Determination of prevalence and antibiotic susceptibility pattern of the bacterial isolates collected from different parts of domestic refrigerators.

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 domestic refrigerators "** has been submitted by me, Fatema Raad Hasan 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 father and mother

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Abstract

Refrigerator form an important link in wide chain of cross contamination which leads to the outbreak of domestic food borne disease.In this study samples were obtained from various parts (drawer,handle,egg tray,surface) of domestic refrigerators of various parts of Mohakhali and Mirpur area, Dhaka, Bangladesh. A total of 37 isolates collected from 8 different parts were identified. Identification of bacteria was done through conventional biochemical tests according to Bergey's Manual of Systematic Bacteriology. Antibiotic susceptibility pattern of all isolates were performed 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)] by using Kirby-Bauer disc diffusion method. A total of 37 isolates had been identified where *Staphylococcus* spp.and *Klebsiella* spp.showed the highest prevalence 8(21.62%), next to *Pseudomonas* spp 5(13.5%).,*E.coli* 5(13.5%), *Salmonella* spp 5 (13.5%), *Bacillus* spp. 2 (5.4%) and *Vibrio* spp. 4 (10.8%). Ampicillin showed the highest resistance percentage (81.08%) for most of the isolates. Out of 37 bacterial isolates, (29.72%) were resistant to more than two antibiotics. We also determined temperature tolerance of the organisms by growing the isolates in different temperature like 45°C,50°C and 55°C. All the isolates showed growth at 45°C but in 50°C and 55°C only (54.05%) and (10.81%) isolates showed viable growth. These results indicate that the presence of these organisms, including potential foodborne pathogens, in domestic refrigerators portends serious health implications. 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

'Household Refrigerator' is also termed as domestic refrigerator is a low temperature appliance used in homes for the preservation and storage of food products .It is one of the most widely practiced methods of controlling microbial growth on perishable product of which temperature specification of four to five (4-5°C) degree celsius is considered desirable. (Ewaoche Itodo,2017). It has also been demonstrated that, perishable food will deteriorate even at refrigerator temperature due to spoilage because of microorganism, enzymes and oxidation. (Haresh khemani,2010)The type of container or wrapping material they are stored in and duration of storage are also important factors that influence the type of microbial growth, toxicity and spoilage of food during refrigerated storage.Although low temperature retards spoilage, but even a sub-freezing temperature of about 7°C does not prevent multiplication of all microorganisms, refrigerated foods are therefore subjected to spoilage by molds, yeasts and bacteria. The internal surface within the domestic refrigerators usually creates an unfavorable environment for many pathogenic bacteria but most of them are capable of growing and surviving at low and cold temperature. If refrigerators are not properly maintained, it becomes a breeding ground for such bacteria. (Haghi, A.K. 2011) Many diseases are caused by microorganisms such as bacteria, viruses and parasitic infestations arising out of food spoiled due to prolonged storage of perishable and semi perishable food items, like *Staphylococcus* bacteria which produces toxin as by-product of growth and multiplication and cause food intoxication. More so, bacteria such as *Escherichia coli, Salmonella spp*, *Shigella spp, Vibrio spp ,Klebsiella spp, Yersinia enterocolitica,Staphylococcus spp,* and parasites such as *Cryptosporidium parvum* and *Giardia lamblia* have been implicated in food and water borne illness. The global incidence of food borne diseases is difficult to estimate, but it has been reported that in 2005 alone, 1.8 million people died from diarrheic diseases. (Tauxe, R.V. 2012)

For example, Kumar et al., (2012) showed that the pathogenic bacteria can survive in refrigerator surfaces and can cause cross contamination.Thus a number of undesirable pathogens such as *Salmonella sp., Citrobacter sp., Shigella sp.,* and *Proteus sp.*,were isolated from the refrigerators. (Kolapo, A.2015) indicates that domestic refrigerators are three times more frequently involved in initiating food borne illness than commercial refrigerators and perhaps as many as 50% of household food borne illness can be attributed to an inappropriate food storage and refrigerator management. (Harder ER.1988)

1.2 Common pathogens able to survive in refrigerated foods:

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. Therefore, although storing foods in the refrigerator is the best way to keep them safe from bacterial contamination, there are also types of bacteria that can grow in cold temperature as well as inside the refrigerators.

Table 1.1 Example of pathogens able to survive in refrigerated foods: (Martin Walker, Port Health Officer,2014)

Microorganism	Common food sources	Survival T (°C)	Optimum growth T
Campylobacter jejuni	Raw chicken, foods contaminated by raw chicken, unpasteurised milk, untreated water	0 – 45 [3]	37 – 42 [3]
Listeria monocytogenes	Ready-to-eat foods, including raw milk, cheeses, ice cream, raw vegetables, fermented raw sausages, raw and cooked poultry, raw meats, raw and smoked fish	-1,5 – 45 [4]	-1,5 – 45 [4]
Yersinia enterocolitica	Raw milk, chocolate milk, water, pork, other raw meats	-1,5 – 44 [5]	-1,5 – 44 [5]
Aeromonas hydrophila	Seafood including oysters, water	-2 – 45 [4]	5 – 25 [4]
Plesiomonas shigelloides	Raw oysters	8 – 44 [5]	37 – 38 [5]
Pseudomonas spp.	Meat, fish, shell fish and dairy products	4 – 43 [6]	37 – 38 [6]
Penicillum, Cladosporium		-3 – 35 [7]	25 – 26 [7]

1.3: Literarture review

Oluwafemi F, Akpoguma S, Oladiran T, Kolapo A (2015) studied the presence of microorganisms which can cause food spoilage and food borne diseases. This study was carried out to examine the consumers' knowledge and hygiene status of household refrigerators in Ibadan, Lagos and Abeokuta. One hundred and eighty households were randomly selected. This study identified the presence of undesirable food related pathogens such as *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Pseudomonas spp., Aspergillus fumigatus, A. niger, Penicillium spp*. which were isolated from 170 refrigerators sampled. *S. aureus* and *E. coli* were the most frequently isolated pathogen in this study and were recovered from 75% of the 170 refrigerators examined. Unlike the othermicrobial pathogens, which principally enter domestic kitchens from previously contaminated raw foods, *S. aureus* as a common inhabitant (up to 50%) of the human nose, throat, and skin is perhaps more likely to contaminate foods and refrigerators by direct or indirect human contact during domestic food handling and storage.

Otu-Bassey, I. B. , Ewaoche, I. S. , Okon, B. F. , & Ibor, U. A. (2017) investigate the microbiological load and the potential risk of refrigerated food and water in Calabar metropolis. In all, 100% of refrigerators sampled showed bacterial contamination, 32% showed fungal contamination, while 8% had parasitic organisms. *Genera* of bacteria isolated in descending order of frequency were: *Staphylococcus aureus* 27.3%, *Escherichia coli* 20.2%, *Shigella* spp 13.0%, *Pseudomonas aeruginosa* 11.9%, *Aeromonas hydrophilia* 8.3%, *Salmonella typhi* 5.9%, *Klebsiella pneumonia* 5%, *Streptococcus pyogenes* 4.7%, and *Proteus mirabilis* 2.3%. Fungal organisms isolated were *Candida albicans* 54%, *Penicillium* spp 43.2% and *Aspergilus flavus* 2.7% while the parasites detected were *Entamoeba histolytica/dispar* 50% and *Ascaris lumbricoides* 50%.The presence of these organisms, including potential foodborne pathogens, in domestic refrigerators portends serious health implications.

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.

1.4 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.

1.5: Aims and objectives:

The objectives of this research work were to demonstrate the prevalence of bacteria in different parts of domestic 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 domestic 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.

Chapter 2: Materials & Methods

2. Materials and methods

2.1 Study Area:

This study was carried out in Mohakhali and Mirpur area in Dhaka city. Samples were obtained from four parts (drawer, handle,surface and egg tray) of each domestic refrigerators.

