BACTERIOLOGICAL ANALYSIS WITH ANTIBIOTIC RESISTANCE PATTERN OF BACTERIA ISOLATED FROM PRAWNS AT DINAJPUR, BANGLADESH

A THESIS

BY

MITA DAS

REGISTRATION NO. 1605135

SEMESTER: JANUARY– JUNE, 2017

SESSION: 2016

MASTER OF SCIENCE (M.S.)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

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Dedicated To My Beloved Parents and Teachers

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The author
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ABSTRACT

Prawns sold in local market could be microbiologically spoiled intrinsically or extrinsically. The research study was attempted to detect Total Bacterial Count (TBC), Total Coliform Counts (TCC), Total Salmonella-Shigella (TSS) Counts and Total Vibrio Counts (TVC). The abundance of bacterial loads was determined from thirty prawn samples. The density of total bacteria was detected in all the prawn samples were significantly higher ($p < 0.001$). Mean±SEM of TBC, TCC, TSS and TVC of prawn samples was 4.49±0.24, 5.20±0.29, 4.60±0.30 and 5.59±0.21 log10 CFU/g respectively. The total bacterial counts (TBC) ranged between 3.8×10^2 CFU/g to 2.8×10^6 CFU/g. The total coliforms counts (TCC) ranged between 4.9×10^2 CFU/g to 3.52×10^7 CFU/g. Furthermore, total Salmonella-Shigella (TSS) counts ranged from 1.65×10^2 CFU/g to 1.3×10^7 CFU/g. The total Vibrio counts (TVC) of thirty samples were ranged between 4.0×10^3 CFU/g to 2.5×10^7 CFU/g. The highest Mean±SEM of TBC, TCC, TSS and TVC were found in Gopalgong bazaar (4.76±0.39 log10 CFU/g), Rail gate bazaar (6.13±0.41 log10 CFU/g), Rail gate bazaar (5.79±0.32 log10 CFU/g) and Rail gate bazaar (5.29±0.40 log10 CFU/g) respectively. But the bacterial load found in prawns did not vary significantly among the three selected local markets of Dinajpur city. A total of thirty prawn samples were examined. All of them were found to be contaminated with different microbes characterized as follows- *E. coli* (100%), *Salmonella* spp. (100%), *Vibrio* spp. (66.7%), *Klebsiella* spp. (40%) and *Shigella* spp. (33.3%). Isolated bacteria were identified using the biochemical tests namely Indole test, Methyl red test, Voges - Proskauer test, Citrate utilization test, Triple sugar

Iron test and MIU test. The results revealed that the bacterial load found to be higher in most of the samples. The study of antibiotic resistance pattern showed a number of pathogenic isolates to be drug-resistant. All of the isolates were found to be 100% resistant against Tetracycline. *E. coli* and *Vibrio* spp. also showed 100% resistant against Cefixime and Erythromycin respectively.

Key words- Prawn, Total Bacterial Count (TBC), Total Coliform Count (TCC), Total Salmonella-Shigella (TSS) Count, Total Vibrio Counts (TVC), Antibiotic resistance.

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LIST OF ABBREVIATIONS AND SYMBOLS

-	:	Negative
%	:	Percentage
/	:	Per
<	:	Less than
>	:	Greater than
+	:	Positive
µg	:	Microgram
µl	:	Micro liter
^{0}C	:	Degree of Celsius
CFU	:	Colony forming units
D_x	:	Dextrose
E. coli	:	*Escherichia coli*
e.g	:	Example
EMB	:	Eosin Methylene Blue
et al.	:	Associated
Etc	:	Etcetera
FAO	:	Food and Agricultural Organization
Fig.	:	Figure
Gm	:	Grams
H_2S	:	Hydrogen sulfide
Hrs	:	Hours
HSTU	:	Hajee Mohammad Danesh Science and Technology University
Lb	:	Pound

Kg	:	Kilogram
KOH	:	Potassium hydroxide
L	:	Lactose
MC	:	MacConkey Agar
Mg	:	Milligram
Min	:	Minutes
MI	:	Milliliter
MIU	:	Motility Indole Urease
Ml	:	Milliliter

LIST OF ABBREVIATIONS AND SYMBOLS (Contd.)

ML	:	Maltose
Mm	:	Millimeter
MN	:	Mannitol
MR	:	Methyl Red
MSA	:	Mannitol Salt Agar
N	:	Number
NA	:	Nutrient agar
NB	:	Nutrient broth
ND	:	Not done
-	:	Negative
No.	:	Number
NS	:	Non-significance
PBS	:	Phosphate Buffer Saline
R	:	Resistant
S	:	Sucrose
S	:	Sensitive
S	:	Significance
Sec	:	Second
SEM	:	Standard error mesn
SL.	:	Serial

spp.	:	Species
Sq	:	Square
SSA	:	Salmonella Shigella Agar
TBC	:	Total bacterial count
TCC	:	Total coliform count
TSS	:	Total Salmonella shigella
TSI	:	Triple sugar iron
TVC	:	Total vibrio count
VP	:	Voges Proskaur
v/v	:	Volume by volume
w/v	:	Weight by volume

APPENDICES

APPENDIX 1

Composition of Different Media

1. Nutrient agar (Hi Media)

Ingredients:

g/L
Peptic digest of animal tissue 5.0
Sodium chloride 5.0
Beef extract 1.5
Yeast extract 1.5
Final pH (at 25^{0}C) 7.4 ± 0.2

2. Eosine methylene blue Agar (Hi Media)

Ingredients:

g/L
Peptic digest of animal tissue 10
Lactose 5.0
Sucrose 5.0
Dipotassium phosphate 2.0
Eosin - Y 0.40
Methylene blue 0.065
Agar 20.0
Final pH (at 25^{0}C) 7.2 ± 0.2

3. MacConkey agar (Hi-media)

Ingredients:
g/L
Peptic digest of animal tissue 17.0
Protease peptone 3.0
Lactose monohydrate 10
Bile salt 1.5
Sodium chloride 5.0
Agar-agar 15.0
Neutral red 0.03
Final pH (at 25^{0}C) 7.1 ± 0.2

4. Deoxycholate Citrate Agar

Component	Amount (g/L)
Meat peptone	10.000
Beef extract	10.000
Lactose monohydrate	10.000
Sodium citrate	20.000
Neutral red	0.020
Sodium deoxycholate	5.000
Ferric citrate	1.000
Agar	13.500

5. Thiosulfate-citrate-bile salts-sucrose agar
g/L

Yeast extract
Proteose Peptone
10.0
Sodium thiosulfate
10.0
Sodium citrate
10.0
Ox gall
5.0
Sodium cholate
3.0
Saccharose
20.0
Sodium chloride
10.0
Ferric citrate
1.0
Bromothymol blue
0.04
Thymol blue
0.04
Agar
15.0

6. Simmon's Citrate Agar

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

7. Mueller Hinton Agar

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

8. TSI agar (Hi Media)

Ingredients:

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

9. MIU medium base (Hi Media)

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

10. MR-VP medium (Hi Media)

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

11. Sugar media

Ingredients:

g/L
a. Peptone water
Bacto-peptone
10.0 gm
Sodium chloride
5.00 gm
0.5% phenol red
0. 10 ml
Distilled water
1000 ml

b. Sugar solutions

Individul sugar
5.00 gm
Distilled water
100 ml

c. Sugar media preparation

Pepton water
4.50 ml
Sugar solution
0.50 ml

12. Peptone water

Ingredients:
g/L
Peptone
1.00 gm
Distilled water
1000 ml

APPENDIX 2

Preparation of reagents

1. Kovacs reagent

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

2. V-P reagent 1

5% alpha –naptholin absolute ethyl alcohol

3. V-P reagent 2

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

4. Phosphate buffered solution

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

5. Methyl red solution

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

6. Phenol red solution

0.2% aqueous solution of phenol red

7. Potassium hydroxide solution

40% aqueous solution of KOH

8. Gram stain solution

- Stock crystal violet

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

- Stock oxalate solution

Ammonium oxalate
1 gm
Distilled water
1000 ml

- Lugols iodine solution

Iodine crystal
1 gm
potassium iodide
2 gm

- Ethyl alcohol
250 ml

- Acetone
250 ml

- Counterstain

Safranine
2.5 ml
Ethyl alcohol (95%)
100 ml

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

CHAPTER: 1

INTRODUCTION

Food security is a complex issue, where fish and fishery products are generally regarded as high risk commodity in respect of pathogen contents, natural toxins threatening illness and other possible contaminants and adulterants.

Fish and Fisheries have been playing a very significant role in nutrition, culture and economy of Bangladesh from time immemorial. Currently, about 80 percent of the animal protein intake in the daily diet of the people comes from fish (Abu Hena Yousuf *et al.* 2008).

Nowadays, prawns play a dominant role in the economy of Bangladesh. Every year it contributes 4.7% to GDP and about 8% to the total export earnings of the country. Therefore, by considering the consumer health safety and economical sustain it is worth to maintain the microbiological quality of the fish (Samia S. *et al.* 2014.). So, to increase fish quality assurance in accordance with microbial load assessment is deemed necessary.

The Food and Agricultural Organization of the United Nations and the World Health Organization (FAO/WHO Codex Alimentarius Commission, 2000) stated that illness due to contaminated food is perhaps the most widespread health problem in the contemporary world and an important cause of reduced economic productivity (Edema M.O.*et al*. 2005).

Biological contaminant such as bacteria, viruses, protozoa, fungi and helminthes constitute the major cause of food borne diseases such as cholera, *E. coli* gastroenteritis, salmonellosis, shigellosis, campylobacteriosis, brucellosis, amoebiasis, typhoid fever and poilomyletis with varying degrees of severity, ranging from mild indisposition to chronic or life threatening illness (Phyllis, E., 2007).

Therefore, for the assurance of consumers European Commission Health and Consumer Protection Directorate General (EC/DG), United States Food and Drug Administration (USFDA) and Canadian Food Inspection

Agency (CFIA) legally mandated in 1999 that the hazard analysis critical control point (HACCP) system and complementary standard sanitation operation procedures (SSOPs) be implemented for processing all purchased food products.

Water and ice quality is also an important factor for good quality fish, because water and ice used for fish processing may contaminate the whole processing plant. EU advised Bangladesh Government to implement the Hazard Analysis Critical Control Point (HACCP) in the processing of fishes (W. H. Andrews and T. S. Hammack, 2001).

Isolation and identification of microbial food contaminants help to understand how infectious agents enter and spread through the food chain and therefore come up with procedures to prevent or minimize exposure of the consumer to such agents.

Consequently, prawns may be contaminated with different types of bacteria such as *Vibrio* spp., *Salmonella* spp., coliform, fecal coliform, streptococci and Staphylococcus spp. Those spoil fishes are responsible for causing cholera and other food borne disease outbreaks (Snowdon *et al.*, 1989; Starutch, 1991; Karunasagar *et al.*, 1994; Cray and Moon, 1995; Wallace *et al.*, 1999; Mobin *et al.*, 2001; WHO, 2012).

The estimation of bacterial numbers in prawn is frequently used to retrospectively assess microbiological quality or to assess the presumptive safety of the product. When total count reaches 10^6 CFU (Colony Forming Units) per gram or milliliter of product, the product is assumed to be at, or nearing, spoilage, according to International Commission on Microbiological Specifications for Foods (ICMSF, 1986.)

Occurrence of antibiotic resistance genes in pathogenic isolates is becoming a serious problem in developing countries where antibiotic misuse is very common. Such antibiotic resistance of the pathogens also be a great threat for the treatment of the diseases, even in the developed countries (Gubala and Proll, 2006; Bhatta *et al.*, 2007; Jakee *et al.*, 2009).

Prawns farmed in both salt water and freshwater are becoming increasingly vulnerable to bacterial infection. As a result, many farmers have made improper use of antibiotics to prevent or treat infections,

leading to the dissemination of antimicrobial-resistant strains in aquatic environments (S. Harakeh *et al*. 2006).

The present study aimed to evaluate the incidence of bacterial load and pathogens in locally available prawn species, to demonstrate the drug-resistance traits of the isolates with a view to provide potential approaches for improving the quality assurance and create awareness among the consumers.

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

i) To find out the Total Bacterial Count (TBC), Total Coliform Counts (TCC), Total Salmonella-Shigella (TSS) Counts and Total Vibrio Counts (TVC) of prawns collected from local fish market of Dinajpur city

ii) To isolate and identify the bacterial pathogens from prawn samples

iii) To detect the antibiotic resistance pattern of identified isolates

CHAPTER: 3
MATERIALS AND MATHODS

The present research work was conducted during January to June, 2017 in the Microbiology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur. The detailed outline of Materials and Methods are given below.

3.1 MATERIALS

3.1.1 Study area and study population

30 Prawn (*Penaeus monodon*) samples were collected from the local fish markets of the Dinajpur city {Bahadur bazaar(10), Rail gate bazaar(10) and Gopalgong bazaar(10)}, Bangladesh. Samples were collected randomly from respective local market for bacteriological analysis with antibiogram study and brought to the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University for laboratory analysis.

3.1.2 Laboratory preparation

All items of glass wares including test tubes, pipettes, cylinder, flasks, conical flasks, glass plate, slides, vials and agglutination test tubes soaked in a household dishwashing detergent solution ('Trix, Recket and Colman Bangladesh Ltd.) for overnight, contaminated glassware's were disinfected in 2% sodium hypo chloride solution prior to cleaning. The glassware were then cleaned by brushing, washed thoroughly and finally sterilized either by dry heat at 160^0 C for 2 hours or by autoclaving for 15 minutes at 121^0 C under 15 lbs pressure per square inch. Autoclaved items were dried in a hot air oven over at 50^0 C. Disposable plastic were (micropipette tips) was sterilized by autoclaving. All the glassware was kept in oven at 50^0 C for future use.

3.1.3 Instrument and apparatus

At research laboratory, Department of microbiology, Hajee Mohammad Danesh Science and Technology University. We used various kinds of apparatus, which are given below-Test tube, Petri dish, Conical flask, Pipette, Micro pipette, Slide, Microscope, Cotton, Immersion oil, Toothpick, Autoclave, Jar, Beaker, Cylinder, Ice box, Balance, Laminar flow, Spirit lamp, Refrigerator, Marking pen, Bacteriological loops etc were used.

3.1.4 Media for culture

3.1.4.1 Liquid media

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

3.1.4.2 Solid media

- Nutrient agar base (Hi-media, India).
- Plate count agar media (Hi-media, India).
- Salmonella-Shigella agar (Hi-media, India).
- Eosin methylene blue (EMB) agar (Hi-media, India).
- MacConkey agar medium (Hi-media, India).
- Thiosulfate-citrate-bile salts-sucrose agar (Hi-media, India).
- Deoxycholate Citrate Agar (DCA) (Hi-media, India).
- Mueller Hinton Agar (Hi-media, India).

3.1.4.3 Media for biochemical test

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

3.1.5 Reagents

- Gram's staining reagent: Crystal violet, Gram's iodine, Acetone and Safranin.
- Alpha-naphthol solution.

- Kovac's reagent.
- Ethyl alcohol (70% and 95%).
- Phosphate Buffered Saline (PBS)
- Physiological Saline Solution (PSS)
- Methylene Blue stain
- Voges-Proskauer (VP) Solution
- Indol Solution
- Methyl Red Solution
- Potassium-di-hydrogen phosphate (0.2M, KH_2PO_4 $2H_2O$)
- Di-sodium hydrogen phosphate (0.2M, $Na_2HPO_4 12H_2O$)

3.1.6 Antimicrobial Sensitivity Discs:

To determine the drug sensitivity pattern of different isolated bacteria different types of commercially available antibiotic discs (Oxoid Ltd., UK) were used. The method allowed for the rapid determination of the efficacy of the drug by measuring the diameter of the zone of inhibition that result from different diffusion of the agent into the medium surrounding the disc. The followings are the antibiotics that were tested against the selected organism.

Table1. Antimicrobial agents with their disc concentration

S/N	Name of antibiotics	Disc concentration (µg/disc)	S/N	Name of antibiotics	Disc concentration (µg/disc)
1.	Cefixime (CFM)	5 µg/disc	6.	Azithromycin (AZM)	15 µg/disc
2.	Tetracycline (TE)	30 µg/disc	7.	Erythromycin (E)	15 µg/disc
3.	Chloramphenicol (C)	30 µg/disc	8.	Penicillin G (P)	10 µg/disc
4.	Ciprofloxacin (CIP)	5 µg/disc	9.	Neomycin (N)	30 µg/disc
5.	Levofloxacin (LE)	5 µg/disc	10.	Vancomycin (VA)	30 µg/disc

Legend: µg = Microgram

3.2 METHODS

3.2.1 Experimental layout

The prawn samples were collected directly from local market of Dinajpur city (Bahadur bazaar, Railgate bazaar and Gopalgong bazaar) for the bacteriological analysis with antibiogram study of the isolated bacteria. The experimental layout illustrated in figure 1.

