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Comparative Microbiological Assessment of Export Oriented Fishes and Locally Marketed Fishes

A dissertation submitted to the Department of Fisheries, University of Dhaka for the partial fulfillment of the requirements for the degree of Master of Science in Fisheries

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Department of Fisheries
University of Dhaka
Dhaka, Bangladesh

Department of Fisheries

University of Dhaka

Dhaka-1000, Bangladesh

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TO WHOM IT MAY CONCERN

This is to certify that **MST. KHADIZA BEGUM** is a student of M.S. Program of 2012-2013 Session under Department of Fisheries of University of Dhaka bearing Examination Roll No.: Curzon-608, Registration no. HA -1492 (2008-2009). As part of her curriculum she has carried out a thesis under our supervision the title of which is "**Comparative Microbiological Assessment of Export Oriented Fishes and Locally Marketed Fishes".**

This is further to certify that it is an original work and suitable for partial fulfillment for the degree of Master of Science in Fisheries, University of Dhaka.

Mohammad Mamun Chowdhury
Supervisor
Associate Professor
Department of Fisheries
University of Dhaka

Mrs. Wahida Haque
Co-supervisor
Associate Professor and Chairperson
Department of Fisheries
University of Dhaka

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TO WHOM IT MAY CONCERN

This is to certify that **MST. KHADIZA BEGUM,** student of M.S. Program under Department of Fisheries, University of Dhaka has carried out a thesis titled "**Comparative Microbiological Assessment of Export Oriented Fishes and Locally Marketed Fishes"** under our direction in the Fish Inspection and Quality Control (FIQC) Laboratory Dhaka, Department of Fisheries (DoF), Matshya Bhaban, Dhaka. This is further to certify that it is an original work and suitable for partial fulfillment for the degree of Master of Science in Fisheries, University of Dhaka.

(Md. Manik Mia)
Fish Inspection and Quality Control Officer
(Laboratory In-charge)
FIQC Laboratory, Dhaka
Department of Fisheries (DoF)
Matshya Bhaban, Dhaka

(Tanzinah Nasrin)
Microbiologist
FIQC Laboratory, Dhaka
Department of Fisheries (DoF)
Matshya Bhaban, Dhaka

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To
my beloved
parents

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Author

Abstract

The present study was conducted for comparative microbiological assessment of export oriented fishes locally marketed fishes. For microbiological assessment five species of fish were selected. These were-Magur (*Clarias batrachus*), Mola (*Amblypharyngodon mola*), Pabda (*Ompak pabda*), Rohu (*Labeo rohita*) and Koi (*Anabas testudineus*). These species were selected because of their high demand in export market and popularity in our country. This study was conducted because the microbiological status of these fishes could be a major public health issue. Fish samples were collected from four fish processing plants, four local fish markets and four super shops. The present study was designed to assess Satndard Plate Counts (SPC), Total Coliform Counts (TCC) and Total Fecal Coliform Counts (TFC) and qualitative analysis of *Salmonella* spp. and *Vibrio cholerae* and antibiotic susceptibility test of pathogenic isolates. The study revealed that SPC in fish processing plant samples ranged from 2.70×10^3 to 4.95×10^5 cfu/g, TCC from <3 to15 MPN/g and TFC from <3 to 7.4 MPN/g. In samples of local fish markets SPC, TCC, TFC ranged from 2.89×10^5 to 2.98×10^7 cfu/g, 15 to 460 MPN/g, 11 to 240 MPN/g, respectively. In fish samples of super shops SPC, TCC, TFC ranged from 5.96×10^4 to 5.16×10^6 cfu/g, <3 to 240 MPN/g, <3 to 120 MPN/g respectively. Among all the fish samples of fish processing plants, local fish markets and super shops, the highest value of SPC was found in Koi fish sample of local market-4 and the lowest value in Mola fish sample of processing plant-2 while TCC was found to be highest in Koi fish samples of local fish market-1 and Pabda fish sample of local fish market-2 and lowest in several fish samples of processing plants and in two fish samples of super shop-1. The highest value of TFC was observed in Koi fish sample of local fish market-1 and Mola fish sample of local fish market-3 whereas the lowest value was found in most of the fish samples of fish processing plants and in two fish samples of super shop-1. *Salmonella* spp. was detected in most of the samples of local fish markets and few samples of super shops and only two samples of fish processing plants were found to be contaminated with *Salmonella* spp. *Vibrio cholerae* was absent in all fish samples of fish processing plants and five samples of local fish markets and three samples of super shops were contaminated with this organism. The bacterial load of fish processing plant samples was almost within the acceptable limit. In this study, the mean value of SPC, TCC and TFC in most of the fish samples were significantly different ($p<0.05$) among fish processing plants and local fish markets, fish processing plants and super shops, but in case of some

fish samples there were no statistically significant difference in mean value of SPC, TCC and TFC among local fish markets and super shops. The study also revealed that the isolated *Salmonella* spp. and *V. cholerae* showed antibiotic resistance and sensitivity to seven antimicrobial agents. Most of the isolates were sensitive to chlorampenicol, tetracycline and erythromycin. The findings of this study indicate that the fish samples of fish processing plants were of good quality for export and consumption, fish samples of local fish markets were more contaminated thus reflects the unhygienic condition of the markets and super shop samples were less contaminated but not as good as fish processing plant samples. The contaminated fish samples could be the potential reservoir for the transmission of pathogenic bacteria causing fish borne disease outbreaks.

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Abbreviations	Details
APW	Alkaline Peptone Water
BFFA	Bangladesh Frozen Food Exporters Association
BGBB	Brilliant Green Bile Broth
BPW	Buffered Peptone Water
CDCP	Center for Disease Control and Preservation
DoF	Department of Fisheries
EC	*E. Coli* Broth
EU	European Union
FAO	Food and Agriculture Organization
FC	Faecal Coliform
FIQC	Fish Inspection and Quality Control
GAP	Good Aquaculture Practice
GHPs	Good Hygiene Practices
GMPs	Good Manufacturing Practices
HACCP	Hazard Analysis and Critical Control Point
HEA	Hecton Enteric Agar
ICMSF	International Committee on Microbial Specification for Foods
ISO	International Organization of Standardization
LFM	Local Fish Market
LSTB	Lauryl Sulphate Tryptose Broth
MHA	Muller Hinton Agar
MKTT	Muller-Kaufment Tetrathionate-novobiocin Broth
NA	Nutrient Agar
NCCLS	National Committee for Laboratory Standards
PCA	Plate Count Agar
PP	Processing Plant
RV	Rappaport Vasiliadis
SNA	Saline Nutrient Agar
SPC	Standard Plate Count
SS	Super Shop

Abbreviations	Details
TBC	Total Bacterial Count
TC	Total Coliform
TCBS	Thiosulphate Citrate Bile Sucrose
TCC	Total Coliform Count
TFC	Total Faecal Coliform Count
TSB	Trypticase Soya Broth
TSI	Triple Sugar Iron
TVBC	Total Viable Bacterial Count
TW	Tryptone Water
UAE	United Arab Emirates
USA	United States of America
USFDA	United States Food and Drug Administration
XLDA	Xylose Lysine Deoxycholate Agar

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CHAPTER 1
INTRODUCTION

Chapter 1
Introduction

1.1 Introduction

Fisheries sector plays an important role in food security, poverty alleviation and economic development of Bangladesh. Fisheries contributes 4.39% of our GDP, 22.76% of agricultural sector and 2.46% to foreign exchange earning. Fish provides about 60% of national animal protein. Total fish production of Bangladesh was 32.62 lakh MT in 2011-12(DoF 2013).

Inland and marine fisheries are the major fisheries sectors of Bangladesh. These sectors support not only the domestic needs but also contribute to world export markets.

Fish body maintains many types of bacterial flora in gills, gut and skin. After the death of fish these bacteria get favorable environment for rapid multiplication. The bacterial flora on fish reflects the aquatic environment which affects the quality and storage life of fishery products (Shewan 1976). The quality of fish can be assessed by the degree of contamination with coliform bacteria. Contamination results mainly form rupturing of fish intestine during poor processing or unhealthy washing. Several studies have suggested that intestinal microflora or contaminations of as a result of enteric bacteria of human or animal origin are responsible for spoilages (Guldreich and Clarke 1966). So, consumption of fish may cause disease due to infection or intoxication. It is believed to be the reflection of the general contamination in the aquatic environment and hence it is obvious that if the growing and harvesting environment of fish is polluted chemically or microbiologically, the fish are also polluted (Boyd 1984). For this reason, quality control of fish and fish products is very much important for consumer acceptance. For being highly perishable foodstuff, fish are needed special care and attention from the catching site to the consumption table.

Fisheries are the second largest export earning sector of Bangladesh. Major export items of fish products are raw shrimp, block frozen, IQF shrimp and white fish, PUD and P&D block frozen shrimp, dry, salted and dehydrated fish, eel, crab and a little quantity of value added fish and shrimp products. Major countries where fish products exported are European Union, USA, Japan, Middle East countries, UAE and Gulf States (BFFEA

2010). To export fishes (processed or frozen) in these countries, we need to comply with the EU standard and USFDA standard.

By the year 1985, a good number of processing plants has developed without biological survey between capture and culture fishery. In the past time most of the factory led the emphasis on quantity rather than quality. Processed products quality depends on the quality of raw materials but it is too difficult to retain freshness of raw materials due to long period of time between harvesting and processing periods. Inadequate processing has resulted in microbial growth which deteriorates food products (FDA 2001). Export market of Bangladesh might be in threat for inadequate processed foods due to contamination by decomposition, high bacterial load, filth, unexpected foreign matters as well as pathogenic microbes (*E. coli, Salmonella, V. cholerae etc*). These activities occur occasionally due to improper implementation of HACCP system as well as poor establishment of GMPs (Good Manufacturing Practices), GHPs (Good Hygienic Practices) and inadequate sanitation procedures. On July 30, 1997, the EU banned imports of fishery products from Bangladesh after the inspection of seafood processing plants. From the inspection serious deficiencies in the infrastructure and hygiene were found in processing establishments. The ban was estimated to cost the Bangladesh shrimp processing sector nearly US$15 million in lost revenues from August to December 1997 (BFFEA 2010).

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Bangladesh frozen fish and shrimp exporters continued facing problems with buyers in the US, the EU and Japan; concerning the safety and quality of their products because many fish processing plants in Bangladesh did not follow the HACCP system and EU hygienic regulations. EU advised Bangladesh Government to implement HACCP system in fish and shrimp processing industries for safe export quality frozen food products. Currently 75 processing industries have EU approval out of 100 (DoF 2013). The major goal of the fish processing industries is to provide safe, wholesome and acceptable products to the consumer. Control of microorganisms is essential to meet this goal. The control is partly exerted through processing and preservation techniques that eliminate microorganisms or prevent their growth.

Domestic markets of Bangladesh support demand of fish of country people. Recently, there are two types of domestic markets in Bangladesh like local fish markets and super

shops. People buy fish from these markets to fulfill their demand. In Bangladesh, super shop has a special site for selling of where modern facilities like sufficient ice, better quality water supply, display tray, uniform salesmen, electric balance, attractive packaging system etc. are available. Whereas, local fish markets have dirty, damp and unhealthy place, unhealthy fish holding baskets, insufficient ice, poor quality water supply, poor storage, display and packaging facilities etc. and this unhealthy conditions encourages microbial contamination from different sources. But collection of fish in both types of market may be from same source and have no special site for production of fish. Most of the retail fish markets and super shops sell freshwater fishes but it takes a while for transportation of fish from different catching sites to the far away markets. There is always a risk of unhygienic handling, transportation and storage and therefore contamination of fish.

In the study, bacteriological status was assessed for standard plate count (SPC), total coliform count (TCC) and total faecal coliform count (TFC) and occurrence of *Salmonella* spp. and *Vibrio cholerae* was examined. Coliforms are gram negative bacteria which ferment lactose and produce gas and acid. Faecal coliforms are generally found in gastrointestinal tract of human and animals. So, if Faecal coliforms are found in fish or fish products, then it can be said that these are contaminated by man or animal excreta. There are three genera of Faecal coliforms, e.g. *Escherichia*, *Klebsiella* and *Enterobacter*.

Salmonella are motile rod and gram negative bacteria. *Salmonella* occurs commonly in domestic animals and birds. Contamination of fish with *Salmonella* is due to growth in polluted waters and poor handling, hygiene and sanitation standards after harvesting.

Vibrio cholerae is gram negative, comma shaped bacteria. *Vibrio* frequently occurs in polluted water. After transmission these organisms multiply rapidly in the intestines of the victims.

Antony *et al.* (2002) assessed that total bacterial load of raw shrimps collected from three seafood processing were almost uniform with 10^4 to 10^5 cfu/g. Raw shrimps from first two plants had lower counts of total bacteria and coliforms than the third one. The

pathogens like *Vibrio cholerae, Salmonella* and *Listeria monocytogenes* were totally absent in raw shrimps.

Oramadike *et al.* (2010) studied microbiological qualities of some frozen fishes available in some reputable supermarkets in Lagos State and reported that total bacterial count ranged between 2.0 $\times 10^3$ to 7.4$\times 10^3$ cfu/g, total coliforms per gram ranged between 0 and 53 MPN/g and did not exceed acceptable total coliforms limit per gram for frozen fish. The sanitary, storage and hygienic conditions of the supermarkets were relatively the same.

Nilla *et al.*(2012) studied that the total bacterial count of marketed Mola (*Amblypharyngodon mola)* ranged from 1.8 ± 0.25 × 104 to 6.5 ± 0.75 × 106 cfu/g for fresh sample and 5.5 ± 0.55 × 103 to 7.0 ± 0.80 × 105 cfu/g for frozen . The highest total coliform count of mola was 8.0 ± 0.55 × 104 and 6.1 ± 0.40 × 103 cfu/g for local market and departmental chain shop, respectively. All fresh and frozen samples were observed having high quantity of *E. coli*. Furthermore, *Salmonella-Shigella* was identified in 67% samples varied from 0.9 ± 0.00 ×102 to 5.3 ± 0.30 × 103 cfu/g whereas *Vibrio* spp. was confirmed in 79% samples. In case of antibiotic sensitivity pattern of the indicator and pathogenic isolates, all of them were resistant to amoxicillin and penicillin.

Rahman *et al.* (2010) studied the variation of bacterial loads among the fishes of various feeding habits. The TBC, TC, FC, FS and total Vibrio counts ranged from 1.72 ± 0.68 × 108 to 7.00 ± 3.39 × 108, 2.49 ± 1.72 × 106 to 6.55 ± 3.00 × 106, 1.58 ± 1.29 × 106 to 2.76 ± 1.42 × 106, 4.83 ± 2.09 × 104 to 1.19 ± 0.46 × 105 and 2.06 ± 0.67 × 103 to 3.68 ± 2.02 × 105, respectively among various feeding groups.

Ali *et al.* (2012) assessed the microbial load of frozen shrimps processed for exporting to different countries of the world and reported that the mean total coliforms was <3 ±0.00 MPN/g in Cooked IQF shrimp, while it was 23.50 ± 13.72 MPN/g in raw block frozen shrimp. Fecal coliforms for both raw block frozen and cooked IQF shrimp were <3 MPN/g.

Noor *et al.* (2013) studied the prevalence of pathogenic microflora along the two major sea fish samples, Rupchanda (*Pampus chinensis*) and Surmai (*Scomberomorus guttatus*)

and reported that the total bacterial count was 2.5×10**6** cfu./g in fish blend samples and the samples were highly contaminated with *Shigella* spp., *Listeria* spp., *Staphylococcus aureus*.

Rahman *et al.* (2012) assessed the health hazard microbes in raw and finished product of coral (*Lates calcarifer*) and reported that total coliform was between 15 MPN/g and 20 MPN/g in the finished product of coral, faecal coliform in raw and finished product of coral was found <3 MPN/g and *Salmonella* spp. and *Vibrio cholerae*, both were absent.

Geldreich and Clarke (1966) made a study of the occurrence, distribution and persistence of coliform, fecal coliform and faecal streptococci in the intestinal tract of freshwater fish. Fecal coliform densities were lowest in blue gills (less than 20/g) and highest in catfish. The occurrence of fecal coliform in fish caught in little Miami River reflected the warm-blooded animal pollution level of the water.

Joarder and Khatun (1995) studied both quantitatively and qualitatively the bacterial contents of scale, skin, muscle or flesh, gills and intestines of four batches of Hilsa fish. Maximum quantities of bacteria were found in the gills (average 6.3×10^6/g). The flesh contained the least amount of bacteria (average 16×10^3/g).

According to Surendran and Iyer (1976) bacterial flora of Tilapia (*O. mossambicus*) was predominantly Gram-positive. The production of H_2S and other volatile sulphides due to spoilage bacteria is another criterion to assess the spoilage potential of bacteria.

Ahmed *et al.* (1997) reported that in Hilsa fish, bacterial load in muscle of 4 days ice stored fish was 2.5×10^2 cfu/g after 20 days when the fishes were organoleptically in unacceptable condition.

Fatema (2005) assessed the bacteriological and heavy metals concentration of Kachki (*Corica soborna*) and Mola (*Amblypharyngodon mola*) fishes both in exportable frozen condition and local market raw condition in Bangladesh. The results revealed that aerobic plate counts (APC) in local market raw fishes were quite high whereas in Kachki fish the highest APC was 8.5×10^7 cfu/g and in Mola fish, 2.4×10^7 cfu/g. In the frozen

Kachki and Mola fishes, the range of APC was 9.1×10^2 to 1.3×10^7 cfu/g and 1.9×10^6 to 8.7×10^7 cfu/g respectively.

