STUDIES ON THE BACTERIAL CONTENTS OF PUBLIC HEALTH CONCERN IN FRESHWATER CULTURED PRAWN (*Macrobrachium rosenbergii*) AND POND WATER

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STUDIES ON THE BACTERIAL CONTENTS OF PUBLIC HEALTH CONCERN IN FRESHWATER CULTURED PRAWN (*Macrobrachium rosenbergii*) AND POND WATER

A Thesis

by

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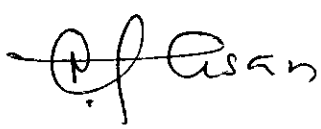
Approved as to Style and Content by



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Prof. Dr. Md. Nazmul Ahsan

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October, 2010.

Declaration

Studies on the bacterial contents of public health concern in freshwater cultured prawn (*Macrobrachium rosenbergii*) and pond water

The results presented in this thesis with the above mentioned title are entirely of the candidate's own investigation and no part of the results are being concurrently submitted for any degree.

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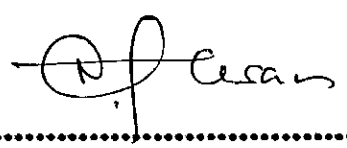
(Signature of the candidate)

Md. Shahjahan Ali

Examination Roll No.: MS. 090602

Session : 2008-2009



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(Signature of the supervisor)

Prof. Dr. Md. Nazmul Ahsan

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October, 2010.

Dedicated

to

My Respectable Parents

Acknowledgement

All the praises belongs to Almighty Allah, the Creator and the Ruler of the universe whose grace and mercy keeps me alive and enables me to purse my education in Fisheries and Marine Resource Technology Discipline of Khulna University and to complete the thesis for the degree of MS in Fish Processing and Quality Control.

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ABSTRACT

Quantitative and qualitative analyses of bacterial content of public health concern of cultured giant freshwater prawn (*Macrobrachium rosenbergii,* De Man, 1879) and pond water collected from the five different farms of Phultala Upazela in the district of Khulna were carried out in this study during November 2009 following the ISO standard methods. Different bacterial parameters of 15 prawn and 15 water samples were tested and total bacterial count (Standard Plate Count) found in prawn ranged from $3.2x10^5$/gm to $5.70x10^5$/ gm while $1.26 x10^4$/ml to $1.72x 10^4$/ml in pond water. On the other hand, total coliforms found in prawn ranged between 43 and 460/ gm, whereas in water 43 to 240/100ml total coliforms were detected. In case of faecal coliforms, the number ranged between 4 and 43 per gm in prawn and 18 and 160 per 100 ml in case of water. *Salmonella* were detected both in prawns and water of two farms while *Vibrio cholerae* was found to be absent in both the prawns and water. It was revealed that *Salmonella spp.* were detected both in prawns and water samples in those ponds, where raw cow dung and poultry faeces were used as manure during grow out period. Total bacterial count, total coliforms and faecal coliforms were also found to be relatively high in prawn and water of those ponds. The practice of use of raw cow dung and poultry faeces as manure and contaminated feed in fresh water prawn farms could have influence on increasing total bacterial load and the enteric bacterial flora as well as may be the source of contamination of pathogenic bacteria like *Salmonella.*

Therefore, according to the findings of the study, it can be is recommended for no use of raw cow-dung and poultry faeces in the fresh water prawn farms during pond preparation and in the grow out period to adapt "Good Aquaculture Practice" and to produce fresh water prawn safe for human consumption.

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CHAPTER I

1. INTRODUCTION

1.1 *Background of the Study:*

Freshwater giant prawn, *(Macrobrachium rosenbergii,* De Man, 1879) is playing an important role for supplying the delicious food and as well as the export item for earning the foreign currency in our country. The culture of giant freshwater prawn is being practiced on commercial scale in several parts of the world specially in south-east Asia. Despite of this, very little attention has been paid to the bacterial flora associated with cultured *M. rosenbergii* (Miyamoto et al, 1983; Anderson et al, 1989) and its influence on the initial quality of this prawn species.

Due to the geographical location, Bangladesh possesses a congenial climatic and soil condition for both freshwater and seawater shrimp culture. Seawater shrimps are cultured in the coastal zone and freshwater prawns are cultured almost all over the country specially in the south and south- west part of the country in the districts of Khulna, Bagerhat, Jessore, Narail, Satkhira, Pirojpur and Patuakhali. For the favourable soil and climatic condition the area of the freshwater prawn culture is expanding every year. There are about 95983 giant freshwater shrimp farms covering the area of 54908 hectors in the districts of Bagerhat, Khulna, Satkhira, Jessore and Narail (Source; department of fisheries). A great number of population are engaged in giant freshwater prawn farming and it is also providing the employment and financial support to change their socio-economic condition. Shrimp and shrimp products are the main component of exportable commodity in the frozen food sector of Bangladesh. Fresh water giant prawn (Golda chingri) is playing an important role in foreign currency earning by export in Bangladesh.

In the year 2008-2009 the total weight of the exported shrimp from Bangladesh was 50368.12 millions metric tons valued at 395.85 million US$ out of which 8689.83 millions metric tons (17.25%) was giant freshwater prawn (*M. rosenbergii*) valued at 97.80 million US$ (24.70%) (Source; department of fisheries).

In Bangladesh, most of the freshwater giant shrimp farms are located in the districts of Khulna, Bagerhat, Satkhira, Jessore and Narail and the culture practice of fresh water giant prawn is almost same. In Bangladesh, most of the farmers culture both freshwater prawn and paddy in the same land. Some of the them also use organic manures like cow-dung and poultry faeces in addition to chemical fertilizers. Some of them also graze their cattle on the dikes of the culture ponds and it's feces are also mixed with pond water by rain runoff. It creates the chance of growth of *Salmonella, Cholera* and other pathogenic bacteria in the ponds. In the foreign markets, fresh water prawns of Bangladesh are rejected mainly due to the contamination of *Salmonella,* and faecal coliforms and rarely for *Vibrio cholerae,* but in the major cases for *Salmonella.* In the fish inspection and quality control laboratory in Khulna, Bangladesh under the department of fisheries, in the year 2008, samples from 2479 consignments (processed freshwater and seawater shrimps and fin fishes) ready for shipment were tested bacteriologically and 31 consignments were rejected due to the presence of *Salmonella,* 147 due to the excess content of total bacterial load (SPC and faecal coliforms). In the year 2009, out of 2770 consignments, 46 consignments were rejected due to the presence of *Salmonella,* 123 due to the excess content of total bacterial load (SPC, and faecal coliforms) in the same laboratory. Among these rejected consignments majority were the consignments of fresh water prawn (*(M. rosenbergii*). The rejection of shrimp consignment both internally and in the foreign market is involved with huge amount of financial loss to the nation which ultimately severely affects the shrimp farmers and all other stakeholders.

Types and levels of bacterial populations associated with farmed giant fresh water prawn (*M. rosenbergii)* are the important indicators for the assessment of quality and safety of prawns. In addition, most diseases in *M. rosenbergii* are caused by opportunistic pathogens which are prevalent in the rearing environment (Janaki Ram & Madhavi 1999).

Therefore, it is essential to investigate the quantitative and qualitative aspects of bacterial flora associated with cultured fresh water prawn and particularly those of public health concern in order to develop a "Good Aquaculture Practice"(GAP) for the production of prawn safe for human consumption and for prevention of prawn disease.

1.2 *Statement of The Problems:*

The initial bacterial load in fish and shrimp plays an important role in quality management of fish and shrimp and the products made thereof. The initial high load of bacteria accelerate the spoilage of fish and shrimp and contamination of pathogenic bacteria like *Salmonella* and *Vibrio cholerae* have a severe bad effect on human health. Contamination of health hazard and pathogenic bacteria is the cause of shrimp and shrimp products rejection by the competent authority nationally and internationally. But the fresh water shrimp farmers have little knowledge about it. Generally, the shrimp farmers do not know about the bacterial status of farmed shrimps and also do not know the sources of bacterial contamination in their culture ponds.

Therefore, to enrich the knowledge of the fresh water shrimp farmers and to develop a good management practice for the production of prawn safe for human consumption and for the prevention of diseases of fresh water giant prawn (*M. rosenbergii)* it is important to study the bacterial contents of prawn and water of the pond during culture.

1.3 *Objective of The Study:*

The overall goal of this study is to determine the bacterial population associated with farmed giant freshwater prawns and their rearing environment. The information obtained from the study would allow a better control over the bacteriological parameters in the farmed freshwater prawn and also a better control on processing to reduce the possible microbial risks. The specific objectives of the study are:

a. To enumerate the total bacterial flora in giant freshwater prawn and pond water.

b. To enumerate the total coliforms bacteria in giant freshwater prawn and pond water.

c. To enumerate the faecal coliforms bacteria in giant freshwater prawn and pond water.

d. To detect the pathogenic bacteria, *Salmonella* and *Vibrio cholerae* in fresh water prawn and pond water.