EXPERIMENTAL LAYOUT

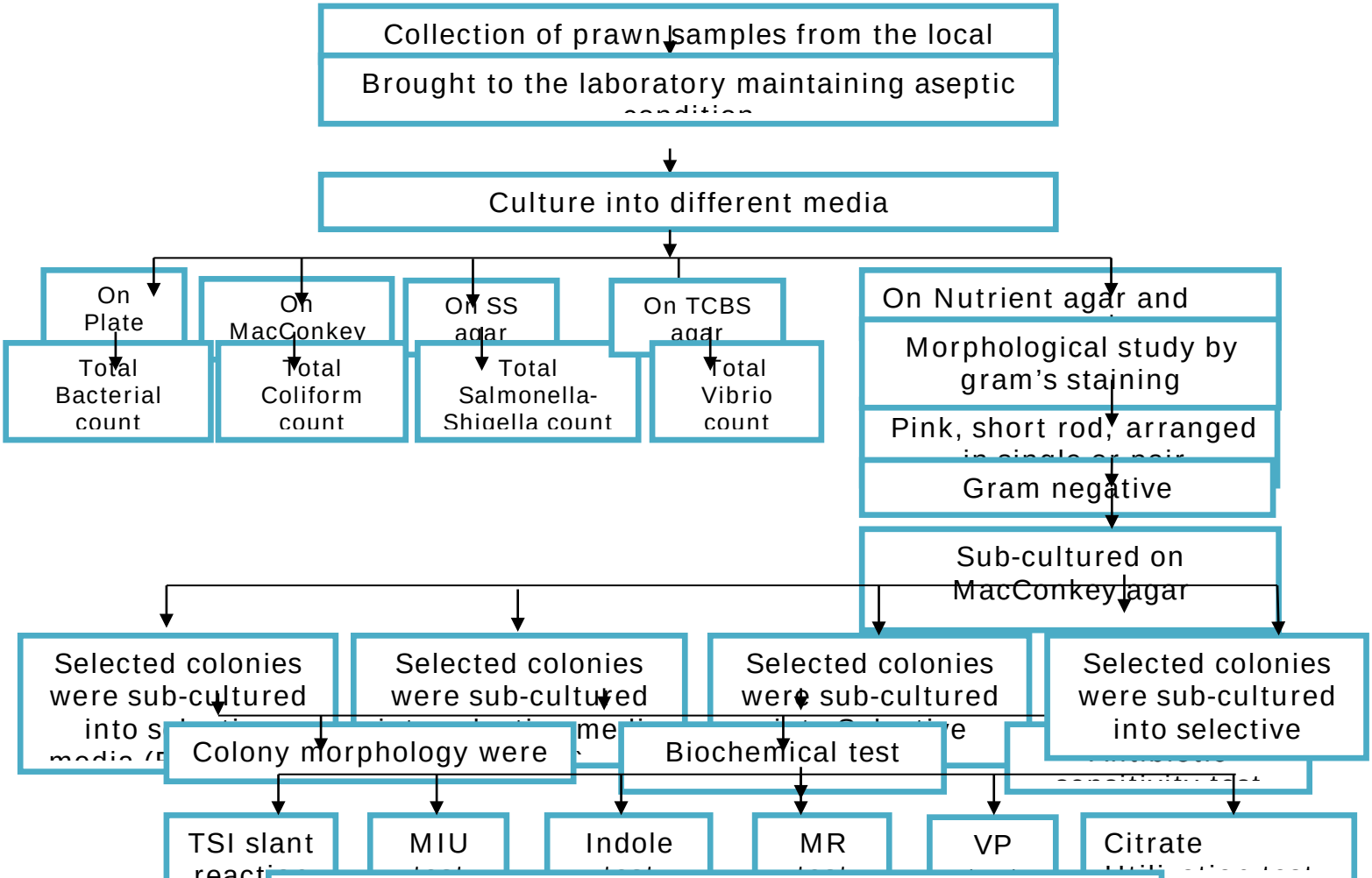

Fig1: Schematic illustration of experimental layout

3.2.2 Preparation of culture media

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

3.2.2.1 Liquid Media

3.2.2.1.1 Nutrient broth

Nutrient broth (NB) was used to grow the organisms from the samples collected from the study areas before performing biochemical test (Cheesebrough, 1985).

13 gram of Bacto-nutrient broth (Difco) was dissolved in 1000 ml of cold distilled water and heated up to boiling to dissolve it completely. The solution was then distributed in tubes, stopper with cotton plugs and sterilized in the autoclave machine at l21°C and 15 pounds pressure per square inch for 15 minutes. The sterility of the medium was judged by incubating overnight at 37°C and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.2.2 Solid media

3.2.2.2.1 Plate count agar

17.5 grams of plate count agar powder was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile petri dish and allowed to solidify. After solidification of the medium was poured in the petri dishes, these were incubated at 37^0C for overnight to check their sterility and used for culture characterization (Carter, 1979).

3.2.2.2.2 Nutrient agar

28.0 grams of nutrient agar powder (Hi-media, India) was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile petridish and allowed to solidify. After solidification of the medium in the petridishes, these were incubated at 37^0C for overnight to check their sterility and used for culture characterization (Carter, 1979).

3.2.2.2.3 Salmonella Shigella Agar

Salmonella Shigella (SS) agar is the selective medium for the isolation of *Salmonella* and *Shigella*. 63.0 grams SS agar powder was dissolved in 1000 ml of distilled water. It was mixed well until a homogeneous suspension is obtained. It was heated with frequent agitation and boiled for one minute. It did not sterilized by autoclaved. It was cooled to 45ºC

and 50° C and distributed in Petri plates and allow the medium to solidify partially uncovered. (HIMEDIA and Leifson et al, 1935)

3.2.2.2.4 Eosin methylene blue agar

Eosin methylene blue (EMB) agar medium was used to observe the growth of *Escherichia coli* (Cheesebrough, 1985).
36 gram of EMB agar base (Hi-media, India) was added to 1000 ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured in to sterile glass. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petridishes partially removed. The sterility of the medium was judged and used or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.2.2.5 MacConkey agar

51.50 grams of dehydrated Bacto-MacConkey agar (Difco) was suspended in 1000 ml of cold distilled water taken in a conical flask and was heated up to boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured sterile glass petridishes. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petridishes partially removed. The sterility of the medium was judged and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

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

TCBS Agar is recommended for the selective isolation and cultivation of *Vibrio cholera* and other enteropathogenic Vibrio's causing food poisoning. 89.08 grams of TCBS agar powder was suspended in 1000 ml distilled water. It was heated to dissolve the medium completely. It did not autoclaved. It was cooled to 50°C and poured into sterile Petri plates (Carter, 1979).

3.2.2.2.7 Deoxycholate Citrate Agar (DCA)

Deoxycholate Citrate Agar is a selective medium recommended for the isolation of enteric pathogens particularly *Salmonella* and *Shigella* species in accordance with European Pharmacopoeia. 69.02 grams of DCA powder was suspended in 1000 ml of purified/distilled water. It was heated to boiling to dissolve the medium completely. Avoided autoclaved and it was cooled to 50°C and poured into sterile Petri plates (HIMEDIA and Leifson et al, 1935)

3.2.2.2.8 Mueller Hinton Agar

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

3.2.2.2.9 MIU medium

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

3.2.3 Reagents preparation

3.2.3.1 Methyl Red-Voges Proskauer broth

A quantity of 3.4 grams of Bacto MR-VP medium was dissolved in 250 ml of distilled water dispensed in 2 ml amount in each test tube and then the

test tubes were autoclaved. After autoclaving, the tubes containing medium were incubated at 37°C for overnight to check their sterility and used for biochemical characterization or stored at 4°C in refrigerator for future use (Cheesbrough, 1984).

3.2.3.2 Methyl red solution

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

3.2.3.3 Voges-Proskauer solution 3.2.3.3.1 Alpha-naphthol solution

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

3.2.3.3.2 Potassium hydroxide solution

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

3.2.3.4 Kovac's reagent

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

3.2.3.5 Phosphate buffered saline solution

For preparation of Phosphate buffered saline (PBS) solution, 8 gram of sodium chloride (NaCl), 2.89 gram of disodium hydrogen phosphate

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

3.2.4 Sample collection and sample processing

Prawn samples (30) from local fish market of Dinajpur city (Bahadur bazaar, Railgate bazaar and Gopalgong bazaar) were collected aseptically and brought to the bacteriology laboratory, Department of Microbiology, HSTU, Dinajpur with necessary precautions for bacteriological examination. At first, Samples were rinsed thoroughly with sterile distilled water. Then 10 g of all part of the samples were homogenized through blending with 90 ml peptone water (Cappuccino and Sherman, 1996). Then 1-10 fold dilutions were performed.

3.2.5 Serial dilution of Sample:

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

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

3.2.6 Enumeration of total bacterial count (TBC)

For the determination of total bacterial count, 1 ml of each ten-fold dilution was transferred and spread on duplicate plate count agar using a sterile pipette for each dilution. The diluted samples were spread as quickly as possible on the surface of the plate with a sterile glass spreader. One sterile spreader was used for each plate. The plates were then kept in an incubator at 37℃ for 24-48 hours. Following incubation, plates exhibiting 30-300 colonies were counted. The average number of colonies in a particular dilution was multiplied by the dilution factor to obtain the total viable count. The total viable count was calculated according to ISO. The results of the total bacterial count were expressed as the number of organism or colony forming units per gram (CFU/gm) of prawn sample.

3.2.7 Enumeration of total coliform count (TCC)

For the determination of total coliform count 1ml of each tenfold dilution was transferred to MacConkey agar. For each dilution five test plates containing MacConkey agar were used. All the agar plates were incubated at 37℃ temperature for 48 hours. The total coliform count was calculated according to ISO. The results of the total coliform count were expressed as the number of organism or colony forming units per gram (CFU/gm) of prawn sample.

3.2.8 Enumeration of total Salmonella- Shigella Count (TSS)

For the determination of total Salmonella-Shigella (TSS) count 1ml of each tenfold dilution was transferred to salmonella-shigella (SS) agar. For each dilution five test plate containing SS agar were used. All the agar plates were incubated at 37℃ temperature for 48 hours. The total salmonella-shigella count was calculated according to ISO. The results of the total salmonella-shigella count were expressed as the number of organism or colony forming units per gram (CFU/gm) of prawn sample.

3.2.9 Enumeration of total Vibrio Count (TVC)

For the determination of total Vibrio (TVC) count 1ml of each tenfold dilution was transferred to TCBS agar. For each dilution five test plate containing TCBS agar were used. All the agar plates were incubated at 37℃ temperature for 48 hours. The total vibrio count was calculated according to ISO. The results of the total vibrio count were expressed as the number of organism or colony forming units per gram (CFU/gm) of prawn sample.

3.2.10 Isolation and identification of bacteria

3.2.10.1 Culture of prawn sample

Media such as Nutient agar, Nutrient broth, MacConkey agar, Eosin Methylene Blue agar (EMB), Salmonella-Shigella (SS) agar, Thiosulfate-citrate-bile salts-sucrose (TCBS) agar and Deoxycholate Citrate Agar (DCA) were used.

3.2.10.2 Culture in ordinary media

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

3.2.10.3 Isolation of bacteria in pure culture

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

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

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

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

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

3.2.10.4 Morphological characterization of organisms by Gram's staining method

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

Technique:

A drop of sterile normal saline was taken on the middle of the clear slide. Then a loopful bacterial suspension (young culture) was transferred to the sterile drop of normal saline and a very thin film was prepared on the slide by spreading uniformly. The film was fixed by passing it gently over flame for two or three times.

- The slide was flooded with crystal violet solution for up to one minute. Wash off briefly with tap water (not over 5 seconds). Drained.
- The slide was flooded with Gram’s Iodine solution, and allow to act (as a mordant) for about one minute. Wash off with tap water. Drained.
- Excess water was removed from slide and blotted, so that alcohol used for decolorization was not diluted. Slide was flooded with 95% alcohol for 10 seconds and washed off with tap water. (Smears that are excessively thick may require longer decolorization. This is the most sensitive and variable step of the procedure, and requires experience to know just how much to decolorize). Drained.
- The slide was flooded with safranin solution and allowed to counter stain for 30 seconds. Washed off with tap water. Drained and blotted with bibulous paper.
- All sides of bacteria were examined under the oil immersion lens.

3.2.10.5 Culture into differential media

3.2.10.5.1 Mac-Conkey agar

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

3.2.10.6 Culture on selective media Media

3.2.10.6.1 Eosin Methylene Blue (EMB) agar:

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

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

3.2.10.6.2 Salmonella -Shigella agar

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

3.2.10.6.3 Deoxycholate Citrate Agar (DCA)

Materials from nutrient broth tubes were inoculated into Deoxycholate Citrate agar containing plates.

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

Samples were inoculated into TCBS agar plates which after incubation, the growth was indicated by smooth, Characteristics yellow colonies.

3.2.11 Microscopic study for identification of *E.coli., Salmonella* spp*., Vibrio* spp*., Klebsiela* spp. and *Shigella* spp. suspected colonies by Gram's staining

Gram's staining was performed by taking colony from selected media to determine the size, shape, and arrangement of bacteria according to the methods described by Merchant and Packer (1967). Stained slides were examined under light microscope at 100 x magnification.

3.2.12 Identification of isolated bacteria by different Biochemical Tests:

Isolated organisms with supported growth characteristics of *E.coli, Salmonella* spp*., Vibrio* spp., *Klebsiela* spp*.* and *Shigella* spp*.* were maintained in pure culture and subjected to biochemical tests.

3.2.12.1 Procedure of Indole test

2 ml of peptone water was inoculated separately with 5 ml of culture of each of the isolated bacteria and incubated for 48 hours. 0.5 ml Kovac's reagent was added, shakes well and examined after 1 minute. A red color ring at the top of the reagent indicated production of the indole by the organisms (Cowan, 1985).

3.2.12.2 Procedure of MR test

The test was performed by inoculating separately a colony of the each of the isolated test organisms in 0.5 ml sterile glucose phosphate broth. After overnight incubation at 37^0C, a drop of methyl red solution was added. A positive methyl red test was shown by the appearance of a bright red color. A yellow or orange color was a negative test (Cowan, 1985).

3.2.12.3 Procedure of VP test

2 ml of sterile glucose phosphate peptone water were inoculated separately with 5ml of each of the isolated organisms and incubated at 37°C for 48 hours. A very small amount (knife point) of creatine was added and mixed. 3 ml of 40% potassium hydroxide were added and shacked well. The bottle cap was removed and left for an hour at room temperature. It was observed closely for the slow development of a pink color for positive cases. In negative cases there was no development of pink color (Cowan, 1985).

3.2.12.4 Procedure of Motility Indole Urease Test (MIU)

MIU media were prepared in test tubes. Then the isolated organisms were inoculated separately into the media by stabbing method with the help of sterile straight wire. Then the test tubes were incubated 37°C overnight. Single stick that is no turbidity throughout the medium indicate gram negative organism (non motile) and turbidity throughout the medium indicate gram positive case (Cowan, 1985).

3.2.12.5 Procedure of Triple Sugar Iron Test (TSI)

Triple sugar iron contains three sugars (Glucose, Sucrose and Lactose). At first TSI agar slant were prepared in a test tube. Then the isolated organisms were inoculated separately into the butt with a sterilized wire and on the slant with a wire loop producing zigzag streaking. The tubes were incubated for 24 hours at 37°C.Yellow color of butt and slant of the test tube indicate fermentation of Glucose, Sucrose and Lactose fermentation and butt shows blacking indicate H_2S production (Cowan, 1985).

3.2.12.6 Citrate utilization test

Simmons citrate agar slants of 2 ml in each vials were prepared by autoclaving at 15 psi $121^{\circ}C$. Using sterile technique, small amount of each of the isolated bacteria from 24-hours old pure culture were inoculated separately into the vials by means of a streak inoculation method with an inoculating needle and the vials were incubated for 48 hours at $37^{\circ}C$ (Cappuccino & Sherman, 2005).

3.2.13 Antibiotic susceptibility test:

3.2.13.1 The Kirby-Bauer disc diffusion method

All aspects of the Kirby-Bauer procedure are standardized to ensure consistent and accurate results. Because of this, a laboratory must adhere to these standards. The media used in Kirby-Bauer testing must be Mueller-Hinton agar at only 4 mm deep, poured into either 100m or 150mm Petri dishes. The pH level of the agar must be between 7.2 and 7.4.

Incubation Procedure

1. Labeled the covers of each of the agar plates with the name of the test organism to be inoculated.
2. Using an aseptic technique, inoculated all agar plates with their respective test organisms as follows:
   - Dip a sterile cotton swab into a well mixed saline test culture and remove excess inoculums by pressing the saturated swab against the inner wall of the culture tube.
   - Using the cotton swab streaked the entire agar surface horizontally, vertically and around the outer edge of the plate to ensure a heavy growth over the entire surface.
3. Allowed all culture plates to dry for about 5 minutes.
4. Using the Sensi-disc-dispenser, applied the antibiotic discs by placing the dispenser over the agar surface and pressing the plunger, depositing the discs simultaneously onto the agar surface. If dispensers are not available, distribute the individual discs at equal distances with forceps dipped in alcohol and flamed.

5. Gently pressed each disc down with the wooden end of a cotton swab or sterile forceps to ensure that the discs adhere to the surface of the agar.
6. Incubated all plate cultures in an inverted position for 24 to 48 hours at 37°C. (Cappuccino and Sherman, 2005)

3.2.13.2 Reading Plates and Interpreting Results

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

3.3 STATISTICAL ANALYSIS

Data obtained for bacterial load was tabulated using Microsoft Excel (MS Excel 2010, Microsoft Corporation). Statistical analyses were done using Statistical Package for the Social Sciences (SPSS version 16.0). Values were expressed as log10 CFU/g appropriate test statistics one way ANOVA using Duncan Multiple Range (DMR) test were used.

CHAPTER: 4

RESULTS

4.1 BACTERIAL COUNTS

4.1.1 Total Bacterial Counts (TBC)

Total Bacterial Count (TBC) is a measure of microbial quality of prawn samples. The result presented in Table 2 showed the total viable bacterial load of thirty prawn samples. The bacterial loads were not uniform and varied quite considerably. Mean±SEM of TBC was 4.49±0.24 log10 CFU/g and all counts was significantly higher ($p<0.001$). The total bacterial count varied with different prawns samples were ranged between 3.8×10^2 CFU/g to 2.8×10^6 CFU/g. The highest count was found in sample no 15 and the lowest count was found in sample no 8. The highest Mean±SEM of TBC was found in Gopalgong bazaar (4.76±0.39 log10 CFU/g) and shown in Table 3.

4.1.2 Total Coliform Counts (TCC)

Coliforms are considered as indicators of quality. Presence of coliforms in high numbers is health hazard causing spoilage of prawns and food borne diseases. Mean±SEM of TCC in prawn samples was 5.20±0.29 log10 CFU/g and all counts was significantly higher ($p<0.001$). The total coliform count (TCC) of thirty samples was ranged between 4.9×10^2 CFU/g to 3.52×10^7 CFU/g. The highest count was found in sample no 15 and the lowest count was found in sample no 17. The highest Mean±SEM of TCC was found in Rail gate bazaar (6.13±0.41 log10 CFU/g) and shown in Table 3.