Das *et al.* (2007) studied the quantitative count of microorganism (bacteria, fungi, yeast) in marketed (local market) indigenous fish species in Bangladeh. The study showed that sampled indigenous fish species contained not only high load of microbial flora but also certain pathogenic bacteria such as *E. coli* and *Salmonella* spp..

Shewan (1961) studied that types and load of microorganism's presence are related to the environment in which fish are caught and processed. Generally fish from tropical water have more bacteria than those from temperate zones.

Sen *et al.* (1966) studied the bacteria from variety of freshwater fish; *Cyprinus carpio var. communis* and reported the presence of micrococci, gram positive and gram-negative rods.

Varga and Anderson (1968) reported that the source of fecal coliform and enterococci in fish products due to improperly sanitized working surface where the microorganisms could survive and proliferate.

Sinha (1991) studied the bacterial flora of marketed Rui and indicated that marketed Rui was found to harbor microorganisms of public health impotance like *Salmonella* spp.

Quaiyum *et. al.* (2012) assessed that in raw product of Chapila, the aerobic plate count (APC) was 2.6×10^5 cfu/g, whereas in frozen products, the load was estimated to be 4.0×10^5 cfu/g. Furthermore, while APC of raw Tengra was 2×10^5 cfu/g, the APCs were 3.3×10^5 cfu/g in frozen product. Total coliform in raw and frozen Chapila was found to be 36.00 ± 2.3 MPN/g and 7.2 ± 1.01 MPN/g, respectively and 27.00 ± 5.57 MPN/g and 9.4 ± 3.75 MPN/g, respectively were found in raw and frozen Tengra samples. Moreover, Fecal coliform in frozen samples of Chapila and Tengra was found within the acceptable limit (<3 MPN/g). *Salmonella* spp. and *Vibrio cholerae* were not detected in any of the raw and frozen Chapila and Tengra samples.

There is a long list of human disease caused by bacteria but the most common pathogenic bacteria associated with diseases of the lower digestive system are given by Tortora *et al*. (1995) as follows:

Table 1: Pathogenecity of some pathogenic bacteria

Disease	Causative agent	Mode of transmission
Staphylococcal food poisoning	*S. aureus*	Ingestion of exotoxin in food, usually improperly refrigerated.
Salmonellosis	*Salmonella* spp.	Ingestion of contaminated food and drink.
Typhoid fever	*S. typhi*	
Cholera	*V. cholerae*	Ingestion of contaminated food and drink.
E. coli gastroenterititis	Enterotoxigenic, enteroinvasive, enteropathogenic and enterohemorrhagic, enteroaggregative stains of *E. coli*	Ingestion of contaminated food and drink.

The widespread use of antibiotics in the aquaculture systems and agricultural sectors in Bangladesh may act as the source of antibiotics diffusion into the sediment. The uncontrolled antibiotics will remain in the sediment and an alternation of micro flora composition of the sediment and antibiotic-resistant bacteria may occur with exerting of selective pressure (Sorum 2006). Sometimes fishes are treated with some antibiotic solutions to extend their shelf life. The broad spectrum antibiotics, tetracyclines, chloramphenicol etc. have been used to extend the shelf life of fish (Balachandran 2001).

Ghosh and Mandal (2010) reported that Multiple Antibiotic Resistances have been found in fish pathogen and bacteria from aquaculture environment with a variety of drug or an uncertain antibiotic usage history. The high level of water contamination with the industrial effluents and agricultural pollutants may magnify the exchange possibilities.

In the present study, five types of fish species were selected for microbiological assessment because these species are not only highly demandable in export market but also popular in our country. So, fish samples were collected from different fish processing plants, local fish markets and super shops. Fish processing plants have special sites for collection of fish such as certain fish farms, and/or landing centre whereas local fish markets and super-shops generally have no such special sites for collection of fish. Processing industry produces export quality fish products by dressing, degutting, partially processed or fully processed block or individually frozen. But local fish markets and super-shops generally collect fish from wholesale markets and bring this to the sites, store with ice and sell them to consumers. Several studies have been done to analyze microbial quality of fresh fishes from local markets but only a few studies have done to assess microbiological quality of fishes from fish processing plants. This study was designed to compare the microbial assessment of export oriented fishes and fishes from local fish markets and super shops. Thus a comparative study of microbial status of export oriented fishes from fish processing plants and fishes from local fish markets and super shops may give a clear idea about health concern. In this study some commercially available antibiotics were used to determine whether the pathogenic isolates were resistant or sensitive against those which might be helpful for effective medication during occasional fish borne disease outbreaks.

1.2 Objectives

This study was aimed for comparative analysis of microbiological assessment of export oriented fishes collected form fish processing plants and fishes collected from local fish markets and super shops.

The specific objectives of this study include:

i) To assess microbiological status of export oriented fishes and locally marketed fishes
ii) To isolate pathogenic bacteria;
iii) To observe antibiotic susceptibility of pathogenic isolates.

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CHAPTER 2
MATERIALS AND METHODS

Chapter 2
Materials and Methods

2.1 Laboratory of Investigation

This study was carried out in Fish Inspection & Quality Control (FIQC) Laboratory, Department of Fisheries (DoF), Matshya Bhaban, Ramna, Dhaka. FIQC laboratory is an accredited laboratory which was accredited by Bangladesh Accreditation Board on 28th May 2013.The laboratory follows ISO 17025 standards.

The duration of this study was from October, 2013 to March, 2014.

2.2 Selection of Fish Samples

For the assessment of microbial status, five types of commercially important fish species were selected because these species have high demand in foreign market, not only that, these are also popular in our country. The selected fish samples are given in the table below:

Table 2: Fish samples

Local Name	Scientific Name
Magur	*Clarias batrachus* (Linnaeus, 1758)
Mola	*Amblyopharyngodon mola* (Hamilton-Buchanan, 1822)
Pabda	*Ompok pabda* (Hamilton-Buchanan, 1822)
Rohu	*Labeo rohita* (Hamilton-Buchanan, 1822)
Koi	*Anabas testudineus* (Bloch, 1795)

Fish species were identified according to Rahman (2005) and Shafi and Quddus(2004). These 5 types of fish species are available in local fish market and super shops of our country and these are also exported to other countries of the world as dressed block frozen.

Fish Samples

Plate 1. Magur (*Clarias batrachus*)

Plate 2. Mola (*Amblyopharyngodon mola*)

Plate 3. Pabda (*Ompok pabda*)

Plate 4. Rohu (*Labeo rohita*)

Plate 5. Koi (*Anabas testudineus*)

2.3 Collection of Fish Samples

Fish samples were collected from four fish processing plants as processed block frozen for bacteriological quality assessment In contrast of samples of fish processing plants, the selected fish samples were also collected from four local fish markets and four super shops for comparative analysis bacterial status.

Table 3: Fish samples collected from different sampling sites

Sampling sites	Name of fish
Fish Processing Plants	Magur
01. Eurocross Frozen Foods Bd. Ltd., Sylhet	Mola
02. Saidowla (Pvt.) Enterprise Ltd., Sunamgonj	Pabda
03. Snow King Frozen Food Ltd., Mirpur-1	Rohu
04. Golden Harvest Ltd., Gazipur	Koi
Local Fish Markets	Magur
01. Kawran Bazar Fish Market	Mola
02. Gulshan Fish Market	Pabda
03. Rampura Fish Market	Rohu
04. Palashi Fish Market	Koi
Super Shops	Magur
01. Agora,Gulshan	Mola
02. Swapna, Gulshan	Pabda
03. Meena Bazar, Bannai	Rohu
04. Family Needs, Uttara	Koi

Export oriented fish samples from processing plants were collected as block frozen & transported to the laboratory using insulated icebox & stored at -18˚ C.

Samples from local fish markets and super shops were collected in sterilized plastic bags asceptically in the morning and transported to the laboratory using icebox and then stored at -18º C.

Collection of Fish Samples

Plate 6 & 7. Export oriented block frozen fish of Processing Plant

Plate 8. Fishes in Local Market

Plate 9. Fishes in Super Shop

Plate 10 & 11. Collection of fish samples

2.4 Microbiological Methods and Analysis

2.4.1 Microbiological Isolation

Bacterial status was assessed from fish samples of fish processing plants, local fish markets and super shops. Bacteriological parameters for examination of fish samples were- SPC, TCC, TFC and qualitative analysis of *Salmonella* spp. and *Vibrio cholerae.*

2.4.2 Culture Media Used for Bacteriological Assessment

a) Bacteriological peptone (Biolife, Italy): Used for serial dilution of fish samples.
b) PCA (Biolife, Italy): Used for standard plate count.
c) LSTB (Biolife, Italy): Used for 1st enrichment of total coliform and faecal coliform
d) BGBB (Biolife, Italy): Used for the final count of total coliform.
e) EC broth (Biolife, Italy): Used for 2nd enrichment of faecal coliform.
f) TW (Biolife, Italy): Used for the confirmation test.
g) APW (Biolife, Italy): Used for 1st enrichment of *Vibrio* spp.
h) TCBS (Biolife, Italy): Used for 2nd enrichment of *Vibrio* spp.
i) BPW (Biolife, Italy): Used for 1st enrichment of *Salmonella* spp.
j) MKTT (Biolife, Italy): Used for 1st solid media for 2nd enrichment of *Salmonella spp.*
k) RV (Biolife, Italy): Used for 2nd solid media for 2nd enrichment of *Salmonella* spp.
l) HEA (Biolife, Italy): Used for 3rd enrichment of *Salmonella* spp.
m) XLDA (Oxoid Ltd., England): Used for 3rd enrichment of *Salmonella* spp.
n) NA (Oxoid Ltd., England): Used for pure culture.
o) TSI agar (Oxoid Ltd., England): Used for the confirmation of *Vibrio cholerae.* and *Salmonella* spp.
p) Urea agar (Oxoid Ltd., England): Used for the confirmation of *Salmonella* spp.
q) TSB (Biolife, Italy): Used for the preservation of isolated organism.
r) MHA (Sigma): Used for antibiotic susceptibility test.

(Media composition and preparation, Appendix B)

2.4.3 Enumeration of Standard Plate Count (SPC):

SPC was enumerated according to ISO 4833:2003.

2.4.3.1 Media Preparation

For enumeration of SPC, Bacteriological peptone plate count agar (PCA) media was used. To dilute the fish sample Bacteriological peptone water was prepared by mixing 1g of media with 1000 ml distilled water (according to the manufacturer's instructions). Then PCA was prepared as mixing 11.75 g of medium with 500 ml of distilled water & heated properly (according to the manufacturer's instructions). Both of the media were, then autoclaved at 121˚C for 15 minutes.

2.4.3.2 Processing of Fish Samples

Fish samples were taken out of the refrigerator & thawed at room temperature. Then samples were cut & 20 g of each sample was blended with 180 ml of sterile bacteriological peptone water in a stomacher blender (Stomacher 400). Then 1 ml of this 10^{-1} dilution was transferred to a screw cap vial containing 10 ml of sterile dilute of bacteriological peptone to make a dilution of 10^{-2}. Then the vial was shaken gently. This process was repeated progressively to prepare of 10^{-3}, 10^{-4} & 10^{-5}.

2.4.3.3 Test Procedures

(i) Each of 1 ml of solution from 10^{-3}, 10^{-4} & 10^{-5} dilutions was plated by pipette into sterile plates.

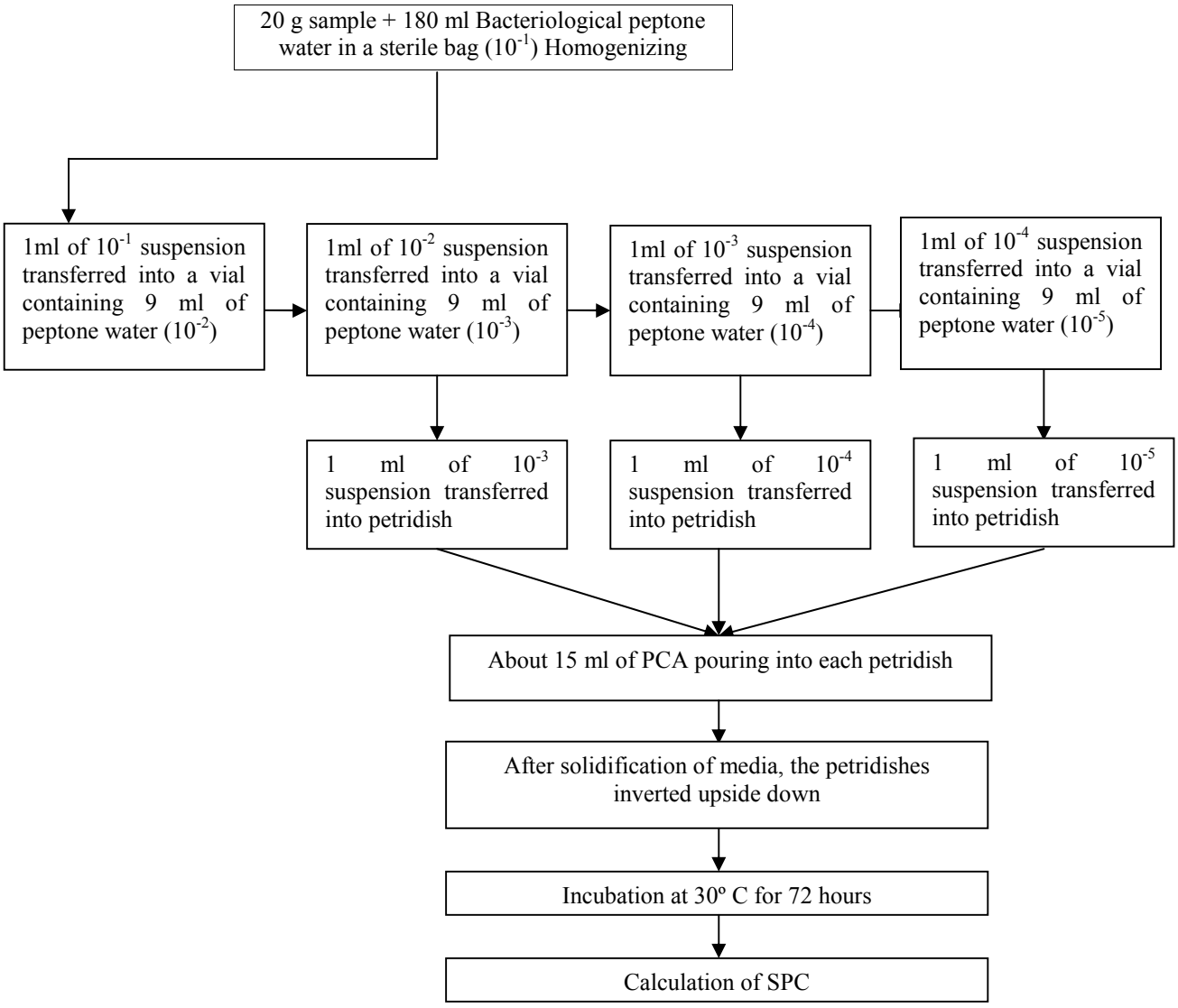
(ii) About 15 ml of sterile PCA was poured into the plates.

(iii) After solidification of the media, the plates were inverted & incubated in incubator (Shel Lab) at 30˚C for 72 hours.

(iv) The total number of bacteria per gram of sample was obtained by multiplying the average number of colonies on Petri dishes by the respective dilution factor.

(v) The total numbers of bacteria found from each Petridis for each dilution were averaged to find a reliable standard plate count (SPC).

