Determination of prevalence and antibiotic susceptibility pattern of the bacterial isolates collected from different parts of restaurant refrigerators of Mohakhali, Dhaka, Bangladesh.

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY

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Declaration

I hereby declare that the thesis project titled "**Determination of prevalence and antibiotic susceptibility pattern of the bacterial isolates collected from different parts of restaurant refrigerators of Mohakhali, Dhaka, Bangladesh"** has been submitted by me, Rokeya Sultana and has been carried out under the supervision of Nazneen Jahan, Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University, Dhaka.

It is further declared that this thesis has been composed solely by me and it has not been submitted, in whole or in part, in any previous institution for a degree or diploma.

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Dedicated to

My Beloved

Father and Mother

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Abstract

Refrigeration is an essential food storage technique and an important link in wide chain of cross contamination which leads to the outbreak of many food borne disease. In this study samples were obtained from various parts (drawer, handle, egg tray, surface) of two restaurant's refrigerators of Mohakhali, Dhaka, Bangladesh. A total of 41 isolates were collected from 8 different parts and identified through conventional biochemical tests according to Bergey's Manual of Systematic Bacteriology. Antibiotic susceptibility pattern was determined by using Kirby-Bauer disc diffusion method for all the 41 isolates against nine commercial antibiotic discs [Ampicillin (10 µg), Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Gentamycin (10 µg), Azithromycin (15µg), Penicillin-G (10 µg), Nalidixic acid (30 µg), Streptomycin (10µg), Tetracycline (30 µg)]. In result, *Staphylococcus spp.* showed the highest prevalence 11 (26.82%), followed by *Salmonella spp.* 7 (17.07%), *Klebsiella spp.* 7 (17.07%), *Vibrio spp.* 6 (14.63%), *Bacillus spp.* 4 (9.75%), *Pseudomonas spp.* 4 (9.75%) *and E.coli 2* (4.87%) respectively. Among the 41 isolates**,** 43.90% were found resistant to more than two antibiotics and highest percentage of resistance was observed against ampicillin (73.17%). On the other hand only 2.43% isolates showed resistance against ciprofloxacin and gentamycin. Temperature tolerance of the organisms also determined by growing the isolates in different temperature like 45°C, 50°C and 55°C. Growth was observed for all organisms at 45°C but in 50°C, 29.26% isolates showed viable growth. In 55º C no growth was observed. These results indicate that the pathogenic bacteria can survive in refrigerator surfaces and can cause cross contamination. It is needed to maintain appropriate food storage and refrigerator management, and proper hand hygiene is recommended.

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List of Abbreviations:

MSA	Mannitol Salt Aagar
MR	Methyl Red
VP	Voges-proskauer
TSI	Triple Sugar Iron
MHA	Muller Hinton Agar
MIU	Motility Indole Urease
µL	Microliter
spp.	Species
WHO	World Health Organization

Chapter 1

Introduction

1.1 Introduction

Microorganisms are ubiquitous occurring nearly everywhere in nature. They occur most abundantly in the presence of nutrients, moisture and temperature suitable for their growth and multiplication (Greenwood, D., R.C.B. Slack. John F.P., (2014). Sixteenth Edition: Medical Microbiology International Edition 2-4. 662-663.) .They are found in the air we breathe, the food we eat and on our body surfaces and other close environments. The presence of microorganisms in a location has various effects that could either be beneficial or harmful and human beings have various ways of resisting invasion by potentially harmful ones.

Refrigerator is a low temperature appliance used in homes and restaurant for the preservation and storage of food products (Haresh Khemani (2010)). Refrigeration is one of the most widely practiced methods of controlling microbial growth on perishable products of which temperature specification of four to five (4-5°C) degree Celsius is considered desirable (Juli Garden-Robinson (2013). Food Freezing Guide). Refrigeration is employed to control the rate of certain chemical and enzymatic reactions as well as the rate of growth of food microorganisms (Scott, E., Bloomfield, S. F., & Barlow, C.G. (1982)); food spoilage slows down as molecular motion slows, which retards growth of bacteria that cause food to spoil.

The pathogenic bacteria can survive in refrigerator surfaces and can cause cross contamination. If refrigerators are not properly maintained, it becomes a breeding ground for such bacteria. Many refrigerators incorrectly adjusted capable of supporting sub optimum but significant growth of mesophilic organisms such as *Staphylococcus aureus* and *Salmonella spp...* Bacterial contamination caused by unwashed raw foods, hands, leaking packages, utensil surface, etc are introduced to domestic refrigerator would directly contaminate other stored foods (Michaels B, Ayers T, Celis M, Gangar V. Inactivation of refrigerator biofilm bacteria for application in the food service environment. Food Sci Technol 2001; 1:169–179).

1.2 Bacteria commonly found in refrigerators

Temperature is an important factor in the growth of microorganisms. The practice of storing food at refrigeration temperature is common for controlling the growth of psychotropic microorganisms, some pathogens, and maintaining product quality. Nevertheless, psychotropic bacteria are commonly associated with spoilage of food at refrigerator temperatures and predominate in refrigerated food spoilage when low water activity, high acidity or packaging conditions select for their growth over bacteria in foods.

Many types of bacteria cause foodborne illnesses which found in the refrigerator. They are mentioned below:

- ***Salmonella,*** a bacterium found in many foods, including raw and undercooked meat, poultry, dairy products, and seafood. *Salmonella* may also be present on egg shells and inside eggs.
- ***Campylobacter jejuni*** *(C. jejuni),* found in raw or undercooked chicken and unpasteurized milk.
- ***Escherichia coli*** *(E. coli),* which includes several different strains, only a few of which cause illness in humans. *E. coli O157:H7* is the strain that causes the most severe illness. Common sources of *E. coli* include raw or undercooked hamburger, unpasteurized fruit juices and milk, and fresh produce.
- ***Listeria monocytogenes*** *(L. monocytogenes),* which has been found in raw and undercooked meats, unpasteurized milk, soft cheeses, and ready-to-eat deli meats and hot dogs.
- ***Vibrio,*** a bacterium that may contaminate fish or shellfish.
- ***Clostridium botulinum (****C. botulinum),* a bacterium that may contaminate improperly canned foods and smoked and salted fish. (NIDDK, 2014)

1.3 Antibiotic resistance

The emergence of antibiotic resistant bacteria in the food chain has become a major area of concern. (Kilonzo-Nthenge, S. N. Nahashon, F. Chen, and N. Adefope. 2008) studied the profiles and patterns of antibiotic resistant bacteria isolated from domestic food. *Enterobacter sakazakii, Shigella spp, Salomonella spp, E. coli, Klebsiella pneumoniae, Klebsiella terrigena, Klebsiella oxytoca, Flavimonas oryzihabitans, Aeromonas hydrophila , Enterobacter cloacea, Enterobacter aerogenes Hafnia alvei1,Kluyvera spp Pantoea* spp were some of the antibiotic resistant bacteria isolated from retail meats and domestic refrigerators. *Enterobacteriaceae* recovered from poultry, beef, swine, fresh produce, and domestic kitchens showed single, double, and triple antibiotic resistance. Results indicated that 49.6% of the isolates were resistant to at least one antibiotic (32.8% to ampicillin, 6.4% to nitrofurantoin, 4% to tetracycline, 3.2% to nalidixic acid, 2.4% to chloramphenicol and 1.7% to trimethoprim). Resistance to multiple antibiotics was observed in 6.4% of the isolates. This study implicates existence of antibiotic resistant *Enterobactericeae* in the domestic refrigerators. (Azevedo, H. Albano, J. Silva, P. Teixeira, 2018).

(Kilonzo-Nthenge A, Chen FC, Godwin SL, 2008) studied the prevalence and the identity of microorganisms in domestic refrigerators. *Klebsiella pneumoniae, Klebsiella oxytoca, Klebsiella terrigena ,Enterobacter sakazakii* ,and *Yersinia enterocolitica* were some of the bacteria isolated from domestic refrigerators. Resistance to antibiotics was most common in erythromycin (39.9%), followed by ampicillin (33.8%), cefoxitin (12.8%), tetracycline (5%), streptomycin (4.0%), nalidixic acid (2.1%), kanamycin (1.4%), and colistin (0.7%).These findings underline the need for greater consumer education regarding proper refrigerator cleaning and safe food handling practices.

1.4 Cross contamination

Cross contamination is the transfer of bacteria from one surface or medium to another (www.pittwater.nsw.gov.au). The most factors in bacterial transfer from one surface to another are moisture, contact time and pressure which can result in higher transfer between surfaces. When bacteria colonize on a rough surface, they are no longer in a direct contact with the transfer surface and thus are not easily transferred (Perez-Rodriguez, 2008). Hands can be a surface upon which bacteria reside for cross contamination to another medium. Contaminated hands are a major source of bacterial transfer in food processing and preparation. Microbial flora found on hands has been categorized in to two types: resident and transient. The resident micro flora consists of organisms that normally are always present on the skin. These are mainly found on the surface of the skin under the superficial cells of the stratum conium. The transmissibility of transient bacteria depends on the species, number of cells on the hand, their survival on the skin and the dermal moisture content. Temporary resident micro flora multiplies and persists for a limited period on the skin (Kampf and Kramer, 2004). Good personal hygiene and scrupulous hand washing can reduce the transfer of fecal microorganisms from hand to mouth and may prevent the spread of potentially transient microorganisms (Shojaei et al., 2006; Allowed et al., 2004; Daniels et al., 2002; Fry et al., 2005; Sneed et al., 2004). Cross contamination by microbial pathogens in the refrigerators play an important role in sporadic and epidemic food borne illnesses.

1.5 Safety from cross contamination:

Refrigerators are breeding grounds of bacteria as different types of food like raw or cooked food are stored within it. It contain objects for spreading the bacteria like drawer and egg tray, which are used regularly by stored food. The surfaces of refrigerators are in contact with hands and food and they contain a whole range of germ.

*Pseudomonas aeruginosa, Staphylococus aureus, Salmonella, Enterobacter, Listeria***.** So, refrigerators should be kept cleaned so that bacteria do not spread to food.

1.6 Literature review

Kumar et al., (2012) estimated the isolation of pathogens from domestic refrigerators was performed to determine the prevalence of pathogenic microorganisms. Samples were obtained from domestic refrigerators of various parts of South India (Vellore district). From the morphological and biochemical characteristics the isolates were identified as *Salmonella sp., Citrobactor sp., Proteus sp., E. coli.* These findings underline the need for greater consumer's education regarding proper cleaning of their refrigerators and safe food handling practices.

Wolde et al., (2015) investigated the prevalence and antibiotic resistance patterns of *Staphylococcus aureus* isolated from refrigerator's drawer used for food container of Jimma town, between October, 2010 and June, 2011. A total of 201 refrigerator's drawer samples from 20 restaurants, 101 hotels, 47 cafeterias and 33 pastry shops were enrolled in this study. Antibiotics susceptibility patterns of *S. aureus* isolates were done using 12 selected antibiotics. Out of 201 samples examined 69(34.3%) refrigerator's drawer were found to have *S. aureus*. Isolation rates of *S. aureus* differed among the food establishment types and it ranged from 30% (restaurants) to 36.4% (hotels). Significant variation in prevalence of *S. aureus* among refrigerator's drawer of restaurants, hotels, pastry shops and cafeterias were revealed. Ampicillin and Streptomycin were the most resisted drugs. Norfloxacin, Amikacin and Ciprofloxacin showed maximum sensitivity. Nine (9) drug resistance patterns were detected among *S. aureus* isolates. There was significant variation in the prevalence of *Staphylococcus aureus*.

1.7 Aims and objectives

The objectives of this research work were to demonstrate the prevalence of bacteria in different parts of refrigerator. Due to emerging incidence of multi-drug resistant organisms this study also aimed at determining the antibiotic resistance profile and detecting the multi-drug resistant organism from the isolated bacterial contaminants.