4.1.3 Total Salmonella-Shigella (TSS) Counts

Microbial quality of prawns is related to salmonella-shigella counts. Mean±SEM of TSS in prawn samples was 4.60±0.30 log10 CFU/g and all counts was significantly higher ($p<0.001$).The total Salmonella-Shigella (TSS) counts of thirty samples were ranged between 1.65×10^2 CFU/g to

1.3×10^7 CFU/g. The highest count was found in sample no 4 and the lowest count was found in sample no 28. The highest Mean±SEM of TSS was found in Rail gate bazaar (5.29±0.40 log10 CFU/g) and shown in Table 3.

4.1.4 Total Vibrio Counts (TVC)

Microbial quality of prawns is related to Vibrio counts. Mean±SEM of TVC in prawn samples was 5.59±0.21 log10 CFU/g and all counts was significantly higher ($p<0.001$).The total Vibrio counts (TVC) of thirty samples were ranged between 4.0×10^3 CFU/g to 2.5×10^7 CFU/g. The highest count was found in sample no 18 and the lowest count was found in sample no 21. The highest Mean±SEM of TVC was found in Rail gate bazaar (5.79±0.32 log10 CFU/g) and shown in Table 3.

Table: 2. Total Bacterial Counts (TBC), Total Coliform Counts (TCC), Total Salmonella-Shigella (TSS) and Total Vibrio Counts (TVC) of 30 prawn samples

Sample (Prawn)		Total Bacterial Count (CFU/g)		Total Coliform Count (CFU/g)		Total Salmonella-Shigella Count (CFU/g)		Total Vibrio Count (CFU/g)	
Bahadur bazaar	S-1	2.4×10^{6}	6.38	5.20×10^{2}	2.71	1.7×10^{2}	2.23	1.3×10^{6}	6.11
	S-2	5.4×10^{2}	2.73	4.3×10^{3}	3.63	1.4×10^{6}	6.14	2.6×10^{4}	4.41
	S-3	2.6×10^{5}	5.41	3.9×10^{6}	6.59	1.6×10^{3}	3.2	1.6×10^{5}	5.20
	S-4	2.7×10^{4}	4.43	3.8×10^{7}	7.57	1.3×10^{7}	7.11	1.5×10^{6}	6.18
	S-5	3.0×10^{3}	3.47	4.12×10^{4}	4.61	1.82×10^{2}	2.26	1.2×10^{7}	7.08
	S-6	3.8×10^{4}	4.58	5.40×10^{2}	2.73	1.62×10^{4}	4.2	2.18×10^{5}	5.33
	S-7	2.84×10^{4}	4.45	4.0×10^{5}	5.6	1.52×10^{5}	5.18	2.0×10^{5}	5.30
	S-8	3.8×10^{2}	2.58	5.1×10^{3}	3.7	1.74×10^{3}	3.24	1.8×10^{7}	7.25
	S-9	2.64×10^{6}	6.42	3.84×10^{4}	4.58	1.52×10^{4}	4.18	2.2×10^{4}	4.34
	S-10	3.3×10^{5}	5.33	4.4×10^{3}	3.64	2.0×10^{2}	2.3	2.06×10^{6}	6.31
	Mean±SEM	4.58±0.43		4.54±0.51		4.00±0.54		5.75±0.32	
	P value	<0.001		<0.001		<0.001		<0.001	
Rail gate bazaar	S-11	4.6×10^{2}	2.66	3.66×10^{5}	5.56	1.34×10^{5}	5.12	3.0×10^{4}	4.48
	S-12	2.5×10^{5}	5.39	4.3×10^{4}	4.63	1.64×10^{3}	3.21	1.2×10^{6}	6.07
	S-13	2.8×10^{4}	4.44	3.6×10^{6}	6.55	1.14×10^{6}	6.05	1.5×10^{6}	6.17
	S-14	4.2×10^{2}	2.62	4.12×10^{5}	5.61	1.54×10^{4}	4.18	1.7×10^{5}	5.23
	S-15	2.8×10^{6}	6.44	3.52×10^{7}	7.55	1.24×10^{5}	5.09	1.8×10^{7}	7.25
	S-16	3.6×10^{3}	3.55	3.64×10^{6}	6.56	0.94×10^{7}	7.02	2.6×10^{4}	4.41
	S-17	4.0×10^{2}	2.6	4.90×10^{2}	2.69	0.8×10^{6}	6.09	2.10×10^{5}	5.32
	S-18	1.9×10^{6}	6.27	2.40×10^{7}	7.38	1.2×10^{4}	4.07	2.5×10^{7}	7.39
	S-19	3.8×10	3.57	2.2×10	7.34	0.9×1	7.04	2.3×1	5.36

		10^3		10^7		10^7		10^5	
	S-20	5.4×10^3	3.73	2.72×10^7	7.43	1.0×10^5	5.0	1.9×10^6	6.27
	Mean±SEM	4.13±0.46		6.13±0.41		5.29±0.40		5.79±0.32	
	P value	<0.001		<0.001		<0.001		<0.001	
Gopalgong bazaar	S-21	2.4×10^6	6.38	3.54×10^5	5.54	1.66×10^2	2.22	4.0×10^3	3.60
	S-22	3.2×10^3	3.5	5.50×10^2	2.74	1.12×10^6	6.04	1.8×10^6	6.25
	S-23	2.2×10^5	5.34	3.52×10^5	5.54	1.3×10^3	3.11	2.4×10^4	4.38
	S-24	3.0×10^4	4.47	4.6×10^3	3.66	0.98×10^7	7.0	3.6×10^4	4.55
	S-25	4.5×10^3	3.65	3.34×10^6	6.52	1.0×10^5	5.0	3.2×10^3	3.50
	S-26	2.12×10^6	6.33	4.0×10^4	4.6	0.6×10^7	7.22	2.9×10^4	4.46
	S-27	5.0×10^2	2.69	3.8×10^4	4.58	1.5×10^4	4.17	3.2×10^5	5.50
	S-28	2.44×10^5	5.38	2.4×10^6	6.38	1.65×10^2	2.21	2.8×10^5	5.44
	S-29	2.8×10^5	5.44	4.02×10^3	3.6	1.04×10^5	5.01	1.9×10^7	7.27
	S-30	2.6×10^4	4.41	2.0×10^6	6.3	1.44×10^3	3.15	1.7×10^7	7.23
	Mean±SEM	4.76±0.39		4.95±0.42		4.51±0.59		5.21±0.43	
	P value	<0.001		<0.001		<0.001		<0.001	

Table: 3. Variation of TBC, TCC, TSS and TVC according to area

Parameter	Bahadur bazaar	Railgate bazaar	Gopalgong bazaar	Level of significance	Total Mean±SEM	Level of significance
TBC	4.58±0.43	4.13±0.46	4.76±0.39	NS	4.49±0.24	S (P<0.001)
TCC	4.54±0.51	6.13±0.41	4.95±0.42	NS	5.20±0.29	S (P<0.001)
TSS	4.00±0.54	5.29±0.40	4.51±0.59	NS	4.60±0.30	S (P<0.001)
TVC	5.75±0.32	5.79±0.32	5.21±0.43	NS	5.59±0.21	S (P<0.001)

(Legends: SEM-Standard error means, NS-Non significance, S- Significance)

4.2 ISOLATION AND IDENTIFICATION OF *E.coli, Salmonella* spp., *Vibrio* spp., *Klebsiella* spp. AND *Shigella* spp BY DIFFERENT BACTERIOLOGICAL METHODS:

E. coli (100%), *Salmonella* spp. (100%), *Vibrio* spp. (66.7%), *Klebsiella* spp. (40%) and *Shigella* spp. (33.3%) were frequently isolated from almost all samples.

Table: 4. Frequency of occurrence of bacteria isolated from prawn samples

Bacterial isolates	Examined	Positive	Rates (%)
E. coli	30	30	100%
Salmonella spp.	30	30	100%
Vibrio spp.	30	20	66.7%
Klebsiella spp.	30	12	40%
Shigella spp.	30	10	33.3%

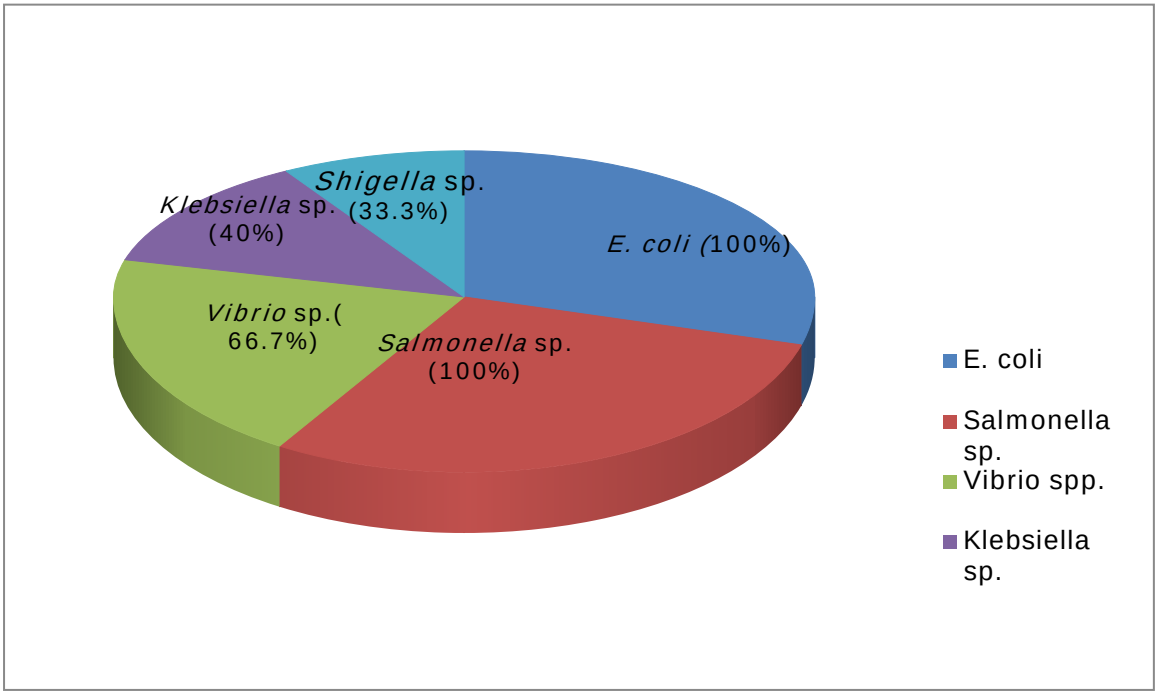

Fig: 2. Pie chart presenting different types of bacteria isolated from prawn samples

4.2.1 Results of cultural examinations:

4.2.1.1 Ordinary media:

4.2.1.1.1 Nutrient agar:

Pale colorless colony was found (Plate-7)

4.2.1.2 Differential media

4.2.1.2.1 MacConkey agar

MacConkey agar plates streaked separately with the organisms from nutrient agar revealed the growth of bacteria after 24 hours of incubation at 37^0C aerobically.

The growth of lactose fermenting organisms was indicated by bright pink colored colonies of on MacConkey agar (Plate-8).

The growth of non lactose fermenting organisms was indicated by pale colored colonies of on MacConkey agar (Plate-9).

4.2.1.3 Selective media

4.2.1.3.1 Eosin methylene blue (EMB) agar

EMB agar plates streaked separately with the lactose fermenter organisms from MacConkey agar revealed the growth of *E. coli* and *Klebsiella* spp. bacteria after 24 hours of incubation at 37^0C aerobically.

The growth of *E.coli* was indicated by smooth, circular, black color colonies with metallic sheen on the agar plate. (Plate-10)

The growth of *Klebsiella* spp. was indicated by smooth, characteristic mucoid lactose-fermenting and pink colored colonies. (Plate-13)

4.2.1.3.2 Salmonella- Shigella (SS) agar

SS agar plates streaked separately with the non lactose fermenting organisms from MacConkey agar revealed the growth of *Salmonella* spp. after 24 hours of incubation at 37^0C aerobically.

The growth of *Salmonella* spp. was indicated by smooth, colorless, usually with black center. (Plate-11)

4.2.1.3.3 Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar

TCBS agar plate streaked separately with the non lactose fermenting organisms from MacConkey agar revealed the growth of *Vibrio* spp. after 24 hours of incubation at 37^0C aerobically.

The growth of *Vibrio* spp. was indicated by yellow colonies. (Plate-12)

4.2.1.3.4 Deoxycholate Citrate Agar (DCA)

DCA agar plate streaked separately with the non-lactose fermenting organisms from MacConkey agar revealed the growth of *Shigella* spp. after 24 hours of incubation at 37^0C aerobically.

The growth of *Shigella* spp. was indicated by colourless (lactose-negative) colonies. (Plate-14)

4.2.1.4 Results of gram's staining

The microscopic examination of Gram's stained smears from EMB agar revealed Gram-negative, pink colored, small rod shaped *E. coli* arranged in single, pairs or short chain. (Plate-15)

The microscopic examination of Gram's stained smears from SS agar revealed Gram-negative, pink colored, small rod shaped *Salmonella* spp. arranged in single, pairs or short chain. (Plate-16)

The microscopic examination of Gram's stained smears from TCBS agar revealed Gram-negative, comma shaped, motile *Vibrio* spp. arranged in single, pairs or short chain. (Plate-17)

The microscopic examination of Gram's stained smears from EMB agar revealed Gram-negative, pink colored, small rod shaped *Klebsiella* spp. arranged in single, pairs or short chain. (Plate-18)

The microscopic examination of Gram's stained smears from DCA agar revealed Gram-negative, pink colored, small rod shaped *Shigella* spp. arranged in single, pairs or short chain. (Plate-19)

4.2.1.5 Results of biochemical tests:

The isolated organisms were confirmed by different biochemical tests.

Table: 5. Identification of *E. coli* by different biochemical tests

Biochemical test	Change of the media	Results	Plate no.
Citrate utilization test	No color change	Negative	20
Indole test	Pink color ring at the top of the media	Positive	21
Triple sugar iron (TSI) test	S-yellow, B-yellow	S-A, B-A, gas (+), H_2S (-)	22
MR test	Red color	Positive	23
VP test	No color change	Negative	24
MIU test	Diffuse, hazy growth, slightly opaque media	Positive	25

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

Table: 6. Identification of *Salmonella* spp. by biochemical test

Biochemical test	Change of the media	Results	Plate no.
Citrate utilization test	No color change	Negative	20
Indole test	No color change	Negative	21
Triple sugar iron (TSI) test	S-Red, B-yellow	S-Al, B-A, gas(+), H_2S (+)	22

MR test	Red color	Positive	23
VP test	No color change	Negative	24
MIU test	Diffuse, hazy growth, slightly opaque media	Positive	25

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

Table: 7. Identification of *Vibrio* spp. by biochemical test

Biochemical test	Change of the media	Results	Plate no.
Citrate utilization test	Prussian blue color	Positive	20
Indole test	Pink color ring at the top of the media	Positive	21
Triple sugar iron (TSI) test	S-Red, B-yellow	S-Al, B-A, gas(-), H_2S (-)	22
MR test	No color change	Negative	23
VP test	Red color	Positive	24
MIU test	Diffuse, hazy growth, slightly opaque media	Positive	25

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

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

Biochemical	Change of	Results	Plate

test	the media		no.
Citrate utilization test	Prussian blue color	Positive	20
Indole test	No color change	Negative	21
Triple sugar iron (TSI) test	S-yellow, B-yellow	S-A, B-A, gas (+), H_2S (-)	22
MR test	No color change	Negative	23
VP test	Red color	Positive	24
MIU test	No color change	Negative	25

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

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

Biochemical test	Change of the media.	Results	Plate no.
Citrate utilization test	No color change	Negative	20
Indole test	Pink color ring at the top of the media	Positive	21
Triple sugar iron (TSI) test	S- Alkaline, B-yellow	S-Al, B-A, gas(-), H_2S (-)	22
MR test	Red color	Positive	23
VP test	No color change	Negative	24

MIU test	No color change	Negative	25

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

4.3 OBSERVATION OF ANTIBIOTIC SENSITIVITY TEST

Antimicrobial susceptibility testing was performed using Muller-Hinton agar (Mumbai, India) plates as recommended by the Clinical and Laboratory Standards Institute.

Table: 10. Antibiotic resistance pattern of the isolated bacteria obtained from prawn.

Antibiotics	*E coli* (n=30)	*Salmonella* spp. (n=30)	*Vibrio* spp. (n=20)	*Klebsiella* spp. (n=12)	*Shigella* spp. (n=10)

	Zone size (mm)	Outcome		Zone size (mm)	Outcome		Zone size (mm)	Outcome		Zone size (mm)	Outcome		Zone size (mm)	Outcome	
Cefixime (CFM)	10	R	100 %	0	R	0%	9	R	75 %	10	R	75%	10	R	70 %
	0	I	0%	0	I	0%	0	I	0%	0	I	0%	0	I	0%
	0	S	0%	23	S	100 %	21	S	25 %	20	S	25%	23	S	30 %
Tetracycline (TE)	8	R	100 %	0	R	100 %	9	R	100 %	0	R	100 %	8	R	100 %
	0	I	0%	0	I	0%	0	I	0%	0	I	0%	0	I	0%
	0	S	0%	0	S	0%	0	S	O%	0	S	0%	0	S	0%
Chloramphenicol (C)	0	R	0%	10	R	10%	0	R	0%	10	R	16.7 %	9	R	20 %
	0	I	0%	0	I	0%	0	I	0%	0	I	0%	15	I	80 %
	25	S	100 %	23	S	90%	27	S	100 %	23	S	83.3 %	0	S	0%
Ciprofloxacin (CIP)	10	R	16.7 %	0	R	0%	0	R	0%	0	R	0%	0	R	0%
	0	I	0%	0	I	0%	20	I	80 %	0	I	0%	0	I	0%
	25	S	83.3 %	32	S	100 %	23	S	20 %	25	S	100 %	31	S	100 %
Erythromycin (E)	0	R	90%	0	R	86.7 %	0	R	100 %	0	R	100 %	0	R	80 %
	0	I	0%	0	I	0%	0	I	0%	0	I	0%	0	I	0%
	25	S	10%	23	S	13.3 %	0	S	0%	0	S	0%	23	S	20 %

Legends: (CFM= Cefixime, TE= Tetracycline, C = Chloramphenicol, CIP= Ciprofloxacin, E= Erythromycin S= sensitive, I= intermediate, R= resistant)

4.3.1 Antibiotic sensitivity test of *E. coli*

The antibiotic sensitivity test revealed that all of the isolated *E. coli* was resistance to Cefixime (100%), Tetracycline (100%) and Erythromycin (90%). The isolates were sensitive to Chloramphenicol (100%) and Ciprofloxacin (83.3%).