Diagram 1: Enumeration of Standard Plate Count (SPC)



Enumeration of SPC

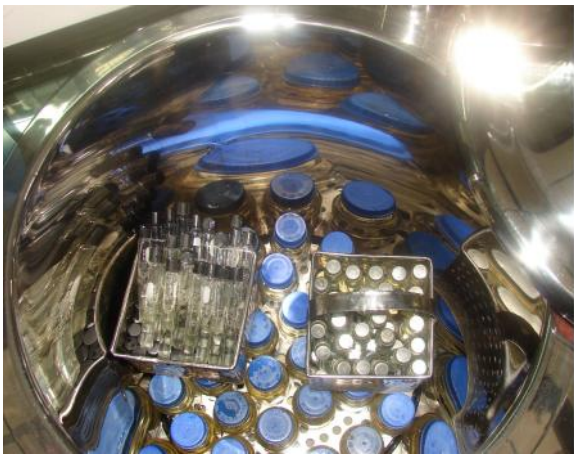


Plate 12. Media Autoclaving

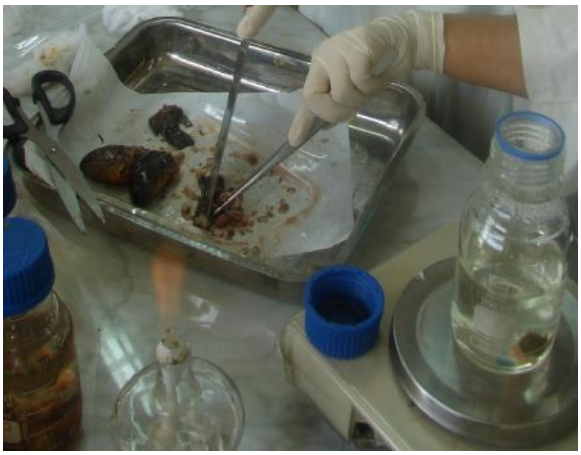


Plate 13. Sample Processing

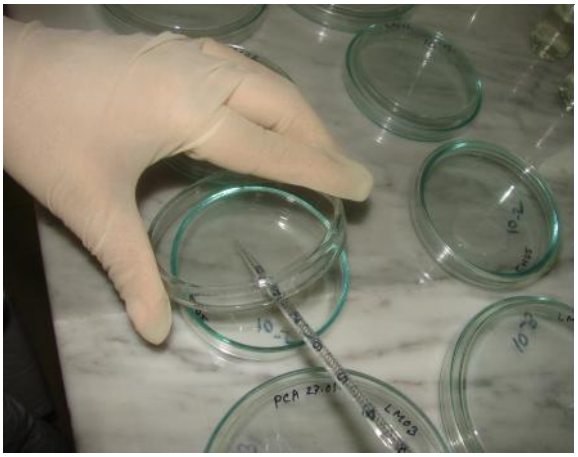


Plate 14. Inoculation of Diluted Sample

Plate 15. Inoculated Plates Poured with PCA

Plate 16. Incubation of Inoculated Sample

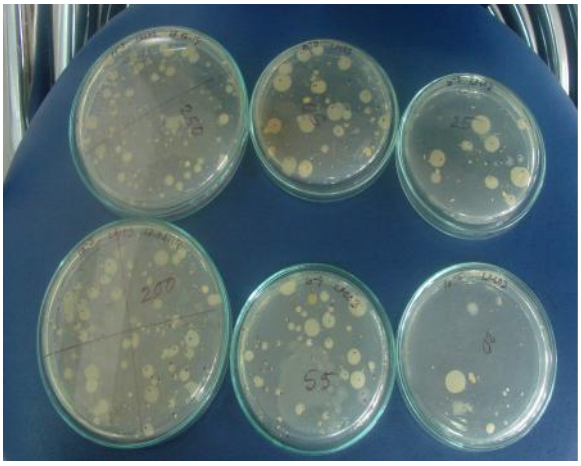


Plate 17. Colonies on PCA Plate

2.4.4 Enumeration of Total Coliforms (TCC)

TCC was enumerated according to ISO 4831:1991

2.4.4.1 Media Preparation

For enumeration of total coliforms LSTB was prepared and transferred into tubes and autoclaved after adding Durham's tubes (Pyrex).

2.4.4.2 Test Procedures

(i) From processed fish samples 1 ml of each of the 10^{-1}, 10^{-2} & 10^{-3} dilutions was transferred into the three separate tubes of LSTB containing Durham's tube.

(ii) The tubes were incubated in an incubator (Binder BD 115) at 37˚C for 48 hours.

(iii) After incubation the positive gas production tubes were recorded.

(iv) For each set of positive LSTB tubes one set of BGBB tubes (for total coliform enumeration) and one set of EC tubes (for total fecal coliforms enumeration 2.4.5) were prepared.

(v) A loopful of broth form each positive LSTB tubes of culture were inoculated into a tube of BGBB and incubated at 37˚C for 24 hours.

(vi) The positive gas production tubes were recorded and the result was computed using MPN chart (Appendix E).

2.4.5 Enumeration of Total Fecal Coliforms (TFC)

TFC was enumerated according to ISO 7251:1993

2.4.5.1 Test Procedure

(i) To enumerate faecal coilforms a loopful broth from the tubes of LSTB that were positive for gas production were transferred to EC containing Durham's tubes.

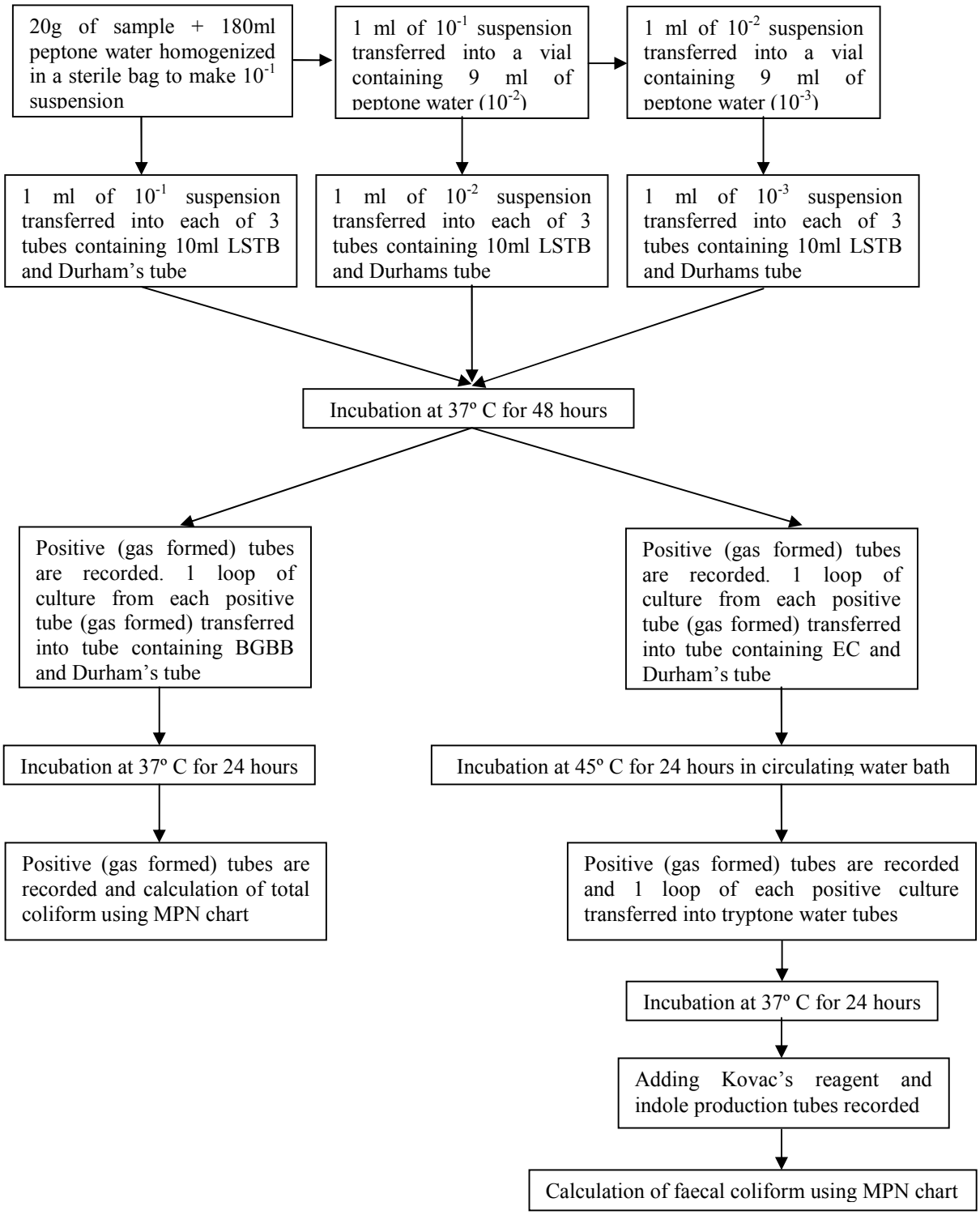
(ii) EC tubes were incubated at 45˚C for 24 hours in circulatory water bath (Shel Lab).

(iii) After incubation, positive gas production tubes were recorded and from each tube a loopful of broth were transferred to sterile tryptone water tube.

(iv) Tubes were incubated at 37˚C for 48 hours in an incubator and after incubation kavac's reagents were added to determine the presence of indole ring. A positive indole reaction indicates the presence of *E. Coli.*

(v) Positive tubes were recorded and the results were computed using MPN chart.(Appendix E)

Diagram 2: Enumeration of Total Coliform and Faecal Coliform



Enumeration of TCC and TFC

Plate 18. Prepared LSTB

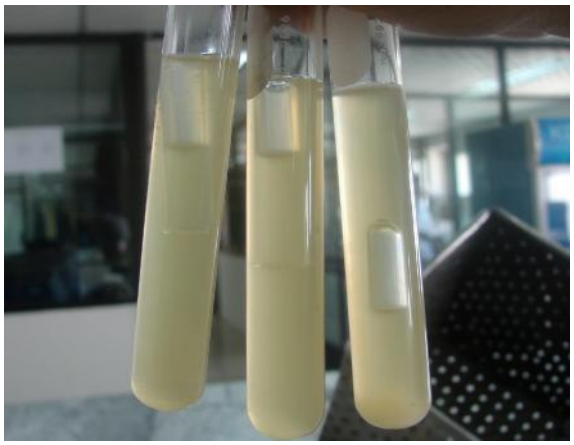


Plate 19. Gas Formation in LSTB after Incubation

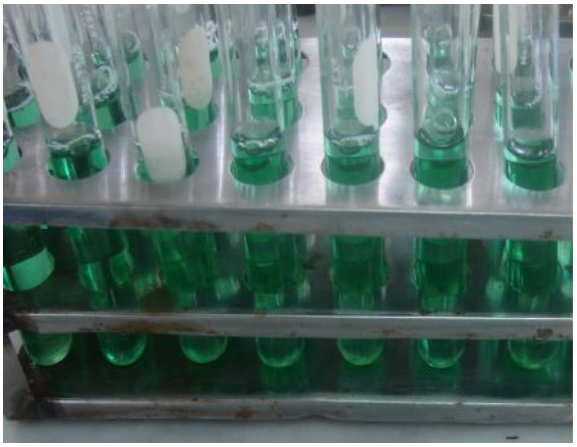


Plate 20. Prepared BGBB

Plate 21. Gas Formation in BGBB after Incubation

Plate 22. Positive Indol (Pink) Reaction in Triptone Water

2.4.6 Detection of *Salmonella spp.*

Presence of *Salmonella* spp was detected according to ISO 6579:2002.

2.4.6.1 Media Preparation

For *Salmonella* spp. isolation at first pre-enrichment medium buffered peptone water (BPW) was prepared by mixing 20g of medium with 1000 ml of distilled water. Then 225 ml of the pre-enrichment broth was transferred to culture bottle and sterilized at 121º C for 15 minutes.

2.4.6.2 Test Procedures

i) For preparation of initial suspension, 25g of sample was taken and diluted with 225 ml of sterile BPW.

ii) This initial suspension was incubated at 37º C for 18 ± 2 hours.

iii) For selective enrichment, RV and MKTTn broth (Appendix B-preparation) were prepared and sterilized.

iv) Then 0.1 ml and 1 ml of the culture obtained after initial suspension incubation were transferred to McCarty's bottle containing 10 ml of the RV broth and MKTTn broth respectively.

v) The inculated RV broth was incubated at 41.5º C for 24 ± 3 hours and inoculated MKTTn broth at 37º C for 24 ± 3 hours.

vi) For identification of *Salmonella spp.* solid media, XLDA and HEA were prepared (Appendix B-preparation).

vii) Then the culture obtained after incubation of RV and MKTTn broth were streaked by means of a loop, both on the surface of XLDA and HEA plates.

viii) After streaking, the plates were inverted and incubated in an incubator (Binder BD115) at 37º C for 24 ± 3 hours.

ix) After incubation, the presence of typical colonies of *Salmonella spp.* were examined and marked on the bottom of the dish.

   a) Typical colonies of *Salmonella* spp. grown on XLD agar have a black centre and a lightly transparent zone of reddish colour.

   b) Typical colonies of *Salmonella* spp. grown on the HE agar have a black round appearance.

2.4.6.3 Pure Culture of Typical *Salmonella* Colonies

For confirmation, the suspected colonies from each selective agar plate were selected and streaked onto the surface of Nutrient Agar (NA) plates inside a laminar cabinet (ESCO) to obtain pure culture. The inoculated NA plates were incubated in an incubator at 37º C for 24 ± 3 hours to develop well isolated colonies.

Pure culture obtained from NA plates were used for biochemical and serological confirmation.

2.4.6.4 Microscopic Examination of Bacterial Morphology

The size shape and arrangement of bacterial isolates were observed through microscopic examination using Gram staining method (Appendix no. F) (Pelczar *et al.* 1993).

2.4.6.5 Biochemical Tests

TSI Test

TSI agar was prepared and sterilized according to manufacturer's instructions (Appendix B). Prepared TSI agar was set into tubes as if it makes a slant and a butt. By means of an inoculating wire, a portion of a isolated colony from pure culture was picked and the medium was inoculated by stubbing the butt and by streaking the slant of the tube. Then tubes were incubated at 37º C for 24 ± 3 hours. After incubation, typical *Salmonella* culture show alkaline (red) slants and acid (yellow) butts with gas formation and formation of H_2S (blackening of the agar).

Urease test

Urea agar was prepared according to manufacturer's instructions (Appendix B). The medium was inoculated by streaking the agar slant and stubbing the butt using a portion of isolated colony from pure culture.

The reaction is positive if the colour of the medium changes to phenol red. But *Salmonella* shows negative reaction.

Indol test

Tryptone medium was prepared for this test. A tube containing 5 ml of the medium was inoculated with suspected colony and incubated at 37º C for 24 ± 3 hours. After incubation 1 ml of kovac's reagent was added.

The formation of a pink colour ring indicates a positive reaction and yellow-brown ring indicates a negative reaction and *Salmonella* is indole negative.

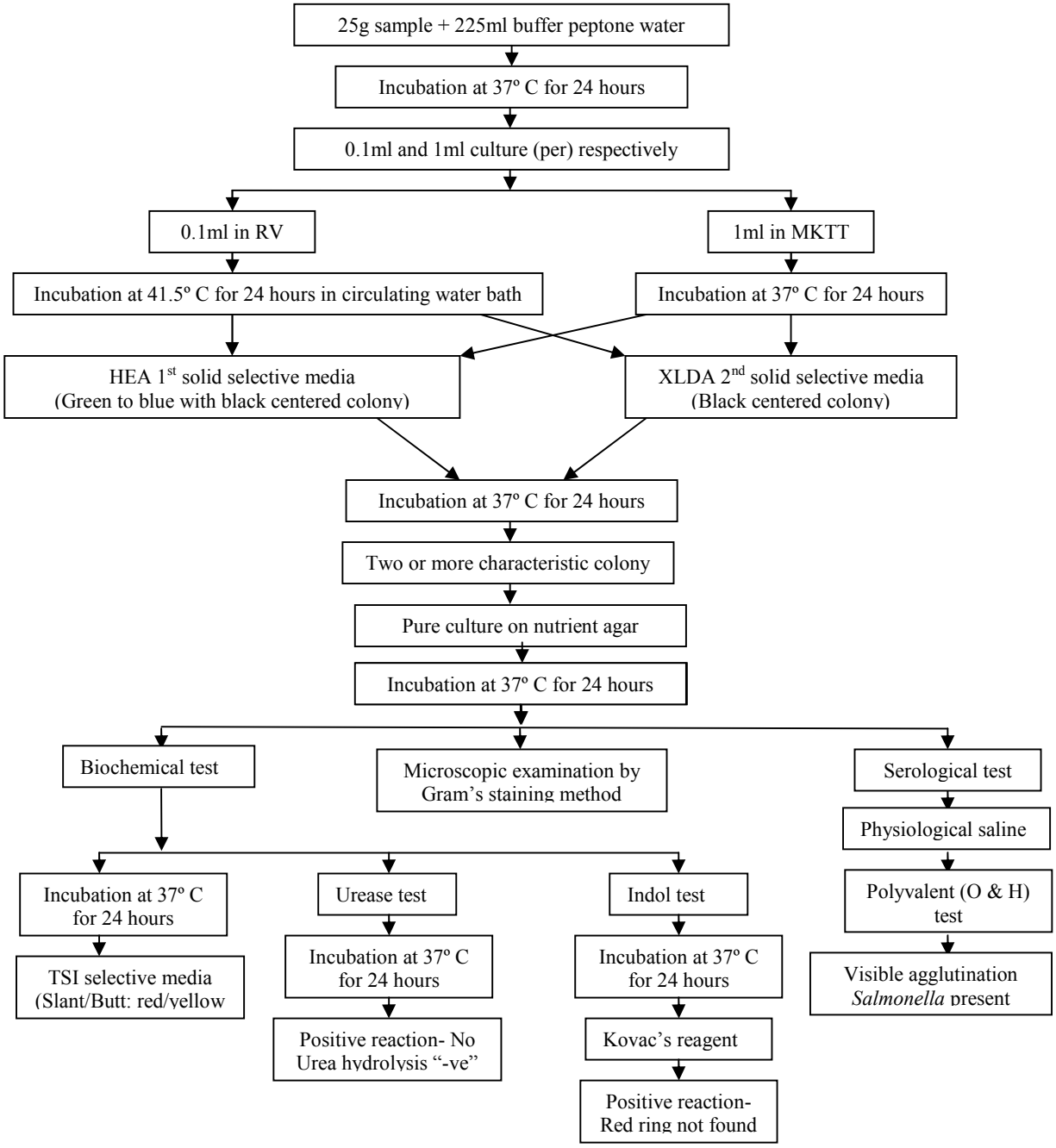
(Interpretation of biochemical confirmation- Appendix G)

2.4.6.6 Serological Test

One drop of anti-O serum was placed onto a clean glass slide. By means of a swab stick, a part of the colony to be tested was dispensed in the serum drop. If agglutination occurs, the reaction is considered positive.

This process was repeated for anti-H serum. Typical *Salmonella* shows positive reaction.

Diagram 3: Detection of *Salmonella* spp.



Detection of *Salmonella* spp.