2.2 Duration:

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

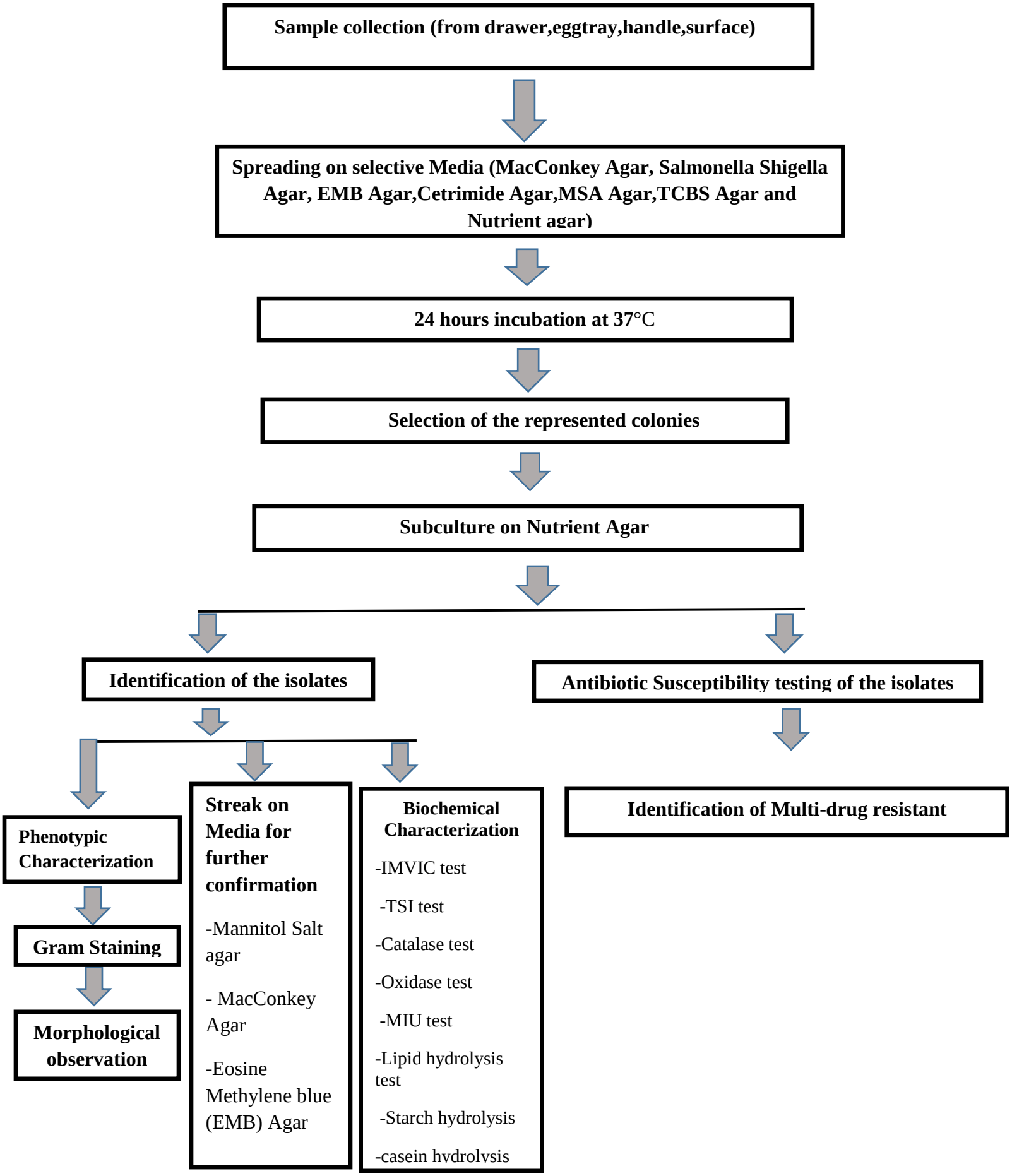
2.3 Sample size:

A total of 8 samples (drawer, handle,surface and egg tray) were collected from the two domestic refrigerators of Mohakhali and Mirpur area.

2.4 Sample collection and processing:

Samples were collected from the refrigerators using the swab-rinse method.In this method samples were taken from the drawer, handle, surface and egg tray of the refrigerator using sterile swab sticks moistened in sterile peptone water and transported back to the Laboratory for analysis within one hour of collection.

2.5 Flow Diagram of the Study Design:



2.6 Isolation,purification and storage of sample:

Sources of 8 samples collected from drawer,surface,handle 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)	08.10.2017	11.00 am	7	S1A1,S1A2,S1A3,S1A4,S1A5, S1A6,S1A7
2	Refrigerator 1 (Part-B-Handle)	08.10.2017	11.00 am	3	S1B1,S1B2,S1B3
3	Refrigerator 1 (Part-C-Egg tray)	08.10.2017	11.00 am	2	S1C1,S1C2
4	Refrigerator 1 (Part-D-Surface)	08.10.2017	11.00 pm	1	S1D1
5	Refrigerator 2 (Part-A-Drawer)	24.10.2017	1.00 pm	9	S2A1,S2A2,S2A3,S2A4,S2A5, S2A6,S2A7,S2A8,S2A9
6	Refrigerator 2 (Part-B-Handle)	24.10.2017	1.00 pm	5	S2B1,S2B2,S2B3,S2B4,S2B5
7	Refrigerator 2 (Part-C-Egg tray)	24.10.2017	1.00 pm	4	S2C1,S2C2,S2C3,S2C4
8	Refrigerator 2(Part-D-Surface)	24.10.2017	1.00 pm	6	S2D1,S2D2,S2D3,S2D4,S2D5, S2D6

After sample collection, samples were spreaded on selective media. Serial dilutions were made for each of the parts from 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 -20ºC.

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. (Cappuccino &Sherman, 2005)

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 (Cappuccino &Sherman, 2005).

2.8.3 Voges-Proskauer (VP) test:

The Voges-Proskauer (VP) test was done to determine if an organism produces acetyl methyl 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. (Cappuccino &Sherman, 2005)

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 citrase.

- 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 colour 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 colour change then it indicates citrate negative result.

2.8.5 Catalase test

The differentiation of bacteria that produce the enzyme catalase from non-catalase producers is achieved using this test. Catalase acts as a catalyst in the breaking down of hydrogen peroxide to Oxygen and water, two to three ml of 3% hydrogen peroxide solution was poured into a test tube. A 24 hour culture of the test organism from the nutrient agar was emulsified in the hydrogen peroxide solution. The release of bubbles immediately indicated a positive test while it was negative when no bubble was formed.

2.8.6 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.

2.8.7 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.

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 tubesby 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 colour of the media will turn to deep pink if the organism is positive for urease test. If yellow colour 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 colour 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 (Cappuccino &Sherman, 2005).

2.8.9 Starch hydrolysis test:

Starch hydrolysis test was done to observe if the microbes can use starch, a complex carbohydrate made from glucose, as a source of carbon and energy for growth. Use of starch is accomplished by an enzyme called alpha-amylase.

- Soluble starch media was dissolved in a small amount of water and was heated slowly with constant stirring. Then all the ingredients were added to it and was transferred into a conical flask and sterilized by autoclaving at 121.5°C.
- The sterilized agar medium was poured into the sterilized Petri plates and was allowed to solidify.
- Each plate was inoculated at the center with the bacterial inoculum.
- Plates were incubated at 37°C for 24–48 hrs.
- To test the hydrolysis of starch, each plate was flooded with iodine.
- An appearance of clear zone around the growth is considered as positive result.(Cappuccino &Sherman, 2005)

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.

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 Antibiotic susceptibility testing (AST):

Antibiotic susceptibility test is done to find the sensitivity or susceptibility and resistance pattern of bacteria to antibiotics.

2.10 Disk diffusion method:

In this research work the antibiotic susceptibility testing of the organisms were performed by Kirby-Bauer disc diffusion method.