Table: 11. Results of antibiotic sensitivity test of *E. coli* (n=30)

Antibacterial agents	Disc concentration (mcg /disc)	No. and Percentages (%) of iso	
		Sensitive	Intermedia
Cefixime	5 mcg/disc	(0) 0%	(0) 0%
Tetracycline	30mcg/disc	(0) 0%	(0)
Chloramphenicol	30 mcg/disc	(30) 100%	(0) 0%
Ciprofloxacin	5 mcg/disc	(25) 83.3%	(0)
Erythromycin	15 mcg/disc	(3) 10%	(0)

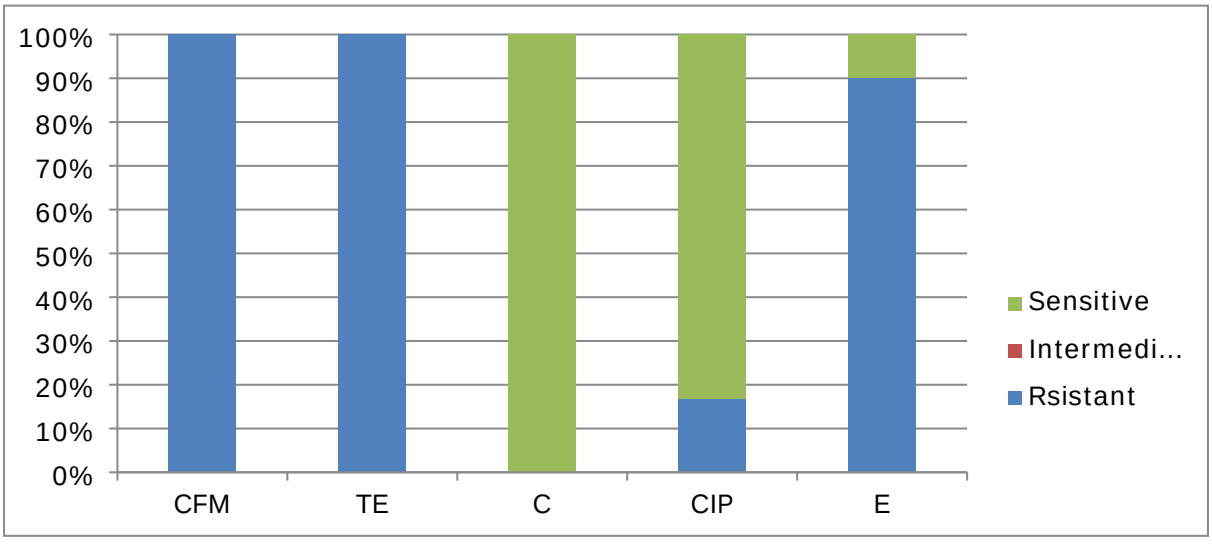

Fig: 3. Column diagram presenting antibiotic sensitivity test of isolated *E. coli*

(Legends: CFM= Cefixime, TE= Tetracycline, C = Chloramphenicol, CIP= Ciprofloxacin, E= Erythromycin).

4.3.2 Antibiotic sensitivity test results of *Salmonella spp.*

The antibiotic sensitivity test revealed that all of the isolated *Salmonella* spp. was resistance to Tetracycline (100%) and Erythromycin (86.7%). The isolates were sensitive to Cefixime (100%), Chloramphenicol (90%) and Ciprofloxacin (100%).

Table: 12. Results of antibiotic sensitivity test of *Salmonella* sp. (n=30)

Antibacterial agents	Disc concentration (mcg /disc)	No. and Percentages (%) of is	
		sensitive	Intermedia
Cefixime	5 mcg/disc	(30) 100%	(0) 0%
Tetracycline	30mcg/disc	(0) 0%	(0)
Chloramphenicol	30 mcg/disc	(27) 90%	(0) 0%
Ciprofloxacin	5 mcg/disc	(30) 100%	(0) 0%
Erythromycin	15 mcg/disc	(4) 13.3%	(0)

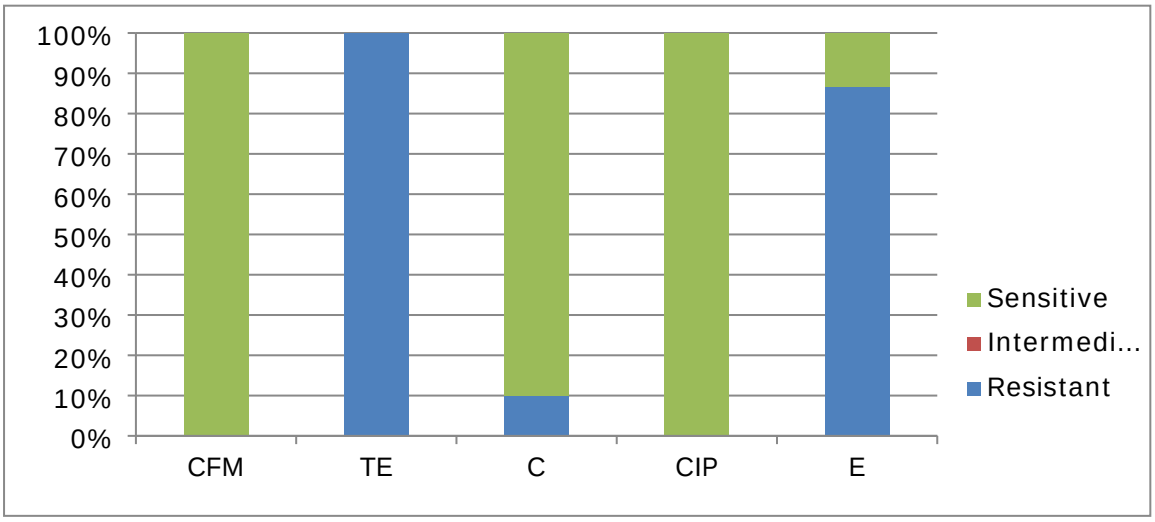

Fig: 4. Column diagram presenting antibiotic sensitivity test of isolated *Salmonella* spp.
(Legends: CFM= Cefixime, TE= Tetracycline, C = Chloramphenicol, CIP= Ciprofloxacin, E= Erythromycin).

4.3.3 Antibiotic sensitivity test results of *Vibrio* spp.

The antibiotic sensitivity test revealed that most of the isolated *Vibrio* spp. was resistance to Cefixime (75%), Tetracycline (100%) and Erythromycin (100%). The isolates were sensitive to Chloramphenicol (100%) while Ciprofloxacin (80%) was found intermediate.

Table: 13. Results of antibiotic sensitivity test of *Vibro* spp. (n=20)

Antibacterial agents	Disc concentration (mcg /disc)	No. and Percentages (%) of is	
		Sensitive	Intermedia
Cefixime	5 mcg/disc	(5) 25%	(0)
Tetracycline	30mcg/disc	(0) 0%	(0)
Chloramphenicol	30 mcg/disc	(20) 100%	(0) 0%
Ciprofloxacin	5 mcg/disc	(4) 20%	(16) 80%

Erythromycin	15 mcg/disc	(0)	0%	(0)

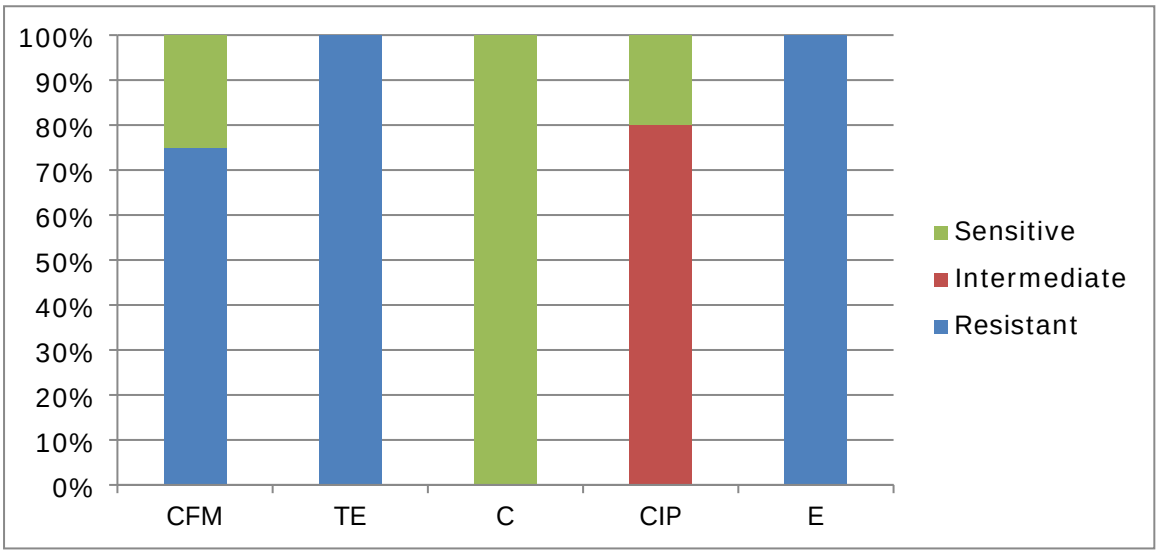

Fig: 5. Column diagram presenting antibiotic sensitivity test of isolated *Vibrio* spp.

(Legends: CFM= Cefixime, TE= Tetracycline, C = Chloramphenicol, CIP= Ciprofloxacin, E= Erythromycin).

4.3.4 Antibiotic sensitivity test results of *Klebsiella spp.*

The antibiotic sensitivity test revealed that most of the isolated *Klebsiella* spp. was resistance to Cefixime (75%), Tetracycline (100%) and Erythromycin (100%). The isolates were found sensitive to Chloramphenicol (83.3%) and Ciprofloxacin (100%).

Table: 14. Results of antibiotic sensitivity test of *Klebsiella* sp. (n=12)

Antibacterial agents	Disc concentration (mcg /disc)	No. and Percentages (%) of isolates		
		Sensitive	Intermediate	Resista
Cefixime	5 mcg/disc	(3) 25%	(0) 0%	(9) 75
Tetracycline	30mcg/disc	(0) 0%	(0) 0%	(12) 10
Chloramphenicol	30 mcg/disc	(10) 83.3%	(0) 0%	(2) 16.
Ciprofloxacin	5 mcg/disc	(12) 100%	(0) 0%	(0) 0

Erythromycin	15 mcg/disc	(0) 0%	(0) 0%	(12) 10

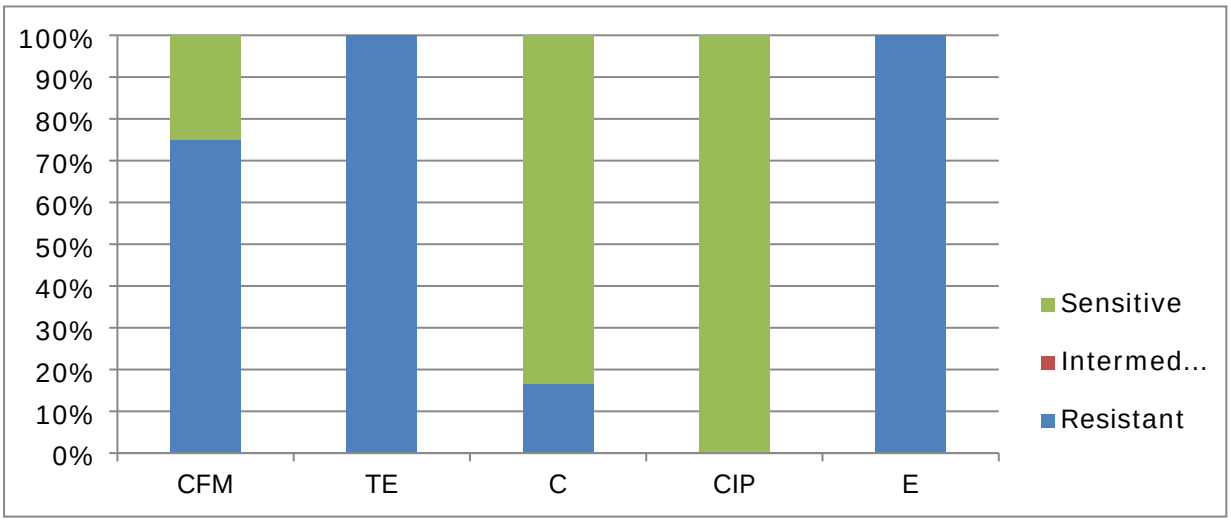

Fig: 6. Column diagram presenting antibiotic sensitivity test of isolated *Klebsiella* spp.

(Legends: CFM= Cefixime, TE= Tetracycline, C = Chloramphenicol, CIP= Ciprofloxacin, E= Erythromycin)

4.3.5 Antibiotic sensitivity test results of *Shigella* spp.

The antibiotic sensitivity test revealed that most of the isolated *Shigella* spp. was resistance to Cefixime (70%), Tetracycline (100%) and Erythromycin (80%). The isolates were sensitive to Ciprofloxacin (100%) and Chloramphenicol (80%) was found intermediate.

Table: 15. Results of antibiotic sensitivity of *Shigella* spp. (n=10)

Antibacterial agents	Disc concentration (mcg /disc)	No. and Percentages (%) of is	
		sensitive	Intermedia
Cefixime	5 mcg/disc	(3) 30%	(0) 0%
Tetracycline	30 mcg/disc	(0) 0%	(0)
Chloramphenicol	30 cg/disc	(0) 0%	(8) 80%
Ciprofloxacin	5 mcg/disc	(10) 100%	(0) 0%

Erythromycin	15 mcg/disc	(2) 20%	(0) 0%

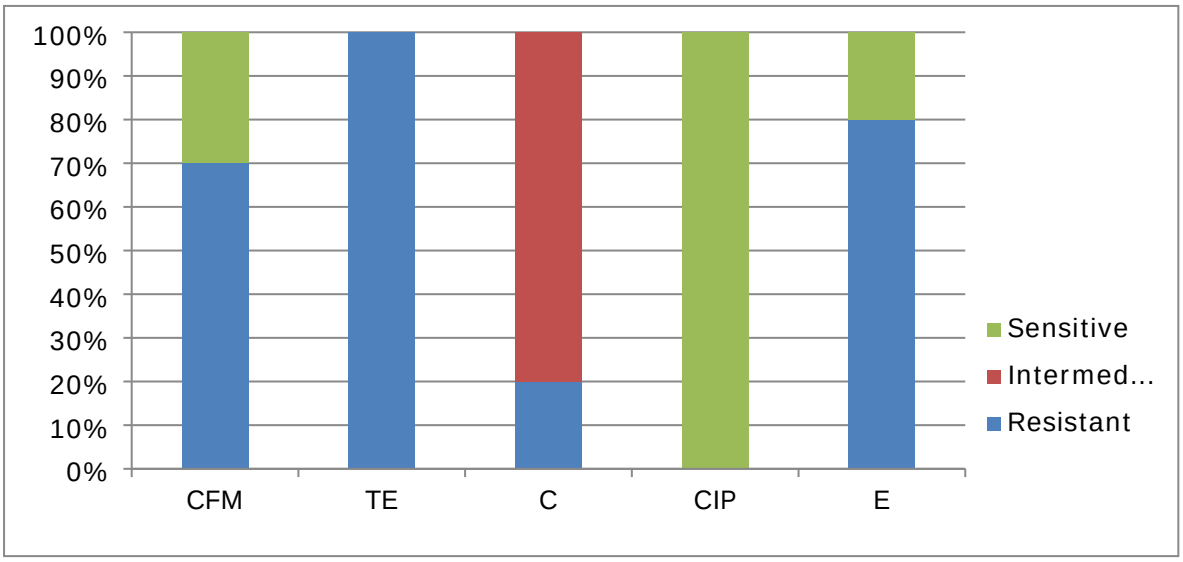

Fig: 7. Column diagram presenting the antibiotic sensitivity test of isolated *Shigella* spp.

(Legends: CFM= Cefixime, TE= Tetracycline, C = Chloramphenicol, CIP= Ciprofloxacin, E= Erythromycin).

Plate 1: Sample of prawn.