On the basis of above context, the objectives of the present study are:

- Isolating the bacterial contaminants present in different parts of refrigerators.
- Identifying and characterizing the bacterial contaminants.
- Determining the prevalence of the isolated organisms.
- Investigating the antibiotic resistance profile of the isolated microorganisms against some commonly used antibiotics and identifying the multi-drug resistant organisms.
- Different temperatures were used to determine the temperature tolerance of the organisms

Chapter 2:

Materials

&

Methods

2. Materials and methods

2.1 Study Area:

This study was carried out from the two restaurant's refrigerators of Mohakhali (BRAC UNIVERSITY cafeteria UB01 and CINNAMON restaurant) area in Dhaka city. Samples were collected from four parts (drawer, handle, surface and egg tray) of each refrigerators.

2.2 Duration:

The study was conducted during the period September-March, 2018.

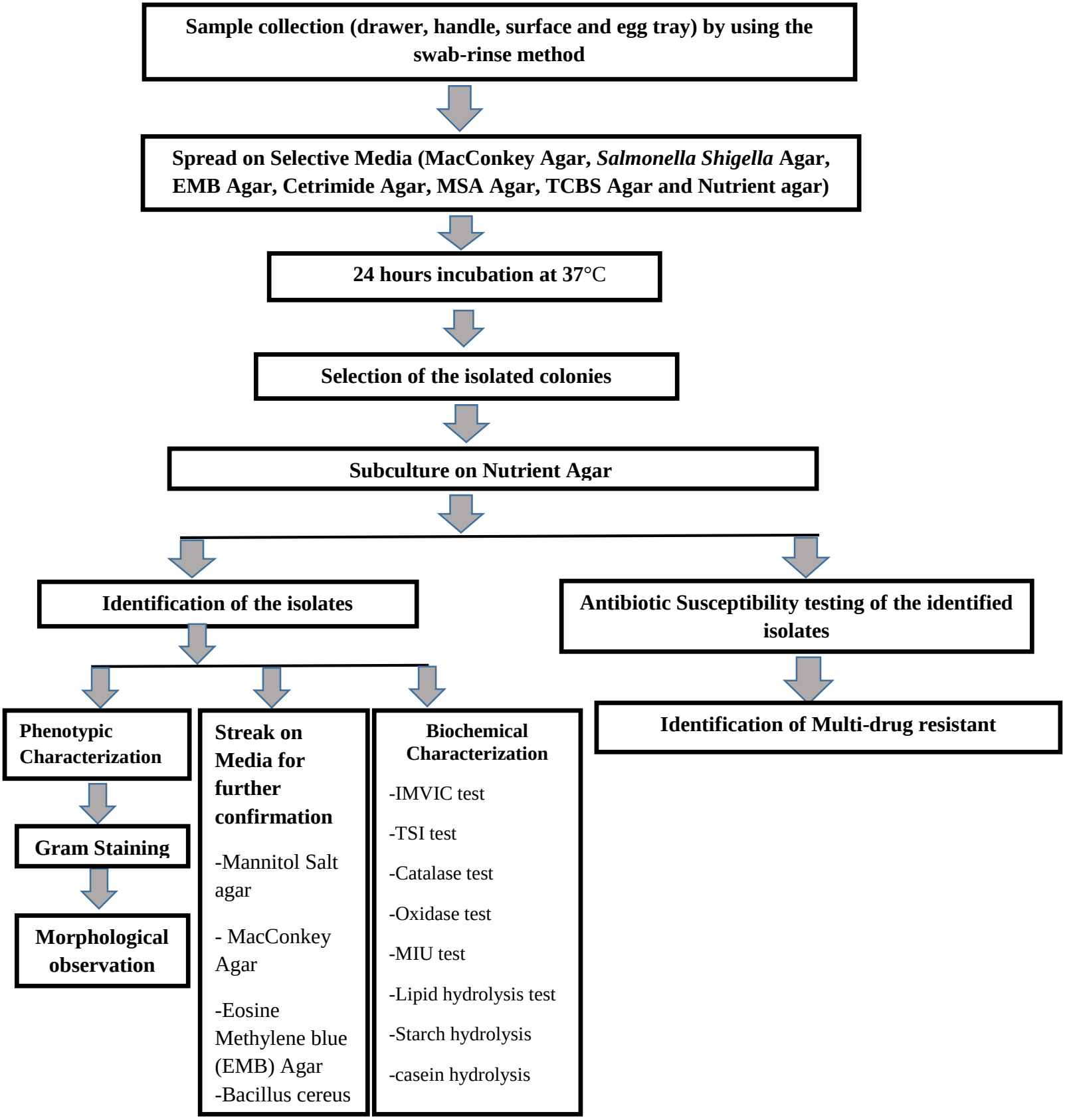
2.3 Sample size:

A total of 8 samples (drawer, handle, surface and egg tray) were collected from the two restaurant's refrigerators of Mohakhali (BRAC UNIVERSITY cafeteria UB01 and CINNAMON restaurant) area.

2.4 Sample collection and processing:

A total 8 samples were collected from the refrigerator's parts (drawer, handle, surface and egg tray) by using the swab-rinse method. In this method samples were taken by using sterile swab sticks moistened in sterile normal saline. Then it was immediately transported to the Microbiology Research Laboratory of BRAC University for further processing and analysis.

2.5 Flow Diagram of the Experimental design:



2.6 Isolation, purification and storage of sample:

Sources of 8 samples collected from drawer, handle, surface and egg tray and their respective collection date, time and number of isolates are mentioned below:

Table 2.1: Sample Collection: Source, Time, Number of the isolates found and their given name in the study

Sample No	Source	Date	Time	Number of the isolates found	Isolates ID
1	Refrigerator 1 (Part-A-Drawer)	06.11.2017	01.00 pm	9	S3A1, S3A2, S3A4, S3A6, S3A9, S3A10, S3A14, S3A15, S3A16
2	Refrigerator 1 (Part-B-Handle)	06.11.2017	01.00 pm	4	S3B3, S3B13, S3B18, S3B20
3	Refrigerator 1 (Part-C-Surface)	06.11.2017	01.00 pm	6	S3C7,S3C8, S3C11, S3C12, S3C17, S3C21
4	Refrigerator 1 (Part-D- Egg tray)	06.11.2017	01.00 pm	2	S3D5, S3D19
5	Refrigerator 2 (Part-A-Drawer)	19.11.2017	01.00 pm	6	S4A1, S4A4, S4A8, S4A9, S4A14, S4A19
6	Refrigerator 2 (Part-B Handle)	19.11.2017	01.00 pm	3	S4B3, S4B13, S4B16
7	Refrigerator 2 (Part-C Surface)	19.11.2017	01.00 pm	9	S4C2, S4C5, S4C7, S4C10, S4C11, S4C12, S4C15, S4C17,S4C18
8	Refrigerator 2(Part-D- Egg tray)	19.11.2017	01.00 pm	2	S4D6, S4D20

After sample collection, samples were purified by using some selective media. Some samples were spreaded on Nutrient Agar after serial dilutions (10^{-1} to 10^{-3}) . Other samples were directly spreaded on some selective media plates (Mannitol Salt agar, TCBS Agar, Eosine Methylene blue Agar, MSA agar, MacConkey Agar, *Salmonella Shigella* Agar) from test tubes. Then the plates were incubated for 24 hours at 37ºC. After incubation, isolated colonies were observed and cfu/ml were calculated for some of the plates and some showed huge growth. Then the colonies were streaked on nutrient agar plates to get pure cultures for storage.

2.7 Long term preservation:

Glycerol stock media was prepared in a sterile eppendorf. For long-term preservation, bacteria was taken from culture plate with sterile inoculating loop and mixed in 500µl Nutrient broth. After that it was incubated for 2 hours at 37ºC. Then 300 µl of sterile glycerol was added to the broth culture and the eppendorf was stored at -20^{0}C.

2.8 Biochemical identification:

Biochemical identification of the isolates was done using methods from Bergey's Manual of Systematic Bacteriology.

2.8.1 Indole test:

Indole production test was done to determine the ability of microorganisms to degrade the amino acidtryptophan by the enzyme tryptophanase.

- For indole test each indole broth containing 6ml of peptone, sodium chloride was taken.
- Using sterile technique, small amount of the experimental bacteria from fresh culture was inoculated into the tubes by means of loop inoculation method with an inoculating loop
- The tubes were then incubated for 24 hours at37ºC.
- In order to detect the indole production, 10 drops of Kovacs reagent was added to all the tubes.

If red reagent layer develops then it indicates indole positive and absence of red color indicates that the substrate tryptophan was not hydrolyzed and it indicates indole negative reaction.

2.8.2 Methyl red (MR) test:

Methyl red test was done to determine the ability of the bacteria to oxidize glucose with the production and stabilization of high concentration of acid end products.

- For methyl red test each MR broth containing 5 ml of dipeptone, dextrose and potassium phosphate was taken.
- Using sterile technique, each tube was inoculated by fresh culture of experimental bacteria by means of loop inoculation method.
- The tubes were then incubated for 48 hours at37ºC.
- After 48 hours, 5 drops of methyl red indicator was added to each tube and the colour of the tubes was observed.
- If red colour develops then it indicates that the organism was capable of fermenting glucose with the production of high concentration of acid.

If orange or yellow colour develops then it indicates methyl red negative result

2.8.3 Voges-Proskauer (VP) test:

The Voges-Proskauer (VP) test was done to determine if an organism produces acetylmethyl carbinol from glucose fermentation.

- For Voges-Proskauer test each VP broth containing dipeptone, dextrose and potassium phosphate was taken.
- Using sterile technique, each tube was inoculated by fresh culture of experimental bacteria by means of loop inoculation method.
- The tubes were then incubated for 48 hours at37ºC.
- After 48 hours, 10 drops of Barritt's reagent A was added to each tube and the tubes were shaken. Then immediately 10 drops of Barritt's reagent B was added and the tubes were shaken.
- The colour was observed after 15-30 minutes of the reagent addition.
- If red colour developed then it indicates that the organism was capable of fermenting glucose with ultimate production of acetyl methyl carbinol and it indicates positive result.
- If no colour developed then it indicates voges- proskauer negative result.

2.8.4 Citrate utilization test:

Citrate utilization test was done to differentiate among enteric organisms on the basis of their ability to ferment citrate as a sole source of carbon by the enzyme citrate.

- For citrate utilization test each vial containing 2.5 ml of Simmons citrate agar was taken.
- Using sterile technique, small amount of the experimental bacteria from 24-hours fresh culture was inoculated into the vials by means of a streak inoculation method with an inoculating loop.
- The vials were then incubated at 37ºC for 24-48 hours.
- After 48 hours incubation, if the Prussian blue color developed then it indicates the citrate positive result which means the organism was capable of fermenting citrate as a sole source of carbon.
- If there was no color change then it indicates citrate negative result.

2.8.5 Triple sugar-iron (TSI) agar test:

Triple sugar iron agar test was done to differentiate between Gram negative enteric bacilli based on their ability to ferment carbohydrate and reduce hydrogen sulfide.

- For TSI test each tube containing TSI agar was taken.
- Using sterile technique, small amount of the experimental bacteria from fresh culture was inoculated into the tubes by means of stab inoculation method with an inoculating needle.
- The tubes were then incubated at 37˚C for 24-48 hours.
- After 24-48 hours the color of both the butt and slant of agar slant cultures were observed.
- The results were recorded based on the following observation.