Table 2.2: List of antibiotics and their disc potency ranges

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:

- Using a sterile loop a colony from the plate was aseptically emulsified in the tube containing sterile saline solution and it was mixed thoroughly to ensure that no solid material from the colony is visible in the saline solution.
- The tube was vortexed properly so that the suspension becomes homogenous.
- Muller Hinton agar plates were prepared.
- A sterile cotton swab was taken and was dipped into the broth culture of the organism.
- The swab was later streaked at least four to six times onto the dried surface of the MHA plate to make a lawn culture and to ensure that the cotton swab is touched entirely on the agar surface.
- After the streaking is complete the plate is allowed to dry for 5 minutes.
- Sterilized forceps were used to place the antibiotic discs.
- After taking the discs, the discs were gently pressed onto the surface of the agar using sterilized forceps.
- Once all the discs were properly placed, the MHA plates were inverted and incubated at 37^0C for 24 hours.

Result interpretation:

- After incubation, the bacterial growth around each disc is observed. If the test isolate is susceptible to a particular antibiotic, a clear area of "no growth" will be observed around that particular disk. The zone around an antibiotic disk that has no growth is referred to as the zone of inhibition since this approximates the minimum antibiotic concentration sufficient to prevent growth of the test isolate.
- A metric ruler is used to measure the diameter of the zone of inhibition for each antibiotic used.
- This zone is measured in mm and compared to a standard interpretation chart used to categorize the isolate as susceptible, intermediately susceptible or resistant.

Table 2.3: 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 Identification:

A total of 8 samples (drawer, handle,surface and egg tray) were collected from the two domestic refrigerators of Mohakhali and Mirpur area.These samples were streaked onto various selective, differential and nutrient agar media to identify organisms present in each sample. Gram positive and Gram negative bacteria were found in the samples. Results were recorded according to their colony morphology and biochemical characteristics of the isolates in different agar media.

3.2 Cultural and morphological characteristics of the bacterial isolates:

In Table 3.1 the color, shape of the colonies on various selective, differential media and the morphology of the bacterial colonies on nutrient agar are explained.

Bacterial isolates	**Agar medium**	**Size**	**Form**	**Pigmentation**	**Margin**	**Elevation**	**Suspected organism**
S1A1	EMB agar	Small	Circular	Metallic green sheen	Entire	Raised	*E.coli*
S1B1	EMB agar	Large	Circular	Pink translucent	Entire	Raised	*Klebsiella spp.*
S1A2	Cetrimide agar	Small	Circular	White,greenish	Undulate	Raised	*Pseudomonas spp*
S1A3	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus spp.*
S1A4	MSA agar	Large	Circular	Yellow	Entire	Convex	*Staphylococcus spp.*
S1B2	MSA agar	Small	Circular	Pink	Entire	Raised	*Staphylococcus spp.*
S1C1	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus spp.*
S1A5	Nutrient agar	Small	Circular	White,moist, glistening	Entire	Raised	*E.coli*

Table 3.1: Cultural and Morphological Characteristics of the Bacterial Isolates from domestic refrigerators.

S1A6	Nutrient agar	Small	Circular	White,slimy, translucent	Entire	Raised	*Klebsiella spp.*
S1A7	Nutrient agar	Large	Circular	White	Entire	Convex	*Bacillus spp.*
S1B3	Nutrient agar	Medium	Circular	White	Entire	Convex	*Salmonella spp.*
S1C2	Nutrient agar	Small	Irregular	Grayish	Undulate	Umbonate	*Salmonella spp.*
S1D1	Nutrient agar	Small	Circular	White	Entire	Raised	*Klebsiella spp.*

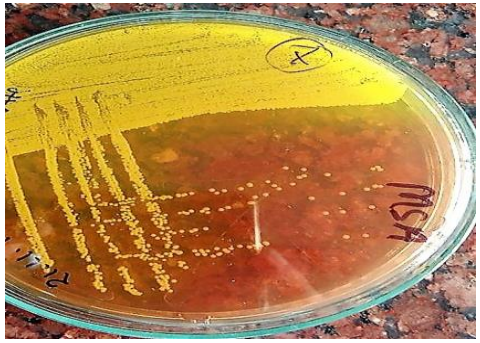
Table 3.1: Cultural and Morphological Characteristics of the Bacterial Isolates from domestic refrigerators

Bacterial isolates	Agar medium	Size	Form	Pigmentation	Margin	Elevation	Suspected organism
S2A1	MacConkey agar	Small	Circular	Medium pink	Entire	Raised	*Klebsiella spp*
S2A2	MacConkey agar	Pin point	Circular	Red	Entire	Convex	*E.coli spp.*
S2A3	MacConkey agar	Large	Irregular	White	Undulate	Umbonate	*Salmonella spp.*
S2C1	MacConkey agar	Small	Circular	Pink translucent	Entire	Convex	*Klebsiella spp.*
S2D1	MacConkey agar	Large	Circular	Pink translucent	Undulate	Umbonate	*Klebsiella spp.*
S2A4	MSA agar	Large	Circular	Yellow	Entire	Convex	*Staphylococcus spp.*
S2A5	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus spp.*
S2B1	MSA agar	Pinpoint	Circular	Pink	Entire	Convex	*Staphylococcus spp.*

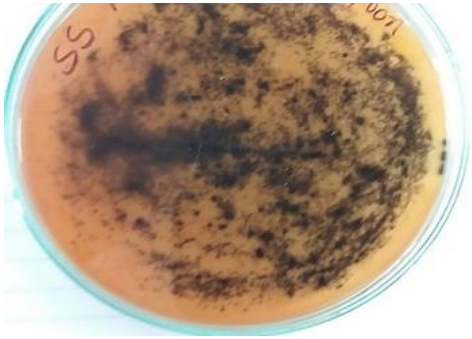
S2A6	SS agar	Small	Circular	Black centered,white colonies	Entire	Raised	*Salmonella spp.*
S2B2	EMB agar	Small	Circular	Metallic green sheen	Entire	Raised	*E.coli*
S2A7	Nutrient agar	Small	Circular	White,slimy, translucent	Entire	Raised	*Klebsiella spp.*
S2B3	Nutrient agar	Large	Circular	White	Entire	Raised	*Klebsiella spp.*
S2C2	Nutrient agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus spp*
S2D2	Nutrient agar	Large	Irregular	White	Undulate	Umbonate	*Bacillus spp.*
S2D3	SS agar	Large	Circular	Black centered,white colonies	Entire	Convex	*Salmonella spp*

Table 3.1: Cultural and Morphological Characteristics of the Bacterial Isolates from domestic refrigerators.

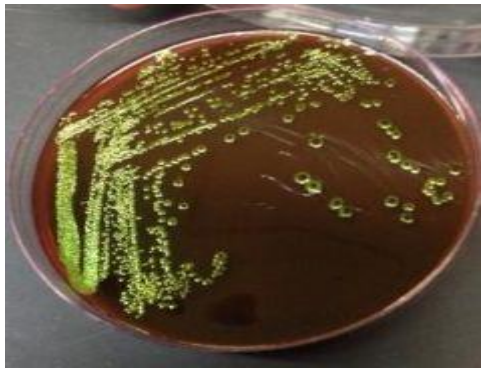
Bacterial isolates	**Agar medium**	**Size**	**Form**	**Pigmentation**	**Margin**	**Elevation**	**Suspected organism**
S2B4	Cetrimide agar	Small	Circular	Greenish	Entire	Convex	*Pseudomonas spp.*
S2C3	Cetrimide agar	Small	Circular	Light yellow	Entire	Raised	*Pseudomonas spp.*
S2D4	Cetrimide agar	Large	Irregular	White	Undulate	Umbonate	*Pseudomonas spp.*
S2A8	Cetrimide agar	Medium	Circular	Greenish	Entire	Raised	*Pseudomonas spp.*
S2C4	TCBS agar	Large	Circular	Yellow	Entire	Raised	*Vibrio spp.*
S2D5	TCBS agar	Small	Circular	Yellow	Entire	Convex	*Vibrio spp.*
S2B5	TCBS agar	Medium	Circular	Green	Entire	Flat	*Vibrio spp.*
S2A9	TCBS agar	Small	Circular	Green	Entire	Convex	*Vibrio spp*
S2D6	EMB agar	Large	Irregular	Metallic green sheen	Undulate	Umbonate	*E.coli*



