 ml of distilled water (Cheesbrough, 1984).

3.5.3 Voges-Proskauer solution

3.5.4 Alpha-naphthol solution

Alpha-naphthol solution was prepared by dissolving 5 grams of 1-naphthol in 100 ml of 95% ethyl alcohol (Cheesbrough, 1984).

3.5.5 Potassium hydroxide solution

Potassium hydroxide (KOH) solution was prepared by adding 40 grams of potassium hydroxide crystals in 100 ml of cold distilled water (Cheesbrough, 1984).

3.5.6 Kovac's reagent

This solution was prepared by mixing 25 ml of concentrated Hydrochloric acid in 75 ml of amyl alcohol and to this mixture 5 grams of paradimethyl- aminohenzyldehide crystals were added. This was then kept in a flask equipped with rubber cork for future use (Merchant and Packer, 1967).

3.5.7 Phosphate buffered saline solution

For preparation of Phosphate buffered saline (PBS) solution, 8 gram of sodium chloride (NaCl), 2.89 gram of disodium hydrogen phosphate (Na_2HPO_4, $12H_2O$), 0.2 gram of potassium chloride (KCl) and 0.2 gram of potassium hydrogen phosphate were suspended in 1000 ml of distilled water. The solution was heated to dissolve completely. The solution was then sterilized by autoclave at 121 °C maintaining a pressure of 15 pounds per square inch for 15 minutes and stored at refrigerator until use. The pH of the solution was measured by a pH meter and maintained at 7.0-7.2 (Cheesbrough, 1984).

3.6 Antibacterial discs

The sensitivity of isolates to antibiotics was determined by disc diffusion technique. The isolates were cultured into peptone water and incubated at 37°C for two hours. A Petri dish containing Muller hinton Agar (MHA) medium, was put in the incubators for 30 minutes to dry and then inoculated with 2 ml volume of the test culture .The inoculated culture was evenly distributed by rotation, the excess inoculums was withdrawn by sterile Pasteur pipette and the plate was left to dry at room temperature for 15 minutes. To determine the drug sensitivity pattern of different isolated bacteria different types of commercially available antibiotic discs (Oxoid Ltd., UK) were placed on the surface of

the inoculated medium with a sterile forceps and pressed gently to ensure good contact with the surface of the medium. The plates were then incubated at 37°C. The followings are the antibiotics that were tested against the selected organism.

Table 1: Antimicrobial agents with their disc concentration

Sl. No.	Name of antibiotics	Disc concentration (µg/disc)
1.	Gentamicin (GEN)	10 µg/disc
2.	Amoxicillin (AMX)	30 µg/disc
3.	Chloramphenicol (C)	30 µg/disc
4.	Ciprofloxacin (CIP)	5 µg/disc
5.	Cefixime (CEF)	5 µg/disc
6.	Azithromycin (AZM)	30 µg/disc
7.	Erythromycin (E)	15 µg/disc
8.	Penicillin G (P)	10 µg/disc
9.	Neomycin (N)	30 µg/disc
10.	Vancomycin (VA)	30 µg/disc

Legend: µg = Microgram

3.7 Methods

3.7.1 Experimental layout

The experimental layout illustrated in figure 1. The entire study is divided into two steps. The first step includes the isolation of bacteria from the collected Tilapia fish samples. The identification were made according to their cultural, staining morphological biochemical properties. The second step is study of antibacterial sensitivity pattern of the isolated bacteria.

Experimental Layout
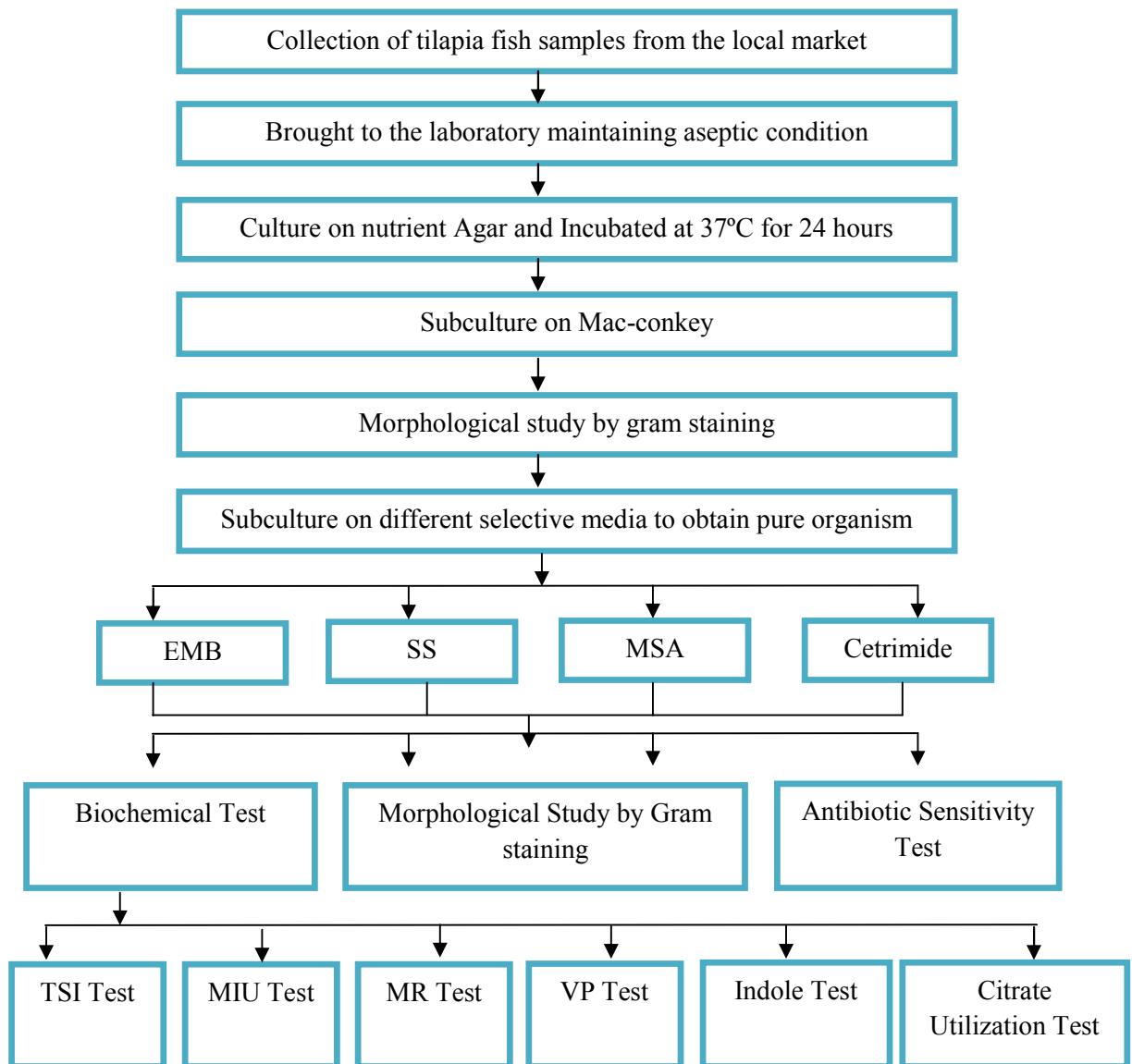
Figure 1: Schematic illustration of experimental layout

3.7.2 Sample collection and sample processing

Tilapia fish samples (60) from local fish market of Dinajpur city (Bahadur bazaar, Railgate bazaar and Gopalgong bazaar) were collected aseptically and brought to the bacteriology laboratory, Department of Microbiology, HSTU, Dinajpur with necessary precautions for bacteriological examination. At first, Samples were rinsed thoroughly with sterile distilled water. Then tilapia fish (gill, skin and intestine) samples were homogenized through blending with 90 ml peptone water (Cappuccino and Sherman, 1996). Then 1-10 fold dilutions were performed.

3.7.3 Dilution of Sample

Serial 10 fold dilutions of each of the samples in a series of dilution tubes were prepared. At first for each of the processed samples 10 sterile test tubes were placed on a test tube holder rack containing 9 ml of 2% buffered peptone water.

1 ml processed sample was mixed with 9 ml of Phosphate buffer solution in the 1st test tube in order to make 10^{-1} dilution. Then 1ml solution from 1st test tube mixed with 2^{ndt} test tube, then from 2nd test tube to 3rd test tube and finally 9th to 10th test tube and 1ml discard from 10th test tube by the help of pipette and in every steps mixing was done properly.

3.7.4 Isolation and identification of bacteria

3.7.4 .1Culture of tilapia fish sample

Media such as Nutient agar, Nutrient broth, MacConkey agar, Eosin Methylene Blue agar (EMB), Salmonella-Shigella (SS) agar, Mannitol salt agar **(**MSA), and Cetrimide Agar were used.

3.7.4.2 Culture in ordinary media

Samples were inoculated separately into ordinary media like nutrient agar, nutrient broth and were incubated at 37°C for overnight. The colonies on primary cultures were repeatedly sub-cultured by streak plate method (Cheesbrough, 1984) until the pure culture with homogenous colonies were obtained.

3.7.4.3 Isolation of bacteria in pure culture

For isolation of bacteria in pure culture, the mixed culture was inoculated into nutrient agar media by streak plate technique to obtain isolated colonies as per:

Step-1: An inoculum was picked up with a sterile loop and spread on an area of the medium in the petridish.

Step-2: The loop was sterilized by being heated as red hot in a flame.

Step-3: The inoculum was spread over the reminder of the plate by drawing the cooled parallel line.

This method was repeated as many times as necessary to obtain a culture containing only one type of colony and usually at least two more times to ensure purity.

3.7.4.4 Culture into differential media

3.7.4.4.1 Mac-Conkey agar

Samples were sub-culture on Mac-conkey agar media and incubated at 37°C for overnight. After that both lactose fermenter bacteria (rose pink color colony) and lactose non fermenter bacteria (pale color colony) were selected.

3.7.4.5 Culture on selective media

3.7.4.5.1 Eosin Methylene Blue (EMB) agar

Samples of positive lactose fermenter were taken and sub-culture on EMB agar media and incubated at 37°C for overnight.

Some EMB agar plate showed slightly circular colonies with dark center metallic sheen. Also in some EMB agar, the growth was indicated by smooth, characteristics mucoid and pink colored colonies which are a consequence of the organism's abundant polysaccharide capsule.

3.7.4.5.2 Salmonella -Shigella agar

Sample of non-lactose fermenter were taken and sub-culture on SS agar media and incubated at 37°C for overnight, which after inoculation, raised, black centered, smooth round colony was present.

3.7.4.5.3 Mannitol salt agar (MSA)

Materials from nutrient agar were inoculated into Mannitol salt agar containing plates and incubated at 37°C for overnight, which after inoculation, raised, yellow, smooth colony was present.

3.7.4.5.4 Cetrimide agar

Samples were inoculated into Cetrimide agar plates which after incubation, the growth was indicated by smooth, Characteristics green pigmented colonies.

3.7.4.6 Microscopic examination

Smears were made from each type of colony on primary culture and from purified colonies. Then fixed by heating and stained by Gram staining method as described by Barrow and Feltham (1993). The stained smears were examined microscopically under oil immersion lens for cell morphology, cell arrangement and staining reaction.

3.7.4.7 Identification of isolates

The purified isolates were identified according to criteria described by Barrow and Feltham (1993). This included staining reaction, cell morphology, cultural characteristics and biochemical characteristics.

3.7.4.8 Biochemical methods for identification of isolated bacteria:

All biochemical tests were performed as described by Barrow and Fellham (1993). They included the following tests:

3.7.4.8.1 Indole production test

The test culture was inoculated into peptone water and incubated at 37°C for 48 h. One ml of Kovacs reagent was added to the tube. The appearance of a pink color in the reagent layer within a minute indicated positive reaction.

3.7.4.8.2 Methyl red test

The test culture was inoculated into glucose phosphate medium and then incubated at 37°C for 48 h. Two drops of methyl red indicator were added and shaken well. A red color indicated positive reaction. Yellow or orange colors indicated negative reaction.

3.7.4.8.3 Voges- Proskauer test

The test culture was inoculated into glucose phosphate medium and incubated at 37°C for 48 h. One ml of cultured medium was transferred aseptically into sterile test tubes, and then 0.6 m1 of 5% alpha-naphthol solution was added, followed by 0.2 m1 of 40% KOH aqueous solution. The test tube was shaken well and kept at slant position for 1 h. A positive reaction was indicated by strong red color.

3.7.4.8.4 Citrate utilization test

The test culture was inoculated onto Simmon's citrate medium, then incubated at 37° C, and examined daily for 7 days. Blue color indicated a positive reaction.

3.7.4.8.5 Motility test

The test culture was inoculated by stabbing with straight wire into the Cragie tube in motility medium and then incubated at 37°C for 24 h. The organism was considered motile if there was turbidity in the medium in and outside the Cragie tube while the growth of nonmotile organism confined inside Cragie tube.