CHAPTER II

2. LITERATURE REVIEW

Though the study of bacterial status of fresh water giant prawn and water in culture pond is important for the safety and quality management of the prawn but very few studies have been dealt with the issue. Naim and Harbi (2005), studied on the quantitative and qualitative analysaes of bacterial flora associated with the digestive tract of the giant freshwater prawn (*M. rosenbergii*) cultured in earthen ponds of Saudi Arabia. Bacterial counts and flora of prawn culture pond water, sediment and prawn along with important physicochemical parameters, were investigated by them. They reported that total viable counts (TVC; mean ± SD) varied between $(1.0 \pm 2.3) \times 10^4$ and $(1.7 \pm 0.8) \times 10^5$ colony-forming units (cfu) per milliliter in pond water, $(5.8 \pm 1.7) \times 10^7$ and $(1.1 \pm 2.9) \times 10^9$ cfu/g in sediment, $(1.5 \pm 0.9) \times 10^5$ and $(1.6 \pm 2.4) \times 10^6$ cfu/cm^2 in the carapace, and $(9.1 \pm 1.5) \times 10^6$ and $(8.7 \pm 1.8) \times 10^7$cfu/g in the digestive tract of freshwater prawns. They also mentioned that *Salmonella* and other species of enterobacteriaceae were abundant bacterial species in pond water.

Kuttanappilly et al (2004), investigated on bacterial microflora associated with farmed freshwater prawn (*M. rosenbergii)* and the aquaculture environment. They studied on the initial counts of bacteria associated with farmed giant freshwater prawn as well as with the water and sediment from two farms located at Kottayam district in Kerala, India. They found that Prawn samples yielded mean microbiological counts of 4.92 log CFU g^{-1} of shell with muscle and 7.78 log CFU g^{-1} of intestine at 30°C. They also found that bacterial numbers in the intestine of prawn were much higher than those in the pond water and faecal coliforms and enterococci were found in significant numbers in *M. rosenbergii.*

They also reported that rearing practices such as feeding and pond fertilization could have influenced the microflora in prawn. Yathavamoorthi et al, (2010) experimented the enteric bacteria and water quality of freshwater prawn *M. rosenbergii* in culture environment at Kerala, India and stated that prawn samples yielded a mean Total Plate Count (TPC) in the range of 4.57 to 6.66 log cfu g-1 and was the highest among all other samples. Prawns followed by water samples had the higher level of enteric indicator organisms. Sediment showed higher count of sulphite reducing clostridia. They also reported that rearing practices such as use of cow-dung as fertilizer and microbiologically contaminated feed could have influenced the enteric flora. They also studied on various physico-chemical parameters of pond water and correlation analysis revealed a significant positive correlation between pollution indicator parameters such as Total Organic Carbon (TOC), Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD) with that of Total Plate Count (TPC) and Total Enterobacteriaceae Count (TEC) of the pond water and prawn samples.

Jeyasekaran et al (2006), worked on "Postharvest microbiology of farm-reared tropical freshwater prawn *(M. rosenbergii)*" and found the initial bacterial load of 10^4 cfu/g. The lactics and *vibrios* were in the range of 10^2 cfu/g, while the *E.coli,* aeromonads, staphylococci, anaerobes, and molds were in the level of 10^1 cfu/g. *Salmonella and Vibrio cholerae* were present in the prawn muscle.

CHAPTER III

3. MATERIAL AND METHOD.

3.1 Description of Sample Site:

In this study to investigate the contents of the bacterial flora of public health significance and concern with the quality of giant freshwater prawn (*M. rosenbergii*), the area was selected where giant freshwater prawns are cultured in a large scale and the farms are situated in cluster forms. Beel Dakatia, located in Phultala and in Dumuria upazila under Khulna district in Bangladesh is the area where the fresh water giant prawns are cultured in a massive way and in cluster forms. At present, the major area of this beel (depressed marshy land) is being used for giant freshwater prawn culture. There are huge clusters of giant freshwater prawn farms in this beel located in the Phultala upazila. There are about a total of 6982 fresh water shrimp farms/ Ghers in the 18 mouza of this upazila. Out of which 14 mouza of three different unions (Shiromoni, Damodor and Jamira) of Phultala upazila are located in the Beel Dakatia comprising of about 6665 freshwater prawn farms. And out of these 14 mouza, the Garakhola mouza of Damodor union has the highest number of giant freshwater shrimp farms, (about 1046) which is located in Beel Dakatia (Source; department of fisheries). Almost all the freshwater prawn farms in this mouza are in a continuous cluster form and the culture practices are almost similar. For this reason, the freshwater prawn farms of this upazila and of this mouza were selected for the purpose of the study. Location of the sampling farms is shown in the Phultala upazila map together with Khulna district map.

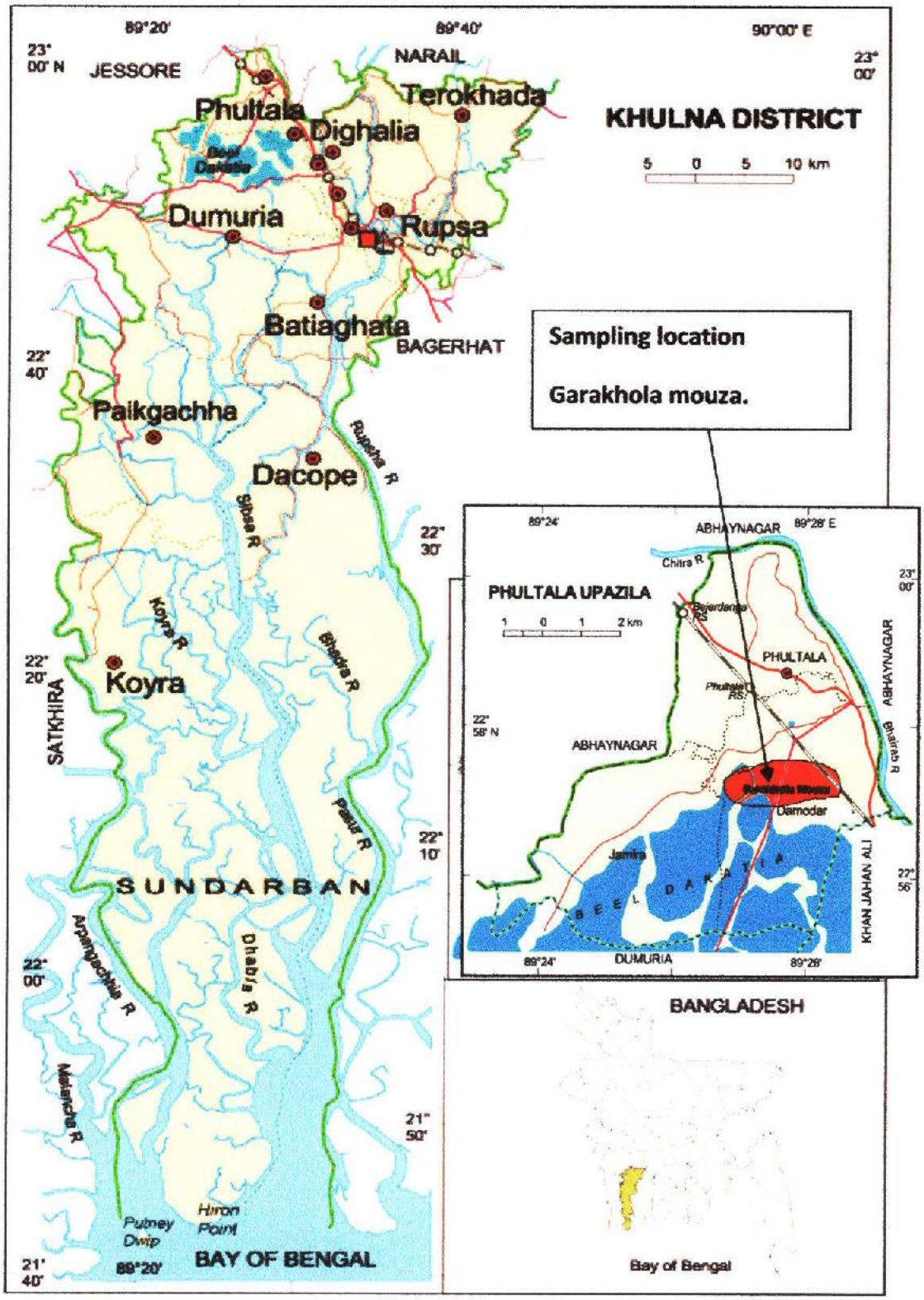

Fig.1. Map of Khulna district and Phultala upazila.

3.2. Sample Collection:

For the purpose of the study 15 samples of harvestable size(10 to 20 prawns / kg) of giant freshwater prawns and 15 samples of pond water were collected from 5 different farms located in Garakhola mouza of Phultala upazila in the district of Khulna on 14.11.2009 from 9.00 am to 11.00 am. The selected sampling farms were situated in the same area and nearly adjacent to each other. Fifteen samples of harvestable size whole prawn were collected from five different ponds comprising of three samples from the same pond of different spots and the total weight of one sample was 500g to 600g consisting of 5 to 8 whole prawns. The prawns were harvested by using cast net and samples were handled with sterilized hand gloves and were collected in sterilized polythene bags by sealing the bags with heat to prevent contamination.