Plate 2: Tenfold dilution of prawn sample

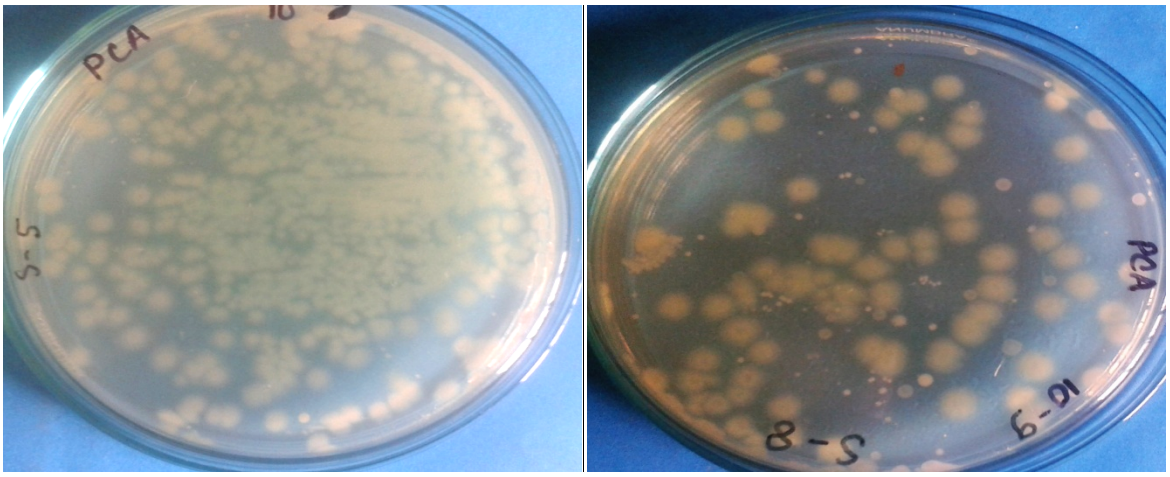

Plate 3: Colony of bacteria in plate count agar for TBC

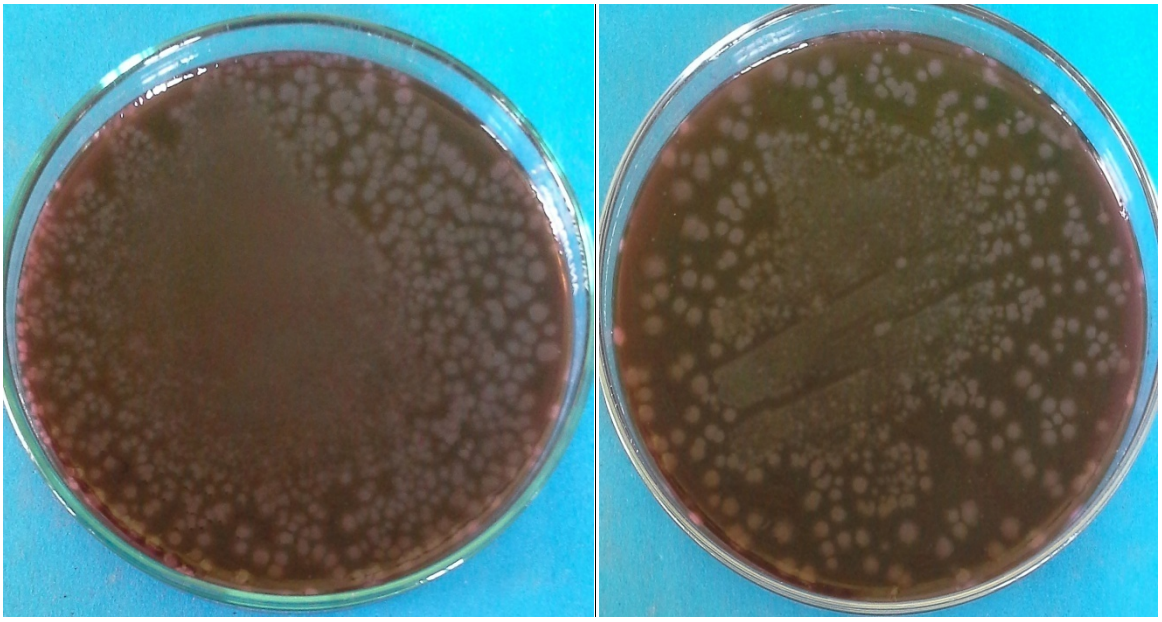

Plate 4: Colony of coliform in MacConkey agar for TCC

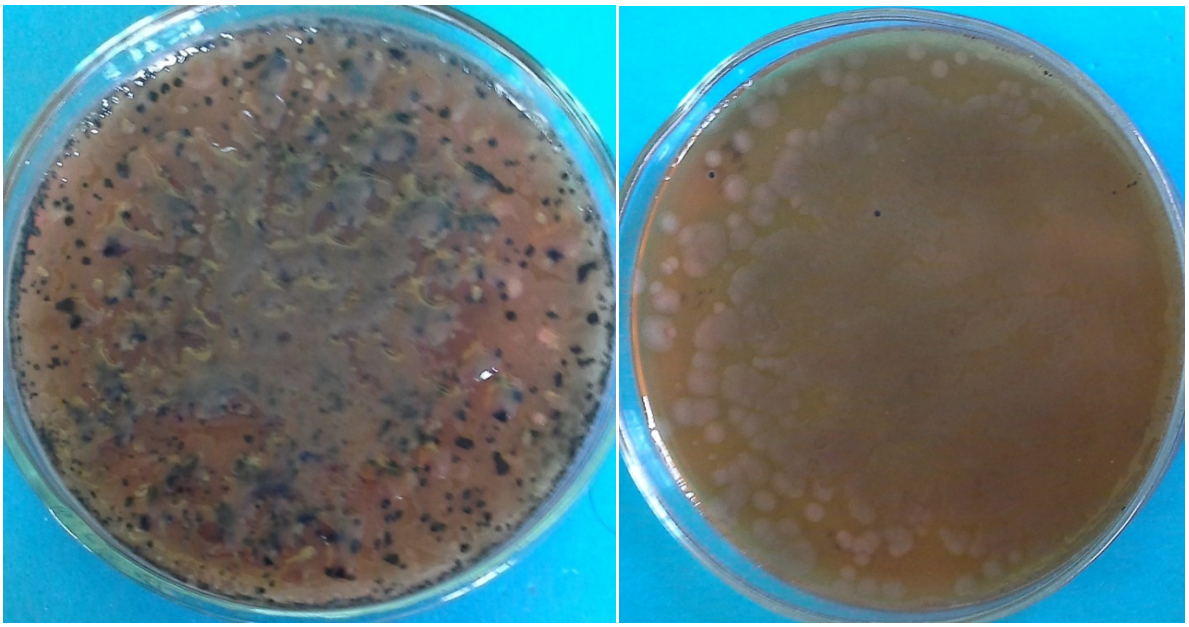

Plate 5: Colony of *Salmonella-Shigella* in SS agar for TSS

Plate 6: Colony of *Vibrio* spp. in TCBS agar for TVC

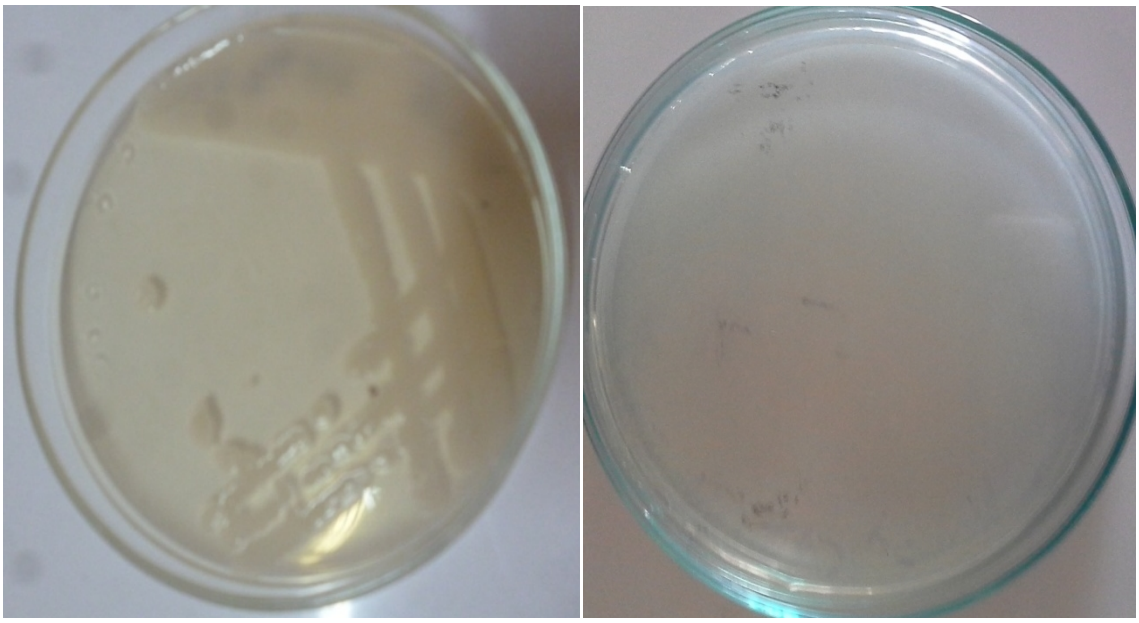

Plate 7: Pale colored colonies on nutrient agar

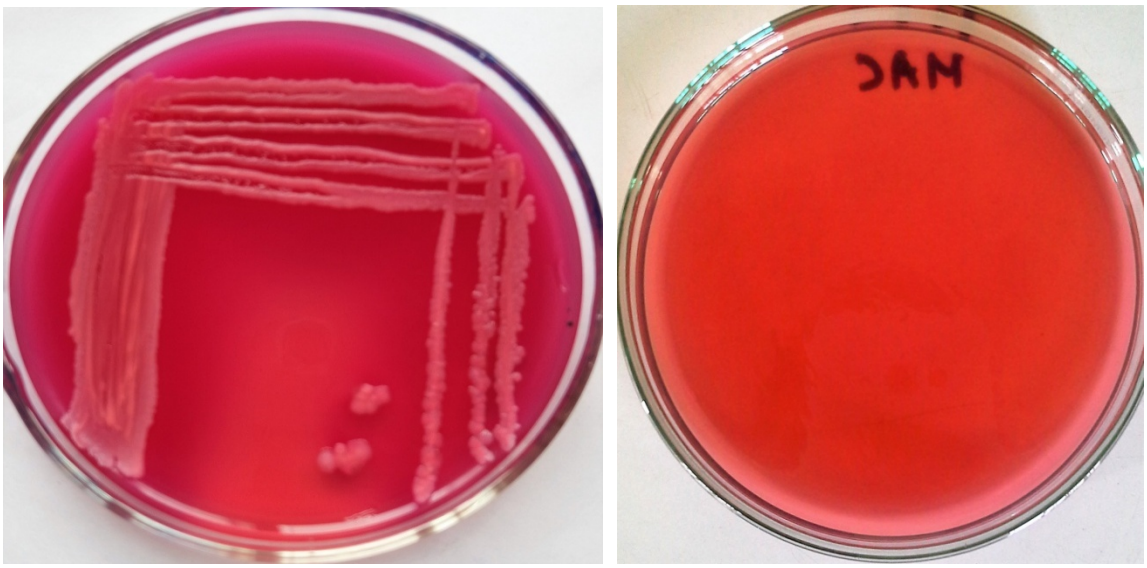

Plate 8: Bright pink colored colonies on MacConkey agar (left) and uninoculated control (right) indicating lactose fermenting bacteria.

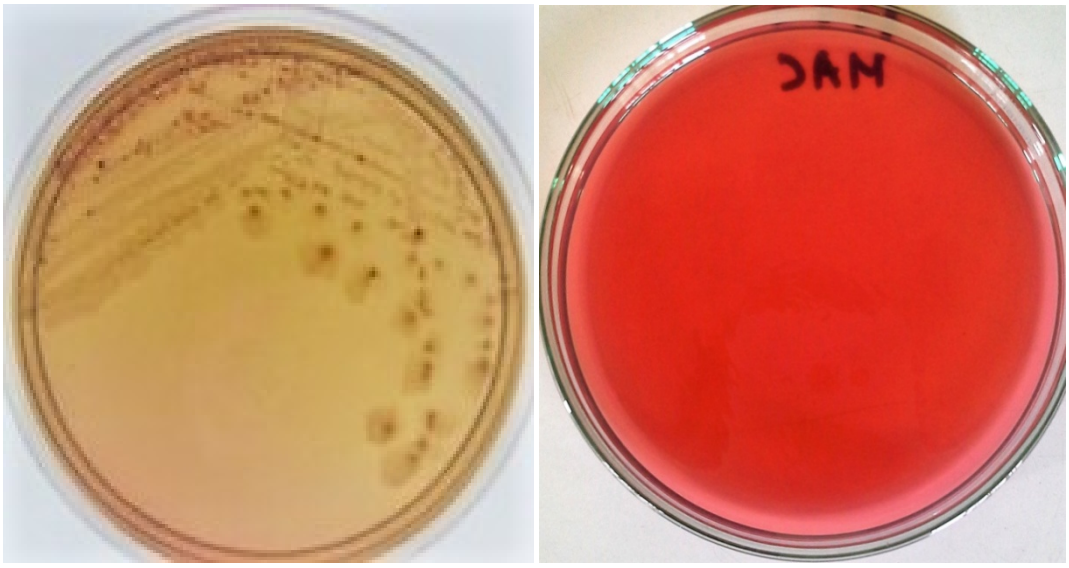

Plate 9: Pale colored colonies on MacConkey agar (left) and uninoculated control (right) indicating non-lactose fermenting bacteria

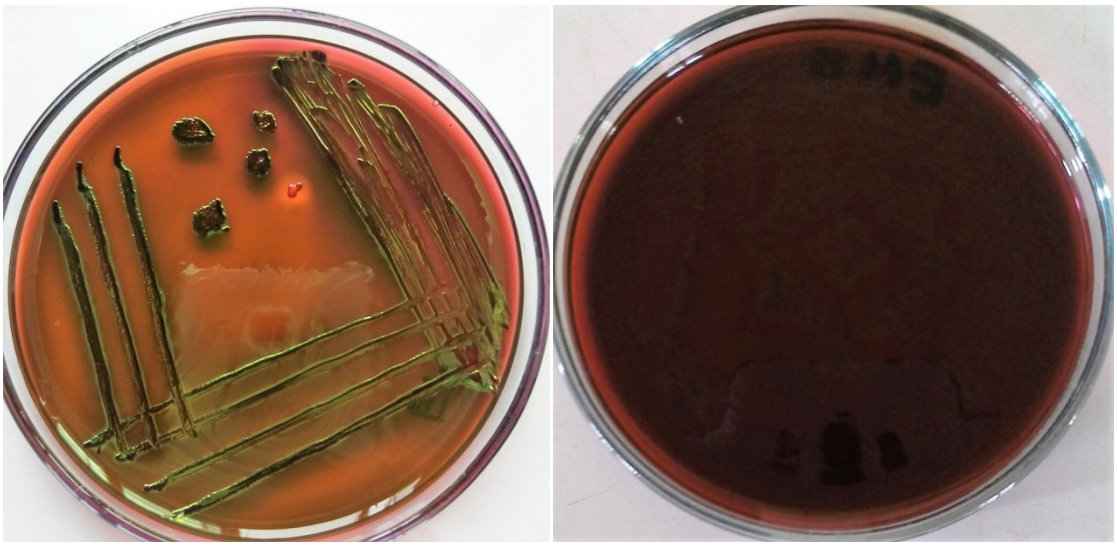

Plate 10: Metallic sheen produced by *E. coli* on EMB agar (left) and uninoculated control (right)

Plate 11: Black centers colonies produced by *Salmonella* spp. on SS agar (left) and un-inoculated control (right).

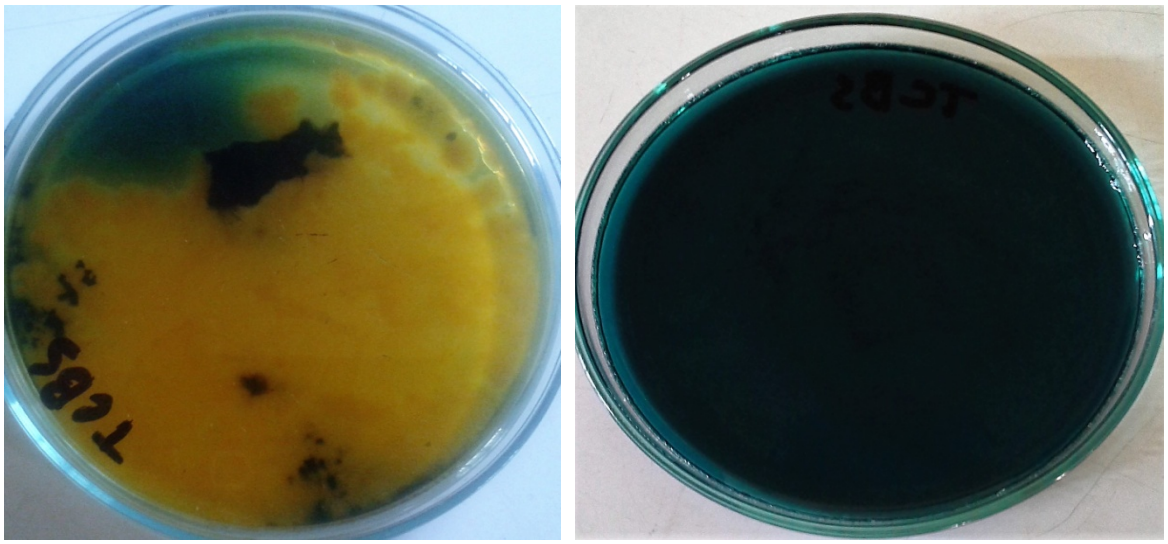

Plate 12: Yellow colonies produced by *Vibrio* spp. on TCBS agar (left) and uninoculated control (right)

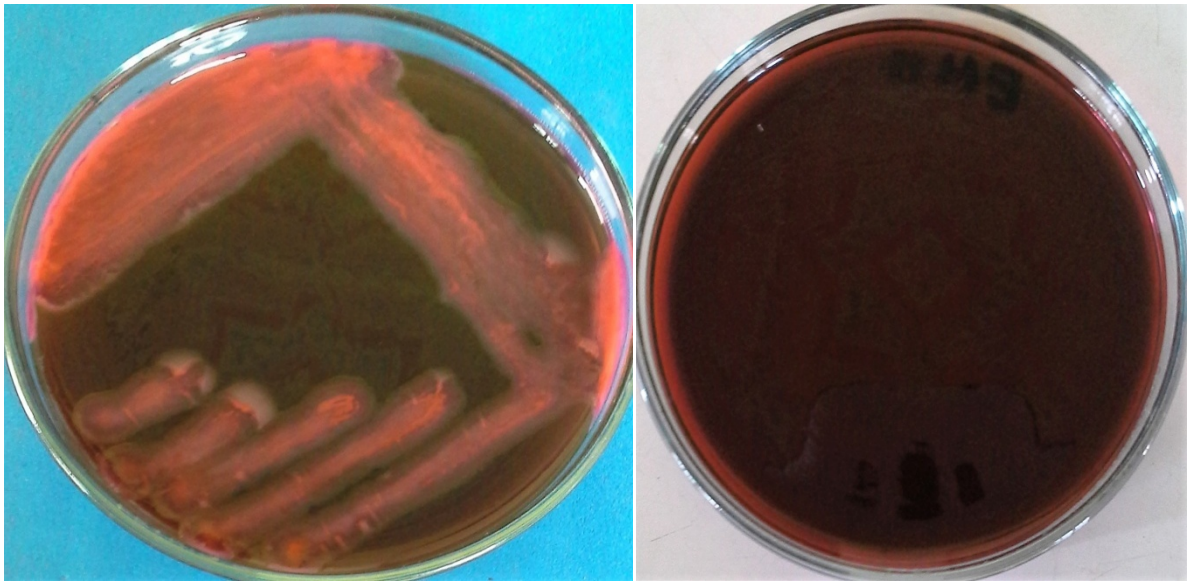

Plate 13: Pink colonies produced by *Klebsiella* spp. on EMB agar (left) and uninoculated control (right).

