Microbiological quality of giant freshwater prawn (*Macrobrachium rosenbergii*) of different local markets and super shops of Dhaka Metropolitan City, Bangladesh

By

Examination Roll NO.: Curzon 603

Session: 2012-2013

Registration NO.: H-1310 (2007-08)

A thesis submitted on the partial fulfillment of the requirements for the degree of Master of Science (MS) in Fisheries

Previous degree: B.S. (Fisheries)

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Certificate

This is to certify that the thesis entitled ‘‘Microbiological quality of giant fresh water prawn (*Macrobrachium rosenbergii*) of different local markets and super shops of Dhaka Metropolitan City” is submitted by Md. Shamim Al Mamun, Roll NO. : Curzon-603, Session: 2012-13, Registration NO.: Ha-1310; has been carried in the laboratory of Department of Fisheries, University of Dhaka out under our supervision.

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

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Abstract

The giant fresh water prawn, *Macrobrachium rosenbergii* (de Man), a leading export item of Bangladesh was studied in terms of pathogenic bacteria contents. The samples were collected from nine different local markets and super shops of Dhaka of Dhaka metropolis namely Newmarket , Karwan Bazar, Swarighat, Palashi Bazar, Ananda Bazar, Kpatan Bazar, Mina Bazar, Shapno supershop and Agora. In microbiological analysis, total viable bacteria, total coliform, total faecal coliform, total *Salmonella* and total *Vibrio* count were ranged from 1.60×10^5 to 1.8×10^7 1.5×10^4 to 1.28×10^6, 6.3×10^2 to 5.7×10^4, 0.0 to 1.30×10^3, 0.0 to 6.8×10^3 cfu/gm, respectively.

In microbiological analysis, significantly higher viable bacteria abundance was found in the sample prawn collected from Newmarket fish Bazar than of other. Total coliform, faecal coliform and total *vibrio* count of examined prawn sampled from Ananda Bazar fish market was significantly higher than that of other markets ($p<0.05$).

Total *Salmonella, Shigella* count found in muscle of prawn sampled from Karwan Bazar fish market was significantly higher than that of others. Collected prawn samples from local market showed relatively high microbial load and presence of pathogens in the tested samples. However, comparatively low level of bacterium was found in the examined super shops. Total coliform, faecal coliform, total *Salmonella* and total *Vibrio* count was nill in the super shop, Shapno and Agora. Low bacterial count was found in the prawn sample of Mina Bazar.

The results of this study constitute an indicator of bacteriological contamination of a variety of fish and shell fishes. The presence of these pathogenic organisms in these samples of local markets could pose a serious threat and hazard to vulnerable people. Thus, fish and shellfish should be adequately cooked before consumption. Good manufacturing practices should always be observed by the trade to minimize the risk of food poisoning associated with the consumption of fish products.

Table of contents

List of Tables

List of Figures

List of Plates

List of Abbreviations

Symbols	**Details**
°C	Degree celcious
ANOVA	Analysis of Variance
Cfu	Colony Forming Unit
cm	Centimeter
DoF	Department of Fisheries
EC	European Commission
EMB	Eosine Methylene Blue
EU	European Union
E.	*Escherichia*
eg	For Example
et al.	and others
FC	Fecal coliform
GDP	Gross Domestic Product
GMP	Good Manufacturing Practice
h	Hour
H_2S	Hydrogen Sulphide
HACCP	Hazard Analysis Critical Control Point
i.e.	That is
Kg	Kilogram
L	Litre
ml	Mililitre
mg	Miligram
M	Molar

mm	Millimeter
MSA	Minitol Salt Agar
µg	Microgram
µl	Microlitre
NA	Nutrient Agar
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NS	Normal Saline
pH	Negative logarithm of hydrogen ion concentration
ppm	Parts per million
S.	*Staphylococcus*
spp.	Species
SSC	*Salmonella* and *Shigella* count
TBC	Total bacterial count
TCBS	Thiosulphate Citrate Bile Salt Sucrose
TC	Total coliform
TEC	Total *E. coli* count
TVC	Total *Vibrio* spp. count
V.	*Vibrio*
(+)	ve Positive
(-)	ve Negative
<	is less than
>	is greater than

Chapter 1
Introduction

1.1 General background

The role of fisheries and livestock sectors in the development of agro-based economy of Bangladesh is very important and promising. They contribute around 8% to national income, which also is 32% of the total agricultural income. About 90% of animal protein in our diet comes from fish and livestock. The fisheries sector contributes 5.10%, of the country's export earnings, 4.91% of its GDP and provides 63% of the national animal protein consumption. The prawn and shrimp sector as a whole is the second largest export industry after readymade garments, generating US$460 million annually and 5.6% of the total value of exports (DOF 2011). There are 1.2 million people employed in prawn and shrimp production and a further 4.8 million household members are associated with the sector (USAID 2006).

It is claimed that the total fish production has increased significantly over the last few decades. Moreover, Bangladesh has about 2.5 million hectares of coastal tidal land under brackish water shrimp culture. Farming area is steadily expanding.

Shrimp is a popular food in worldwide and Bangladesh is no exception. Here Black Tiger Shrimp (*Penaeus monodon*) or bagda and golda (*Macrobrachium rosenbergii)* are cultivated .Bangladesh shrimp industry is geared for export, and more than 80% of the total national shrimp producd ends up on foreign markets. The most important is the Black Tiger Shrimp which contribute about 70% of all cultured shrimp. Golda or giant river prawn contributes about 15%. However, Bangladesh, meanwhile, under a self imposed ban on export of shrimps (especially prawn) to the European Union (EU), a decision taken on May 2009, after 54 rejections were made from late 2008 to early 2009 due to a “Rapid Alert” notice, which circulates information on food safety problems of our export in EU. In the early seventies, Bangladesh entered the world export market for shrimp and since then this crustacean has suddenly become a very high-priced commodity. In recent decades, due to an increased international demand, shrimp has become one of the most important export products. The major markets for the Bangladeshi shrimp have been the USA, UK, Belgium, Germany and Japan. From 2002 - 03 to 2007 - 08 the quantity exported to the USA was on the increase. The quantity of

shrimp exported to the USA was 26% of the total exported in 2002 - 03 which increased to 46% in 2007 - 08. It was only the USA where export quantity continuously increased. The quantity of shrimp exported to the UK decreased from 29 to 21% during this period. Belgium also showed a similar downward trend (BFFEA 2009).

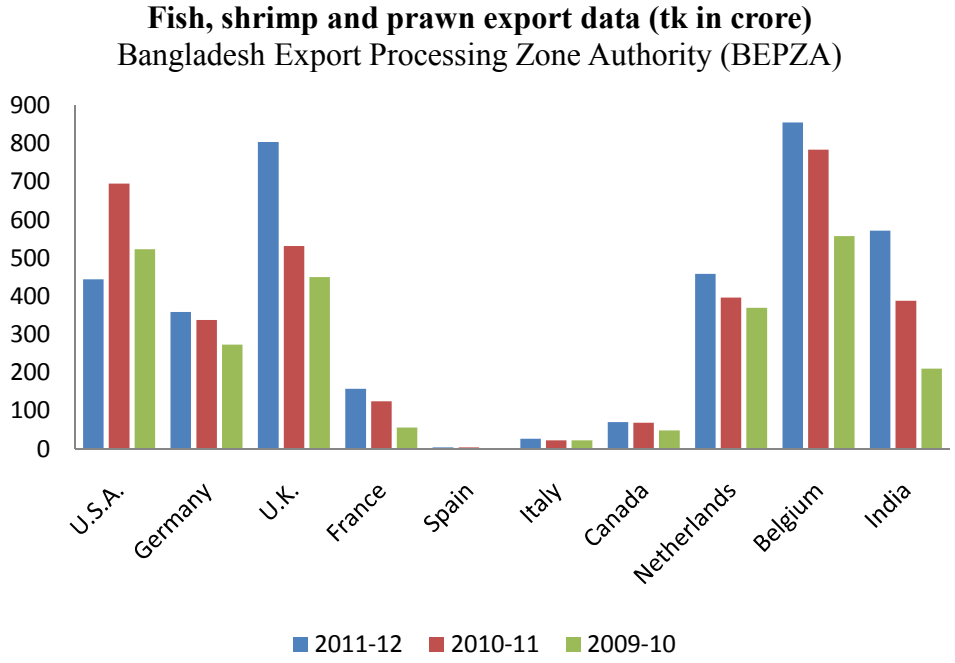

Figure 1.1 Export trend countries wise

Golda or giant fresh water prawn is available in our local markets. Bangladesh is con sidered one of the most suitable countries in the world for giant freshwater prawn (*Macrobrachium rosenbergii* De Man 1879) or golda farming, because of its favorable resources and agro-climatic conditions. A sub-tropical climate and a vast area of water bodies provide a unique opportunity for the production of *Macrobrachium* spp. Twenty-four species of freshwater prawns including 10 species of Macrobrachium are found in Bangladesh. However, only *M. rosenbergii* has significant aquaculture potential and is commercially cultured (Akand & Hasan 1992; Ahmed 2001; Muir 2003a). Freshwater prawn (*M. rosenbergii*) farming is currently one of the most important sectors of the national economy and during the last two decades, its development has attracted considerable attention because of its export potential.

However shrimp has already ranked the second position in earning foreign exchanges in Bangladesh, but very often due to small size, bacterial load or presence of chemical residue fishery products are rejected by the importer countries. So the production and identification of quality fishery products have become a major concern.