Staphylococcus spp. on MSA

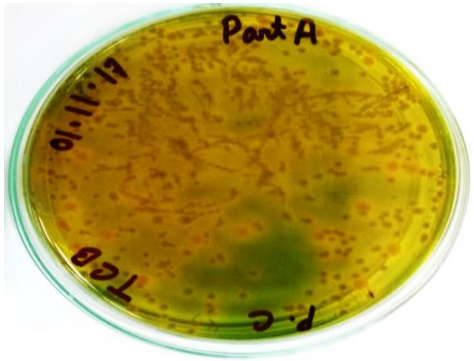


Salmonella spp. on SS Agar

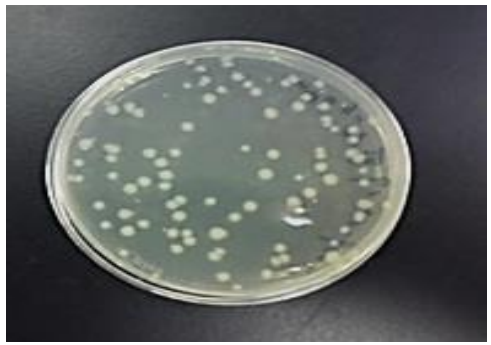


E.coli on EMB Agar

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 3.2: Biochemical characteristics of the bacteria isolated from domestic refrigerators of Mohakhali area.

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	H_2S	Gas	Maltose	Gas	Motility	Indole	Urease					
S1A1	-	rod	+	+	-	-	Y/Y	+	+	+	-	+	+	+	+	+	-	+	-	-	-	***E.coli* spp.**
S1B1	-	rod	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	+	-	-	***Klebsiella* spp.**
S1A2	-	rod	-	-	-	+	R/R	+	-	-	-	-	+	-	+	+	+	+	+	+	+	***Pseudomonas* spp.**
S1A3	+	cocci	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	+	-	-	***Staphylococcus* spp.**
S1A4	+	cocci	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	+	-	-	***Staphylococcus* spp.**
S1B2	+	cocci	-	+	-	-	Y/Y	+	+	+	-	-	+	-	-	-	+	+	+	-	+	***Staphylococcus* spp.**
S1C1	+	cocci	-	+	-	-	Y/Y	+	+	+	-	-	+	+	-	-	+	+	+	-	+	***Staphylococcus*spp.**
S1A5	-	rod	+	+	-	-	Y/Y	+	+	+	-	+	+	+	+	+	-	+	-	-	-	***E.coli* spp.**
S1A6	-	rod	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	+	-	+	***Klebsiella* spp.**

Table 3.2: Biochemical characteristics of the bacteria isolated from domestic refrigerators of Mohakhali area.

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	H_2S	Gas	Maltose	Gas	Motility	Indole	Urease					
S1A7	+	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	-	-	-	-	+	+	+	+	***Bacillus spp.***
S1B3	-	rod	-	+	-	+	Y/Y	+	+	+	+	-	+	+	+	-	-	+	-	-	-	***Salmonella spp.***
S1C2	-	rod	-	+	-	+	Y/Y	+	+	+	+	-	+	+	+	-	-	+	-	-	-	***Salmonella* spp.**
S1D1	-	rod	-	-	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	+	-	-	***Klebsiella spp***

Table 3.3: Biochemical characteristics of the bacteria isolated from domestic refrigerators of Mirpur area.

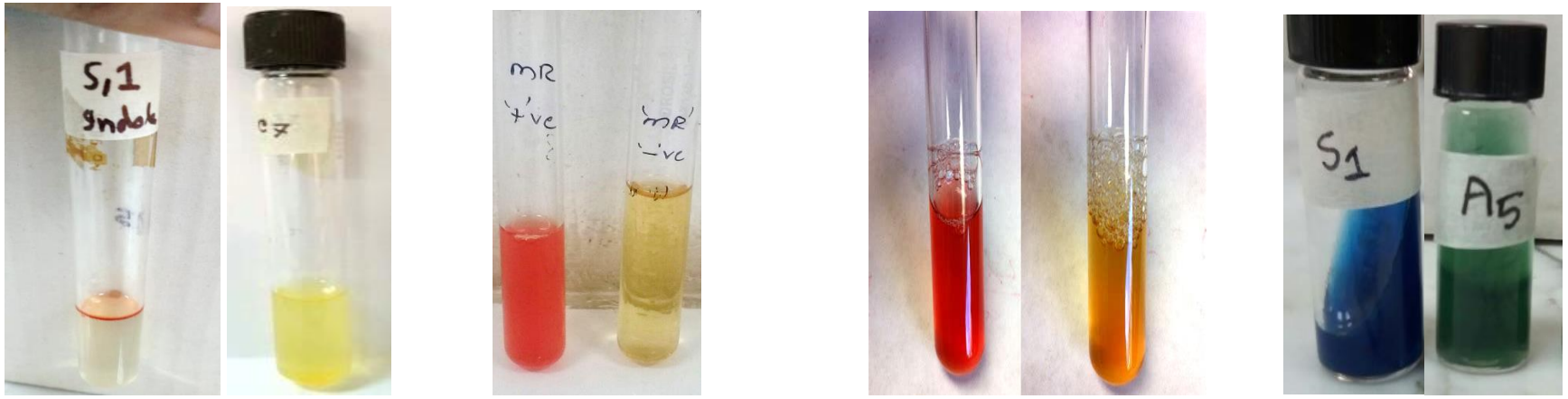
Isolates ID	Gram staining		Indole Test	Methyl red Test	VP Test	Citrate test	Triple Sugar Iron Test						Carbohydrate fermentation		MIU Test			Catalase Test	Lipid Hydrolysis	Starch Hydrolysis	Casein hydrolysis	Suspected Organism
	Gram reaction	Shape					Slant/Butt	Glucose	Lactose	Sucrose	H_2S	Gas	Maltose	Gas	Motility	Indole	Urease					
S2A1	-	rod	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	+	-	-	***Klebsiella* spp.**
S2A2	-	rod	+	+	-	-	Y/Y	+	+	+	-	+	+	+	+	+	-	+	-	-	-	***E.coli* spp.**
S2A3	-	rod	-	+	-	+	Y/Y	+	+	+	+	-	+	+	+	-	-	+	-	-	-	***Salmonella* spp.**
S2C1	-	rod	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	+	-	-	***Klebsiella* spp.**
S2D1	-	rod	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	+	-	-	***Klebsiella* spp.**
S2A4	+	cocci	-	+	-	-	Y/Y	+	+	+	-	-	+	-	-	-	+	+	+	-	-	***Staphylococcus* spp.**
S2A5	+	cocci	-	+	-	-	Y/Y	+	+	+	-	-	+	+	-	-	+	+	+	-	-	***Staphylococcus*spp.**
S2B1	+	cocci	-	+	-	-	Y/Y	+	+	+	-	-	+	+	-	-	+	+	+	-	-	***Staphylococcus* spp.**
S2A6	-	rod	-	+	-	+	Y/Y	+	+	+	+	-	+	+	+	-	-	+	-	-	-	***Salmonella* spp.**

Table 3.3: Biochemical characteristics of the bacteria isolated from domestic refrigerators of Mirpur area.

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	H_2S	Gas	Maltose	Gas	Motility	Indole	Urease					
S2B2	-	rod	+	+	-	-	Y/Y	+	+	+	-	+	+	+	+	+	-	+	-	-	-	***E.coli* spp.**
S2A7	-	rod	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	+	-	-	***Klebsiella* spp.**
S2B3	-	rod	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	+	-	-	***Klebsiella* spp.**
S2C2	+	cocci	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	+	-	-	***Staphylococcus* spp.**
S2D2	+	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	-	-	-	-	+	+	+	+	***Bacillus* spp.**
S2D3	-	rod	-	+	-	+	Y/Y	+	+	+	+	-	+	+	+	-	-	+	-	-	-	***Salmonella*spp.**
S2B4	-	rod	-	-	-	+	R/R	+	-	-	-	-	+	+	+	+	-	+	-	-	-	***Pseudomonas* spp.**
S2C3	-	rod	-	-	-	+	R/R	+	-	-	-	-	+	+	-	-	+	+	+	-	+	***Pseudomonas* spp.**
S2D4	-	rod	-	-	-	+	R/R	+	-	-	-	-										***Pseudomonas* spp.**

Table 3.3: Biochemical characteristics of the bacteria isolated from domestic refrigerators of Mirpur area.