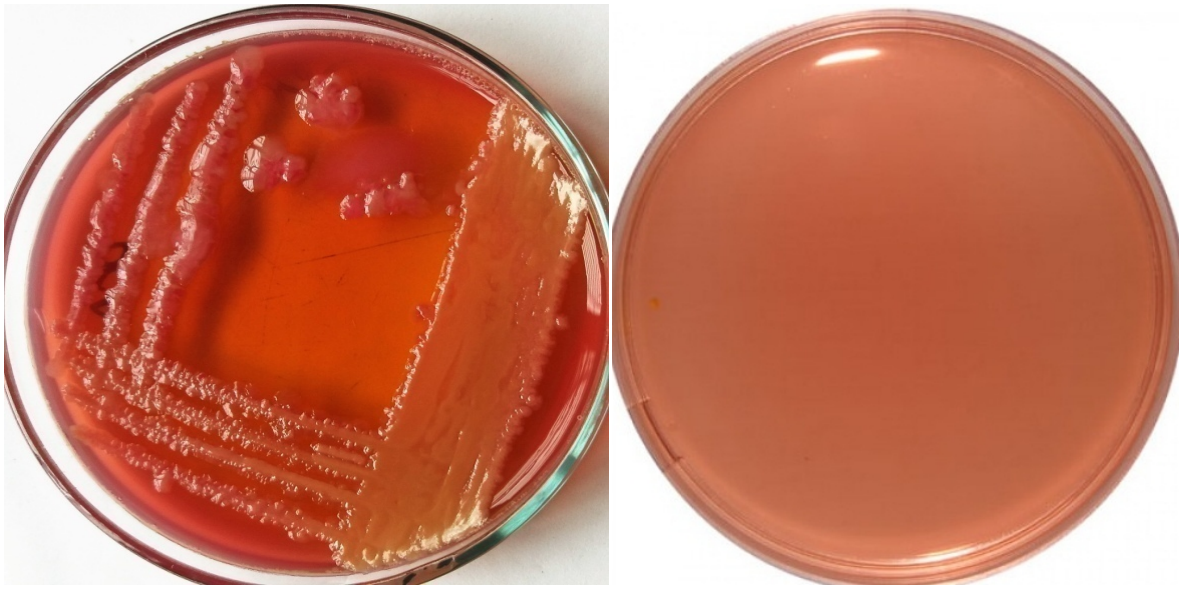

Plate 14: Colorless colonies produced by *shigella* spp. on DCA agar (left) and uninoculated control (right)

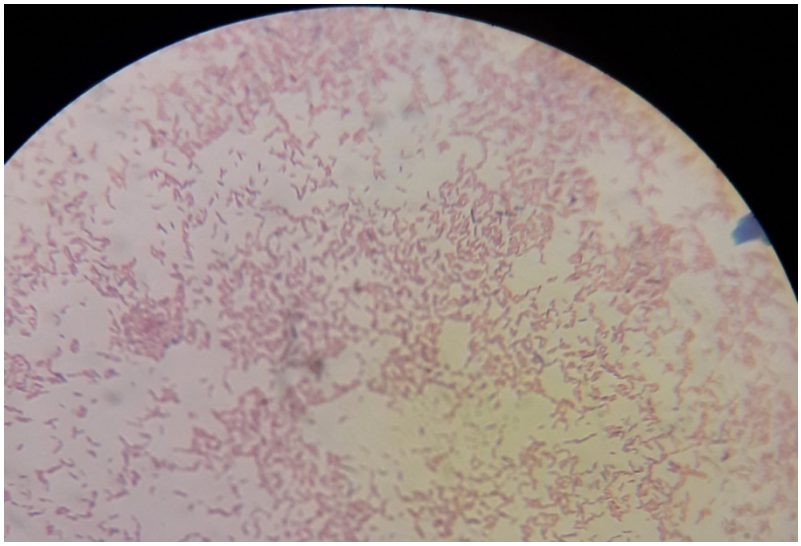

Plate15: *E. coli* showing at 100x magnification (Gram's staining).

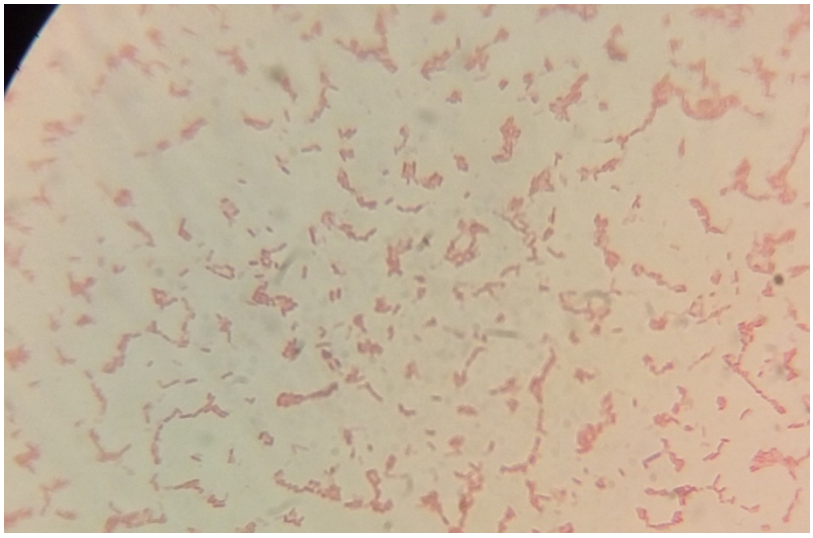

Plate16: *Salmonella* spp. showing at 100x magnification (Gram's staining)

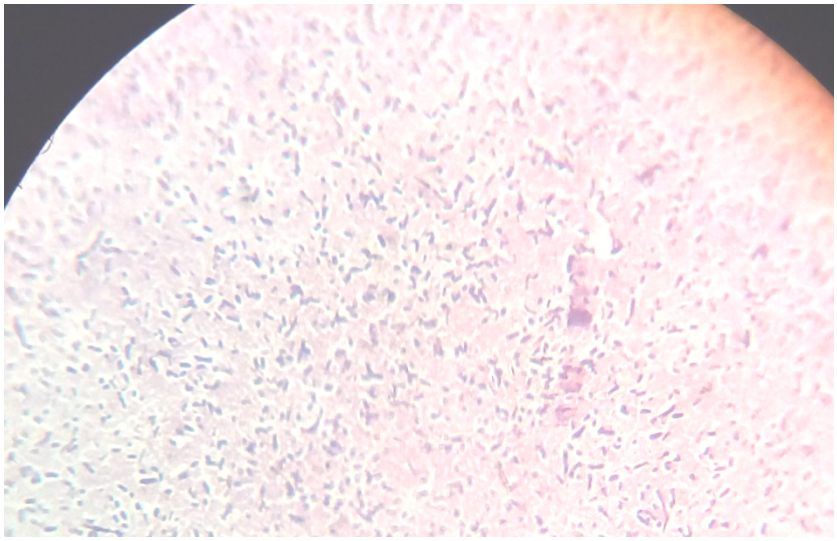

Plate 17: *Vibrio* spp. showing at 100x magnification (Gram's staining)

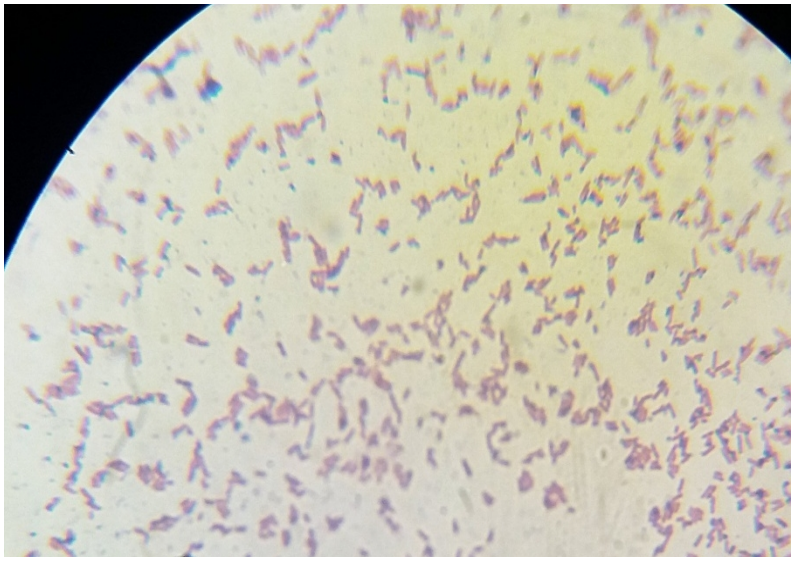

Plate 18: *Klebsiella* spp. showing at 100x magnification (Gram's staining)

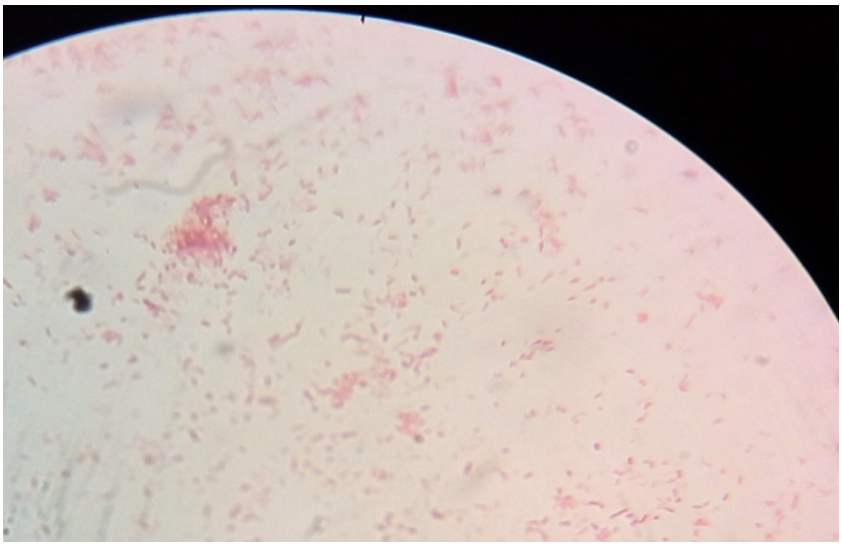

Plate 19: *Shigella* spp. showing at 100x magnification (Gram's staining)

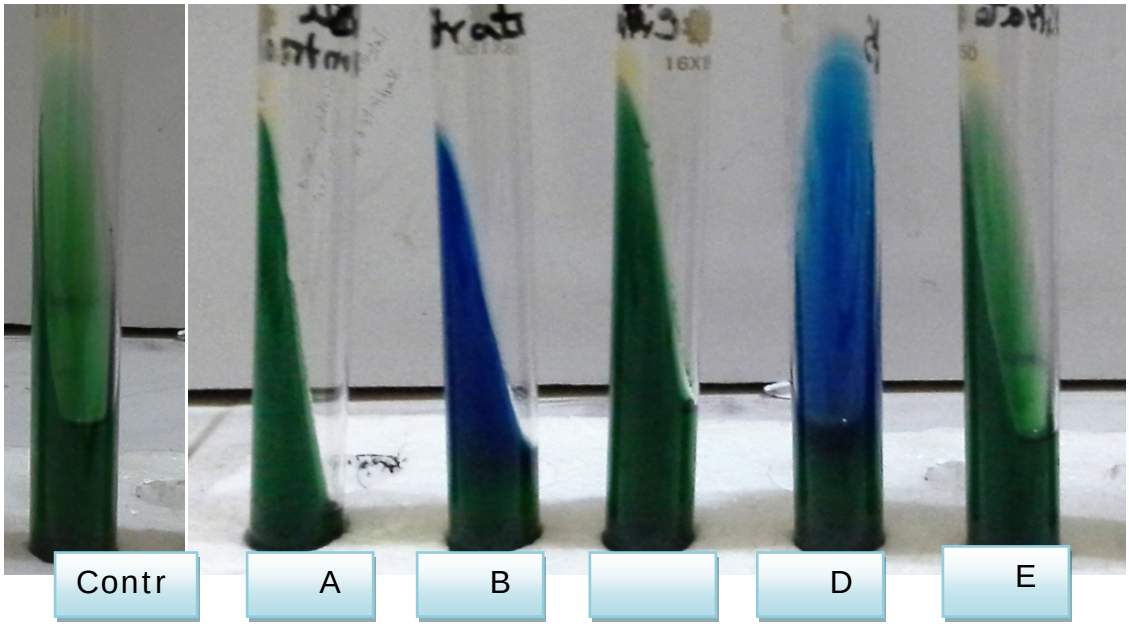

Plate 20: Citrate utilization test results (right) A= *E.* coli (negative), B= *Klebsiella* spp. (positive), C= *Salmonella* spp. (negative*)*, D *= Vibrio* spp. (positive) E= *Shigella* spp. (negative), and uninoculated control (left)

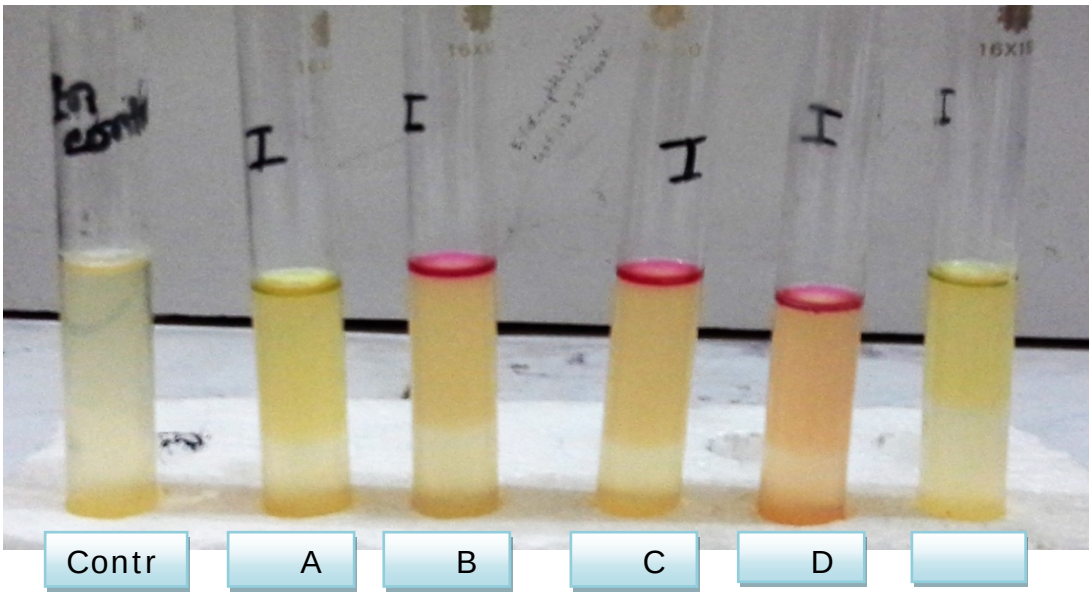

Plate 21: Indole test results (right) A= *Klebsiella* spp. (negative), B= *E.* coli (positive), D= *Vibrio* spp. (positive), *Shigella* spp. (positive), E *= Salmonella* spp. (negative) and uninoculated control (left)

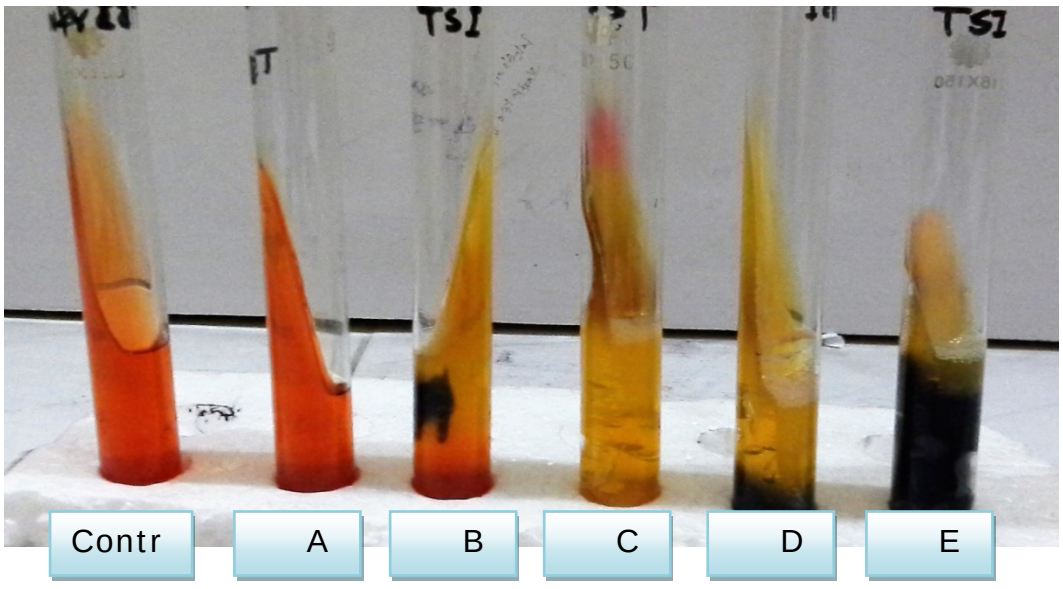

Plate 22: TSI test results (right) A= *E.* coli [S-A, B-A, gas (+), H_2S (-)], B= *Klebsiella* spp. [S-A, B-A, gas (+), H_2S (-)] C= *Vibrio* spp. [S-Al, B-A, gas(-), H2S (-)], D= *Shigella* spp.[S-Al, B-A, gas(-), H_2S (-)] E= *Salmonella* spp. [S-Al, B-A, gas(+), H_2S (+)]and uninoculated control (left)