Fish is a good source of protein. Its popularity high because of its high biological value in term of high protein retention in the body, low cholesterol level and presence of essential amino acids (Emikpe *et al.* 2011). Fish are generally regarded as safe, nutritious and beneficial but aquaculture products have sometimes been associated with certain food safety issues (WHO, 2007). Fish take a large number of bacteria into their gut from water sediment and food (Emikpe *et al.* 2011). It has been well known that both fresh and brackish water fishes can harbor human pathogenic bacteria particularly the coliform group (Emikpe *et al.* 2011). Fecal coliform in fish demonstrates the level of pollution in their environment because coliform are not named flora of bacteria in fish (Emikpe *et al.* 2011).Fish contamination can also be linked to raw material, personnel, processing tools such as forklifts through leakage, opening in building and pests. Some pathogens may even become established in the processing plants from niches where they can survive for a long period of time (Adebayo-Tayo *et al.* 2012b). The tissue of a healthy fish is normally considered sterile until bacterial inversion that leads to spoilage. According to Adams and Moses (2008), the normal bacterial load of the surface slime of fish can range from 10^2 – 10^7 cfu/cm^2 and the Gills and Intestines can range up to 10^3 and 10^7cfu/g respectively.

Shellfish is a food substrate for some zoonotic vibrios of which these microorganisms, cause food poisoning and diarrhea in human (Merwad *et al.*2011). Shellfish make an excellent substrate for the microorganisms to live in the aquatic habitats due to loose texture of their flesh (Merwad *et al.* 2011).When the aquatic system is contaminated with pathogenic *Vibrio*, these bacteria become part of shellfish microflora (Colakoglu *et al.* 2006). Concerning the zoonotic aspect, the hazardous pathogenic *Vibrio* causes life threatening food borne infections and poses a considerable public health threat as agents of sporadic and epidemic human infections to be represented an important microbial group in the field of food safety (Espineira *et al.* 2010, Merwad *et al.* 2011).So there is a number of food borne diseases related to shellfish consumption.

1.2 Microbial status of giant river prawn *(Macrobrachium rosenbergii)*

Seafood related disease outbreaks have been reported almost throughout the world including countries like Japan, U.S, India and U.K. International Committee for Microbiological Food Safety (ICMFS) has devised permissible counts for various pathogens in different food products. Presence of these pathogens above the acceptable level is usually rejected by the importing country as unfit for human consumption, so to assess the microbiological quality of seafood in any part of the world has become significant to avoid health hazards and also economic losses. The economic losses due to spoilage are rarely quantified but a report by the US National Research Council Committee (FND/NRC) estimated that one-fourth of the world food supply is lost through microbial activity alone (EEC, 1992).

The aerobic mesophilic flora of prawn is dominated by gram-negative bacteria belonging to genera *Vibrio, Moraxella, Acinetobacter* and *Pseudomonas/Shewanella* and family Enterobacteriaceae. Among Gram-positive bacteria, *Micrococcus, Staphylococcus* and *Bacillus* are found to a lesser extent. Faghri *et al*. (1984) Bacteria belonging to the genera *Vibrio*, *Aeromonas*, *Pseudomonas*, *Alteromonas*, *Flavobacterium*, *Spirillum*, *Moraxella*, *Pasteurella* and *Photobacterium* are all reportedas probable agents involved in shell disease syndrome incrustaceans (Getchell 1989). *A. hydrophila, A. sobria* and *A. caviae* have been described as emergent food-borne pathogen of increasing importance causing gastroenteritis (Kirov 1997). Potentially, *Aeromonas* spp. can become a serious food problem as many of them can grow at refrigeration temperatures. *A. hydrophila* is also often found in association with disease outbreaks in aquaculture production (Nielsen *et al.* 2001). Handling and cross contamination might be a health hazard, particularly with susceptible populations.

1.2.1 Pathogenicity of Vibrio

Pathogenic *Vibrios* have been a public health concern for seafood consumers and have been cause of import bans, detentions and rejections in international fish trade (Wafaa *et al.* 2011). The family Vibrionaceae is autochthonous to aquatic environments including estuarine, coastal waters and sediments worldwide, and some species are wellknown pathogens of marine organisms including fish and shellfish (Merwad *et al.* 2011Species such as *V. cholerae, V. parahaemolyticus, V.vulnificus, V. alginolyticus, V. mimicus, V.*

fluvialis, V. furnissii, V. metschnikovii, V. hollisae and *V. damsel* are human pathogens (Adeleye *et al.* 2010). They account for a significant proportion of human infections such as gastroenteritis, usually associated with consumption of raw or undercooked seafood, wound infections, septicemia and ear infections (Adeleye *et al.* 2010).Most of these *vibrios* secreteenterotoxins in foods, water or in the gastrointestinal tract. The presence of other species of *Vibrio* (*Vibrio parahaemolyticus, Vibrio fluvialis,* and *Vibrio mimicus*) has been repeatedly reported on shellfishes by Colakogu *et al.* (2006). It enters human hosts via wound infections or consumption of raw shellfish (primarily oysters), and infections frequently progress to septicemia and death in susceptible individuals.

1.2.2 Enterobacteriaceae

Enterobacteriaceae are a large, diverse heterogeneous group of rod shaped gram negative bacilli that survive under aerobic conditions and normally inhabit the intestine of man and animals; some are motile while some others are not. *Enterobacteriaceae* were isolated from gills, skin, muscles and the intestine of randomly collected fishes. Thampuran *et al*. (2005) reported that the microbial quality of the tilapia indicated that all tissue samples except muscle tissues were contaminated with fecal coliform where *Escherichiacoli* was the most common contaminant and is often encountered in high numbers.

1.2.3 Salmonella

Contamination of seafood with *Salmonella* is a major public health concern. The presence of *Salmonella* in seafood has been reported in Vietnam, India, Sri Lanka, Thailand, Taiwan and Japan (Ponce *et al.* 2008; Wafaa *et al.* 2011). During a 9-year study (1990–1998), the Food and Drug Administration noted an overall incidence of *Salmonella* in 7.2% of 11,312 samples from imported and 1.3% of 768 samples from domestic U.S. seafood (Wafaa *et al.* 2011).

1.2.4 Escherichia coli

Escherichia coli cause dysentery. Most strains are harmless but some causes diarrhea (ICMSF, 2002). Normal fish and human skin is a complex organ and the bacterial populations associated with it are complex in kind and number. The skin supports the growth of both aerobic and anaerobic bacteria (Adebayo-Tayo *et al.* 2006, 2009).

1.2.5 Contamination with *Staphylococcus spp*

Staphylococcus aureus, a mesophile have been implicated in food poisoning outbreak of some food material (Adebayo-Tayo *et al.* 2009). Odunfa (1988 cited by Adebayo-Tayo *et al.* 2006, 2009) reported that *S. aureus* levels of 10^8/ml are considered potential hazardous to consumers. The presence of *S. aureus* is an indication of contamination by food handlers and 80% of them are being harbored by man as normal micro flora (Adebayo-Tayo *et al.* 2009). *S. aureus*known for production of heat stable enterotoxin and potentials for multiple antibiotic resistances when they get into the living tissue makes the product of immense epidemiological danger (Adebayo-Tayo *et al.* 2009).

1.2.6 Contamination with *Pseudomonas spp*

Pseudomonas sp on the other hand is prevalent among patients with wounds, burns, cystic fibrosis are likely to have introduced into the environment by swimmers and infected individuals. The contamination may be as a result of human activities such as deposition of faecal matters, washing, bathing, discharge of effluents into the rivers were these fish are harvested from.

1.2.7 Contamination with *Bacillus sp*

Bacillus sp causes a toxin-medicated disease rather than infection such as diarrhea and emetic illness characterized by nausea and vomiting (Adebayo-Tayo *et al.* 2006, 2009). The occurrences of *Bacillus* sp. in fish can be said to be as a result of prevalence of their spores in the environment (Adebayo-Tayo *et al.* 2009). *Bacillus* species are spore formers whose spores could survive high temperatures of processing. The organisms are present in most raw materials used in food manufacturing at concentration of 10^3/g or less. The infectious dose has been estimated to be 10^5/g (Adebayo-Tayo *et al.* 2009)

1.2.*8 Klebsiella species*

*Bac*teria from the genus *Klebsiella* causes numerous infections in human, which are often treated with β–lactam antibiotics (Amin *et al.* 2009). A variety of nosocomial and community acquired (food borne) infections are caused by *K. pneumoniae*, one of the most deadly pathogens of *Enterobactericeae* (Amin *et al.* 2009, Udeze *et al.* 2012b).

K. pneumoniae is an enteric Gram negative bacillus causing hospital-acquired infections and infections in debilitated or immune-compromised patients accounting for up to 10% of all nosocomial bacterial infections. (Amin *et al.* 2009, Udeze *et al.* 2012b). Udeze *et al.* (2012) carried out a study to find out if *Klebsiella pneumonia*bacteria isolate can survive in the fish (immunity of fish) and observation of the public health hazard.

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1.3 Research Needs

Fish and Shellfish not only transmit disease to man but are themselves subject to many diseases and capable of transmitting many of the established food borne microbial infections and intoxications (Emikpe *et al.* 2011). One of the major risks involves the consumption of raw or undercooked crab that may be naturally contaminated by food borne pathogens present in the marine environment. Such risk is further increased if the food is mishandled during processing where pathogens could multiply exponentially under favorable conditions. Sea foods are prone to bacterial contamination, especially filter feeders such as mussels, and oysters, which concentrate these bacteria in their filtration systems and, therefore, are ideally suited to trap all bacteria and viruses, pathogenic or otherwise, that live in the water (Popovic *et al.* 2010, Wafaa *et al.* 2011).