3.8 Antibiotic susceptibility test

Susceptibility of isolates of *Escherichia coli*, *salmonella spp, Staphylococcus spp* and *Pseudomonas spp.* To different antimicrobial agents was performed to determine the drug sensitivity pattern and to interpret their disease potential. This method allows for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that results from diffusion of the agent into the medium throught the disc. The overnight nutrient broth cultured organism spread uniformly with the help of sterile glass spreader. Antibacterial disc were applied aseptically to the surface of the plate at an appropriate arrangement with the help of sterile forceps and incubated at hours, aerobically (Carter, 1979).Antibiotic sensitivity pattern of isolated *Escherichia coli*, *salmonella spp, Staphylococcus spp* and *Pseudomonas spp* were performed against 10 commonly used antibiotics belonging to different groups. After incubation plates were arnined and diameters of the zones of inhibition for individual antibacterial agents were designated as sensitive, intermediate and resistant.

3.8.1 Reading Plates and Interpreting Results

After 24 hours of incubation, each plate was examined. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones oh inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies were apparent, the inoculum was too light and the test must be repeated. The diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disc. Zones were measured to the nearest whole millimeter, using sliding calipers or a ruler, which was held on the back of the inverted Petri plate. The Petri plate was held a few inches above a black, nonreflecting background and zones are measured in millimeter (mm) from the upper surface of the agar illuminated with reflected light, with the cover removed (EUCAST, 2015).

3.9 Maintenance of stock culture

3.9.1 Agar slant method

The stock culture was maintained following the procedures of choudhury *et al.* (1987) isolated and identified bacteria were inoculated into nutrient agar slants and incubated at 37°C for 24 hours and then examine for growth. One slant was used for an individual isolate. Then the sterile mineral oil was poured into the tube until the colonies were covered completely. The tube were sealed off with paraffin and kept at room temperature for future use seed. By this method, bacteria can be preserved with no deviation of their original charecters for few months (Buxton and Fraser, 1977)

3.9.2 Sterile buffered glycerin method

Sterile buffered glycerin (20%) was prepared by mixing 20 parts of pure glycerin and 80 parts of PBS. Then aloopful of thick bacterial culture was mixed with 20% Sterile buffered glycerin in small vials and was preserved at -20°C.This method is more appropriate for preserving bacteria with no deviation of their original characters for several years (Buxton and Fraser, 1977).

CHAPTER 4

RESULTS

The results describe the isolation, identification and characterization of bacteria isolated from different part of the Tilapia fish. Results obtained from the study are presented in the Tables 2 to 14 and described below under the following headings:

4.1 Isolation and identification of bacteria:

A total of 60 samples, 20 gill, 20 skin and 20 intestine samples of apparently Healthy Tilapia fishes were collected from the local fish markets of Dinajpur city, Bangladesh. (Table 2).

All the collected samples showed bacterial growth on Nutrient agar and MacConkey agar medium. The 60 samples gave 77 isolates. The isolates were both Gram negative bacteria and Gram positive bacteria. The isolates were 31 (40.26%) *Escherichia coli*, 10(12.99%) *salmonella spp.*, 23 (29.87%) *Staphylococcus spp.* and 13 (16.88%) *Pseudomonas spp.* (Table 3).

4.1.1 Bacteria isolated from gills

The number of samples collected from gills was 20 samples. Out of these 20 samples, all samples showed positive growth, and they yielded 22 isolates. The 22 bacterial isolates comprised both Gram-negative and Gram positive bacteria.

The 22 isolates bacteria were 9 (40.91%) *Escherichia coli*, 4(18.18%) *salmonella spp.*, 6(27.27%) *Staphylococcus spp.* and 3(13.64%) *Pseudomonas spp.* (Table 4)

4.1.2 Bacteria isolated from intestine:

The number of samples collected from intestine was 20 samples. Out of these 20 samples, all samples showed positive growth, and they yielded 26 isolates. The 26 bacterial isolates comprised both Gram-negative and Gram positive bacteria.

The 26 isolates bacteria were 10 (38.46%) *Escherichia coli*, 3 (11.55%) *salmonella spp*, 9 (34.62%) *Staphylococcus spp,* and 4 (15.38%) *Pseudomonas spp.* (Table 5).

4.1.3 Bacteria isolated from skin

The number of samples collected from skin was 20 samples. Out of these 20 samples, all samples showed positive growth, and they yielded 29 isolates. The 29 bacterial isolates comprised both Gram-negative and Gram positive bacteria.

The 29 isolates bacteria were 12 (41.38%) *Escherichia coli*, 3 (10.34%) *salmonella spp.*, 8 (27.59%) *Staphylococcus spp.* and 6 (20.69%) *Pseudomonas spp.* (Table 6).

Table 2: Summary of samples collected from Tilapia Fish

Samples	Number of Samples	Grand Total
Gill	20	
Intestine	20	60
Skin	20	

Table 3: Summary of isolated bacteria from different samples of Tilapia fish

Samples	No. of isolated bacteria			
	Escherichia coli	*Salmonella spp*	*Staphylococcus spp*	*Pseudomonas spp*
Gills (20)	9	4	6	3
Intestine (20)	10	3	9	4
Skin (20)	12	3	8	6
Total	31	10	23	13

Table 4: Frequency of occurrence of Bacteria isolated from 20 Gill samples of Tilapia fish

Bacterial species	Number of isolates	Isolation Percentage
Escherichia coli	9	40.91%
Salmonella spp	4	18.18%
Staphylococcus spp	6	27.27%
Pseudomonas spp	3	13.64%
Total	22	100%

Table 5: Frequency of occurrence of Bacteria isolated from 20 Intestine samples of Tilapia fish

Bacterial species	Number of isolates	Isolation Percentage
Escherichia coli	10	38.46%
Salmonella spp	3	11.54%
Staphylococcus spp	9	34.62%
Pseudomonas spp	4	15.38%
Total	26	100%

Table 6: Frequency of occurrence of Bacteria isolated from 20 Skin samples of Tilapia fish

Bacterial species	Number of isolates	Isolation Percentage
Escherichia coli	12	41.38%
Salmonella spp	3	10.34%
Staphylococcus spp	8	27.59%
Pseudomonas spp	6	20.69%
Total	29	100%

4.2 Cultural, microscopic and biochemical reactions of the isolates

4.2.1 Results of cultural examinations

4.2.1.1 Ordinary media

4.2.1.1.1 Nutrient agar

Pale colorless colony was found.

4.2.1.2 Differential media

4.2.1.2.1 MacConkey agar

MacConkey agar plates streaked separately with the organisms from nutrient agar revealed the growth of bacteria after 24 hours of incubation at 37°C aerobically.

The growth of lactose fermenting organisms was indicated by bright pink colored colonies of on MacConkey agar.

The growth of non-lactose fermenting organisms was indicated by pale colored colonies of on MacConkey agar.

4.2.1.3 Selective media

4.2.1.3.1 Eosin methylene blue (EMB) agar

EMB agar plates streaked separately with the lactose fermenter organisms from MacConkey agar revealed the growth of *E. coli* bacteria after 24 hours of incubation at 37°C aerobically.

The growth of *E.coli* was indicated by smooth, circular, black color colonies with metallic sheen on the agar plate.

4.2.1.3.2 Salmonella- Shigella (SS) agar

SS agar plates streaked separately with the non-lactose fermenting organisms from MacConkey agar revealed the growth of *Salmonella* spp. after 24 hours of incubation at 37°C aerobically.

The growth of *Salmonella* spp. was indicated by smooth, colorless, usually with black center.

4.2.1.3.3 Cetrimide (CET) Agar

CET agar plate streaked organisms from MacConkey agar revealed the growth of *Pseudomonas spp.* after 24 hours of incubation at 37°C aerobically.

The growth of *Pseudomonas spp.* was indicated by green pigment colonies.

4.2.1.3.4 Manitol Salt Agar (MSA)

MSA agar plate streaked separately with the organisms from MacConkey agar revealed the growth of *Staphylococcus spp.* after 24 hours of incubation at 37°C aerobically.

The growth of *Staphylococcus spp.* was indicated by pale yellowish colonies.

4.2.1.4 Results of gram's staining

The microscopic examination of Gram's stained smears from EMB agar revealed Gram-negative, pink colored, small rod shaped *E. coli* arranged in single, pairs or short chain.

The microscopic examination of Gram's stained smears from SS agar revealed Gram-negative, pink colored, small rod shaped *Salmonella spp.* arranged in single, pairs or short chain.

The microscopic examination of Gram's stained smears from CET agar revealed Gram-negative, rod shaped, motile *Pseudomonas spp.* arranged in single, pairs or short chain.

The microscopic examination of Gram's stained smears from MSA agar revealed Gram-positive, parple colored, small cocci shaped *Staphylococcus spp.* arranged in pairs or clusters.

4.2.1.5 Results of biochemical tests:

The isolated organisms were confirmed by different biochemical tests.

Table 7: Identification of *E. coli* by different biochemical tests

Biochemical test	Change of the media	Results
Citrate utilization test	No color change	Negative
Indole test	Pink color ring at the top of the media	Positive
Triple sugar iron (TSI) test	S-yellow, B-yellow	S-A, B-A, gas (+), H_2S (-)
MR test	Red color	Positive
VP test	No color change	Negative
MIU test	Turbidity and changing of color of media	Positive

(Legends: S=Slant, B=Butt, A = Acid, MR = Methyl-Red test, VP = Voges-Proskauer test, MIU= Motility Indole Urease**,** + = Positive reaction, - = Negative reaction).

Table 8: Identification of *Salmonella spp.* by biochemical test

Biochemical test	Change of the media	Results
Citrate utilization test	No color change	Negative
Indole test	No color change	Negative
Triple sugar iron (TSI) test	S-Red, B-yellow	S-Al, B-A, gas(+), H_2S (+)
MR test	Red color	Positive
VP test	No color change	Negative
MIU test	No turbidity and no changing of color of media	Negative

(Legends: S=Slant, B=Butt, A = Acid, Al- Alkaline, MR = Methyl-Red test, VP = Voges-Proskauer test, MIU= Motility Indole Urease**,** + = Positive reaction, - = Negative reaction).

Table 9: Identification of *Staphyloccus spp.* by biochemical test

Biochemical test	Change of the media	Results
Citrate utilization test	No color change	Negative
Indole test	No color change	Negative
Triple sugar iron (TSI) test	S-Red, B-yellow	S-Al, B-A, gas(-), H_2S (-)
MR test	red color	Positive
VP test	Red color	Positive
MIU test	No turbidity and no changing of color of media	Negative

(Legends: S=Slant, B=Butt, A = Acid, Al- Alkaline, MR = Methyl-Red test, VP = Voges-Proskauer test, MIU= Motility indole urease**,** + = Positive reaction, - = Negative reaction).

Table 10: Identification of *Pseudomonas spp*. by biochemical test

Biochemical test	Change of the media	Results
Citrate utilization test	Prussian blue color	Positive
Indole test	No color change	Negative
Triple sugar iron (TSI) test	S-yellow, B-yellow	S-A, B-A, gas (+), H_2S (-)
MR test	No color change	Negative
VP test	No color change	Negative
MIU test	Turbidity and changing of color of media	Positive

(Legends: S=Slant, B=Butt, A = Acid, Al- Alkaline, MR = Methyl-Red test, VP = Voges-Proskauer test, MIU= Motility indole urease**,** + = Positive reaction, - = Negative reaction).

4.6 Results of antibiotics sensitivity tests

Antimicrobial susceptibility testing was performed using Muller-Hinton agar (Mumbai, India) plates as recommended by the Clinical and Laboratory Standards Institute. Four (4) isolates of *E. coli, Salmonella spp, Staphylococcus spp,* and *Pseudomonas spp* were subjected to antibiotic sensitivity tests. The results of antibiotics sensitivity tests are presented in Table 11, 12, 13 and 14.

Table 11: Results of antibiotic sensitivity test of *E. coli*

Antibacterial agents	Disc concentration (μg/disk)	Diameter of zone of inhibition(mm)			Interpretation
		Sensitive	Intermediate	Resistance	
Gentamicin (GEN)	10 μg/disc	19	-	-	S
Amoxicillin (AMX)	30 μg/disc	-	-	-	R
Chloramphenicol (C)	30 μg/disc	22	-	-	S
Ciprofloxacin (CIP)	5 μg/disc	-	-	-	R
Cefixime (CFM)	5 μg/disc	-	-	-	R
Azithromycin (AZM)	30 μg/disc	18	-	-	S
Erythromycin (E)	15 μg/disc	-	-	-	R
Penicillin G (P)	10 μg/disc	-	-	-	R
Neomycin (N)	30 μg/disc	-	15		I
Vancomycin (VA)	30 μg/disc	-	-	-	R

The antibiotic sensitivity test revealed that all of the isolated *E. coli* was resistant to Amoxicillin, Ciprofloxacin, Cefixime, Erythromycin, Penicillin G and Vancomycin. The isolates were sensitive to Gentamicin, Azithromycin and Chloramphenicol.

S = Sensitive; R = Resistant; - = No zone of inhibition.