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/But t	Glucose	Lactose	Sucrose	H_2S	Gas	Maltose	Gas	Motility	Indole	Urease					
S2A8	-	rod	-	-	-	+	R/R	+	-	-	-	-	+	-	+	+	+	+	+	+	+	***Pseudomonas* spp.**
S2C4	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	+	+	-	-	+	+	-	-	***Vibrio* spp.**
S2D5	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	+	+	-	-	+	+	-	-	***Vibrio*spp.**
S2B5	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	+	+	-	-	+	+	-	-	***Vibrio* spp.**
S2A9	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	+	+	-	-	+	+	-	-	***Vibrio*spp.**
S2D6	-	rod	+	+	-	-	Y/Y	+	+	+	-	+	+	+	+	+	-	+	-	-	-	***E.coli* spp.**



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

Yellow slant Yellow butt | Red slant Red butt | Red slant Yellow butt | Yellow slant Yellow butt (gas produced) | MIU test (Urease –ve, Non-motile) | MIU test (Urease+ve, Non-motile)

Figure 3.2: Biochemical test results of bacterial isolates

Catalase test positive

Starch hydrolysis (positive) Starch hydrolysis (negative)

Casein hydrolysis negative positive(clear zone)

Lipid hydrolysis positive negative

Figure3.2: Biochemical test results of bacterial isolates

After observing the cultural and morphological characteristics of bacterial isolates and performing the biochemical tests, 40 isolates have been identified.The isolates that have been confirmed include *Staphylococcus* sp., *Pseudomonas* spp., *Bacillus* spp., *E.coli*, *Vibrio* spp., *Salmonella* spp.,and *Klebsiella* spp.. The total number and the percentage of the isolates obtained from the samples are shown in table 3.3and figure 3.4

Table 3.4: Prevalence of bacteria species isolated from domestic refrigerators.

Bacterial isolates	**Number of the isolates**	**Total bacterial isolates**	**% Prevalence**
Staphylococcus spp.	8	37	21.62
Pseudomonas spp.	5		13.5
Bacillus spp.	2		5.4
Vibrio spp.	4		10.8
Salmonella spp.	5		13.5
Klebsiella spp.	8		21.62
E.coli	5		13.5

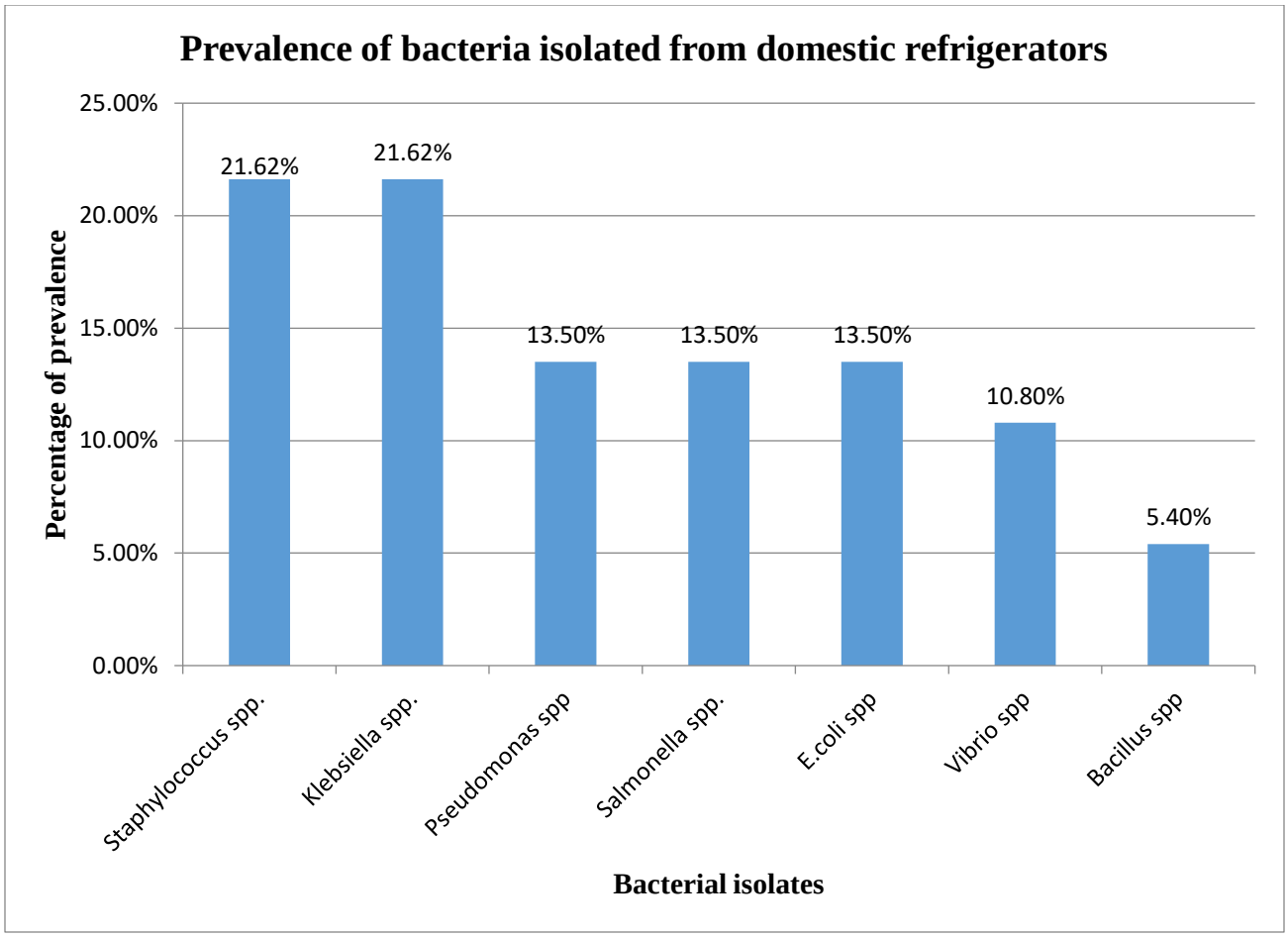


Figure 3.3: Percentage of prevalence of isolated bacteria from domestic refrigerators.

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 3.4 and Figure 3.5

Table 3.5: Distribution of the isolates according to Gram's Reaction

Gram's Reaction	**Number of isolates found**	**Percentage (%)**
Gram positive	10 (out of 37)	27.07%
Gram negative	27 (out of 37)	72.97%