The environment has a great influence on the bacterial flora of freshly caught shrimp and prawns. During handling, bacteria present on the body surface or in the intestine can be entered the prawn meat and cause contamination. Aquatic crustaceans always exercise large number of microbes into their body parts from water, sediments and food. There are several reports on bacterial infection of the golda (*Macrobrachium rosenbergii*). The increase demand of mud crab production and their value for export, attention should be given to their microbiological quality and concerning food safety for human consumption.

The importers are much concerned about the nutritional value and microbial quality of fish products. The quality of processed products largely depends on the raw materials and method of handling. Along the entire production chain maintenance of hygienic condition and proper handling is very important. That's why the study of microbial (especially bacterial) flora and proximate composition is necessary to ensure the quality of the product. The presence of indicator organisms will prove the low quality of the product.

1.4 Rationale

Contamination of freshwater and shell fish by harmful bacteria is of great concern from a public health view point .There is no constant monitoring system in fisheries sector in our country .The fish marketing system in Dhaka city is very obsolete ,un systemic and unscientific. Only a few mobile courts have been arrived to check the presence of formalin cit on food stuff.

Vibriosis, coliform, *salmonell*a etc. are very common prevalent zoonotic diseases in aquaculture in Bangladesh. There is no quality maintain system in the fish markets of Dhaka city. For the assurance of public health it's necessary to determine the quality of fish and shell fish product found in different markets in Dhaka Metropolitan City.

In Bangladesh reports on the aquatic bacterial population study and the distribution of bacteria in different parts of different fish and shell fishes are still unknown .Therefore, to minimize the risk of public health it is necessary to have a complete study on microbial load of Giant river prawn in different fish market in Dhaka Metropolitan City.

1.5 Objectives

The overall objective of the present study was to determine the microbiological quality of Giant freshwater prawn in Dhaka Metropolitan City.

The specific objectives were:

i. Enumeration of aerobic hetetrophilic ,enteric and related bacteria
ii. Isolation ,characterization and identification of bacteria
iii. Possible biochemical app
iv. Compare the quality of prawns of local fish markets and super shops.

Chapter 2
Materials and Methods

2.1 Experimental fish

In the present study, *Macrobrachium rosenbergii* were examined. The samples were obtained from nine different markets namely Newmarket, Karwan Bazar, Kpatan Bazar, Swarighat, Palashi Bazar, Shapno supershop, Agora, Mina Bazar and Aanondo Bazar. Only muscle sample were examined for each specimen.

2.2 Sampling

Samples were collected in sterilized plastic bag aseptically following methods of American Public Health Association (APHA, 1998) from different fish markets of Dhaka metropolitan city. Samples were collected early in the morning during the period of October 2013 to December 2013. Samples were then transported in the laboratory, using icebox within one hour.

2.3 Laboratory of investigation

All experiments were carried out in the Aquatic Laboratory, Department of Fisheries, University of Dhaka.

2.4 Media used for isolation

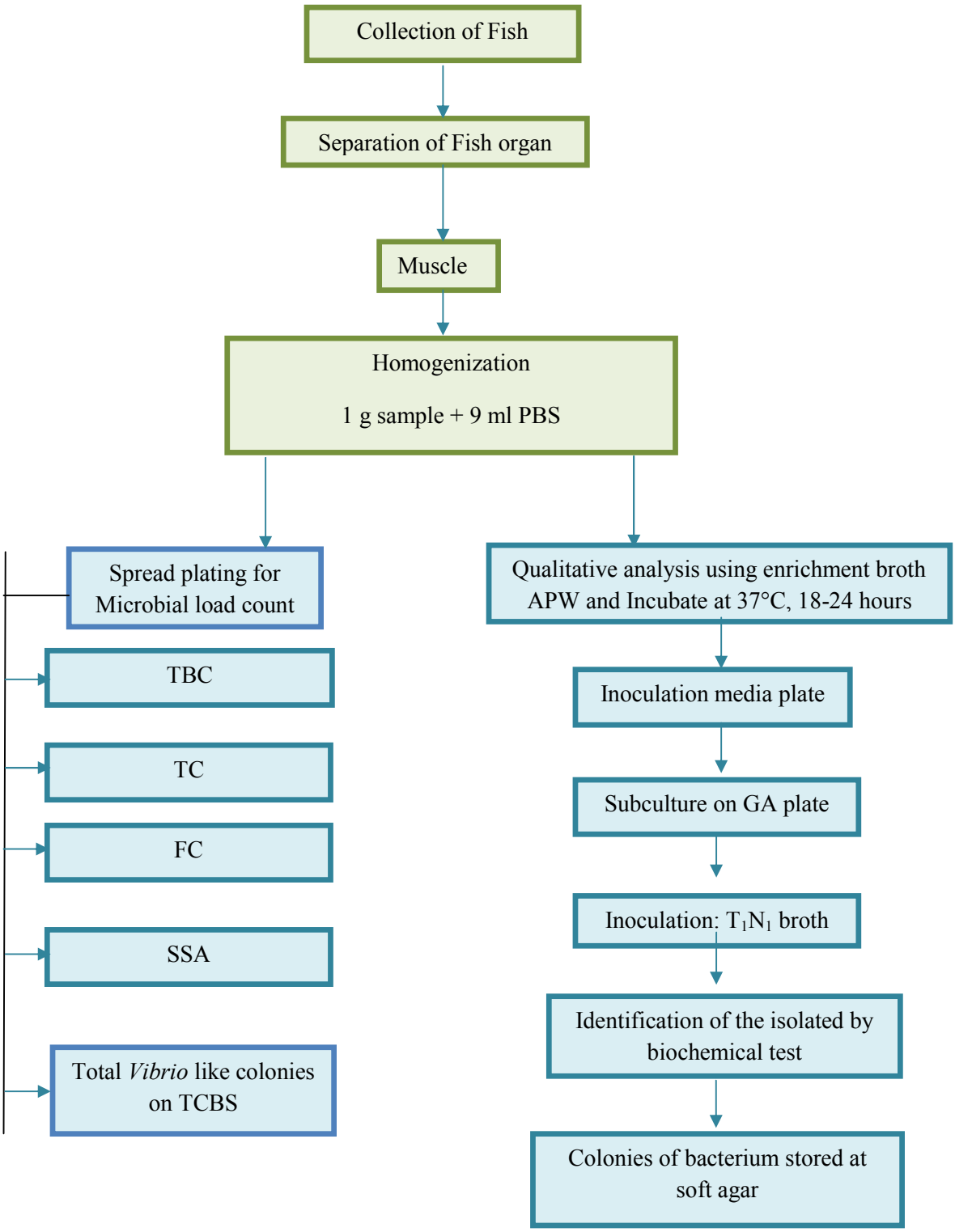
i) **Nutrient Agar:** For total aerobic mesophilic plate count
ii) **mFC Agar:** Total and faecal coliform that gives blue colonies
iii) **MacConkey Agar:** *Enterobacteriaceae*. Lactose fomenters give pink colonies.
iv) **Thiosulphate Citrate Bile Salt Sucrose (TCBS) Agar:** Pathogenic *Vibrio* gives yellow colony while the non-pathogenic is green
v) **SS Agar:** *Salmonella* gives dark black centered colony and *Shigella* pink

2.5 Processing of samples

The prawn samples were processed within 2 h of collection following aseptic techniques. First, the samples were washed with sterile phosphate buffer saline (PBS) to remove sand, detritus as well as microorganisms attached to the surface of fish. Then only the muscle samples were collected aseptically following the method of APHA (1998). The

collected samples were separately homogenized with PBS solution using homogenizer and were then used for microbial load count and pathogen specific enrichment.

Flow chart- 2.5. 1: Processing of fish sample and identification of bacterial spp.



2.6 Plating procedure for enumeration of bacterial load

2.6.1 Total bacterial count (TBC)

Sampled fish was frozen at -20º C until laboratory analysis. Laboratory analyses were done within a weak. Dehydrated nutrient agar (OXOID, USA) was used for preparation of culture medium. A sub-sample of 1 g was taken from homogenized tissue of each sample and mixed with 9 mL PBS solution to prepare 10^{-1} dilution. Then, subsequent serial dilutions were prepared for 10^{-2} to10^{-5} plate count. The spread plate method was used to enumerate the total bacteria following Hasan and Bart (2006). A sub-sample of 100 µL samples from each dilution with three replicates was used to count bacteria as colony forming unit (CFU) per g of sampled fish. CFU/g for 3 replicates were initially averaged and used for final calculation. All equipment and chemicals used were sterilized properly prior to use.

2.6.2 Total Coliform (TC)

Total coliforms are *E. coli, Klebsiella, Citrobacter ,Enterobacter*. Coliforms are gram negative, rod shaped, non-spore forming bacteria capable of growth in the presence of bile salt or surface active agents with similar growth inhibiting properties, which are cytocrome –oxidase negative and able to ferment lactose at either 35°C or 37°C with the production of acid, gas and aldehyde within 24 to 48 hours.

For the enumeration of total coliform samples were diluted serially. Membrane faecal coliform (mFC) agar plate was used for the enumeration of coliforms. For this purpose 100µl from the above diluted solution were spread on the mFC plate. Then incubated at 37°C for 18 to 24 hours. Blue colonies were considered as total coliform (APHA, 1998). Finally, colony forming unit (CFU) was calculated.

2.6.3 Fecal Coliform (FC)

Those that have the same properties at a temperature of 44 or 44.5°C are described as faecal (thermo tolerant) coliform organisms (*E. coli* & *Klebsiella*).

