DETECTION AND ANTIBIOGRAM STUDY OF BACTERIA ISOLATED FROM DRIED AND COOKED FISH

A THESIS

BY

DR. MD. MUNIRUZZAMAN

REGISTRATION NO. 1705430

SEMESTER: JULY-DECEMBER, 2018

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MASTER OF SCIENCE (M.S.)
IN
VETERINARY PUBLIC HEALTH AND FOOD HYGIENE

DEPARTMENT OF MICROBIOLOGY

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HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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DEDICATED TO MY BELOVED PARENTS AND TEACHERS

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ABSTRACT

Fishes are very rich source of animal proteins. The soft tissues of fish and aquatic environment are extremely susceptible to microbial contamination which have a significant zoonotic importance. This study was carried out to detect and antibiogram study of pathogenic bacteria isolated from dried and cooked fish. For this purpose, the present research work was conducted during the period of January to October, 2018. A total of 79 samples were collected from different local market. Out of 79 samples 54 samples were from dried fish and 25 from cooked fish samples. In this research there were 18 different types of dried fish and 6 types of cooked fish were used as a sample. All the microbiological work was done by different bacteriological laboratory methods and purified isolates were identified according to gram's staining reaction, cell morphology, cultural characteristics, biochemical and antibiotic susceptibility test. All most 100% dried and 20% cooked fish sample were contaminated. In this research work seven different species and 168 isolates were identified from dried fish and these were *Escherichia coli* 21.43% (36), *Vibrio* spp. 18.45% (31), *Staphylococcus* spp.17.86% (30), *Pseudomonas* spp.17.86% (30), *Salmonella* spp.12.5% (21), *Shigella* spp. 8.93% (15) and *Klebsiella* spp. 2.97% (5). In cooked fish 9 isolates were identified and species were *Escherichia coli* 66.66% (6) and *Shigella* spp. 33.34% (3). Total viable count varies from 1.28×10^{7}CFU/g to 3.74×10^{9}CFU/g. The highest concentration was found in Loitta fish and lowest in Ruhi from dried fish. Most species of bacteria isolated were resistant to amoxicillin, penicillin-g, kanamycin, azithromycin, cefuroxime sodium, cephalexin, nalidixic acid, cephradine 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 dried fish related pathogen to animal and humans.

Key Words: *Fish borne diseases, dried fish, cooked fish, public health hazard from fish. Microbial quality of dried and cooked fish.*

CONTENTS

CONTENTS (Contd.)

CONTENTS (Contd.)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Contd.)

LIST OF ABBREVIATIONS

-	:	Negative
%	:	Percentage
+	:	Positive
µg	:	Microgram
^0C	:	Degree of Celsius
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 become an increasingly important source of protein, necessary for the maintenance of good health. The quality of fish is of major concern to the food processors, consumers, and public health authorities (Pal *et al*., 2016). Fish and Fish products are not only nutritional importance but also importance in global market as foreign currency earner for a number of countries in the world (Sohana & Karim.,2016). Human food habit dramatically changes day by day on fish in the last three decades, especially in East Asia (mainly in China) and in the Near East/North African region. Recent report shows that global per capita fish consumption is 20 kg per year which provides 6.7% of all protein consumed by human all over the world (FAO, 2016). Fish revolution also occurs in Bangladesh about 25-fold growth in farmed fish market over the last three decades (Ahmad, 2017). Bangladesh is considered one of the most suitable regions for fisheries in the world, with the world's largest flooded wetland and the third largest aquatic biodiversity in Asia after China and India. Bangladesh mainly exports ten categories of fishery products (Frozen freshwater fish, frozen marine water fish, frozen shrimp, chilled fish, live fish, dry fish, salted dehydrate, live kusia, live crab, and fish scale/shrimp scull) to more than 55 countries. Only in 2016-17, Bangladesh earns BDT 42876.40 million by exporting almost 68.31 Thousand MT of fish and fisheries products. This success is due to export of quality shrimp introducing HACCP procedure and traceability regulation according to the requirement of European Union (EU) and USA. (Shamsuzzaman *et al*., 2017)

According to the Department of Fisheries (DoF, 2017), fisheries contribute 3.69 percent of Bangladesh's GDP and over 23 percent of agricultural GDP. With an average fish intake of 53 grams per person a day, fish now account for 60 percent of protein supply for the entire population (DoF, 2016). In Seventh Five Year Plan (7FYP) Bangladesh Government has taken 5 major goal, and the 5^{th} is Improved food safety- **a.** Good Aquaculture Practices (GAP) and Good Manufacturing Practices (GMP) at all stages of fish/shrimp supply chain to comply international market. **b.** Food safety measures for domestic markets. From the food safety point of view provision of safe sound and wholesome fish and fish products are more essential for control the contamination of fish. Quality of fish deteriorates due to a complex process of physical chemical and

microbial changes in the content of fish. Fish of good quality should have bacterial count less than 105 per gram. The greatest risk to human health occurs due to the consumption of raw, inadequately cooked or insufficiently processed fish, and fish products (Pal *et al.*, 2016).

Bacteria are the main fish-borne zoonotic agents (diseases transmitted from fish to human vice versa), infection is typically acquired through abrasions, cuts, or penetrating wounds in the skin when handling infected fish or fomites (Boylan, 2011). Human infections caused by pathogens transmitted from fish or the aquatic environment are mostly depending on the season, patients contact with fish and surrounding environment, dietary habits and the immune system status of the exposed individual. The infection source may be fish kept for food or as a hobby (aquarium fish). Some bacterial species are facultatively pathogenic for both fish and humans. They may be isolated from fish without apparent symptoms of the disease. Human infections and intoxications with the following bacteria have been recorded: *Mycobacterium* spp., *Streptococcus iniae, Photobacterium damselae, Vibrio alginolyticus, V. vulnificus, V. parahaemolyticus, V. cholerae, Erysipelothrix rhusiopathiae, Escherichia coli, Aeromonas* spp., *Salmonella* spp., *Staphylococcus aureus, Listeria monocytogenes, Clostridium botulinum, C. perfringens, Campylobacter jejuni, Delftia acidovorans, Edwardsiella tarda, Legionella pneumophila*, and *Plesiomonas shigelloides* (Novotny *et al.*, 2004). The results of numerous studies indicate that fish possess bacterial populations on or in their skin, gills, digestive tract, and light-emitting organs (Austin, 2006).

Fish borne zoonotic diseases can be transmitted directly by consuming raw and improperly cooked fish meat or indirectly via contaminated water from the surroundings of infected fish. Due to lack of available data about microbiological quality of fish it is difficult to achieve this goal. We are also facing problem to meet up the requirement HACCP protocol in the processing of frozen fishes for export. Therefore, the purpose of the present study was to investigate the microbiological quality of dried and cooked fish. The study also investigates the significant effect of antibiotic sensitivity pattern of the isolated bacteria.

Research objectives:

- To evaluate of microbial load of cooked and dried fish samples.
- To isolate and identify the bacterial pathogens in fish samples.
- To observe antimicrobial sensitivity pattern of the isolated bacteria.
- To create awareness among local beneficiaries and consumers.

CHAPTER 2

REVIEW OF LITERATURE

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

Edris *et al*., (2017) studied that bacterial conditions of fish products with E.O.S, and its hazards on public health. A total of 60 samples of fish products (smoked fish: herring and smoked salmon–semi cooked fish: fish finger and breaded shrimp) (15 of each) were collected from different retail markets for bacteriological examination. The average of APC, Coliform, *Escherichia coli*, Mould and yeast and *Staphylococcal aureus* counts (log_{10} cfu/g) were 4.17 ± 0.12, 2.92 ± 0.16, 2.19 ± 0.23, 3.96 ± 0.14 and 1.72 ± 0.21 for herring, respectively, 3.16 ± 0.19, 2.69 ± 0.13, 1.22 ± 0.16, 2.22 ± 0.18 and 1.06 ± 0.06 in smoked salmon, respectively, 2.78 ± 0.12, 2.02 ± 0.22, 1.59 ± 0.22, 2.14 ± 0.15 and 1.24 ± 0.24 in fish finger, respectively, and 2.60 ± 0.13, 2.33 ± 0.14, 1.46 ± 0.23, 1.96 ± 0.20, 0 ± 0 in breaded shrimp, respectively. The incidence of food poisoning organisms (*Salmonella* and *Listeria monocytogenes* and *Vibrio parahaemolyticus* also investigated and no one of them was isolated in the examined samples.

Sivaraman *et al*., (2016) isolate organisms from sun dried and salted fish products are such as mesophilic bacteria, halotolerant & halophilic micrococci, spore-forming bacteria, some Gram-positive and lactic acid bacteria, mainly *Bacillus* spp., *Micrococcus* spp., *Halobacterium* spp., *Halococcus* spp., *Staphylococcus aureus, E. coli*, *Salmonella* spp. and *Shigella* spp.

Armani *et al*., (2016) observed that over the past few years, the demand for the introduction of fish products in public canteens (schools, hospitals and nursing-homes) has grown due to their good nutritional proprieties. The particular health conditions and sensitivity of some groups of consumers exposes them to greater risks of food poisoning. It is therefore important to monitor the raw materials that end up in mass catering implementing strategies of mass catering control, both with self-monitoring strategies and with regular controls performed by the competent health authorities. The purpose of this study is to assess the overall quality of seafood dealt out from public catering services located in Northeast Italy. In this paper we illustrate the results of microbiological analysis performed on 135 fish samples (58% of samples were raw fishes, 27% cooked fishes, 6% raw fish products, 9% cooked fish products) and species

identification performed on 102 fish samples. Additionally, 135 environmental swabs were collected to determine the effectiveness of cleaning and sanitation of food contact (cutting boards, cooking equipment and food processing surfaces) and non-contact (refrigerator wall and handle, tap lever) surfaces. Of raw seafood samples, 24% had total aerobic mesophilic bacteria count >105 CFU/g and for Enterobacteriaceae the fecal contamination was excluded since no Salmonella spp. and Escherichia coli were isolated. Just 3.8% of raw seafood samples resulted positive for Listeria monocytogenes. The results of swab samples of cooking utensils and surfaces showed that sanitation practices should be improved. Molecular analysis for fish species identification revealed a mislabeling for 25% of sampled fishes. The results of this survey can provide valuable information for monitoring and surveillance programs for the control of quality of fish and fish products.

Bibi *et al.,* (2015) Determined the occurrence of salmonella in freshwater fish. Smoked fishes serve as a vehicle for the transmission of *Salmonella* that exists on skin, in gills and intestine. The absence of a suitable hygiene programed 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* spp., *E. coli, Klebsiella* spp., *Proteus* spp. and *Brucella* spp. 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* spp. 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 were 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.

Abdollahzadeh *et al.*, (2014) observed that Food poisoning caused by *Listeria monocytogenes* leads to a 30% rate of mortality among patients. The antibacterial activity of cinnamon, thyme, and rosemary essential oils (EOs) and shallot and turmeric extracts was tested against *L. monocytogenes* using agar well and disc diffusion techniques. Results showed that thyme EO had the highest antimicrobial activity, followed by cinnamon and rosemary EOs, respectively. The antilisterial activity of thyme EO at 0.4%, 0.8%, and 1.2% levels, nisin at 500 or 1000 IU/g level, and their combination against *L. monocytogenes* was examined in minced fish samples. The antilisterial properties of nisin were also investigated in cooked minced fish treatments. Nisin at 500 or 1000 IU/g in the minced fish meat demonstrated bacteriostatic activity against *L. monocytogenes.* The use of thyme EO at 0.8% and 1.2% reduced the *L. monocytogenes* viable count below 2 log cfu/g after 6 days. Furthermore, simultaneous use of thyme EO at 0.8% and 1.2%, and nisin at 500 or 1000 IU/g level, reduced the *L. monocytogenes* viable count below 2 log cfu/g after the second day of storage. The antilisterial activity of nisin in the cooked minced fish samples was slightly stronger than that of the raw group.

Longast *et al.*, (2012) investigated salt dried marine fishes for observed microbial quality. Conventional method employed in salt drying of fishes that are intended for human consumption are facing serious health hazards due to improper and unscientific methods. In conventional method they were isolated fecal coliforms and *Vibrio* spp. Which is an alarming situation that warrants the need for incorporating hygienic and scientific ways of salt drying.

Sultana *et al.*, (2010) performed a study was to determine the load of pathogenic bacteria contaminating different dried fishes collected from retail market and khamar. A total of 25 different dried fishes collected from Bashkhali, Anwara, Karnafuli Khamar, K.R. market and Seshmore were analyzed in the Bacteriology Laboratory, Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh. Each sample was inoculated in plate count agar, EMB agar media and mannitol salt agar media to determine TVC, TCC and TSS. Among the dried fishes of three areas of Chittagong, the highest mean TVC and TSC was found 90.00±1.00 and 86.33±1.52 in

Churi. On the other hand, the lowest TVC and TSC were 30.67±0.58 and 31.67 ± 1.53 in Rikshaw and Chingry respectively. Similarly, highest TVC and TSC was 113.00±2.65 and 83.00±2.65 in Chapa and lowest TVC and TSC was 94.67± 0.58 and 53.00±2.65 in Challa of two areas of Mymensingh district. There were significant differences among TVC and TSC. No coliform bacteria were isolated from dried fishes collected from different areas. Highest TVC and TSC were found in dried fish of Sheshmor of Mymensingh in case some dried fishes TVC and TSC were found nil. It may be concluded that bacterial load in dried fish of Chittagong region is less compare to that of Mymensingh region and lowest bacterial load was found in dried fishes of Karnafuli Khamar compare to other regions of collection.

Soliman *et al*., (2010) carried out 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.

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 fishes were discussed based on their potential pathogenic effect toward public health and their role to enhance rapid spoilage of fishes.

Kumar *et al.,* (2008) reported that Escherichia coli is one of the most common causes of bloodstream infections associated with high mortality.The quality deterioration of foods during processing, storage and distribution is mainly caused by microorganisms. The kind of microorganisms present in foods is closely connected to the microflora of the environment. Dry fishes used for the present study were *Sardinella gibbosa, Terapon theraps, Terapon* spp., *S.longiceps, Sphyraena* spp., *Sardinella fimbriata, Upeneus* spp., *Thryssa setirostris, Lutjanus vitta, Sillago siham, Gerres filamentosus, Stolephorus japonicus, Lethrinus* spp., *Sardinella albella and Mugil cephalus*. It was also observed that the *E. coli* strains isolated from all the fishes were found to be resistant to the three antibiotics, Vancomycin, Bacitracin and Penicillin G. The study also reveals that the *E. coli* strains isolated from at least seven of the fish samples were found to be sensitive to Neomycin, Streptomycin and Chloramphenicol. So the present work reveals that the salt-dried fishes sold in Tuticorin fish markets are contaminated with fungi and pathogenic bacteria like *E. coli.* The *E. coli* strains has developed a high resistance pattern to a few of the antibiotic tested. So public awareness (fisherman, fish workers and vendors) on sanitary and hygienic practices to stress the importance of quality is of utmost importance. Landing sites should be maintained clean.

Bagge-Ravn *et al.,* (2003) showed that the microflora adhering to the processing equipment during production and after cleaning and disinfecting procedures was identified in four different processing plants. A total of 1009 microorganisms was isolated from various–agar plates and identified. A stepwise procedure using simple phenotypic tests was used to identify the isolates and proved a fast way to group a large collection of microorganisms. *Pseudomonas*, *Neisseriaceae*, Enterobactericeae, *Coryneform*, *Acinetobacter* and lactic acid bacteria dominated the microflora of cold-smoked salmon plants, whereas the microflora in a plant processing semi-preserved herring consisted of *Pseudomonas, Alcaligenes* and Enterobactericeae. *Psychrobacter*, *Staphylococcus* and yeasts were found in a caviar processing plant. Overall, many microorganisms that are often isolated from fish were also isolated from the fish processing plants.

Azam *et al.,* (2003) examined organoleptic, microbiological and biochemical qualities of four dried fish samples (*Mugil cephalus, Scoliodons horrakowah, Harpodon nehereus and Setipinna phasa)* were assessed in summer and winter season and their qualities were compared. The organoleptic score of the sample were higher than summer season.