Plate 23. Incubation of Inoculated BPW

Plate 24. Inoculation of First Enrichment Culture in RAV and MKTT

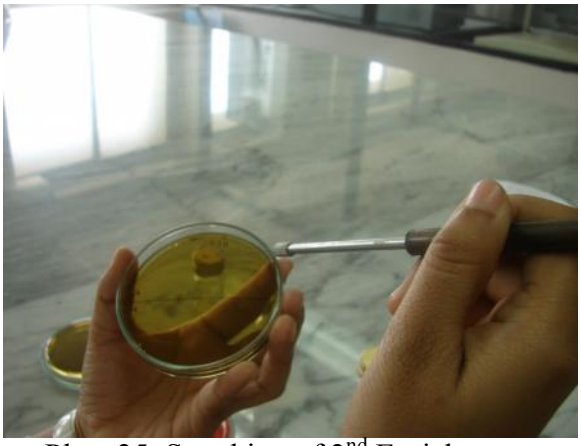


Plate 25. Streaking of 2nd Enrichment Culture on HEA

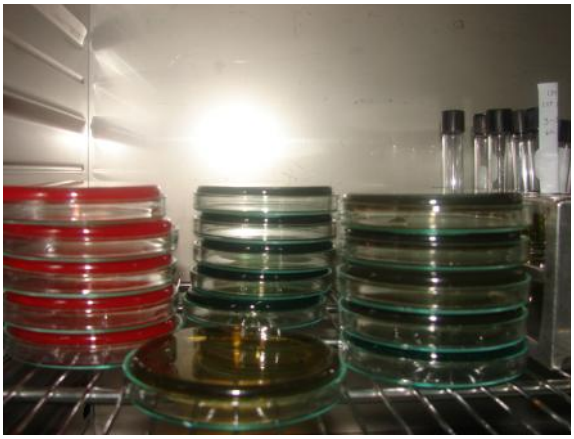


Plate 26. Incubation of Inoculated XLDA and HEA

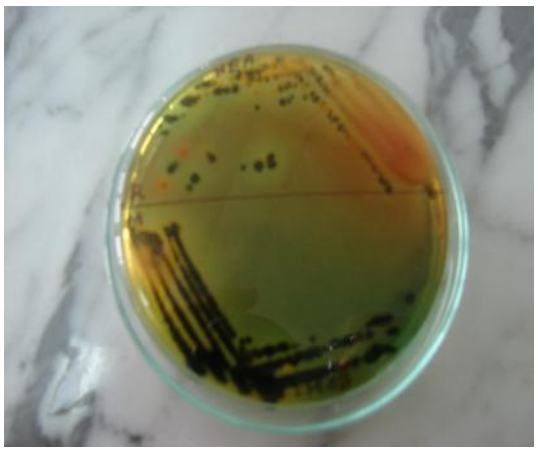


Plate 27 & 28. Typical *Salmonella* Colonies on XLDA and HEA

Detection of *Salmonella* spp. (Continued)

Plate 29. Streaking of Suspected Colonies to Obtain Pure Culture inside Laminar Cabinet

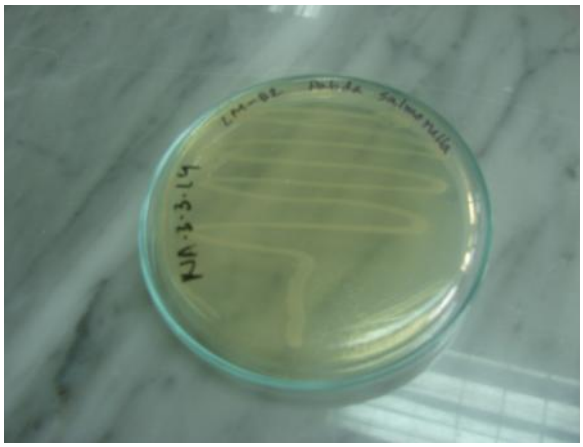


Plate 30. Pure Culture Plate of *Salmonella*

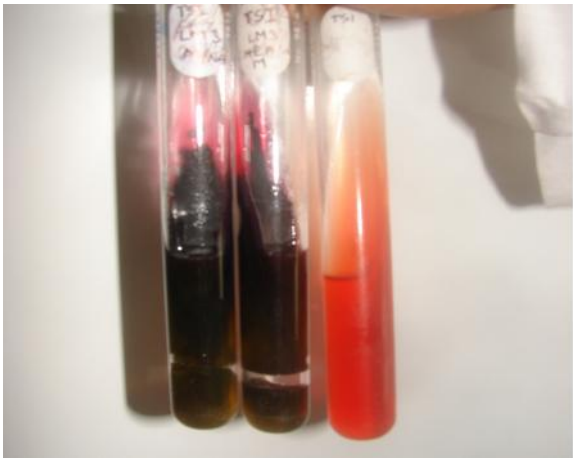


Plate 31. TSI Test: Positive (first and middle tube), Control (third tube)

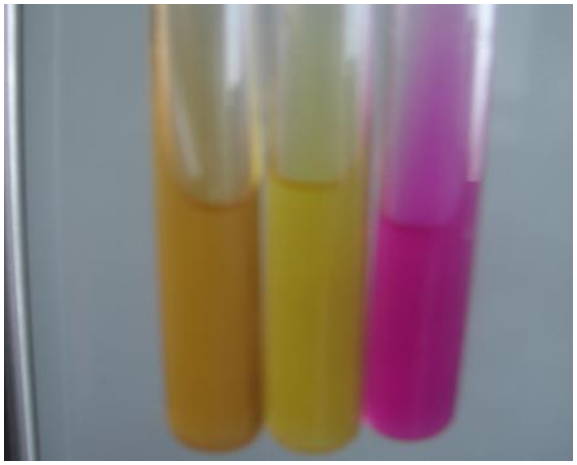


Plate 32. Urease Test: Control (first tube), Negative (middle tube), Positive (third tube)

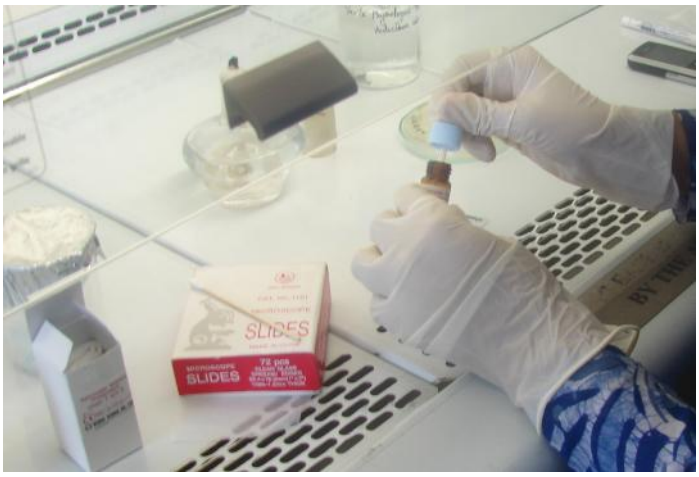


Plate 33. Serological Test

2.4.7 Detection of *Vibrio cholerae*

Presence of *Vibrio cholerae* was detected according to ISO/TS 21872-1:2007.

2.4.7.1 Media Preparation

For *Vibrio cholerae* isolation at first saline Alkaline Peptone Water (APW) was prepared by mixing 20g of medium with 1000 ml of distilled water. Then 225 ml of the first enrichment broth was transferred to culture bottle and sterilized at 121º C for 15 minutes.

2.4.7.2 Test Procedures

(i) For preparation of first enrichment, 25g of sample was taken and diluted with 225 ml of sterile APW. This suspension was incubated in an incubator at 37º C for 6 hours.

(ii) For second enrichment, 1ml culture of first enrichment was transferred to McCarty's bottle containing 10ml of sterile APW.

(iii) For selective enrichment, TCBS agar was prepared and sterilized. Then the culture of second enrichment was streaked by means of a loop on the surface of TCBS agar plates. After streaking, the plates were inverted and incubated in an incubator at 37º C for 24 ± 3 hours. After incubation, the presence of typical colonies of *Vibrio cholerae* were examined and marked on the bottom of the dish.

- Typical colonies of *Vibrio cholerae* grown on TCBS agar are smooth, yellow and 2 mm to 3 mm in diameter.

.

2.4.7.3 Pure Culture of Typical Colonies

For confirmation, the suspected colonies from each selective agar plate were selected and streaked onto the surface of saline nutrient agar (SNA preparation- Appendix B) plates inside a laminar cabinet (ESCO) to obtain pure culture. The inoculated plates were incubated at 37º C for 24 ± 3 hours to develop well isolated colonies.

Pure culture obtained from saline nutrient agar plates were used for biochemical test.

2.4.7.4 Microscopic Examination of Bacterial Morphology

The size shape and arrangement of bacterial isolates were observed through microscopic examination using Gram staining method (Appendix no. F) (Pelczar *et al.* 1993).

2.4.7.5 Biochemical Tests

Oxidase Test

The presence of cytochrome oxidase was detected by Kovac's oxidase test (Kovac 1956). A piece of Whatman filter paper was soaked with 1% solution of N'N'N'N'-tetramethyl- p –phynylenadiamine dihydrochloride. A portion of colony of the test organism was picked up with a sterile cotton swab stick and touched onto the paper with impregnated reagent. The test is considered positive if the colour turns to mauve, violet or deep purple within 10 seconds.

TSI Test:

Saline TSI agar was prepared and sterilized according to manufacturer's instructions (Appendix B). Prepared TSI agar was set into tubes as if it makes a slant and a butt. By means of an inoculating wire, a portion of a isolated colony from pure culture was picked and the medium was inoculated by stubbing the butt and by streaking the slant of the tube. Then tubes were incubated at 37º C for 24 ± 3 hours. After incubation, typical *Vibrio cholerae* show an acid slant (yellow) and acid butt (yellow) without formation of hydrogen sulfide or gas.

Indol Test:

Saline tryptone medium was prepared for this test. A tube containing 5 ml of the medium was inoculated with suspected colony and incubated at 37º C for 24 ± 3 hours. After incubation 1 ml of kovac's reagent was added.

The formation of a pink colour ring indicates a positive reaction and *Vibrio cholerae* is indole positive.

Halotolerance Test

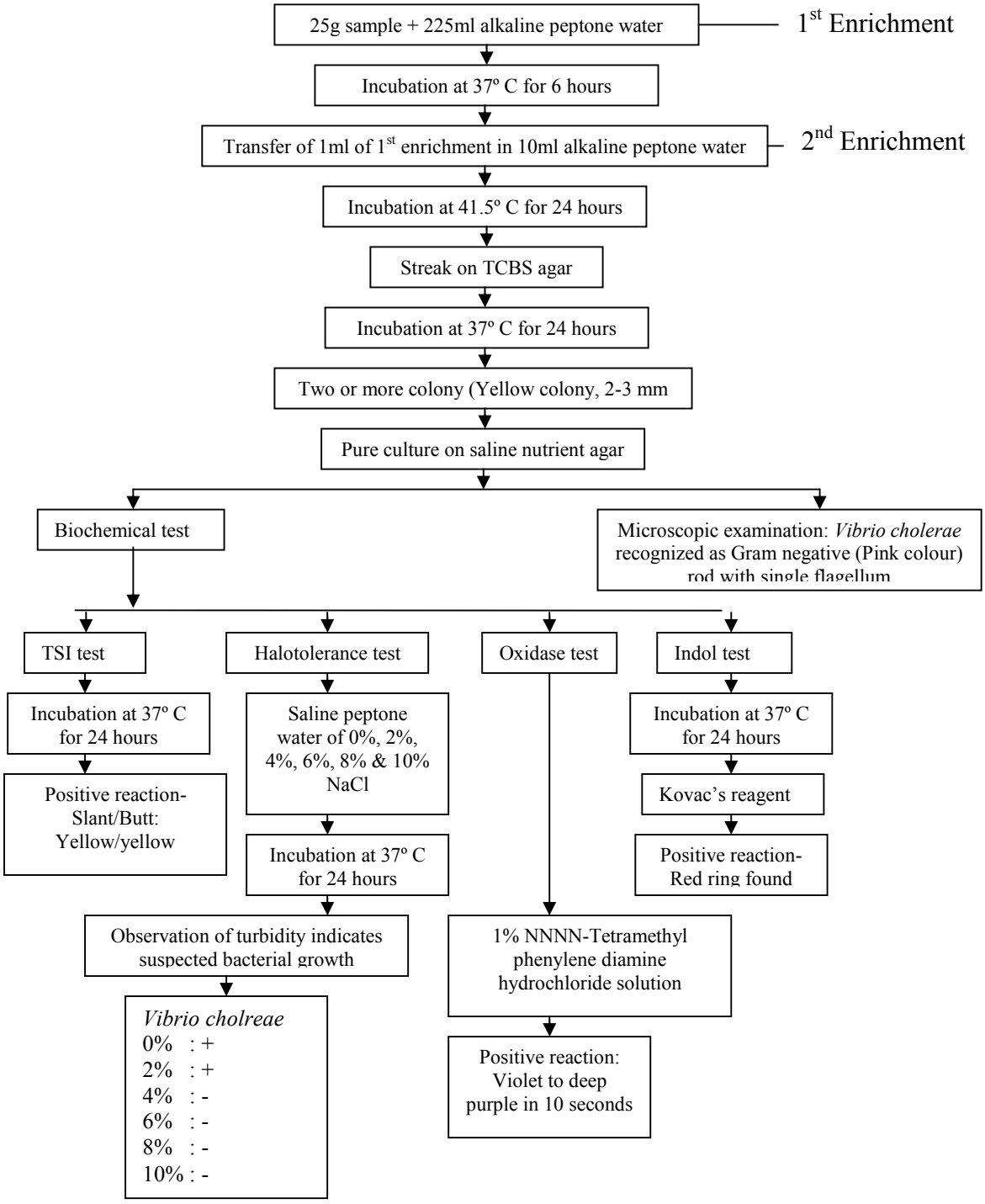
First, a series of peptone waters with increasing salt (NaCl) concentration: 0%, 2%, 4%, 6%, 8% and 10% was prepared.

A suspension with the colony to be identified was prepared and lightly inoculated each of the tubes (with a loopful) and incubated at 37 ºC for 24 h ± 3 h.

Observation of turbidity in 0% and 2% NaCl concentration indicates that the suspected *Vibrio cholerae* is present.

(Interpretation of biochemical confirmation- Appendix G)

Diagram 4: Detection of *Vibrio cholerae*



Detection of *Vibrio cholerae*

Plate 34. Inoculation of 1st Enrichment Culture in APW

Plate 35. Incubation of Inoculated APW in Circulatory Waterbath

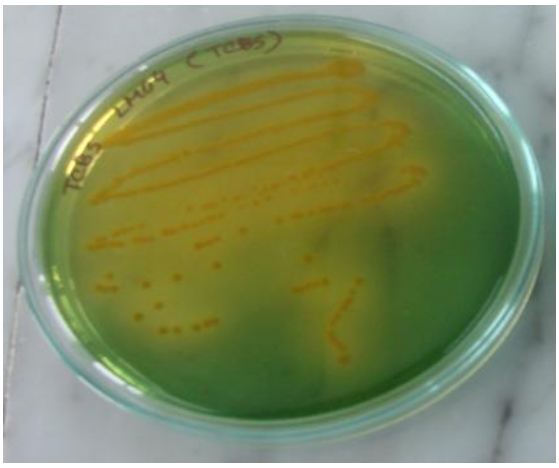


Plate 36. Colonies of *V. cholerae* on TCBS after Incubation

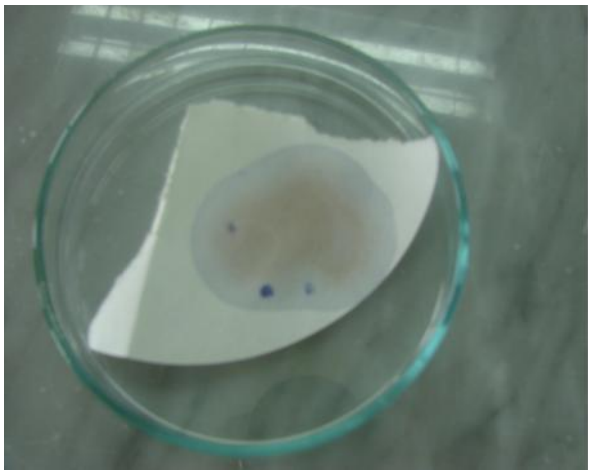


Plate 37. Oxidase Test

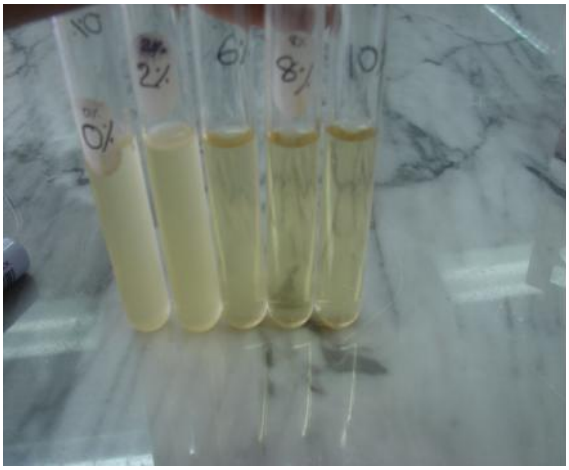


Plate 38. Halotolerance Test

Plate 39. Indol Test

2.5. Maintenance and Preservation of Culture Strain

For long-term preservation, selected and identified bacterial strain were stored in Trypticase soya broth with 16% glycerol and stored from without significant loss of viability at – 20^0C until further study.