For the enumeration of fecal coliform same procedure was followed that described for the enumeration of total coliform except the incubation temperature. In case of fecal coliform plates were incubated at 44 to 44.5°C for overnight and enumerated accordingly.

2.6.4 Total Vibrio like colonies on TCBS

A sub-sample of 100 µl of homogenized samples was spreaded on TCBS plate for counting the Vibrio like colonies. All the colonies of yellow and green color after 18-24 h of incubation were counted as cfu/g of sampled fish.

Procedures for the isolation of *Vibrios*

Enrichment for the isolation of *Vibrios* from Fish samples:

I. Incubate all the samples, i.e., already inoculated into enrichment media (APW) at 37^{o}C for 6 hours
II. After enrichment, take 1-3 loopfuls of the enrichment broth cultures and streak on TCBS and TTGA plates.
III. Incubate the TTGA and TCBS plates for 18-24 hours at 37^{o}C for selective growth of the members of Vibrionaceae.
IV. Pick up the suspected characteristic colonies with a sterile needle or straight wire and subculture on gelatin agar (GA) plates.

Carry on the multi-test biochemical screening for each of the suspected isolates. The presumptive identifications were confirmed by additional tests following standard procedures.

Each of the laboratory tests described below was used to confirm the identification of the organisms that was found in the samples. Specific biochemical reactions, cell morphology, motility, formative metabolism, utilization of glucose, production of catalase, lack of production of indophenoloxidase, reactions to salinity or reduction of nitrates to nitrites help classify members of the *vibrio* family.

Identification of the Members of Vibrionaceae

After incubation, select characteristic colonies on TCBS (yellowish medium size), TTGA (transparent, black centered, gelatinase positive). Sub culture on non-selective GA plates and screen for the presence of cytochrome oxidase, and gelatinase activity. All the colonies giving a positive cytochrome oxidase and gelatinase activity were then identified biochemically and serologically.

2.7 Procedures for identification

a. Pure culture preparation

Before biochemical identification all the suspected bacterial colonies should be obtain without any contamination from other bacteria or fungi. Obtain pure cultures of test organisms by carefully picking up one isolated colony and subculturing on gelatin agar (GA) plates following overnight incubation at 37^{o}C for 18-24 hours.

b. Observation of colony morphology

Different types of colonies in various media were observed. Morphological characteristics including shape, size, surface, edge, elevation colour etc. were studied.

c. Gram Staining

Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50-90% of cell envelope), which are stained purple by crystal violet, whereas Gram-negative bacteria have a thinner layer (10% of cell envelope), which are stained pink by the counter-stain(Wikipedia). There are four basic steps of the Gram staining:

1. Applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture.
2. The addition of iodine, which binds to crystal violet and traps it in the cell,
3. Rapid decolorization with alcohol
4. Counterstaining with safranin

d. Biochemical tests

I) Oxidase test

Principle: This test depends on the presence of cytochrome oxidase in bacteria that will catalyze the transport of electrons between electron donors and redox dye. Tetramethyl-p-phenylene diamine dihydrochloride in the reagent is reduced to deep purple color. This test is used for the screening of Pseudomonas, Vibrio, Neisseria, Brucella and Pasteurella, which give positive test. Enterobacteriaceae are oxidase negative.

Procedure: A piece of filter paper was placed in a petri dish and 3 drops of freshly prepared oxidase reagent (1% solution of tetramethyl-p-phenylene diamine dihydrochloride) was added. Using a sterile glass rod, a colony of test organisms from a culture plate was picked and smeared it on the filter paper.

Interpretation: Oxidase positive organisms gave blue color within 5-10 seconds, and in oxidase negative organisms, color did not change.

II) Catalase Test

Procedure: A small drop of normal saline was placed on a slide.With a sterilized and cooled inoculating loop, a small amount of the culture from the nutrient agar plate was picked.it was smeared to make a smooth suspension..With a Pasteur pipette, one drop of hydrogen peroxide over the test smear was placed. Observe the fluid over the smears for the appearance of gas bubbles.

Interpretation: Catalase positive organisms gave gas bubbles within 5-10 seconds, and in catalase negative organisms, no change occurred.

III) KIA test

Procedure: With needle a colony was picked and stabbed through the center of the agar to the bottom of the tube. Then the surface of the slant was streaked. Kligler slants were incubated for 24 hours.

Interpretation: Lactose (+) organisms yielded a yellow slant and lactose (-) organisms yielded a red slant. Glucose (+) organisms yielded a yellow buttH_2S (+) organisms blacken the lower portion of the tube due to formation of iron sulfide.

IV) Motility Detection

Procedure: In a test tube containing MIU mediuma wired loop was used to stab the medium. Test tubes were incubated for 24 hrs.

Interpretation: Non-motile organisms which lack flagella formed a single line of growth that does not spread into the surrounding area. While a motile bacterium grewaroundthe stab line producing a hazy zone.

V) Indole test (tryptone broth)

Principle: Tryptophan hydrolysis -Some bacteria split tryptophan into indole and pyruvic acid using the hydrolyses called tryptophanase. Indole can be detected with Kovac's reagent (Indole reagent). This test is very important in differentiating *E. coli* (indole positive) from some closely related enteric bacteria. It also differentiates *Proteus mirabilis* (indole negative) from all other *Proteus* species (indole positive). Tryptone broth is used for this test as it contains a large amount of tryptophan.

Procedure: A loopful of bacteria was inoculated into a tryptone broth and incubated for 48 hours.

Interpretation: A few drops of Indole reagent was added to the broth culture (tryptone broth). A positive result had a red layer at the top. A negative result had a yellow or brown layer.

VI) Citrate test (Simmon's Citrate slant)

Principle: Simmon's citrate agar tests for the ability of an organism to use citrate as its sole source of carbon. This media contains a pH indicator called bromthymol blue. The agar media changes from green to blue at an alkaline pH.

Procedure: A loopful of bacteria was streaked onto a citrate agar slantnot stabbing the butt. It was incubated for 24 to 48 hours with loose cap, longer for *Bacillus* species.

Interpretation: A positive reaction was indicated by a slant with a Prussian blue color. A negative slant remained green.

VII) Methyl Red test (MRVP broth)

Principle: Mixed acid fermentation - Many gram-negative intestinal bacteria can be differentiated based on the products produced when they ferment the glucose in MR-VP medium. *Escherichia, Salmonella*, and *Proteus* ferment glucose to produce lactic, acetic, succinic, and formic acids and CO_2, H_2, and ethanol. The large amounts of acids

produced lowers the pH of the medium - Methyl red (a pH indicator) will turn red when added to the medium if the organism was a mixed acid fermented. Many of these organisms also produce gas.

Procedure: A loopful of bacteria was inoculated into MRVP broth and incubated for 3 to 5 days.

Interpretation: A few drops of methyl red were added to the turbid broth. A positive result had a distinct red layer at the top of the broth. A negative result had a yellow layer.

VIII) Voges-Proskauer test

Principle: Organisms that are negative in the methyl red test may be producing 2, 3 butanediol and ethanol instead of acids. These non-acid products do not lower the pH as much as acids do. *Enterobacter, Serratia* and some species of *Bacillus* produce these substances. There is no satisfactory test for determining production of 2, 3 butanediol. A precursor of 2,3 butanediol called acetoin can be detected with Barritt's reagent.

Procedure: A loopful of bacteria was inoculated into MRVP broth and incubated for 3 to 5 days.

Interpretation: The VP A reagent (15 drops) and 5 drops of the VP B reagentwas added to the 1 ml of broth culture. With a positive reaction the medium changed to pink or red indicating that acetone was present. With a negative reaction the broth did not change color or was copper colored. At least 15 minutes waited for color to develop before calling the test negative.

IX) Nitrate Reduction Test

Principle: Nitrate broth is used to determine the ability of an organism to reduce nitrate (NO3) to nitrite (NO2) using the enzyme nitrate reductase. It also tests the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen.

Procedure: After incubating the nitrate broth with target colony, a dropperful of sulfanilic acid and α-naphthylamine was added.

Interpretation: The medium turned red after the addition of the nitrate reagents was considered a positive result for nitrate reduction. When the medium did not turn red after the addition of the nitrate reagents, a small amount of powdered zinc was added. If the tube was turned red after the addition of the zinc, it meant that unreduced nitrate was present. Therefore, a red color on the second step was a negative result. If the medium did not turn red after the addition of the zinc powder, then the result was called a positive

complete. If no red color forms, there was no nitrate to reduce. Since there was no nitrite present in the medium, either, that means that denitrification took place and ammonia or molecular nitrogen were formed.

X) Salt tolerance test

Procedure: This test was done on nutrient agar media supplemented with varying amounts of sodium chloride (0%, 1%, 3%, 6%, 8%, 10%). Tubes containing 3.0 ml broth are inoculated with test organism grown in T_1N_1 broth for 3-4 hours at 37^{o}C. Growth is observed visually after 24 and 48 hours of incubation at 37^{o}C. The cloudy appearance of the whole tube after shaking indicated positive growth.

Interpretation: The cloudy appearance of the whole tube after shaking indicated positive growth.

e. Identification of the isolates

All the isolates were identified following Bergey's manual of determinative bacteriology.