The standard plate count (SPC) ware acceptable in both seasons. Total coliform count of sample summer and winter were found to vary between <3to 4 $MPNg^{-1.}$

Valdimarsson et al., *(1998)* reported that Iceland was a major producer of cold-water shrimp, *Pandalus borealis*. In recent years considerable attention has been given to improve hygiene in the factories producing cooked, peeled and frozen shrimp. To keep track of the bacteriological status of the end product, shrimp from most of the factories is routinely analysed bacteriologically by the request of shrimp exporters. This paper reports on the results of a bacteriological analysis of 7913 samples of shrimp from 26 Icelandic factories over a 6-year period. The results showed that the geometric mean of APC (at 35°C) was 1718 per g and 57% of the samples had APC under 1000 per g. Some 70% of the samples had less than one coliform per g and 99.9% of the samples had less than one faecal coliform per g. *Staphylococcus aureus* was detected in less than 0.2% of the samples. The results show improvement in bacterial profiles, mainly total coliforms, over the 6-year period. Overall, the results show acceptable bacterial numbers in the finished product, indicating a high level of hygiene. *Listeria* spp. were, however, found in 270 of 3331 samples examined or 8.1%. Species identification was done on 49 of the 270 positive samples. The proportion of *L. monocytogenes* was found to be 26.5%.

Vishwanath *et al.,* (1998) showed a comparative study of the biochemical, nutritional and microbiological quality of the fresh (FF) and smoked (SF) symbranchoid mud eel fish, *Monopterus albus* was carried out. SF had lower percentages of total protein (79.0 vs 76.0), pure protein (66.7 vs 57.1), lipid (10.74 vs 9.82) and ash (7.00 vs 6.00) contents than FF. The pH values of SF were more acidic than those of FF (6.90 vs 7.25). Digestibility and protein efficiency ratio (PER) were significantly less (P<0.05) in SF. Total plate counts of bacteria (TPC) and fungi (TFC) were 10^6–10^7 g^{-1} and 10^2 g^{-1}, respectively in FF and 10^9–10^{10} g^{-1} and 10^5 g^{-1}, respectively, in SF. *Salmonella* and *E. coli* were not present in any samples examined. However, coliform bacteria, *Staphylococcus aureus* and faecal *Streptococci* were detected in both. Seven genera of fungi were present in FF, the dominant one being *Fusarium* sp. Five genera of fungi were detected in SF, out of which *Penicillium* was dominant.

Gram *et al.,* (1996) observed spoilage of fresh and lightly preserved fish products is caused by microbial action. This paper reviews the current knowledge in terms of the microbiology of fish and fish products with particular emphasis on identification of specific spoilage bacteria and the qualitative and quantitative biochemical indicators of

spoilage. *Shewanella putrefaciens* and *Pseudomonas* spp. are the specific spoilage bacteria of iced fresh fish regardless of the origin of the fish. Modified atmosphere stored marine fish from temperate waters are spoiled by the CO_2 resistant *Photobacterium phosphoreum* whereas Gram-positive bacteria are likely spoilers of CO_2 packed fish from fresh or tropical waters. Fish products with high salt contents may spoil due to growth of halophilic bacteria (salted fish) or growth of anaerobic bacteria and yeasts (barrel salted fish). Whilst the spoilage of fresh and highly salted fish is well understood, much less is known about spoilage of lightly preserved fish products. It is concluded that the spoilage is probably caused by lactic acid bacteria, certain psychrotrophic *Enterobacteriaceae* and/or *Photobacterium phosphoreum*. However, more work is needed in this area.

Prasad et al., (1994) analyzed chemical and microbiological qualities of 75 samples of dry fish from local markets were studied. In most of the samples, moisture content was found to be higher and salt content lower as compared with ISI specifications for cured dried fish. Potential pathogens, Escherichia coli and coagulase positive staphylococci, were isolated from some of the samples.

Farber *et al.,* (1991) investigated a limited sampling of fish products at both the wholesale and retail levels demonstrated that ready-to-eat fish products such as shrimp and smoked salmon are often contaminated with *Listeria monocytogenes*. This study shows that growth of the organism at 4°C occurred on artificially inoculated cooked crabmeat, lobster, shrimp, and smoked salmon. The organism generally grew better on crab and lobster. *L. monocytogenes* was also observed to multiply slowly on naturally contaminated shrimp. Given the low levels of *L. monocytogenes* found on cooked fish products and their relatively short shelf life, unless these products are temperature abused, *Listeria* contaminated fish should not represent a serious health hazard.

John *et al.,* *(*1988) reported a microbial profile of dry foods in Ghana including smoke dried herrings, salt dried tilapia, salt dried trigger fish, gari, kokonte and okra was evaluated. Okra had the highest aerobic count of 42×10^6, followed by kokone 16–20×10^4, smoke dried herrings 0.2–4×10^4, salt dried tilapia 3–4×10^3, salt dried trigger fish 3– 44×10^2 and gari 3–34×10^2. Anaerobic count was low for all the samples except smoke dried herrings 7–9.5×10^2. Aspergillus and Penicillium were the predominant molds. Coliform count was low for salt dried fish and gari, but higher for smoke dried herrings 2–25×10^2, kokonte 11–29×10^2 and okra 31 – 47×10^2.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Study Area and Duration

The present research work was carried out on dried and cooked fish available in the local market in Dinajpur city. The laboratory works was conducted in the laboratory of the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, during the period from January to December, 2018.

3.1.2 Collection of samples

A total 79 samples were collected from local market and different restaurant in Dinajpur city. Out of 79 samples 54 samples were dried fish and 25 were cooked fish samples. There were 18 different types of dried fish and 6 different types of cooked fish product samples. Then the samples were brought to the laboratory of the Department of Microbiology, Faculty Veterinary and Animal Science of Hajee Mohammad Danesh Science and Technology University, Dinajpur, for detection and antibiogram study of bacterial pathogens from these samples.

Figure 1: Sample Collection

3.1.3 Media use for Culture

The media and reagents that have been used for the isolation and identification of the bacteria are mentioned below.

3.1.3.1 Liquid media

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

3.1.3.2 Solid media

- Nutrient Agar Base (Hi-media, India).
- Plate Count Agar
- 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).

3.1.3.3 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

3.1.4 Reagents

- Gram's staining reagent: (Crystal violet, Gram's iodine, Acetone alcohol 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
- Indole Solution
- Methyl Red Solution

- Potassium-di-hydrogen phosphate (0.2M, KH_2PO_4 $2H_2O$)
- Di-sodium hydrogen phosphate (0.2M, $Na_2HPO_4 12H_2O$)

3.1.5 Antibiotics Sensitivity Test

3.1.5.1 Kirby-bauer (K-B) antibiotic sensitivity testing materials:

- Mueller-Hinton or Nutrient Plates
- Antibiotics Disks
- Stock Cultures of experimental bacteria
- Bunsen Burner
- Inoculation loop or needle
- Forceps
- Sterile Swab
- Incubator

3.1.5.2 Antimicrobial Sensitivity Discs:

To determine the drug sensitivity pattern of different bacterial isolate with different types of commercially available antimicrobial discs (Oxoid Ltd., UK) were used. In **Table-1** represent the antibiotics that were tested against, the selected organisms with their disc concentration.

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.	Cefuroxime Sodium (CXM)	30 µg/disc
4.	Cephalexin (CN)	30 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.	Nalidixic Acid (NA)	30 µg/disc
10.	Colistin (CL)	10 µg/disc
11.	Streptomycin (S)	10 µg/disc
12	Kanamycin (K)	30µg/disc
13	Cefradine (CH)	25 µg/disc

- Legend: µg = Microgram

3.1.6 Instruments and appliances

Phase contrast microscope, digital Colony Counter, digital balance, gripper bag, cotton, hand gloves, plastic syringe (5 ml), micropipette (1 ml, 500 µl, 10- 20µl,1µl), glass slides, magnifying glass, marker pen, ice-box, spirit lamp, cover slips, inoculating loop test tube and rack, autoclave, refrigerator, conical flask etc.

3.1.7 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.

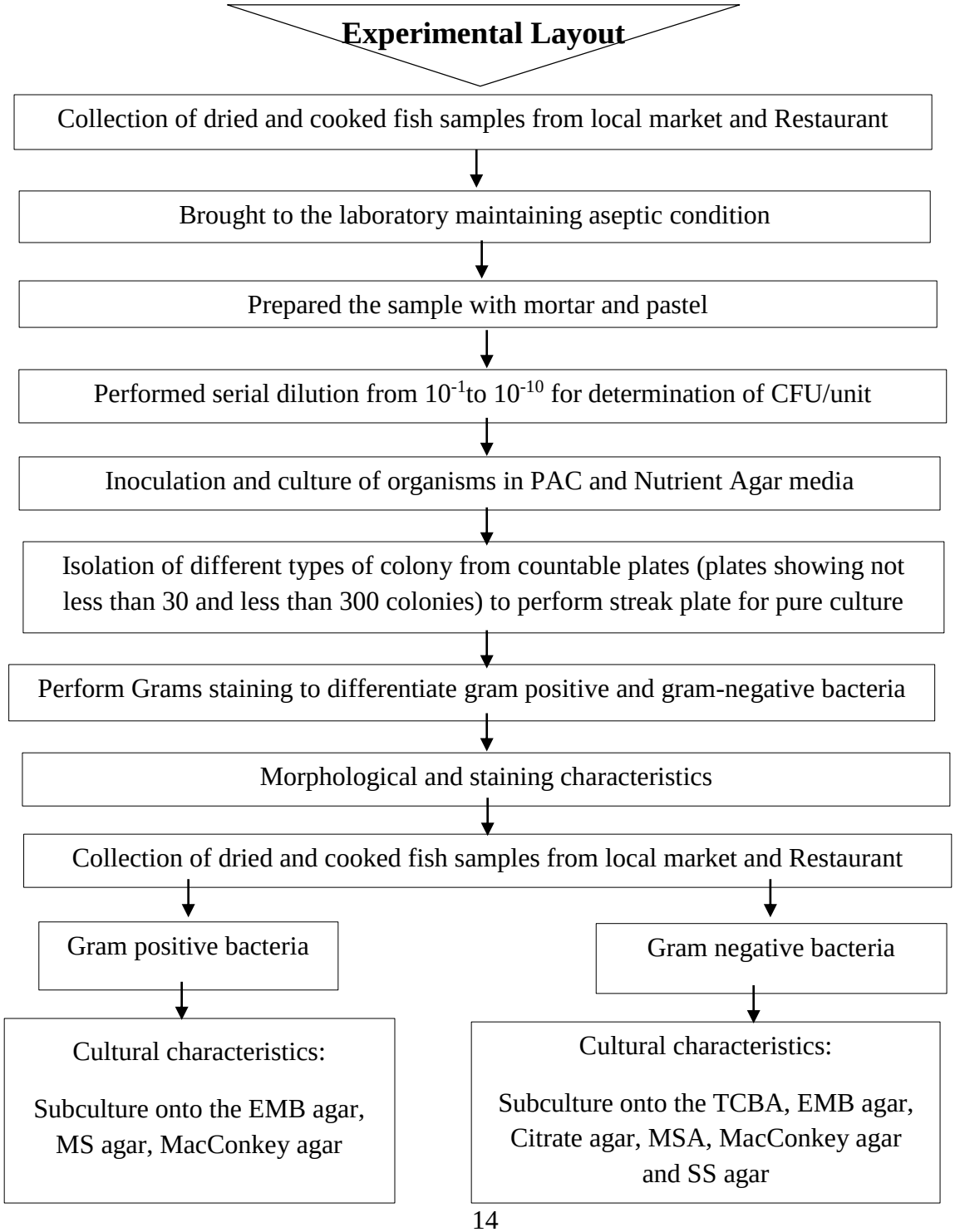
3.2 Methods

3.2.1 Laboratory preparation

All items of glassware including test tubes , pipettes, cylinder, flasks, conical flasks, glass plate , slides ,vials and agglutination test tubes soaked in a household dishwashing detergent solution for overnight, contaminated glassware were disinfected in 2% sodium hypo chloride solution prior to cleaning .The glassware were then cleaned by brushing ,washed thoroughly and finally sterilized either by dry heat at 160°C for 2 hours or by autoclaving for 15 minutes at 121°C under 15 lbs pressure per square inch. Autoclaved items were dried in a hot air oven at 50°C.Disposable plastic ware (micropipette tips) was sterilized by autoclaving. All the glassware was kept in oven at 50°C for future use.

3.2.2 Experimental layout

The experimental works were divided into two steps: The first step was performed for the isolation and identification of the organisms of collected samples using cultural, staining and biochemical characteristics. The second step was conducted for the determination of antibiotics sensitivity and resistant pattern of isolated organisms of various samples by using different antibiotics discs available in the market. The experimental layout illustrated in f**igure 1**.



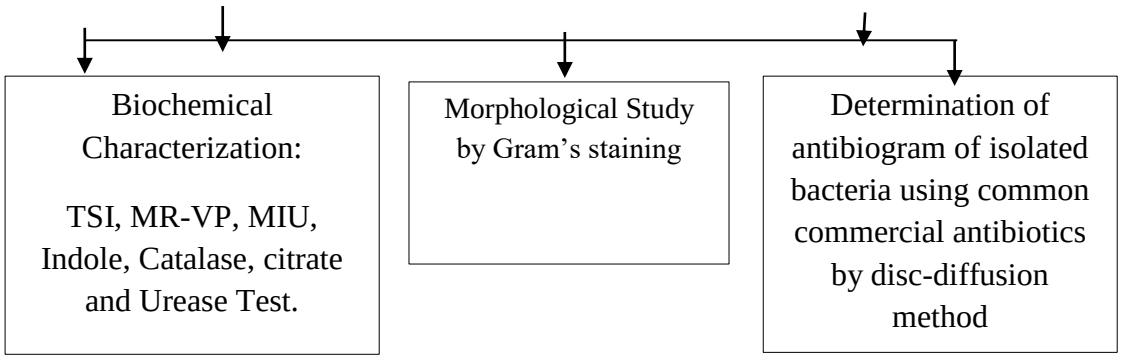


Figure 2: The schematic illustration of the experimental layout

3.2.3 Preparation of culture media

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

3.2.3.1 Liquid Media

a) 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 m1 volumes into clean bottles, and then sterilized by autoclaving at 121 °C (15 lb./inch^2) for 15 minutes.

b) 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.

3.2.3.2 Solid media

a) Nutrient agar

Twenty-eight 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).

b) Plate Count Agar (PCA)

Add 17.5g to 1 liter of distilled water. Dissolve by bringing to the boil with frequent stirring, mix and distribute into final containers. Sterilize by autoclaving at 121°C for 15 minutes. After autoclaving, the medium was poured into each sterile petri dish and allowed to solidify. After solidification of the medium in the petri dishes, these were incubated at 37°C for overnight to check their sterility and used for cultural characterization or stored at 4°C refrigerator for future use (Cater 1979).

c) 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).

d) Eosin methylene blue agar

Eosin methylene blue (EMB) agar medium was used to observe the growth of *Escherichia coli* (Cheesebrough, 1985). 36 grams 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).

e) Salmonella Shigella Agar

Salmonella *S*higella (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 sterilize 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)

f) Mannitol salt agar

111 grams Mannitol Salt Agar base powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely (necessary calculation was done for required number of plates). The medium was then sterilized by autoclaving at 1.2 kg/cm2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 450- 500C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass Petri dishes (large sized) to form thick layer there. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the Petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

g) 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).

h) 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).

i) Thiosulfate-citrate-bile salts-sucrose (TCBS) agar

Suspend 89.08 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely without using autoclave. Then Cool to 45-50°C. Mix well and pour into sterile Petri plates. (Kobayashi 1963)

j) 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).

k) Urease test:

Urea Agar was developed by Christensen in 1946 for the differentiation of enteric bacilli. The urease test is used to determine the ability of an organism to split urea, through the production of the enzyme urease. This test is used to differentiate organisms based on their ability to hydrolyze urea with the enzyme urease.

Suspend 24.01 grams in 950 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 10 lbs. pressure (115°C) for 20 minutes. Cool to 45-50°C and aseptically add 50 ml of sterile 40% Urea Solution (FD048) and mix well. Dispense into sterile tubes and allow to set in the slanting position. Do not overheat or reheat the medium as urea decomposes very easily.

3.2.4 Reagents preparation

a) 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).

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.

b) Voges-Proskauer solution

Alpha-naphthol solution

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

Potassium hydroxide solution

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

c) Indole reagent

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).

d) 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 (KH2PO4) 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).

3.2.5 Sample collection and sample processing

54 samples of available 18 types dried fishes are collected from different local market of Dinajpur city. All samples 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 samples were homogenized through blending with 90 ml peptone water (Cappuccino and Sherman, 1996). Then 1-10-fold dilutions were performed.

Darikana dry Fish

Mass of Darikana dry Fish

Puti Fish

Chingri Fish

Figure 3: Preparation of dried and Cooked fish sample

3.2.6 Serial dilution of Sample

10g of each fish sample were weighed aseptically and homogenized in 90ml sterile PBS water. Then, serial dilutions were made by mixing 1.0ml of the suspension in 9.0ml sterile PBS water to obtain 10^{-1} dilution. The dilution was then made to 10^{-2}, and 10^{-6} diluents.