Table 12: Results of antibiotic sensitivity test of *Salmonella spp.*

Antibacterial agents	Disc concentration (µg/disk)	Diameter of zone of inhibition(mm)			Interpretation
		Sensitive	Intermediate	Resistance	
Gentamicin (GEN)	10 µg/disc	23	-	-	S
Amoxicillin (AMX)	30 µg/disc	-	-	-	R
Chloramphenicol (C)	30 µg/disc	-	-	-	R
Ciprofloxacin (CIP)	5 µg/disc	-	16	-	I
Cefixime (CFM)	5 µg/disc	22	-	-	S
Azithromycin (AZM)	30 µg/disc	24	-	-	S
Erythromycin (E)	15 µg/disc	-	-	-	R
Penicillin G (P)	10 µg/disc	-	-	-	R
Neomycin (N)	30 µg/disc	18	-	-	S
Vancomycin (VA)	30 µg/disc	-	-	-	R

The antibiotic sensitivity test revealed that all of the isolated *Salmonella spp* was resistant to Amoxicillin, Erythromycin, Penicillin G, Chloramphenicol and Vancomycin. The isolates were sensitive to Gentamicin, Cefixime, Azithromycin and Neomycin.

S = Sensitive; R = Resistant; - = No zone of inhibition.

Table 13: Results of antibiotic sensitivity test of *Staphylococcus spp*

Antibacterial agents	Disc concentration (μg/disk)	Diameter of zone of inhibition(mm)			Interpretation
		Sensitive	Intermediate	Resistance	
Gentamicin (GEN)	10 μg/disc	21	-	-	S
Amoxicillin (AMX)	30 μg/disc	-	-	-	R
Chloramphenicol (C)	30 μg/disc	18	-	-	S
Ciprofloxacin (CIP)	5 μg/disc	22	-	-	S
Cefixime (CFM)	5 μg/disc	21	-	-	S
Azithromycin (AZM)	30 μg/disc	22	-	-	S
Erythromycin (E)	15 μg/disc	-	18	-	I
Penicillin G (P)	10 μg/disc	-	-	-	R
Neomycin (N)	30 μg/disc	-	14	-	I
Vancomycin (VA)	30 μg/disc	-	-	12	R

The antibiotic sensitivity test revealed that all of the isolated *Staphylococcus spp.* was resistant to Amoxicillin, Penicillin G, And Vancomycin. The isolates were sensitive to Gentamicin, Cefixime, Ciprofloxacin, Azithromycin and Chloramphenicol.

S = Sensitive; R = Resistant; - = No zone of inhibition.

Table 14: Results of antibiotic sensitivity test of *Pseudomonas spp*

Antibacterial agents	Disc concentration (μg/disk)	Diameter of zone of inhibition(mm)			Interpretation
		Sensitive	Intermediate	Resistance	
Gentamicin (GEN)	10 μg/disc	19	-	-	S
Amoxicillin (AMX)	30 μg/disc	-	-	-	R
Chloramphenicol (C)	30 μg/disc	-	-	8	R
Ciprofloxacin (CIP)	5 μg/disc	39	-	-	S
Cefixime (CFM)	5 μg/disc	-	-	-	R
Azithromycin (AZM)	30 μg/disc	20	-	-	S
Erythromycin (E)	15 μg/disc	-	-	-	R
Penicillin G (P)	10 μg/disc	-	-	-	R
Neomycin (N)	30 μg/disc	-	-	12	R
Vancomycin (VA)	30 μg/disc	-	-	-	R

The antibiotic sensitivity test revealed that all of the isolated *Pseudomonas spp. was* resistant to Amoxicillin, Chloramphenicol, Cefixime, Erythromycin, Penicillin G, Neomycin and Vancomycin. The isolates were sensitive to Gentamicin, Azithromycin, and Ciprofloxacin.

S = Sensitive; R = Resistant; - = No zone of inhibition**.**

Figure 2: Tilapia fish

Plate 1: Gills

Plate 2: Intestine

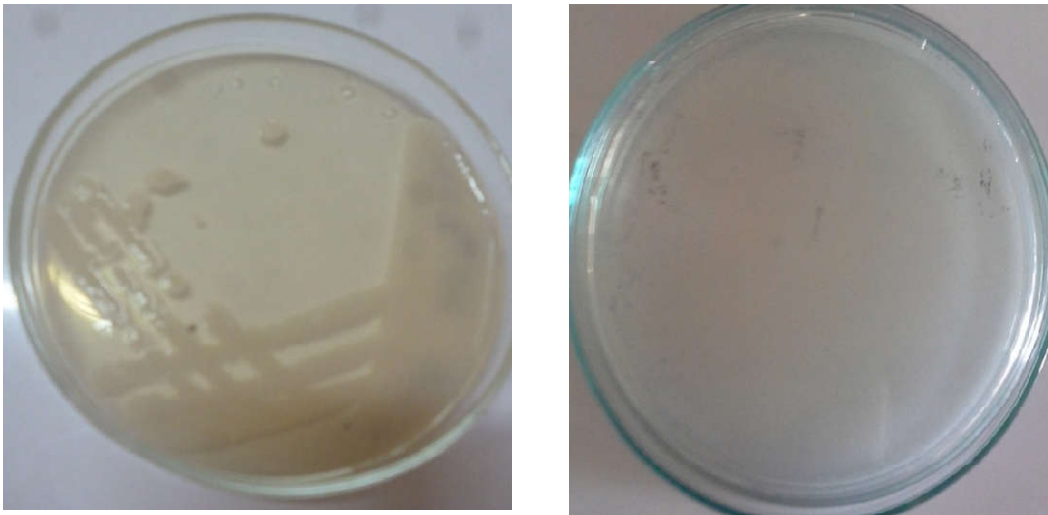**Plate 3:** Tenfold dilution of fish samples

Plate 4: Pale colored colonies on nutrient agar (left) and uninoculated control (right)

Plate 5: Bright pink colored colonies on MacConkey agar (left) and uninoculated control (right) indicating lactose fermenting bacteria.
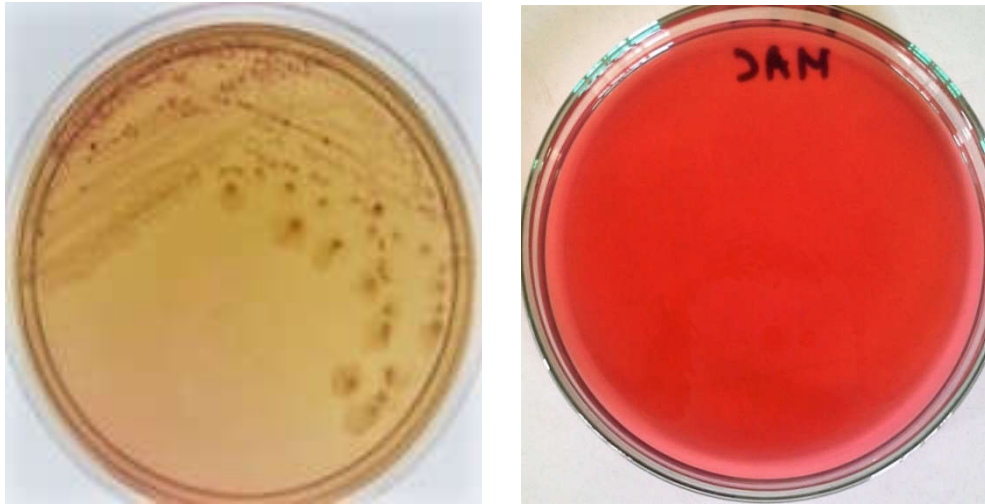
Plate 6: Pale colored colonies on MacConkey agar (left) and uninoculated control (right) indicating non-lactose fermenting bacteria.
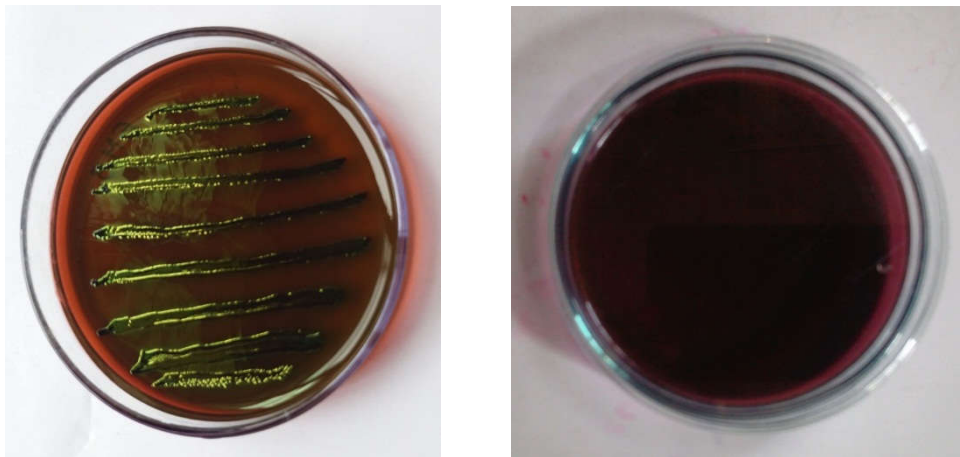
Plate 7: Metallic sheen produced by *E. coli* on EMB agar (left) and uninoculated control (right).

Plate 8: Black centers colonies produced by *Salmonella* spp. on SS agar (left) and un-inoculated control (right).