2.7 Statistical analysis

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

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

List of Plates

Plate 1 Bacterial colonies on MacConkey agar media

Plate 2 Bacterial colonies on TCBS agar media

Plate 3 Bacterial colonies on mfc agar media

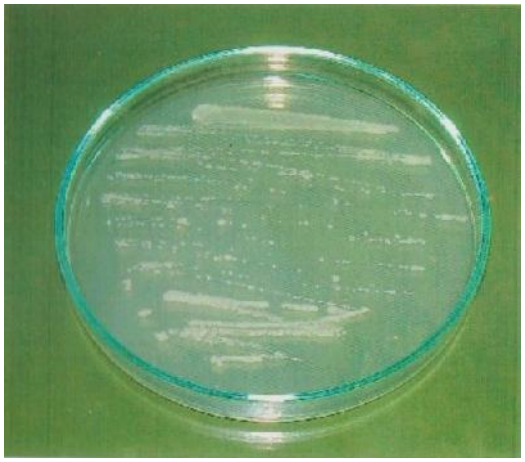


Plate 4 TBC (Total bacterial count) in NA

Plate 5 Different media used for bacterial analysis

Plate 6 Simmon's citrate test

Plate 7 KIA test

Plate 8 Biochemical properties of vibrio spp.

Chapter 3
Result

3.1 Bacterial density of giant fresh water prawn samples

In this study, a total of 27 samples of the giant fresh water prawn were analyzed for total viable bacterial count (TBC), total coliform count (TC), total faecal coliform count (FC), total *Salmonella* count (TSC) and total *Vibrio* count (TVC). The results are presented in Table 1.

Tabl 1. Quantitative analysis of different bacterial groups in giant fresh water prawn samples collected from local markets.

SL no.	Sources	TBC Cfu/g	Total vibrio count cfu/g	TC Cfu/g	FC Cfu/g	TSC Cfu/g
1	Newmarket	$1.80\pm1.20\times10^{7a}$	$5.36\pm0.34\times10^{3ab}$	$1.06\pm0.29\times10^{6ab}$	$8.23\pm0.76\times10^{3ab}$	$6.7\pm0.91\times10^{2bc}$
2	Karwan Bazar	$1.49\pm0.10\times10^{6b}$	$7.20\pm0.49\times10^{2b}$	$2.60\pm0.15\times10^{5ab}$	$1.30\pm0.68\times10^{4ab}$	$1.30\pm0.23\times10^{3a}$
3	Swarighat	$3.93\pm0.37\times10^{6a}$	$2.50\pm0.35\times10^{3b}$	$2.40\pm0.16\times10^{5ab}$	$1.63\pm1.10\times10^{4ab}$	$4.06\pm0.90\times10^{2cd}$
4	Palashi Bazar	$1.40\pm0.17\times10^{7ab}$	$7.06\pm5.50\times10^{2b}$	$2.60\pm2.00\times10^{5ab}$	$1.66\pm1.33\times10^{4ab}$	$6.90\pm2.1\times10^{d}$
5	Aanondo Bazar	$6.46\pm1.79\times10^{6b}$	$6.80\pm1.10\times10^{3a}$	$1.28\pm.41\times10^{6a}$	$5.70\pm0.1\times10^{4a}$	$8.86\pm0.44\times10^{3ab}$
6	Kpatan Bazar	$7.13\pm1.2\times10^{5b}$	$2.60\pm0.80\times10^{3b}$	$4.80\pm3.60\times10^{5ab}$	$2.70\pm2.00\times10^{4ab}$	$1.65\pm0.66\times10^{3ab}$
7	Shapno supershop	$1.67\pm0.70\times10^{4b}$	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
8	Mina Bazar	$2.20\pm1.03\times10^{5b}$	0.00±0.00	$1.50\pm0.46\times10^{4b}$	$6.30\pm2.40\times10^{2b}$	0.00±0.00
9	Aagora	$4.03\pm1.7\times10^{5}$	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Graphical presentation of TBC (Total Bacterial Count), TVC (Total *Vibrio* count), TC (Total coliform), and FC (Fecal Coliform), total *Salmonella* count (TSC) of prawn collected from 9 different markets of Dhaka city.

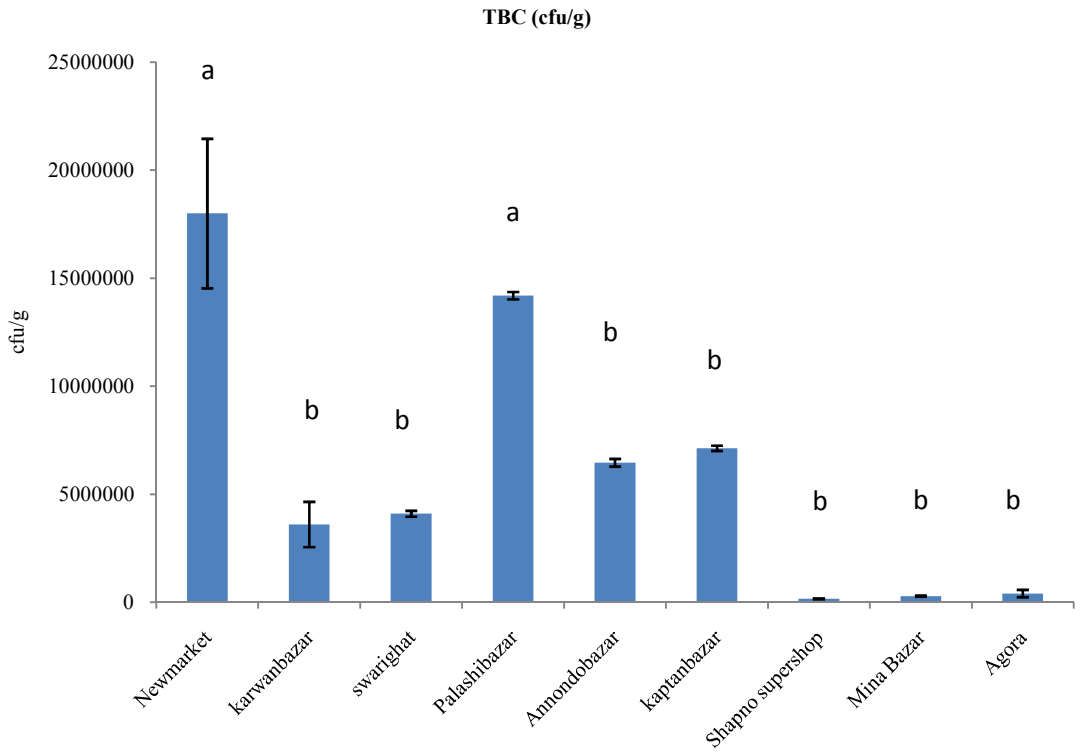


Figure 1 Density of total bacteria found in different prawn meat collected from nine different markets of Dhaka city

Significantly higher bacterial count was detected in prawn meat sampled from new market than that of others ($p<0.05$). Similar density of total bacteria was found in prawn sampled collected from Karwan Bazar, Swarighat, Palashi Bazar, Ananda Bazar, Kpatan Bazar, Mina Bazar, Shapno supershop, Aagora. Comparatively low bacterial count was observed in the sampled of three different super shop.

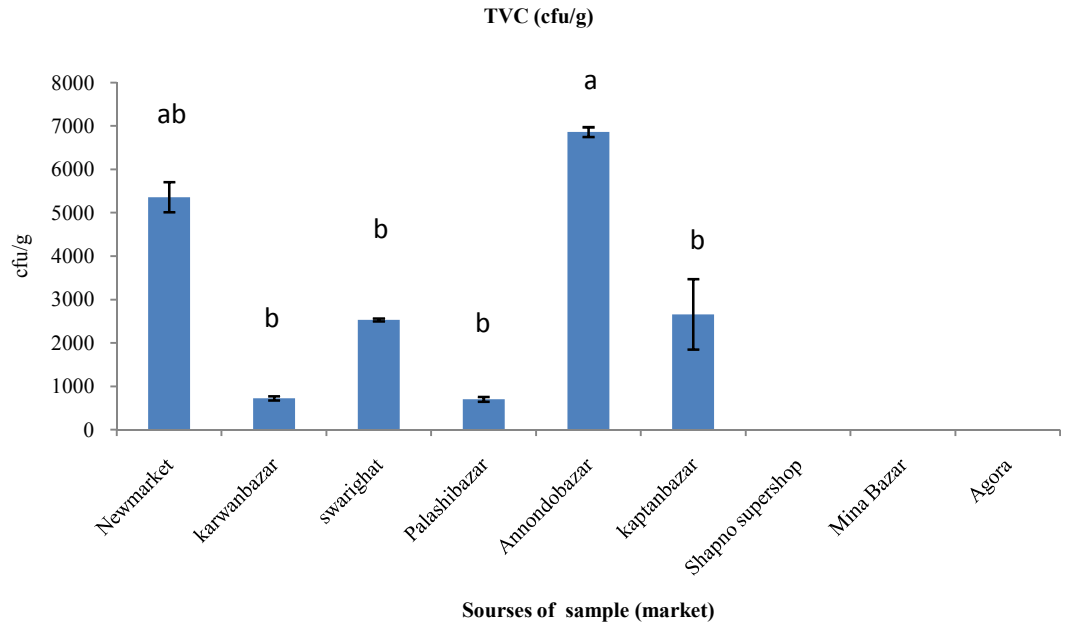


Figure 2 Density of total *vibrio* spp (cfu/g) found in different prawn meat collected from nine different markets of Dhaka city

Total vibrio count observed in muscle of prawn sampled from Ananda Bazar fish market was significantly higher than that of others ($p<0.05$). However, similar abundance was detected in prawn meat collected from Karwan Bazar, Swarighat, Palashi Bazar, Ananda Bazar, Kpatan Bazar . Vibrio count was nill in the prawn sampled of three super shops namely Mina Bazar, Shapno and Agora.