Table 2.2: Interpretation of Triple sugar iron (TSI) test result

Result	Interpretation	Symbol
Yellow slant/yellow butt	Glucose and lactose and/or sucrose fermentation with acid accumulation in slant and butt.	A/A
Red slant/yellow butt	Glucose fermentation with acid production. Proteins catabolized aerobically (in the slant) with alkaline products (reversion).	K/A
Red slant/red butt	No fermentation. Peptone catabolized aerobically and anaerobically with alkaline products. Not from *Enterobacteriaceae*.	K/K
Red slant/no change in butt	No fermentation. Peptone catabolized aerobically with alkaline products. Not from *Enterobacteriaceae*.	K/NC
No change in slant / no change in butt	Organism is growing slowly or not at all. Not from *Enterobacteriaceae*.	NC/NC
Black precipitate in the agar	Sulfur reduction. (An acid condition, from fermentation of glucose or lactose and/or sucrose, exists in the butt even if the yellow color is obscured by the black precipitate.)	H_2S
Cracks in or lifting of agar	Gas production.	G

2.8. 6 Catalase test:

Catalase test was done to determine the ability of the bacteria to degrade hydrogen peroxide by producing the enzyme catalase.

- For catalase test a sterile microscopic slide was taken.
- A drop of the catalase reagent3% Hydrogen peroxide was placed on the glass slide
- Using a sterile inoculating loop, a small amount of bacteria from 24-hour pure culture was placed onto the reagent drops of the microscopic slide

An immediate bubble formation indicated a positive result and no bubble formation indicated catalase negative result (Reiner, 2010).

2.8.7 Oxidase test

This is particularly useful for the differentiation of pseudomonas from other Gram-negative bacteria. A strip of whatmann filter paper was impregnated with 10% aqueous solution of *tetra methyl-p-phenylene-diamine hydrochloride*. A wire loop was used to pick a colony of the test organism and rubbed on the wet filter paper impregnated with the oxidase reagent. Development of purple or deep blur colorations after about 5 minutes was recorded as a positive reaction.

2.8.8 MIU (Motility-indole-urease) test:

MIU test was done for determining the motility of bacteria, indole production and urea degradation by means of the enzyme urease.

- Using sterile technique, small amount of the experimental bacteria from fresh culture was inoculated into the tubes by means of stab inoculation method with an inoculating needle
- The tubes were then incubated for 24 hours at 37ºC.
- The growth of the organism would spread throughout the test tube from downward to the upward of the test tube, if the organism is motile.
- The color of the media will turn to deep pink if the organism is positive for urease test. If yellow color develops then it indicates urease negative result.

To confirm the indole test, five drops of Kovac's reagent was added following overnight incubation. Then the color of the media was examined and the results were recorded. Formation of a rose red ring at the top indicates a positive result. A negative result can have a yellow or brown layer.

2.8.9 Starch Hydrolysis test:

This test was performed to determine if the microbe can use starch as a source of carbon and energy for growth and this use of starch was accomplished by an enzyme called alpha-amylase.

- Using sterile technique, Starch agar plates were inoculated with the test organism by using a sterile inoculating loop.
- Then the plates were incubated for 24 hours 37°C and after that Grams Iodine was added in the plates and allowed to stand for 10 minutes.
- Media will turn blue-black indicating the absence of starch-splitting enzymes and representing a negative result. If the starch has been hydrolyzed, a clear zone of hydrolysis will surround the growth of the organism and this is a positive result.

2.8.10 Casein hydrolysis test:

This test was done to determine the ability of microorganisms to excrete hydrolytic extracellular enzymes capable of degrading the protein casein.

- Using sterile technique, skim milk agar plates were inoculated with the test organism by using a sterile inoculating loop.
- Then the plates were incubated for 24 hours at 37°C.

- If the organisms secrete proteases, it will exhibit a zone of proteolysis which is demonstrated by a clear area surrounding the bacterial growth. It represents a positive result. In the absence of protease activity, the medium surrounding growth of the organism remains opaque which is a negative result. (Cappuccino &Sherman, 2005)

2.8.11 Lipid Hydrolysis

- This media tests for the ability of an organism to break down and use a vegetable lipid (tributyrin) present in the agar plates.
- If an organism is able to secrete lipase the lipid can be hydrolyzed. The media usually contains spirit blue or methylene blue as an indicator.
- Use of the lipid can be observed as a zone of clearing around areas of growth.
- The zone has to be transparent for the test to be considered positive; color changes are not considered to be positive.

2.9 Antimicrobial assay using antibiotic disc:

Kirby-Bauer disc diffusion method was used to determine the susceptibility of clinical isolates. About 9-10 different antibiotic discs were used for identifying antibiotic sensitive and resistant bacteria. Antibiotics those were used in the study are given in table.

Table 2.3: List of antibiotics and their potency

Serial no	Antibiotic	Disc code	Disc potency (µg)
1	**Ampicillin**	**AM**	**10**
2	**Azithromycin**	**AZM**	**15**
3	**Ciprofloxacin**	**CIP**	**5**
4	**Chloramphenicol**	**C**	**30**
5	**Gentamycin**	**CN**	**10**
6	**Penicillin-G**	**P**	**10**
7	**Nalidixic acid**	**NA**	**30**
8	**Streptomycin**	**S**	**10**
9	**Tetracycline**	**TE**	**30**

Steps performed in antibiotic susceptibility test:

The steps of the work are given beneath:

1. Standardized inoculum of 0.5 McFarland (approximate cell count density: 1.5x108) turbidity standard was prepared by taking 1-2 colonies of organisms.

2. Using sterile cotton swabs, each of the test bacterial strains were lawn cultured on properly labelled Mueller Hinton Agar plates.

4. After lawn culture, sterile forceps were used to carefully pick up antibiotic disks from the stacks and were placed very carefully on the lawn culture.

5. Care was taken to ensure that the disks are well-spaced in order to prevent overlapping of inhibition zones.

6. Then the plates were incubated at 37°C for 24 hours.

7. After incubation antibiotic susceptibility was determined by measuring the diameter of inhibition zone in millimeter.

8. Finally, result was interpreted according to the table given below (table: 2.4).

Table 2.4: Chart used in the result interpretation of antibiotic susceptibility testing.

Serial no	Antibiotic	Inhibition Zone diameter (in mm)		
		Resistant	**Intermediate**	**Susceptible**
1	**Ampicillin**	≤11	12-13	≥14
2	**Azithromycin**	≤13	14-17	≥18
3	**Ciprofloxacin**	≤15 / ≤20	16-20/ 21-30	≥21 / ≥31
4	**Chloramphenicol**	≤12	13-17	≥18
5	**Gentamycin**	≤12	13-14	≥15
6	**Penicillin-G**	≤23	-	≥29
7	**Nalidixic acid**	≤13	14-18	≥19
8	**Streptomycin**	≤14	15-20	≥21
9	**Tetracycline**	≤11	12-14	≥15

Chapter 3: Results

RESULTS

3.1: Bacterial isolation

Samples (drawer, handle, surface and egg tray) were collected from the two restaurant's refrigerators of Mohakhali (BRAC UNIVERSITY cafeteria UB01 and CINNAMON restaurant). These samples were streaked onto various selective, differential and nutrient agar media to identify organisms present in each sample. Both gram positive and gram negative bacteria were found in the two samples. Results were recorded according to their colony morphology and biochemical characteristics of the isolates in different agar media.

3.2 Bacterial Identification:

3.2 Cultural and morphological characteristics of the bacterial isolates:

In Table 3.2 the colour, shape of the colonies on various selective, differential and enriched media and the morphology of the bacterial colonies on nutrient agar are explained.

Table 3.1: Cultural and Morphological Characteristics of the Bacterial Isolates on various Selective, Differential, Enriched media and Nutrient Agar from BRAC UNIVERSITY cafeteria UB01

Isolates ID	Growth on Selective, Differential and Enriched Media						Colony morphology on Nutrient Agar					Suspected organism
	Mac-Conkey Agar	Mannitol Salt Agar (MSA)	Eosine Methylene Blue Agar (EMB)	Cetrimide agar	Salmonella-Shigella Agar (SS Agar)	TCBS Agar	Size	Color	Form	Margin	Elevation	
S3A1		-	-	White, greenish	-	-	Small	White	Circular	undulate	Raised	*Pseudomonas spp*
S3A2				White, greenish	-		Small	White	Circular	Undulate	Raised	*Pseudomonas spp*
S3B3	-		Pink coloured mucoid colonies				Large	Creamy	Circular	Entire	Flat	*Klebsiella spp.*
S3A4			Pink coloured mucoid colonies				Large	creamy	Circular	Entire	Flat	*Klebsiella spp.*
S3D5					Black centered colonies		Large	white	Circular	Entire	Convex	*Salmonella spp*
S3A6					Black centered colonies		Large	white	Circular	Entire	Convex	*Salmonella spp*

Isolates ID	Growth on Selective, Differential and Enriched Media						Colony morphology on Nutrient Agar					Suspected Organism
	Mac-Conkey Agar	Mannitol Salt Agar (MSA)	Eosine Methylene Blue Agar (EMB)	Cetrimide agar	Salmonella-Shigella Agar (SS Agar)	TCBS Agar	Size	Color	Form	Margin	Elevation	
S3C7		-		-	Black centered colonies	-	Large	white	Circular	Entire	convex	*Salmonella spp*
S3C8	Pink coloured mucoid colonies		Pink coloured mucoid colonies	-	-	-	Large	Creamy	Circular	Entire	Flat	*Klebsiella spp.*
S3A9						Green coloured colonies	Small	White	Circular	Entire	Flat	*Vibrio spp.*
S3A10		Small, yellow coloured colonies					Small	yellow	Circular	Entire	Convex	*Staphylococcus spp*
S3C11	-	-	-				Large	White	irregular	undulant	urbanite	*Bacillus spp.*
S3C12		Small, yellow coloured colonies					Small	Yellow	Circular	Entire	Convex	*Staphylococcus spp.*

Isolates ID	Growth on Selective, Differential and Enriched Media						Colony morphology on Nutrient Agar					Suspected Organism
	Mac-Conkey Agar	Mannitol Salt Agar (MSA)	Eosine Methylene Blue Agar (EMB)	Cetrimide agar	Salmonella -Shigella Agar (SS Agar)	TCBS Agar	Size	Color	Form	Margin	Elevation	
S3B13	-	Small, yellow coloured colonies	-	-	-	-	Small	Yellow	Circular	Entire	Convex	*Staphylococcus spp.*
S3A14		Small, yellow coloured colonies			-		Small	Yellow	Circular	Entire	Convex	*Staphylococcus spp.*
S3A15		Small, yellow coloured colonies					Small	Orange	Circular	Entire	Flat	*Staphylococcus spp.*
S3A16							Large	White	Irregular	Undulant	Umbonate	*Bacillus spp.*
S3C17		Small, yellow coloured colonies					Small	Orange	Circular	Entire	Flat	*Staphylococcus spp.*

Isolates ID	Growth on Selective, Differential and Enriched Media						Colony morphology on Nutrient Agar					Suspected Organism
	Mac-Conkey Agar	Mannitol Salt Agar (MSA)	Eosine Methylene Blue Agar (EMB)	Cetrimide agar	Salmonella -Shigella Agar (SS Agar)	TCBS Agar	Size	Color	Form	Margin	Elevation	
S3B18		Small, yellow coloured colonies					Small	Orange	Circular	Entire	Flat	*Staphylococcus spp.*
S3D19	-	-	-	Black centered colonies	-	-	Large	White	Circular	Entire	Convex	*Salmonella spp*
S3B20			Pink colonies		-		Large	Creamy	Circular	Entire	Flat	*Klebsiella spp.*