Plate 9: Yellow colonies produced by *Staphylococcus* spp. on MSA agar (left) and uninoculated control (right).
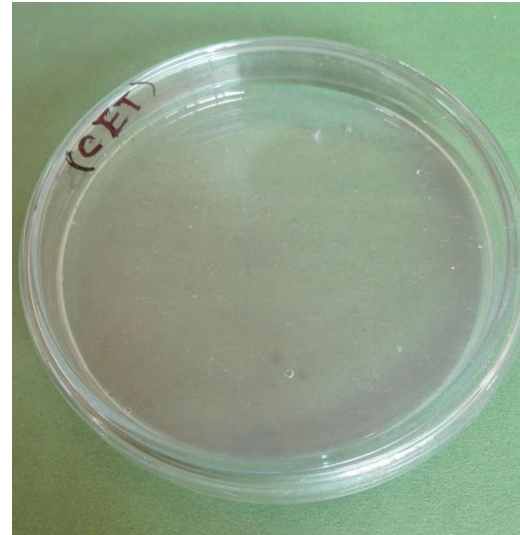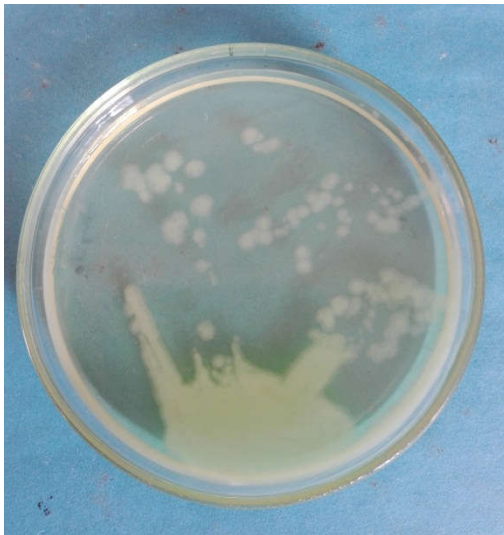
Plate 10: Green pigment colonies produced by *Pseudomonas spp.* on Cetrimide agar (left) and uninoculated control (right).
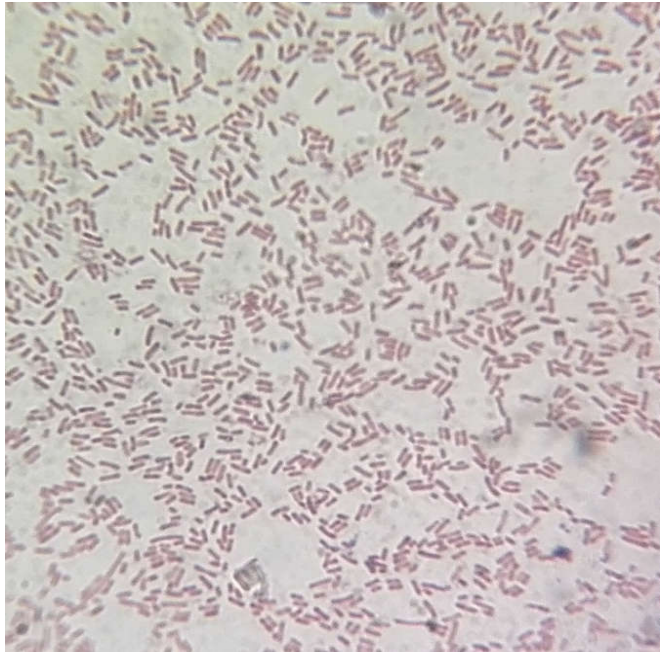
Plate 11: *E.coli* showing Gram negative short rods arranged singly or paris
(Gram's staining)
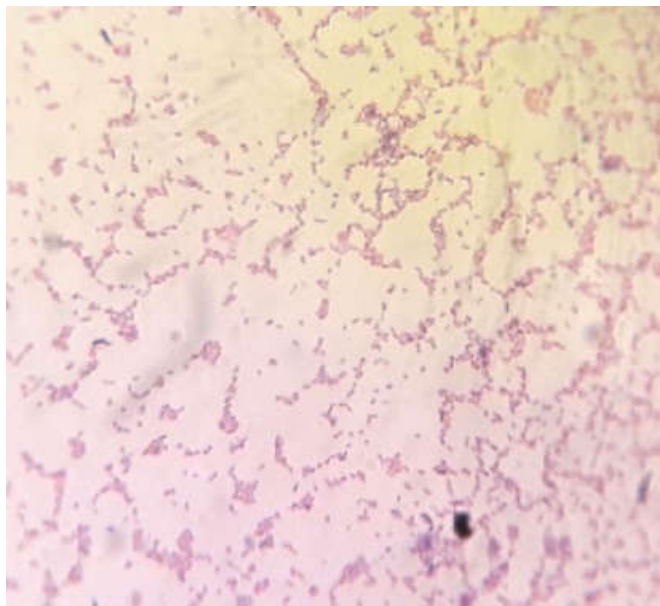
Plate 12: *Salmonella spp.* showing Gram negative very short plum rods arranged singly
or paris (Gram's staining)
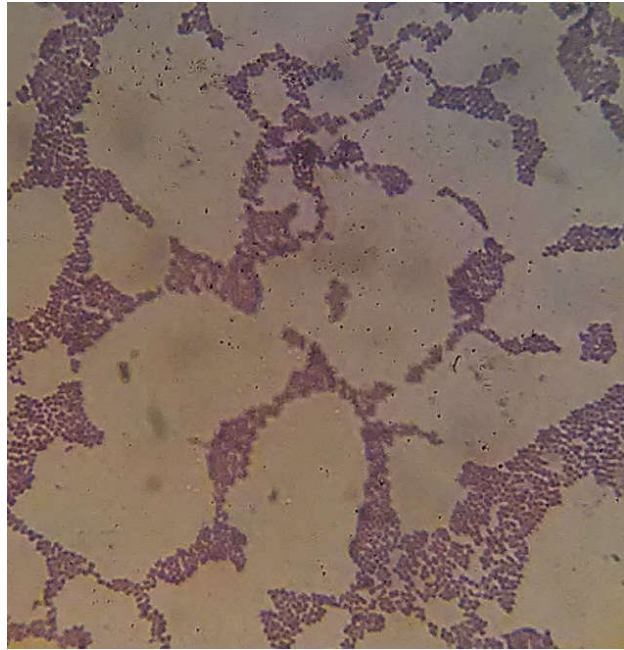
Plate 13: *Staphylococcus spp.* showing Gram positive small coccus arranged in cluster (Gram's staining)
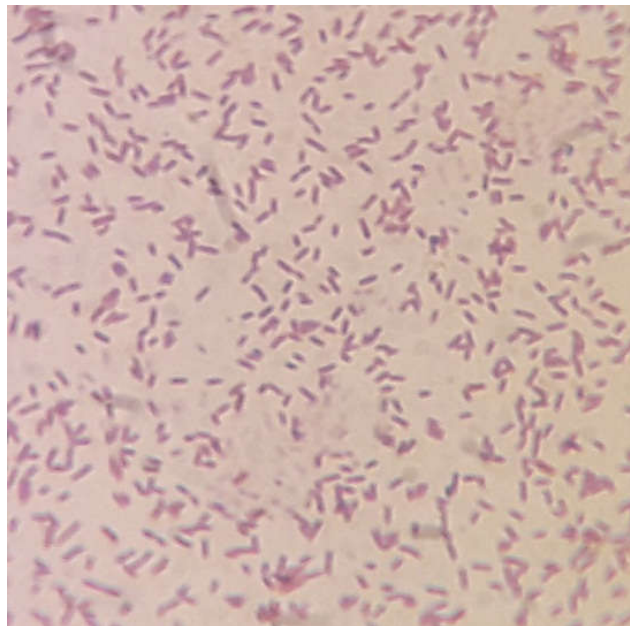
Plate 14: *Pseudomonas spp.* showing Gram negative small rods arranged singly or paris (Gram's staining)
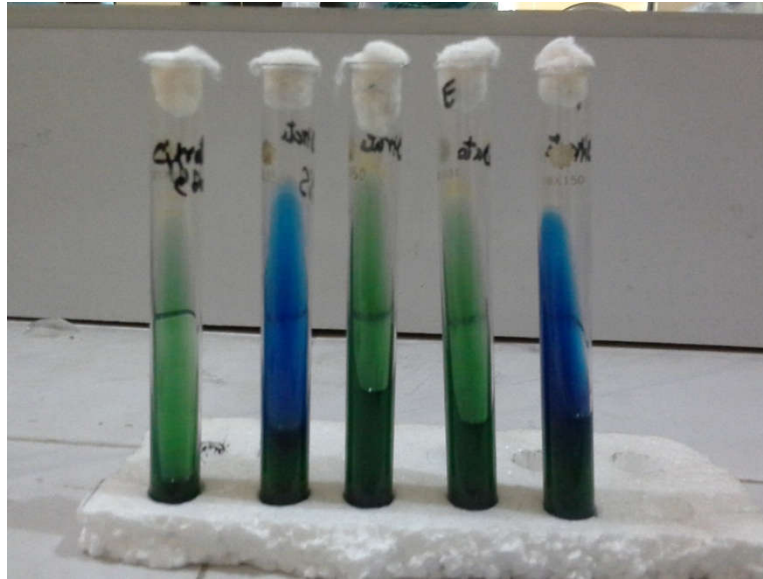
Plate 15: Citrate utilization test results (right) *Salmonella spp.* (positive), *E. coli* (negative), *Staphylococcs spp.* (negative*), Psedomonas spp.* (positive) and uninoculated control (left).

Plate 16: TSI test results (right) *Pseudomonas spp.* [S-Al, B-A, gas(-), H2S (-)], *E. coli* [S-A, B-A, gas (+), H_2S (-)], *Staphylococcus spp.* [S-A, B-A, gas (+), H_2S (-)] , *Salmonella spp.* [S-Al, B-A, gas(+), H_2S (+)]and uninoculated control (left).

Plate 17: Indole test results (right) *Salmonella spp.* (negative), *Staphylococcus spp.* (negative) , *E.* coli (positive), *Pseudomonas spp.* (negative), and uninoculated control (left).
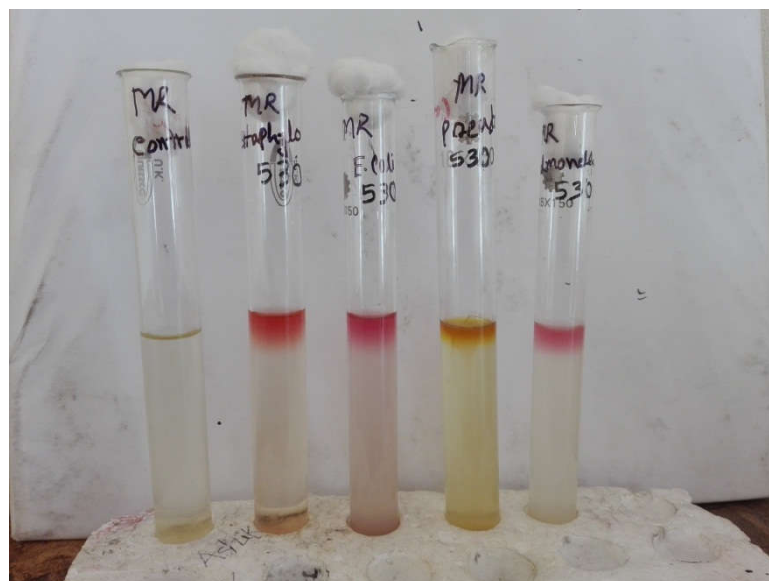
Plate 18: MR test results (right) *Staphylococcus spp.* (positive),   *E.* coli (positive), *Pseudomonas spp.* (negative) and *Salmonella spp.* (positive) and uninoculated control (left).
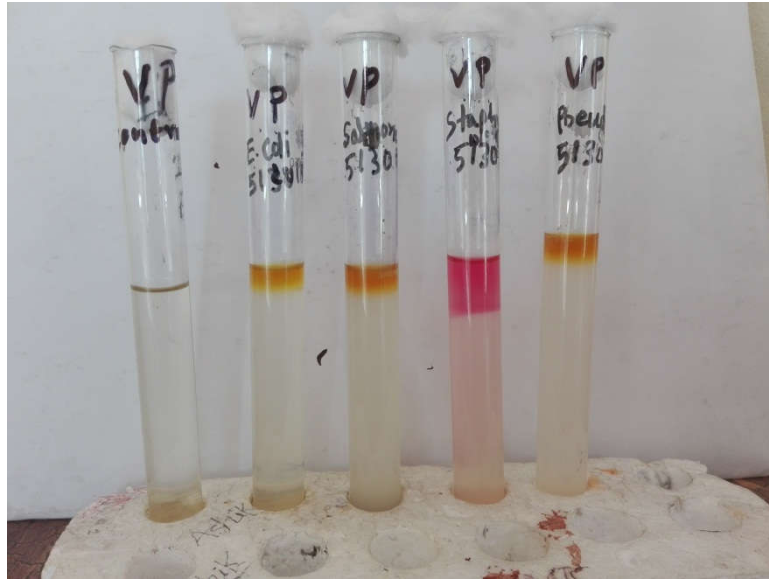
Plate 19: VP test results (right) *E.* coli (negative), *Salmonella spp.* (negative*)*, *Staphylococcus spp*. (positive), *Pseudomonas spp*. (negative) and uninoculated control (left).

Plate 20: MIU test results (right*) Staphylococcus spp.* (negative),   *Pseudomonas spp.* (positive), *E.* coli (positive), *Salmonella spp*. (negative) and uninoculated control (left).