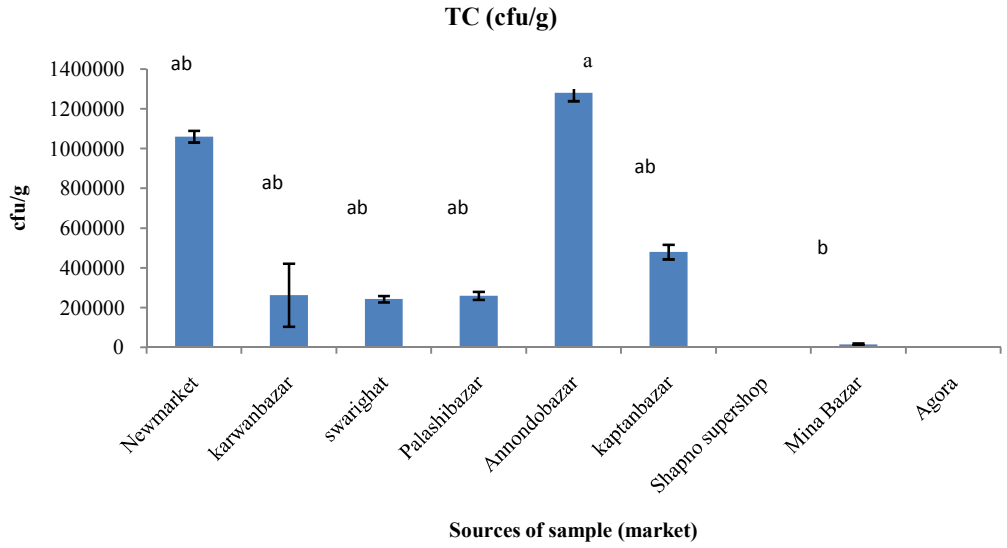


Figure 3 Density of total coliform (cfu/g) found in different prawn meat; collected from nine different markets of Dhaka city

Bloom of coliform bacterium in MacConkey agar was significantly higher bacterial colony was detected in Ananda bazar fish market ($p<0.05$). However, similar density was found in prawn meat collected from Karwan Bazar, Swarighat, Palashi Bazar, Aanondo Bazar, Kpatan Bazar, Mina Bazar, Shapno supershop, Agora. Total coliform count was nill in the prawn sampled of two super shop Shapno and Agora.

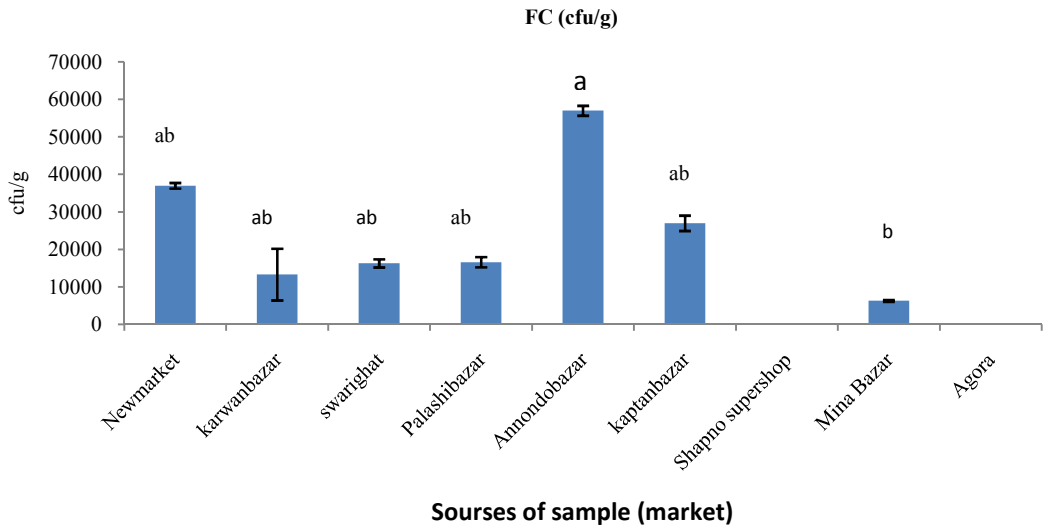


Figure 4 Density of fecal coliform (cfu/g) in different prawn meat collected from nine different markets of Dhaka city

Fecal coliform bacterium was significantly higher in Ananda bazar fish market.However, similar density was found in prawn meat collected from Karwan Bazar, Swarighat, Palashi Bazar, Aanondo Bazar, Kpatan Bazar, Mina Bazar. .No fecal colimorm count was found in the prawn sampled of two super shop Shapno and Agora.

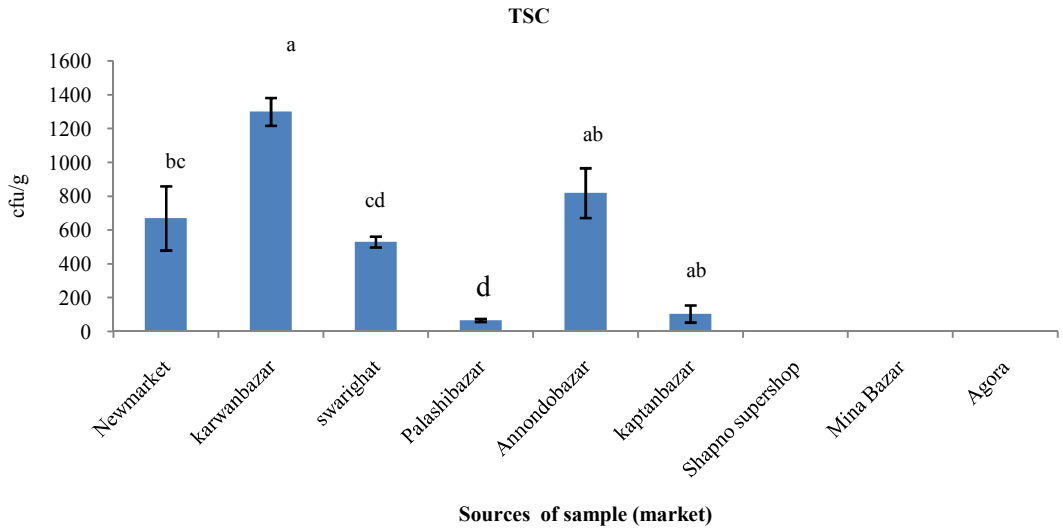


Figure 5 Total *Salmonella, Shigella* counts of Prawn meat collected from nine different markets of Dhaka city

Total *Salmonella, Shigella* count found in muscle of prawn sampled from Karwan Bazar fish market was significantly higher than that of others($p<0.05$) . However, similar abundance was detected in prawn meat collected from from Karwan Bazar, Swarighat, Palashi Bazar, Aanondo Bazar, Kpatan Bazar, Mina Bazar, Shapno supershop, Agora. Super shops were no abundance *Salmonella, Shigella*.

3.2 Identification of the isolated bacteria

3.2.1 Cultural characteristics of the microorganisms

Cultural characteristics of the microorganisms were shown in the table 2

Table 2 Cultural characteristics of microorganisms

Medium	Colour	Form	Margin	Elevation	Gram's reaction
NA	White	Irregular	Undulate	Convex	+
	Yellow	Circular	Entire	Flat	-
SS	Pink	Circular	Entire	Convex	-
	Black	Circular	Entire	Raised	-
TCBS	Yellow	Circular	Entire	Raised	-
	Green	Circular	Entire	Raised	-
	Dark Green	Circular	Entire	Raised	-
MSA	Yellow	Circular	Entire	Raised	+
mFC	Blue	Circular	Entire	Convex	-
EMB	Pink	Circular	Entire	Raised	-

3.2.2 Isolation and selection of the isolates

During study period, total 26 colonies were primarily isolated. These colonies comprised of all heterotrophic, enteric and related bacteria. Twenty-one isolates screened and finally 8 was selected from different sources (Table 3) and purified for detail study towards identification.

Table 3 Sources of the isolates

Isolates	Identified bacteria
NA 1	*Bacillus spp*
NA 2	*Klebsiella*
SS 1	*Salmonella*
SS 2	*Shigella*
TCBS 2	*Vibrio alginolyticus*
TCBS 3	*Vibrio parahaemolyticus*
MSA 1	*Staphylococcus aures*
mFC 1	*E. coli*
EMB 1	*E. coli*

Identification of bacteria was based on different biochemical tests and Gram's reaction results. Both Gram positive and Gram negative bacteria were obtained in the study (Table 4 and 5).

Table 4 Physiological and biochemical characteristics of the Gram positive isolates.

Isolate no.	*Catalase*	*Deep glucose agar*	*Voges proskauer Test*	*Methtyl red*	*Gas from glucose*	*Casen*	*Starch*	*Citrate*	*Propionate*	*Phenylalanine*	*Nitrate reduction*	*Indole*	*Motiliy*	*Provisional identification*
S2/NA/3/1	+	FA	-	-	-	+	+	+	+	-	-	-	-	*Bacillus spp*
S2/MSA/1′/1	+	SA	+	+	-	-	+	+	+	-	+	-	-	*Staphylococcus aureus*
S3/MSA/1/1	+	SA	-	+	-	-	-	+	+	-	-	-	+	*Staphylococcuc aureus*
S5/MSA/2/1	+	SA	+	+	-	-	-	+	+	-	+	-	-	*Staphylococcuc aureus*

'+' sign indicates catalase activity positive ; Voges-Proskauer positive; Methyl red produced ; Gas not produced from glucose; Casein, Gelatin and Starch hydrolyzed; citrate and propionate utilized; Nitrate reduced; Indole produced; Tyrosine degraded; motile; lecithinase produced.