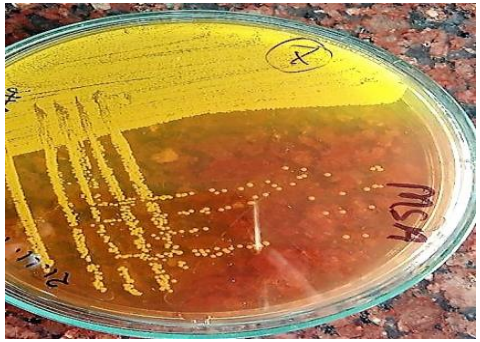
Table 3.2: Cultural and Morphological Characteristics of the Bacterial Isolates on various Selective, Differential, Enriched Media and Nutrient Agar from CINNAMON restaurant

Isolates ID	Growth on Selective, Differential and Enriched Media						Colony morphology on Nutrient Agar					
	Mac-Conkey Agar	Mannitol Salt Agar (MSA)	Eosine Methylene Blue Agar (EMB)	Cetrimide agar	Salmonella -Shigella Agar (SS Agar)	TCBS Agar	Size	Color	Form	Margin	Elevation	Suspected Organism
S4A1		Small, Yellow coloured colonies					Small	Orange	Circular	Entire	Flat	*Staphylococcus spp.*
S4C2		Small, yellow coloured colonies					Small	Orange	Circular	Entire	Flat	*Staphylococcus spp.*
S4B3	-	-	-	-	-	-	Large	White	Irregular	Undulant	Umbonate	*Bacillus spp.*
S4A4					-	Yellow coloured colonies	Small	White	Circular	Entire	Flat	*Vibrio spp.*
S4C5						Yellow coloured colonies	Small	white	Circular	Entire	Flat	*Vibrio spp.*
S4D6					Black centered colourless colonies		Large	White	Circular	Entire	Convex	*Salmonella spp.*

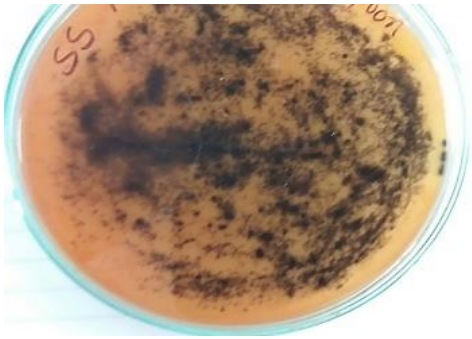
Isolates ID	Growth on Selective, Differential and Enriched Media						Colony morphology on Nutrient Agar					Suspected Organism
	Mac-Conkey Agar	Mannitol Salt Agar (MSA)	Eosine Methylene Blue Agar (EMB)	Cetrimide agar	Salmonella -Shigella Agar (SS Agar)	TCBS Agar	Size	Color	Form	Margin	Elevation	
S4C7						Yellow coloured colonies	Small	White	Circular	Entire	Flat	*Vibrio spp.*
S4A8	-		Metallic Green sheen colonies				small	Creamy	Circular	Entire	Raised	*E.coli*
S4A9	-		Metallic Green sheen colonies	-	-	-	Medium	Creamy	Circular	Entire	Raised	*E.coli*
S4C10			Pink coloured mucoid colonies		-		Large	Creamy	Circular	Entire	Flat	*Klebsiella spp.*
S4C11				White, greenish			Small	white	Circular	Undulate	Raised	*Pseudomonas spp*

Isolates ID	Growth on Selective, Differential and Enriched Media						Colony morphology on Nutrient Agar					Suspected Organism
	Mac-Conkey Agar	Mannitol Salt Agar (MSA)	Eosine Methylene Blue Agar (EMB)	Cetrimide agar	Salmonella -Shigella Agar (SS Agar)	TCBS Agar	Size	Color	Form	Margin	Elevation	
S4C12					Black centered colonies		Medium	White	Circular	Entire	Convex	*Salmonella spp.*
S4B13			pink coloured colonies				Small	Creamy	Circular	Entire	Convex	*Klebsiella spp*
S4A14			pink coloured colonies				Small	Creamy	Circular	Entire	Convex	*Klebsiella spp*
S4C15				White, greenish	-	-	Small	white	Circular	Undulate	Raised	*Pseudomonas spp*
S4B16					-		Large	white	Irregular	Undulate	Umbonate	*Bacillus spp.*

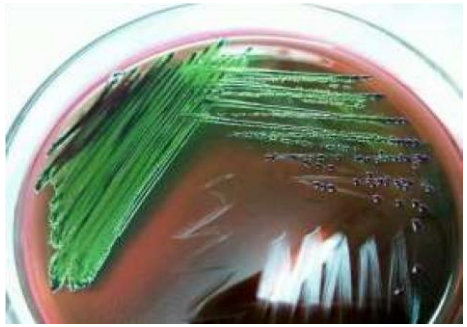
S4C17		**Small, pink coloured colonies**					**Small**	**Orange**	**Circular**	**Entire**	**Flat**	***Staphylococcus spp.***
S4C18						**Yellow coloured colonies**	**Small**	**White**	**Circular**	**Entire**	**Flat**	***Vibrio spp.***
S4A19						**Yellow coloured colonies**	**Small**	**White**	**Circular**	**Entire**	**Flat**	***Vibrio spp.***
S4D20					**Black centered colonies**		**Large**	**White**	**Circular**	**Entire**	**Convex**	***Salmonella spp.***
S4C21					**Black centered colonies**		**Large**	**White**	**Circular**	**Entire**	**Convex**	***Salmonella spp.***



Staphylococcus spp. on MSA

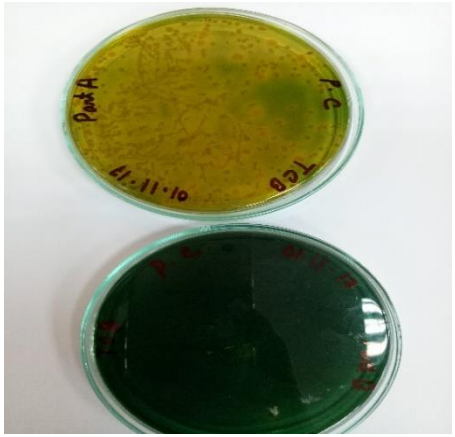


Salmonella spp. on SS Agar

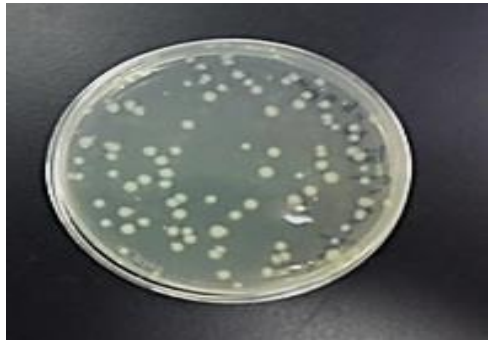


E.coli on EMB Agar

Klebsiella spp.on MacConkey Agar



Vibrio spp. on TCBS Agar



Pseudomonas spp.on Cetrimide Agar

Figure 3.1: Bacterial growth on various selective media.

3.3 Biochemical characteristics of the bacterial isolates:

Table 3.3: Biochemical characteristics of the bacteria isolated from BRAC UNIVERSITY cafeteria UB01

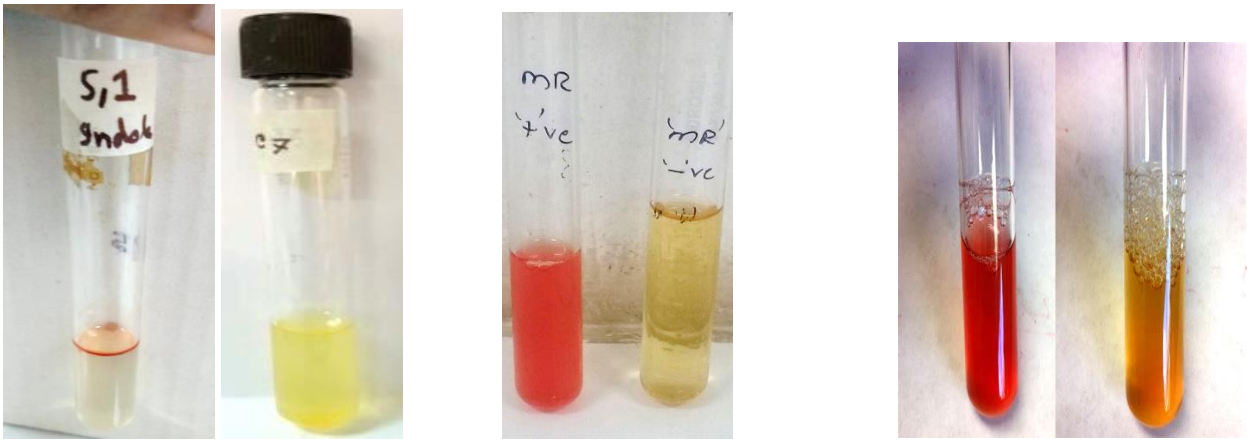
Isolates ID	Gram staining		Indole Test	Methyl red Test	VP Test	Citrate test	Triple Sugar Iron Test						Carbohydrate fermentation		MIU Test			Catalase Test	Lipid hydrolysis	Starch Hydrolysis	Casein Hydrolysis	Suspected Organism
	Gram	Shape					Slant/Butt	Glucose	Lactose	Sucrose	H2S	Gas	Maltose	Gas	Motility	Indole	Urease					
S3A1	-	Rod	-	-	-	+	R/R	+	-	-	-	-	+	-	+	+	+	+	+	+	+	*Pseudomonas spp*
S3A2	-	Rod	-	-	-	+	R/R	+	-	-	-	-	+	-	+	+	+	+	+	+	+	*Pseudomonas spp*
S3B3	-	Rod	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	+	-	-	*Klebsiella spp.*
S3A4	-	Rod	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	+	-	-	*Klebsiella spp.*
S3D5	-	Rod	-	+	-	+	Y/Y	+	+	+	+	-	+	+	+	-	-	+	-	-	-	*Salmonella spp*
S3A6	-	Rod	-	+	-	+	Y/Y	+	+	+	+	-	+	+	+	-	-	+	-	-	-	*Salmonella spp.*
S3C7	-	Rod	-	+	-	+	Y/Y	+	+	+	+	-	+	+	+	-	-	+	-	-	-	*Salmonella spp*
S3C8	-	Rod	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	+	-	-	*Klebsiella spp.*
S3A9	-	Rod	-	-	-		Y/Y	+	+	+	-	-	+	+	+	-	-	+	+	-	-	*Vibrio spp.*
S3A10	+	cocci	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	+	-	-	*Staphylococcus spp.*

S3C11	**+**	**Rod**	**-**	**-**	**-**	**-**	**Y/Y**	**+**	**+**	**+**	**-**	**-**	**+**	**-**	**-**	**-**	**-**	**+**	**+**	**+**	**+**	***Bacillus spp.***
S3C12	**+**	**cocci**	**-**	**+**	**+**	**+**	**Y/Y**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**-**	**-**	**+**	**+**	**+**	**-**	**-**	***Staphylococcus spp.***
S3B13	**+**	**cocci**	**-**	**+**	**+**	**+**	**Y/Y**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**-**	**-**	**+**	**+**	**+**	**-**	**-**	***Staphylococcus spp.***
S3A14	**+**	**cocci**	**-**	**+**	**+**	**+**	**Y/Y**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**-**	**-**	**+**	**+**	**+**	**-**	**-**	***Staphylococcus spp.***
S3A15	**+**	**cocci**	**-**	**+**	**+**	**+**	**Y/Y**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**-**	**-**	**+**	**+**	**+**	**-**	**-**	***Staphylococcus spp.***
S3A16	**+**	**Rod**	**-**	**-**	**-**	**-**	**Y/Y**	**+**	**+**	**+**	**-**	**-**	**+**	**-**	**-**	**-**	**-**	**+**	**+**	**+**	**+**	***Bacillus spp.***
S3C17	**+**	**cocci**	**-**	**+**	**+**	**+**	**Y/Y**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**-**	**-**	**+**	**+**	**+**	**-**	**-**	***Staphylococcus spp.***
S3B18	**+**	**cocci**	**-**	**+**	**+**	**+**	**Y/Y**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**-**	**-**	**+**	**+**	**+**	**-**	**-**	***Staphylococcus spp.***
S3D19	**-**	**Rod**	**-**	**-**	**+**	**+**	**R/Y**	**+**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**-**	**-**	**+**	**-**	**-**	**-**	***Salmonella spp.***
S3B20	**-**	**Rod**	**-**	**+**	**+**	**-**	**Y/Y**	**+**	**+**	**+**	**-**	**-**	**+**	**-**	**-**	**-**	**+**	**+**	**+**	**-**	**-**	***Klebsiella spp.***
S3C21	**+**	**cocci**	**-**	**+**	**+**	**+**	**Y/Y**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**-**	**-**	**+**	**+**	**-**	**-**	**-**	***Staphylococcus spp.***