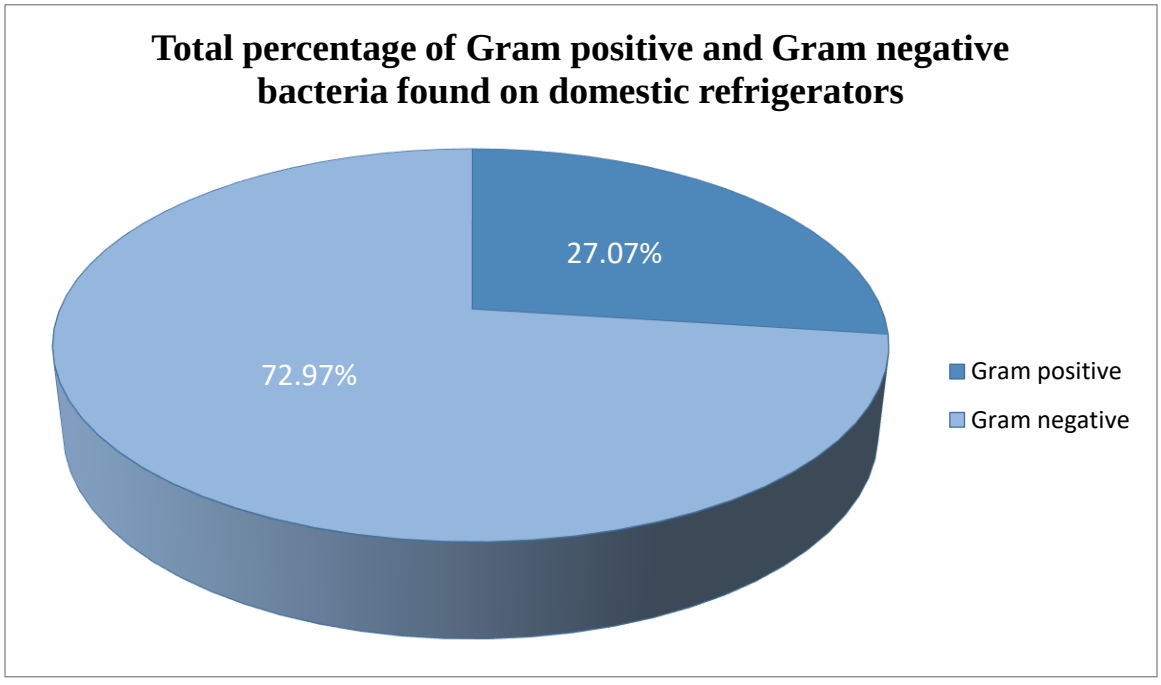


Figure 3.4: Total percentage of Gram positive and Gram negative bacteria identified from domestic refrigerators.

3.4 Antibiotic susceptibility test:

After identifying and confirming the organisms, the isolates were selected for antibiotic susceptibility test. About nine antibiotics were used and the sensitive and resistance pattern of the isolates to these antibiotics were determined. The interpretation of each bacterium either resistant, intermediate or susceptible to antibiotic is shown in shown in Table 3.6.

Table 3.6: Antibiotic susceptibility pattern of various organisms isolated from domestic refrigerators.

Isolates Id	Suspected organism	Penicillin	Ciprofloxacin	Chloramphenicol	Ampicillin	Gentamycin	Streptomycin	Tetracycline	Nalidixic acid	Azithromycin
		INP	INP	INP	INP	INP	INP	INP	INP	INP
S1A1	***E.coli* spp.**	R	S	S	R	S	I	S	S	S
S1B1	***Klebsiella* spp.**	R	S	S	R	S	I	S	S	S
S1A2	***Pseudomonas* spp.**	R	S	S	R	S	S	I	S	S
S1A3	***Staphylococcus* spp.**	R	S	S	R	S	S	S	S	S
S1A4	***Staphylococcus* spp.**	R	S	R	R	S	S	S	R	S
S1B2	***Staphylococcus* spp.**	S	S	S	S	S	S	S	I	S
S1C1	***Staphyloccus* spp.**	R	S	S	R	S	S	S	S	S
S1A5	***E.coli* spp.**	R	S	S	R	S	S	S	S	S
S1A6	***Klebsiella* spp.**	R	S	I	R	I	I	S	S	I
S1A7	***Bacillus* spp.**	R	S	S	R	S	I	I	S	S
S1B3	***Salmonella* spp.**	R	S	S	S	S	S	R	R	S
S1C2	***Salmonella* spp.**	R	S	S	S	S	I	S	R	S
S1D1	***Klebsiella* spp.**	R	I	S	R	S	I	S	S	S

Table 3.6: Antibiotic susceptibility pattern of various organisms isolated from domestic refrigerators.

Isolates Id	Suspected organism	Penicillin	Ciprofloxacin	Chloramphenicol	Ampicillin	Gentamycin	Streptomycin	Tetracycline	Nalidixic acid	Azithromycin
		INP	INP	INP	INP	INP	INP	INP	INP	INP
S2A1	***Klebsiella* spp.**	R	S	S	R	S	I	S	S	S
S2A2	***E.coli* spp.**	R	S	S	R	S	I	S	S	S
S2A3	***Salmonella* spp.**	R	S	S	R	S	I	S	I	S
S2C1	***Klebsiella* spp.**	R	S	S	R	S	S	S	S	I
S2D1	***Klebsiella* spp.**	R	S	S	S	S	S	S	S	S
S2A4	***Staphylococcus* spp.**	S	S	S	S	S	S	I	R	S
S2A5	***Staphylococcus* spp.**	R	S	S	R	S	S	S	R	R
S2B1	***Staphylococcus* spp.**	I	S	S	R	S	R	I	S	S
S2A6	***Salmonella* spp.**	R	I	S	R	S	S	S	R	S
S2B2	***E.coli* spp.**	R	S	S	R	R	I	S	S	S
S2A7	***Klebsiella* spp.**	R	S	S	R	I	S	S	S	S
S2B3	***Klebsiella* spp.**	I	S	S	R	S	I	S	S	I
S2C2	***Staphylococcus* spp.**	S	S	S	R	S	S	S	R	S
S2D2	***Bacillus* spp.**	R	S	S	R	S	I	I	S	S
S2D3	***Salmonella* spp.**	S	S	S	S	S	S	S	S	S
S2B4	***Pseudomonas* spp.**	R	S	S	S	S	S	I	R	S
S2C3	***Pseudomonas* spp.**	R	R	S	R	S	S	S	R	R

S2D4	***Pseudomonas* spp.**	R	S	R	R	S	R	I	R	S
S2A8	***Pseudomonas* spp.**	R	S	S	R	S	S	S	R	S
S2C5	***Vibrio* spp.**	I	S	S	R	S	S	R	R	S
S2D5	***Vibrio*spp.**	I	R	S	R	S	S	S	R	S
S2B5	***Vibrio*spp.**	I	S	S	R	R	S	S	R	S
S2A9	***Vibrio* spp.**	R	S	S	R	S	I	S	R	S
S2D6	***E.coli***	R	S	S	R	S	I	S	S	S
INP= Interpretation, S= Sensitive, I= Intermediate, R=Resistant										

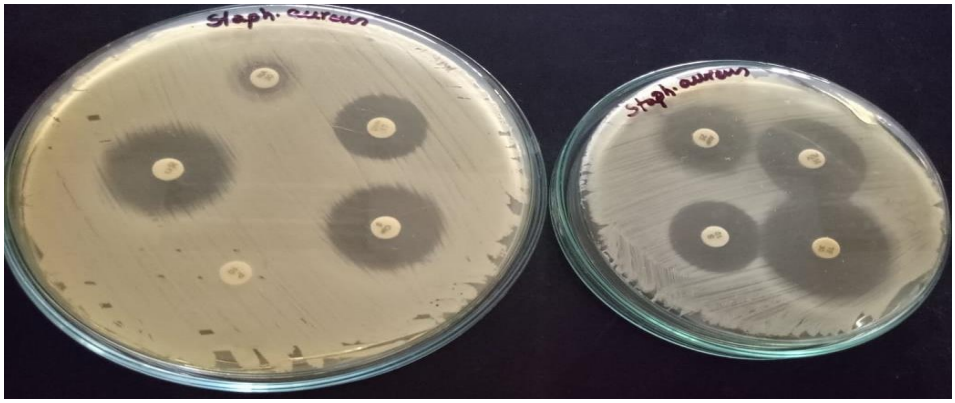


Figure 3.5: Antibiotic susceptibility test of *Staphylococcus* spp.

3.2.1 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 3.6 and in figure 3.6

Table 3.7: Antibiotic resistance pattern of total 37 bacterial isolates.