'-' Sign indicates, Voges-Proskauer negative; Methyl red negative; Gas not produced from glucose; casein, gelatin and starch not hydrolysed; citrate and propionate not utilized; Indole not formed; Tyrosine not degraded; No deamination of phenylalanine; non motile; no lecithinase produced;

SA=strict aerobes, FA= facultative anaerobes

Table 3.9 Physiological and biochemical characteristics of isolated (Gram-negative) strains.

Isolate No.	Catalae	KIA				MIU			MR	VP	Hydrolysis		Oxidase	KOH solubility	Deep glucose agar	Provisionally identified bacteria
		Slant	Butt	H_2S	Gas	Motility	Indole	Urease			Casein	Starch				
S3/EMB/1/6	+	**A**	**A**	-	+	-	-	-	-	+	-	-	-	+	**FA**	*Klebsiella*
S5/EMB/2/1	+	**A**	**A**	+	-	+	-	+	-	+	-	-	-	+	**FA**	*E. coli*
S3/TCBS/3	+	**K**	**A**	-	+	+	-	+	+	-	-	-	-	+	**FA**	*Vibrio alginolyticus*
S3/TCBS/4	+	**K**	**A**	-	+	-	+	-	-	+	+	+	+	+	**FA**	*Vibrio parahaemolyticus.*
S3/SS/2/1	+	**K**	**A**	-	-	+	-	-	+	-	-	-	-	+	**FA**	*Salmonella*
S4/SS/1/2	+	**K**	**A**	-	-	+	-	-	+	+	-	-	-	+	**FA**	*shigella sp*

Chapter 4
Discussion

According to Liton (1990), most of the investigation and scientific studies besed on microbiology of fish foods through 1970s addressed mainly to the spoilage and associated biochemical mechanisms. But in subsequent years increase of food poisoning particularly due to consumption of marine and freshwater fishes alarmed the public and the community of scientist and technologists concerned. Emphasis science then has been shifted to investigation on microbiological quality of different fishes a food.
In Bangladesh few studies have carried count microbial quality of most common marine and fresh water fishes. The present study has been conducted to assess the microbial condition and identification of the isolates of fresh water prawns of different fish markets in Dhaka city. For this purpose giant river prawn or golda of different markets were examined. The samples were obtained from nine different markets of Dhaka metropolis namely Newmarket , Karwan Bazar, Swarighat, Palashi Bazar, Ananda Bazar, Kpatan Bazar, Mina Bazar, Shapno supershop and Agora.

Raw fishes are highly perishable protein that contains normal bacterial flora from their environment in addition to the contaminants occurred during harvesting and handling of the products. The living fish carry populations of predominantly gram-negative psychrotrophic bacteria on their external skin, nearly 10^2-10^3 bacteria per gram. Coliforms could be absent or present at very low density and *salmonella, shigella*, *Vibrio* and other entric pathogens are usually not found as these organisms are not the normal flora of the fishes or their environment. However, in the present study the prawn samples of different sources were highly contaminated with total aerobics bacteria as well as total coliform, fecal coliform *E. coli* and *vibrio spp*.

During this investigation bacterial load and types were studied for two important categories viz,(1) aerobic heterotrophic bacteria and (2) enteric and related bacteria. In almost all fishes the highest count was found in local fish markets. The examined super shops were free from toxic bacteria and low abundance of other bacterium.

Fish of good quality should have bacterial count less than 10^5 per gram. Contamination of fish from enteric bacteria of human and animal origin may be responsible for various food borne diseases (Emikpe *et al.* 2011).

The results of the bacterial analysis of prawns bought from nine markets in Dhaka city indicate high levels of contamination with pathogenic bacteria. When bacterial load is 106/gm or higher in food and food products, those foods are considered as spoiled (Conseulo *et al.* 1983).The total aerobic bacteria counts (TAB) exceeded the minimum acceptable limit ($<10^5$ cfu/g) for total aerobic counts. The high total aerobic counts is an indication of reduced shelf-life for the prawn meats while the high coliform counts is an indication of potential food infection/ intoxication (Buchanan 1991).

All of the tested samples from local markets were contaminated with coliform.. The total coliform count varied from 1.28×10^6 to 1.8×10^5 cfu/gm .So total coliform counts of the prawn samples were above the acceptable limit (coliform $<10^2$ cfu/g, according to Whong *et al*.(2003) Presence of coliform in food indicates sewage contamination.The total faecal coliform count varied from 0.0 to 5.70×10^4 cfu/gm.

Salmonella was found in 50% of the tested samples and the highest level recorded was 1.30×10^3 cfu/gm. Out of the 9 samples, five were contaminated with *Vibrio* and highest level was 6.80×10^3 cfu/gm. This result refers that the concerned giant fresh water prawns had an exposure to very unhygienic condition of different stages. It may start from hatcheries and other subsequent processes like farming area (gher, pond), processing station, transportation and market place. Health status of working personnels and their poor sanitation practices are also responsible.Prevalence of microbial load in shrimp was also reported (Yousuf *et al.* 2008).

The presence of *Escherichia coli* in all the samples of local markets except the super shop markets may be suggestive of faecal contamination due to poor hygiene and sanitation. *E. coli* has been implicated in human diarrheal particularly type 0157:H7 (Nester *et al.* 2007).

There is no indication that fish food is an important indication of *E. coli* infection. According to FDA and ICMSF (1986) guidelines the MPN of *E. coli* should be less than

10 and 3 respectively per gram of fish. Pathogenic *E. coli* produce disease in gut which may vary in the severity from extremely mild to severe and possibly life-treating depending on a number of factors such type of pathogenic strain , susceptibility to victim and degree of exposure.

The presence of *Pseudomonas, Salmonella* and *Shigella* in the prawn samples may be due to exposure to flies (Mensah *et al.* 2002) and prevailing poor sanitary conditions in the markets. *Salmonella* and *Shigella* are well-known important human pathogens (Olonitola *et al.* 2006). Their presence is unacceptable because of their attendant health risks (Mensah *et al.* 2002).

Fish and shellfish are prone to bacterial contamination and could cause health risk to consumers (Wafaa *et al.* 2011). The food poisoning associated with consumption of fish and shellfish either raw or slightly cooked, contaminated with *Vibrio* spp. causes intestinal infection characterized by diarrhea, abdominal cramps, sickness, vomiting, fever and severe headache (Merwad *et al. 2008*, Espineira *et al. 2010)*. The samples of fish and shellfish analyzed bacteriological showed varying degree of *Vibrio* contamination.

Vibrio parahaemolyticus is responsible for human gastroenteritis worldwide and sporadic cases and outbreaks occur regularly in Asia as well as in other countries (Serichantalergs *et al*. 2007).

Marine and fresh water invertebrates are constantly exposed to high concentration of microorganisms. Shrimps have been implicated in *Vibrio parahaemolyticus* food poisoning, Cholera, Salmonellosis, Shigellosis and Yersinia food infection. Deaths from staphylococcal food poisoning have been reported. The report also asserted that the offending organism, *Staphylococcus aureus* grow rapidly and produces enterotoxins between 66°F and 99°F (20°C and 37°C) and that the staphylococcal enterotoxins are highly resistant to heat. Bergdoll reported that the normal temperature used in cooking will not destroy the toxins and foods containing staphylococcal enterotoxins and they usually look and taste normal.

Chapter 5
Conclusion and recommendation

5.1 Conclusion

The study has provided novel information on the abundance and characteristic of bacterial flora isolated from prawn sample collected from nine different local markets and super shops of Dhaka Metropolitan City such as Newmarket, Karwan Bazar, Swarighat, Palashi Bazar, Aanondo Bazar, Kpatan Bazar, Mina Bazar, Shapno super shop and Agora.

Collected prawn samples from local market showed relatively high microbial load and presence of pathogens in the tested samples which indicated that the samples had exposure to an unhealthy environment. In microbial point of view comparatively better quality of prawns were found in the super shops in Dhaka Metropolitan City.

Presence of *E. col , Salmonella, vibrio spp* in the sample local fish markets prawns indicates a possibility of typhoid, diarrhea and other food born diseases through ingestion contaminated prawn. Salmonella in prawn samples indicate a major public health concerns.

5.2 Recommendation

1. Further study should be conducted on seasonal occurrences in those fish markets to identify the possible reservoirs of different bacteria
2. Research is needed to see the occurrences in other fish and shell fishes of different markets
3. Further in situ studies are needed in other coastal areas of Bangladesh
4. Investigation are needed to see the pathogenic organisms to evaluate whether a fish sample are safe for consumption
5. The microbial load of prawns can also be improved through regular disinfection of catching gears or working equipment, and brief immersion of caught prawns in disinfecting solution such as brine water to reduce the microbial load on the fish before storing at cold temperature or sold to the public
6. Before transport to the market the bacteriological quality of fish and shell fishes should be tested

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Appendices 1

Microbiological media

Media used were prepared by standard methods using appropriate compositions. Components used were high grade and were produced either by Sigma or DIFCO, USA. All media were sterilized by autoclaving for 15 minutes. The composition used for different media have been shown below

Alkaline Peptone Water (APW)

Ingredients	Amount (g/L)
Peptone	10 g
NaCl	10 g
Distilled water	1 L

Adjust pH to 8.5 ± 0.2 after dissolving ingredients. Dispense 10 mL portions into tubes and autoclave 10min at 121ºC.

Oxidase Reagent

N,N,N',N'-tetramethyl -p-phenylenediamine.2HCl	0.1 g
Distilled water	10 mL

Use this reagent on the day of preparation.