Fifteen water samples were collected from the same five different ponds from which the prawn samples were collected comprising of three samples from the same pond from three different spots (surface, middle and bottom). Screw caped sterilized glass bottles were used for collecting the water samples and the sample size was 500 ml of water for each unit sample. Collected samples (prawn and water) were kept in separate insulated sample collection box instantaneously and the temperature was maintained below 5^0c using ice in the sampling box. Samples were carried to the Fish Inspection and Quality Control laboratory, in Khulna within one hour after sampling. Bacteriological examination of the prawn samples were started on the same day while the water samples were kept in the refrigerator below 5^0C until the start of analysis on the following day.

3.3 Collection of Information About Prawn Farms:

Information about the freshwater prawn farms and culture practice were recorded during sample collection using a tabular data-sheet given in the appendices. (Appendix-03)

3.4 : Analytical Method :

The experiments of bacteriological parameters of collected samples were performed in the microbiological laboratory of Fish Inspection and Quality Control Office in Khulna under the Department of Fisheries, Government of Bangladesh. Bacteriological parameters of prawn and water samples were determined by following the ISO methods.

3.5 Enumeration of Standard Plate Count (SPC) of Freshwater Prawn:

The aerobic plate count provides an estimate of the number of viable bacteria present in food according to the media used and the time and the temperature of incubation. This method is based on the assumption that the bacterial cells present in a sample mixed with an agar media form visible and separated colonies. This is obtained by mixing decimal dilutions of the food samples homogenate with the media. After incubation of the plates at 30^0C for 72 hrs. the number of mesophilic aerobic bacteria per gram of food sample is calculated from the number of colonies obtained in selected Petri dishes at the specific level of dilutions giving a significant result. The procedure was followed according to the ISO method 4833 (Third edition).

3.5.1 Sample Preparation and Dilution:

i. Twenty gm. of shrimp muscle with shell and digestive tract (except stomach) was taken aseptically from the different prawn of the same sample into a sterile sampling bag and 180 ml of 0.1% sterilized Peptone solution was added to it. The sample was blended for two minutes by a stomacher lab blender. This was considered 1^{st} decimal dilution (0.1).

ii. The prawn homogenates were mixed by shaking and 1.0 ml of the homogenates

from the each sample were taken by a pipette into 1st McCartney bottle containing 9.0 ml of the 0.1% sterilized peptone solution and mixed carefully. This resulted in 2nd decimal dilution (0.01).

iii. From the decimal dilution described in (ii), 1.0 ml of diluted homogenates from the each sample were transferred to the 2nd McCartney bottle containing 9.0 ml of the 0.1% sterilized peptone solution to make 3rd decimal dilution, (0.001) and

iv. The process was repeated and 4th (0.0001) and 5th (0.00001) decimal dilutions were made

v. Following the same procedure (i to iv) 15 samples were prepared.

vi. For each dilution separate sterile pipette was used and the homogenates were mixed well thoroughly.

3.5.2 Pour Plating:

i. One ml of the sample homogenates from the 3rd, 4th and 5th dilution from each of 15 samples were transferred by pipette into each of the appropriately marked and sterile duplicate Petri dishes (size 100 mm dia)

ii. Approximately 15 ml of the plate count agar (kept at 45^0C in a water bath) was Poured into each Petri dishes within shortage possible time of original dilution.

iii. The sample dilutions and agar media were mixed thoroughly and uniformly by rotating the Petri dishes clockwise and anticlockwise.

iv. Then the agar media were allowed to solidify.

3.5.3 Incubation:

The Petri dishes with solidified agar were incubated at 30^0C for 72 hrs. in the incubator.

3.5.4 Counting of Colonies:

After the specified period of incubation bacterial colonies (15 t0 300) of the each Petri dishes were counted , using the colony counting equipment.

3.5.5. Calculation of Colonies:

The incubated Petri dishes containing 15 to 300 colonies at each dilution were counted. The numbers of bacteria (N) per gram of sample was calculated using the following equation:

$$N = \frac{\sum C}{(n_1 + 0.1n_2)\, d}$$

Where

$\sum C$, is the sum of colonies counted on all the dishes retained.

n_1, is the number of dishes retained in the first dilution.

n_2 , is the number of dishes retained in the second dilution.

d is the dilution factor corresponding to the first dilution.

The result of the number of micro-organisms per gram of sample, expressed as a number, multiplied by 10^x , where x is the appropriate power of 10.

3.6 *Enumeration of Standard Plate Count (SPC) in Pond water of* Freshwater Prawn.

3.6.1 Sample Preparation and Dilution:

Twenty ml. of sample water was taken aseptically from the each sample and was transferred to screw caped bottle containing 180 ml of 0.1% sterilized peptone solution measuring by the sterilized cylinder. The samples were mixed properly by shaking the bottle carefully. This made 1st decimal dilution (0.1). Following the same procedure described in section (3.5.1), 15 samples were prepared and diluted.

3.6.2 Pour Plating:

Pour plating was performed by following the procedure as narrated in section 3.5.2

3.6.3 Incubation:

The petridishes with solidified agar were incubated at 30^0C for 72 hrs. in the incubator.

3.6.4 Counting of Colonies:

After the specified period of incubation bacterial colonies (15 to 300) of the each Petri dishes were counted , using the colony counting equipment.

3.6.5 Method of Calculation of Colonies:

The colonies were counted following the method described in section (3.5.5).

3.7 Enumeration of Total Coliforms and Faecal Coliforms of prawn by Most Probable Number (MPN) Method:

This method is based on MPN procedure using Lauryl Sulphate Triptose Broth (LSTB) in a presumptive test, followed by confirmation of gas positive tubes using Brilliant-Green Lactose Bile Broth (BGLBB), each being incubated at 37^0C$\pm 1^0$C and 44 ^{O}C$\pm 0.5^0$C for 48hrs respectively for LSTB and BGLBB and formation of indole in triptone water. The procedure was followed according to the ISO 4831 (third edition) of bacteriological method.

3.7.1 Sampling and Preparation of Homogenate:

i. Twenty gm. of shrimp muscle with shell and digestive tract was taken aseptically from the different prawn of the same sample size into a sterile sampling bag and 180 ml of 0.1% peptone solution was added to it. The samples were blended for two minutes by a stomacher lab blender. This made 1st decimal dilution (0.1).

ii. The sample homogenates of 1st dilution were mixed by shaking and 1ml was transferred into a McCartney bottle containing 9 ml of the 0.1 % peptone water and were mixed carefully by aspirating 5 times with a pipette. This made 2nd dilution (0.01).

iii. From the 2nd dilution, 1ml was transferred with another pipette to another McCartney bottle containing 9 ml of 0.1 % peptone water for 3rd dilution (0.001).

iv. All dilutions were shaked carefully after addition of the inoculums in each 9ml bottles.

v. Following the above procedure (i to iv) 15 samples were prepared.

3.7.2 Inoculation:

i. Each of the 3 tubes of LST broth (containing inverted Durham's tubes) was inoculated with 1 ml of food homogenate from the 1st dilution.

ii. Each of the 3 tubes of LST broth (containing inverted Durham's tubes) was inoculated with 1 ml of food homogenate from the 2nd dilution.

iii. Each of the 3 tubes of LST broth (containing inverted Durham`s tubes) was inoculated with 1ml of food homogenate from the 3rd dilution.

iv. Sterilized LSTB tubes and separate sterilized pipettes were used for each dilution.

3.7.3 Incubation:

The inoculated 9 LSTB tubes of all the 15 samples were incubated at 37 ^{0}C for 48 hrs.

3.7.4 Reading of Enrichment tubes (presumptive test):

After incubation, the LSTB tubes showing gas production were recorded.

3.7.5 Calculation of Total Coliforms Using(MPN) Chart:

Total number of coliforms was calculated matching the number of gas positive tubes of the respective decimal dilution to the Most Probable Number (MPN) table in appendixes (Appendix-01).

3.7.6 Enumeration of Faecal Coliform :

Faecal coliform was enumerated using the gas positive inoculums of the LSTB tubes narrated in 3.7.4 following the steps stated below:

i. 1 loop full of inoculums from each of the gas positive LSTB tubes of the specific dilution was transferred to the 10 ml of BGLBB tube containing inverted Durham's tubes and 10ml triptone water (TW) tube separately. This was done aseptically.

ii. The inoculated BGLBB tubes and TW tubes were incubated at 44 0c for 48 hrs. in the circulating water bath.

iii. After incubation, the number of gas forming BGLBB tubes along with the TW tubes of the specific dilution were marked.

iv. 0.2 to 0.5 ml of Kovac's (Indole) reagent was poured to TW tubes and shaked gently and kept for 1 minute. Deep red asile acetate ring formed on the broth surface of some TW tubes.

v. Both gas forming tubes of BGLBB and red ring (indole) forming tubes of Triptone Water were considered as positive respectively for specific dilutions.

vi. All the media and the apparatus were sterilized before use.

3.7. 7 Calculation of Faecal Coliforms by (MPN) chart:

Total number of faecal coliforms was enumerated by comparing the common number of gas positive tubes of BGLB and red rings of the TW tubes with MPN chart shown in appendixes (Appendix-01).