53
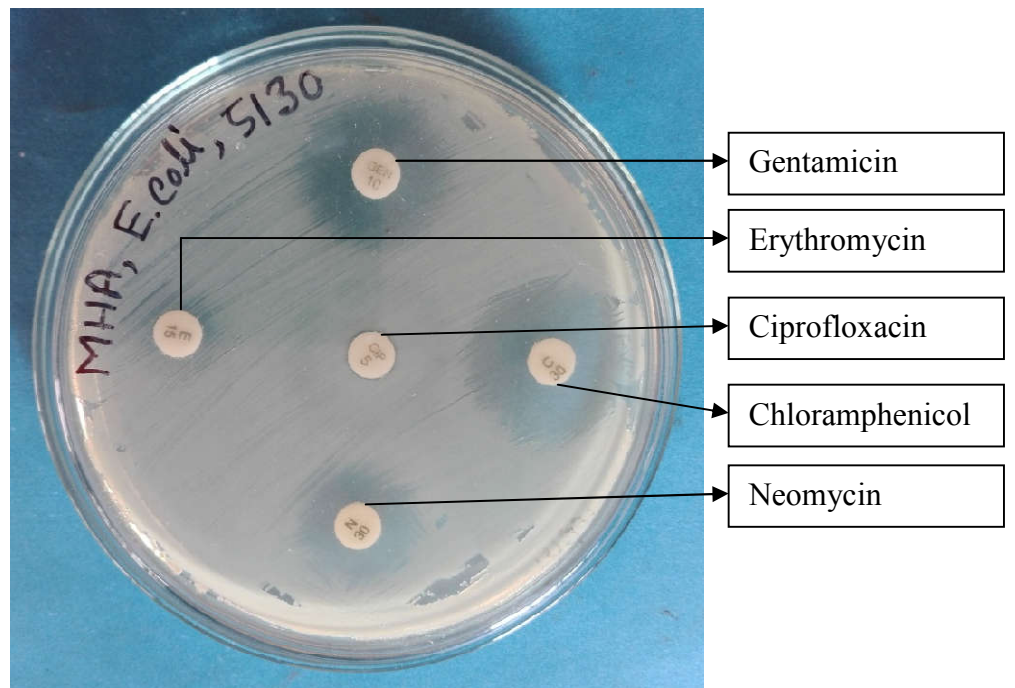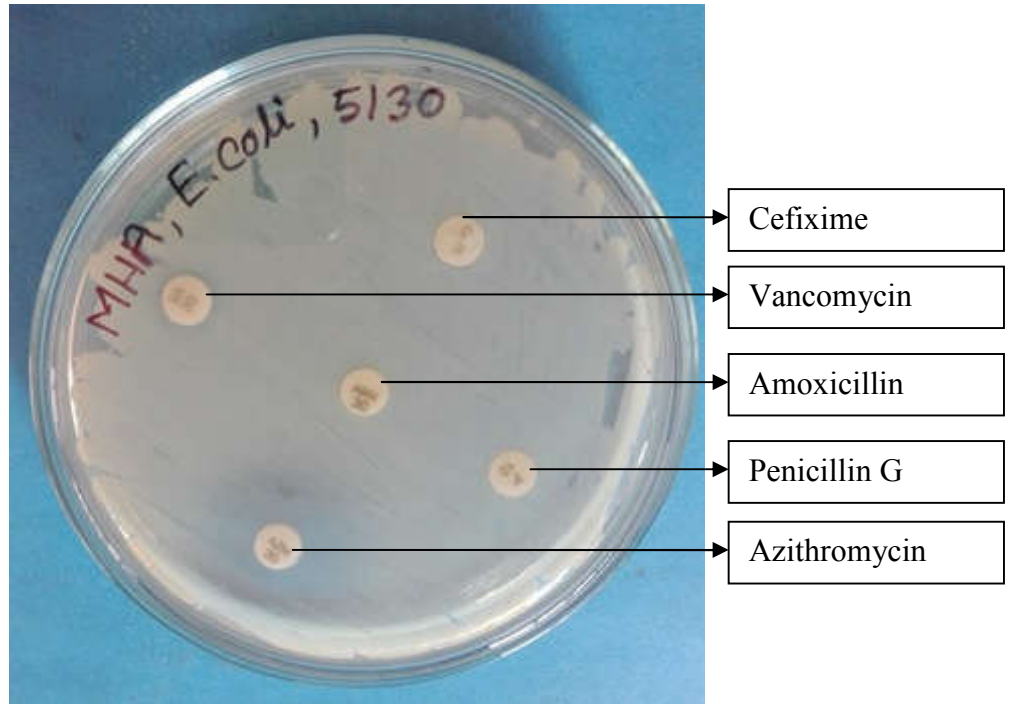
Plate 21: Antibiotic sensitivity test results of *E. coli* on Mueller-Hinton agar.
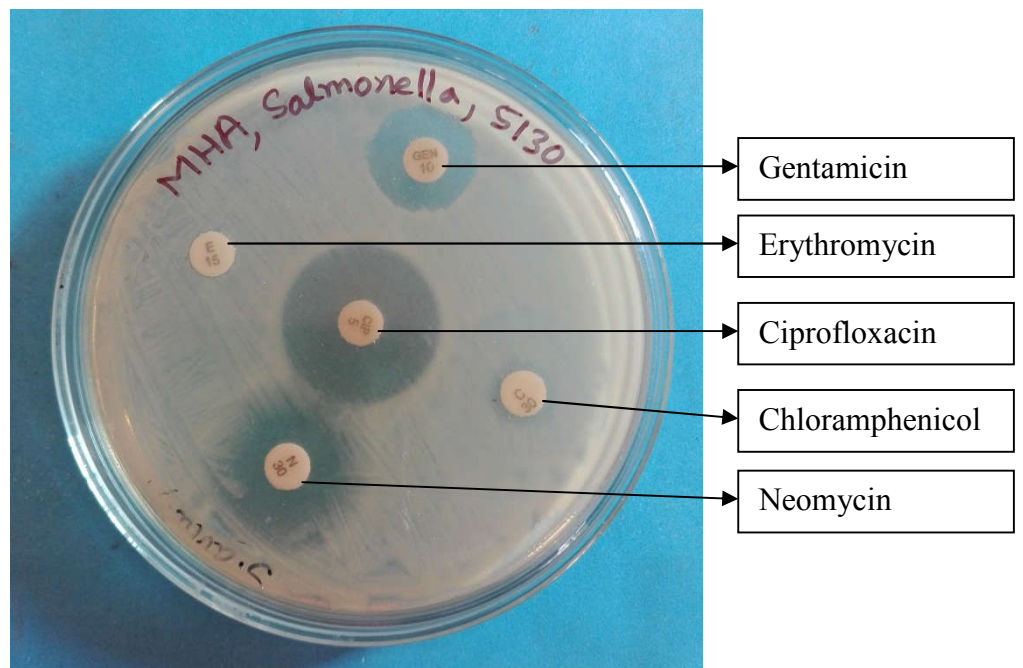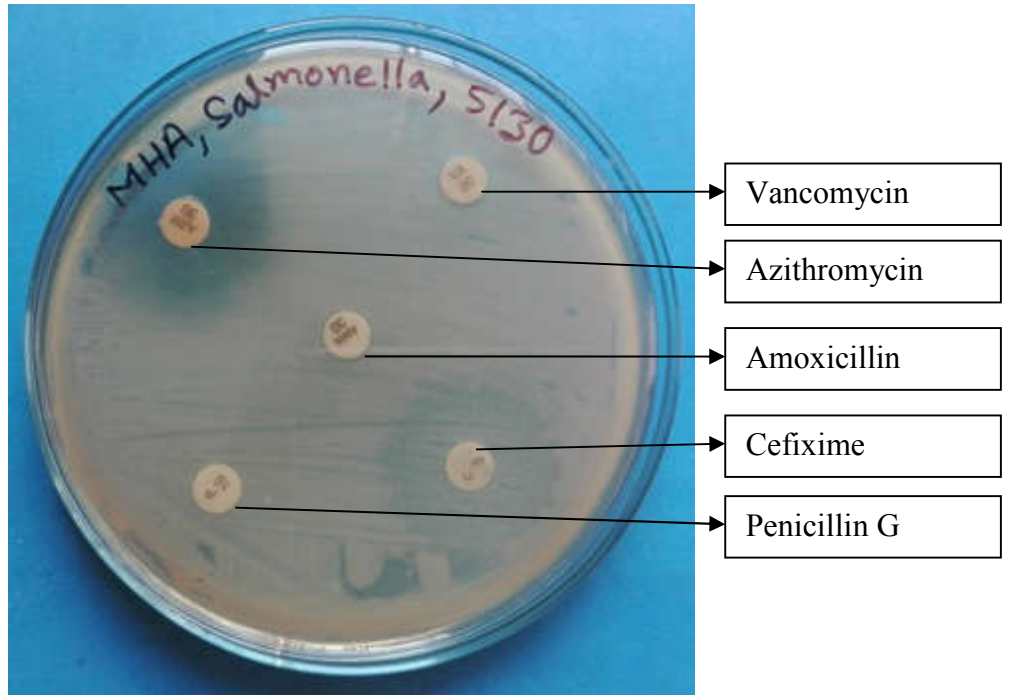
Plate 22: Antibiotic sensitivity test results of *Salmonella spp*. on Mueller-Hinton agar.
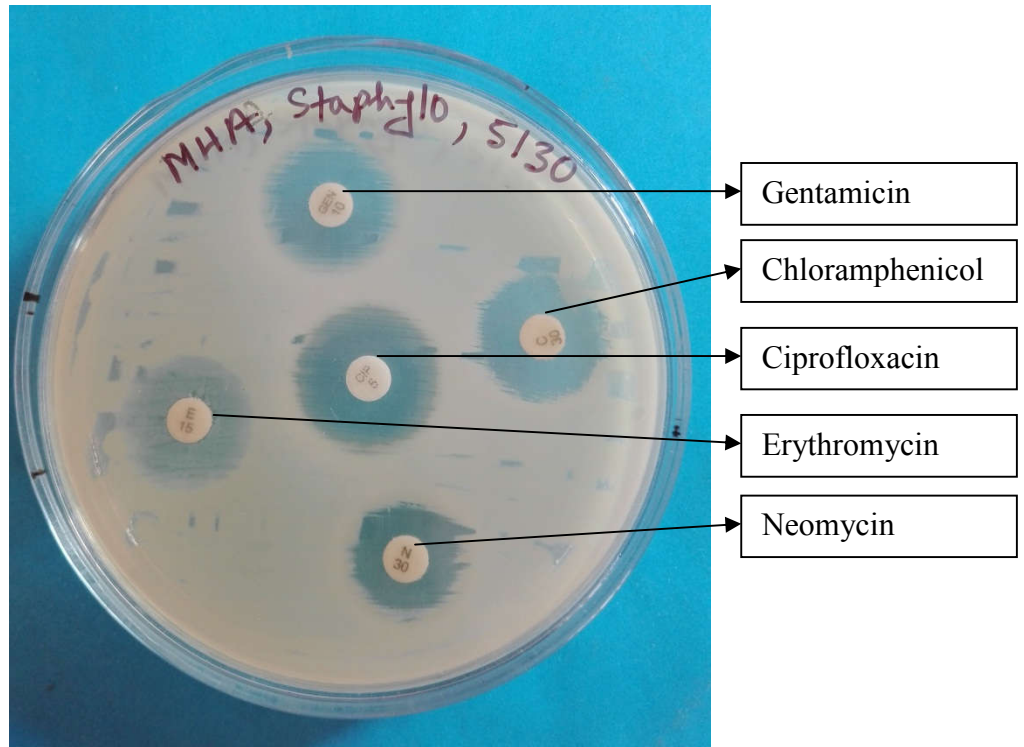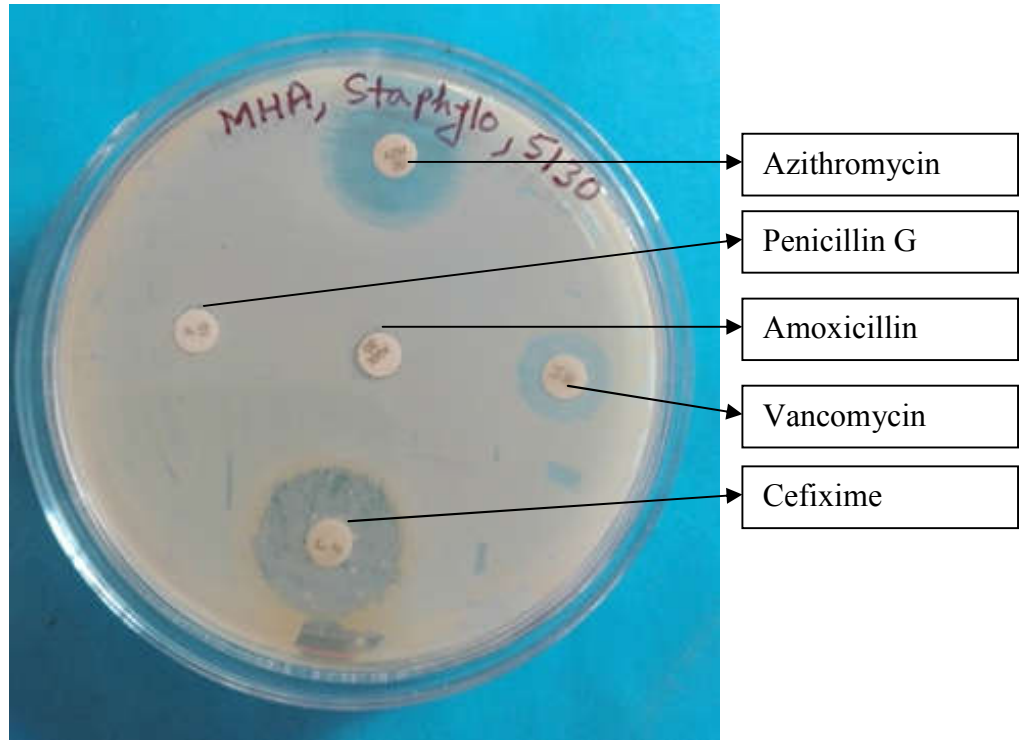
Plate 23: Antibiotic sensitivity test results of *Staphylococcus spp.* on Mueller-Hinton agar