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.

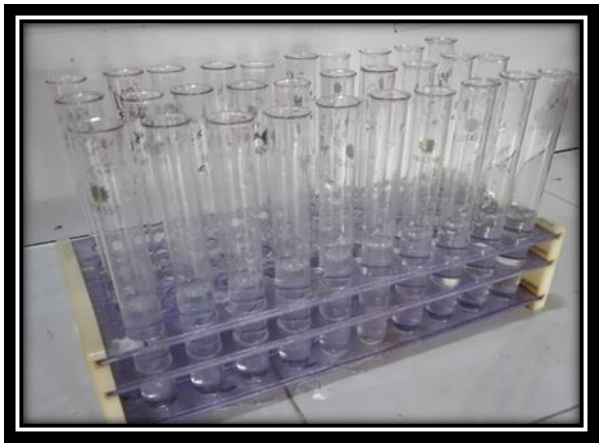


Figure 4: Serial Dilation

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 2ndt test tube, then from 2nd test tube to 3rd test tube and finally 5th to 6th test tube and 1ml discard from 7th test tube by the help of pipette and in every step, mixing was done properly.

3.2.7 Enumeration of total viable count (TVC)

50µl of each fivefold dilution was transferred and spread onto **Plate Count Agar** using a micropipette for each dilution for the determination of total bacterial count. The diluted samples were spread as quickly as possible on the surface of the plate. The plates were kept in an incubator at 37$_0$C for 24 hrs. After incubation, plates exhibiting 30-300 colonies were counted. The average number of colonies in particular dilution was multiplied by the dilution factor to obtain the total viable count. The total viable count was calculated according to ISO (1995). The results of the total bacterial count were expressed as the number of colonies forming units (CFU) per ml of food samples.

Number of cells per ml=number of colonies × Dilution factor

3.2.8 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.

3.2.9 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 petridishes.

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.

3.2.10 Morphological characteristics of organism by gram's staining method

The gram staining was followed to study the morphological and staining characterization of bacteria and provide information about the presumptive bacterial identification as per recommendation of (Cowan and Steel 1979).

Procedure:

- A loopful sterile distilled water was placed in the center of a clean sterile slide.
- A small colony was picked up with a bacteriological loop and was mixed with distilled water of a slide.
- The colony was made to thin smear on a slide.
- The smears were fixed by air driving.
- 0.5% of crystal violet solution was then applied on the for two minutes
- Then washout with clean water
- Grams iodine was then added to act as mordant for one minute 24
- Then washed out with clean water
- Acetone alcohol was then added to decolorize for 1-2 seconds.
- washed out with clean water
- Safranin was as counter a stain and allowed for one minute.
- Washed out with water.
- Then the slide was blotted with blot paper and was allowed to dry. The slide was examined under microscope with high power objective (100X) using with immersion oil.

Grams staining observation:

- Gram Positive: Dark purple.
- Gram Negative: Pale to dark red.
- Cocci: Round shape.
- Bacilli: Rod shape.

3.2.11 Culture into differential media

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.

3.2.12 Culture on selective media

3.2.12.1 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.

3.2.12.2 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.

3.2.12.3 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.

3.2.12.4 Cetrimide agar

Cetrimide Agar is the selective agent as it inhibits the growth of the accompanying microbial flora. Typical *Pseudomonas* spp. colonies are greenish or yellowish green in color (Cheesbrough, 1985).

3.2.12.5 Thiosulfate-citrate-bile salts-sucrose (TCBS) agar

Samples were inoculated int TCBS agar plates after incubation (37^0c for overnight) the yellow pigmented colonies indicated positive test for *Vibrio species*.

3.2.13 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.

3.2.13.1 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.

3.2.13.2 Biochemical methods for identification of isolated bacteria:

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

1 Catalase test
2 Indole test
3 Methyl test
4 Voges-Proskauer (VP) test
5 Triple sugar iron (TSI)
6 Citrate utilization test
7 Urease test
8 Motility indole and ornithine decarboxylate (MIO) test

3.2.13.2 .1 Catalase test

The present of catalase is determined by its ability to break down peroxide into water and oxygen, releasing bubbles of oxygen. This test is used to differentiate those bacteria that produce the enzyme catalase, such as *staphylococci*, from non-catalase producing bacteria such as *streptococci* by (Cheesbrough, 1985).

Procedure:

a) Picked up a colony of the bacteria from a plate and transferred the colony on a glass slide in a drop of water.
b) Placed a few drops of 3% H2O2 (dilute 30% commercial solution (1: 10) over the culture.

Observations: Positive- Immediate strong bubbling; **Negative-** No formation of bubble.

3.2.13.2.2 Indole test

The test organism was cultured in a medium containing tryptophan. The organisms break down tryptophan and indole are released. It was detected by the action of Kovac's reagent or Ehrlich reagent (formation of red colored compound). This test was important for the identification of Enterobacteria such as *Escherichia coli, Pseudomonas vulgaris* etc. by (Cheesbrough, 1985).

Procedure

(a) Tryptophan containing broth was inoculated with bacteria.

(b) The tube was incubated at 37° C for 24 hours.

(c) Added 0.5 ml of the Kovac's reagent after the bacterial growth.

(d) If indole positive within a 30 second a red color ring appeared at the junction of medium in the tube

(e) Negative: No color development or slightly pink color.

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.

3.2.13.2.3 Methyl red (MR) test

This test was performed to differentiate Enterobacteria. Some Enterobacteria when cultured in buffered glucose peptone water, ferment glucose to produce sufficient acidity, which gives red color with methyl red indicator (PH range: 4.4-6.2, Color change: red yellow) by (Cheesbrough, 1985).

Procedure

(a) Sterile MR-VP broth was inoculated with the test organism and following incubation at 370 c for 24 hours.

(b) Few drops of methyl red solution were added.

Observations:

- A distinct red color indicated MR positive test
- Yellow or orange color indicated a negative result.

3.2.13.2.4 Voges Proskauer (VP) test

The test organisms were cultured in glucose phosphate peptone water for 24 hours. Acetone formed was converted to diacetyl. It was converted to a pink compound by the action of creatine. This test was used to assist in the differentiation of Enterobacteria such as *Vibrio cholerae*, *Klebsiella* spp. and some strains of Enterobacter (Cheesbrough,1985).

Procedure

a) Sterile MR-VP broth was inoculated with the test organism and following incubation at 37° C for 24 hours.
b) After incubation, 5 drops of napthol solution and 5 drops of KOH solution were added.

Observations: The development of a bright red or pink-red color was recorded as a positive Result

3.2.13.2.5 Citrate utilization test

The test organism was cultured in a medium containing sodium citrate, an ammonium salt and bromothymol blue indicator. The organisms use citrate (the only source of28

nitrogen). The citrate utilization is followed by alkaline reaction (change of the color form light to blue) and growth in the medium was indicated by appearance of turbidity. This test was performed in the identification of Enterobacteria by (Cheesbrough, 1985).

Procedure

a) A loop of bacteria was spread across the surface of the agar.
b) Kept the tubes at 370C for 24 hours for incubation.
c) Examined the tubes for the result.

Observations:

Positive: - produce blue color,
Negative: - no color

3.2.13.2.6 MIU (Motility Indole Urease) test

MIU is a semisolid medium designed for detection in Enterobacteriaceae of urease activity, motility, and indole production by Cheesbrough, 1985).

Procedure

Inoculate tubes with a pure culture by stabbing the center of the column of medium to greater than half the depth. Incubate tubes for 18-48 hours at 35 ± 2 °C in aerobic atmosphere.29

a) Motility was observed by growth extending from the line of inoculum or diffuse turbidity of the medium. No motile organisms grow only along the line of inoculation.
b) Urease activity was observed by a change of color to red.
c) Indole production is indicated by the formation of a pink to red color after the addition of three or four drops of Kovac's reagent to the surface of the medium.

Observations: A negative reaction is indicated by the development of a yellow color. The red color of phenol red in alkaline pH did not interfere because of the acidity of Kovac’s reagent. By (Cheesbrough, 1985)

3.2.13.2.7 Triple sugar Iron (TSI) agar slant

65 grams TSI agar base powder was mixed in 1000 ml of cold distilled water in a flask and mixed thoroughly, then heated to boiling for dissolving the medium completely

(necessary calculation was done for required number of test tubes).The medium was then sterilized by autoclaving for 15 minutes at 121°c maintaining a pressure of 1.2 kg/.Then 20/10 ml of medium was poured into each sterilized test tubes and allowed to cool and to solidify (kept in horizontal position). After solidification test tube were used for biochemical characterization and incubated at 37°c for 24 hours.

Procedure

(a) A loop of bacteria was spread across the surface of the agar.
(b) A needle of bacteria was inserted (stabbed) into the bottom (butt) of the tube.
(c) Kept the tubes at 37^0C for 24 hours for incubation.
(d) Examined the tubes for the result.

Table 2: Triple sugar Iron (TSI) agar slant

Result (Slant/butt	**Symbol**	**Interpretation**
Red/yellow	K/A	Glucose fermentation only
Yellow/Yellow	A/A	Glucose and lactose and/or sucrose fermentation
Red/Red	K/K	No fermentation
Yellow/Yellow with bubbles	A/A, G	Glucose and lactose and/or sucrose fermentation; Gas produced
Red/Yellow with bubbles	K/A, G	Glucose fermentation only; Gas produced
Red/Yellow with bubbles and black precipitation	K/A, G H_2S	Glucose fermentation only; Gas produced H2S Produced
Red/Yellow with black precipitation	K/A, H_2S	Glucose fermentation only; H_2S Produced
Yellow/Yellow with black precipitation	A/A, H_2S	Glucose and lactose and/or sucrose fermentation; H_2S Produced
No Change/ No Change	NC/NC	No fermentation

Note: A=acid production: K= alkaline reaction: G=gas production: H_2S= Hydrogen sulfide production.

3.2.13.2.8 Urease test

Urea is the product of decarboxylation of amino acids. Hydrolysis of urea produces ammonia and CO_2. The formation of ammonia alkalinizes the medium, and the pH shift is detected by the color change of phenol red from light orange at pH 6.8 to magenta (pink) at pH 8.1. Rapid urease-positive organisms turn the entire medium pink within 24 hours.

Weakly positive organisms may take several days, and negative organisms produce no color change or yellow as a result of acid production.

Procedure:

1. Streak the surface of a urea agar slant with a portion of a well-isolated colony or inoculate slant with 1 to 2 drops from an overnight brain-heart infusion broth culture.
2. Leave the cap on loosely and incubate the tube at 35°-37°C in ambient air for 48 hours

Observations:

Positive Reaction: Development of an intense magenta to bright pink color

Negative Reaction: No color change

3.2.14 Antibiotic susceptibility test

Antibiotic sensitivity assay of isolated bacteria, to determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to various antimicrobial compounds was determined in vitro by using the standardized agar disc-diffusion method known as the Kirby Bauer (K-B) (Hudzicki J., 2009), Labeled the covers of each of the agar plates with name of the test organisms was inoculated.

(a) Using sterile technique, inoculated all agar plates with their respective test organisms as follow:

1 Dipped a sterile cotton swab into a well-mixed saline test culture and removed excess inoculums by pressing the saturated swab against the inner wall of the culture tube.

2 Using the swab streaked the entire agar surface horizontally, vertically, and around the outer edge of the plate to ensure a heavy growth over the entire surface.

(b) Allowed all culture plates to dry for about 5 minutes.

(c) Distributed the individual antibiotic discs at equal distance with forceps dipped in alcohol and flamed.

(d) Gently pressed each disc down with the wooden end of the cotton swab or sterile forceps to ensure that the discs adhered to the surface of the agar.

(e) The plates were then inverted and incubated at 370 C for 24 hours.

(f) After incubation, the plates were examined and the diameter of the zones of complete inhibition was measured in mm.

(g) The zone diameter for individual anti-microbial agents was used to determine susceptible, intermediate, and resistant categories by referring to an interpreting table

3.2.15 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). Table 3 represent interpretation result of antibiotic sensitivity test.

Table 3: Reading Plates and Interpreting Results

Antimicrobial agent active against bacteria (Disc Code)	**Potency** (µg/disc)	**Zone Diameter Nearest Whole mm**		
		Resistant ≤	**Intermediate**	**Susceptible** ≥
Gentamicin (GEN) *Entrobacteriaceae* *Pseudomonas aeruginosa.* *Acinetobacter* spp. *Staphylococcus* spp.	10 µg/disc	12	13-14	15
Amoxicillin (AMX) *Entrobacteriaceae* *Staphylococcus* spp..	30 µg/disc	13	14-17	18
		19	----	20
Cefuroxime Sodium (CXM) Entrobacteriaceae Staphylococcus spp.	30 µg/disc	14	15-17	18
Cephalexin (CN) All Organisms	30 µg/disc	14	15-17	18
Cefixime (CEF) Entrobacteriaceae	5 µg/disc	15	16-18	19
Azithromycin (AZM) Staphylococcus spp. Streptococcus spp.	30 µg/disc	13	14-17	18
Erythromycin (E) Staphylococcus spp. Enterococcus spp.	15 µg/disc	13	14-22	23
Penicillin G (P) Entrobacteriaceae. Acinetobacter spp. Pseudomonas aeruginosa	10 µg/disc	17	18-20	21
		17	-	18
Nalidixic Acid (NA) Entrobacteriaceae	30 µg/disc	13	14-18	19
Colistin (CL)	10 µg/disc	12	-	14
Streptomycin (S) Entrobacteriaceae	10 µg/disc	11	12-14	15
Kanamycin (K) Entrobacteriaceae Staphylococcus spp.	30µg/disc	13	14-17	18
Cefradine (CH)	25 µg/disc	15	16-18	19

3.2.16 Maintenance of stock culture

Stock culture is a culture of a microorganism maintained solely for the purpose of keeping the microorganism in a viable condition by subculture, as necessary, into fresh medium.

3.2.16.1 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 was 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 characters for few months (Buxton and Fraser, 1977)

3.2.16.2 Sterile buffered glycerin method

Sterile buffered glycerin (20%) was prepared by mixing 20 parts of pure glycerin and 80 parts of PBS. Then a loopful 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 of microbial loads, staining, cultural, biochemical, antibiotic sensitivity pattern and percentage of incidence of isolated bacteria are presented in different table and described below under the following headings:

4.1 Result of Total Viable Count (TVC):

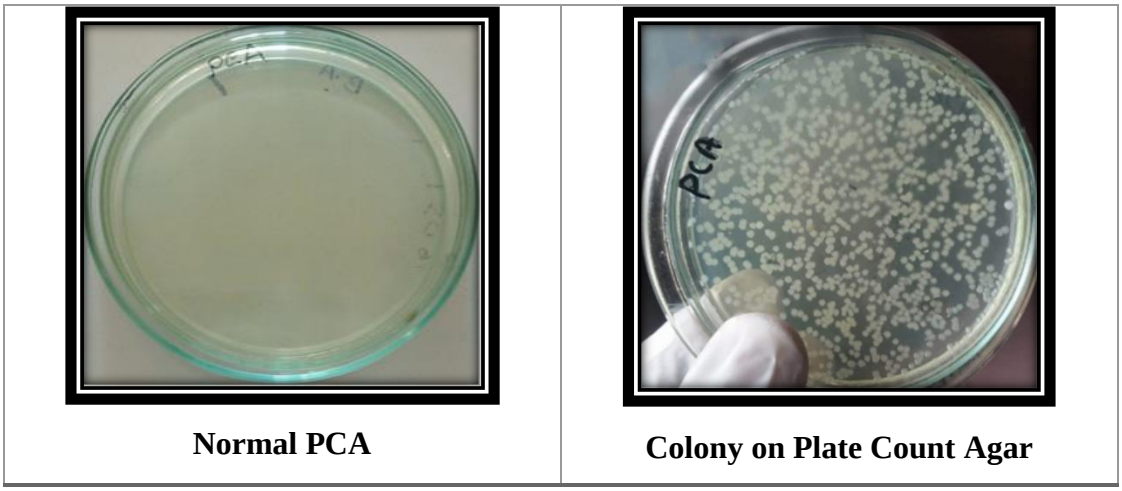
After preparation of tenfold dilution all 50µl sample were spread Plate Count Agar (PCA) media up to dilution 10^{-6}. Then the number of colonies was observed and recorded (in the form of CFU/gm) of samples are presented in Table-4 for dried fish sample and Table-5 for cooked fish.