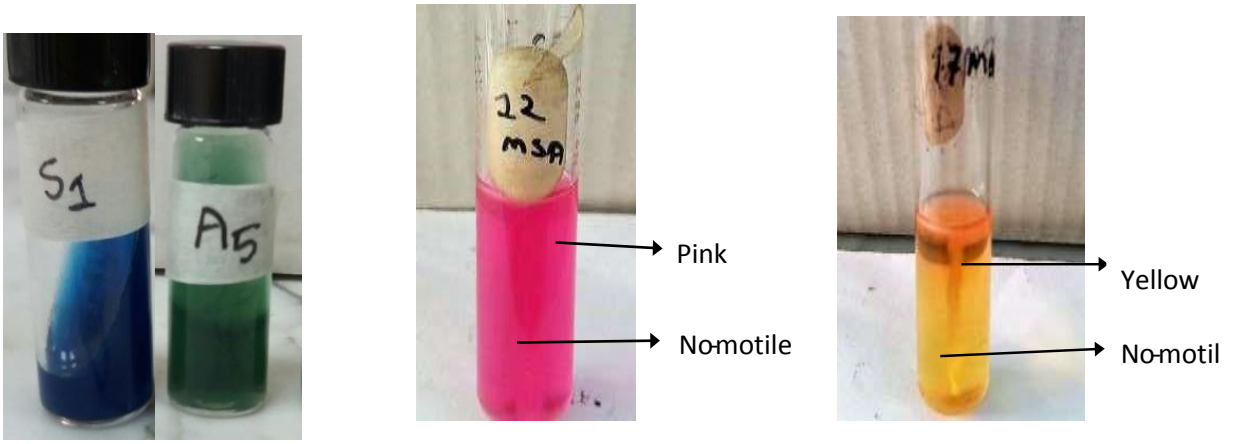
Table 3.4: Biochemical characteristics of the bacteria isolated from CINNAMON restaurant

Isolates ID	Gram staining		Indole Test	Methyl red Test	VP Test	Citrate test	Triple Sugar Iron Test						Carbohydrate fermentation		MIU Test			Catalase Test	Lipid Hydrolysis	Starch Hydrolysis	Casein hydrolysis	Suspected Organism
	Gram reaction	Shape					Slant/Butt	Glucose	Lactose	Sucrose	H2S	Gas	Maltose	Gas	Motility	Indole	Urease					
S4A1	+	cocci	-	+	+	+	Y/Y	+	+	+	-	-	+	-	-	-	+	+	+	-	-	*Staphylococcus spp.*
S4C2	+	cocci	-	+	-	-	Y/Y	+	+	+	-	-	+	-	-	-	+	+	+	-	-	*Staphylococcus spp.*
S4B3	+	Rod	-	-	-	-	R/Y	+	-	-	-	-	+	-	-	-	-	+	-	-	-	*Bacillus spp.*
S4A4	-	Rod	-	-	-	-	Y/Y	+	+	+	-	-	+	+	+	-	-	+	+	-	-	*Vibrio spp.*
S4C5	-	Rod	-	-	+	+	Y/Y	+	+	+	-	+	+	+	+	-	-	+	+	-	-	*Vibrio spp*
S4D6	-	Rod	-	+	-	+	Y/B	+	+	+	+	+	+	-	+	-	-	+	-	-	-	*Salmonella spp.*
S4C7	-	Rod	-	+	-	+	Y/Y	+	+	+	-	-	+	-	+	-	-	+	+	-	-	*Vibrio spp.*
S4A8	-	Rod	-	-	+	+	Y/Y	+	+	+	-	+	+	+	+	-	-	+	-	-	-	*E.coli*
S4A9	-	Rod	-	-	+	+	Y/Y	+	+	+	-	+	+	+	+	-	-	+	-	-	-	*E.coli*
S4C10	-	Rod	-	-	+	+	Y/Y	+	+	+	-	+	+	+	+	-	-	+	+	-	-	*Klebsiella spp.*

S4C11	**-**	**Rod**	**-**	**-**	**+**	**+**	**Y/Y**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**+**	**-**	**-**	**+**	**+**	**-**	**-**	***Pseudomonas spp***
S4C12	**-**	**rod**	**-**	**+**	**+**	**+**	**Y/Y**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**-**	**-**	**+**	**+**	**-**	**-**	**-**	***Salmonella spp.***
S4B13	**-**	**Rod**	**-**	**-**	**-**	**-**	**Y/Y**	**+**	**+**	**+**	**-**	**-**	**+**	**-**	**+**	**-**	**-**	**+**	**+**	**-**	**-**	***Klebsiella spp.***
S4A14	**-**	**Rod**	**-**	**-**	**-**	**-**	**Y/Y**	**+**	**+**	**+**	**-**	**-**	**+**	**+**	**+**	**-**	**-**	**+**	**+**	**-**	**-**	***Klebsiella spp.***
S4C15	**-**	**Rod**	**-**	**+**	**-**	**+**	**B/B**	**+**	**+**	**+**	**+**	**+**	**+**	**-**	**+**	**-**	**-**	**+**	**-**	**-**	**-**	***Pseudomonas spp***
S4B16	**-**	**Rod**	**-**	**+**	**-**	**+**	**Y/B**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**-**	**-**	**+**	**+**	**-**	**-**	***Bacillus spp.***
S4C17	**-**	**rod**	**+**	**+**	**-**	**-**	**Y/Y**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**-**	***Staphylococcus spp.***
S4C18	**-**	**rod**	**-**	**-**	**+**	**+**	**Y/Y**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**-**	**-**	**+**	**+**	**+**	**-**	**-**	***Vibrio spp.***
S4A19	**-**	**rod**	**-**	**-**	**+**	**+**	**Y/Y**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**+**	**-**	**-**	**+**	**+**	**-**	**-**	***Vibrio spp.***
S4D20	**-**	**rod**	**-**	**+**	**-**	**+**	**Y/Y**	**+**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**-**	**-**	**+**	**-**	**-**	**-**	***Salmonella spp.***



Indole(positive) Indole(negative) Methyl red positive and negative VP test positive negative



Citrate positive negative MIU test (Urease +ve, Non-motile) MIU test (Urease -ve, Non-motile)

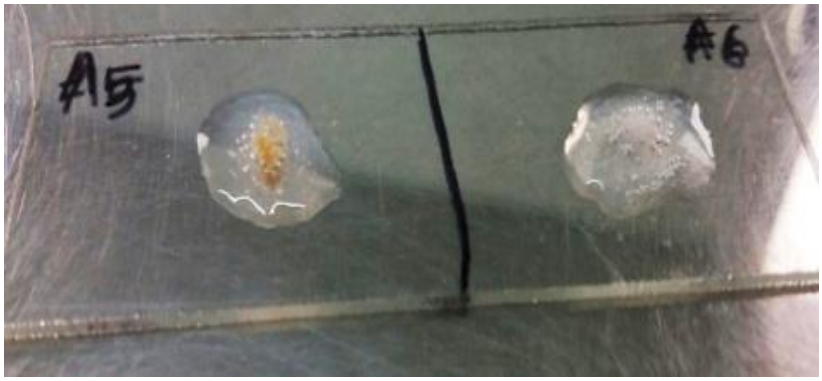
Figure 3.2: Biochemical test results of bacterial isolates

Yellow slant and butt

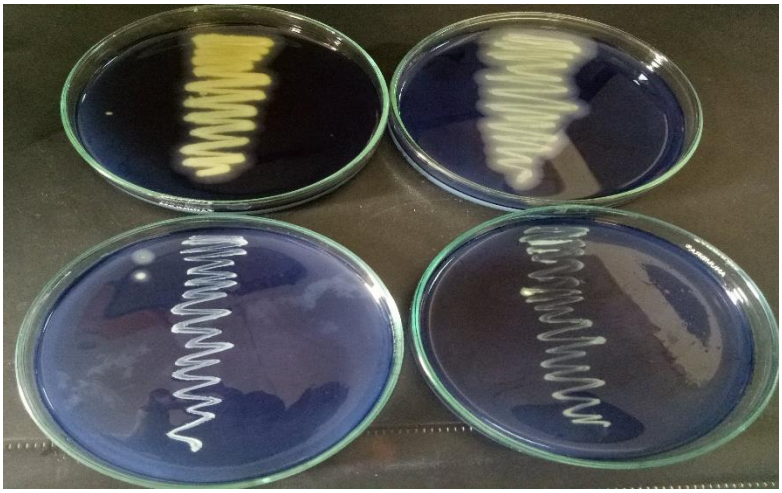
Red slant, yellow butt

Yellow slant, yellow butt gas produce

Red slant, red butt



Catalase test positive



Starch hydrolysis (positive)

Starch hydrolysis (negative)

Lipid hydrolysis positive **negative**

Casein hydrolysis negative **positive (clear zone)**

After observing the cultural and morphological characteristics of bacterial isolates and performing the biochemical tests, 41 isolates could have been identified from 8 different samples collected from two restaurant's refrigerators of Mohakhali (BRAC UNIVERSITY cafeteria UB01 and CINNAMON restaurant). The isolates that have been confirmed include *Staphylococcus* sp., *Bacillus* spp., *E.coli*, *Vibrio* spp., *Salmonella* spp., *Klebsiella* spp. and *Pseudomonas* spp. The total number and the percentage of the isolates obtained from the samples are shown in table.