3.8 Enumerationof Total Coliforms and Faecal Coliforms in Pond Water of Freshwater Prawn By MPN Method:

This method is based on an MPN procedure using double strength and single strength Lauryl Sulphate Triptose Broth (LSTB) in a presumptive test, followed by confirmation of gas positive tubes using Brilliant-Green Lactose Bile Broth (BGLBB), each being incubated at $37^0C \pm 1^0C$ for 48hrs. and formation of indole in triptone water. Tubes those show acid and gas are considered as, presumptive positive. Computation of the probable of coliforms is done as per probability table according to Me Crady, for water and ice. ISO method 4831 (Third edition) was followed in the procedure.

3.8.1 Enumeration of Total Coliforms by (MPN) Method Using Eleven Tubes Technique.

i. Fifty ml of water sample was added to 50 ml of double strength LSTB medium in one tube containing durham's tube in inverted condition for indication of gas.

II. Ten ml of sample water was added to each 10 ml of double strength LSTB medium in 5 tubes containing durham's tube in inverted condition for indication of gas.

iii. One ml of sample water was added to each 10 ml of single strength LSTB medium in 5 tubes containing durham's tube in inverted condition for indication of gas.

iv. Fifteen samples were prepared by following procerdure from (i to iii) .

3.8.2 Incubation:

The inoculated 11, LSTB tubes for each samples were incubated at 37^0C for 48 hrs.

3.8.3 Reading of Enrichment Tubes (presumptive test):

After incubation the tubes showing gas production were recorded.

3.8.4 Calculation for Total Coliform by Using (MPN) Chart:

Total number of coliform bacteria was calculated matching the number of gas positive tubes of the respective decimal dilution to the MPN chart water that is shown in appendixes (Appendix- 02)

3.8.5 Enumeration of Faecal Coliforms:

Faecal coliforms were enumerated using the gas positive inoculums of the LSTB tubes (recorded in section 3.8.4 (A) following the steps stated below:

i. One loop-full of inoculums from each of the gas positive LSTB tubes of the specific dilution was transferred to the 10ml of BGLBB tubes containing Durham's tubes and 10ml of triptone water (TW) tube separately and aseptically.

ii. The inoculated BGLBB tubes and TW tubes were incubated at 44 ^{0}C for 48 hrs. in the circulating water bath.

iii. After incubation, the number of gas forming BGLBB tubes along with the triptone water tubes of the specific dilution were marked.

iv. 0.2 to 0.5 ml of Kovac's (Indole) reagent was poured to TW tubes and shaked gently and kept for 1 minute.

v. Deep red asile acetate ring formed on the broth surface of the TW tubes.

vi. Both gas forming tubes of BGLBB and red ring (indole) forming tubes of Triptone Water were considered as positive respectively for specific dilutions.

3.8.6 Calculation for Faecal Coliforms:

Total number of faecal coliform bacteria was enumerated by comparing the common number of gas positive tubes of BGLB and red rings of the TW tubes with MPN chart shown in appendixes (Appendix-02)

3.9. Detection of *Salmonella* in Freshwater Prawn.

The method used for the detection of *Salmonella* is a qualitative method and the result is reported as *Salmonella* detected or not detected in the certain quantity of sample taken. The procedure was followed according to the ISO method , 6579 (Fourth edition).

3.9.1 Preparation of Sample Homogenate:

Tweenty five gms of the mixed shrimps including shell and digestive tract was weighed cutting from the different shrimps of each sample with knife and forceps aseptically in to a sterile stomacher poly bag and 225ml of sterilized buffered peptone water was added to it. Each sample was blended by the stomacher for 120 sec. 15 samples homogenates were prepared following the same procedure

3.9.2 Pre -enrichment :

Blended sample homogenates (25 gm sample blended with 225 ml BPW) were transferred aseptically to sterile 500ml screw cap bottles and these were incubate at 37 ^{0}C for 18 hrs.

3.9.3 Selective Enrichment:

After incubation 0.1 ml of each pre-enrichment broth were transferred to 10 ml RVS (Rappaport -vassiliadis soya peptone) medium and 1ml to 10 ml of MKTTn (Muller Kauffmann Tetrathionate novobiocin) medium and were incubated at 41.5 ^{0}C and 37 ^{0}C respectively for about 24 hrs.

3.9.4 Selective Plating out :

After incubation of the Selective enrichment the culture or the inoculums from the RVS & MKTTn broth were inoculated by streaked out by means of a loop on the surface of

the both large-size Petri dish (140mm dia) of sterile XLD & HEA, agar media to obtain the well-isolated colonies. The streaked agar Petri dishes were incubated in inverted position at 37^0C for 24 hrs .

3.9.5 Selection of Colonies for Confirmation:

After incubation of the selective plating media the colonies of the XLD and HEA were examined and Sample codes P_3S_1, P_3S_3, P_5S_1 and P_5S_2 were selected for confirmation test on the basis of the following colony character:

-Typical colonies of *Salmonella* grown on XLD agar have a black centered and a lightly transparent zone of pink to reddish color due to the color change of the indicator. *Salmonella*, H_2S negative variants (e.g.S. Paratyphi) grown on XLD agar are yellow with or without blackening.

-Typical colonies of *Salmonella* grown on HEA agar are bluish green with or without a black center.

For the confirmation test, one prominently characterized typical colony was selected from each of the XLD and HEA agar plates. The selected colonies were streaked on the surface of the sterile pre-dried nutrient agar plates, in a manner to develop the isolated colonies. The inoculated plates were incubated at 37^0C for about 24 h. These pure cultures were used for biochemical confirmation test.

3.9.6 Biochemical Confirmation Test:

3.9.6.1 TSIA (Triple Sugar Iron Agar) and LIA (Lysine Decarboxylate Iron Agar) Test:

The pure cultures of suspected colony from the nutrient agar were streaked on the slant surface and stabbed to the butt in the both TSI agar and LIA media test tubes .

The agar media with test tubes were incubate at 37 ^{0}C for 24 hrs. After incubation of the TSI and LIA tubes colour change in the media were interpreted as follows:

Sample code	Agar medium	Reaction in the tubes		Comment	Decision
		Slant	Butt		
P_3S_1	TSIA	Red slant (alkaline)	Yellow with acid .	Positive reaction for salmonella	Salmonella may be present.
	LIA	No change of media colour	Deep purple	Do	Do
P_3S_3,	TSIA	Red slant (alkaline) and black with H_2S gas	black with H_2S gas	Do	Do
	LIA	purple colour	black colour	Do	Do
P_5S_1	TSIA	Red slant (alkaline)	Yellow with acid .	Do	Do
	LIA	No change of media colour	black colour	Do	Do
P_5S_2 .	TSIA	Red slant (alkaline)	black with H_2S gas	Do	Do
	LIA	No change of media colour	Deep purple colour	Do	Do

Typical *Salmonella* cultures show alkaline (red) slants and acid (yellow) butts with gas formation (bubbles) and formation of hydrogen sulfide (blackening of the agar) in about 90% of the cases.

3.9.6.2 Urea Agar Test:

Pure cultures of sample code P_3S_1, P_3S_3, P_5S_1 and P_5S_2 from the nutrient agar were streaked to the urea agar slants surface of the test tubes and were incubated at 37 ^{0}C for 2 to 4 hrs. During incubation, the tubes were examined at intervals. If the reaction is positive, splitting of urea liberates ammonia, which changes the color of phenol red to rose pink and later to deep cerise . The reaction is often apparent after 2 to 4 hrs. The suspected samples were test for urea agar for confirmation and the colour of the urea was not changed that indicates the presence of *Salmonella spp.*

3.9.6.3. Indole (reaction) Test:

The pure bacterial cultures from the nutrient agar of the suspected colony were inoculated in test tubes containing 5ml of the tryptone medium. It was incubated at 37^{0}C for 24 hrs. After incubation, one ml of the Kovac's reagent was added to it. The formation of a red ring indicates negative reaction and yellow or brown ring indicates positive reaction for salmonella. Suspected samples were tested for indole reaction for confirmation and all the tubes showed positive reactions and yellow ring was formed. That was the indication of presence of *Salmonella spp.*

3.9.6.4 Decision:

Since, all the selective tests and biochemical tests perform for the detection of *Salmonella* showed positive and characteristic reactions for *Salmonella,* therefore, it was decided that the detected bacteria was *Salmonella spp.*

3.10 Detection of *Salmonella* in Pond Water of Freshwater Prawn.

For the detection of *Salmonella spp.* in pond water of freshwater prawn ISO method , 6579 (Fourth edition) was followed.

3.10.1. Preparation of Sample Homogenate:

Twenty five ml of water from the each sample was taken aseptically by a measuring cylinder and was added to 225ml of sterilized buffered peptone water in screw caped bottle. The bottles were shaked well to mix the sample water with buffered peptone water.

3.10.2 Pre-enrichment :

Same procedure was followed as described in section (3.9.2).

3.10.3 Selective Enrichment:

Same procedure was followed as described in section (3.9.3)

3.10.4 Selective Plating out :

Same procedure was followed as described in section (3.9.4).

3.10.5 Selection of colonies for Confirmation:

After incubation of the selective plating media the colonies of the XLD and HEA were examined and the samples code P_3B and P_5B were selected for confirmation test on the basis of the following colony character as described in section 3.9.5 .