Table 4: TVC result of dried fish sample:

Local Name	Dried Fish Name (Scientific Name)	Dilution	Number of Colonies	Total Viable Count cfu/gm
Boal	Wallago Catfish *Wallago attu*	10^{-4}	Over 300	TNTC
		10^{-5}	204	4×10^{8}
		10^{-6}	160	3.2×10^{9}
Shol/Shoal	Snauchead Murrel/ Snakehead fish *Channa striata*	10^{-4}	212	4.24×10^{7}
		10^{-5}	180	3.6×10^{7}
		10^{-6}	153	3.06×10^{9}
Darikana/Dariki	Slender rasbora *Rasbora daniconius*	10^{-4}	240	4.8×10^{7}
		10^{-5}	80	1.6×10^{8}
		10^{-6}	32	6.4×10^{8}
Churi	Churi Fish	10^{-4}	88	1.76×10^{7}
		10^{-5}	60	1.2×10^{8}
		10^{-6}	38	7.6×10^{8}
Ilish	Hilsa Shad/Hilsa fish *Tenualosa ilisha*	10^{-4}	76	1.52×10^{7}
		10^{-5}	40	8.0×10^{7}
		10^{-6}	32	6.4×10^{8}
Baspata	Sind Danio *Devario devario*	10^{-4}	80	1.6×10^{7}
		10^{-5}	44	8.8×10^{7}
		10^{-6}	Less 30	TFTC
Ruhi	Rohu Carp/ Rohu *Labeo rohita*	10^{-4}	64	1.28×10^{7}
		10^{-5}	Less 30	TFTC
		10^{-6}	Less 30	TFTC
Rupchada	Elongate glassy perchlat *Pampus chunesis*	10^{-4}	187	3.74×10^{7}
		10^{-5}	87	1.74×10^{8}
		10^{-6}	63	1.26×10^{9}
Bhetki	Barramund/Koral *Lates calarifer*	10^{-4}	222	4.44×10^{7}
		10^{-5}	166	3.32×10^{8}
		10^{-6}	123	2.46×10^{9}

Local Name	Dried Fish Name (Scientific Name)	Dilution	Number of Colonies	Total Viable Count cfu/gm
Mola/ Ulfa	Mola craplet *Amblypharyngodon mola*	10^{-4}	203	4.06×10^{7}
		10^{-5}	76	1.52×10^{8}
		10^{-6}	47	9.4×10^{8}
Cheli	Silver razorbe belly minnow *Salmostoma acinaces*	10^{-4}	141	2.82×10^{7}
		10^{-5}	120	2.4×10^{8}
		10^{-6}	105	2.1×10^{9}
Baim	Tixe track spiny ell *Mastacebelus armatus*	10^{-4}	179	3.58×10^{7}
		10^{-5}	121	2.42×10^{8}
		10^{-6}	Less 30	TFTC
Lakhoa/ lokkha	Indian threadfin *Polynemus indicus*	10^{-4}	Over 300	TNTC
		10^{-5}	105	2.1×10^{8}
		10^{-6}	Less 30	TFTC
Loitta/ Lotey	Bombay Duck *Harpadon nehereus*	10^{-4}	Over 300	TNTC
		10^{-5}	290	5.8×10^{8}
		10^{-6}	212	4.24×10^{8}
Kechhki	Ganges river sprat *Corica soborna*	10^{-4}	Over 300	TNTC
		10^{-5}	256	5.12×10^{8}
		10^{-6}	98	1.96×10^{9}
Taki	Spotted Snakehead *Channa punctatus*	10^{-4}	Over 300	TNTC
		10^{-5}	284	5.68×10^{8}
		10^{-6}	138	2.76×10^{9}
Chingri	Shrimp/ Prawn *Penaneus monodon*	10^{-4}	167	3.34×10^{7}
		10^{-5}	54	1.08×10^{8}
		10^{-6}	Less 30	TFTC
Chapila	Ganges river/ Indian river shad *Gadusia chapra*	10^{-4}	Over 300	TNTC
		10^{-5}	269	5.38×10^{8}
		10^{-6}	187	**3.74×10^{9}**

Note: TNTC= Too numerous to count; TFTC =Too Few to Count



Normal PCA **Colony on Plate Count Agar**

Figure 5: Colony Count in Plate Count Agar

Table 5: TVC result of cooked fish sample

Sample No	Cooked Fish Local Name	Dried Fish Name (Scientific Name)	Dilution	Number of Colonies	Total Viable Count cfu/gm
S_1R_1	Mirigel/Mirka	Mirgal Carp/ *Cirrhinus cirrhinus*	10^{-2}	89	1.78×10^{5}
S_2R_2	Chingri+Ptato Fry	Shrimp/ Prawn *Penaneus monodon*	10^{-3}	71	1.42×10^{6}
S_3R_3	Ruhi	Rohu Carp/ Rohu *Labeo rohita*	10^{-2}	63	1.26×10^{5}
S_4R_4	Puti	Punti/*Puntius sphore*	10^{-3}	171	3.42×10^{5}
S_5R_5	Ruhi	Rohu Carp/ Rohu *Labeo rohita*	10^{-2}	98	1.96×10^{5}
S_6R_6	Bata+ Potato	*Labeo bata*	10^{-2}	123	2.48×10^{5}

4.2 Result of isolation and identification of bacteria:

Isolation and identification of bacteria was made by microscopic characteristic examination and different biochemical examination from the pure culture of the organisms. Seven species of bacteria such as *Escherichia coli, Salmonella* spp.., *Pseudomonas* spp., *Vibrio* spp., *Staphylococcus* spp., *Shigella* spp. and *Klebsiella* spp. were isolated from dried fish but two species of bacteria such as *Escherichia coli* and *Shigella* spp. isolated from cooked fish respectively. Following **Table-6** represent summary of bacteria isolates from dried fish samples and **Table-7** of cooked fish samples.

Table 6: Summary of isolated bacteria from different dried fish samples:

Fish Sample	**Isolated Presumptive Organisms**	**Fish Sample**	**Isolated Presumptive Organisms**	**Fish Sample**	**Isolated Presumptive Organisms**
Boal	*Escherichia coli* *Salmonella* spp. *Pseudomonas* spp. *Vibrio* spp.	Rup Chads	*Escherichia coli* *Staphylococcus* spp. *Salmonella* spp. *Pseudomonas* spp. *Vibrio* spp.	Cheli	*Escherichia coli* *Staphylococcus* spp. *Pseudomonas* spp.
Shol/Shoal	*Escherichia coli* *Staphylococcus* spp. *Shigella* spp. *Pseudomonas* spp. *Vibrio* spp.	Bhetki	*Escherichia coli* *Staphylococcus* spp. *Salmonella* spp.	Baim	*Escherichia coli* *Salmonella* spp.
Baspata	*Escherichia coli*	Mola/ Ulfa	*Escherichia coli* *Salmonella* spp.	Lakhoa/ lokkha	*Escherichia coli* *Staphylococcus* spp. *Salmonella* spp. *Vibrio* spp.
Darikana/ Dariki	*Staphylococcus* spp. *Shigella spp.* *Pseudomonas* spp.	Ruhi	*Escherichia coli* *Staphylococcus* spp. *Pseudomonas* spp. *Vibrio* spp.	Loitta/ Lotey	*Klebsiella* spp. *Vibrio* spp. *Shigella spp.*
Ilish	*Escherichia coli* *Staphylococcus* spp. *Salmonella* spp. *Pseudomonas* spp. *Vibrio* spp.	Churi	*Escherichia coli* *Staphylococcus* spp. *Pseudomonas* spp. *Vibrio* spp.	Kechhki	*Staphylococcus* spp. *Klebsiella* spp. *Shigella spp.* *Pseudomonas* spp.
Taki	*Vibrio* spp.	Chingri	N/B	Chapila	*Shigella spp.* *Vibrio* spp. *Pseudomonas* spp.

Table 7: Summary of isolated bacteria from different cooked fish samples:

Cooked Fish Sample	**Isolated Presumptive Organisms**
Mirigel/Mirka	*Escherichia coli*
Chingri+Ptato Fry	*Escherichia coli, Shigella* spp.
Ruhi	*Escherichia coli*
Puti+Potato	*Escherichia coli, Shigella* spp.
Silver Carp	*Escherichia coli*
Bata+ Potato	*Escherichia coli, Shigella spp.*

4.3 Frequency of isolated bacterial organism:

From 54 dried fish samples of 18 types fish showed 100% bacterial contamination. Total isolated organism was 168 from dried fish. In case 25 samples of cooked fish 20% were found contaminated, total isolated organisms in number was 9. Following Table-7 and Table-8 represent the frequency of bacterial isolate gradually.

Table 8: Frequency of occurrence of Bacteria isolated dried fish samples.

Bacterial species	Number of isolates	Isolation Percentage
Escherichia coli	36	21.43%
Klebsiella spp.	5	2.97%
Salmonella spp.	21	12.5%
Shigella spp.	15	8.93%
Staphylococcus spp.	30	17.86%
Pseudomonas spp.	30	17.86%
Vibrio spp.	31	18.45%
Total	168	100%

Table 9: Frequency of occurrence of Bacteria isolated from cooked fish samples.

Bacterial species	Number of isolates	Isolation Percentage
Escherichia coli	6	66.66
Shigella spp.	3	33.34

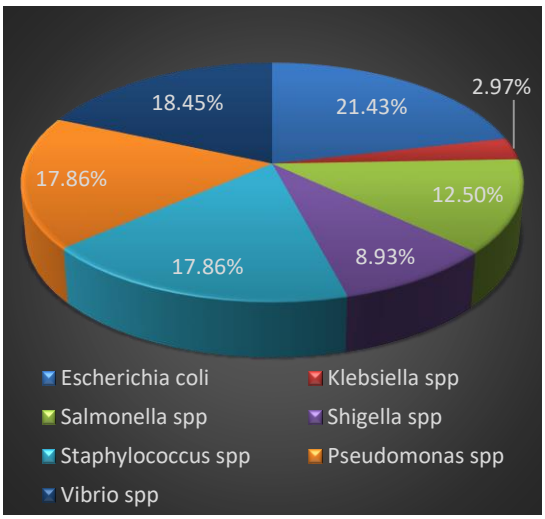


Figure 6: Frequency of isolated bacteria from Dried Fish

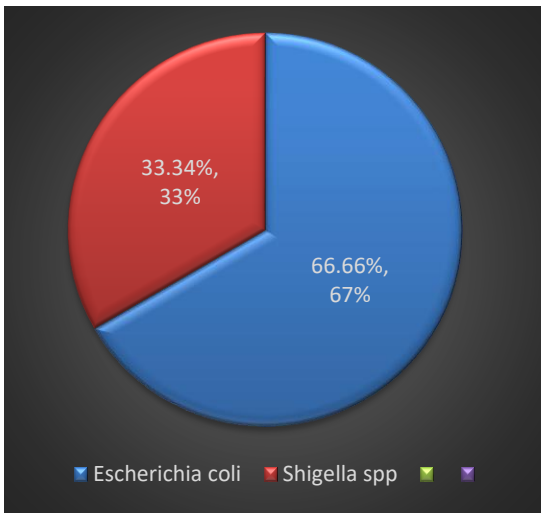


Figure 7: Frequency of isolated bacteria from Cooked Fish

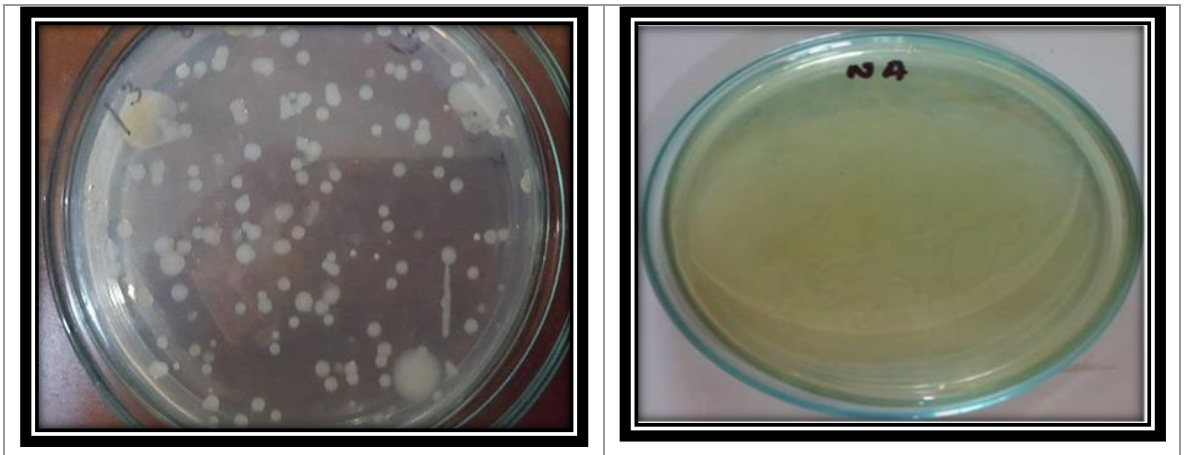
4.4 Cultural, microscopic and biochemical reactions of the isolates

4.4.1 Results of cultural examinations

Cultural characteristics of each type of bacteria isolated from different dried and cooked fish sample were studied for the determination of size, shape and colony characteristics in various bacteriological media. The staining property of primary culture of each of the different samples indicated the presence of more than one type of bacteria in the same smear. The pure cultures of the organism from each mixed culture were obtained by repeated streak plate method using different simple and selective solid media for study. The individual cultural characteristics of bacterial isolates are presented in table-9 The cultural characteristics of *Klebsiella* spp., *Staphylococcus* spp., *Shigella spp. Salmonella* spp., *Pseudomonas* spp., *Vibrio* spp. and *E. coli* exhibited on the media are presented in following figure.

4.4.1.1 Ordinary media

Nutrient agar: Pale colorless colony was found.



Colonies in Nutrient Agar | **Nutrient Agar**

Figure 8: Bacteria in Nutrient Agar

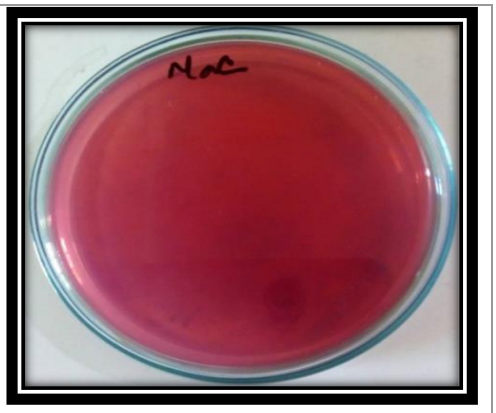
4.4.1.2 Differential media

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.

Colonies in MaC Agar Agar



Control MaC Agar

Figure 9: Colony in MacConkey Agar

4.4.1.3 Selective media

a. Eosin methylene blue (EMB) agar

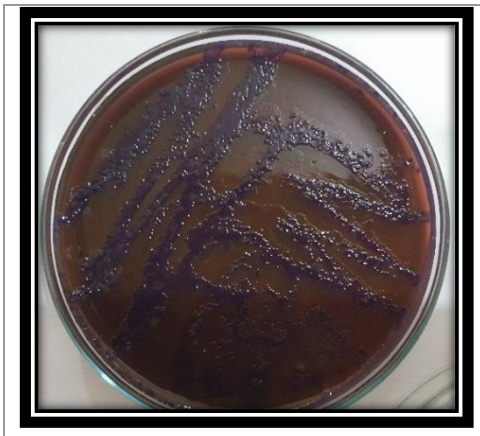
EMB agar plates streaked separately with the lactose fermenter organisms from MacConkey agar revealed the growth of *E. coli* bacteria after 24 hours of incubation at 37°C aerobically.

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

b. Salmonella- *Shigella* (SS) agar

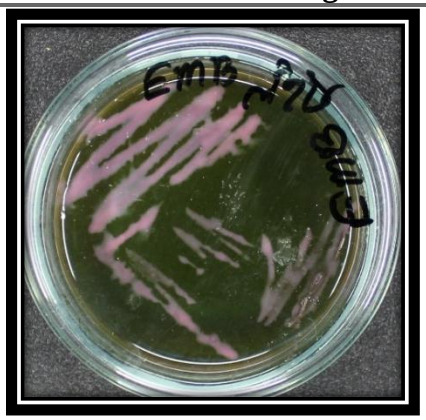
SS agar plates streaked separately with the non-lactose fermenting organisms from MacConkey agar revealed the growth of *Salmonella* spp. after 24 hours of incubation at 37°C aerobically.