Table 3.5: Prevalence of bacteria species isolated from restaurant's refrigerators

Bacterial isolates	**Number of the isolates**	**Total bacterial isolates**	**% Prevalence**
Staphylococcus spp.	11	41	26.82
Bacillus spp.	4		9.75
Vibrio spp.	6		14.63
Salmonella spp.	7		17.07
Klebsiella spp.	7		17.07
E.coli	2		4.87
Pseudomonas spp.	4		9.75

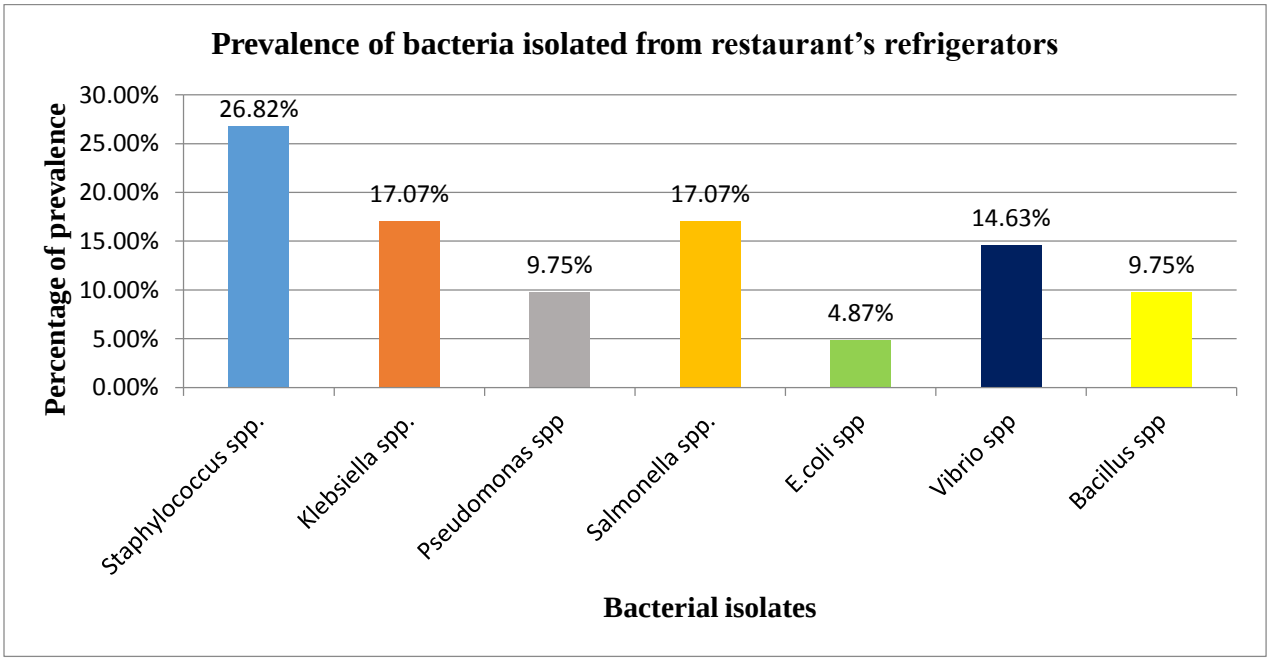


Figure 3.3: Percentage of prevalence of isolated bacteria from restaurant's refrigerators

Among the identified isolates, both the Gram positive and Gram negative organisms were found. The Gram positive organisms that have been identified include *Staphylococcus* spp and *Bacillus* spp. The Gram negative organisms that have been identified include *E.coli*, *Klebsiella* spp, *Vibrio* spp, *Pseudomonas spp* and *Salmonella* spp. The differentiation, number and the percentage of the identified bacterial isolates based on Gram reaction are shown in Table 3.5 and Figure 3.6.

Table 3.6: Distribution of the isolates according to Gram's Reaction

Gram's Reaction	Number of isolates found	Percentage (%)
Gram positive	15 (out of 41)	36.58
Gram negative	26 (out of 41)	63.49

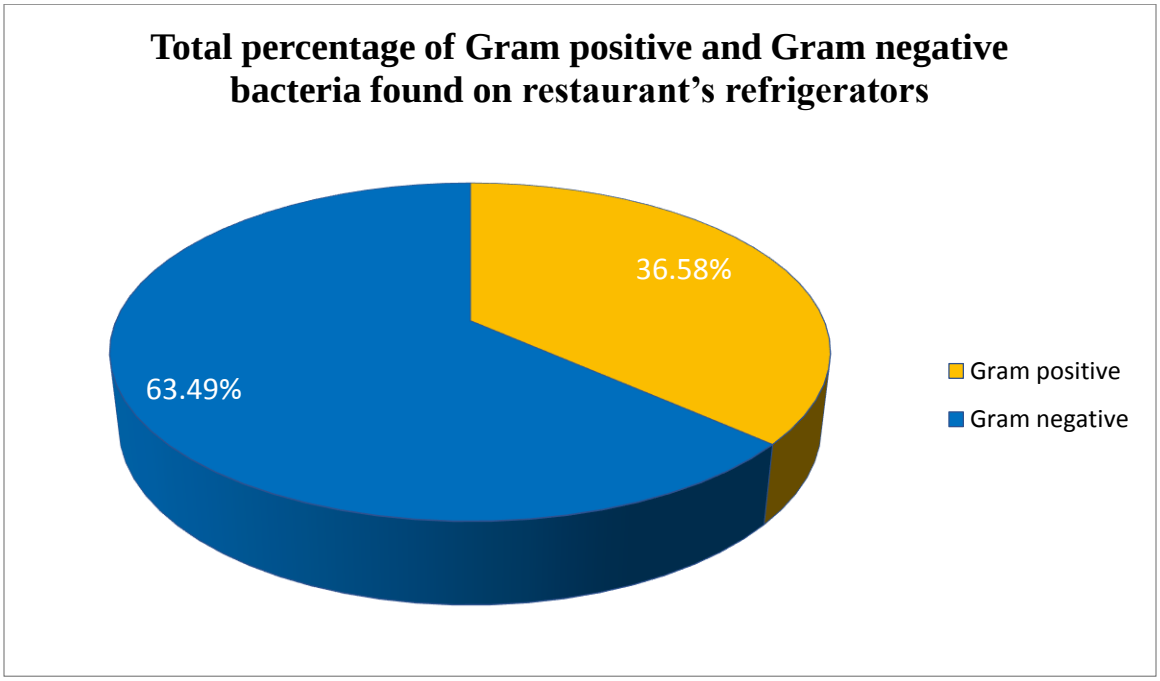


Figure 3.4: Total percentage of Gram positive and Gram negative bacteria identified from restaurant's refrigerators

3.4 Antibiotic susceptibility test:

After identifying and confirming the organisms, the isolates were selected for antibiotic susceptibility test. About nine antibiotics were used for each of the forty one isolates isolated from restaurant's refrigerators of Mohakhali (BRAC UNIVERSITY cafeteria UB01 and CINNAMON restaurant). The sensitive and resistance pattern of the isolates to these antibiotics were determined.

Table 3.7: Antibiotic susceptibility pattern of various organisms isolated from restaurant's refrigerators (BRAC UNIVERSITY cafeteria UB01)

Isolates Id	Suspected organism	Penicillin	Ciprofloxacin	Chloramphenicol	Amoxicillin	Gentamycin	Streptomycin acid	Tetracycline	Nalidixic acid	Azithromycin
		INP	INP	INP	INP	INP	INP	INP	INP	INP
S3A1	*Pseudomonas spp.*	R	S	R	R	S	S	S	S	R
S3A2	*Pseudomonas spp.*	R	S	S	R	S	I	S	I	R
S3B3	*Klebsiella spp.*	R	S	S	R	S	S	S	S	S
S3A4	*Klebsiella spp.*	R	S	S	R	S	S	S	S	I
S3D5	*Salmonella spp.*	R	S	S	R	S	I	S	S	I
S3A6	*Salmonella spp.*	S	S	S	S	S	S	S	S	S
S3C7	*Salmonella spp.*	R	S	S	R	S	S	S	R	S
S3C8	*Klebsiella spp.*	R	S	S	R	S	S	I	I	S
S3A9	*Vibrio spp.*	I	S	I	S	R	I	S	S	S
S3A10	*Staphylococcus spp.*	I	S	S	R	S	R	R	S	R
S3C11	*Bacillus spp.*	R	S	S	S	S	S	R	I	S
S3C12	*Staphylococcus spp.*	S	S	S	S	S	I	S	S	R
S3B13	*Staphylococcus spp.*	R	S	I	S	S	I	S	S	R

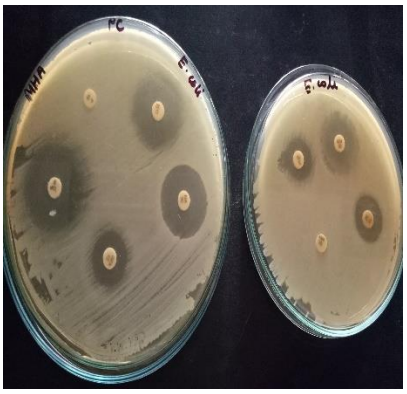
S3A14	***Staphylococcus spp.***	**R**	**S**	**S**	**R**	**S**	**R**	**S**	**I**	**R**
S3A15	***Staphylococcus spp.***	**R**	**S**	**S**	**I**	**S**	**S**	**S**	**R**	**S**
S3A16	***Bacillus spp.***	**R**	**S**	**I**	**R**	**S**	**S**	**S**	**S**	**S**
S3C17	***Staphylococcus spp.***	**I**	**S**	**S**	**R**	**S**	**R**	**S**	**S**	**I**
S3B18	***Staphylococcus spp.***	**R**	**S**	**R**	**R**	**S**	**S**	**S**	**S**	**R**
S3D19	***Salmonella spp.***	**R**	**R**	**I**	**R**	**S**	**S**	**R**	**R**	**S**
S3B20	***Klebsiella spp.***	**R**	**S**	**S**	**R**	**S**	**S**	**S**	**R**	**S**
S3C21	***Staphylococcus spp.***	**R**	**S**	**S**	**R**	**S**	**R**	**S**	**I**	**R**
INP= Interpretation, S= Sensitive, I= Intermediate, R=Resistant										

Table 3.8: Antibiotic susceptibility pattern of various organisms isolated from restaurant's refrigerators (CINNAMON restaurant)

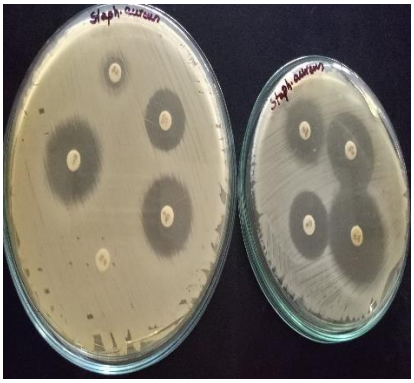
Isolates Id	**Suspected organism**	**Penicillin**	**Ciprofloxacin**	**Chloramphenicol**	**Amoxicillin**	**Gentamycin**	**Streptomycin acid**	**Tetracycline**	**Nalidixic acid**	**Azithromycin**
		INP	**INP**	**INP**	**INP**	**INP**	**INP**	**INP**	**INP**	**INP**
S4A1	***Staphylococcus* spp.**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**R**
S4C2	***Staphylococcus* spp.**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
S4B3	***Bacillus* spp.**	**R**	**S**	**I**	**R**	**S**	**S**	**S**	**S**	**S**
S4A4	***Vibrio* spp.**	**I**	**S**	**S**	**R**	**S**	**S**	**R**	**R**	**S**
S4C5	***Vibrio* spp.**	**I**	**S**	**S**	**R**	**S**	**S**	**R**	**R**	**S**
S4D6	***Salmonella* spp.**	**R**	**I**	**S**	**R**	**S**	**S**	**S**	**R**	**S**
S4C7	***Vibrio* spp.**	**I**	**S**	**S**	**R**	**S**	**S**	**R**	**R**	**S**

S4A8	***E.coli***	**R**	**S**	**S**	**R**	**S**	**I**	**S**	**S**	**S**
S4A9	***E.coli***	**R**	**S**	**S**	**R**	**S**	**I**	**S**	**S**	**S**
S4C10	***Klebsiella* spp.**	**I**	**S**	**S**	**R**	**S**	**I**	**S**	**S**	**I**
S4C11	***Pseudomonas* spp.**	**R**	**S**	**S**	**S**	**S**	**R**	**I**	**R**	**S**
S4C12	***Salmonella* spp.**	**R**	**I**	**S**	**R**	**S**	**S**	**S**	**R**	**S**
S4B13	***Klebsiella* spp.**	**R**	**S**	**S**	**R**	**I**	**S**	**S**	**S**	**S**
S4A14	***Klebsiella* spp.**	**R**	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**I**
S4C15	***Pseudomonas* spp.**	**R**	**S**	**S**	**R**	**S**	**R**	**I**	**S**	**S**
S4B16	***Bacillus* spp.**	**R**	**S**	**S**	**R**	**S**	**I**	**I**	**S**	**S**
S4C17	***Staphylococcus* spp.**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**R**
S4C18	***Vibrio* spp.**	**I**	**S**	**S**	**R**	**S**	**S**	**R**	**R**	**S**
S4A19	***Vibrio* spp.**	**R**	**S**	**S**	**R**	**S**	**I**	**S**	**R**	**S**
S4D20	***Salmonella* spp.**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
INP= Interpretation, S= Sensitive, I= Intermediate, R=Resistant										