| Vancomycin |
| Azithromycin |
| Amoxicillin |
| Cefixime |
| Penicillin G |

| Chloramphenicol |
| Neomycin |
| Ciprofloxacin |
| Gentamicin |
| Erythromycin |
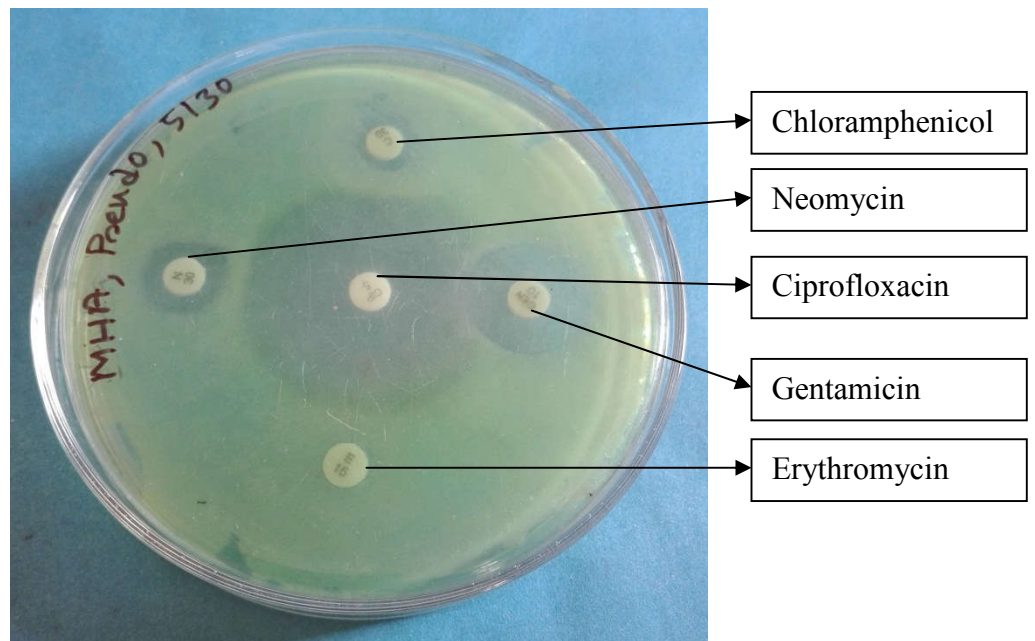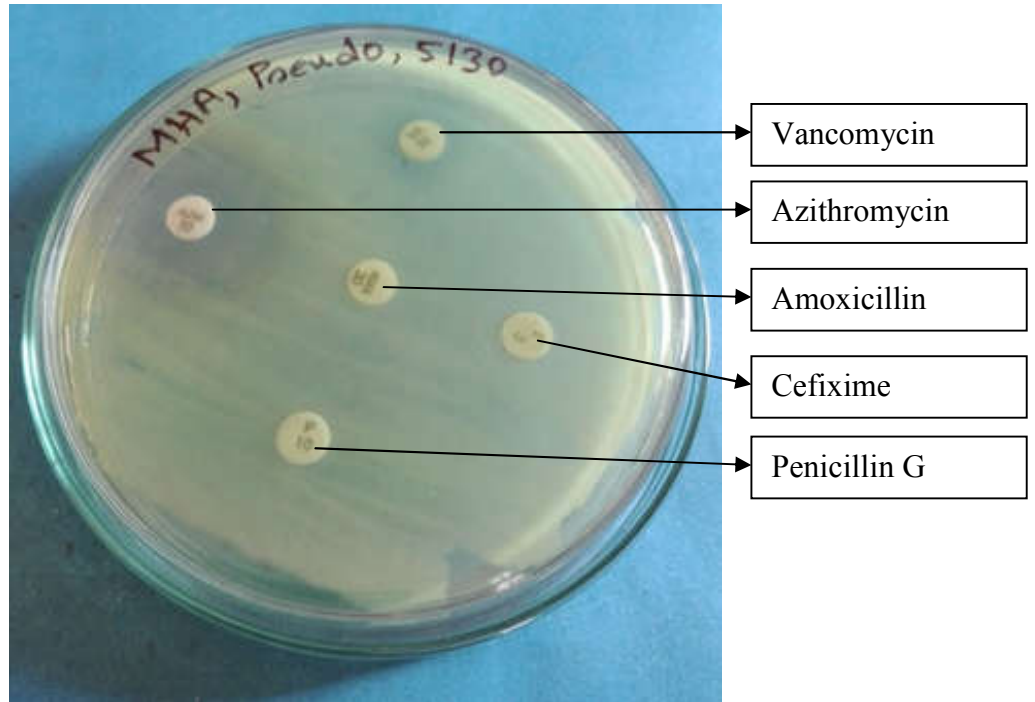
Plate 24: Antibiotic sensitivity test results of *Pseudomonas spp.* on Mueller-Hinton agar

CHAPTER 5

DISCUSSION

The present study was chosen and carried out for the isolation and identification of bacterial pathogens of Tilapia fishes collected from the local fish markets of the Dinajpur city {Bahadur bazaar(10) and Gopalgong bazaar(10)}, Bangladesh. A total of 20 gills samples, 20 intestine samples and 20 skin samples were collected and examined for the isolation, identification and antibiogram study of the isolated bacteria.

Local fish market is the sources of bacterial contamination and fish culture in pond increase bacterial growth rate of fish. There are several billions of bacteria that are present in environment of local market including both pathogenic and nonpathogenic species, the normal flora and the opportunistic ones. The bacteria isolated from fishes included *Escherichia coli*, *Staphylococcus spp*, *Salmonella spp*, *Enterobacter cloacae*, *Klebsiella spp*, *Proteus spp*. and *Pseudomonas spp* Fish ponds could be a source of many bacteria that isolated from Tilapia fishes in this study.

In this study, 04 different types of bacteria were isolated from a total 60 samples collected from tilapia fishes. In this work the bacterial isolates were obtained from gills, skin and intestines. All samples showed bacterial growth and gave 77 isolates. Out of 77 isolates 31(40.26%) were found positive for *Escherichia coli*, 10(12.99%) *salmonella spp*, 23 (29.87%) *Staphylococcus spp*, and 13 (16.88%) *Pseudomonas spp*.

In this study *Pseudomonas spp* was isolated from gills,skin and intestines of apparently healthy *Oreochromis niloticus* fishes this finding is identical with previous investigation in Sudan which reported the presence of *Pseudomonas spp* in gills and intestines of *Oreochromis niloticus* fishes (Hnadi, 2008). This result is also in agreement with several authors' studies (Ryser *et al.*, 1984; Wakabayashi *et al.*, 1996; Mohammed, 1999; Tripathy *et al.*, 2007; Chen and Kou, 1987), they reported the presence of *Pseudomonas aeruginosa* from different parts of a number of freshwater fish species. *Pseudomonas aeruginosa* was detected on the surface and muscle lesions of several afflicted fish species (Kar *et al.*, 1990; Mohammed, 1999).

In man *P. aeruginosa* causes between 10–20% of infection in most hospitals. *Pseudomonas* infection is especially prevalent among patient with burn wounds, cystic

fibrosis and acute leukemia. The most serious infection cause by Pseudomonas includes malignant external otitis, endopthalmitis, endocarditis, meningitis, pneumonia and septiceamia (Gerald *et al*., 1983).

Escherichia coli was isolated from gills and intestines of apparently healthy *Oreochromis niloticus* fishes in the present investigation this is in agreement with previous study in Sudan by Hnadi (2008) who reported the presence of *Escherichia. coli* in gills and intestines of *Oreochromis niloticus* fish. *Escherichia coli* was also reported from intestines and gills of different freshwater fish species (Austin and Al-Zahrani, 1988; Al-Harbi, 2003; Guzman *et al*., 2004; Khan, 1987; Kasing *et al*., 1999; Nieto *et al*., 1984; Mohammed, 1999). Most *E. coli* strains are important human pathogens, but some, such as serotype O157:H7 can cause serious food poisoning (Vogt and Dippold, 2005).

In the present study *salmonella spp*. was isolated from gills and intestines of *Oreochromis niloticus* fishes also *Salmonella* spp. Were present in all parts of the fishes(Cahill, 1990). The prevalence of *Salmonella* in different body parts of fishes was studied by Mohamed Hatha and Lakshmanaperumalsamy in 1997.The presence of Salmonella spp. Indicates faecal contamination of water from which the fishes were collected. The Freshwater lake, farmed and market fishes were 31%, 5% and 10 to 28%, respectively (Mohamed Hatha and Lakshmanaperumalsamy, 1997). (Adams *et al*., 1999) has demonstrated that fish and fish products are only occasionally associated with Salmonella and that filter feeding shell fish harvested from polluted water have been identified as higher risk products.

In the present investigation *Staphylococcus spp* was isolated from gills and intestines of *Oreochromis niloticus* fishes also in Sudan Hnadi (2008) reported the presence of *Staphylococcus* spp in gills and intestines of two freshwater fish species. Many authors (Austin and Al- Zahrani, 1988; Kasing *et al*., 1999; Nieto *et al*., 1984) reported the presence of *Staphylococcus* species in intestines and gills of different fish species. There is strong possibility that, the fish may obtain this bacterium from ponds water because chicken manure was used as fertilizers in ponds.

In this investigation, fish pathogenic bacteria, *Pseudomonas spp*., *Escherichia coli, salmonella spp, Staphylococcus spp* were isolated from Tilapia (*Oreochromis niloticus)* fishes collected from the local fish market. This indicates these bacterial pathogens might

cause serious infection leading to considerable economic losses in fishes when environmental condition altered in local market and fish's resistance was reduced.

Bacterial pathogens of public health importance, *Pseudomonas spp.*, *Escherichia coli, salmonella* spp*, Staphylococcus* spp were isolated in this study from Tilapia (*Oreochromis niloticus)* fishes, this indicates these fishes could be a source of infection and the possibility of transmission of these pathogens to workers in fish industry and consumers. Generally human contract fish-borne bacterial disease through ingestion of contaminated fish tissue in *Escherichia coli* infection or by contaminated water and injection of the organism into puncture wounds or abrasions in *Aeromonas hydrophila* infections, although the transmission of others bacterial species has not been documented, the potential for human infection does exist among individual who handle diseased fish (Stoskopf, 1993).

Antibiotic sensitivity test of 4 different types of bacterial isolates to 10 different antibiotics such as gentamycin, azithromycin chloramphenicol, amoxicillin, penicillin G. vancomycin, erythromycin, neomycin, cefixime and ciprofloxacin were studied. The antibacterial sensitivity of isolates in this study to antibiotics was variable. The isolates in this study were sensitive to gentamycin, azithromycin chloramphenicol and ciprofloxacin, while they were resistance to amoxicillin, penicillin G. vancomycin and erythromycin this finding is identical with Hasina karki *et al*., (2013), they also reported all isolated from fish revealed resistance to penicillin and sensitive to gentamicin. The bacterial isolates were sensitive to ciprofloxacin, which is gentamycin and this is in line with the findings of (Jawahar, 2011) whose findings were similar with bacterial human pathogens highly sensitive to ciprofloxacin, gentamycin and chloramphenicol.

The antibacterial resistance observed in the isolated *Pseudomonas spp.*, *Escherichia coli, salmonella spp, Staphylococcus spp.* Might be due to indiscriminate use of those antibacterial agents in fishes where they cultured or rapid chromosomal mutation and the specific plasmid DNA. The sensitivity test were perform by disc diffution method using 10 different antibiotic discs. This will provide guideline to the fermars to select appropriate antibiotics to reduce the economic losses through selecting the sensitive antibiotics.

The result of isolation, identification, biochemical test and antibiotic sensitivity of the bacteria isolated from Tilapia fish in the present study indicates that the microbial

contamination play an important role for the development of diseases in Tilapia fish and Some of these pathogens have tendancy to transmit to man who eat fish meat or deal with fish and fish products.

Detailed further study in connection with the present research work might be:

1. Molecular characterization of the bacteria associated with fish diseases needs further study.
2. Detail genomic analysis of the isolates to obtain the idea about molecular basis of pathogenecity and drug resistance.
3. Further study on the influenced of bacteria of artificial supplementary feeds taken by fish.
4. Good management of environment surrounding fish population in ponds is necessary to prevent the fish from bacterial diseases.

CHAPTER 6

SUMMARY AND CONCLUSION

The present study investigated the Tilapia fishes of local fish markets which had been found to common human bacterial pathogens. *Escherichia coli, Salmonella spp., Pseudomonas spp. and Staphylococcus spp.* were isolated from most of the samples. The presence, in large populations of these bacterial pathogens indicates high levels of contamination in the fishes. It was indicating that these fish samples have not been protected from the microbial spoilage during handling, packaging, storage, and transport. Appropriate maintenance of microbiological quality is thus a vital aspect of quality control measures of such fishes. The fish act as a reservoir of human pathogens and the presence of highly pathogenic agents such as *Salmonella* species cause health hazard. Opportunistic pathogens is a potential health risk/hazard to human beings and may cause diseases to susceptible individuals especially the immune-compromised consumers potentially pathogenic to humans, in the fish suggest that if they are improperly handled, undercooked or consumed raw may contribute to the spread of the pathogens in the community. Further examination of fish especially for the presence of pathogens, during handling, storage and up to the very point of consumption is needed for the protection and maintenance of community health by keeping food borne diseases to a minimum. The study of antibiogram revealed that the most of the pathogens were found to be resistant to commonly used antibiotics. Most of the isolates were resistance against Amoxicilin, Penicilin G, Vancomycin and Erythromycin

Conclusively, this research has brought to light those bacterial species associated with fresh Tilapia fish and has shown that they are potentially pathogenic to humans. Hence adequate measures should be taken in processing the fish before consumption.

So, it is necessary to give more attention to the quality and safety aspects of Tilapia fish products related to the harvesting, handling, processing and packaging. If proper cooking time and temperature is not maintained then the pathogens can survive in the cooking product and caused hazard to the consumers.

In the context of this study, it may be concluded that,

i. The presence of *E.coli*, *Salmonella spp., Staphylococcus spp.* and *Pseudomonas spp*. in most of the samples are public health concern.

ii. All of the 04 types of bacteria isolated from Tiapia fishes are opportunistic, which can cause diseases under certain environmental stress condition.

iii. High bacterial contamination can cause different type of bacterial diseases in fish.

iv. The presence of bacteria in all samples indicates that consumption of Tilapia fish without proper cooking is harmful and also accelerated the public health threat.

v. The antibiotic resistance properties of isolated bacteria can cause serious health hazards because of ineffective treatment of the sufferers by the commonly prescribed antibiotics.

REFERENCES

Abou El-Atta, M.E. (2003). Efficiency of polymerase chain Reaction (P.C.R.) in diagnosis of some bacterial fish pathogens. Ph.D. Thesis in Vet. Sci (Bacteriology, immunology and mycology), pp. 44-63.

Adam, A.J. and Tobaias, W.J. (1999). Red Mang rove proproot habitat as a finfish nursery area; a case study of salt rivea bay, st. Croix, USVI. Proc Gulf Caribb fish inst, 46: 22-46.

Al-Harbi, A.H. (1994). First isolation of Streptococcus spp. From hybrid tilapia (*Oreochromisniloticus* and *O. aureus*) in Saudi Arabia. *Aquaculture*, 128: 195-201.