Antibiotics	**Penicillin**	**Ciprofloxacin**	**Chloramphenicol**	**Ampicillin**	**Gentamycin**	**Streptomycin**	**Tetracycline**	**Nalidixic acid**	**Azithromycin**
No of isolates resistant to tested antibiotics	28	2	2	30	2	2	2	15	2
Percentage of isolates resistant to antibiotics	75.67	5.4	5.4	81.08	5.4	5.4	5.4	40.5	5.4

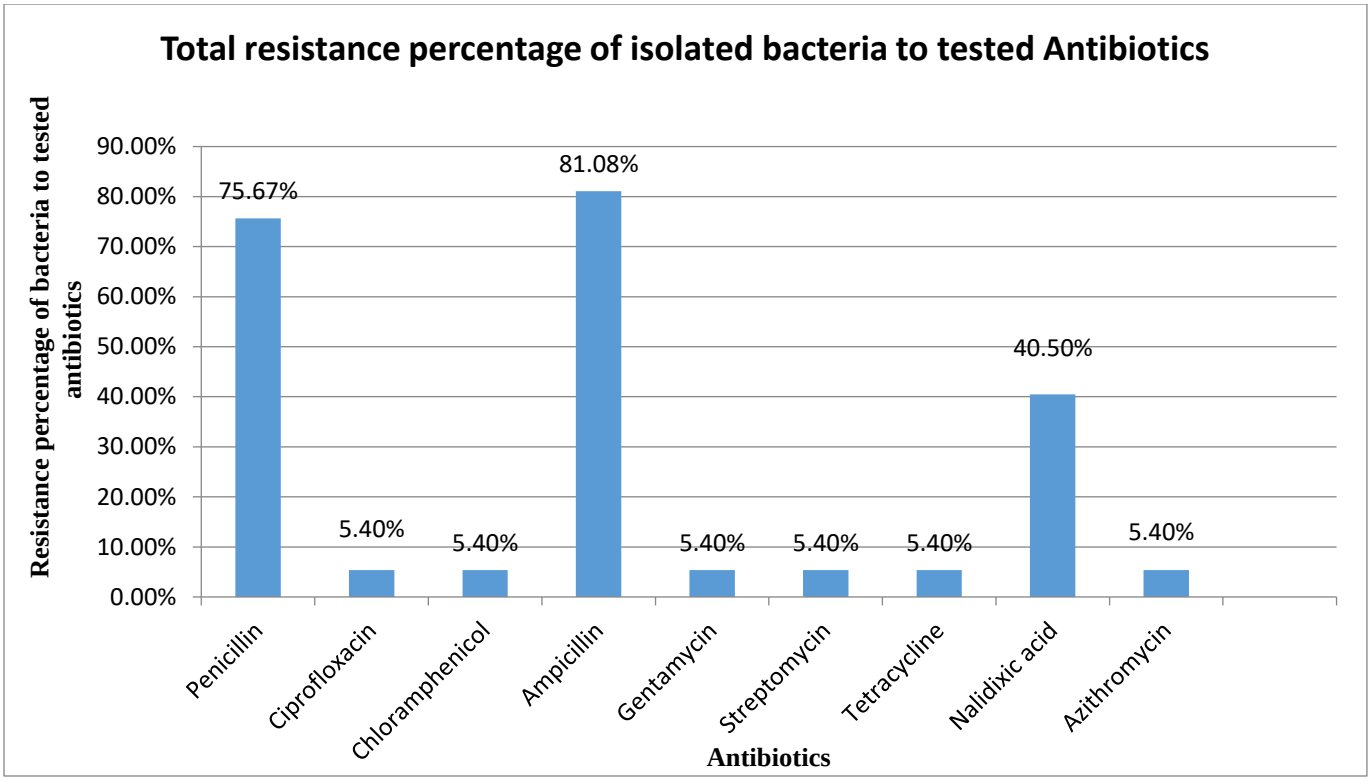


Figure 3.6: Resistance percentage of the isolated bacteria to tested antibiotics

3.2.2: 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 Table3.8

Table 3.8: 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 (11)
37	29.72

3.3 Temperature tolerance of the tested organism:

Temperature tolerance of the organisms was determined by growing the isolates in different temperature like 45°C,50°C and 55°C. All the isolates showed growth at 45°C but in 50°C and 55°C, 54.05% and 10.81% isolates showed viable growth respectively.

Table 3.9: Temperature tolerance of bacterial isolates:

Isolates ID	45°C	50°C	55°C
S1A1 (*E.coli*)	+	+	+
S1B1 (*Klebsiella spp*)	+	+	-
S1A2 (*Pseudomonas spp*)	+	_	-
S1A3(*Staphylococcus spp*)	+	_	-
S1A4 (*Staphylococcus spp*)	+	_	-
S1B2 (*Staphylococcus spp*)	+	_	-
S1C1 (*Staphylococcus spp*)	+	_	-
S1A5 (*E.coli*)	+	+	-
S1A6 (*Klebsiella spp*)	+	+	-
S1A7 (*Bacillus spp*)	+	+	-
S1B3 (*Salmonella spp*)	+	+	-
S1C2 (*Salmonella spp*)	+	+	-
S1D1 (*Klebsiella spp*)	+	+	-

Isolates ID	45°C	50°C	55°C
S2A1 (*Klebsiella spp*)	+	+	-
S2A2 *(E.coli*)	+	+	+
S2A3 (*Salmonella spp*)	+	+	-
S2C1(*Klebsiella spp*)	+	+	-
S2D1(*Klebsiella spp*)	+	+	-
S2A4 (*Staphylococcus spp*)	+	_	-
S2A5 (*Staphylococcus spp*)	+	_	-
S2B1 (*Staphylococcus spp*)	+	_	-
S2A6 (*Salmonella spp*)	+	+	-
S2B2 (*E.coli*)	+	+	+
S2A7 (*Klebsiella spp*)	+	+	-
S2B3 (*Klebsiella spp*)	+	+	-
S2C2 (*Staphylococcus spp*)	+	_	-
S2D2 (*Bacillus spp*)	+	+	_
S2D3 (*Salmonella spp*)	+	+	_
S2B4 (*Pseudomonas spp*)	+	_	_
S2C3 (*Pseudomonas spp)*	+	_	_
S2D4 (*Pseudomonas spp*)	+	_	_
S2A8 (*Pseudomonas spp*)	+	_	-
S2C5 (*Vibrio spp*)	+	_	-

Isolates ID	45°C	50°C	55°C
S2D5 (*Vibrio spp*)	+	–	–
S2B5 *(Vibrio spp*)	+	–	-
S2A9 (*Vibrio spp*)	+	–	-
S2D6 (*E.coli*)	+	+	+

(+) = growth (-) = no growth

Table:3.10 Total number of positive bacterial growth:

Total bacterial isolates	**Bacterial growth at 45**°C	**Bacterial growth at 50**°C	**Bacterial growth at 55**°C
37	37 (100%)	20 (54.05%)	4 (10.81%)

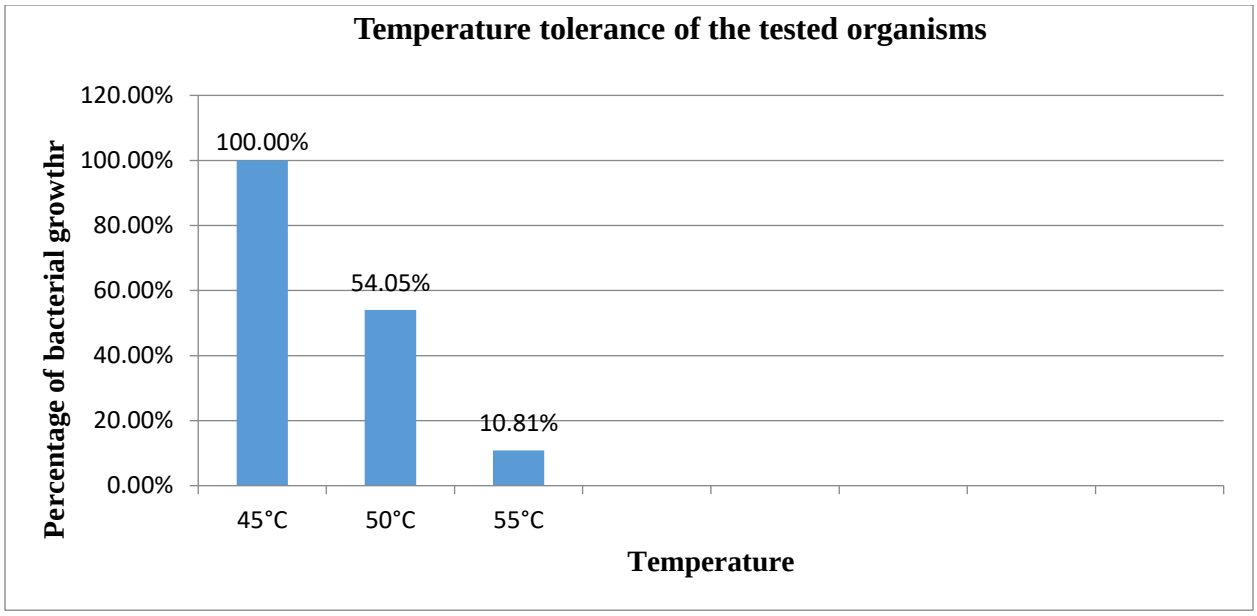


Figure 3.7: Temperature tolerance percentage of the tested organisms.