0% NaCl solution

Peptone	1g
NaCl	0 g
Dislilled water	100mL
pH adjusted to	7.4

3% NaCl solution

Ingredients for 100mL

Peptone	1g
NaCl	3 g
Dislilled water	100mL
pH adjusted to	7.4

6% NaCl solution

Ingredients for 100mL

Peptone	1g
NaCl	6 g
Dislilled water	100mL
pH adjusted to	7.4

8% NaCl solution

Ingredients for 100mL

Peptone	1g
NaCl	8 g
Dislilled water	100mL
pH adjusted to	7.4

10% NaCl solution

Ingredients for 100mL

Peptone	1g
NaCl	10 g
Dislilled water	100mL
pH adjusted to	7.4

Stock solutions

Normal saline

Ingredients	Amount (g/1)
NaCl (sigma)	8.5
Distilled water	1.0

pH was adjusted to 7.8.

Nutrient Agar

Ingredients	Amount (g/ml)
Powder nutrient agar	5.6
Distilled water	200

mFC Agar

Ingredients	Amount (g/ml)
Powder mFC agar	10.42
Distilled water	200
Rosolic acid	few drops

Rosolic Acid

Ingredients	Amount (g/ml)
Rosolic acid powder	0.5
Sodium Hydroxide	0.2
Distilled water	5.0

Minitol Salt Agar

Ingredients	Amount (g/ml)
Powder MSA	22.2
Distilled water	200

MacConkey Agar

Ingredients	Amount (g/ml)
Powder MacConkey agar	10.4
Distilled water	200

Eosin Methylene Blue Agar

Ingredients	Amount (g/ml)
Powder EMB agar	7.2
Distilled water	200

SS Agar

Ingredients	Amount (g/ml)
Powder SS agar	12.6
Distilled water	200

TCBS Agar

Ingredients	Amount (g/ml)
Powder TCBS agar	17.6
Distilled water	200

Nutrient Broth

Peptone	0.25 g
NaCl	0.25 g
Beef extract	0.15 g
Distilled water	50 mL

Indole Broth

Peptone	0.50 g
NaCl	0.25 g
Distilled water	50 mL

MRVP Broth

Peptone	1.05 g
Dextrose	0.75 g
Potassium phosphate	0.75 g

Distilled water 150 mL

Nitrate reduction Broth

Peptone 0.50 g

NaCl 0.50 g

Beef extract 0.30 g

$KNO_3$0.50 g

Agar powder 0.10 g

Distilled water 100 mL

Methyl red reagent

Ethyl alcohol 95% 60 ml

Methyl red 0.10 g

Distilled water 40 ml

Nitrate reduction reagents

Solution A

Sulfanilic acid 0.8 ml

Acetic acid 100 ml

Solution B

Alpha Napthalamine 0.5 g

Acetic acid 100 ml

5N Acetic acid

Glacial Acetic acid 63 ml

Distilled water 137 ml

Appendices 2

Case Summaries[a]

				TBC	TVC	TC	FC	SSA
Sources of sample	New market	1		34000000.00	4800.00	880000.00	6700.00	730.00
		2		46000000.00	6000.00	670000.00	9000.00	490.00
		3		4000000.00	5300.00	1630000.00	9000.00	790.00
		Total	N	3	3	3	3	3
			Mean	28000000.0000	5366.6667	1060000.0000	8233.3333	670.0000
			Std. Error of Mean	12489995.99680	348.01022	291376.04569	766.66667	91.65151
	karwan Bazar	1		320000.00	400.00	.00	.00	1170.00
		2		570000.00	1700.00	550000.00	23000.00	980.00
		3		3600000.00	60.00	240000.00	17000.00	1760.00
		Total	N	3	3	3	3	3
			Mean	1496666.6667	720.0000	263333.3333	13333.3333	1303.3333
			Std. Error of Mean	1054139.98649	499.73326	159199.38582	6887.99277	234.82854
	Swarighat	1		3200000.00	2000.00	.00	.00	460.00
		2		4500000.00	3200.00	540000.00	37000.00	530.00
		3		4100000.00	2400.00	190000.00	12000.00	230.00
		Total	N	3	3	3	3	3
			Mean	3933333.3333	2533.3333	243333.3333	16333.3333	406.6667
			Std. Error of Mean	384418.75316	352.76684	158149.01552	10898.52182	90.61518
	Palashi Bazar	1		15200000.00	1800.00	130000.00	6000.00	34.00
		2		10900000.00	.00	650000.00	44000.00	66.00
		3		16600000.00	320.00	.00	.00	109.00
		Total	N	3	3	3	3	3
			Mean	14233333.3333	706.6667	260000.0000	16666.6667	69.6667
			Std. Error of Mean	1714966.79592	554.41661	198578.28011	13775.98555	21.72812

	1		8600000.00	5000.00	720000.00	34000.00	970.00
	2		7900000.00	6700.00	2100000.00	80000.00	870.00
	3		2900000.00	8900.00	1020000.00	57000.00	820.00
Annondo Bazar	Total	N	3	3	3	3	3
		Mean	6466666.6667	6866.6667	1280000.0000	57000.0000	886.6667
		Std. Error of Mean	1794745.41680	1128.91295	419046.53680	13279.05619	44.09586
	1		960000.00	2800.00	240000.00	14000.00	1180.00
	2		560000.00	1200.00	.00	.00	950.00
	3		620000.00	4000.00	1200000.00	67000.00	1040.00
Kaptan Bazar	Total	N	3	3	3	3	3
		Mean	713333.3333	2666.6667	480000.0000	27000.0000	1056.6667
		Std. Error of Mean	124543.61128	811.03500	366606.05560	20404.24792	66.91620
	1		140000.00	.00	.00	.00	.00
	2		60000.00	.00	.00	.00	.00
	3		300000.00	.00	.00	.00	.00
Shapno supershop	Total	N	3	3	3	3	3
		Mean	166666.6667	.0000	.0000	.0000	.0000
		Std. Error of Mean	70553.36830	.00000	.00000	.00000	.00000
	1		370000.00	.00	14000.00	1100.00	.00
	2		22000.00	.00	24000.00	500.00	.00
	3		270000.00	.00	8000.00	300.00	.00
Mina Bazar	Total	N	3	3	3	3	3
		Mean	220666.6667	.0000	15333.3333	633.3333	.0000
		Std. Error of Mean	103442.95261	.00000	4666.66667	240.37009	.00000
	1		400000.00	.00	.00	.00	.00
	2		110000.00	.00	.00	.00	.00
	3		700000.00	.00	.00	.00	.00
Aagora	Total	N	3	3	3	3	3
		Mean	403333.3333	.0000	.0000	.0000	.0000
		Std. Error of Mean	170326.48388	.00000	.00000	.00000	.00000

	Total	N	27	27	27	27	27
		Mean	6181555.5556	2095.5556	400222.2222	15466.6667	488.1111
		Std. Error of Mean	2097946.52642	491.14592	107747.16280	4392.19717	97.69915

a. Limited to first 100 cases.

Homogeneous Subsets

TBC

	Sources of sample	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	Shapno supershop	3	166666.6667	
	Mina Bazar	3	220666.6667	
	Aagora	3	403333.3333	
	Kaptan Bazar	3	713333.3333	
	karwan Bazar	3	1496666.6667	
	Swarighat	3	3933333.3333	
	Annondo Bazar	3	6466666.6667	
	Palashi Bazar	3	14233333.3333	14233333.3333
	New market	3		28000000.0000
	Sig.		.373	.399

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

TVC

	Sources of sample	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	Shapno supershop	3	.0000		
	Mina Bazar	3	.0000		
	Aagora	3	.0000		
	Palashi Bazar	3	706.6667		

	karwan Bazar	3	720.0000		
	Swarighat	3	2533.3333		
	Kaptan Bazar	3	2666.6667	2666.6667	
	New market	3		5366.6667	5366.6667
	Annondo Bazar	3			6866.6667
	Sig.		.059	.054	.608

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

TC

	Sources of sample	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	Shapno supershop	3	.0000	
	Aagora	3	.0000	
	Mina Bazar	3	15333.3333	
	Swarighat	3	243333.3333	243333.3333
	Palashi Bazar	3	260000.0000	260000.0000
	karwan Bazar	3	263333.3333	263333.3333
	Kaptan Bazar	3	480000.0000	480000.0000
	New market	3	1060000.0000	1060000.0000
	Annondo Bazar	3		1280000.0000
	Sig.		.085	.096

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

FC

	Sources of sample	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	Shapno supershop	3	.0000	
	Aagora	3	.0000	
	Mina Bazar	3	633.3333	
	New market	3	8233.3333	8233.3333
	karwan Bazar	3	13333.3333	13333.3333
	Swarighat	3	16333.3333	16333.3333
	Palashi Bazar	3	16666.6667	16666.6667
	Kaptan Bazar	3	27000.0000	27000.0000
	Annondo Bazar	3		57000.0000
	Sig.		.647	.066

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

TSC

	Sources of sample	N	Subset for alpha = 0.05			
			1	2	3	4
Tukey HSD[a]	Shapno supershop	3	.0000			
	Mina Bazar	3	.0000			
	Aagora	3	.0000			
	Palashi Bazar	3	69.6667			
	Swarighat	3	406.6667	406.6667		
	New market	3		670.0000	670.0000	
	Annondo Bazar	3			886.6667	886.6667
	Kaptan Bazar	3			1056.6667	1056.6667
	karwan Bazar	3				1303.3333
	Sig.		.112	.567	.147	.098

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.