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

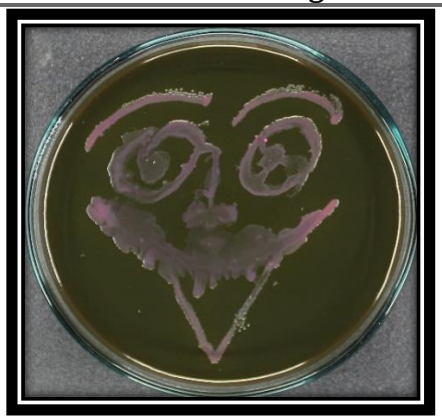


Colonies in EMB Agar

Control EMB Agar



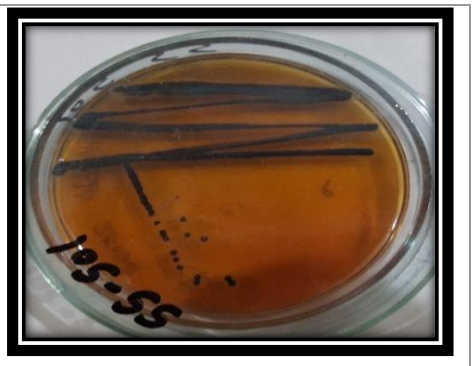
Colonies in EMB agar



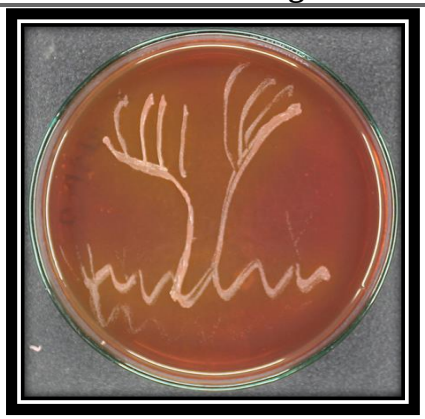
Colonies in EMB agar

Figure 10: Colonies on EMB agar

Controlo SS Agar



Colonies in SS Agar



Colonies in SS Agar

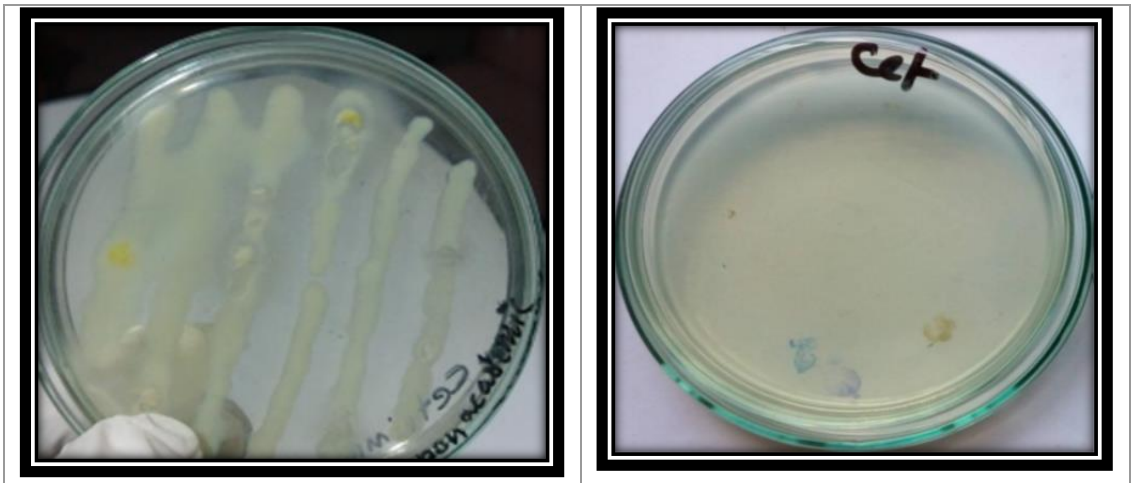
Colonies in SS Agar (cooked)

Figure 11: Colonies on SS Agar

c. 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.



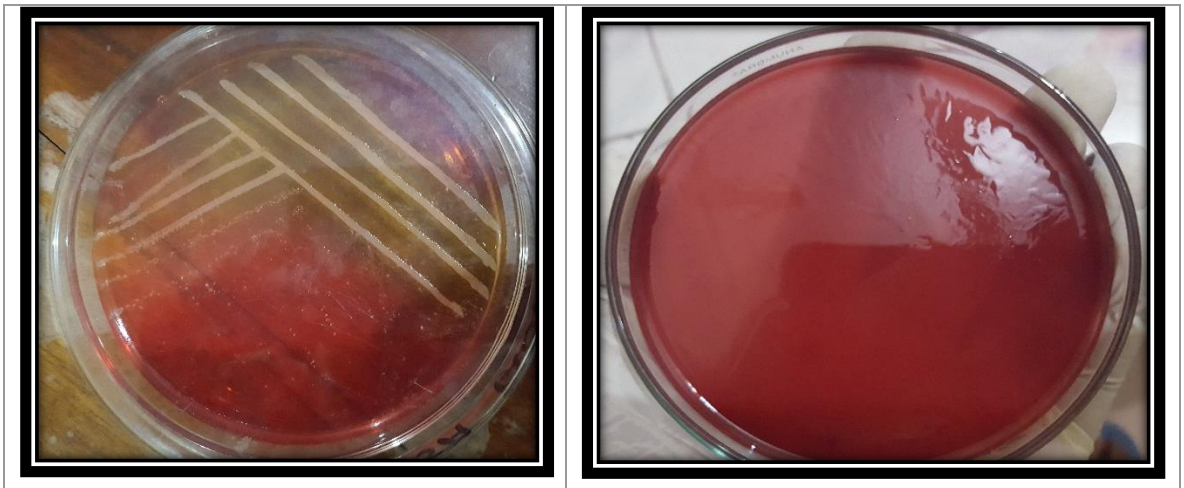
Colonies in CET Agar | **Control CET Agar**

Figure 12: Colonies on Cetrimide Agar

d. 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.

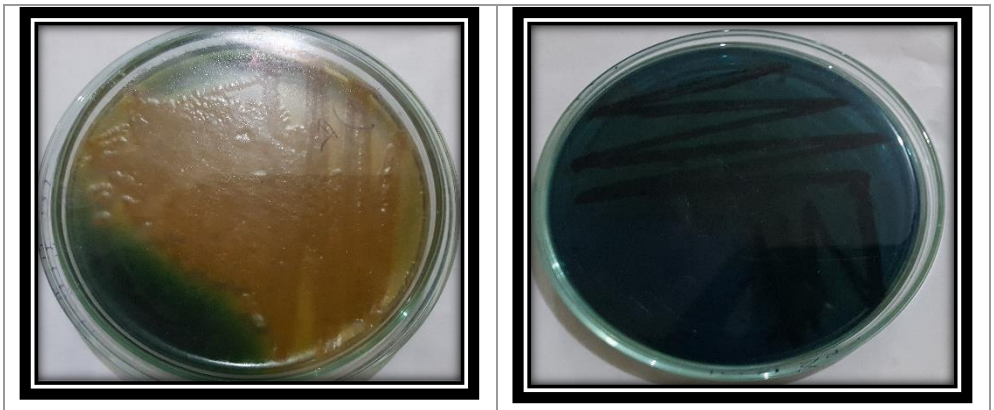


Colonies in MSA | **Control MSA**

Figure 13: Colonies on MSA agar

e. Thiosulfate-citrate-bile salts-sucrose (TCBS) agar

Samples were inoculated int TCBS agar plates after incubation (370c for overnight) the yellow pigmented colonies indicated positive test for *Vibrio* spp.



TCBS Agar with colonis | **TCBS Agar without Colony**

Figure 14: Colonies on TCBS Agar

Table 10: Cultural characteristics of the bacterial isolates

Sl. No	Suspected case of Bacteria	Name of Media	Cultural Characteristics
01	*Klebsiella* spp.	MacConkey agar	Large, mucoid, bright pink lactose fermented colony
02	*Staphylococcus* spp.	MS agar	Medium yellowish colony
03	*Shigella* spp.	SS Agar	Small non-lactose fermented colony
04	*Salmonella* spp.	SS Agar	Small non-lactose fermented with black center colony
05	*Escherichia. coli*	EMB agar,	Metallic sheen (greenish black) - colony
06	*Pseudomonas* spp..	CET agar	green pigment colonies
07	*Vibrio* spp..	TCBS agar	yellow pigmented colonies

a. Legends:

MS=Manitol salt, EMB = Eosin methylene blue, SS=*Salmonella Shigella,* TCBS=Thiosulfate-citrate-bile salts-sucrose. CET= Cetrimide.

4.4.2 Result of staining characteristics of Bacterial isolates

The staining characteristics of the isolated organisms were determined according to Gram's staining technique. Morphological and staining characteristics of bacteria recorded from the swab samples by Gram's staining are presented in Table-10 and following figures.

Table 11: Morphological and staining properties of isolated bacteria by Gram's staining

Sl. No	Bacterial isolates	Shape	Arrangement	Gram's Staining character
1.	***Staphylococcus* spp.**	Cocci in shape	Arranged in cluster	Gram (+)
2.	***Klebsiella* spp.**	Rod in shape	Single, pairs or cluster	Gram (-)
3.	***Escherichia coli***	Rod in shape	Single, pair or in short chain	Gram (-)
4.	***Salmonella* spp.**	Rod in shape	Single or pair	Gram (-)
5.	***Pseudomonas* spp.**	Rod in shape	Arranged in single	Gram (-)
6.	***Vibrio* spp.**	Rod in shape	Arranged in single	Gram (-)
7.	***Shigella spp.***	Rod in shape	Single or pair	Gram (-)

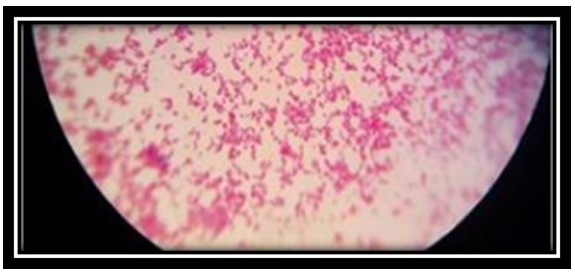


Figure 15: *Klebsiella* spp.

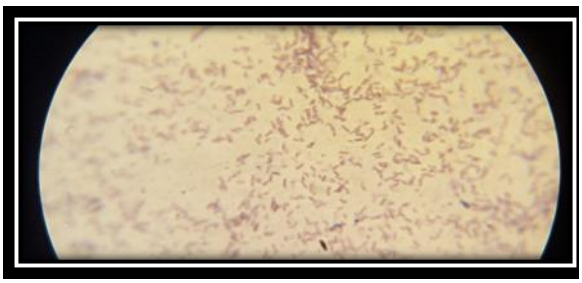


Figure 16: *Salmonella* spp..

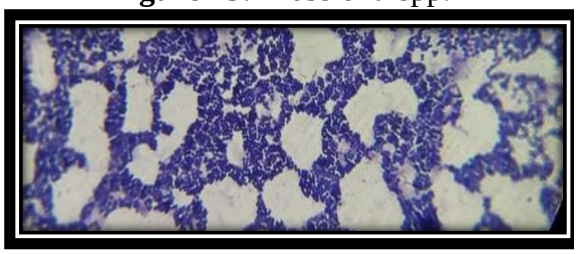


Figure 17: *Staphylococcus* spp.

Figure 18: *E. coli*

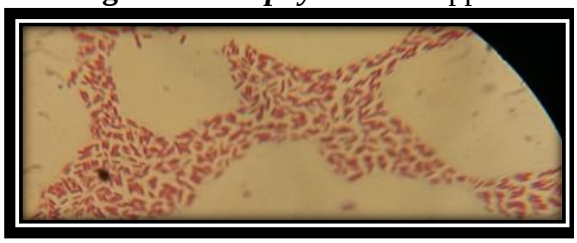


Figure 19: *Shigella* spp.

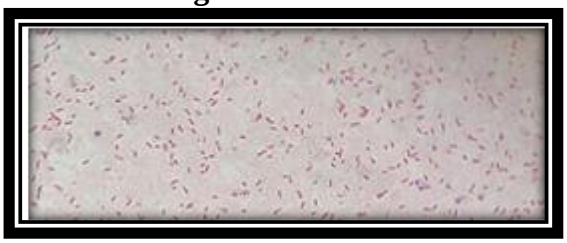


Figure 20: *Pseudomonas* spp..

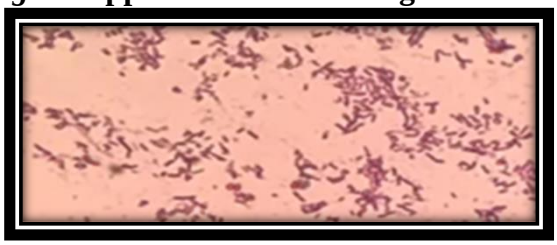


Figure 21: *Vibrio* spp.

4.4.3 Results of biochemical tests

The isolated organisms were confirmed by different biochemical tests. Following **table 11-17** represent the results obtain from different biochemical test for different fish sample.

Table 12: Identification of *E. coli* by different biochemical tests

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

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

Table 13: Identification of *Klebsiella* spp. by different biochemical tests

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

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

Table 14: Identification of *Salmonella* spp. by biochemical test

Biochemical test	**Change of the media**	**Results**
Catalase test	Gas production	Positive
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 15: Identification of *Shigella* spp. by biochemical test

Biochemical test	**Change of the media**	**Results**
Catalase test	Gas production	Positive
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
Urease test	No color change	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 16: Identification of *Pseudomonas* spp. by biochemical test

Biochemical test	**Change of the media**	**Results**
Catalase test	Bubble produced	Positive
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
Urease test	No color change	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 17: Identification of *Vibrio* spp. by biochemical test

Biochemical test	**Change of the media**	**Results**
Citrate utilization test	Prussian blue color	Positive
Indole test	Color change	Positive
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
Urease	No color change	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 18: Identification of *Staphylococcus* spp. by biochemical test

Biochemical test	**Change of the media**	**Results**
Catalase test	Bubble produced	Positive
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).

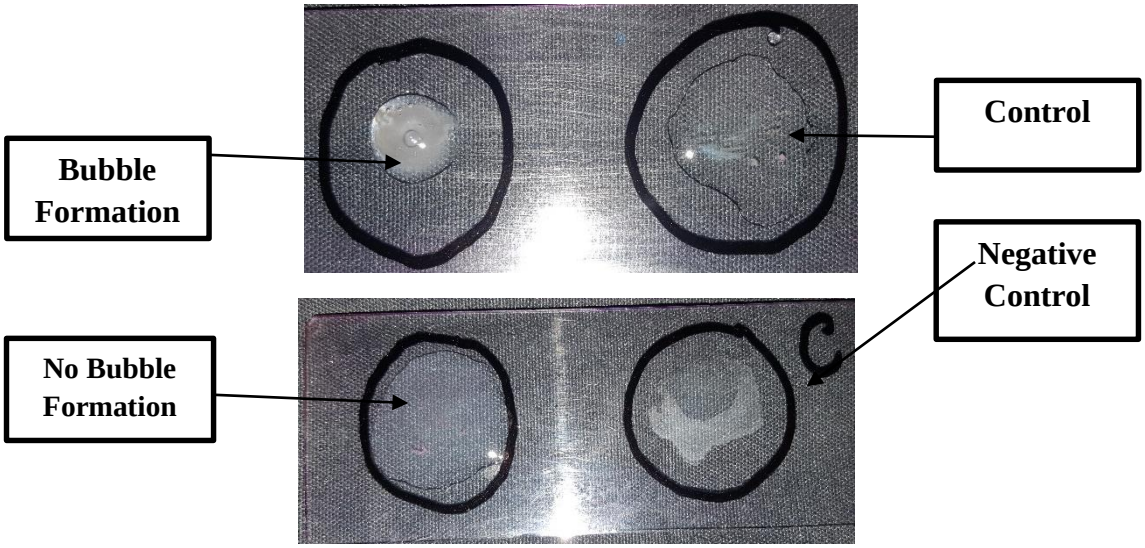


Figure 22: Catalase test, Bubble formation (Positive); No Bubble formation (Negative)

Biocehmical tests result of of *Salmonella* spp.