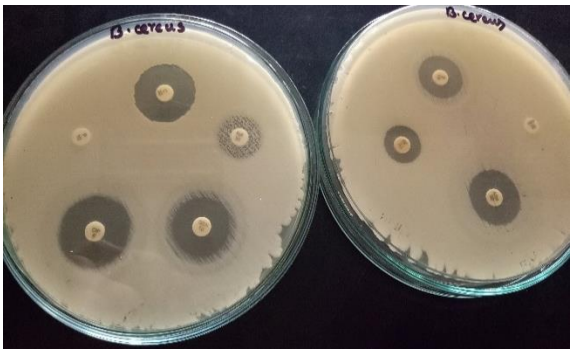
Pseudomonas spp



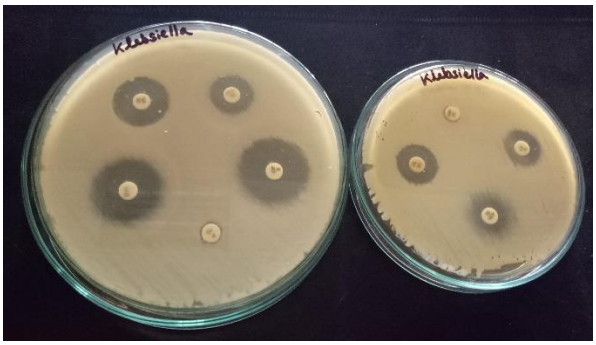
E.coli



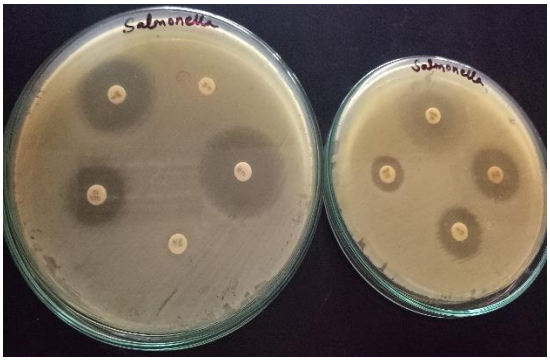
Staphylococcous spp.



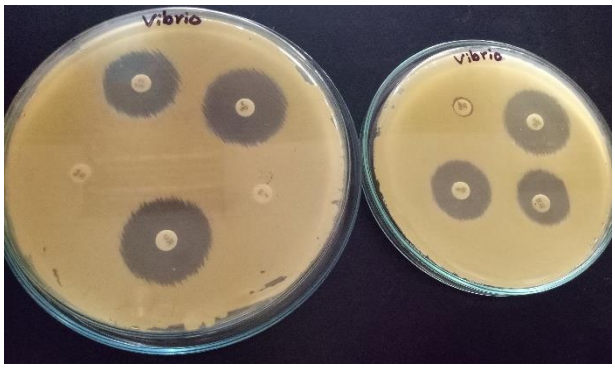
Bacillus spp.



Klebsialla spp



Salmonella spp.



Vibrio spp.

Figure 3.5: Antibiotic susceptibility test

3.5 Resistance pattern of the organisms to the tested antibiotics:

After determining the antibiotic resistant organisms, their percentage of the resistance to the antibiotics tested was also determined which are shown in Table 3.9 and in figure 3.3

Table 3.9: Antibiotic resistance pattern of total 41 bacterial isolates.

Antibiotics	**Penicillin**	**Ciprofloxacin**	**Chloramphenicol**	**Ampicillin**	**Gentamycin**	**Streptomycin**	**Tetracycline**	**Nalidixic acid**	**Azithromycin**
Number of isolates resistant to tested antibiotics	28	1	2	30	1	6	7	12	10
Percentage of isolates resistant to antibiotics	68.29	2.43	4.87	73.17	2.43	14.63	17.07	29.26	24.39

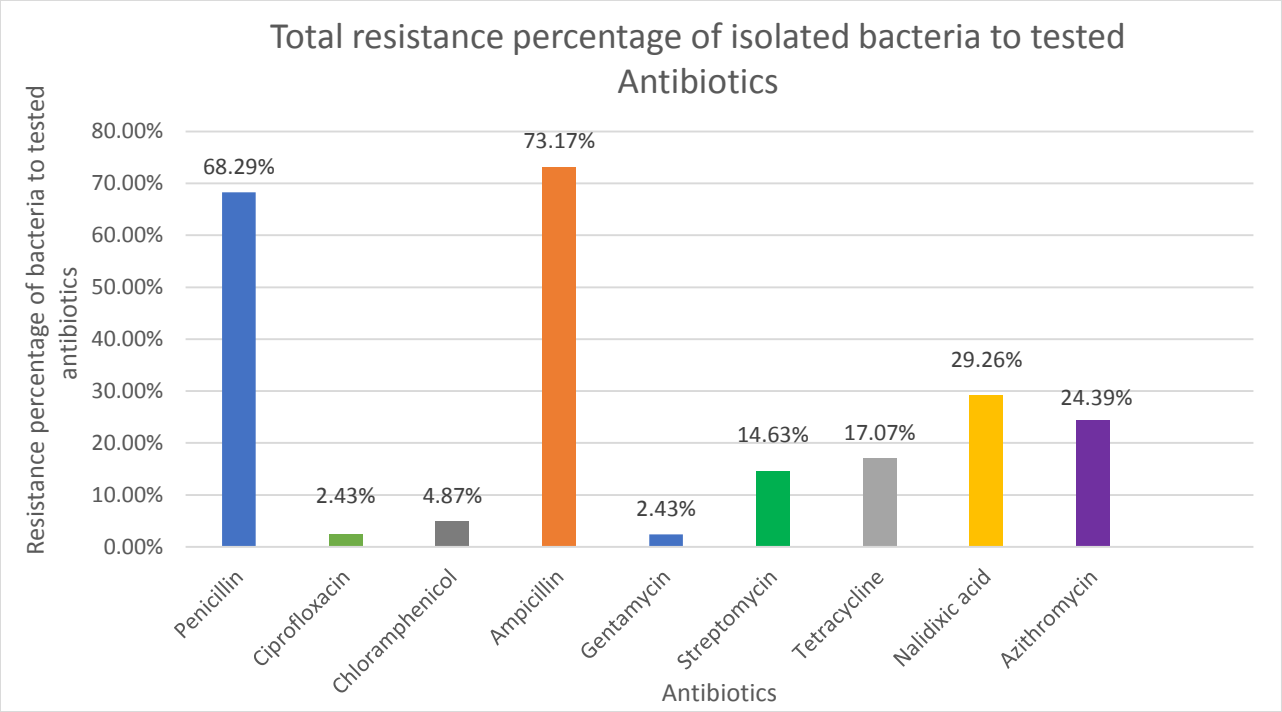


Figure 3.6: Resistance percentage of the isolated bacteria to tested antibiotics

3.6: Prevalence of Multiple drug resistant organisms:

After observing the antibiotic resistance pattern of the organisms, it was found that organisms were resistant to more than two antibiotics. Their total number and percentage are given below in Table3.10

Table 3.10: Total number and percentage of the isolates resistant to more than two antibiotics:

Total bacterial isolates	Percentage of isolates resistant to more than two antibiotics (18)
41	43.90

3.7 Temperature tolerance of the tested organism:

Different temperature like 45°C, 50°C and 55°C were used to determine the temperature tolerance of the organisms. In 45°C all organisms could grow but in 50°C and 55°C, 29.26% and 0% isolates showed viable growth respectively.

Table 3.11: Temperature tolerance of bacterial isolates:

Isolates Id	Suspected organism	45°C	50°C	55°C
S3A1	***Pseudomonas spp.***	+	-	-
S3A2	***Pseudomonas spp.***	+	_	-
S3B3	***Klebsiella spp.***	+	_	-
S3A4	***Klebsiella spp.***	+	+	-
S3D5	***Salmonella spp.***	+	+	-
S3A6	***Salmonella spp.***	+	_	-
S3C7	***Salmonella spp.***	+	+	-
S3C8	***Klebsiella spp.***	+	+	-
S3A9	***Vibrio spp.***	+	-	-
S3A10	***Staphylococcus spp.***	+	-	-
S3C11	***Bacillus spp.***	+	+	-
S3C12	***Staphylococcus spp.***	+	-	-

S3B13	***Staphylococcus spp.***	+	-	-
S3A14	***Staphylococcus spp.***	+	-	-
S3A15	***Staphylococcus spp.***	+	-	-
S3A16	***Bacillus spp.***	+	-	-
S3C17	***Staphylococcus spp.***	+	-	-
S3B18	***Staphylococcus spp.***	+	-	-
S3D19	***Salmonella spp.***	+	+	-
S3B20	***Klebsiella spp.***	+	-	-
S3C21	***Staphylococcus spp.***	+	-	-

Isolates Id	Suspected organism	45°C	50°C	55°C
S4A1	***Staphylococcus* spp.**	+	-	-
S4C2	***Staphylococcus* spp.**	+	-	-
S4B3	***Bacillus* spp.**	+	+	-
S4A4	***Vibrio* spp.**	+	+	-
S4C5	***Vibrio* spp.**	+	_	-
S4D6	***Salmonella* spp.**	+	_	-
S4C7	***Vibrio* spp.**	+	_	-
S4A8	***E.coli***	+	+	-
S4A9	***E.coli***	+	+	-
S4C10	***Klebsiella* spp.**	+	+	-
S4C11	***Pseudomonas* spp.**	+	-	-
S4C12	***Salmonella* spp.**	+	_	-
S4B13	***Klebsiella* spp.**	+	-	_
S4A14	***Klebsiella* spp.**	+	+	_
S4C15	***Pseudomonas* spp.**	+	_	_
S4B16	***Bacillus* spp.**	+	_	_
S4D17	***Staphylococcus* spp.**	+	_	_
S4C18	***Vibrio* spp.**	+	_	-
S4A19	***Vibrio* spp.**	+	_	-
S4D20	***Salmonella* spp.**	+	_	_

(+) = growth (-) = no growth

Table: 3.12 Total number of positive bacterial growth:

Total bacterial isolates	Bacterial growth at 45°C	Bacterial growth at 50°C	Bacterial growth at 55°C
41	**41 (100%)**	**12 (29.26%)**	**No growth**

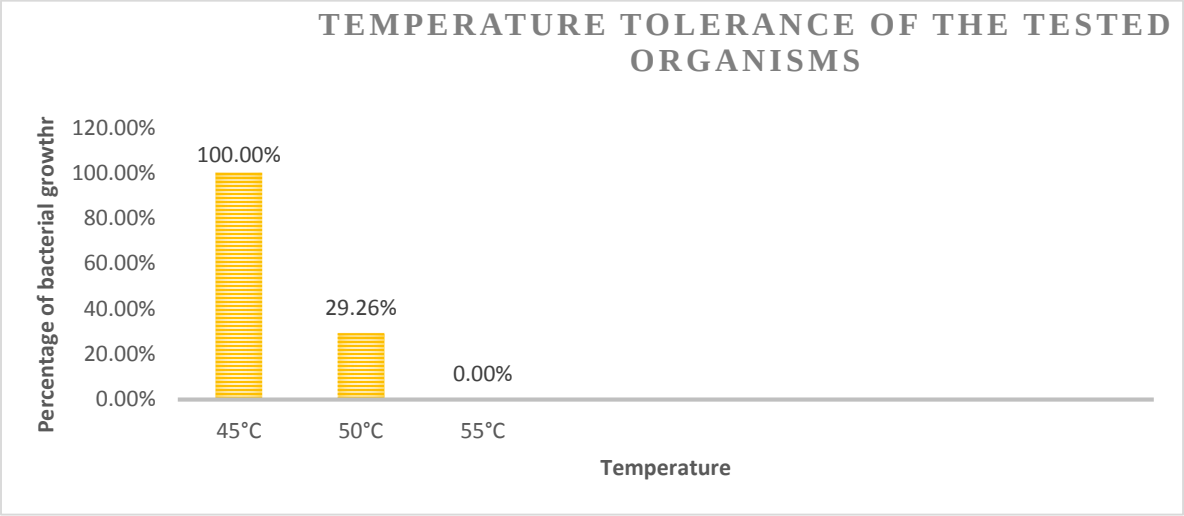


Figure 3.7: Temperature tolerance percentage of the tested organisms.