2.6. Antibiotic Susceptibility Test

Antibiotic susceptibility test of the isolated organisms was done by disc diffusion method using the Kirby-Bauer technique (Bauer *et al*. 1966) and as per recommendation of NCCLS (NCCLS 1997).

2.6.1. Antimicrobial Agent Used

Following seven antimicrobial agents were used for determined antibiotic suscetibility of isolated organism:

- Ampicillin (10µg)
- Ciprofloxacin (5µg)
- Chloramphenicol (10µg)
- Ceftriazone (30µg)
- Erythromycin (15µg)
- Tetracycline (30µg)
- Trimethoprine-sulfomethoxazole (25µg)

2.6.2. Test Procedures

Muller-Hinton agar was prepared from a commercially available dehydrated base according to the manufacture's instruction. Immediately after autoclaving, the media was allowed to cool to 45^0C to 50^0C in a water bath. Then the steps were as follows:

i) In the antimicrobial disc diffusion susceptibility test, a standardized amount of pure culture was used as inoculum and to standardize the inoculum density Barium chloride turbidity standard, equivalent to a 0.5 McFarland Standard (Appendix C) was used.

ii) The plates were then heavily inoculated with that standardized inoculum by means of a cotton swab stick and the inoculum was allowed to dry for 5 minutes at room temperature with the lid closed.

iii) Then the discs were applied by a sterile forceps 15mm away from the edge of the Petri dish and having a space of 25-35mm in between two discs.

iv) The plates were incubated overnight at 37^0C in an incubator.

v) After 16 to 18 hours of incubation, each plate was examined and inhibition zones were measured in millimeter (mm) by using a ruler over the surface of the plate with the lid open

vi) The zone of inhibition was interpreted by antibiotic zone size as recommended by NCCLS and was categorized into two groups, namely sensitive (S) and resistant (R).

Preservation of Isolated Organism and Antibiotic Susceptibility Test

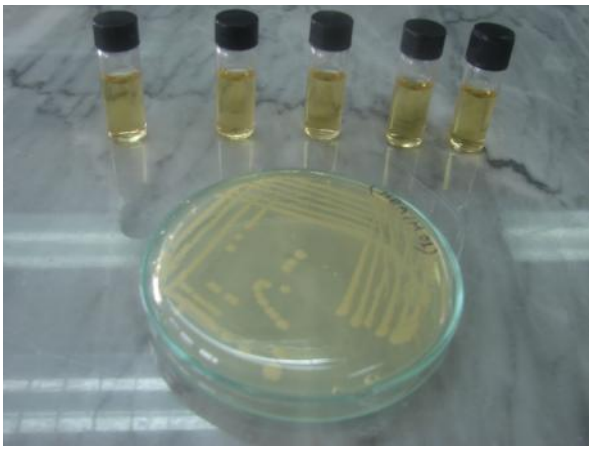


Plate 40. Preservation of Isolated Organism in TSB

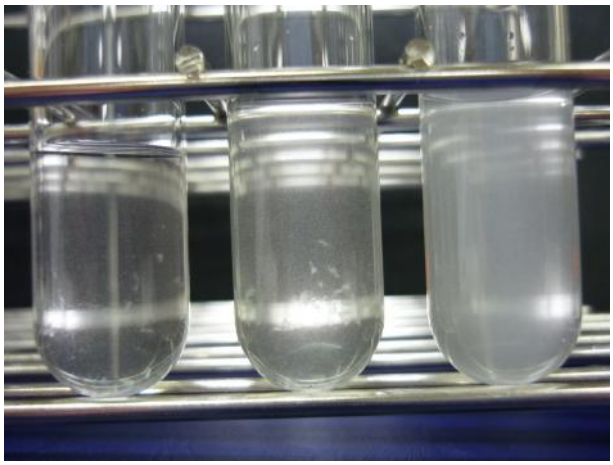


Plate 41. McFarland Standard

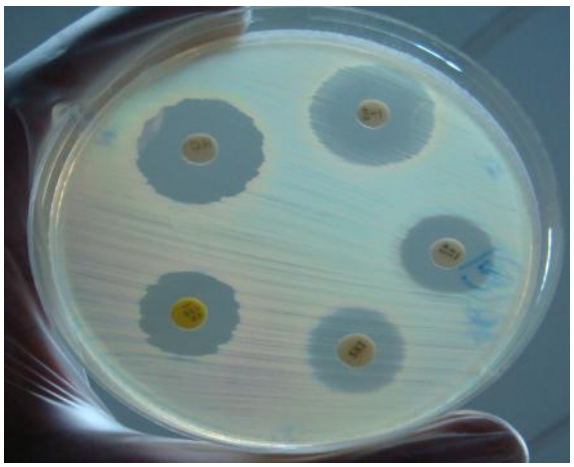


Plate 42. Antibiotic Susceptibility Test

2.7 Statistical Analysis

The means of bacterial load were compared using ANOVA followed by Tukey's post hoc for multiple comparisons. The software used were:

i. Statistical software IBM SPSS statistics 20 was used to analyze the data with the level of significance at $p<0.05$
ii. Microsoft Office Excel 2007 was used to make graphs.

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CHAPTER 3

RESULTS

Chapter 3
Results

3.1. Standard Plate Count (SPC) cfu/g

Standard Plate Counts (SPC) cfu/g of 5 types of export oriented fish samples collected from four fish processing plants and fish samples from four local fish market and four super shops are given in the table below:

Table 4. Standard Plate Counts (SPC) cfu/g of fish samples collected from fish processing plants, local fish markets and super shops.

Fish Samples / Sampling Sites	Magur	Mola	Pabda	Rohu	Koi
Fish Processing Plant-1	4.20×10^4	6.04×10^4	4.95×10^5	9.13×10^4	4.70×10^4
Fish Processing Plant-2	2.70×10^5	2.32×10^4	1.67×10^5	5.75×10^4	1.59×10^5
Fish Processing Plant-3	3.09×10^4	2.58×10^5	2.68×10^4	3.00×10^4	3.18×10^5
Fish Processing Plant-4	5.18×10^4	5.36×10^4	6.30×10^4	1.80×10^5	3.41×10^5
Local Fish Market-1	2.65×10^6	3.12×10^6	6.38×10^5	5.53×10^6	3.84×10^6
Local Fish Market-2	1.55×10^7	4.56×10^6	3.52×10^6	4.76×10^6	8.69×10^5
Local Fish Market-3	4.07×10^6	2.89×10^5	6.02×10^5	9.81×10^5	7.03×10^6
Local Fish Market-4	5.21×10^5	6.84×10^5	3.09×10^5	6.94×10^6	2.98×10^7
Super Shop-1	2.50×10^5	3.32×10^5	3.26×10^5	2.56×10^5	2.91×10^5
Super Shop-2	3.36×10^5	5.70×10^5	7.81×10^5	4.49×10^5	5.16×10^6
Super Shop-3	2.44×10^6	3.23×10^5	5.96×10^4	2.50×10^5	1.76×10^6
Super Shop-4	5.20×10^5	5.04×10^5	4.44×10^5	3.60×10^6	6.57×10^5

In this study standard plate count (SPC) was found to be the highest in Pabda collected from fish processing plant-1 and that was 4.95×10^5 *cfu/g*, the lowest value was found in Mola sample of processing plant-2. But SPC of all samples were within the acceptable limit.

In the samples of local fish markts, the highest value of SPC was 2.89×10^7 *cfu/g* which was found in Koi sample collected form local fish market-4 and the lowest value was

found in Mola sample of local fish market-2 that was 2.89×10^5 *cfu/g*. Other samples exceeded the limit of 5×10^5 *cfu/g*.

The highest value of SPC in samples of super shops was 5.16×10^6 *cfu/g* which was found in Koi sample of super shop-4 and the lowest was 5.96×10^4 *cfu/g* in Pabda sample collected from super shop-3.

3.2. Total Coliform Count (TCC) MPN/g

Total Coliform Counts (TCC) MPN/g of 5 types of export oriented fish samples collected from four fish processing plants and fish samples from four local fish market and four super shops are given in the table below:

Table 5. Total Coliform Count (TCC) MPN/g of fish samples collected from fish processing plants, local fish market and super shops.

Fish Samples / Sampling Sites	Magur	Mola	Pabda	Rohu	Koi
Fish Processing Plant-1	<3	15	3.6	9.2	<3
Fish Processing Plant-2	<3	<3	3.6	<3	3
Fish Processing Plant-3	<3	9.2	7.4	9.2	7.4
Fish Processing Plant-4	3.6	3.6	3.6	<3	<3
Local Fish Market-1	93	150	75	43	460
Local Fish Market-2	210	120	460	93	210
Local Fish Market-3	240	240	43	21	120
Local Fish Market-4	75	27	75	15	210
Super Shop-1	<3	240	9.2	<3	29
Super Shop-2	43	120	27	11	150
Super Shop-3	36	75	14	35	120
Super Shop-4	36	75	64	29	240

In the samples of fish processing plants, Total coliform count (TCC) was the highest in Mola sample of processing plant-1, which was 15 MPN/g and the lowest value was <3

MPN/g which was found in several samples of all the processing plants. All values of TCC were within the acceptable limit.

TCC, in local fish market samples, was highest in Koi sample of local fish market-1 and Pabda sample of local fish market-2, that was 460 MPN/g and the lowest 15 MPN/g was found in Rohu sample collected from local fish market-4.

In super shop samples, TCC was highest in Mola sample of super shop-1 and Koi of super shop-4, which was 120 MPN/g and the lowest value was found in Magur and Rohu samples collected from super shop-1 and that was <3 MPN/g.

3.3 Total Fecal Coliform Count (TFC) MPN/g

Total Fecal Coliform Counts (TFC) MPN/g of 5 types of export oriented fish samples collected from four fish processing plants and fish samples from four local fish market and four super shops are given in the table below:

Table 6. Total Fecal Coliform Counts (TFC) MPN/g of fish samples collected from fish processing plants, local fish market and super shops.

Fish Samples / Sampling Sites	Magur	Mola	Pabda	Rohu	Koi
Fish Processing Plant-1	<3	7.4	<3	<3	<3
Fish Processing Plant-2	<3	<3	<3	<3	<3
Fish Processing Plant-3	<3	<3	<3	3.6	<3
Fish Processing Plant-4	<3	<3	<3	<3	<3
Local Fish Market-1	43	150	43	15	240
Local Fish Market-2	64	75	93	15	150
Local Fish Market-3	150	240	11	21	43
Local Fish Market-4	75	21	23	9.2	21
Super Shop-1	<3	36	3.6	<3	15
Super Shop-2	23	120	15	11	23
Super Shop-3	29	27	9.2	21	38
Super Shop-4	21	21	23	11	11

In processing plant samples, the highest value of total faecal coliform (TFC) was 7.4 MPN/g which was found in Mola sample of processing plant-1 and the lowest value was

<3 MPN/g which was found in almost all the samples except Mola of processing plant-1 and Rohu of processing plant-3.

The highest TFC, in local fish market samples, was found in Koi and Mola samples of local fish market-1 and local fish market-3 respectively, which was 240 MPN/g and the lowest value was 9.2 MPN/g found in Rohu sample of local fish market-4.

In super shop samples, TFC was highest in Mola sample of super shop-2, which was 120 MPN/g and the lowest value was found in Magur and Rohu samples collected from super shop-1 and that was <3 MPN/g.

3.4. Occurrence of *Salmonella* spp.

Occurrence of *Salmonella* spp. in five types of export oriented fish samples collected from four fish processing plants and fish samples from four local fish market and four super shops is given in the table below:

Table 7: Occurrence of *Salmonella* spp. in fish samples collected from fish processing plants, local fish markets and super shops .

Fish Samples / Sampling Sites	Magur	Mola	Pabda	Rohu	Koi
Fish Processing Plant-1	Absent	Absent	Absent	Absent	Absent
Fish Processing Plant-2	**Present**	Absent	Absent	**Present**	Absent
Fish Processing Plant-3	Absent	Absent	Absent	Absent	Absent
Fish Processing Plant-4	Absent	Absent	Absent	Absent	Absent
Local Fish Market-1	**Present**	Absent	Absent	**Present**	Absent
Local Fish Market-2	Absent	**Present**	**Present**	**Present**	**Present**
Local Fish Market-3	**Present**	**Present**	Absent	Absent	Absent
Local Fish Market-4	Absent	**Present**	**Present**	**Present**	Absent
Super Shop-1	Absent	**Present**	Absent	Absent	**Present**
Super Shop-2	Absent	**Present**	Absent	**Present**	Absent
Super Shop-3	**Present**	Absent	**Present**	Absent	Absent
Super Shop-4	Absent	**Present**	Absent	**Present**	Absent

Salmonella spp. was absent in almost all the samples except Magur and Rohu of processing plant-2.

Salmonella spp. was present in most of the samples of all the local fish markets except Mola, Pabda and Koi of local market-1, Magur of local market-2, Pabda, Rohu, Koi of local market-3 and Magur and Koi of local market-4.

Salmonella spp. was present in Mola and Koi samples of super shop-1, Mola and Rohu samples of super shop-2, magur and Pabda samples of super shop-3 and Mola and Rohu samples of super shop-4.

3.5. Occurrence of *Vibrio cholerae*

Occurrence of *Vibrio cholerae* in five types of export oriented fish samples collected from four fish processing plants and fish samples from four local fish markets and four super shops is given below:

Table 8: Occurrence of *Vibrio cholerae* in fish samples collected from fish processing plants, local fish markets and super shops .

Fish Samples / Sampling Sites	Magur	Mola	Pabda	Rohu	Koi
Fish Processing Plant-1	Absent	Absent	Absent	Absent	Absent
Fish Processing Plant-2	Absent	Absent	Absent	Absent	Absent
Fish Processing Plant-3	Absent	Absent	Absent	Absent	Absent
Fish Processing Plant-4	Absent	Absent	Absent	Absent	Absent
Local Fish Market-1	Absent	**Present**	Absent	Absent	**Present**
Local Fish Market-2	Absent	Absent	Absent	Absent	Absent
Local Fish Market-3	Absent	Absent	Absent	**Present**	**Present**
Local Fish Market-4	Absent	Absent	Absent	Absent	**Present**
Super Shop-1	Absent	Absent	Absent	Absent	Absent
Super Shop-2	Absent	Absent	Absent	Absent	**Present**
Super Shop-3	Absent	Absent	Absent	Absent	Absent
Super Shop-4	Absent	Absent	**Present**	Absent	**Present**

In processing plant samples, *Vibrio cholerae* was absent in all the samples.

Vibrio cholerae, in local market samples, was present in Mola, and Koi samples collected from local fish market-1, Rohu and Koi samples of local fish market-3 Koi of local fish market-4.

In this study *Vibrio cholerae* was present in Koi sample of super shop-2 and Pabda and Koi samples collected from super shop-4.

3.6 Comparative Analysis of Bacterial Load

Comparative analysis of bacterial load associated with export oriented fish samples collected from fish processing plants and fish samples of local fish markets and super shops are shown in the graphical presentation below.

SPC (Mean Log Value)

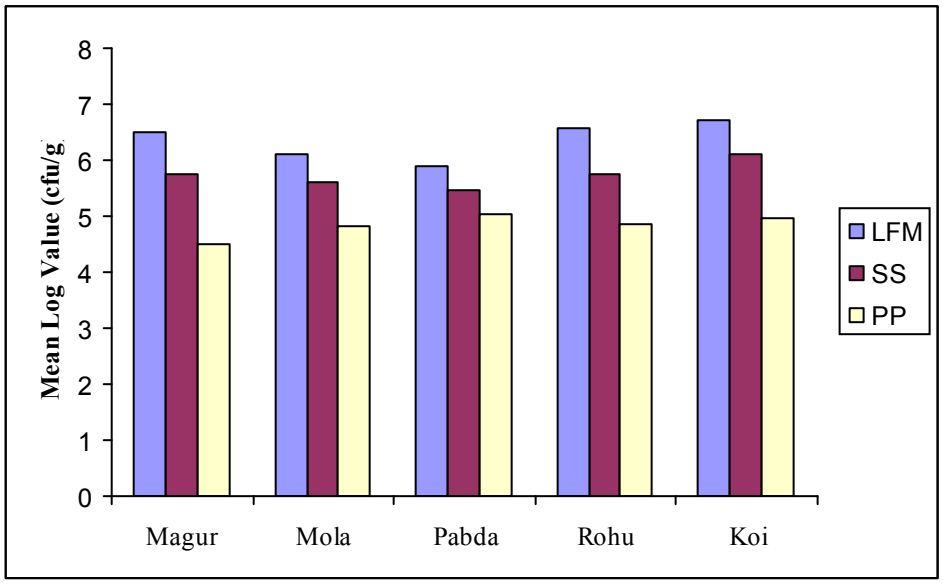

Fig. 1. Comparative analysis of SPC (mean log value) in fish samples collected from local fish markets (LFM), super shops (SS) and fish processing plants (PP).

Figure 1 is showing a comparative analysis among mean log values of SPC (*cfu/g*) of five types of fish samples collected from local fish markets (LFM), super shops (SS) and fish processing plants (PP). From the diagram it is obvious that in all types of fish samples collected from local fish markets, standard plate counts (SPC) were higher than

super shops and fish processing plants. The SPC in Magur fish sample collected from fish processing plants was the lowest, whereas in the Koi fish samples collected from local fish markets and super shops it was highest. In case of Mola fish sample, the mean log value of SPC were highest in samples collected from local fish markets while the lowest were in the samples collected from fish processing plants which was a little higher in the samples collected from super shops. SPC in Pabda fish samples collected from super shops was fewer than the samples collected from local fish markets but comparatively higher than the samples collected from fish processing plants. SPC in Rohu fish sample was found highest in the samples of local fish markets and the lowest in the samples of fish processing plants which was lesser than the samples collected from super shops. SPC in Koi fish samples collected from super shops and local fish markets were higher than the samples of fish processing plants.