Methyl Red Test

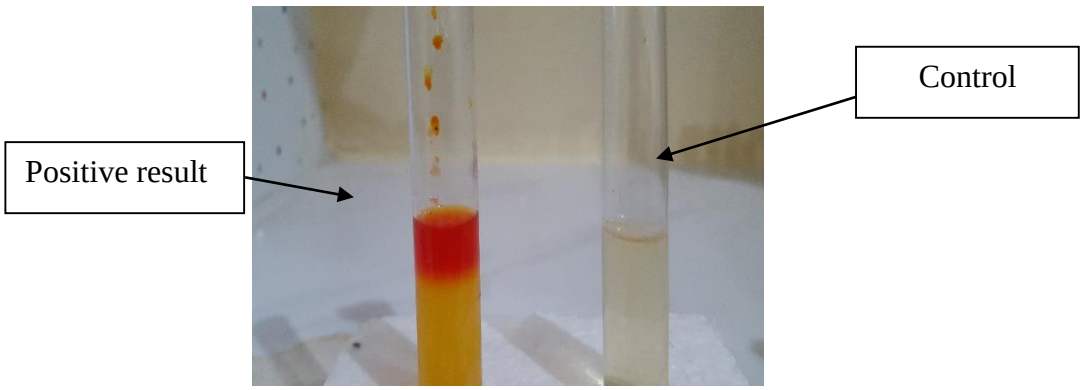


Figure 23: *Salmonella* spp. showing positive result (Right) on Methyl Red test with control (Left)

Voges-Proskauer Test

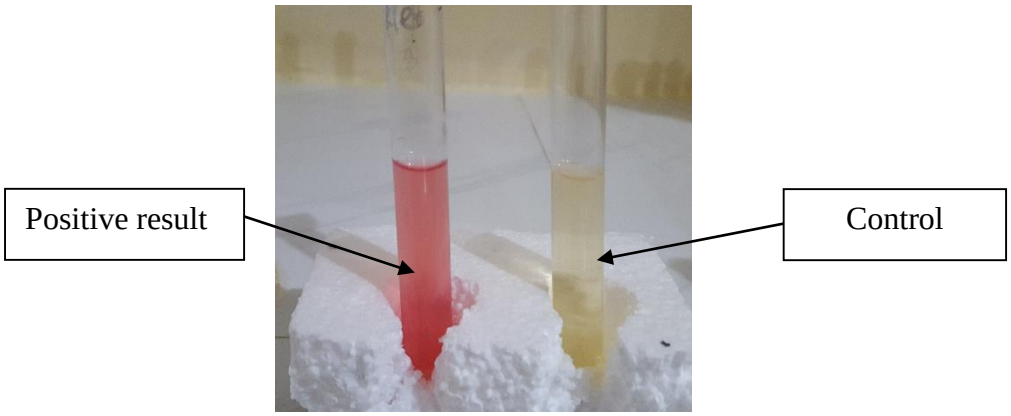


Figure 24: *Salmonella* spp. showing positive result (Right) on Voges-Proskauer test with control (Left)

Triple Sugar Iron (TSI) Test

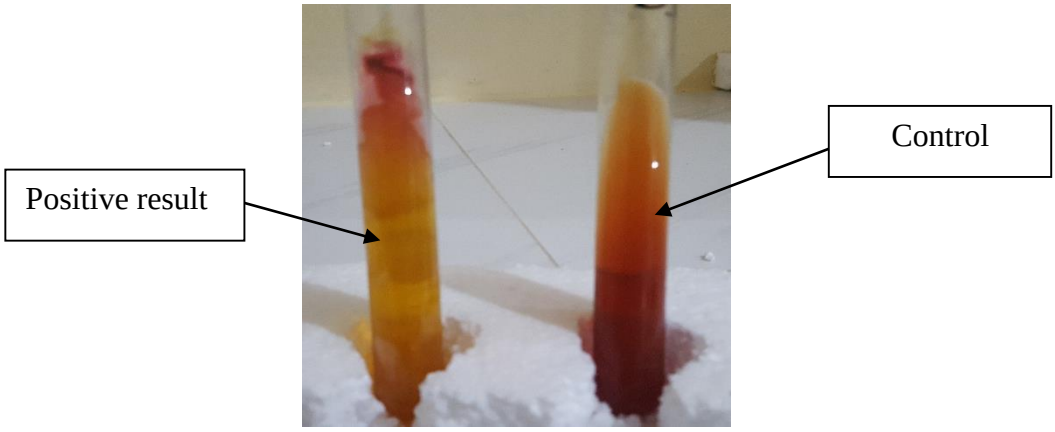


Figure 25: *Salmonella* spp. showing positive result (Right) on Triple sugar iron (TSI) test with control (Left)

Citrate Utilization Test

Figure 26: *Salmonella* spp. showing positive result (Right) on citrate utilization test with control (Left)

Indole Test

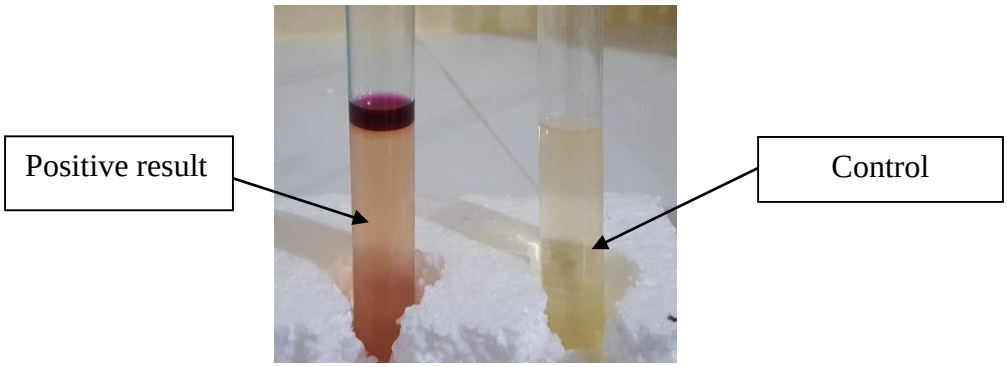


Figure 27: *Salmonella* spp. showing positive result (Right) on Indole test with control (Left)

Biocehmic tests result of *Klebsiella* spp. in.

Methyl Red Test

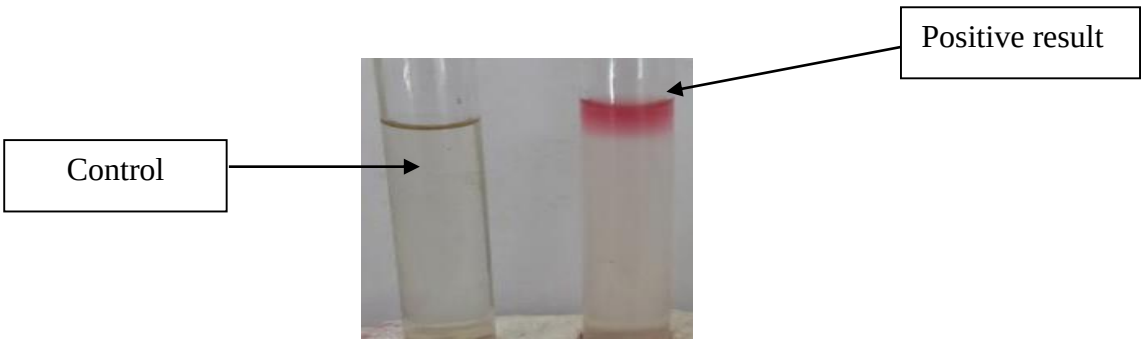


Figure 28: *Klebsiella* spp. showing positive result (Right) on Methyl red test with control (Left)

Voges-Proskauer Test

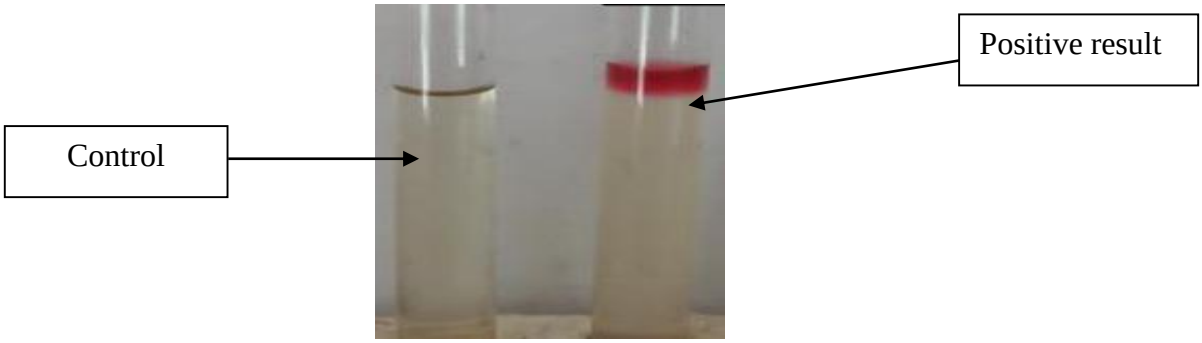


Figure 29: *Klebsiella* spp. showing positive result (Right) on Voges-Proskauer test with control (Left)

Triple Sugar Iron (TSI) Test

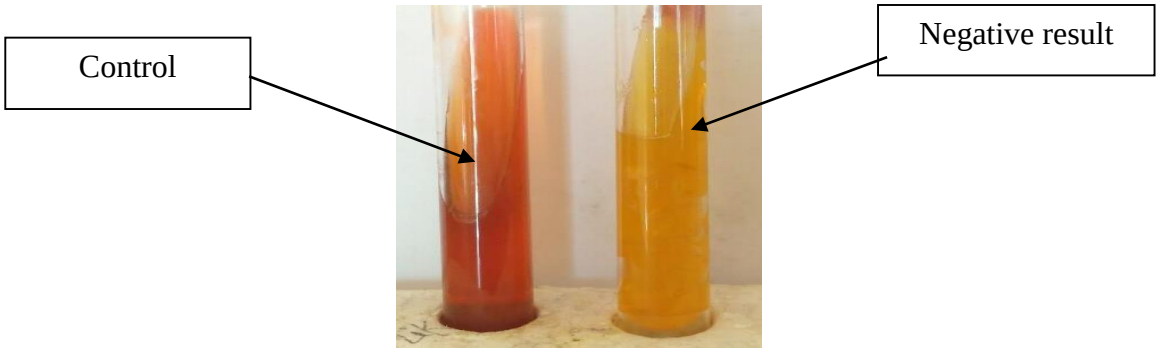


Figure 30: *Klebsiella* spp. showing negative result (Right) on Triple sugar iron (TSI) test with control (Left)

Citrate Utilization Test

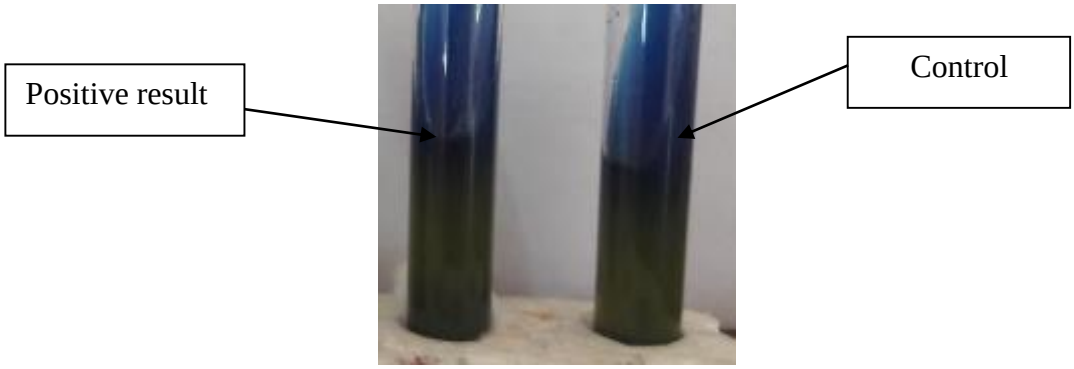


Figure 31: *Klebsiella* spp. showing positive result (Left) on citrate utilization test with control (Right)

Indole test

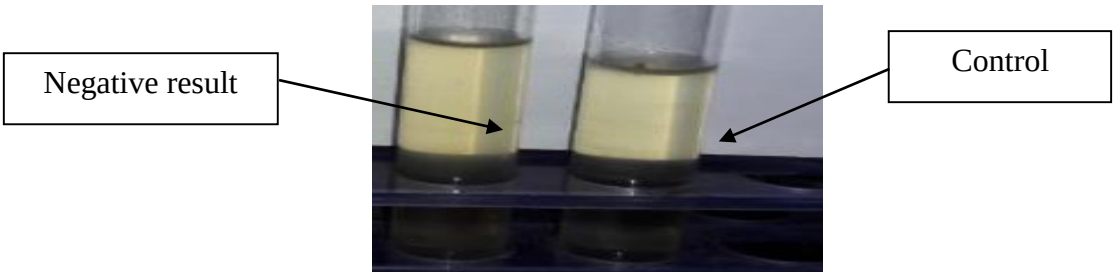


Figure 32: *Klebsiella* spp. showing negative result (Left) on Indole test with control (Right)

Biocehmic tests result of *Shigella spp.* in.

Methyl Red Test

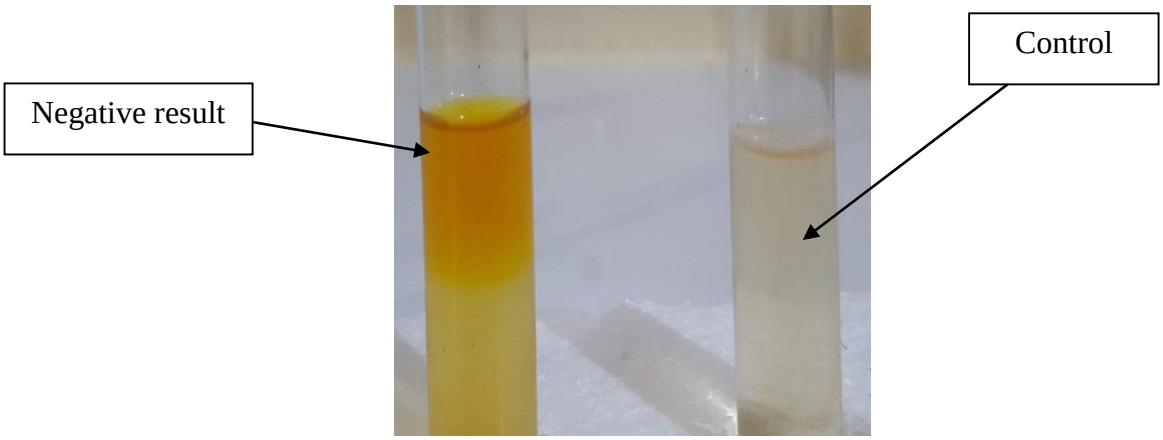


Figure 33: *Shigella* spp. showing negative result (Right) on Methyl red test with control (Left)

Voges-Proskauer Test

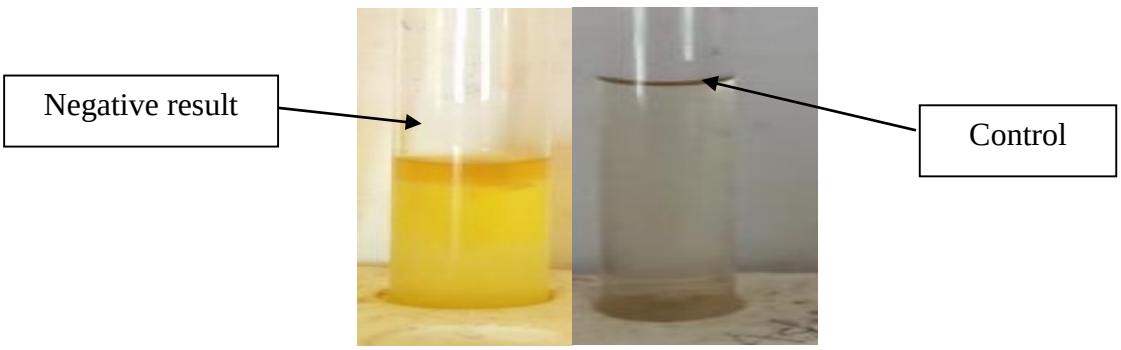


Figure 34: *Shigella* spp. showing Negative result (Left) on Voges-Proskauer test with control (Right)

Triple Sugar Iron (TSI) Test

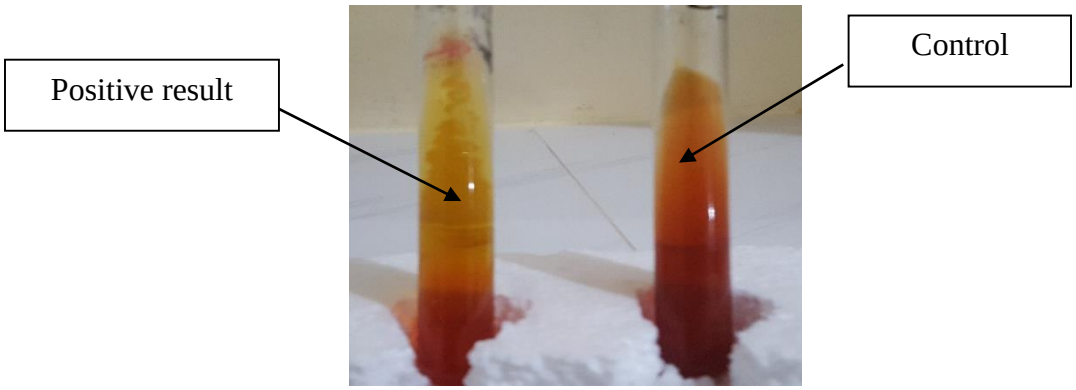


Figure 35: *Shigella* spp. showing Positive result (Right) on Triple sugar iron (TSI) test with control (Left)