Al-Harbi, A.H. and Uddin, N. (2003). Quantitative and qualitative studies on bacterial flora of hybrid tilapia (*Oreochromis niloticus* and *O. aureus*) cultured in earthen ponds in Saudi Arabia. *Aquaculture Research*, 34: 43 - 48.

Al-Harbi, A.H. and Uddin, M.N. (2005). Bacterial diversity of tilapia (*Oreochromis niloticus*) cultured in brackish water in Saudi Arabia. *Aquaculture*, 250: 566-572.

Allen, D.A., Austin, B. and Colwell, R.R. (1983). Numerical taxonomy of bacterial isolates associated with of freshwater fishery. *Journal of general microbiology*, 129: 2043-2062.

Aly, S.E.M. (1994). Pathological studies on some fish in Suez Canal area. Ph.D. Thesis (pathology) Fact. of Vet. Med. Suez. Canal University.

Angka, S.L and Lioe, K.G. (1982). Control of bacterial hemorrhagic septicaemia in cultured fish. Bul. Perel Inst. Pertan Bogor, 3(3): 13-14.

Ashiru, A.W., Uaboi-Egbeni, P.O., Oguntowo, J.E. and Idika, C.N. (2011) Isolation and Antibiotic Profile of *Aeromonas* Species fromTilapia Fish (*Tilapia nilotica*) and Catfish (*Clarias betrachus*). *Pakistan Journal of Nutrition*, 10 (5): 982-986.

Austin, B. and Al-Zahrani, A.M.J. (1988). The effect of antimicrobial compounds on the gastrointestinal microflora of rainbow trout. *Journal Fish Biology*, 33: 1-14.

Austin, B. and Austin, D.A. (1987). Bacterial Fish Pathogens, Disease of Farmed and Wild Fish. Ellis Horwood, Chichester.

Barrow, G. and Feltham, R.K.A. (1993). Manual for the identification of medical bacteria, 3rd Edition. Cambridge University press. p. 331.

Belton, B., Karim, M., Thilsted, S., Murshed-E-Jahan, K., Collis, W. and Phillips, M. (2011). Studies and Reviews 2011-53. The World Fish Center. Penang, Malaysia. p. 71.

Bibi, F., Qaisrani, S.N., Ahmad, A.N., Akhtar, M., Khan, B.N. and Ali, Z. (2015). Occurrence of *Salmonella* in freshwater fishes: *A Review the Journal of Animal & Plant Sciences*, 25(3 Supp. 2): 303-310.

Boari, C.A., Pereira, G.I., Valeriano, C., Silva, B.C., Morais, V.M.D., Figueiredo, H.C.P. and Piccoli, R.H. (2008). Bacterial ecology of tilapia fresh fillets and some factors that can influence their microbial quality. Food Science and Technology (Campinas), 28(4): 863-867.

Budiati, T., Rusul, G., Wan-Abdullah, W.N., Ahmad, R. and Arip, Y.M. (2015). Microbiological Quality of Catfish (Clarias Gariepinus) and Tilapia (Tilapia Mossambica) Obtained from Wet Markets and Ponds in Malaysia. *Journal of Aquaculture Research Development*, 6 (1): 100-291.

Buxton, A. and Fraser, G. (1977). *Escherichia coli*. In: *Animal Microbiology*. Vol 1, Blackwell Scientific Publications, London, pp. 78-80.

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

Cahill, M.M. (1990). Bacterial flora of fishes: a review. *Microbiology Ecology*, 19(1): 21-41.

Cappuccino, J.G. and Sherman, N. (1996). Microbiology- A Laboratory Manual. 4th edn. California: The Benjamin/Cummings Publishing Co., Inc.

Carter, G.R. (1979). Diagnostic Procedures in Veterinary Bacteriology and Mycoplasma. 3rd edn. Charles C, Thomas Publicher U.S.A., pp. 398-417.

Cheesbrough, M. (1984). Medical laboratory manual for tropical countries. Microbiology, English Language Book Socity, London, 2(35): 40-57.

Chen, H.Y. and Kou, G.H. (1987). Studies on bacterial drug resistance in aquaculture I Drug resistance of bacteria in pond reared eels (*Anguilla japonica*). The Memoir of Bacteriology and Immunology in Fish Disease, 10: 12-24.

Choudhury, K.A., Amin, M.M., Sarker, A.J., Ali, M. and Ahmad, A.R. (1987). Immunization of chickens against fowl cholera with oil adjuvanated broth culture vaccine. *Bangladesh Veterinary Journal*, 21(1-2): 63-73.

Dangwetngam, M., Suanyuk, N., Kong, F. and Phromkunthong, W. (2016). Serotype distribution and antimicrobial susceptibilies of Streptococcus agalactiae isolated from infected cultured tilapia (*Oreochromis niloticus*) in Thailand: Nine-year perspective. *Journal of medical microbiology*, 65(3): 247-254.

Donkeng, N.N., Maiwore, J., Ngoune, L.T., Montet, D. and Mbofung, C.M.F. (2011). Characterization of the bacterial flora of tilapia (*Oreochoromis niloticus*) harvested from four lakes in the north of Cameroon. *African Journal of Biotechnology*, 10(71): 16016-16023.

Douglas, D. (2007). Identifying fresh water Aquarium fish disease.

Edris, A.M., Amany, M.S. and Michael, F.A. (2014). Incidence of *Listeria monocytogenes* in fresh tilapia nilotica fish. *Benha veterinary medical journal*, 26(1): 120-126.

Eissa, I.A.M., Badran, A.F., Diab, A.S. and Abdel-Rahman, A.M. (1996). "Some studies on prevailing bacterial diseases among cultured tilapias in Abbassa fish farms". Proceedings of the 3rd Vet. Med. Congress, Zagazig University, Egypt, pp. 185-202.

Eissa, N.M.E., Abou, E., Ghiet, E.N., Shaheen, A.A. and Abbass, A. (2010). Characterization of Pseudomonas Species Isolated from Tilapia *"Oreochromis niloticus"* in Qaroun and Wadi-El-Rayan Lakes, Egypt. Global Veterinaria, 5 (2): 116-121.

Emikpe, B.O., Adebisi, T. and Adedeji, O.B. (2011). Bacteria load on the skin and stomach of Clarias Gariepinus and Oreochromis Niloticus from Ibadan, South West Nigeria: Public health implications. *Journal of Microbiology and Biotechnology Research*, 1: 52-59.

Etyemez, M. and Balcazar, J.L. (2016). Isolation and characterization of bacteria with antibacterial properties from Nile tilapia (*Oreochromis niloticus*). *Research in veterinary science*, 105: 62-64.

EUCAST (2015). Breakpoint tables for interpretation of MICs & zone diameters, version 5.0.

FAO. (2007). (Food and Agriculture Organization of WHO). The state of the world Fisheries and Aquaculture.

Fatima, R., Hussain, Q., Nadeem, S.G. and Hakim, S.T. (2014). Bacteriological Analysis of Nile Tilapia Fish (*Oreochoromis niloticus*). J*ournal of Biological Research & Applied Sciences*, 5(1): 30-35.

FRSS. (2012). A Comprehensive Study on High Value Fish (Tilapia, Pangas and Koi) in Bangladesh.

Gerald, P.B., Ricardo, B.V. and Fainstein, L.J. (1983). Infections caused by *Pseudomonas aeruginosa.* Reviews of Infectious Diseases, 5 (2): 279-313.

Gisain, M., Yusoff, S.M. and Abdullah, Z. (2010). Isolation and Identification of Pathogenic Bacteria from Red Tilapia in Cage-Cultured System and its Environment. 5th Proceedings of the Seminar in Veterinary Sciences, pp. 5-8.

Goncalves, J.R., Braum, G., Fernandes, A., Biscaia, I., and Bastardo, J. (1992). Aeromonas hydrophila fulminate pneumonia in a fit young man Tborax, 47: 482-483.

Grema, H.A., Geidam, Y.A., Suleiman, A., Gulani, I.A. and Birma, R.B. (2015). Multi-Drug Resistant Bacteria Isolated from Fish and Fish Handlers in Maiduguri, Nigeria. *International Journal of Animal and Veterinary Advances*, 7(3): 49-54.

Guzman, M.C., Bistoni, M.A., Tamagnini, L.M. and Gonzalez, R.D. (2004). Recovery of *Escherichia coli* in fresh water fish, *Jenynsia multidentata* and *Bryconamericus iheringi. Water Research*, 38: 2368–2374.

Henrietta, U., Thomas, A., Ifeanyichukwu, I., Emmanue, N. and Chika, E. (1016). Cluster Analysis of Aerobic Heterotrophic Bacteria from Clarias gariepinus and Tilapia zillii in Unwana River, Ebonyi State, Nigeria. *International Journal of Environmental & Agriculture Research,* ISSN: [2454-1850]

Hi-Media and Leifson (1935). J. Path, bact., pp. 40:581.

Hnadi, G.B.B. (2008). *Microbial contaminations of some fresh water fish species* (*Oreochromis niloticus*, *Clarias* spp and *Synodontis* spp). B.Sc. (Hon) Department of fisheries, University of Juba.

Huicab-Pech, Z.G., Castaneda-Chavez, M.R. and Lango-Reynoso, F. (2017). Pathogenic Bacteria in *Oreochromis Niloticus* Var. Stirling Tilapia Culture. *Fish Aquaculture Journal*, *an open access journal*, ISSN: 2150-3508.

Jawahar, A.T. (2011). Food safety hazards related to emerging antibiotic resistant bacteria in cultured Freshwater fishes of Kolkata, India. *Advance Journal of Food Science and Technology*, 3 (1): 69-72.

Jimoh, W.A., Oladele-Bukola, M.O., Adebayo, M.D., Yusuff, A.A., Azeez, A.F. and Salami. (2013). Microbial Flora of the Gastro-Intestinal Tract Of Nile Tilapia (*Oreochromis niloticus*) Caught From River Dandaru, Ibadan. *Nigerian Journal of Fisheries*, 10 (1 & 2).

Kamei, Y., Sakata, T. and Kakimoto, D. (1985). Microflora in the alimentary tract of tilapia: characterization and distribution of anaerobic bacteria. *Journal of General Applied Microbiology,* 31: 115-124.

Kaneko, S. (1971). Microbiological study of fresh fish. New Food Industry, 13:76–80.

Kapute, F., Likongwe, J., Kang'ombe, J., Kiiyukia, C. and Mpeketula, P. (2012). Quality Assessment of Fresh Lake Malawi Tilapia (*Chambo*) Collected from Selected Local and Super Markets in Malawi. *Internet Journal of Food Safety*, 14: 113-121.

Kar, D., Michael, R.G., Kar, S. and Changkija, S. (1990). Studies on fish epidemics from Assam. *Journal of Indian Fish Association*, 20: 73-75.

Karki, H., Mustafa, A., Master, A.L. and Dhawale, S. (2013). Antibiotic Resistant Bacteria in the Gut of Hatchery-reared Tilapia and Coho Salmon. *Universal Journal of Microbiology Research*, 1(3): 43-46.

Kasing, A., Asiah, M.Y. and Kumbang, J. (1999). Distribution of bacteria in tropical freshwater fish and ponds. *International Journal of Environmental Health Research*, 9 (4): 285-292.

Kato, C.D., Mugaanyi, M.B., Majalija, S., Tamale, A., Musisi N.L. and Sengooba A. (2016). Isolation and Identification of Potential Probiotics Bacteria from the Gut of *Oreochromis niloticus* and *Clarias gariepinus* in Uganda. *British Microbiology Research Journal*, 17(5): 1-8.

Khan, S.M., Nunjurul, H. and Shafi, M. (1987). Study of the bacteriological flora in the alimentary canal of *Clarias batrachus* and *Heteropneustes fossilis* in Bangladesh. Bangladesh Association for the Advancement of Science, Dhaka (Bangladesh). Proceedings of the 12th Annual Bangladesh Science Conference, pp. 35-36.

Ladipo, O., Fabiyi and Fatula, G.T. (1981). Marketing and distribution of fish in Nigeria. Report submitted to the federal development of fisheries, Lagos. p. 35.

Lara-Flores, M., Aguirre-Guzman, G., Balan-Zetina, S.B., Sonda-Santos, K.Y. and Zapata, A.A. (2014). Identification of Mycobacterium Agent Isolated from Tissues of Nile tilapia (*Oreochromis niloticus*). *Turkish Journal of Fisheries and Aquatic Sciences*, 14(2): 575-580.

Leisner. J.J., Vancanneyt, M., Rusul, G., Pot, B., Lefebvre, K., Fresi, A. and Tee, L.T (2001). Identification of lactic acid bacteria constituting the predom, nating microflora in an acid feamented condiment (tempoyak) popular in Malaysia. *International Journal of food microbiology*, 63: 147-157.

Mandal, S.C., Hasan, M., Rahman, M.S., Manik, M.H., Mahmud, Z.H. and Islam, M.S. (2009). Coliform bacteria in Nile Tilapia, Oreochromis niloticus of shrimp-Gher, pond and fish market. *World Journal of Fish and Marine Science*, 1(3): 160-166.