3.10.6 Biochemical Confirmation Test:

3.10.6.1 TSIA (Triple Sugar Iron Agar) and LIA (Lysine Decarboxylate Agar) Test:

The pure cultures from the nutrient agar of sample codes P_3S and P_5S were streaked on the slant surface and stabbed to the butt in the both TSI agar and LIA agar test tubes. The agar media with test tubes were incubate at 37^0C for 24h. After incubation of the TSI and LIA tubes colour change in the medium was interpreted as follows:

Sample code	Agar media	Reaction in the tube		Comment	Decision
P_3B	TSIA	**Slant**	**Butt**	Positive reaction for salmonella	Salmonella may be present.
		Red slant (alkaline)	Yellow with acid		
	LIA	Purple colour	Deep purple	Do	Do
P_5B	TSIA	Slant black with H_2S	Yellow with acid	Do	Do
	LIA	No change of media colour	Black colour	Do	Do

3.10.6.2 Urea Agar Test:

Pure cultures from the nutrient agar of sample codes P_3B and P_5B were streaked to the urea agar slant surface of the test tubes and were incubated at 37^0C for 2 to 4 hrs. After incubation the tubes were examined at intervals. If the reaction is positive, splitting of urea liberates ammonia, which changes the color of phenol red to rose pink and later to deep cerise The reaction is often apparent after 2 to 4 hrs. The suspected samples were test for urea agar for confirmation and the colour of the urea was not changed that indicates the presence of *Salmonella spp.*

3.10.6.3 Test for Indole Reaction:

Suspected samples were tested for indole reaction for confirmation following the procedure described in section 3.9.6.3 and all the tubes showed characteristic positive reactions of *Salmonella* . That was the indication of presence of *Salmonella spp.*

3.10.7 Decision:

Since, all the selective tests and in all the biochemical tests perform for the detection of *Salmonella* show positive reactions of Salmonella, therefore, it was decided that the detected bacteria was *Salmonella spp.*

3.11 Detection of *Vibrio cholerae* in Freshwater Prawn:

For the detection of *Vibrio cholerae* in freshwater prawn ISO method, 21872-1 was followed. Detection of *V. cholerae* requires 5 successive stages. These are:

-First enrichment in a selective liquid media.

-Second enrichment in a selective liquid media.

-Plating on solid selective isolation media.

-Identification of colonies.

-Confirmation test

3.11.1 Preparation of Sample Homogenate:

Twenty five gms. of whole shrimp was weighed cutting from the different shrimps of each sample with knife and forceps aseptically in to a sterile stomacher poly bag and 225ml of sterilized alkaline peptone water (APW) was added to it. Each sample was blended by the stomacher for 120 sec. Fifteen samples homogenates were prepared following the same procedure.

3.11.2 First Selective Enrichment :

Blended sample homogenates were transferred aseptically to sterile 500 ml screw caped bottles and these bottles were incubate at 37 ^{0}C for 6 hrs.

3.11.3 Second Selective Enrichment:

i. One ml of the incubated culture was taken from the surface of the flask and was transferred into a test tube of 10 ml of alkaline saline peptone water.

ii. The test tubes containing alkaline saline peptone water was incubated at 41.5 ^{0} C for 18 hrs.

3.11.4 Plating on Solid Selective Isolation Media:

A 5mm loop-full of culture from the incubated alkaline saline peptone water was streaked on the surface of the dry petri plates (140mm) of TCBS (Thiosulphate citrate bile salt sucrose) agar, the selective media used for *V. cholera* detection in a manner to grow isolated colonies. The TCBS agar plates were incubated at 37 °C for 24 hrs.

3.11.5. Identification of Colonies:

After incubation, the TCBS agar plates were checked for characteristics colony appearances for *V. cholerae*. Typical colonies of *V. cholerae* are smooth, yellow (Sucrose positive) and 2 to 3 mm. in diameter and slightly flattened with opaque centers and translucent peripheries. The incubated TCBSA plates were checked but no characteristic colonies of *V. cholerae* were found.

3.11.6 Decision:

No characteristic colonies of *V. cholerae* were found in any of the TCBS agar plates (the selective agar medium for *V. cholerae*) of the fifteen prawn samples. So the confirmatory tests were not done and therefore, it was decided that *V. cholerae* were found to be absent in the collected prawn samples.

Detection of *Vibrio cholerae* in the Pond Water of Fresh water Prawn.

For the detection of *V. cholerae* in the pond water of fresh water cultured prawn, ISO method mentioned in section 3.11 was followed.

3.12.1 Preparation of Sample Homogenate:

Twenty five ml of water sample from each of the collected sample was transferred to 225ml of sterilized alkaline peptone water (APW) in a screw caped bottle measuring by sterilized pipette. The bottles were shaked well to mix the sample water with alkaline peptone water. Fifteen sample homogenates were prepared following the same procedure.

3.12.2. First Selective Enrichment :

The sample homogenates (25 ml of sample water with 225 ml APW) with screw cap bottles were incubate at 37 ^{0}C for 6 hrs.

3.12.3 Second Selective Enrichment :

Same procedure was followed as described in section 3.11.3

3.12.4 Plating on Solid Selective Isolation Media:

For the plating on solid selective isolation media same procedure was followed as described in section 3.11.4

3.12.5 Identification of colonies:

After incubation the TCBS agar plates were checked for characteristics colony appearances for *V. cholerae*. Typical colonies of *V. cholerae* are smooth, yellow (Sucrose positive) and 2 to 3 mm in diameter and slightly flattened with opaque centers and translucent peripheries. The incubated TCBSA plates were checked but no characteristic colonies of *V. cholerae* were found.

3.12.6 Decision:

No characteristic colonies of *V. cholerae* were found in any of the TCBS agar plates (the selective agar medium for *V. cholerae*) of the fifteen water samples. So the confirmatory tests were not done and therefore, it was decided that *V. cholerae* were found to be absent in the collected water samples.

CHAPTER IV

4. Results and Discussion:

In this study quantitative and qualitative analyses of bacterial content of public health concern of cultured giant freshwater prawn *(M. rosenbergii)* and pond water were carried out. For the purpose of study 15 prawn samples and 15 water samples were collected from the five different farms and investigated to detect public health concern bacteria (*Salmonella* and *Vibrio cholerae*) and to enumerate the total bacterial count (SPC), total coliforms and faecal coliforms. The bacteriological tests were performed following the ISO standard methods and the results of the study have been illustrated in table 1 and 2.

In the freshwater prawn samples, SPC (Standard Plate Count) found in the range of $3.20x10^5$ to $5.70x10^5$/gm, total coliforms 43 to 460/gm and faecal coliforms 4 to 43/gm. The highest number of bacterial count $5.70x10^5$/gm found in the sample no. P_3S_1, collected from pond no.3 where as the lowest $3.20x10^5$/gm in the sample no. P_4S_3, collected from the pond no.4. The highest number of total coliforms (460/gm) and faecal coliforms (43/gm) were enumerated in the sample no P_3S_1 and P_5S_1 collected from the pond no.3 and 5 respectively. On the other hand, the lowest number of total coliforms 43/mg and faecal coliforms 4/gm found in the sample no.P_2S_2 collected from the pond no 2. *Salmonella* were detected in the four prawn samples (P_3S_1 and P_3S_3) collected from the pond no.3 and in the samples (P_5S_1 and P_5S_2) collected from the pond no. 5. *Vibrio cholerae* was not detected in any of the prawn samples that has been demonstrated in the table 1.

Table 1: Bacterial flora found in cultured freshwater prawn.

Pond No.	Sample code	SPC/gm	Total coliform/gm	Faecal coliform/gm	*Salmonella* /25 gm	*V. cholerae* / 25gm
1.	P_1S_1	$4.10x10^5$	150	15	Absent	Absent
	P_1S_2	$4.00x10^5$	93	4	Absent	Absent
	P_1S_3	$4.70x10^5$	210	21	Absent	Absent
2.	P_2S_1	$5.00x10^5$	150	21	Absent	Absent
	P_2S_2	$3.40x10^5$	43	4	Absent	Absent
	P_2S_3	$4.20x10^5$	120	9	Absent	Absent
3.	P_3S_1	$5.70 x10^5$	460	43	**Present**	Absent
	P_3S_2	$4.30 x10^5$	150	15	Absent	Absent
	P_3S_3	$5.50 x10^5$	210	21	**Present**	Absent
4.	P_4S_1	$3.60 x10^5$	210	15	Absent	Absent
	$P_4 S_2$	$4.00 x10^5$	150	9	Absent	Absent
	P_4S_3	$3.20 x10^5$	240	28	Absent	Absent
5.	P_5S_1	$5.05 x10^5$	460	43	**Present**	Absent
	P_5S_2	$5.30 x10^5$	240	28	**Present**	Absent
	P_5S_3	$4.50 x10^5$	210	15	Absent	Absent

P, pond; S, sample.