Citrate Utilization Test

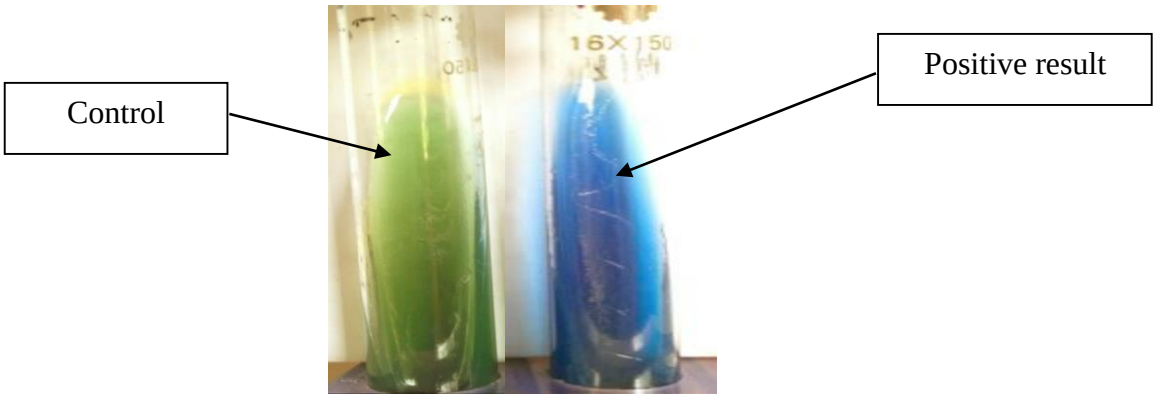


Figure 36: *Shigella* spp. showing positive result (Right) on citrate utilization test with control (Left)

Indole test

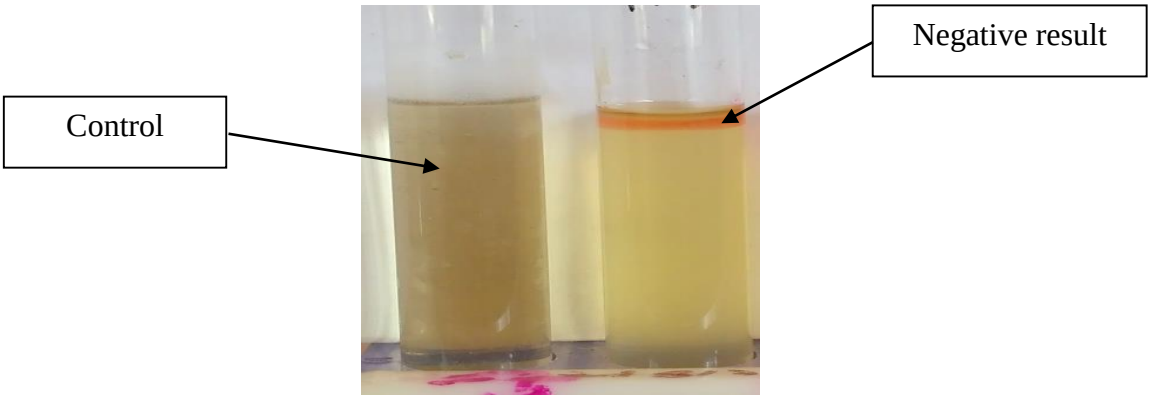


Figure 37: *Shigella* spp. showing negative result (Right) on indole test with control (Left)

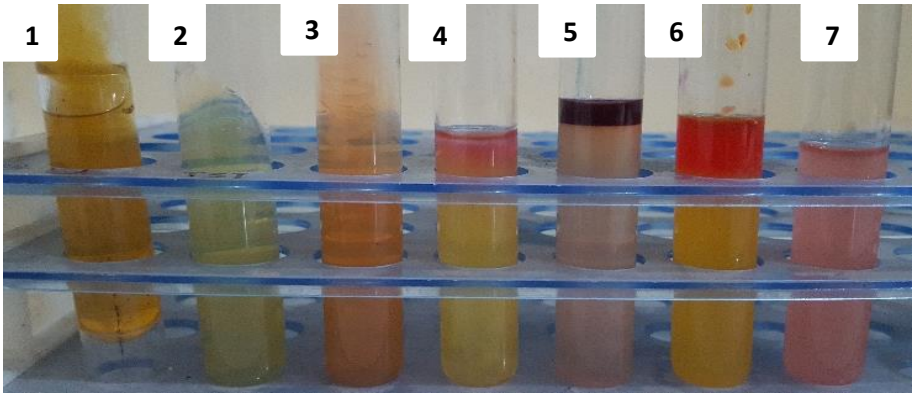


Figure 38: Biochemical Test Results *Escherichia coli.* (1.TSI=Yellow butt and slant with gas, 2. Citrate utilization= Negative, 3. Urease=Negative, 4.MIU=Positive, 5. Indole= Positive, 6.MR=Positive, 7. VP= Negative)

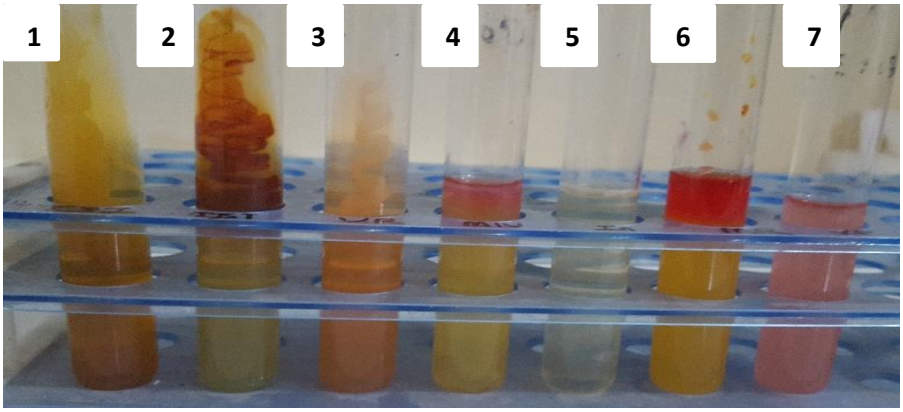


Figure 39: Biochemical Test Results *Staphylococcus* spp. (1.TSI=Slant and Butt both acidic, 2. Citrate utilization=Negative, 3. Urease=Positive, 4. MIU=Negative, 5. Indole=Negative, 6.MR=Positive, 7.VP=Positive)

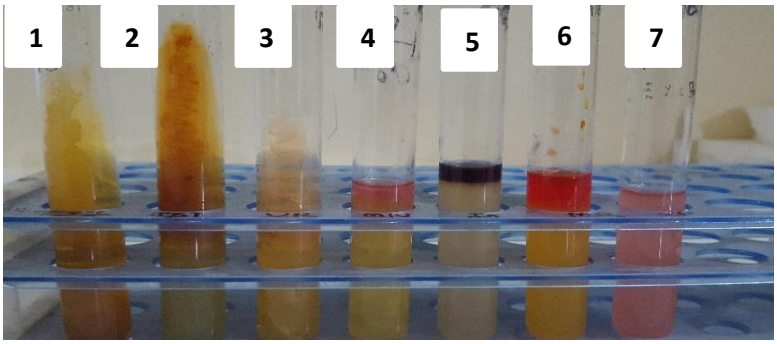


Figure 40: Biochemical Test Results *Vibrio* spp. (1. TSI= Yellow slant and butt, 2. Citrate utilization= Positive, 3. Urease=Negative, 4.MIU= Positive,

5. Indole= Positive, 6. MR=Negative, 7. VP= Negative)

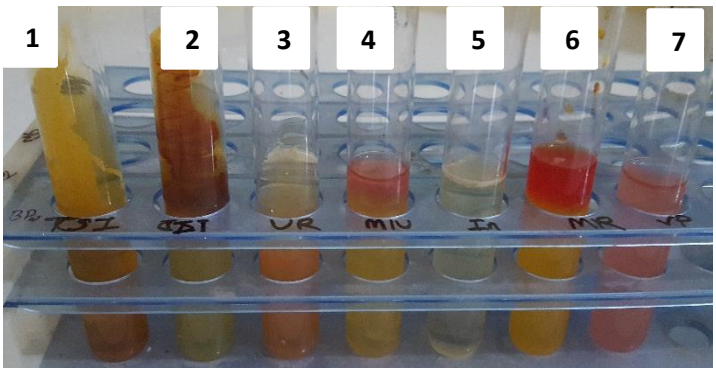


Figure 41: Biochemical Test results *Pseudomonas* spp. (1.TSI= Yellow butt and slant, 2. Citrate utilization=Positive, 3. Urease=Negative, 4.MIU=Positive,

5. Indole=Negative, 6.MR=Negative, 7. VP=Negative)

4.4.4 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. Seven (7) isolates of *E. coli*, *Shigella* spp.., *Klebsiella* spp.., *Salmonella* spp., *Staphylococcus* spp., *Vibrio* spp. and *Pseudomonas* spp. were subjected to antibiotic sensitivity tests for dried fish sample. Two isolates *E. coli* and *Shigella* spp.., The results of antibiotics sensitivity tests are presented in Table18,19 and 20.

Table 19: Results of antibiotic sensitivity test for gram negative organisms:

Bacterial Species	Antibacterial agents and Diameter of zone of inhibition(mm) with Interpretation								
	GEN	S	CH	E	CFM	CN	K	NA	CL
E. coli	45(S)	37(S)	R	R	R	R	R	R	22(S)
Shigella spp.	18(S)	16(S)	R	R	14(I)	R	22(S)	17(I)	19(S)
Klebsiella spp.	35(S)	16(S)	R	R	R	R	R	R	27(S)
Salmonella spp.	28(S)	9(R)	R	16(I)	12(R)	R	R	R	26(S)
Pseudomonas spp.	19(S)	R	8(R)	39(S)	R	R	20(S)	R	R
Vibrio spp..	24(S)	20(s)	R	17(I)	R	R	20(S)	15(I)	R

Note: GEN= Gentamicin, S=Streptomycin, CH=Cephradine, E=Erythromycin, CFM=Cefixime, K=Kanamycin, NA=Nalidixic Acid and Cl=Colistin.

I=Intermediate, S=Susceptible and R=Resistance

Table 20: Results of antibiotic sensitivity test for gram positive organism

Bacterial Species	Antibacterial agents Diameter of zone of inhibition(mm) Interpretation								
	GEN	S	E	AMX	CL	AZM	P	CXM	CN
Staphylococcus spp.	23(S)	18(S)	18(I)	R	14(S)	22(S)	R	R	R

Note: G=Gentamycin, S= Streptomycin, E=Erythromycin, AMX=Amoxicillin, CL=Clindamycin, AZM =Azithromycin, P=Penicillin G CXM=Cefuroxime Sodium, CN=Cephalexin.

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

Table 21: Result of antibiotic sensitivity test for gram negative organism isolated from cooked fish

Bacterial Species	Antibacterial agents and Diameter of zone of inhibition(mm) with Interpretation								
	GEN	S	CH	E	CFM	CN	K	NA	CL
E. coli	41(S)	11(R)	R	R	R	R	R	22(S)	22(S)
Shigella spp.	18(S)	16(S)	R	R	14(I)	R	22(S)	17(I)	19(S)

Note: GEN= Gentamicin, S=Streptomycin, CH=Cephradine, E=Erythromycin, CFM=Cefixime, K=Kanamycin, NA=Nalidixic Acid and Cl=Colistin.

I=Intermediate, S=Susceptible and R=Resistance

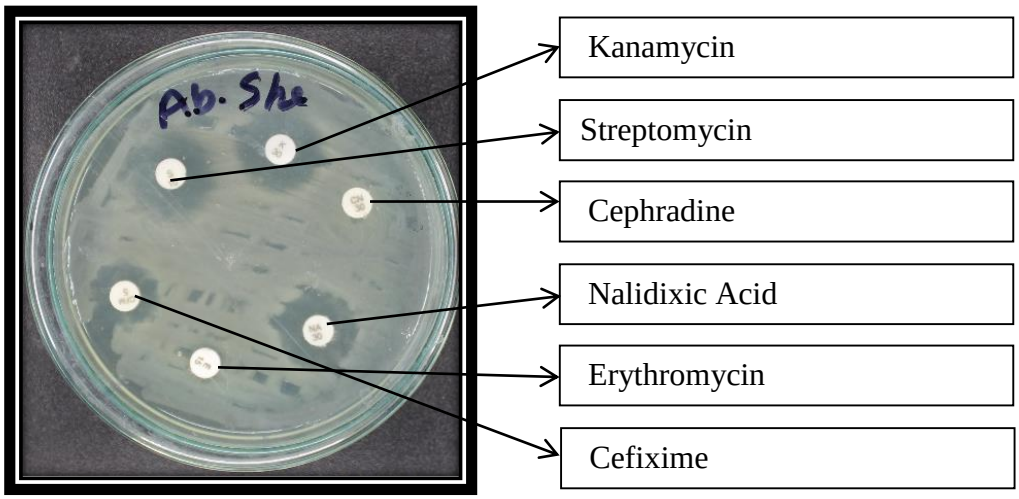


Figure 42: Anitibiotic sensitivity test of *Shigella* spp.

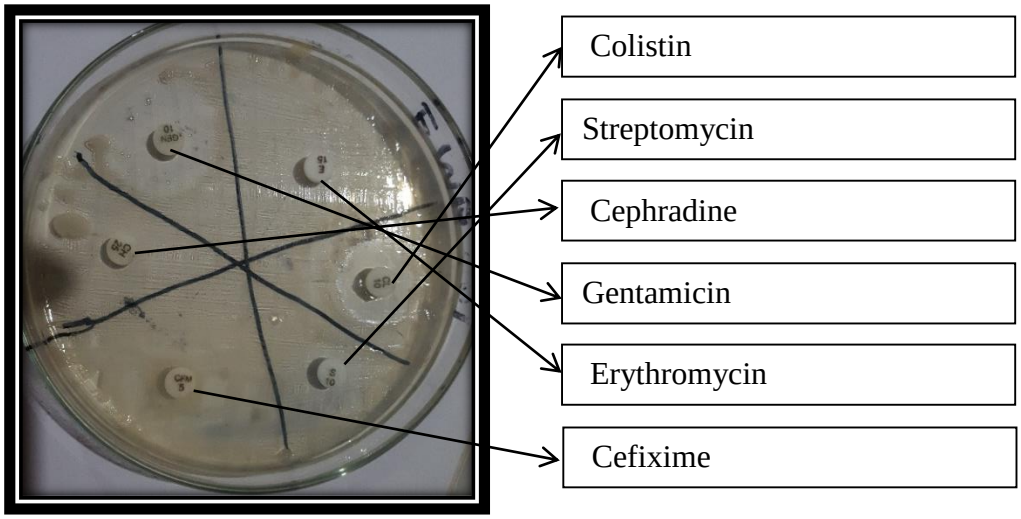


Figure 43: Anitibiotic sensitivity test of *E. coli* (Cooked Fish)

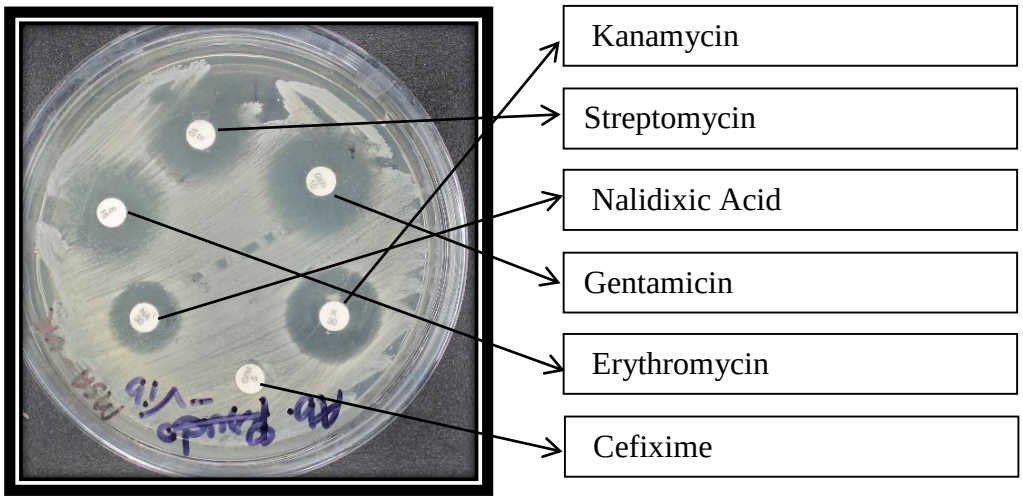


Figure 44: Anitibiotic sensitivity test of *Vibrio* spp.