TCC (Mean Value)

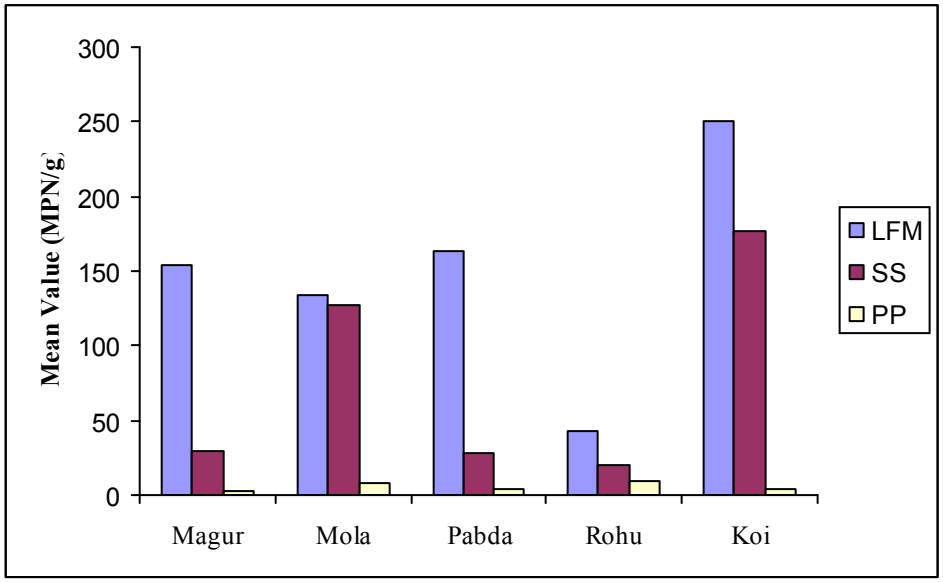

Fig. 2. Showing comparative analysis of TCC (mean value) in fish samples collected from local fish markets (LFM), super shops (SS) and fish processing plants (PP).

Figure 2 is showing a comparison among the mean values of TCC (MPN/g) of five types of fish samples collected from different local fish markets (LFM), super shops (SS) and fish processing plants. The diagram is making it clear that in all types of fish samples collected from fish processing plants, the total coliform counts (TCC) were lowest in

comparison with the fish samples collected from super shops and local fish markets. Among all the samples, the TCC in Koi fish sample collected from local fish markets was highest whereas the TCC in Magur fish sample of fish processing plants was lowest. The TCC in Magur fish sample of super shops is higher than the sample of fish processing plants but considerably lower than the samples collected from local fish markets. In case of Mola fish samples, the total coliform count (TCC) was almost same in the samples collected from super shops and local fish markets which was much lower in the samples collected from fish processing plants. While the TCC in Pabda fish samples collected from super shop was much lower that the samples of local fish markets, still it was higher than the samples collected from fish processing plants. The TCC in Rohu fish sample was lower in the samples collected from super shops than the samples collected from local fish markets where the TCC was highest but it was also lowest in the samples collected from fish processing plants. In the Koi fish samples collected form fish processing plants the TCC is considerably lower than the samples collected from super shops.

TFC (Mean Value)

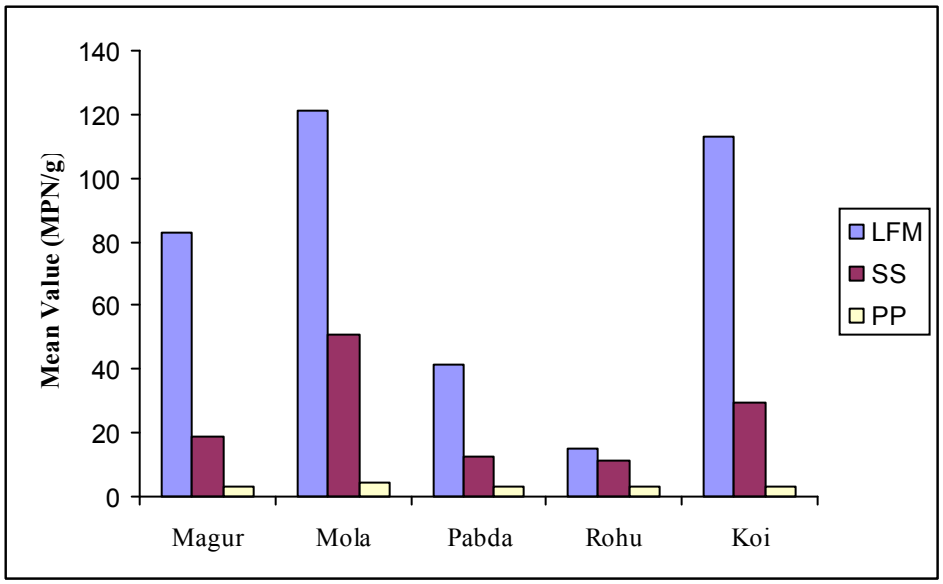

Fig 3. Showing comparative analysis of TFC in fish samples collected from local fish markets (LFM), super shops (SS) and fish processing plants (PP).

This figure is showing a comparative analysis among mean values of total faecal coliform counts (TFC) (MPN/g) of five types of fish samples collected from different

local fish markets (LFM), super shops (SS) and fish processing plants (PP). The diagram is showing that the mean value of TFC were higher in Mola, Koi and Magur samples collected from different local fish markets while the value is lowest in Magur, Pabda and Koi samples collected from different fish processing plants. Among the samples collected from super shops, the TFC was highest in Mola and lowest in Rohu and the lowest mean value of TFC among the samples of all fishes collected from fish processing plants. From this figure it is also clear that the value of TFC in different fish samples collected from fish processing plants are showing stable and lowest results whereas it is very much unstable and higher in case of the fish samples collected from super shop and local fish markets.

Bacterial load were statistically analysed using ANOVA followed by Tukey's Post Hoc for multiple comparisons at $p < 0.05$ level of significance (Appendix H).

3.7 Antibiotic susceptibility of pathogenic isolates

Table 9. Antibiotic susceptibility of pathogenic isolates

Antibiotics	Salmonella spp. n=21		Vibrio cholerae n=8	
	R(%)	S(%)	R(%)	S(%)
Ampicillin	17(81%)	4(19%)	6(75%)	2(25%)
Ciprofloxacin	12(57.14%)	9(42.86%)	1(12.5%)	7(87.5%)
Chloramphenicol	8(38%)	13(62%)	2(25%)	6(75%)
Ceftriazone	6(28.6%)	15(71.4%)	5(62.5%)	3(37.5%)
Erythromycin	7(33.33%)	14(66.67%)	3(37.5%)	5(62.5%)
Tetracycline	10(47.62%)	11(52.38%)	2(25%)	6(75%)
Trimethoprine-sulfomethoxazole	3(14.3%)	18(85.7%)	8(100%)	0(0%)

n= Number of isolates, R= Resistant, S= Sensitive

The above mentioned table summarizes the antibiotic susceptibility pattern of twenty-one *Salmonella* spp. and eight *Vibrio cholerae* isolates against seven antimicrobial agents.

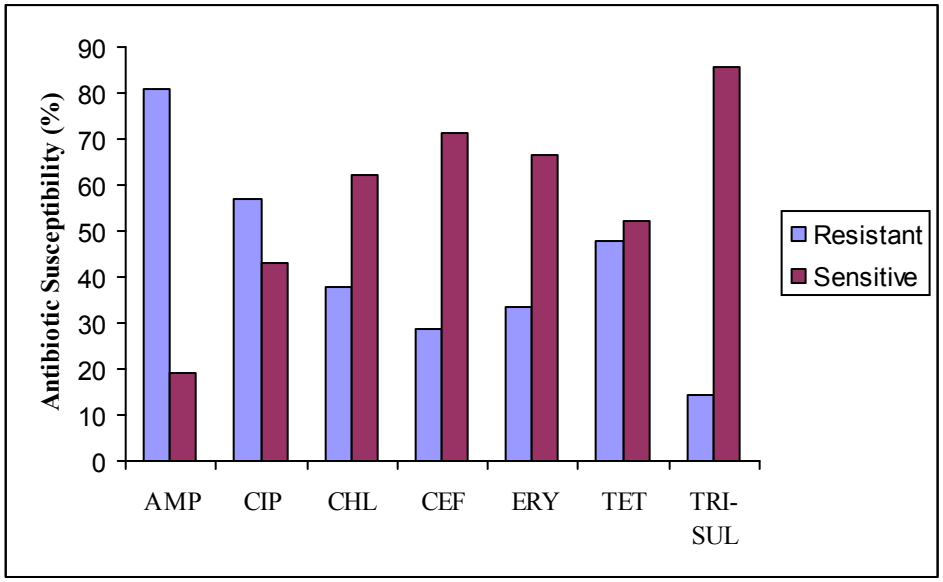

Fig. 4. Antibiotic susceptibility (%) of *Salmonella spp.*

Most of the isolates of *Salmonella spp.* were resistant to ampicillin (AMP) and ciprofloxacin (CIP), and sensitive to Chloramphenicol (CHL), Ceftriazone (CEF), Tetracycline (TET), Erythromycin (ERY) and Trimethoprine-sulfomethoxazole (TRI-SUL).

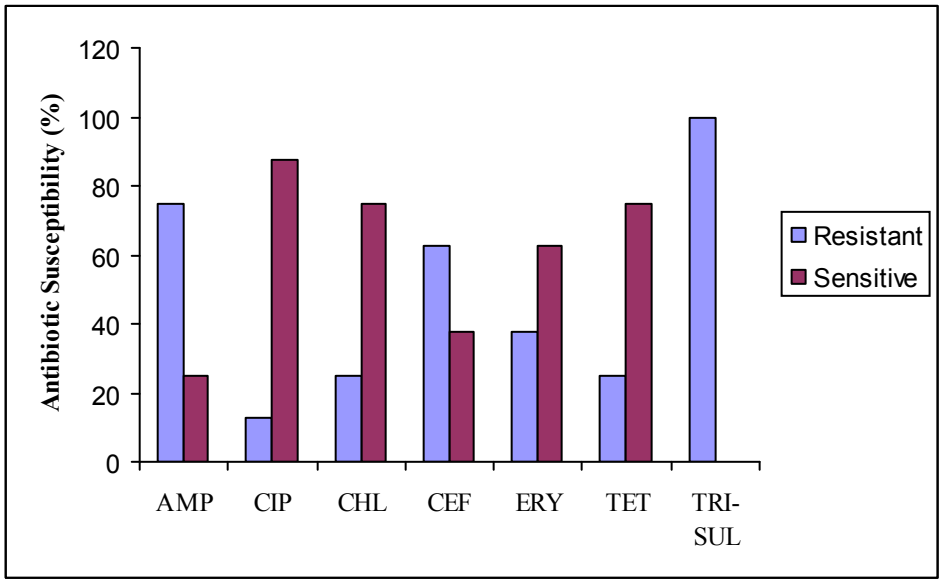

Fig. 5. Antibiotic susceptibility (%) of *Vibrio cholerae*

Most of the isolates of *V. cholerae* were resistant to ampicillin (AMP), Ceftriazone (CEF) and Trimethoprine-sulfomethoxazole (TRI-SUL), and sensitive to Ciprofloxacin (CIP), Chloramphenicol (CHL), Tetracycline (TET) and Erythromycin (ERY).

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CHAPTER 4
DISCUSSION

Chapter 4
Discussion

Raw fish are highly perishable protein source that contain normal bacterial flora from their environments in addition to the contaminants occurred during harvesting and handling of the products. The living fishes carry populations of predominantly Gram-negative psychotropic bacteria on their external skin. Coliforms could be absent or present in very low density and *Salmonella, Shigella, Vibrio* and other enteric pathogens are usually not found as these organisms are not the normal flora of the fishes or of their environment (FAO 1979).

According to the guideline of ICMSF (1986) acceptable limit of standard plate count (SPC) for white fish is 5×10^5 cfu/g and acceptable limit of total coliform and faecal coliform are 100 MPN/g and <3 MPN/g respectively. *Salmonella* spp. and V*ibrio cholerae* (including non-01/non-0139) should be absent (ICMSF 1986).

Begum *et. al.* (2010) assessed highest total bacterial count (1.9×10^8 cfu/g) in Sarpunti collected from local market and lowest (2.0×10^4 cfu/g) in Ayr fish collected from super shop. Highest *Pseudomonas* count (4.80×10^7cfu/g) was found in Sarpunti of local market and lowest (1.35×10^3 cfu/g) in both Rui and Ayr fish of supershop. Highest total coliform (>240MPN/g) was found in a number of samples of local markets whereas lowest count (0.9 MPN/g) in Rohu fish of super shop Similarly, highest count (110 MPN/g) of fecal coliform was found in many samples of local markets . Pathogenic bacteria, *Salmonella* and *Vibrio* spp. were mostly present in samples of local markets but usually absent in samples of super shops.

In the present study the export oriented fish samples from different fish processing plants were of good quality. But *Salmonella* spp. was present in Magur and Rohu fish samples collected from processing plant-2. As the processing plants follow proper EU guidelines for handling, processing and storage of fish, polluted aquaculture environment might be responsible for the presence of *Salmonella* spp. In other fish processing plants the fish samples were below the acceptable limit of SPC, TCC and TFC according to ICMSF guidelines, and *Salmonella* spp. and *Vibrio cholerae* were absent. So these plants were able to fulfill the requirements of international export guidelines. The bacterial loads

were within the acceptable limits and pathogenic isolates were more or less absent in these fish processing plants because these plants follow international export guidelines and HACCP system for handling, processing and preservation of fish and fish products.

On the other hand, from the result it was found that the bacteriological condition of local fish markets were not so good as they showed higher counts in all the bacterial parameters. Whereas, the bacterial load and occurrence of pathogenic isolates were comparatively lower in super shop samples. Bacteriological condition of super shops was good in comparison with local fish markets. From the result it might be pointed out that the processing, handling and storage condition of super shops is good and the quality of fish is better than the local markets. It might also indicate better quality preservation, handling and hygiene and sanitary maintenance of super shops than local markets but not as much satisfactory as fish processing plants. This high quality rate of fish processing plants might be the reflection of their compliance with HACCP and EU guidelines which are not followed by any local fish market or super shop.

The bacterial load on newly caught fish depends on the environment in which it is caught rather that on the fish species (Shewan 1961).The presence of coliform group (*E. coli*) in higher range suggests contamination of the samples before or during handling, processing and marketing. Lower load of TCC and TFC in samples of super shops indicates low range of contamination than local fish markets. FC is present highly in diarrheal stools of infected persons. So, the unwashed hands of infected food handlers forgetting to wash hands with soap after using the bathroom may also contaminate food (CDCP 2010).

Salmonella is highly pathogenic and this is the major reason for isolation of such bacteria from fish samples. Most of the samples of local fish markets and super shop were contaminated by *Salmonella* spp. whereas *Salmonella* was absent in almost all the samples of fish processing plants except two samples of processing plant-2. The environment acts as main source of this organism in aquaculture products rather than poor standards of hygiene and sanitation. But external contamination may also be the source of the occurrence of these bacteria in fish (Huss 1994).

Presence of *V. cholerae* can be a cause of infection to the consumer. This organism can cause cholera or diarrheal diseases. In the present study *V. cholerae* was qualitatively analysed and found in few samples of local fish market and super shop and was absent in all the samples of fish processing plants. So, proper care should be taken to avoid contamination of fish products with *V. cholerae* during handling, processing and preservation.

The significance of public health is dependent on the health status of the consumer as well as on the concentration and on the virulence of the pathogen (Lhafi and Kuhne 2007). According to the European Commission Regulation (2005), foodstuffs should not contain microorganisms or their toxins or metabolites in quantities that present an unacceptable risk for human health. The safety of foodstuffs is mainly ensured by a preventive approach, such as implementation of good hygiene practice and application of HACCP principles.

In this study the mean bacterial load of each fish sample collected from fish processing plants, local fish markets and super shops were compared using ANOVA followed by Tukey's Post Hoc Test at $p<0.05$ level of significance. Based on the analysis of data there were significant variation in SPC, TCC and TFC of Magur fish samples among fish processing plants, local fish markets and super shops. In multiple comparisons, there was no significant difference in SPC of Magur fish samples among local fish markets and super shops and in case of TCC and TFC there were no statistically significant difference among fish processing plants and super shops samples.

In case of Mola fish samples, there were no significant difference in SPC and TFC among fish processing plants, local fish markets and super shops but TCC was significantly different. In multiple comparisons, there was no significant difference in SPC, TCC and TFC of Mola fish samples among fish processing plants and local fish markets, fish processing plants and super shops, local fish markets and super shops.

In Pabda fish samples, SPC and TFC were significantly different among fish processing plants, local fish markets and super shops but there was no significant difference in TCC.

SPC and TFC of Rohu fish samples were significantly different. In multiple comparisons, SPC was significantly different among fish processing plants and local fish markets, fish processing plants and super shops but there was no significant difference among local fish markets and processing plants and TCC and TFC were significantly different among local fish markets and super shops and fish processing plants and local fish markets respectively.