Meidong, R., Doolgindachbaporn, S., Sakai, K. and Tongpim, S. (2017). Isolation and selection of lactic acid bacteria from Thai indigenous fermented foods for use as probiotics in tilapia fish *Oreochromis niloticus*. Aquaculture, *Aquarium, Conservation & Legislation-International Journal of the Bioflux Society* (AACL Bioflux), 10(2): 455-463.

Merchant, I.A. and Packer, R.A. (1967). Veterinary bacteriology and virology. 7th edition, The Iowa State University Press, Ames, Iowa, United State of America, pp. 211-305.

Mhango, M., Mpuchane, S.F. and Gashe, B.A. (2010). Incidence of indicator organisms, opportunistic and pathogenic bacteria in fish. *African Journal of Food, Agriculture, Nutrition and Development*, 10(6): 4202-4218.

Mohamed, H.A. and Lakshmanaperumalsamy, P. (1997). Prevalence of *Salmonella* in fish and crustaceans from markets in Coimbatore, South India. Food Micro., 14(2): 111-116.

Mohammed, X.S.A. (1999). Quality monitoring of some farm in Kafr Elsheikh. *Association of Medical Journal*, 41: 152- 161.

Molinari, L.M., De Oliveira, S.D., Pedroso, R.B., De Lucas Rodrigues Bittencourt, N., Nakamura, C.V., Ueda-Nakamura, T. and Dias Filho, B.P. (2003). Bacterial microflora in the gastrointestinal tract of Nile tilapia, Oreochromis niloticus, cultured in a semi-intensive system. Acta Scientific Biological Science, 25(2): 267-271.

Musefiu, T.A., Obuko, E.B. and Bolarinwa, A.O. (2011). Isolation and Identification of Aerobic Bacteria Flora of the Skin and Stomach of Wild and Cultured *Clarias Gariepinus* and *Oreochromis Niloticus* from Ibadan, Southwest Nigeria. *Journal of Applied Sciences Research*, 7(7): 1047-1051.

Newaj-Fyzul, A., Mutani, A., Ramsubhag, A. and Adesiyuu, A. (2008). Prevalence of Bacterial Pathogens and their anti-microbial resistance in Tilapia and their Pond water in Trinidad. Zoonoses and public Health, 5(5): 206-213.

Nieto, T.P., Toranzo, A.E. and Barja, J.L. (1984). Comparison between the bacterial flora associated with fingerling rainbow trout cultured in two different hatcheries in the north-west of Spain. *Aquaculture*. 42: 193-206.

Roberts, R.J. and Horne, M.T. (1978). Bacterial meningitis in farmed rainbow trout salmogarirdneri Richrdson, affected with chronic pancreatic necrosis. *Journal of fish diseases*, 1: 157-164.

Rocha, R.D.S., Leite, L.O., Sousa, O.V.D. and Vieira, R.H.S.D.F. (2014). Antimicrobial susceptibility of Escherichia coli isolated from fresh-marketed Nile Tilapia (*Oreochromis niloticus*). *Journal of pathogens*, pp. 1-5.

Romana-Eguia, M.R.R., Ikeda, M., Basiao, Z.U. and Taniguchi, N. (2004). Genetic diversity in farmed Asian Nile and red hybrid tilapia stocks evaluated from microsatellite and mitochondrial DNA analysis. *Aquaculture*, 236:131-150.

Ryser, E.T., Marth, E.H. and Taylor, S.L. (1984). Histamine production by psychrotrophic pseudomonads isolated from fish. *Journal of Food Protection*, 47: 378-380.

Saqr, S., Khaliel, R. and Ibrahim, M.S. (2016). Antibiotic Resistance and Virulence Genes of *E. coli* Isolated From Fresh Nile Tilapia (*Oreochromis Niloticus*) in El-Behera Governorate, Egypt. *Alexandria Journal for Veterinary Sciences*, 48 (2): 83-90.

Shayo, S.D., Mwita, C.J. and Hosea, K.M. (2012). Virulence of *Pseudomonas* and *Aeromonas* bacteria recovered from *Oreochromis niloticus* (Perege) from Mtera hydropower Dam; Tanzania. *Scholars Research Library Annals of Biological Research*, 3 (11): 5157-5161.

Shinkafi, S.A. and Ukwaja, V.C. (2010). Bacteria Associated with Fresh Tilapia Fish (*Oreochromis niloticus*) Sold at Sokoto Central Market in Sokoto, Nigeria. *Nigerian Journal of Basic and Applied Science*, 18(2): 217-221.

Shirak, A., Cohen-Zinder, M., Barroso, R.M., Seroussi, E., Ron, M. and Hulata, G. (2009). DNA bar-coding of Israeli indigenous and introduced cichlids. *Israeli Journal of Aquaculture*, 61: 83-88.

Sichewo, P.R., Gono, R.K., Muzvondiwa, J.V. and Sizanobuhle, N. (2013). Isolation and Identification of Pathogenic Bacteria in Edible Fish: A Case Study of Fletcher Dam in Gweru, Zimbabwe. I*nternational Journal of Science and Research (IJSR)*, India Online ISSN: 2319-7064.

Silva, H.S.K. and Widanapathirana, S. (1984). The Nature of the Aerobic Gastrointestinal Bacteria of Cichlid Fish *Sarotherrodon mossambicus* {Peters) and *Tilapia Nilotica* (Linneaus) Grown under Captivity. J. Nara, 31: 73-79.

Soliman, M.K., Khalil, R.H., Saad, T.T., El-Gamal, M.H.L. and Gebril, A.E. (2010). Isolation and Identification of *E. coli* from Cultured Freshwater Fish. *Journal of the Arabian Aquaculture Society*, 5(1): 311-325.

Son, R., Rusul, G., Sahilah, A.M., Zainuri, A., Raha, A.R. and Salmah, I. (1997). Antibiotic resistance and plasmid profile of *Aeromonas hydrophila* isolates from cultured fish, Telapia (*Telapia mossambica*). Letters in Applied Microbiology, 24: 479-482.

Stoskopf, M.K. (1993). Zoonotic diseases. In: *Fish medicine*. W. B. Saunders Company. pp. 214- 217.

Sugita, H., Tokuyama, K. and Deguchi, Y. (1985). The intestinal microflora of carp, Cyprinus carpio, grass carp, Ctenopharyn godonidella, and tilapia Sarotherodon niloticus. Bulletin of the. *Japanese Society of Scientific Fisheries*, 51: 1325-1329.

Thillaimaharani, K.A., Logesh, A.R., Sharmila, K., Magdoom, B.K. and Kalaiselvam, M. (2012). Studies on the intestinal bacterial flora of tilapia *Oreochromis mossambicus* (Peters, 1852) and optimization of alkaline protease by *Virgibacillus pantothenticus*. *Journal of Microbiology and Antimicrobials*, 4(5): 79-87.

Tiamiyu, A.M., Soladoye, M.O., Adegboyega, T.T. and Adetona, M.O. (2015). Occurrence and Antibiotic Sensitivity of Bacterial Strains Isolated from Nile Tilapia, *Oreochromis niloticus* Obtained in Ibadan, Southwest Nigeria. *Journal of Biosciences and Medicines*, 3: 19-26.

Tripathy, S., Kumar, N., Mohanty, S., Samanta, M., Mandal, R.N. and Maiti, N.K. (2007). Characterization of *Pseudomonas aeruginosa* isolated from freshwater culture systems. *Microbiological Research*, 162 (4): 391-396

Vogt, R.L. and Dippold, L. (2005). *Escherichia coli* O157:H7 outbreak associated with consumption of ground beef. Public Health Reports, 2: 174–178.

Wakabayashi, H. and Egusa, S. (1972). Characteristics of *Pseudomonas spp.* from an epizootic of pond-cultured eels. Bull. Japan Soc. Scient. Fish, 38: 577-587.

Wang, K.Y., Chen, D.F., Huang, L.Y., Lian, H., Wang, J., Xiao, D. and Lai, W.M. (2013). Isolation and characterization of Streptococcus agalactiae from Nile Tilapia Oreochromis niloticus in China. *African Journal of Microbiology Research*, 7(4): 317-323.

Weinstein, M.R., Litt, M., Kertesz, D.A., Wyper, P., Rose, D., Coulter, M., McGeer, A., Facklam, R., Ostach, C., Willey, B.M., Borczyk, A. and Low, D.E. (1997). Invasive infections due to a fish pathogen, Streptococcus iniae. *England Journal of Medicine*, 337: 589-594.

WHO. (2007). Food safety issues associated with products from aquaculture. T.R.S. No. 883, World Health Organization, Geneva, Switzerland.

Yagoub, S.O. (2009). Isolation of *Enterobacteriaceae* and *Pseudomonas spp.* from raw fish sold in fish market in Khartoum state. *Journal of Bacteriology Research*, 1(7): 85-88.

Younes, A.M., Fares, M.O., Gaafar, A.Y. and Laila, A.M. (2016). Isolation of *Vibrio alginolyticus* and *Vibrio vulnificus* Strains from Cultured *Oreochromis niloticus* Around Qarun Lake, Egypt. Global Veterinaria 16 (1): 01-05.

Zapata, A.A. and Lara-Flores, M. (2013). Antimicrobial Activities of Lactic Acid Bacteria Strains Isolated from Nile Tilapia Intestine (*Oreochromis niloticus*). *Journal of Biology and Life Science*, 4 (1): 2157-6076.

Zlotkin, A., Chilmonczyk, S., Eyngor, M., Ghittino C. and Hurvitz, A. (2003). Trojan horse effect: phagocyte-mediated Streptococcus iniae infection of fish. Infect Immun, 71 (5): 2318-2325.

Composition of Different Media

1. Nutrient agar (Hi Media)

Ingredients:	g/L
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (at 250C)	7.4 ± 0.2

2. Eosine methylene blue Agar (Hi Media)

Ingredients:	g/L
Peptic digest of animal tissue	10
Lactose	5.0
Sucrose	5.0
Dipotassium phosphate	2.0
Eosin - Y	0.40
Methylene blue	0.065
Agar	20.0
Final pH (at 250C)	7.2 ± 0.2

3. MacConkey agar (Hi-media) Ingredients:

	g/L
Peptic digest of animal tissue	17.0
Protease peptone	3.0
Lactose monohydrate	10
Bile salt	1.5
Sodium chloride	5.0
Agar-agar	15.0
Neutral red	0.03
Final pH (at 250C)	7.1 ± 0.2

4. Simmon's Citrate Agar

Component	Amount (g/L)
Magnesium sulphate	0.2
Ammoniun dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bacto bromo thymol blue	0.08

5. Mueller Hinton Agar

Component	Amount (g/L)
Beef infusion	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Final pH(at 25°C)	7.3±0.1

6. TSI agar (Hi Media)

Ingredients:	g/L
Peptic digest of animal tissue	10.00
Casein enzymic hydrolysate	10.00
Yeast extract	3.00
Beef extract	3.00
Lactose	10.00
Sucrose	10.00
Dextrose	1.00
Sodium chloride	5.00
Ferrous sulphate	0.20
Sodium thiosulphate	0.30
Phenol red	0.024
Agar	12.00
Final pH(at 25°C)	7.4 ± 0.2

7. MIU medium base (Hi Media)

Ingredients:	g/L
Casein enzymic hydrolysate	10.00
Dextrose	1.00
Sodium chloride	5.00
Phenol Red	0.01
Agar	2.00
Final pH(at 25°C)	6.8 ± 0.2

8. MR-VP medium (Hi Media)

Ingredients:	g/L
Buffered peptone	7.00
Dextrose	5.00
Dipotassium phosphate	5.00
Final pH (at 25°C)	6.9 ± 0.2

APPENDIX 2

Preparation of reagents

1. Kovacs reagent

P-dimethyl aminobenzal dehyde	5 gm
Amylalcoho	l75 gm
Conc. HCL	25 ml

2. V-P reagent 1

5% alpha –naptholin absolute ethyl alcohol

3. V-P reagent 2

40% potassium hydroxide containing 0.3creatine. The ingredients were dissolved by heating gently over steam bath. When in solution add 0.05gm of cotton blue dye.

4. Phosphate buffered solution

Sodium chloride	8 gm
Disodium hydrogen phosphate	2.8 gm
Potassium chloride	0.2 gm
Potassium hydrogen phosphate	0.2 gm
Distilled water to make	1000 ml

5. Methyl red solution

Methyl red	0.05 gm
Ethanol (absolute)	28 ml
Distilled water	22 ml

6. Phenol red solution

0.2% aqueous solution of phenol red

7. Potassium hydroxide solution

40% aqueous solution of KOH

8.Gram stain solution

Stock crystal violet

Crystal violet	10 gm
Ethyl alcohol (95%)	1000 ml

Stock oxalate solution

Ammonium oxalate	1 gm
Distilled water	1000 ml

Lugols iodine solution

Iodine crystal	1 gm
Potassium iodide	2 gm
Ethyl alcohol	250 m
Acetone	250 m

Counterstain

Safranine	2.5 ml
Ethyl alcohol (95%)	100 ml

Safranine working solution

The stock safranine is diluted 1:4 with distilled water.