In case of prawn, three samples were collected from three different spots of the same pond. From the table 1 it is evident that the results of standard plate count of the three samples collected from the same pond were very close to each other. Similarly the results of total coliforms and faecal coliforms were found within little difference to each others of the samples collected from the same pond. The bacterial loads (SPC, total coliforms & faecal coliforms) of all the prawn samples found in the study and depicted in

table. 1 appeared to be almost similar among the samples collected from the 5 different ponds. It is presumably due to the fact that the ponds were situated in a cluster and very adjacent to each others and the water sources of the farms were the same. From the table 1 it is also evident that the total coliforms and faecal coliforms were higher in those samples in which SPC were also higher. *Salmonella* were also found in those samples that contain the higher bacterial loads (both SPC and coliforms). It also relates with the phenomena of abundance of natural bacterial flora in the pond water.

In the water samples, collected from the freshwater shrimp ponds SPC (Standard Plate Count) found in the range of $1.26x10^4$ to$1.72x10^4$/ml,total coliforms within 43 to 240/100ml and faecal coliforms 18 to160/100ml. The highest number of bacterial load (SPC) $1.72x10^4$ / ml found in the sample no P_3B collected from pond no. 3, on the other hand the lowest number, $1.26x10^4$/ ml in the sample P_4M collected from the pond no. 4. The highest number of total coliforms 240/100ml and faecal coliforms 160/100 ml were enumerated in the sample no.P_3B and P_5B collected from the pond no.3 and 5 respectively while the lowest number of total coliforms 43/100ml and faecal coliforms 18/100ml found in the sample no. P_1M collected from pond no.1. *Salmonella* were detected in the two water samples, P_3B collected from pond no. 3 and P_5B collected from pond no.5. *V. cholerae* was not found in any of the fifteen water samples that have been demonstrated in the table 2.

Table 2. Bacterial flora found in pond water of freshwater prawn.

Pond No.	Sample code	SPC/ml	Total coliforms/100 ml	Faecal coliform/ 100ml	Salmonella/ 25ml	V. cholerae/ 25ml
1	P_1S	$1.50x10^4$	160	35	Absent	Absent
	P_1M	$1.40x10^4$	43	18	Absent	Absent
	P_1B	$1.61x10^4$	240	92	Absent	Absent
2.	P_2S	$1.52x10^4$	160	54	Absent	Absent
	P_2M	$1.41 x10^4$	160	18	Absent	Absent
	P_2B	$1.54 x10^4$	240	92	Absent	Absent
3.	P_3S	$1.49 x10^4$	240	92	Absent	Absent
	P_3M	$1.35 x10^4$	160	18	Absent	Absent
	P_3B	$1.72 x10^4$	240	160	**Present**	Absent
4	P_4S	$1.37 x10^4$	160	35	Absent	Absent
	P_4M	$1.26 x10^4$	160	30	Absent	Absent
	P_4B	$1.40x10^4$	240	92	Absent	Absent
5	P_5S	$1.50 x10^4$	160	54	absent	Absent
	P_5M	$1.48x10^4$	90	30	Absent	Absent
	P_5B	$1.60 x10^4$	240	160	**Present**	Absent

***P,** Pond , **S,** Surface, **M,** Middle and **B,** Bottom*

In case of pond water, three samples were collected from the three layers (surface, middle and bottom) from three different spots of the same pond. The results of standard plate count (SPC), of the three samples collected from the same pond were very close to each other. From the table 2 it is evident that the total bacterial loads (SPC, total coliforms and faecal coliforms) found higher in all the samples collected from the bottom and also from

the surface. On other hand, lower in all the samples collected from the middle layer of the ponds. From the table 2 it is also evident that the bacterial loads in between the bottom water and surface water samples, all the bottom water samples contain higher bacterial load than that of the surface water samples. It may happened due to that the most of the organic materials, debris and living organisms including prawn exist in the bottom of the pond that act as substratum for the bacterial flora. There are also similarities among the results of water samples collected from the different ponds having no great difference and is evident from the table 2. The result also shows that the pond water that contains higher bacterial loads also contains the higher count of total coliforms and faecal coliforms. *Salmonella* were detected in the two water samples P_3B and P_5B collected from the bottom layers of the pond no.3 and pond no.5 respectively. Total bacterial loads were also enumerated higher in the samples collected from the same ponds (pond no.3 and 5) in which *Salmonella* was detected.

From the farm information data sheet in the appendixes (Appendix-03), it is known that dried cow-dung was used during pond preparation and raw cow-dung and poultry faeces were used as manure in rearing period in the pond no. 3 and pond no.5. Results in the table 1 and 2 show that the bacterial loads (SPC, total coliforms and faecal coliforms) found higher and also *Salmonella* were found in both prawn and water samples collected from those ponds where cow-dung and poultry feces were used. Therefore, cow-dung and poultry faeces may the possible source of *Salmonella* and may have the role to increase the bacterial load. This finding relates to the findings of (Austin & Allen-Austin 1985; González, Lòpez, García, Prieto & Otero 1999), they mentioned that the composition and level of the microorganisms associated with the intestinal tract is related to the environment as well as to the food consumed by fish .

There have been few studies conducted on the enumeration and detection of bacterial flora associated with giant freshwater cultured prawn (*M. rosenbergii)* and in farms water. The Standard Plate Count (SPC), total coliforms, faecal coliforms enumerated and the detection of *Salmonella* in the present study found almost similar to those reported for farmed giant freshwater prawn (*M. rosenbergii*) cultured in earthen ponds in Saudi Arabia (Naim and Harbi, 2005), "Bacterial microflora associated with farmed freshwater prawn (*M. rosenbergii)* and the aquaculture environment". (Kuttanappilly et al. 2004), "Enteric Bacteria and Water Quality of Freshwater Prawn (*M. rosenbergii)* in Culture Environment from Kerala", India (Yathavamoorthi et al. 2010).

Total bacterial count and coliforms observed in the present study were also comparable to that reported for farmed freshwater prawn in India, (Nayyarahamed & Karunasagar, 1995); (Surendran et al,1995); (Lalitha & Gopakumar,1996) and (Surendran & Narayanan, 2000). In the present study, faecal coliforms levels in *M. rosenbergii* were high as previously reported for tiger prawn farms in India (Surendran *et al.* 1995) . This microbial group is important in foods as indicator of hygienic quality of foods and also as spoilage flora, (Gennari, Tomaselli & Cotrona 1999). If the influent water does not contain high numbers of these organisms, incidence of such high numbers of these organisms in prawn may be attributed to the feed or animal manure commonly used to fertilize ponds. The composition of the bacterial microflora found in *M. rosenbergii* farms is typical of freshwater environments and as generally recognized, is dominated by Gram-negative bacteria (Austin & Allen-Austin ,1985, Lalitha K.V. & Gopakumar K. 1996).

(Tran Thi Tuyet Hoa et al, Vietnam and Austin (1987), studied on the diseases in giant fresh water prawn *(M. rosenbergii)* and found that four types of *vibrios* are associated with vibriosis in giant fresh water prawns. These are V*ibrio cholerae, Vibrio alginolyticus* and *Vibrio carchariae. (*Bethsy Rodriquez and Gina Conry et al, 2010) conducted a pathobiological study on cultured fresh water prawn and found *Vibrio anguillarum* and other enterobacteriaceae but not *Salmonella* and *V. cholerae*. The results found in this study are comparable and similar to the results of the studies that have been conducted in different countries by different researchers that has already been discussed other than *V. cholerae.*

(Jeyasekaran &Ayyappan, 2006), and (Naim & Harbi, 2005), reported that they found *Vibrios* in the fresh water shrimp muscle but did not mentioned whether it was *Vibrio cholerae.* (Ahmed & Harbi, 2003), cited in their report that they found *Vibrio alginoliticus* in fresh water prawn in Saudi Arabia. (Kuttanappilly et al. 2004), repoted in their study that they detected *Vibrio cholerae* in the fresh water shrimp farm in Karala, India. Therefore, a few researchers reported that they have detected *V. cholerae* in fresh water shrimp farms but most of the researchers have reported only the genus of the *Vibrios* but did not mention the species.

In the year 2009, a total of 2770 consignments processed shrimps and fish products of different fish processing plants were tested bacteriologically in the Fish Inspection and Quality Control (FIQC) laboratory, Khulna, under the department of fisheries and *Salmonella* were detected only in 46 consignments and no *V. cholerae* was detected (Source; Department of Fisheries). Therefore, the detection of *Salmonella* and absence of *V. cholerae* in this study correlates with the result findings of most of researchers and also with the results of FIQC laboratory of Khulna, Bangladesh.

CHAPTER V

5. CONCLUSION

This study revealed that farmed giant freshwater prawns, (*M. rosenbergii)* contain pathogenic bacteria and significant number of total and faecal coliforms bacterial flora. Detection of *Salmonella* in the fresh water prawn plays a very important role because presence of this species of bacteria in freshwater prawns can cause detrimental effects on human health. In this study it was found that total bacterial load including enteric bacteria was higher and *Salmonella* was detected both in prawn and in pond water in those ponds where cow-dung and poultry faeces were used. Therefore, use of cow-dung and poultry faeces may be the possible source of pathogenic bacteria in freshwater prawn farms. Further more studies are needed to prove the postulation.