In case of Koi fish samples, SPC and TCC were significantly different among fish processing plants, local fish markets and super shops but there was no significant difference in TFC.

In most of the fish samples, the mean value of SPC, TCC and TFC were significantly different among fish processing plants, local fish markets and super shops whereas in a few fish samples there were no statistically significant difference in mean values of bacterial counts among local fish markets and super shops.

This study gives a clear perspective on the variation of bacterial load and occurrence of *Salmonella* and *V. cholerae* comparatively in five types of fish samples collected from fish processing plants, four local fish markets and four super shops. This study also reveals that the bacteriological state of fish processing plants was better than the two types of domestic markets and bacterial conditions of super shops were better than local markets but not better than fish processing plants. The handling and processing methods, species variation and also the aquaculture practices in fish farm might be responsible for the reasons of variations in microbial quality.

The bacterial ecology of fish products is connected to environmental factors such as water pollution, fish feed quality, hygienic procedures of slaughter, handling, transport, commercialization and storage conditions. In freshwater aquaculture, the microbial load in the water used for cultivation is closely connected to several factors such as bacterial ecology of supply water, environment fish feed, soil and water table (Begum *et al.* 2010).

The natural flora of fish consists of bacteria which are harmless to human beings. However, during handling after catch fish get contaminated with various kinds of terrestrial bacteria, many of which are of great sanitary significance because of their

pathogenecity in humans. The chances of contamination with such bacteria are very high unless a very high degree of sanitation is maintained during handling, processing and storage and transport practices until the fish reaches the consumer.

The uncontrolled and irregular use of antibacterial agents in aquaculture systems and agricultural sectors is responsible for the occurrence of the multiple antibiotic resistance traits among the fish pathogens (Keys *et al.* 1986). In this study the pathogenic isolates- *Salmonella* spp. and *Vibrio cholerae* were tested against seven antimicrobial agents to observe whether they were sensitive or resistant to those agents. From the result is has been found that most of the isolates of *Salmonella spp.* were resistant to ampicillin and ciprofloxacin, and sensitive to Chloramphenicol, Ceftriazone, Tetracycline, Erythromycin and Trimethoprine-sulfomethoxazole and most of the isolates of *V. cholerae* were resistant to ampicillin, Ceftriazone and Trimethoprine-sulfomethoxazole, and sensitive to Ciprofloxacin, Chloramphenicol, Tetracycline and Erythromycin. This result suggests that commercially available fish may facilitate the dissemination of the antibiotic resistant bacteria (Ryu *et al.* 2012). For this reason, effective medication might be restricted during fish born disease outbreaks and antibiotic sensitivity might be helpful for effective medication.

From this research it can be concluded that, to produce better quality fishes and to avoid public health risks due to fish born disease outbreaks, it is necessary to follow the Good Aquaculture Practice (GAP) and Good Hygienic Practices (GHPs) concerning fish production, handling of the catch, icing, post-harvesting procedure and storage.

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CHAPTER 5

SUMMARY

Chapter 5
Summary

This study was designed for comparative microbiological assessment of export oriented fishes collected form different fish processing plants and locally marketed fishes from local fish markets and super shops. The whole study can be summarized as follows:

5.1 Materials and Methods

Five types of fish samples were selected and collected from four fish processing plants, four local fish markets and four super shops. The methodology was designed to assess SPC, TCC, TFC, qualitative analysis of *Salmonella* spp. and *V. cholerae* and antibiotic susceptibility test of pathogenic isolates.

Media Used: Bacteriological peptone, PCA, LSTB, BGBB, EC broth, TW, APW, TCBS, BPW , MKTT, RV, HEA, XLDA, NA, TSI agar, Urea agar, TSB and MHA.

SPC: SPC was enumerated according to ISO 4833: 2003. Test procedure and diagram of the whole test has been described in page number 14 and 15 respectively.

TCC: TCC was enumerated according to ISO 4831: 1991. Test procedure and diagram of the whole test has been described in page number 17 and 19 respectively.

TFC: TFC was enumerated according to ISO 7251: 1993. Test procedure and diagram of the whole test has been described in page number 17 to 19.

***Salmonella* spp.:** *Salmonella* spp. was detected according to ISO 6579: 2002. Test procedure and diagram of the whole test has been described in page number 21 and 24 respectively.

Vibrio cholerae: *Vibrio cholerae* was detected according to ISO/TS 21872-1: 2007. Test procedure and diagram of the whole test has been described in page number 27 and 29 respectively.

Antibiotic Susceptibility Test: Antibiotic Susceptibility of pathogenic isolates was tested by disc diffusion method using Kirby-Bauer technique and as recommendation of NCCLS. Test procedure has been given in page number 31.

5.2 Results and discussion

Among all the samples of fish processing plants, local fish markets and super shops SPC, TCC, TFC ranged from 2.70×10^3 to 2.98×10^7 cfu/g, <3 to 460 MPN/g and <3 to 240 MPN/g respectively. The results of SPC, TCC and TFC has been enumerated in Table 4 (page number 35), Table 5 (page number 36) and Table 6 (page number 37) respectively.

Occurrence of *Salmonella* spp. and *Vibrio cholerae* has been detailed in Table 7 (page number 38) and Table 8 (page number 39) respectively.

Results of antibiotic susceptibility test of pathogenic isolates has been give in Table 9 (page number 43).

Based on data analysis, in most of the cases there are significant difference ($p<0.05$) in mean values of SPC, TCC and TFC of fish samples among fish processing plants, local fish markets and super shops.

From the results it might be said that most of the fish samples of fish processing plants were of good quality and most of the fish samples of local fish markets were contaminated while the fish samples of super shops were less contaminated than the fish samples of local fish markets but not as good as the fish samples of fish processing plants, the reasons of which have been illustrated in Chapter 4.

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CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

Chapter 6
Conclusion and Recommendations

6.1 Conclusion

The present study provides a clear perspective of the bacteriological quality of five types of commercially available fish species which are imperative not only for consumption by local population but also possessing high export demand. The study revealed that the export oriented fish samples collected from four different fish processing plants were of better quality. Whereas the fish samples collected from local fish markets were more contaminated with high bacterial load than those of the super shops due to unhygienic condition of the handling and marketing area. The study also revealed that the presence of antibiotic resistant bacteria may create ecological and public health implications and antibiotic sensitivity may reduce human health risk.

The findings of the present study indicate that the hygienic condition of the fish processing plant was good and the quality of block frozen export oriented fishes was good for export and consumption whereas the hygienic and sanitation condition of domestic markets were not good enough, among which the hygienic condition of local fish markets was very poor compared to fish processing plants and super shops and contaminated fish could be the potential reservoirs for transmission of pathogenic bacteria and rapid spoilage of the fishes.

6.2 Recommendations

This study revealed that the export oriented fish samples collected from fish processing plants were of good quality because these plants follow HACCP system and international export guidelines. On the other hand fish samples of local fish markets were contaminated and super shop samples less contaminated than local fish markets but were not as good as fish samples of processing plants. So, it is necessary to implement HACCP system in super shops as well as in local fish markets to ensure good quality of fish.

In the present study, *Salmonella* spp. and *Vibrio cholerae* were isolated from tested fish samples. So, further study should be conducted on genomic sequencing.

The present study was conducted through collection of fish samples from only fours fish processing plants of Dhaka, Sylhet and Sunamgonj, four local fish markets and four super shops of Dhaka Metropolitan city. So, further study should be conducted for other areas of Bangladesh to analyze the overall situation.

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APPENDICES

Appendices

Appendix A: Questionnaire for Sample Collection from Local Market and Super Shop

1. Location: Area:
2. Date: No. of Seller:
3. Time:
4. Type of Seller:
   ☐ Wholesaler ☐ Paikar ☐ Retailer ☐ Others
5. Where do the fish come from?
   ☐ Farm ☐ Local Market ☐ Others
6. If local market, then from which one?
   ☐ Karwan Bazar ☐ Others
7. How fish are transported?
   ☐ Rickshaw ☐ Motor vehicle ☐ Others
8. What kind of boxes are used for transportation?
   ☐ Bamboo ☐ Metal ☐ Plastic ☐ Others
9. Where do the ice come from?
   ☐ Factory ☐ Others
10. How fish are stored?
    ☐ Icing ☐ Freezing ☐ Others
11. From where the water are supplied in the market?
    ☐ WASA ☐ Ground water ☐ Others
12. Ice supply (Market):
13. Kind of fish selling platform:
    ☐ Cemented ☐ Wooden ☐ Others
14. Water drainage system in the market:
    ☐ Poor ☐ Very poor ☐ Good ☐ Excellent
15. Condition of sanitation:
    ☐ Poor ☐ Very poor ☐ Good ☐ Excellent

Appendix B: Media Composition and Preparation

1. Bacteriological peptone:

Composition:

Bacteriological Peptone	1.0 g
Water	1000 ml

Preparation:

Ingredients in above quantities are mixed well for making homogenous dilution.

2. Plate count agar (PCA)

Composition:

Enzymatic digestion of casein	5.0 g
Yeast extract	2.5 g
Glucose, anhydrous ($C_6H_{12}O_6$)	1.0 g
Agar	9 g to 18 g
Water	1000 ml

Preparation:

The manufacturer's instruction is followed to prepare liquid medium from commercial dehydrated complete medium.

The pH is adjusted, if necessary, so that after sterilization it is 7.0 ± 0.2 at 25 ˚

3. Lauryl sulphate tryptose broth (LSTB selective enrichment medium)

Composition:

	single-strength medium
Tryptone	20 g
Lactose	5 g
Dipotassium hydrogen phosphate (K_2HPO_4)	2.75 g
Potassium dihydrogen phosphate (KH_2PO_4)	2.75 g
Sodium chloride	5 g
Sodium lauryl sulphate ($CH_3(CH_2)_{11}OSO_3Na$)	0.1 g
Water	1000 ml

Preparation:

Dehydrated complete medium is dissolved in water, sometimes by heating if required, and the medium is dispensed in quantities of 10 ml into tubes containing Durham's tubes and media in test tubes are then sterilized in an autoclave set at 121 ºC for 15 min. Care is taken so that Durham tubes do not contain air bubbles after sterilization.

4. EC Broth (confirmation medium)

Composition:

Tryptose or trypticase	20 g
Lactose	5.0 g
Bile salts No. 3	1.5 g
Dipotassium hydrogen phosphate (K_2HPO_4)	4.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.5 g
Sodium chloride	5.0 g
Water	1000 ml

Preparation:

Mentioned components or dehydrated complete medium is dissolved in water, if necessary heating is applied to medium for dissolving homogenously. Also, pH is adjusted if necessary so that after sterilization it is 7.2 at 25 ºC.

The medium is dispensed in quantities of 10 ml into test tubes containing Durham tubes and then sterilized in autoclave set at 121 ºC for 15 min. Care is taken so that Durham tubes do not contain air bubbles after sterilization.

5. Tryptone water

Composition:

Tryptone	10.0 g
Sodium chloride	5.0 g
Water	1000 ml

Preparation:

The components or dehydrated complete medium are dissolved in water, by heating if necessary. Then pH is adjusted, if necessary so that after sterilization it is 7.3 at 25 ºC. The medium is dispensed in quantities of 5 ml to 10 ml into tubes and sterilized for 15 min in an autoclave set at 121

6. Buffered peptone water

Composition:

Enzymatic digest of casein	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)	9.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.5 g
Water	1000 ml

Preparation:

The components or dehydrated complete medium is dissolved in the water, by heating if necessary, adjusted the pH if necessary, so that after sterilization it is 7.0±0.2 at 25 ºC. The medium is dispensed of suitable capacity into culture bottle to obtain the portions necessary for the test and sterilized in an autoclave set at 121 ºC for 15 min.

7. Rappaport-Vassiliadis medium with soya (RVS broth)

Composition:

Enzymatic digest of soya	5.0 g
Sodium chloride	8.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.4 g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.2 g
Water	1000 ml

Preparation:

The components are dissolved in the water by heating to about 70 C if necessary. The solution is prepared on the day of preparation of the complete RVS medium.

8. Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn)

Base medium composition:

Meat extract	4.3 g
Enzymatic digest of casein	8.6 g
Sodium chloride (NaCl)	2.6 g
Calcium carbonate ($CaCO_3$)	38.7 g
Sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$)	47.8 g
Ox bile for bacteriological use	4.78 g
Brilliant green	9.6 mg
Water	1000 ml

Preparation:

The dehydrated basic components or the dehydrated complete medium is dissolved in the water by boiling for 5 min, adjusted the pH if necessary so that it is 8.2 ± 0.2 at 25 ºC. Then thoroughly mix the medium. The basic medium may be stored for 4 weeks at 3 ºC ± 2 ºC.

8.1. Iodine-iodine solution

Composition:

Iodine	20.0 g
Potassium iodide (KI)	25.0 g
Water	100 ml

Preparation:

Completely dissolving the potassium iodide in 10 ml of water, then adding the iodine and dilute to 100 ml with sterile water. No heat is applied. The prepared solution is stored in the dark at ambient temperature in a tightly closed container.

8.2. Novobiocin solution

Composition:

Novobiocin sodium salt	0.04 g
Water	5 ml

Preparation:

The novobiocin sodium salt is dissolved in the water and sterilized by filtration, then stored for up to 4 weeks at 3 ºC ± 2 ºC.

8.3. Complete medium

Composition:

Base medium	1000 ml
Iodine-iodine solution	20 ml
Novobiocin solution	5 ml

Preparation

Aseptically 5 ml of the novobiocin solution added to 1000 ml of base medium. Mixed, then added 20 ml of the iodine-iodine solution. Mixed well and dispensed the medium aseptically into sterile flasks of appropriate capacity to obtain the portion necessary for the test.

9. Xylose lysine deoxycholate agar (XLD agar)

Base medium Composition:

Yeast extract powder	3.0 g
Sodium chloride (NaCl)	5.0 g
Xylose	3.75 g
Lactose	7.5 g
Sucrose	7.5 g
L-Lysine hydrochloride	5.0 g
Sodium thiosulfate	6.8 g
Iron (III) ammonium citrate	0.8 g
Phenol red	0.08 g
Sodium deoxycholate	1.0 g
Agar	9 g to 18 g
Water	1000 ml

Preparation:

Dissolved the dehydrated base components or the dehydrated complete base in the water by heating, with frequent agitation, until the medium starts to boil. Care is taken to avoid overheating.

10. Hecton Enteric Agar (HEA)

Composition:

Peptic digest of meat	12.0 g
Yeast extract	3.0 g
Lactose	12.0 g
Sucrose	12.0 g
Salicin	2.0 g
Bile salts	9.0 g
Sodium chloride	5.0 g
Sodium thiosulfate	5.0 g
Ferric ammonium citrate	1.5 g
Bromthymol blue	65.0 mg
Acid fuchsin	40.0 mg
Bacteriological agar	13.5 g

Preparation:

76 g of medium is suspended in 1 litre of distilled water, mixed well and dissolved by hitting with frequent agitation and boiled for above 20 minutes until complete dissolution. Without autoclaving the medium is cooled to 55º C-60º C and poured into petri-dishes.

11. Nutrient agar

Composition:

Meat extract	3.0 g
Peptone	5.0 g
Agar	9 g to 18 g
Water	1000 ml

Preparation:

Dissolved the dehydrated components or dehydrated complete medium in the water, by heating if necessary. Adjusted the pH if necessary, so that after sterilization it is 7.2±0.2 at 25 ºC.

Transfered the medium into containers of appropriate capacity Sterilize in an autoclave set at 121 ºC for 15 min.

Transfer about 15 ml of the melted medium to sterile small Petri dishes

12. Triple sugar/iron agar (TSI agar)

Composition:

Peptone	20.0 g
Meat extract	3.0 g
Yeast extract	3.0 g
Sodium chloride (NaCl)	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Glucose	1.0 g
Iron (III) citrate	0.3 g
Sodium thiosulfate	0.3 g
Phenol red	0.024 g
Agar	9 g to 18 g
Water	1000 ml

Preparation:

Dissolved the components or dehydrated base medium in the water, by heating if necessary. Adjust the pH if necessary, so that after sterilization it is 7.4±0.2 at 25 ºC.

Dispensed the medium in quantities of 10 ml in tubes of appropriate capacity. Sterilized in an autoclave set at 121 ºC for 15 min.

Left to solidify in an inclined position so as to obtain a butt of around 2.5 cm in depth.

13. Urea agar (Christensen)

Base medium Composition:

Peptone	1.0 g
Glucose	1.0 g
Sodium chloride (NaCl)	5.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	2.0 g
Phenol red	0.012 g
Agar	9 g to 18 g
Water	1000 ml

Preparation:

Dissolve the components or dehydrated base medium in the water, by heating if necessary.

Adjust the pH if necessary, so that after sterilization it is 7.4±0.2 at 25 ºC.

Sterilize in an autoclave set at 121 ºC for 15 min.

13.1. Urea solution

Composition:

Urea	400 g
Water, to a final volume of	1000 ml

Preparation

Dissolve the urea in water. Sterilize by filtration and check the sterility. Or, commercially prepared urea solution (40%) is used.

13.2. Complete medium

Composition

Base	950 ml
Urea solution	50 ml

Preparation

Add, under aseptic conditions, the urea solution to the base, which has been previously melted and then cooled to 44 ºC to 47 ºC. Dispense the complete medium into sterile tubes (7.8) in quantities of 10 ml. Allow to set in a sloping position.