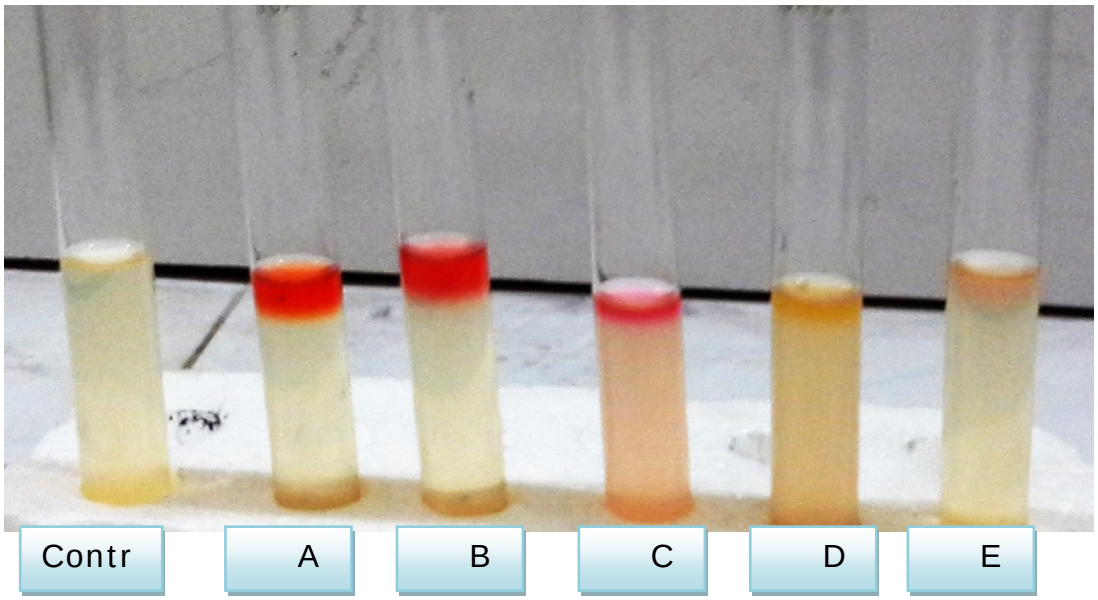

Plate 23: MR test results (right) A= *E.* coli (positive), B= *Shigella* spp. (positive), C= *Salmonella* spp. (positive*)*, D= *Klebsiella* spp. (negative), E= *Vibrio* spp. (negative) and uninoculated control (left)

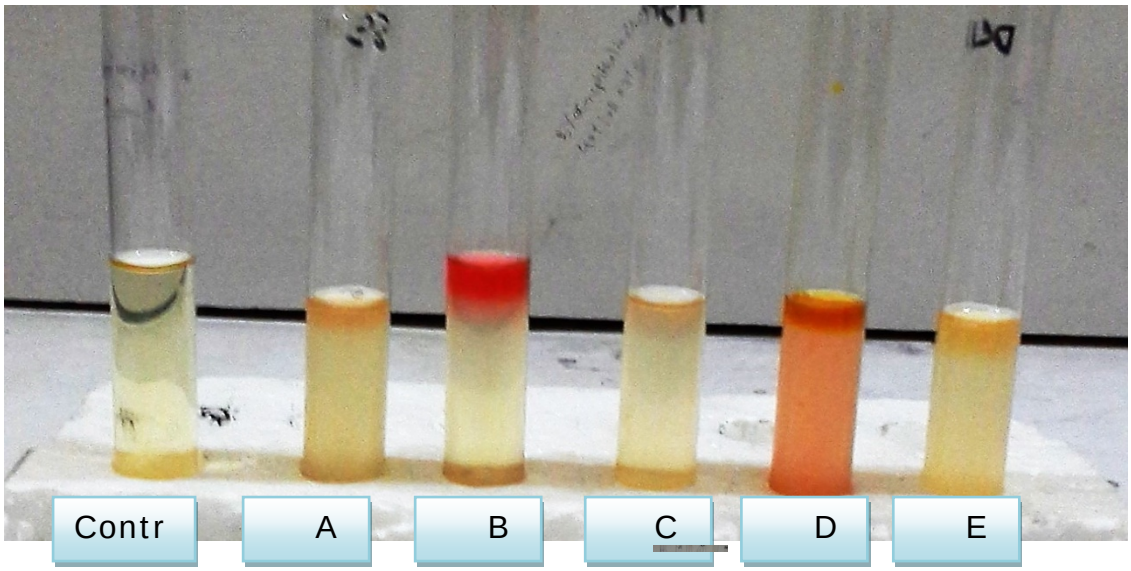

Plate 24: VP test results (right) A= *E.* coli (negative) B= *Klebsiella* spp. (positive), C= *Salmonella* spp. (negative), D= *Vibrio* spp. (positive), E = *Shigella* spp. (negative) and uninoculated control (left)

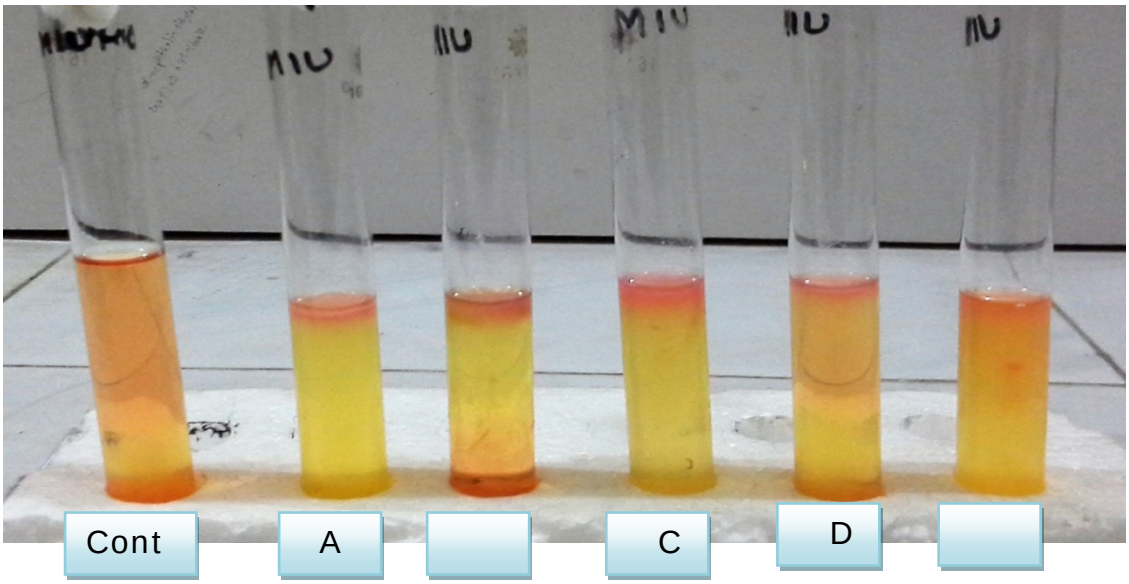

Plate 25: MIU test results (right) A= *Klebsiella* spp. (negative), B= *E.* coli (positive), C= *Shigella* spp. (negative), D= *Salmonella* spp. (positive), E = *Vibrio* spp. (positive) and uninoculated control (left)

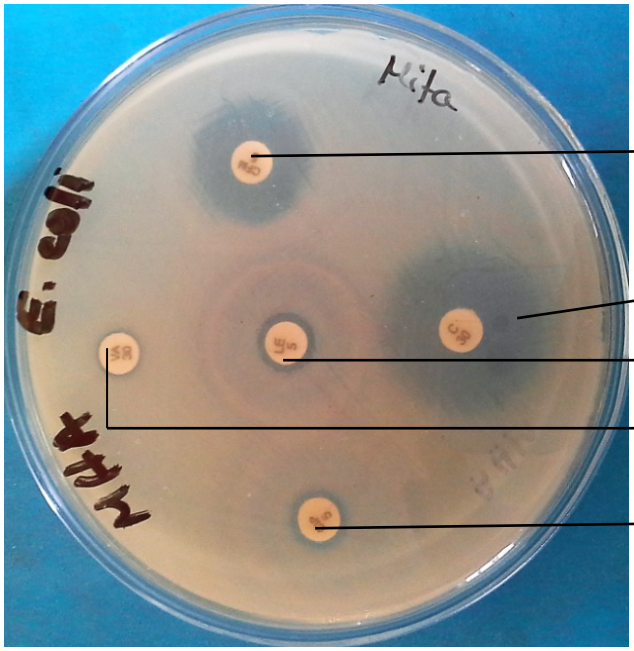

Plate 26: Antibiotic sensitivity test results of *E. coli* on Mueller-Hinton agar

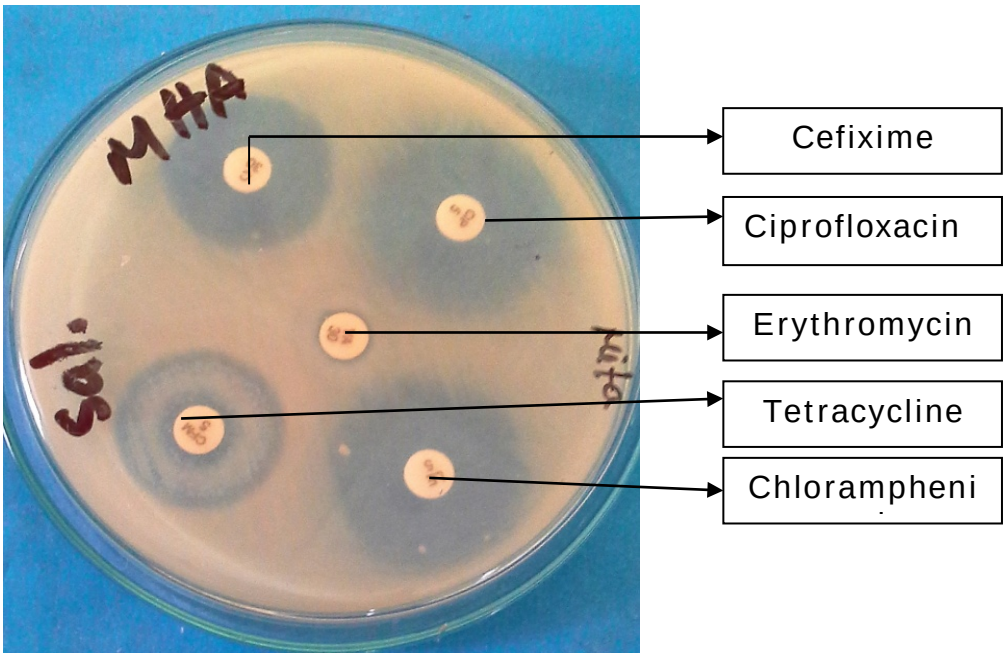

Plate 27: Antibiotic sensitivity test results of *Salmonella* spp. on Mueller-Hinton agar

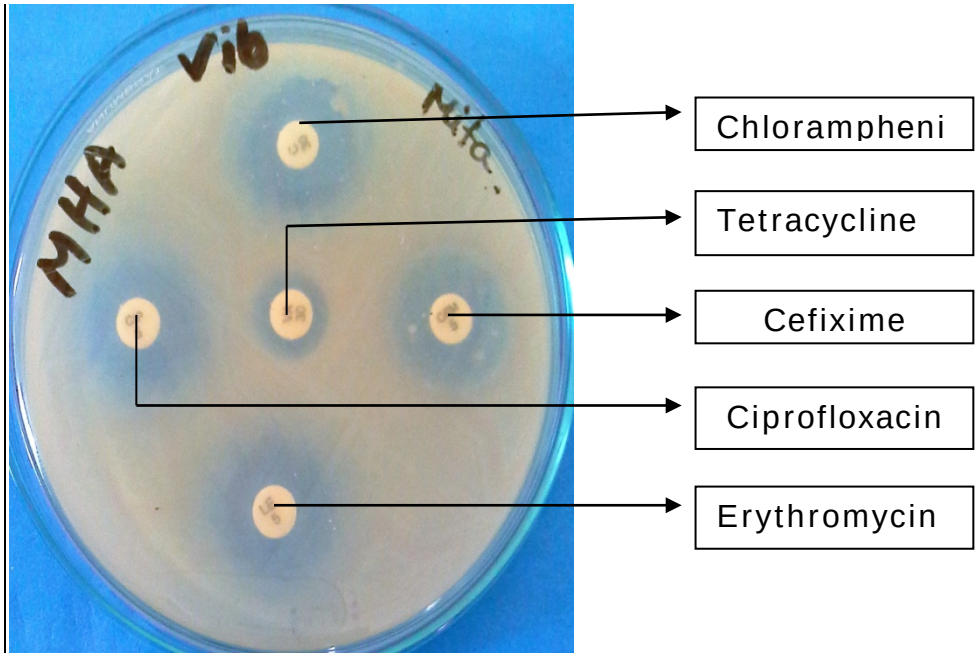

Plate 28: Antibiotic sensitivity test results of *Vibrio* spp. on Mueller-Hinton agar

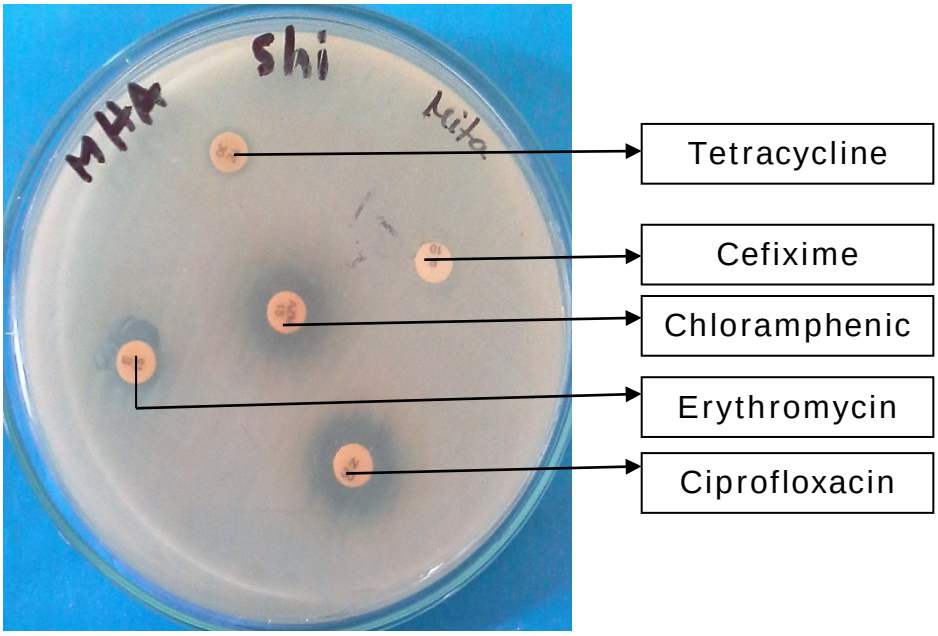

Plate 29: Antibiotic sensitivity test results of *Shigella* spp. on Mueller-Hinton agar

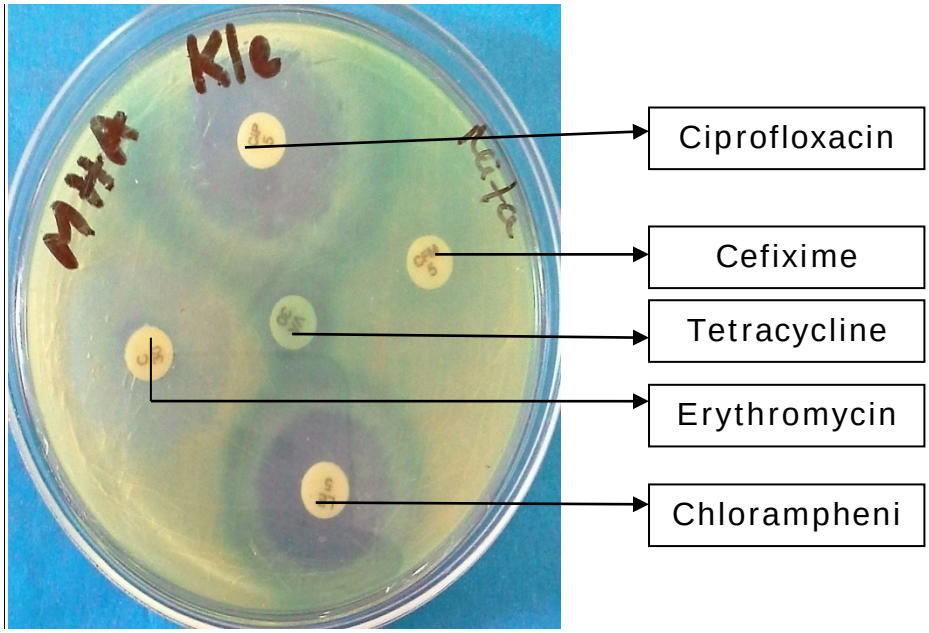

Plate 30: Antibiotic sensitivity test results of *Klebsiella* spp. on Mueller-Hinton agar

CHAPTER: 5

DISCUSSION

Fish and fish products are one of the favorite food items in Bangladesh. However, fish borne diseases may put the overall public health at a serious risk. By considering the public health significance, present study endeavored to emphasis the bacterial quality analysis of prawn samples. The present study reflected the Total Bacterial Count (TBC), Total Coliform Count (TCC), Total Salmonella-Shigella (TSS) Count, Total Vibrio Count (TVC) and antibiotic resistance pattern of the bacteria isolated from prawn samples.

Bacterial load in different prawn samples were shown in the table-2. From the results it is clear that all the prawn samples contain a significant amount of microorganisms. Mean±SEM of TBC in all prawn samples was 4.49±0.24 log10 CFU/g and all counts was significantly higher ($p<0.001$). The total bacterial count was within a range between 3.8×10^2 CFU/g to 2.8×10^6 CFU/g. The highest count was found in sample no 15 and the lowest count was found in sample no 8. Total bacterial counts for most the samples were generally high exceeding the limit of 1.0×10^2 CFU/g. Abu Hena Yousuf *et al*. (2008) reported that the total viable count of prawn was 1.08×10^2 to 1.2×10^5CFU/ml. Ramesh Babu K *et al*. (2017) also found the load of viable bacteria in prawn samples, were ranged between 1.82×10^2cfu/g to 3.23×10^2cfu/g. Najmus S. Khan *et al.* (2012) found that the total bacterial count of prawn ranged between 4.37×10^5 to 0.328×10^5 CFU/g.