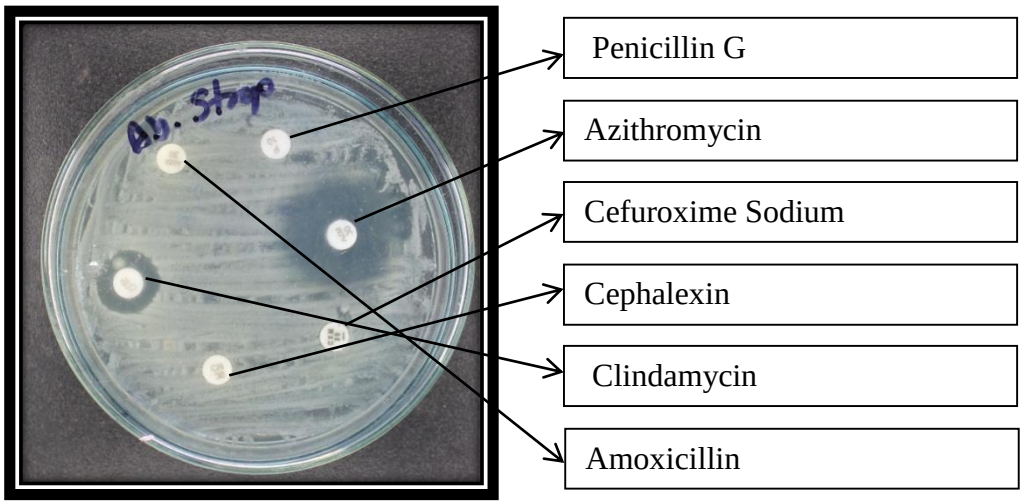


Figure 45: Anitibiotic sensitivity test of *Staphylococcus* spp.

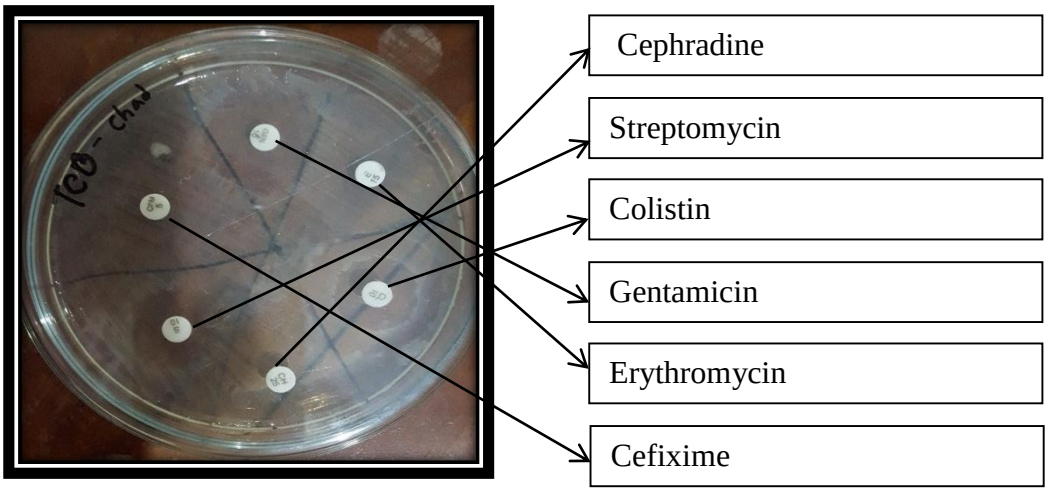


Figure 46: Anitibiotic sensitivity test of *Vivrio* spp.

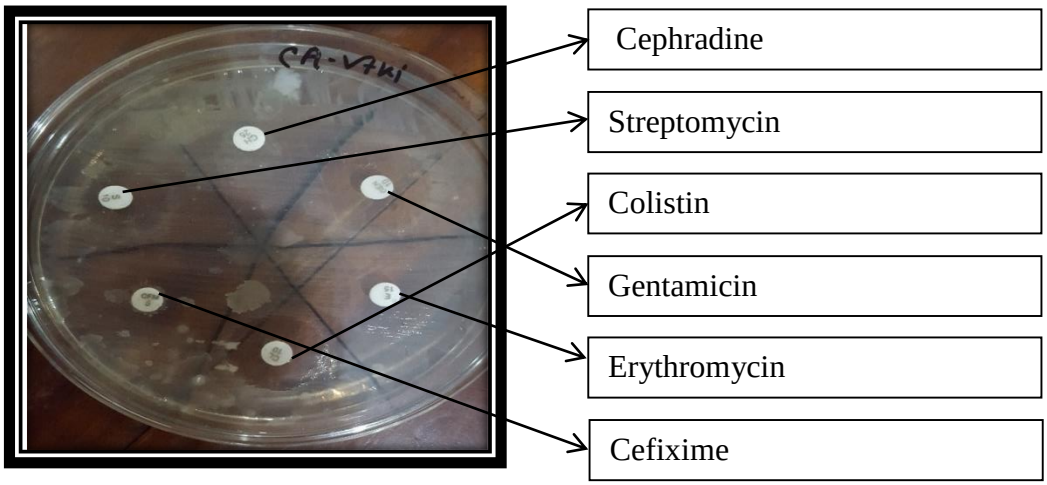


Figure 47: Anitibiotic sensitivity test of *Pseudomonas* spp.

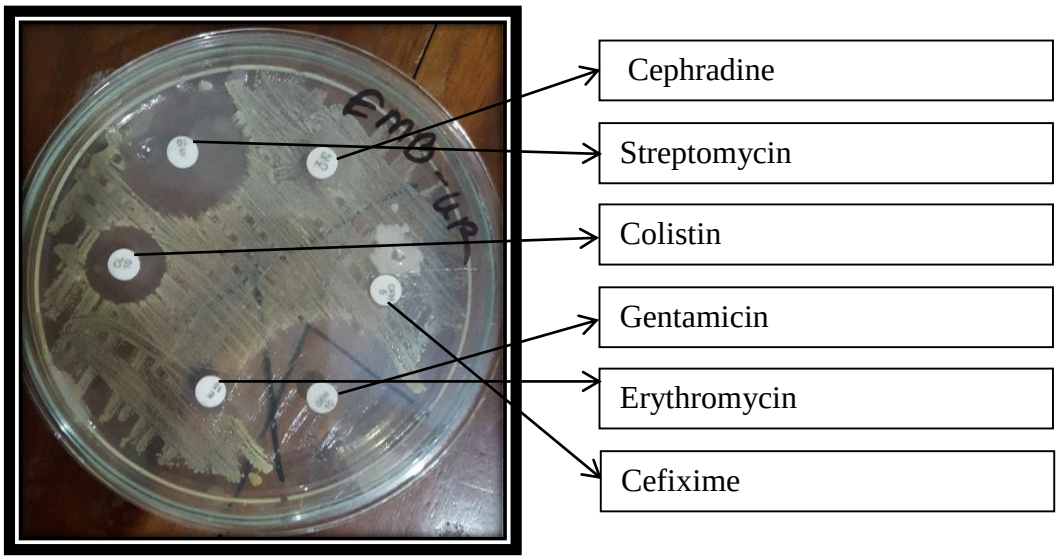


Figure 48: Anitibiotic sensitivity test of *E. coli* (Dried Fish)

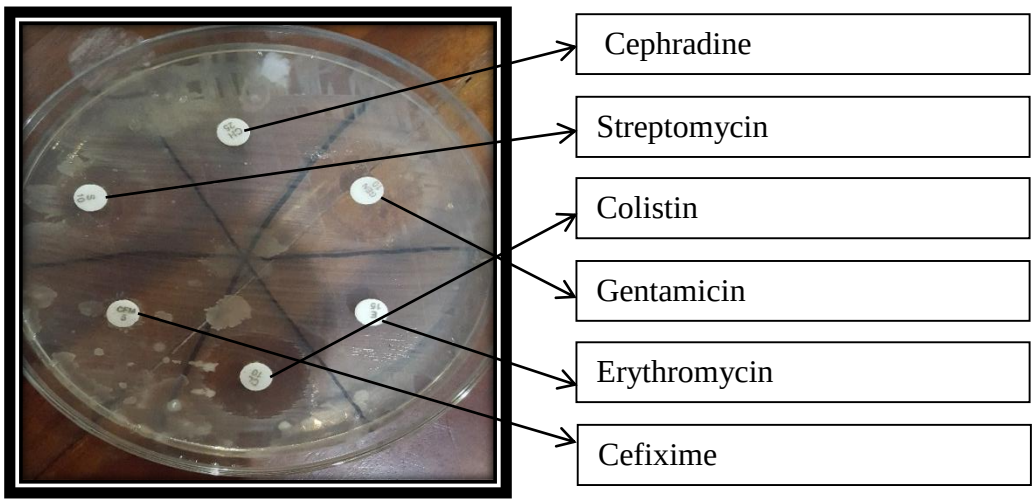


Figure 49: Anitibiotic sensitivity test of *Salmonella* spp.

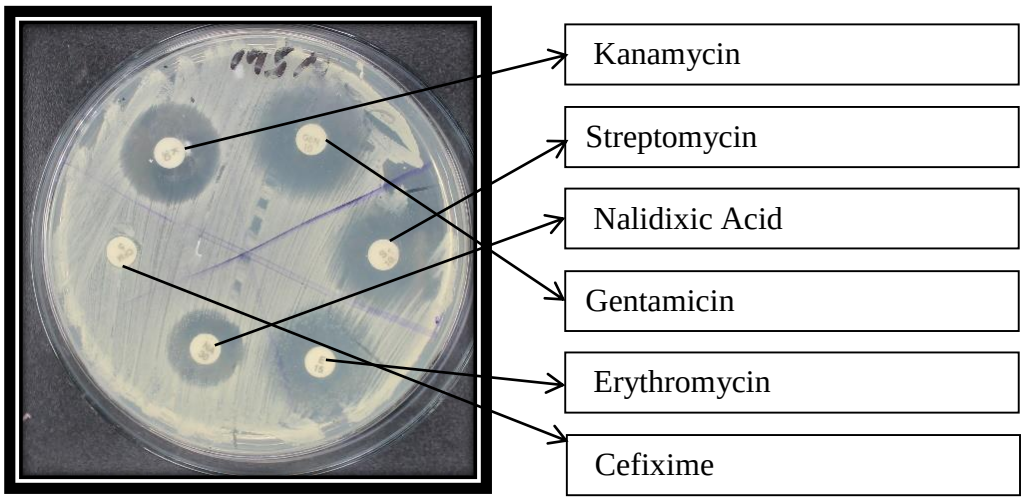


Figure 50: Anitibiotic sensitivity test of *Klebsiella* spp.

CHAPTER 5

DISCUSSION

The experiment was carried out for detection and antibiogram study of bacterial pathogens isolated from 79 fish samples. Out of the 79 fish samples 54 samples were collected from dried fish and 25 samples were collected from cooked fish. There were 18 different types dried fish sample collected from the local fish markets and 6 different types cooked fish product from 15 different restaurants of Dinajpur city. For this study a series of test ware conducted for isolation, identification and antibiogram study of the isolated bacteria. For total aerobic viable microbial load detection Total Viable Plate Count was performed (TVC) in Plate Count Agar (PCA) media and counted by digital colony counter (Start Doc-It Colony Counter). The TVC result vary from 1.28×10^7CFU/g to 3.74×10^9CFU/g. Highest microbial load found in Lottya and lowest TVC count in Ruhi dried fish sample. This study indicated that the different dried fish samples showed wide range of TVC which exceed accept level. Hazard Analysis and Critical Control Point-Total Quality Management (HACCP-TQM) technical guidelines rates microbial quality for raw foods containing aerobic plate count of $<10^4$ cfu/g as "Good", 10^4-5×10^6 cfu/g as "Average", 5×10^6-5×10^7 cfu/g as "Poor" and $>5\times10^7$cfu/g as "Spoilt" Which can be said to be extremely hazardous for public health.

All samples were inoculated into various selective media such as Eosin Methylene Blue (EMB) agar, MacConkey agar, Salmonella *Shigella* (SS) agar, and Mannitol Salt (MS) agar, Cetrimide agar and Thiosulfate Citrate Bile Salts Sucrose (TCBA). Among 54 dried samples all (100%) and 20% of 25 cooked fish sample had found contaminated. A total of 168 bacterial isolates belong to seven genera (*staphylococcus* spp., *Vibrio* spp., *Escherichia coli, Klebsiella* spp., *Salmonella* spp., *Pseudomonas* spp. *and Shigella* spp.) were identified from dried fish. The prevalence was 21.43%*Escherichia coli*,18.45% *Vibrio* spp., 17.86% *Staphylococcus* spp., 17.86%*Pseudomonas* spp., 12.5% *Salmonella* spp., 8.93% *Shigella* spp. and 2.9% *Klebsiella* spp. In case of cooked fish samples 9 bacterial isolates of two species (*E. coli* and *Shigella* spp.) were found with a prevalence 66.66% *Escherichia coli* and 33.34% *Shigella* spp.

Our findings are in agreement with findings of **Sulieman *et al.*,** (2014) in which they isolated *Escherichia coli*, *Staphylococcus* spp., *Salmonella* spp., *Enterobacter cloacae*,

Klebsiella spp., *Proteus* spp., *Bacillus cereus*, *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Pseudomonas* spp. from dried fish. Logesh *et al.*, (2012) also studied the microbiological quality of salted dried Marin fish according to their study they isolated *Escherichia coli, Staphylococcus* spp., *Salmonella* spp. and *Vibrio cholerae* which are known as human pathogenic organisms.

Above all reports are more or less similar to this experiment. Result of the present study indicates that all of the seven different types of bacteria were commonly present in dried cooked fish sample. The in vitro antibiotic sensitivity test of 7 different types of bacterial isolates to 9 different antibiotics for gram negative organisms such as gentamicin, streptomycin, cephalexin, erythromycin, cefixime, kanamycin, Cephradine, nalidixic acid, Colistin and 9 for gram positive organism such as gentamycin, Streptomycin, Erythromycin, Amoxicillin, Colistin, Azithromycin, Penicillin G, Cefuroxime Sodium and Cephalexin. The antibacterial sensitivity of isolates in this study to antibiotics was variable.

Antimicrobial sensitivity test result showed that all of the sample harbor multidrug resistant food borne bacteria which might cause public health hazards if these antibiotic resistant transfer to human. Hence, it is recommended that a closer supervision of such food type should be carried out by relevant authorities to avoid any future pathogen outbreaks.

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 pathogenicity and drug resistance.

CHAPTER 6

SUMMARY AND CONCLUSION

The present study was conducted for detection and antibiogram study of the bacteria isolated from different dried fishes and cooked fish samples. Presence of coliforms in the sample might be due to poor quality of water, unhygienic processing, handling, packaging transport, surrounding places and poor personal hygiene of personals involve in fish and fish product processing unit. Most dried fish producer and restaurant cook are illiterate and they did not have a clear hygienic knowledge about the preparation, storage and serving of the food. The results of this study suggested that a good source of animal protein may not be healthy due to lack of hygienic measures, dirty utensils, and cook ‘s hygiene. These factors contributing many species of bacteria but major pathogens are *E. coli, Salmonella* spp. *Shigella* spp., *Klebsiella* spp., *Staphylococcus* spp., *Pseudomonas* spp. and *Vibrio* spp. Basic and main source of bacterial infection is poor hygienic measures and this problem may be solved by improving supervision in food handling procedure, extended consumer education on transmission of enteric food borne diseases and food safety risks. So that fish drying and cooking should be manufactured under Good Hygienic Practices and conservation practices should be developed in order to minimize the microbial contamination of food.

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

i. The presence of *E. coli*, *Salmonella* spp., *Staphylococcus* spp., *Shigella* spp., *Vibrio* spp. and *Pseudomonas* spp. in most of the samples are public health concern.
ii. All of the 7 types of bacteria isolated from dried fishes are opportunistic, which can cause diseases under certain environmental stress condition.
iii. The presence of bacteria in all samples indicates that consumption of contaminated dried fish without proper cooking is harmful and also accelerated the public health threat.
iv. The antibiotic resistance properties of isolated bacteria can cause serious health hazards because of ineffective treatment of the sufferers by the commonly prescribed antibiotics.

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APPENDICES

APPENDIX 1

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. Eosin 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. Simon's Citrate Agar

Component	Amount (g/L)
Magnesium sulphate	0.2
Ammonium 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

Safranin	2.5 ml
Ethyl alcohol (95%)	100 ml

Safranin working solution

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

THE END