14. Tryptone/Tryptophan medium

Composition:

Tryoptone	10.0 g
DL-Tryptophan	1.0 g
Sodium chloride (NaCl)	5.0 g
Water	1000 ml

Preparation:

Dissolve the components or complete dehydrated medium in the water, by heating if necessary. Adjust the pH if necessary, so that after sterilization it is 7.5±0.2 at 25 ºC.

Dispense the medium in quantities of 5 ml into tubes of appropriate capacity . Sterilize in an autoclave set at 121 ºC for 15 min.

15. Alkaline peptone water (APW)

Composition:

Peptone	20.0 g
Sodium chloride	20.0 g
Water	1000 ml

Preparation:

Dissolve the components in the water, by heating if necessary. Adjust the pH if necessary, so that after sterilization it is 8.6±0.2 at 25 ºC.

Dispense the medium in quantities required for the examination, into flasks or tubes of sufficient capacities and Sterilize in an autoclave set at 121 ºC for 15 min.

16. Thiosulfate citrate bile and sucrose (TCBS) agar

Composition

Peptone	10.0 g
Yeast extract	5.0 g
Sodium citrate	10 g
Sodium thiosulfate	10 g
Iron (III) citrate	1.0 g
Sodium chloride (NaCl)	10 g
Dried bovine bile	8.0 g
Sucrose	20.0 g
Bromothymol blue	0.04 g
Thymol blue	0.04 g
Agar	8.0 g to 18.0 g
Water	1000 ml

Preparation

Dissolve the components or complete dehydrated medium in the water by bringing to the boil. Adjust the pH if necessary, so that after sterilization it is 8.6±0.2 at 25 ºC. No autoclave is required.

17. Saline nutrient agar (SNA)

Composition:

Meat extract	5.0 g
Peptone	3.0 g
Sodium chloride (NaCl)	10.0 g
Agar	8 g to 18 g
Water	1000 ml

Preparation:

Dissolve the dehydrated components or dehydrated complete medium in the water, by heating if necessary. Adjust the pH if necessary, so that after sterilization it is 7.2±0.2 at 25 ºC.

Transfer the medium into containers of appropriate capacity. Sterilize in an autoclave set at 121 ºC for 15 min.

18. Saline triple sugar iron (TSI) agar

Composition:

Peptone	20.0 g
Meat extract	3.0 g
Yeast extract	3.0 g
Sodium chloride (NaCl)	10.0 g
Lactose	10.0 g
Sucrose	10.0 g
Glucose	1.0 g
Iron (III) citrate	0.3 g
Phenol red	0.024 g
Agar	8 g to 18 g
Water	1000 ml

Preparation:

Dissolve the components or dehydrated base medium in the water, by heating if necessary. Adjust the pH if necessary, so that after sterilization it is 7.4±0.2 at 25 ºC.

Dispense the medium in quantities of 10 ml in tubes of appropriate capacity. Sterilize in an autoclave set at 121 ºC for 15 min.

19. Trypticase Soya Broth (TSB)

Composition:

Ingredients	Amount
Trypticase (Pancreatic Digest of Casein)	17 g
Papic Digest of soyabean meal	3.0 g
NaCL	5.0 g
Di-hasic Potassium phosphate	2.5 g

P^H 7.3 ± 0.2 at 25º C

Preparation:

30g of medium is mixed with 1 litre of distilled water and autoclaved at 121º C for 15 minutes.

20. Muller-Hinton Agar

Composition:

Ingredients	Amount
Beef in fusion	2.0 g
Bacto casarn--.o acid (technical)	17.5 g
Starch	1-5 g
Bacto agar	17.5µ
Distilled water	1 litre

$P^H = 7.3 \pm 0.1$

Sterilized at 121º C for 15 minutes

Preparation:

Muller-Hinton agar was prepared from a commercially available dehydrated base according to the manufacture's instruction. Immediately after autoclaving, the media was allowed to cool to 45^0C to 50^0C in a water bath. Freshly prepared and cooled medium was poured into the sterile Petri dishes to give a uniform depth of approximately 4mm.

Appendix C: Reagents

Gram Staining reagents

Ingredients	Amount
Crystal violate Solution A	
Crystal violate (90% dye content)	2.0g
Ethyl Alcohol (95%)	20 ml
Crystal violate Solution B	
Ammonium oxalate	0.8g
dH_2O	80 ml
Mixed solution A and B	
Gram's Iodine	
Iodine	1g
Potassium iodide	2g
dH_2O	300 ml
Ethyl Alcohol (95%)	
Ethyl Alcohol (100%)	95 ml
dH_2O	5 ml
Safranin	
Safranin O	0.25 ml
Ethyl Alcohol (95%)	10 ml
dH_2O	100 ml

Oxidase reagent

Ingredients		Amount
1%	N`N`N`-tetraniethyl-p-phynylenadiamine dihydroclilortde	100 mg/ 100 ml of distilled water

Kovac's reagent

Ingredients	Amount
p-dimethyaminobenzaldehyde	5.P g
Amyl alcohol	75 ml
Hydrochloric acid (concentrated)	25 ml

McFarland standard 0.5

Ingredients	Amount
$BaCL_2$ (0.048 M/L)	1.175% (w/v)
H_2SO_4 (0.36 N)	1.00% (v/v)

Physiological saline:

Sodium chloride was weighted 8.5 g/l and transferred to a leak-proof bottle pre-marked to hold 1 litre. Distilled water was added to the 1 litre mark, and mixed well until the salt was fully dissolved. The mixture was sterilized by autoclaving at 121º C for 15 minutes. The bottle was stored at room temperature.

Appendix D: SPC Calculation

$N = \sum C / V(n1 + 0.1\ n2\ )d$

Where,

$\sum C$ is the sum of the colonies counted on all the dishes retained from two successive dilutions, at least one of which contains 15 colonies;

V is the volume of inoculum applied to each dish, in milliliter;

n1 is the number of dishes retained at the first dilution;

n2 is the number of dishes retained in the second dilution;

d is the dilution factor corresponding to the first dilution retained

Appendix E: MPN Index

For 3 Tubes each at 0.1, 0.01 and 0.001 g inocula, the MPNs per gram of samples

Number of positive results			MPN/g
0.1	0.01	0.001	
0	0	0	< 3
0	0	1	3
0	1	0	3
0	1	1	6.1
0	2	0	6.2
0	3	0	9.4
1	0	0	3.6
1	0	1	7.2
1	0	2	11
1	1	0	7.4
1	1	1	11
1	2	0	11
1	2	1	15
1	3	0	16
2	0	0	9.2
2	0	1	14
2	0	2	20
2	1	0	15
2	1	1	20
2	1	2	27
2	2	0	21
2	2	1	28
2	2	2	35
2	3	0	29
2	3	1	36
3	0	0	23
3	0	1	38
3	0	2	64
3	1	0	43
3	1	1	75
3	1	2	120
3	1	3	160
3	2	0	93
3	2	1	150
3	2	2	210
3	2	3	290
3	3	0	240
3	3	1	460
3	3	2	1100
3	3	3	> 1100

Appendix F: Methods of Gram Staining

Microscopic examination of bacterial morphology:

1. From the incubated culture plate after 24 hours incubation at 37º C, a portion of the culture was taken with sterile loop and suspended in a drop of saline over a clean glass slide and mixed.
2. A thin smear was made on it and the smear was allowed to dry in air.
3. The smear was fixed by slightly heating the under surface of the slide over a gas burner.
4. Crystal violate stain was flooded over the smear and kept for one minute and then washed with distilled water.
5. The smear was flooded with gram's iodine and kept for one minute and then washed with distilled water.
6. The slide was decolourised by applying ethanol for 10 seconds and rinsed well with distilled water.
7. Finally the smear was covered with safranine for 1 minute and subsequently washed off thoroughly with distilled water.
8. The slide was kept for air-drying before microscopy.
9. The dried smear was examined under the microscope using oil immersion objective for stained bacteria.

Appendix G: Interpretation of Biochemical Test

1. Interpretation of biochemical tests of *Salmonella* spp.

Test	Salmonella strain
TSI acid from glucose	+
TSI gas from glucose	+
TSI acid from lactose	+
TSI acid from sucrose	+
TSI hydrogen sulfide produced	+
Urea hydrolysis	−
Production of indole	−

2. Interpretation of biochemical tests of *V. cholerae*

Tests (media containing 1% of NaCl)	V. cholerae
Oxidase	+
Production of gas (glucose)	-
Lactose	-
Sucrose	+
Production of indole	+
Growth in peptone water with	
0 % NaCl	+
2 % NaCl	+
6 % NaCl	-
8 % NaCl	-
10 % NaCl	-

Appendix H: Data analysis for samples collected from Fish Processing Plants, Local Fish Market and Super Shop

SPC (Log value) cfu/g, TCC MPN/g, TFC MPN/g

Magur:

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
SPC	Between Groups	9.870	2	4.935	15.930	.001
	Within Groups	2.788	9	.310		
	Total	12.659	11			
TCC	Between Groups	52301.527	2	26150.763	10.966	.004
	Within Groups	21462.270	9	2384.697		
	Total	73763.797	11			
TFC	Between Groups	14336.000	2	7168.000	9.363	.006
	Within Groups	6890.000	9	765.556		
	Total	21226.000	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable		(I) Sources	(J) Sources	Mean Difference (I-J)	Std. Error	Sig.
SPC	Tukey HSD	Fish Processing Plant	Local Fish Market	-2.18500*	.39358	.001
			Super Shop	-1.44000*	.39358	.013
		Local Fish Market	Fish Processing Plant	2.18500*	.39358	.001
			Super Shop	.74500	.39358	.196
		Super Shop	Fish Processing Plant	1.44000*	.39358	.013
			Local Fish Market	-.74500	.39358	.196
TCC	Tukey HSD	Fish Processing Plant	Local Fish Market	-151.35000*	34.53040	.005
			Super Shop	-26.35000	34.53040	.734
		Local Fish Market	Fish Processing Plant	151.35000*	34.53040	.005
			Super Shop	125.00000*	34.53040	.014
		Super Shop	Fish Processing Plant	26.35000	34.53040	.734
			Local Fish Market	-125.00000*	34.53040	.014
TFC	Tukey HSD	Fish Processing Plant	Local Fish Market	-80.00000*	19.56471	.007
			Super Shop	-16.00000	19.56471	.702
		Local Fish Market	Fish Processing Plant	80.00000*	19.56471	.007
			Super Shop	64.00000*	19.56471	.024
		Super Shop	Fish Processing Plant	16.00000	19.56471	.702
			Local Fish Market	-64.00000*	19.56471	.024

Mola:

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
SPC	Between Groups	.716	2	.358	.581	.579
	Within Groups	5.550	9	.617		
	Total	6.266	11			
TCC	Between Groups	40550.007	2	20275.003	4.402	.046
	Within Groups	41456.190	9	4606.243		
	Total	82006.197	11			
TFC	Between Groups	27936.827	2	13968.413	3.742	.066
	Within Groups	33593.520	9	3732.613		
	Total	61530.347	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable		(I) Sources	(J) Sources	Mean Difference (I-J)	Std. Error	Sig.
SPC	Tukey HSD	Fish Processing Plant	Local Fish Market	-.54250	.55527	.609
			Super Shop	-.05250	.55527	.995
		Local Fish Market	Fish Processing Plant	.54250	.55527	.609
			Super Shop	.49000	.55527	.664
		Super Shop	Fish Processing Plant	.05250	.55527	.995
			Local Fish Market	-.49000	.55527	.664
TCC	Tukey HSD	Fish Processing Plant	Local Fish Market	-126.55000	47.99085	.064
			Super Shop	-119.80000	47.99085	.079
		Local Fish Market	Fish Processing Plant	126.55000	47.99085	.064
			Super Shop	6.75000	47.99085	.989
		Super Shop	Fish Processing Plant	119.80000	47.99085	.079
			Local Fish Market	-6.75000	47.99085	.989
TFC	Tukey HSD	Fish Processing Plant	Local Fish Market	-117.40000	43.20077	.056
			Super Shop	-46.90000	43.20077	.546
		Local Fish Market	Fish Processing Plant	117.40000	43.20077	.056
			Super Shop	70.50000	43.20077	.282
		Super Shop	Fish Processing Plant	46.90000	43.20077	.546
			Local Fish Market	-70.50000	43.20077	.282

Pabda:

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
SPC	Between Groups	.826	2	.413	2.166	.171
	Within Groups	1.717	9	.191		
	Total	2.543	11			
TCC	Between Groups	58541.040	2	29270.520	2.196	.167
	Within Groups	119952.810	9	13328.090		
	Total	178493.850	11			
TFC	Between Groups	3389.840	2	1694.920	3.694	.067
	Within Groups	4129.440	9	458.827		
	Total	7519.280	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable		(I) Sources	(J) Sources	Mean Difference (I-J)	Std. Error	Sig.
SPC	Tukey HSD	Fish Processing Plant	Local Fish Market	-.62250	.30886	.164
			Super Shop	-.17250	.30886	.845
		Local Fish Market	Fish Processing Plant	.62250	.30886	.164
			Super Shop	.45000	.30886	.355
		Super Shop	Fish Processing Plant	.17250	.30886	.845
			Local Fish Market	-.45000	.30886	.355
TCC	Tukey HSD	Fish Processing Plant	Local Fish Market	-158.70000	81.63360	.182
			Super Shop	-24.00000	81.63360	.954
		Local Fish Market	Fish Processing Plant	158.70000	81.63360	.182
			Super Shop	134.70000	81.63360	.275
		Super Shop	Fish Processing Plant	24.00000	81.63360	.954
			Local Fish Market	-134.70000	81.63360	.275
TFC	Tukey HSD	Fish Processing Plant	Local Fish Market	-39.50000	15.14640	.067
			Super Shop	-9.70000	15.14640	.802
		Local Fish Market	Fish Processing Plant	39.50000	15.14640	.067
			Super Shop	29.80000	15.14640	.176
		Super Shop	Fish Processing Plant	9.70000	15.14640	.802
			Local Fish Market	-29.80000	15.14640	.176

Rohu:

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
SPC	Between Groups	5.784	2	2.892	15.373	.001
	Within Groups	1.693	9	.188		
	Total	7.477	11			
TCC	Between Groups	2791.227	2	1395.613	2.803	.113
	Within Groups	4481.440	9	497.938		
	Total	7272.667	11			
TFC	Between Groups	298.580	2	149.290	5.769	.024
	Within Groups	232.900	9	25.878		
	Total	531.480	11			

Post Hoc Tests

Multiple comparisons

Dependent Variable		(I) Sources	(J) Sources	Mean Difference (I-J)	Std. Error	Sig.
SPC	Tukey HSD	Fish Processing Plant	Local Fish Market	-1.70000*	.30671	.001
			Super Shop	-.89000*	.30671	.042
		Local Fish Market	Fish Processing Plant	1.70000*	.30671	.001
			Super Shop	.81000	.30671	.063
		Super Shop	Fish Processing Plant	.89000*	.30671	.042
			Local Fish Market	-.81000	.30671	.063
TCC	Tukey HSD	Fish Processing Plant	Local Fish Market	-36.90000	15.77875	.101
			Super Shop	-13.40000	15.77875	.684
		Local Fish Market	Fish Processing Plant	36.90000	15.77875	.101
			Super Shop	23.50000	15.77875	.340
		Super Shop	Fish Processing Plant	13.40000	15.77875	.684
			Local Fish Market	-23.50000	15.77875	.340
TFC	Tukey HSD	Fish Processing Plant	Local Fish Market	-11.90000*	3.59707	.022
			Super Shop	-8.35000	3.59707	.104
		Local Fish Market	Fish Processing Plant	11.90000*	3.59707	.022
			Super Shop	3.55000	3.59707	.603
		Super Shop	Fish Processing Plant	8.35000	3.59707	.104
			Local Fish Market	-3.55000	3.59707	.603

Koi:

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
SPC	Between Groups	6.099	2	3.050	10.173	.005
	Within Groups	2.698	9	.300		
	Total	8.797	11			
TCC	Between Groups	121091.727	2	60545.863	6.269	.020
	Within Groups	86925.270	9	9658.363		
	Total	208016.997	11			
TFC	Between Groups	27973.167	2	13986.583	4.023	.056
	Within Groups	31287.750	9	3476.417		
	Total	59260.917	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable		(I) Sources	(J) Sources	Mean Difference (I-J)	Std. Error	Sig.
SPC	Tukey HSD	Fish Processing Plant	Local Fish Market	-1.72750*	.38715	.004
			Super Shop	-1.08500*	.38715	.049
		Local Fish Market	Fish Processing Plant	1.72750*	.38715	.004
			Super Shop	.64250	.38715	.272
		Super Shop	Fish Processing Plant	1.08500*	.38715	.049
			Local Fish Market	-.64250	.38715	.272
TCC	Tukey HSD	Fish Processing Plant	Local Fish Market	-245.90000*	69.49231	.016
			Super Shop	-130.65000	69.49231	.200
		Local Fish Market	Fish Processing Plant	245.90000*	69.49231	.016
			Super Shop	115.25000	69.49231	.272
		Super Shop	Fish Processing Plant	130.65000	69.49231	.200
			Local Fish Market	-115.25000	69.49231	.272
TFC	Tukey HSD	Fish Processing Plant	Local Fish Market	-110.50000	41.69183	.062
			Super Shop	-18.75000	41.69183	.896
		Local Fish Market	Fish Processing Plant	110.50000	41.69183	.062
			Super Shop	91.75000	41.69183	.124
		Super Shop	Fish Processing Plant	18.75000	41.69183	.896
			Local Fish Market	-91.75000	41.69183	.124