Most of the prawn samples in our study were found to be unfavorable for consumption because many of them showed the presence of coliforms (*E. coli* and *Klebsiella* spp.). Mean±SEM of TCC in all prawn samples was 5.20±0.29 log10 CFU/g and all counts was significantly higher ($p<0.001$). The total coliform count was found to be higher ranged which was between 4.9×10^2 CFU/g to 3.52×10^7 CFU/g in all parts of the prawn sample. The highest count was found in sample no 15 and the lowest count was found in sample no 17. According to ICMSF (1986), total

coliform limits can be $1.0x10^2$ CFU/g in the food, but most of the prawn samples were exceeding the limit. Abu Hena Yousuf *et al.* (2008) showed the presence of coliform in prawn which was ranged between $5x10^2$ to $4.4x10^4$cfu/ml. Ramesh Babu K. *et al.* (2017) also found $2.14\times10^2$to 5.22×10^2cfu/g coliform in prawn. In comparison with these studies, large numbers of coliforms were found in our study. The total coliform counts were shown in table 2 and all of the samples were found to exceed the ICMSF standards.

Salmonella and *Shigella* may cause human disease such as food poisoning through the production of toxins. A few reports have shown the prevalence of Salmonella-Shigella counts in prawn samples. Abu Hena Yousuf *et al.* (2008) found total Salmonella-Shigella counts ranged between $0.26x10^2$ to $0.96x10^4$cfu/ml. In our study, the total Salmonella-Shigella (TSS) counts of prawn samples were ranged between 1.65×10^2 CFU/g to 1.3×10^7 CFU/g.The highest count was found in sample no 4 and the lowest count was found in sample no 28. Mean±SEM of TSS in prawn samples was 4.60±0.30 log10 CFU/g and all counts was significantly higher ($p<0.001$).

Microbial quality of prawns is related to *Vibrio* counts. Mean±SEM of TVC in prawn samples was 5.59±0.21 log10 CFU/g and all counts was significantly higher ($p<0.001$).The total vibrio counts (TVC) of thirty samples were ranged between 4.0×10^3 CFU/g to 2.5×10^7 CFU/g. The highest count was found in sample no 18 and the lowest count was found in sample no 21.

The Mean±SEM of TBC found in prawn from Bahadur bazaar, Railgate bazaar and Gopalgong bazaar were 4.58±0.43 log10 CFU/g, 4.13±0.46 log10 CFU/g and 4.76±0.39 log10 CFU/g respectively. The Mean±SEM of TCC found in prawn from Bahadur bazaar, Rail gate bazaar and Gopalgong bazaar were 4.54±0.51 log10 CFU/g, 6.13±0.41log10 CFU/g and 4.95±0.42 log10 CFU/g respectively. The Mean±SEM of TSS found in prawn from Bahadur bazaar, Rail gate bazaar and Gopalgong bazaar were 4.00±0.54 log10 CFU/g, 5.29±0.40 log10 CFU/g and 4.51±0.59 log10 CFU/g respectively. The Mean±SEM of TVC found in prawn from

Bahadur bazaar, Rail gate bazaar and Gopalgong bazaar were 5.75±0.32 log10 CFU/g, 5.79±0.32 log10 CFU/g and 5.21±0.43 log10 CFU/g respectively. But the bacterial load found in prawns did not varied significantly among these three local markets.

The pathogenic profile in this study conferred that the overall bacteriological quality of the prawn samples was not satisfactory. In most of the cases, the pathogenic load exceeded safety limit (ICMSF, 1986) which might have a great effect on overall public health. The routes of contamination might be unhygienic handling, contaminated water source, improper packaging, transportation and storage which were supported by Antony *et al.* (2002).

The study revealed that the bacterial pathogens isolated from prawn samples were *E. coli* (100%), *Salmonella* spp. (100%), *Vibrio* spp. (66.7%), *Klebsiella* spp. (40%) and *Shigella* spp. (33.3%). Abu Hena Yousuf *et al.* (2008) also isolated *Vibrio* sp. (43.75%) and *Salmonella* sp. (25%), *Shigella* sp. (12.5%) and *Staphylococcus auerus (*6.25%) from prawn and shrimp samples.

N. Narasimhan *et al.* (2013) isolated different bacterial strains such as *Aureobacterium faciens, Aeromicrobium erythreum, Bacillus subtilis, Escherichia coli, Vibrio cholarae, Enterobacter aerogens, Micrococcus Luteus, Pseudomonas putida, Pseudomonas aeruginosa and Enterococcus pseudo avium* from muscle tissues of prawn samples (*P.monodon)*.

The antibiotic sensitivity was indicated by diameter of the Zone of growth inhibition by specific antibiotic supported by EUCAST, 2015.

Samia et al. (2013) used a new aspect on their investigation related of antibiogram study of the pathogenic isolates found in the prawn samples and found that isolated *Klebsiella* spp. was resistant against Chloramphenicol (40%) and sensitive to Ciprofloxacin (90%) and on the other hand isolated *Shigella* spp. was resistant against Chloramphenicol (58%) and sensitive to Ciprofloxacin (90%).

Thomas M. Berry *et al.* found that most of the isolates were resistance against Oxytetracycline, Ampicillin, Erythromycin and sensitive to Chloramphenicol and Ormetoprim-sulfadimethoxine.
In this study, *E. coli* showed resistance against Cefixime (100%), Tetracycline (100%), Erythromycin (90%) and sensitive to Chloramphenicol (100%) and Ciprofloxacin (83.3%).

Salmonella spp. showed resistance against Tetracycline (100%), Erythromycin (86.7%) and sensitive to Cefixime (100%), Chloramphenicol (90%) and Ciprofloxacin (100%). *Vibrio* spp. showed resistance to Cefixime (75%), Tetracycline (100%), Erythromycin (100%) and sensitive to Chloramphenicol (100%) while Ciprofloxacin (80%) was found intermediate. *Klebsiella* spp. showed resistance to Cefixime (75%), Tetracycline (100%), Erythromycin (100%) and sensitive to Chloramphenicol (83.3%) and Ciprofloxacin (100%). *Shigella* spp. showed resistance to Cefixime (70%), Tetracycline (100%), Erythromycin (80%) and sensitive to Ciprofloxacin (100%) while Chloramphenicol (80%) was found intermediate.
In this study, all of the isolates were found to be 100% resistant against Tetracycline. Cefixime and Erythromycin also found resistant in most of the isolates.
During this study, it was observed that collected samples from local fish market were more contaminated. The fact is that, all products such as fish and prawns are stored together in local market; as a result cross contamination may occur. Not only that but also long time storage until sold and sometimes improper storage condition due to technological disruption can be responsible for microbial contamination of prawns.

CHAPTER: 7
CONCLUSION

Overall, the present study investigated the prawns of local fish markets which had been found to harbor a huge array of pathogenic microorganisms. *Escherichia coli, Salmonella* spp.*, Vibrio* spp.*, Klebsiella* spp*. and Shigella* spp. were isolated from most of the samples. It was indicating that these prawn samples have not been protected from the microbial spoilage during handling, packaging, storage, and transport. Appropriate maintenance of microbiological quality is thus a vital aspect of quality control measures of such prawns. The study of antibiogram revealed that the most of the pathogens were found to be resistant to commonly used antibiotics. Most of the isolates were resistance against Cefixime, Tetracycline and Erythromycin.

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

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

i. The presence of *E.coli*, *Salmonella* spp.*, Vibrio* spp., *Klebsiella* spp and *Shigella* spp. in most of the samples are public health concern.
ii. Total bacterial count, total coliform counts, total salmonella-shigella count and total vibrio count were successfully performed from different prawn samples.
iii. High counts of bacteria in all prawn samples indicate that consumption of prawn without proper cooking is harmful and also accelerated the public health threat.
iv. The antibiotic resistance properties of isolated bacteria can cause serious health hazards because of ineffective treatment of the sufferers by the commonly prescribed antibiotics.

REFERENCES

Abu Hena Muhammad Yousuf, Md. Kawser Ahmed, Sabina Yeasmin, Nazmul Ahsan, Md. Mujibur Rahman and Md. Monirul Islam(2008). Prevalence of Microbial Load in Shrimp, Penaeus monodon and Prawn, Macrobrachium rosenbergii from BangladeshWorld Journal of Agricultural Sciences 4 (S): 852-855.

Antony, M. M. Jeyasekaran, G. Shakila, R. J. and Shanmugam, S. A. (2002). Microbiological quality of raw shrimps processed in seafood processing plants of Tuticorin, Tamil Nadu, India. Asian Fisheries Science 15 (1): 33-41.

Anwar Hossain, Shankar Chandra Mandal, Mohammad Shamsur Rahman, Md. Mofizur Rahman and Mahmud Hasan (2010). Microbiological Quality of Processed Frozen Black Tiger Shrimps in Fish Processing Plant. World Journal of Fish and Marine Sciences 2 (2): 124-128.

applications. Toronto, Ontario, Canada, University of Toronto Press (2nd edition).

Babalola and A. A. Ogun (2008). Microbial studies on frozen shrimps processed in Ibadan and Lagos, Nigeria. Scientific Research and Essay 3 (11): 537-546.

Bauer AW, Kirby WMM, Strerris JC and Turk M (1999). Antibiotic susceptibility testing by a standard singledisk method. American Journal of Clinical Pathology, 45: 493-496.

Bauer, A. W., Kirby, W. M. M., Sherris, J. C. and Tierch, M. (1966). Antibiotic susceptibility testing by a standardized single disc method. American Journal of Clinical Pathology 45 (4): 493-496.

Bhatta, D. R., Bangtrakulnonth, A., Tishyadhigama, P., Saroj, S. D., Bandekar, J. R., Hendriksen, R. S. and Kapadnis, B. P. (2007). Serotyping, PCR, phage-typing and antibiotic sensitivity testing of *Salmonella* serovars isolated from urban drinking water supply systems of Nepal. Letters in Applied Microbiology 44 (6): 588-594.

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

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

Cheesbrough, M. (1984). Medical laboratory manual for tropical countries. 1st edn. Vol. 02 Microbiology, English Language Book Socity, London, 35:40-57.

Cowan, S.T. and Steel, K.T. (1985). Manual for the Identification of Medical Bacteria. 2nd edition. Cambridge University Press, London, 22-122.

Cray, W. C. J. and Moon, H. W. (1995). Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. Applied Environmental Microbiology 61 (4): 1586-1590.

Edema,M.O., A.M. Omemu and M.O. Bankole, (2005). Microbiological safety and quality of ready-to-eat foods in Nigeria. University of Agriculture, Abeokuta, : 26.

EUCAST (2015). Breakpoint tables for interpretation of MICs & zone diameters, version 5.0, valid from 01.01.2015.

Euloge Y. Kpoclou, Victor B. Anihouvi, Paulin Azokpota, Mohamed M. Soumanou, Georges Daube, Caroline Douny, François Brose, Marie-Louise Scippo, D. Joseph Hounhouigan, (2013). Microbiological and Physico-Chemical Quality of Smoked Shrimp, An Expanding Food Condiment in Beninese Local Markets. Food and Public Health, 3(6): 277-283.

European Commission, (2002). Opinion of the Scientific Committee on Food on the risks to human health of Polycyclic Aromatic Hydrocarbons in food, SCF /CS /CNTM /PAH/29 Final. (http:// europa. eu. int/ comm/ food /fs /sc/scf/out153_en.pdf).

FAO/WHO Codex Alimentarius Commission. (2000). Recommended Codes of Practice: Shrimps/Prawns (CAC/RCP 17-1978); Quick Frozen Shrimps/Prawns (Rev. 1-1995).

Fatma Hassan Mohamed Ali (2011). Quality Evaluation of Some Fresh and Imported Frozen Seafood. Advance Journal of Food Science and Technology 3(1): 83-88.

G. M. M. Anwarul Hasan, Md. Sabir Hossain, Sahana Parveen and Meher Nigad Nipa (2012). Microbiological Assessment of Rui (*Labeo rohita*), Catla (*Catla catla*), Tilapia *(Oreochromis mossambicus*) of Cultured Ponds and Different Markets of Bangladesh. International Journal of Science and Research 3.:358.

Gubala, A. J. and Proll, D. F. (2006). Molecular-Beacon Multiplex Real-Time PCR Assay for Detection of *Vibrio cholerae*. Applied Environmental Microbiology 72 (9): 6424–6428.

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

International Commission on Microbiological Specifications for Foods (ICMSF),(1986). Microorganisms in foods 2-Sampling for microbiological analysis: principles & specific

Jakee, J. E., Moussa, E. I., Mohamed, K. F. and Mohamed, G. (2009). Using Molecular Techniques for Characterization of *Escherichia coli* Isolated from Water Sources in Egypt. Global Veterinaria 3 (5): 354-362.

Karunasagar, I., Pai, R., Malathi, G. R., and Karunasagar, I. (1994). Mass mortality of *Penaeus monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. Aquaculture 128: 203-209.

Leera Solomon, Chimezie J. Ogugbue and Gideon C. Okpokwasili (2013). Antibiotic Resistance Profiles of Bacteria Associated with Fresh and Frozen Shrimp (*Palaemonetes* sp.) and Their Public Health Significance. International Journal of Scientific Research in Knowledge 1(10): 448-456.

Mauro Faber de Freitas Leitão and Daniel de Pinho Astacio Rios (2000). Microbiological and chemical changes in freshwater prawn

(*macrobrachium rosembergii*) stored under refrigeration. Brazilian Journal of Microbiology. 31:178-183.

Md. Abdul Quaiyum, Md. Mofizur Rahman, Bhakta Supratim Sarker, Md. Masud Alam, Najmus Sakib Khan, Mohammad Shamsur Rahman and Rokeya siddiqui (2012). Microbiological quality assessment of Chapila (*Gudusia chapra*) and Tengra (*Mystus vittatus*) in Bangladesh. Stamford Journal of Microbiology, 2(1).

Md. Golam Sarwer, Sayed Abdus Sadik, Ahnaf Tahmid and Md. Mahmudul Hasan Rony (2016). Microbial quality assurance of finished, cooked and raw shrimp (*Penaeus monodon*) products of a marine fish processing industry of Bangladesh. Journal of Entomology and Zoology Studies; 4(6): 263-267.

Md. Rokibul, H., Mrityunjoy, A., Eshita, D., 1Kamal, K. D., Tasnia, A., Muhammad, A. A., Kazi., K. F. and Rashed, N. (2013). Microbiological study of sea fish samples collected from local markets in Dhaka city. International Food Research Journal 20(3): 1491-1495.

Md. Shakir Hossain, Mehedi Mahmudul Hasan, Md. Golam Sarwer and Shuva Bhowmik (2015). Comparative analysis of microbiological status between raw and ready-to-eat product of black tiger shrimp (Penaeus monodon). International Journal of Biosciences 6 (8): 43-49.

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

Mobin SMA, Chowdhur MB, Islam MS, Uddin MN (2001). Status of bacterial flora in the intestine of two freshwater fish. Bangladesh Journal of Life Science, 13 (1&2): 149-155.

N. Narasimhan, T. Ravimanickam, M. Sukumaran, R. Ravichelvan, R. Ravichandran and D. Madhavan (2013). Pathogenic bacteria isolated from tiger prawn *penaeus monodon* in shrimp culture ponds at east coast of thanjavur district tamil nadu, india. International journal of research in biological sciences; 3(2): 98-101.

Nadia A. Abd-El-Aziz and Y.G. Moharram (2016). Microbiological quality of imported frozen shrimp in Egypt. Annals of Agricultural Science 61(1):35–40.

Najmus Sakib Khan, M.R. Islam, M. Belal Hossain, M. Abdul Quaiyum, M. Shamsuddin and Jiban Krishna Karmaker (2012). Comparative Analysis of Microbial Status of Raw and Frozen Freshwater Prawn (Macrobrachium rosenbergii). Middle-East Journal of Scientific Research 12 (7): 1026-1030.

Phyllis, E., (2007). Food safety: new perspectives. ASM Press. Virginia,USA., : 414.

Ramesh Babu K., Govinda Rao V., Krishna N. M., Geetha S. and Kakara R. R. (2017). Assessment of Bacteriological Quality in Selected Commercially Important Shrimps of Visakhapatnam, East Coast of India. International Journal of Microbiology and Biotechnology; 2(2): 102-105.

S. Harakeh, H. Yassine, and M. El-Fadel, (2006). “Antimicrobialresistant patterns of *Escherichia coli* and *Salmonella* strains in the aquatic Lebanese environments,” *Environmental Pollution*. 143 (2):269–277.

Samia, S., Galib, H. T., Tanvir, A. S., Basudeb, C. S., Md. Walliullah, Tasnia, A., Md. Sakil, M., Afsana, F. N., Sadia, K. P., Kamal, K. D., Mrityunjoy, A., Nusrat, J. U., Tasmina, R. and Rashed, N. (2014). Microbiological quality analysis of shrimps collected from local market around Dhaka city. International Food Research Journal 21(1): 33-38.

Snowdon, J. A., Cliver, D. O. and Converse, J. C. (1989). Land disposal of mixed human and animal wastes: a review. Waste Management Research 7: 121-134.

Starutch, D. (1991). Survival of pathogenic microorganisms and parasites in excreta, manure sand sewage sludge. Review of Science and Technology 10 (3): 813-846.

Thomas M. Berry., Douglas I. Park and Donald V. Lightner (1994). Comparison of the Microbial Quality of Raw Shrimp from China,

Ecuador, or Mexico at Both Wholesale and Retail Levels. Journal of Food Protection, 57(2):150-153.

Titik Budiati, Gulam Rusul, Wan Nadiah Wan-Abdullah, Rosma Ahmad and Yahya Mat Arip(2015). Microbiological Quality of Catfish (Clarias Gariepinus) and Tilapia (Tilapia Mossambica) Obtained from Wet Markets and Ponds in Malaysia. J Aquac Res Development, 6:1.

W. H. Andrews and T. S. Hammack (2001) “Food sampling and preparation of sample homogenate,” in United States Food and Drug Administration (US FDA) Bacteriological Analytical Manual, chapter 1, United States Food and Drug Administration, Silver Spring, Md,USA,2001,http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm063335.htm.

Wallace, B. J., Guzewich, J. J., Cambridge, M., Altekruse, S. and Morse, D. L. (1999). Seafood-Associated Disease outbreaks in New York, 1980-1994. American Journal of Preventive Medcine 17 (1): 48-54.

World Health Organization (WHO) 2012. Alzheimers disease: the brain killer. Downloaded from http://www.searo.who.int/en/section1174/section1199/section1567/section1823-8066.htm on 26/7/2012.