Suggestions can be given to the freshwater prawn farmers to refrain from the use of raw cow-dung and poultry faeces in the farms and to regulate the bacterial loads in the prawn aquaculture system by adopting "Good Aquaculture Practice" to produce shrimp safe for human. The shrimp farmers should also be advised to wash the harvested shrimps with iced cold water and to use ice in shrimp to maintained at a temperature below 5°C during storing and transportation. If these suggestions are followed it will be possible to produce freshwater prawns safe for human consumption and it will also certinly enhance to increase foreign currency earnings by exporting prawns .

CHAPTER VI

6. References

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CHAPTER VII
7. Appendices

Appendix-01:

Table 3. Most Probable Number (MPN) Chart for Calculation for Total Coliforms and faecal coliforms. by using MPN index, Dilutions : 0.1, 0.01 and 0.001

A. Combination of positives	MPN per gram	B. Combination of positives	MPN per gram
0 0 0	<3	0 0 2	6
0 0 1	3	0 0 3	9
0 1 0	3	0 1 1	6
1 0 0	4	0 1 2	9
1 0 1	7	0 1 3	12
1 1 0	7	0 2 0	6
1 1 1	11	0 2 1	9
1 2 0	11	0 2 2	12
2 0 0	9	0 2 3	16
2 0 1	14	0 3 0	9
2 1 0	15	0 3 1	13
2 1 1	20	0 3 2	16
2 2 0	21	0 3 3	19
2 2 1	28	1 0 2	11
3 0 0	23	1 0 3	15
3 0 1	39	1 1 2	15
3 0 2	64	1 1 3	19
3 1 0	43	1 2 1	15
3 1 1	75	1 2 2	20
3 1 2	120	1 2 3	24
3 2 0	93	1 3 0	16
3 2 1	150	1 3 1	20
3 2 2	210	1 3 2	24
3 3 0	240	1 3 3	29
3 3 1	460	2 0 2	20
3 3 2	1100	2 0 3	26
3 3 3	> 1100	2 1 2	27
		2 1 3	34
		2 2 2	35
		2 2 3	42
		2 3 1	36
		2 3 2	44
		2 3 3	53
		3 1 3	160
		3 2 3	290

A. REF: USFDA, Bacteriological analytical manual, 6th Edition, 1984.

Appendix-02:

Table 4 : MPN index for various combinations of positive and negative results when one 50 ml portion, five 10 ml portions and five 1 ml portions are used.

Number of positive tubes			MPN/100 ml	Number of positive tubes			MPN/100 ml
50 ml	10 ml	1 ml		50 ml	10 ml	1 ml	
0	0	0	1	1	2	2	10
0	0	1	1	1	2	3	12
0	0	2	2	1	3	0	8
0	1	0	1	1	3	1	11
0	1	1	2	1	3	2	14
0	1	2	3	1	3	3	18
0	2	0	2	1	3	4	21
0	2	1	2	1	4	0	13
0	2	2	4	1	4	1	17
0	3	0	3	1	4	2	22
0	3	1	5	1	4	3	28
0	4	0	5	1	4	4	35
1	0	0	1	1	4	5	43
1	0	1	3	1	5	0	24
1	0	2	4	1	5	1	35
1	0	3	6	1	5	2	54
1	1	0	3	1	5	3	92
1	1	1	5	1	5	4	160
1	1	2	7	1	5	5	240
1	1	3	9				
1	2	0	5				
1	2	1	7				

Reference : Standard methods for the analysis of water and ice, 1971, 14 th Edition, APHA.

Information About the Sample Collected Shrimp Farms : (Appendix-03) Table- 5

Location of farms: District- Khulna , Upazila- Fultala, Union: Damodor Mouza: Garakhala . **Nature of Farms:** All old.

Pond No.	Area& depth of water	Source of water	Pond preparation treatment	Fertilizer& manure used	Chemical used	Medicine used	Feed used	Cultivation on dike	Grazing animal ondike	Culture period	Species cultured
1	2	3	4	5	6	7	8	9	10	11	12
1	1.0 ha. 5 feet in canal & 4 feet in plain land	Rain and under - ground water	Lime, Rotenone, bleaching powder,	Urea , TSP	Lime, Oxy-flow	None	Oil cake, rice polish, fish meal, flour, Wheat, Snail meat	Vegetables	None	5 months	Prawn (Golda) & carps
2	1.2 ha. 5 feet in canal & 3.5 feet in plain land	Do	Lime, Rotenone, bleaching powder	Urea , TSP	Lime, Potash, Oxy-flow	None	Oil cake, rice polish, Prawn feed, flour, Wheat, snail meat	None	None	5 months	Do
3	1.05 ha 5 feet in canal & 3 feet in plain land	Do	Lime, Rotenone,	Urea , TSP, **Cow-dung, poultry faeces**	Lime, Oxy-flow	None	Oil cake, rice polish, fish meal, flour, Wheat, snail meat	Vegetables	None	6 months	Do
4	1.0 ha 5 feet in canal & 3 feet in plain land	Do	Lime, Rotenone, bleaching powder	Urea , TSP	Lime, Oxy-flow	None	Oil cake, rice polish, prawn feed, flour, Wheat, snail meat	None	None	5 months	Do
5	0.80 ha 4 feet in canal & 3.5 feet in plain land	Do	Lime, Rotenone,	**Cow-dung, poultry faeces** Urea , TSP	Lime, Oxy-flow	None	Oil cake, rice polish, prawn feed, flour, Wheat, snail meat	Vegetables	None	6 months	Do

Source; Interview with the prawn farmers during sample collection.

Appendix-04.

Composition and preparation of culture media and reagents.

A. Peptone salt solution (Bacto-peptone):

A.1. Composition:

Enzymatic digest of casein	1.0 g
Sodium chloride	8.5 g
Distilled water	1000 ml

A.1.2 Preparation: The media was prepared according to the guide line of the manufacturer.

B. Alkaline peptone water (APW):

B.1 Composition:

Peptone	20.0 g
Sodium chloride (NaCl)	20.0 g
Water	1000 ml

B. 1.2 Preparation : The media was prepared according to the guide line of the manufacturer.

C. Buffered peptone water :

C.1 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*

C.1.2 Preparation: The media was prepared according to the guide line of the manufacturer.

D. Plate count agar (PCA).

D.1 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
Distilled water	*1000 ml*

D.1.2 Preparation: The media was prepared according to the guide line of the manufacturer.

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

E.1 Solution A:

E.1.1 Composition:

Enzymetic 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

E.1.2 Preparation: The media was prepared according to the guide line of the manufacturer.

The solution shall be prepared on the day of preparation of the complete RVS medium.

E.2 Solution B:

E.2.1 Composition:

Magnesium chloride hex hydrate ($MgCl_2.6H_2O$)	400.0 g
water	1 000 ml

E .2.2. Preparation: The media was prepared according to the guide line of the manufacturer.

E.3 Solution C

E.3.1 Composition:

Malachite green oxalate	0.4 g
Water	100 ml

E.3.2 Preparation: The media was prepared according to the guide line of the manufacturer.

The solution may be kept in a brown glass bottle at room temperature for at least 8 months.

E.4 Complete medium

E.4.1 Composition:

Solution A	1000 ml
Solution B	100 ml
Solution C	10 ml

E.4.2 Preparation : The media was prepared according to the guide line of the manufacturer.

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

F.1 Base medium:

F.1.2 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	9.6mg
Water	1000 ml

F.1.2.3 Preparation: The media was prepared according to the guide line of the manufacturer.

F.2 Iodine-iodide solution:

F2.1 Composition:

Iodine	20.0 g
Potassium iodide (KI)	25.0 g
Water	100.00 ml

F.2.2 Preparation: The media was prepared according to the guide line of the manufacturer.

F.3 Novobiocin solution:

F.3.1 composition:

Novobiocin sodium salt	0.04 g
Water	5.0 ml

F.3.1.2 Preparation : The media was prepared according to the guide line of the manufacturer.Store for up to 4 weeks at $3^0C + 2^0C$.

F.4 Complete medium:

F.4.1 Composition:

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

F.4.2 Preparation : The media was prepared according to the guide line of the manufacturer.

G. Xylose lysine deoxycholate agar (XLD agar).

G.1 base medium:

G.1.1 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

G.1.2 Preparation: The media was prepared according to the guide line of the manufacturer.

H. Nutrient agar.

H.1 Compositon:

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

H.1.2 Preparation : The media was prepared according to the guide line of the manufacturer.

I. Triple sugar/iron agar (TSI agar).

I.1 Composition:

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

I.1.2 Preparation : The media was prepared according to the guide line of the manufacturer.

J. Urea agar (Christensen)

J.1 Base medium

J.1.1 Composition :

Peptone	1.0 g
Glucose	1.0 g
Sodium chloride (NaCl)	5.0 g
Potassium dihydrogen phosphate (KH2PO4)	2.0 g
Phenol red	0.012 g
Agar	9 g to 18 g
Water	1000 ml

J.1.2 Preparation: The media was prepared according to the guide line of the manufacturer.

J.2 Urea solution

J.2.1 Composition:

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

J.2.1.2 Preparation: The media was prepared according to the guide line of the manufacturer.

J.3 Complete medium:

J.3.1 Composition:

Base	950 g
Urea solution	50 g

J.3.1.2 Preparation: The media was prepared according to the guide line of the manufacturer.