Chapter 4: Discussion and Conclusion

Discussion

The microorganisms live in every part of the biosphere, and some of them are even capable of growing at low temperatures, including those below the freezing point. These microorganisms live in the sea or in high mountains, but unfortunately also in the refrigerators, where they may spoil or, as pathogens, contaminate foods.

In this research, a total of 41 isolates had been identified from 8 different samples collected from different parts (drawer, handle, surface and egg tray) of two restaurant's refrigerators of Mohakhali. This study revealed high level of bacterial contaminants on restaurant's refrigerators which were contaminated with considerable number of Gram positive bacteria and Gram negative bacteria. However, Gram negative bacteria (63.49%) were found to occur more than Gram positive bacteria (36.58%). Out of 41 bacterial isolates, *Staphylococcus spp.* showed the highest prevalence 11 (26.82%), next to *Salmonella* spp. 7 (17.07%), *Klebsiella* spp. 7 (17.07%), *Vibrio* spp. 6 (14.63%), *Bacillus* spp. 4 (9.75%), *Pseudomonas* spp. 4 (9.75%) and *E.coli* 2 (4.87%).

The levels of pollution saw in refrigerators are probably going to be affected by a scope of components including the nature and levels of introductory sullying, the cleanliness of those getting ready and setting nourishments into the refrigerators, and the effectiveness and recurrence of refrigerators upkeep and cleaning. These undesirable organisms may have entered the refrigerators from unwashed raw foods, leaking packages from improperly packed foods (meats, eggs, and milk), unclean hands, through an opened refrigerator door, warm temperature, and unclean container surfaces introduced into the refrigerator. These can cause direct or cross contamination of other stored foods and persist in internal surfaces (Kumar, M. Rohini; Rishu, Apurva, Bhawna; Osborne, Jabez W. 2012) and, if ingested, may result in food borne illness.

Staphylococcus aureus and *Salmonella* spp. were the most frequently isolated bacteria in this study but the frequency of *Staphylococcus aureus* (26.82%) is higher which enter domestic kitchens, on previously contaminated raw foods. *S. aureus*, as a typical occupant (up to 50%) of the human nose, throat, and skin is maybe more likely to defile nourishment and refrigerators by immediate or backhanded human contact during by dealing sustenance with handling. As a gram-

positive bacteria, it is generally resistant to drying and become dominant than more desiccation-sensitive organisms, especially in the low water activity conditions which prevail in refrigerators.

According to this study, determination of antibiotic susceptibility pattern revealed that 18(43.90%) of the bacterial isolates tested were resistant to at least two antibiotics among the 41 bacterial isolates. Out of the nine tested antibiotics, ampicillin was found to be least effective because 73.17% bacterial isolates showed resistance to ampicillin. The resistance percentage of the isolates to other antibiotics include penicillin (68.29%) , nalidixic acid (29.26%), chloramphenicol (4.87%), azithromycin (24.39%), streptomycin (14.63%), tetracycline (17.07%), both ciprofloxacin and gentamycin (2.43%). This finding correlates to the work of Wolde and Bacha, (2015) who investigated the prevalence and antibiotic resistance patterns of *Staphylococcus aureus* in food establishments of Jimma town and the most resisted drugs were Norfloxacin, Amikacin and Ciprofloxacin showed maximum sensitivity.

Moreover, in this study, analyzing the temperature tolerance of the organisms in different temperatures like 45°C, 50°C and 55°C were followed. At 45°C all organisms showed growth, whereas, only 29.26% isolates survived in 50°C. Inhibition of growth at 55º C indicates that most of the isolates in this study is mesophilic.

From questionnaires, this study also revealed that erratic electricity supply was quite common which affected the temperature regimes of the refrigerators. This resulting change in the refrigerator temperature might allow the growth of mesophilic organisms which can be pathogenic and therefore increases the risk of food borne disease.

Conclusion

The present study indicated that restaurant's refrigerators can be very important sources of potential pathogens which cause various foodborne illnesses. The presence of *Staphylococcus* spp., *Bacillus* spp., *Vibrio* spp., *Salmonella* spp., *Klebsiella* spp., *E.coli* and *pseudomonas* spp. indicates poor personal hygiene. Additionally, the frequent introduction of contaminants vehicles (fruits, vegetables, meats, poultry and eggs) to refrigerators without previous washing, which was a common practice noted among study participants, promotes a continuous inoculation. The presence of microorganisms in the refrigerator affects the microbial quality of the food kept in it. Besides this, multidrug resistant bacteria that can be easily spread during food preparation, via refrigerators are a serious threat to human health. The characterization of refrigerator's associated microbial communities, in combination with future experimental studies to show specific treatment effect on survivability, growth and transmission of bacteria on particular refrigerator's surfaces, with improve our understanding of the microbial ecology of refrigerators.

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Appendices

Appendix- I

Media compositions:

The composition of all media used in the study is given below:

Nutrient Agar

Component	Amount (g/L)
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

Saline

Component	Amount (g/L)
Sodium Chloride	9.0

Nutrient broth

Component	Amount (g/L)
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH	7.4±0.2 at 25ºC

Mannitol Salt Agar

Component	Amount (g/L)
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	75.0
D-mannitol	10.0
Phenol red	0.025
Agar	15.0
Final pH	7.4 ± 0.2 at 25°C

Cetrimide Agar

Component	Amount (g/L)
Pancreatic Digest of Gelatin	20.0
Potassium Sulfate	10.0
Magnesium Chloride	1.4
Cetyltrimethylammonium Bromide	0.3
Glycerin	10.0
Agar	13.6
Final pH	7.2± 0.2 at 25°C

Salmonella Shigella Agar

Component	Amount (g/L)
Peptic digest of animal tissue	15.0
Proteose peptone	5.0
Dextrose	1.0
Lead acetate	0.2
Sodium thiosulphate	0.08
Agar	15.0

Final pH	7.0± 0.2 at 25°C

MacConkey Agar

Component	Amount (g/L)
Peptic digest of animal tissue	1.5
Casein enzymatic hydrolysate	1.5
Pancreatic digest of gelatin	17.0
Lactose	10.0
Bile salt	1.50
Crystal violet	0.001
Neutral red	0.03
Agar	15.0
Final pH	7.1 ± 0.2 at 25°C

TCBS Agar

Component	Amount (g/L)
Proteose peptone	10.0
Yeast extract	5.0
Sodium thiosulphate	10.0
Sodium citrate	10.0
Oxgall	8.0
Sucrose	20.0
Sodium chloride	10.0
Ferric citrate	1.0
Bromo thymol blue	0.04
Thymol blue	0.04
Agar	15.0
Final pH	8.6± 0.2 at 25°C

Eosine Methylene Blue Agar (EMB):

Component	Amount (g/L)
Peptone	10.0
Dipotassium Phosphate	2.0
Lactose	5.0
Sucrose	5.0
Eosin yellow	0.14
Methylene Blue	0.065
Agar	13.50
Final pH	7.1 ± 0.2 at 25°C

Muller Hinton Agar

Component	Amount (g/L)
Beef, dehydrated infusion form	300
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH	7.3± 0.1 at 25°C

Simmon's Citrate Agar

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

Methyl Red -Voges Proskauer(MR-VP) Media

Component	Amount (g/L)
Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
Final pH	7.0

Triple Sugar Iron Agar (TSI)

Component	Amount (g/L)
Bio-polytone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Phenol red	0.0125
Agar	13.0
Final pH	7.3

Motility Indole Urease (MIU) Agar

Component	Amount (g/L)
Tryptone	10
Phenol red	0.1
Agar	2.0
Sodium chloride	5.0
pH (at 25°C)	6.8 ± 0.2 at 25°C

Indole broth

Component	Amount (g/L)
Peptone	10.0
Sodium chloride	5.0

Phenol Red Maltose Broth

Component	Amount (g/L)
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	5.0
Maltose	5.0
Phenol red	0.018
pH (at 25°C)	7.4 ± 0.2 at 25°C

Starch Agar

Component	Amount (g/L)
Meat extract	3.0
Peptic digest of animal tissue	5.0
Starch, soluble	2.0
Agar	15.0
pH (at 25°C)	7.2 ± 0.1 at 25°C

Skim Milk Agar

Component	Amount (g/L)
Skim milk powder	28.0
Casein enzymic hydrolysate	5.0
Yeast extract	2.5
Dextrose	1.0
Agar	15.0
pH (at 25°C)	7.0 ± 0.2 at 25°C

Appendix – II

Reagents and buffers

Gram's iodine (300 ml)

To 300 ml distilled water, 1 g iodine and 2 g potassium iodide was added. The solution was mixed on a magnetic stirrer overnight and transferred to a reagent bottle and stored at room temperature.

Crystal Violet (100 ml)

To 29 ml 95% ethyl alcohol, 2 g crystal violet was dissolved. To 80 ml distilled water, 0.8 g ammonium oxalate was dissolved. The two solutions were mixed to make the stain and stored in a reagent bottle at room temperature.

Safranin (100ml)

To 10 ml 95% ethanol, 2.5 g safranin was dissolved. Distilled water was added to the solution to make a final volume of 100 ml. The final solution was stored in a reagent bottle at room temperature.

Kovac's Reagent (150 ml)

To a reagent bottle, 150 ml of reagent grade isoamyl alcohol, 10 g of p-dimethylaminobenzaldehyde (DMAB) and 50 ml of HCl (concentrated) were added and mixed. The reagent bottle was then covered with an aluminum foil to prevent exposure of reagent to light and stored at 4°C.

Methyl Red (200 ml)

In a reagent bottle, 1 g of methyl red powder was completely dissolved in 300 ml of ethanol (95%). 200 ml of destilled water was added to make 500 ml of a 0.05% (wt/vol) solution in 60% (vol/vol) ethanol and stored at 4°C.

Barrit's Reagent A (100 ml)

5% (wt/vol) a-naphthol was added to 100 ml absolute ethanol and stored in a reagent bottle at 4°C.

Barrit's Reagent B (100 ml)

40% (wt/vol) KOH was added to 100 ml distilled water and stored in a reagent bottle at 4°C.

Catalase Reagent (20 ml 3% hydrogen peroxide)

From a stock solution of 35 % hydrogen peroxide, 583 µl solution was added to 19.417 ml distilled water and stored at 4°C in a reagent bottle.

Urease Reagent (50 ml 40% urea solution)

To 50 ml distilled water, 20 g pure urea powder was added. The solution was filtered through a HEPA filter and collected into a reagent bottle. The solution was stored at room temperature.

Appendix-III

Instruments

Autoclave	Model: WIS 20R Daihan Scientific Co. ltd, Korea
Laminar airflow cabinet	Model-SLF-V, vertical, SAARC group Bangladesh
Incubator	Model-0SI-500D, Digi system Laboratory Instruments Inc. Taiwan
Vortex Mixer	Digi system Taiwan, VM-2000
Electronic Balance	RADWAG Wagi ELEktroniczne Model: WTB 200
Refrigerator (4°C)	Model: 0636 Samsung