K. L-Lysine decarboxylation medium :

K.1 Composition

L-Lysine monohydrochloride	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
Bromocresol purple	0.015 g
Water	1000 ml

K.1.2 Preparation: The media was prepared according to the guide line of the manufacturer.

Appendix-05: Pictures of sample collection and bacteriological experiments.

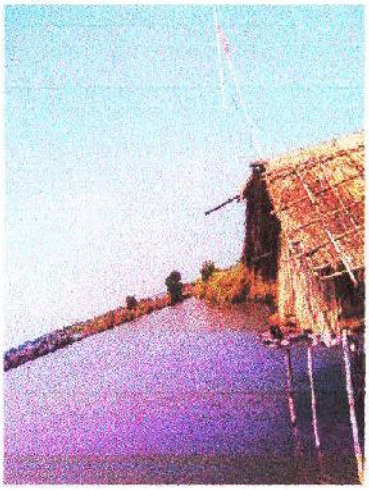
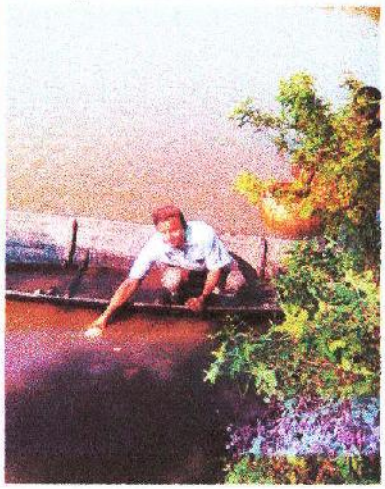


Fig.2. One of the sampling farms **Fig.3**. Collection of water sample, **Fig 4**. Sampling box



Fig.5 Enumeration of SPC,**Fig. 6** Incubation of TCBSA for *V. cholera* **Fig7**. Inoculation of selective enrichment for *Salmonella.*


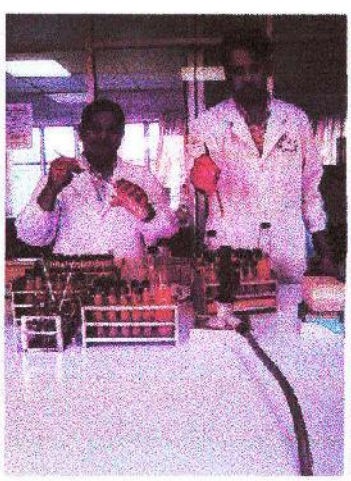
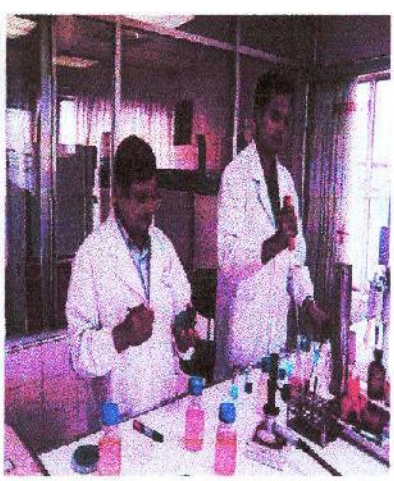

Fig.8. Coliform test of prawn **Fig.9**. Coliform test of water **Fig.10**. Inoculation of Salmonella on selective agar media.

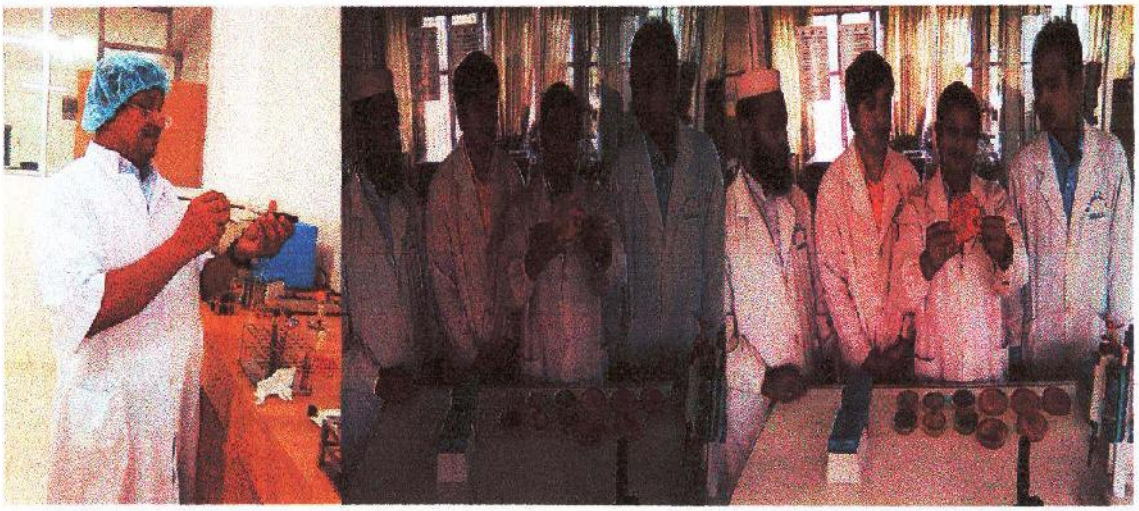

Fig.11 Streaking for Salmonella on selective agar **Fig 12.**Identification of *Salmonella* colony on selective agar ,

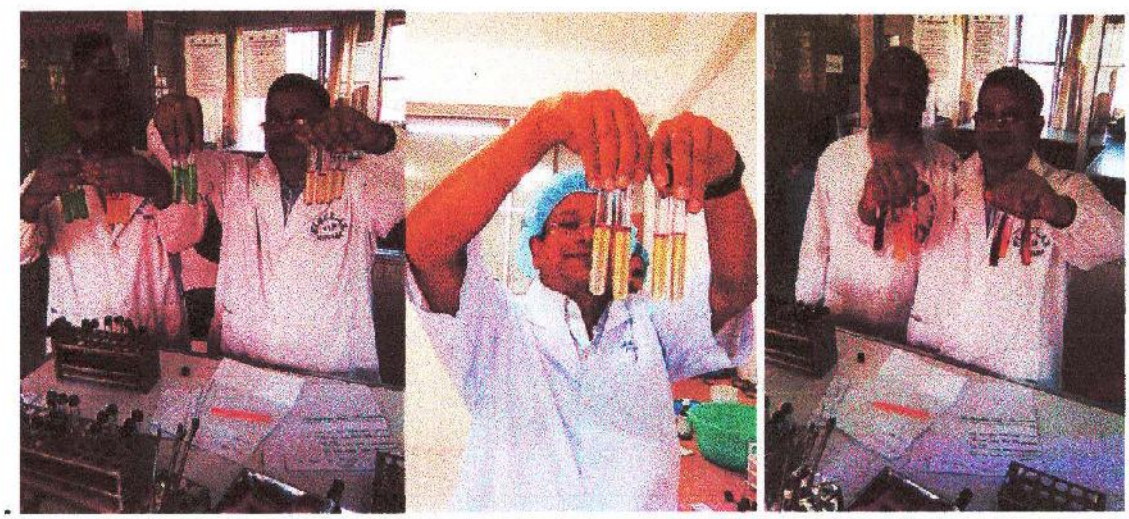

Fig.13 Faecal coliform test **Fig.14**. Indole test. **Fig.15.** Biochemical test of Salmonella

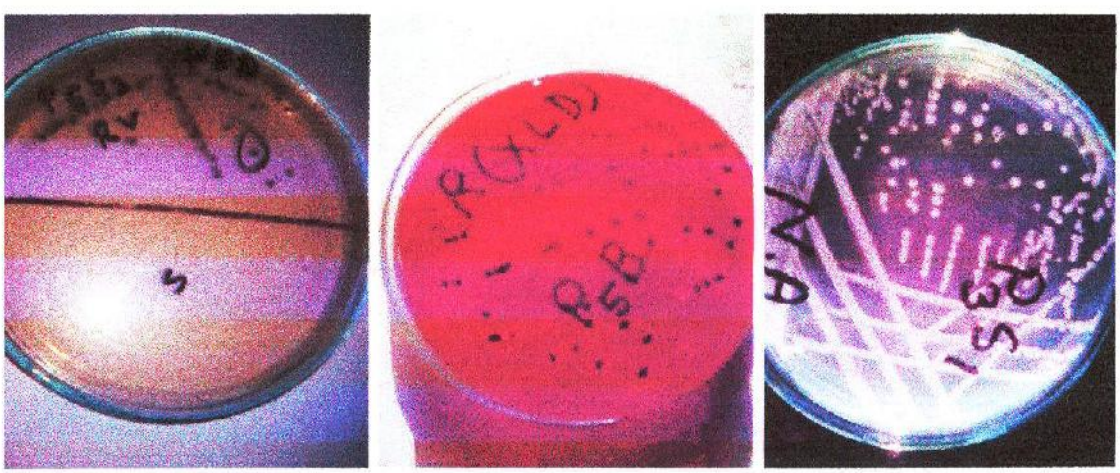

Fig.16. Characteristic colonies of Salmonella on selective agar plates **(HEA,XLD&NA)**