Chapter 4: Discussion and Conclusion

Discussion

The levels of contamination observed in domestic refrigerators are likely to be influenced by a range of factors including the nature and levels of initial contamination introduced on contaminated foods, the presence and absence of effective packaging, the hygiene of those preparing and placing foods into the refrigerator, and the efficiency and frequency of refrigerator maintenance and cleaning(U.S Food and Drug Administration,2017).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 and, if ingested, may result in food borne illness (Kumar,2012).

In this study, a total of 37 isolates had been identified from 8 different samples collected from different compartments (drawer, egg tray, handle,surface) of domestic refrigerator. The isolates have been confirmed after observing the cultural and morphological characteristics of bacterial isolates in different selective and differential media and performing the conventional biochemical tests. Out of 37 bacterial isolates, both *Staphylococcus* spp.and *Klebsiella* spp.showed the highest prevalence 8 (21.62%), next to *Pseudomonas* spp 5(13.5%).,*E.coli* 5(13.5%), *Salmonella* spp 5 (13.5%), *Bacillus* spp. 2 (5.4%) and *Vibrio* spp. 4 (10.8%).

Staphylococcus spp and *Klebsiella* spp were the most frequently isolated bacteria in this study but the frequency of *Staphylococcus aureus* (21.62%) is higher than some previous reported detection, e.g. 5% (Scott *et al.,* 1982).Unlike the other microbial pathogens which principally enter domestic kitchens, on previously contaminated raw foods, *S. aureus,* as a common inhabitant (up to 50%) of the human nose, throat, and skin is perhaps more likely to contaminate foods and refrigerators by direct or indirect human contact during domestic food handling and storage(Ananya Mandal 2012). Bacteria belonging to the genus *Klebsiella* frequently cause human nosocomial infections. In particular, the medically most important *Klebsiella* species, *Klebsiella pneumoniae,* accounts for a significant proportion of hospital-acquired urinary tract infections, pneumonia, septicemias, and soft tissue infections.(Podschun R, Ullmann U.1998).

Escherichia coli is widely accepted indicator of fecal contamination, suggesting that the refrigerator interior surfaces are frequently contaminated by import of contaminated raw foods or by poor personal hygiene. Although some strains of *E.coli* are harmless, Enterohaemorrhagic *E.coli* are capable of producing one or more toxins and a particular serotype 0157:H7 have been associated with haemorrhagic colitis and traveler's diarrhea (Adams, M.R. & Moss, M.O.2008).

Other frequently occurring bacteria like *Salmonella sp.* is a frequent contaminant of many retail foods, and possess public health challenges in terms of potential cross contamination to food and food preparation surfaces during routine food preparation (Kumar et al.,2012). *Pseudomonas* sp. presence and could be attributed to improperly packed food sources (raw milk) or have been already growing inside the refrigerator due to improper sanitation and is quite harmful to human health (Spiers, J. P., Anderton, A., & Anderson, J.G. 1995). The main route of transmission of *Vibrio* pathogens to man is through drinking of contaminated water and consumption inadequately cooked aquatic food products. Continuous monitoring of *Vibrio* pathogens among environmental freshwater and treated effluents is expected to help reduce the risk associated with the early detection of sources of infection, and also aid our understanding of the natural ecology and evolution of *Vibrio* pathogens (Osunla CA, Okoh AI.2017).

The lowest bacteria isolated, *Bacillus* sp. (5.4%), are also associated with food borne illness because *Bacillus* sp. endospores are partially resistant to pasteurization, dehydration, gamma radiation, and other physical stresses used in food processing, and their adhesive characteristics promote biofilm-forming capability on a variety of substrates in dairy operations.(Alyssa A.Grutsch,2018).

In comparing the overall microbial pathogens it was found that the drawer had the highest distribution of microbial pathogens in comparison with handle, egg tray and surface of domestic refrigerator.

In this study, antibiotic susceptibility pattern of bacteria isolated from domestic refrigerators were determined against 9 antibiotics. 81.08% isolates showed resistance against Ampicillin. The percentage of resistance to other antibiotics was ciprofloxacin (5.4%),chloramphenicol (5.4%), azithromycin (5.4%), gentamycin (5.4%), streptomycin(5.4%), tetracycline (5.4%),nalidixic acid (40.5%), penicillin (75.67%).Out of 37 bacterial isolates, 54.05% (20 out of 37) showed resistant to two antibiotics. Results showed that, most of the isolates were resistance against ampicillin.

Wolde and Bacha, (2015) investigated the prevalence and antibiotic resistance patterns of *Staphylococcus aureus* in food establishments of Jimma town and the most resisted drugs Norfloxacin, Amikacin and Ciprofloxacin showed maximum sensitivity. In this study all the isolates (100%) were multiple resistant to at least three antimicrobials being used. This figure is much higher than earlier reports from different studies in the country. It is also higher than reports of other studies from other parts of the world that showed most of the isolates were found to be sensitive for the antibiotics used. This result revealed that the isolates were highly resistant to Penicillin G, Ampicillin, Streptomycin, Chloramphenicol, Kanamycin and Methicillin.

Most households had erratic electricity supply and this affected the temperature regimes of the refrigerators. This resulting change in the refrigerator temperature will allow the growth of mesophilic organisms which can be pathogenic and therefore increases the risk of food borne disease (Oluwafemi, F., Akpoguma. S, Oladiran. T & Kolapo, A.2015). Investigation showed that most refrigerators do not work optimally because temperature will change when there is no adequate supply of power to the refrigerators. This may not be the case for some householders that get alternative source to power their refrigerators in some selected rich communities and some industrial areas. (Adams, M.R. & Moss, M.O.2008)

In this study,bacterial growth was observed at 45°C,50°C and 55°C. All bacterial isolates showed growth at 45°C whereas, only (54.05%) and (10.81%) isolates survived in 50°C and 55°C respectively.

It is impossible to completely exclude food pathogens from the kitchens; however their spread, growth in survival can be controlled with correct food storage and preparation of practices and regular cleaning and disinfection of food contact site. As we rely more and more on refrigeration as a means of food preservation it is crucial to aware the public about the refrigeration better handlings. The importance of temperature control and regular efficient cleaning should be communicated to the public.

Conclusion

This study has shown that food pathogens can survive on domestic refrigerators surfaces and therefore pose a risk of cross-contamination. Thus a number of potential food related pathogens including *Staphylococcus* spp, *Escherichia coli*, *Salmonella* spp, *Vibrio* spp, *Pseudomonas* spp, *Klebsiella* spp ,*Bacillus* spp, indicates poor personal hygiene.It is observed that irregular power supply, poor level of cleanliness, constant opening of the refrigerator doors greatly accounted for the presence of microbial pathogens and spoilage of food items stored in the refrigerators.As we rely more and more on refrigeration as a means of food preservation, it is crucial that the public be made aware that the refrigerator can in fact represent a significant niche for the persistence and dissemination of food borne pathogens. The importance of temperature control and regular efficient cleaning regimes need to be communicated to the public so that, effective management and cleaning of refrigerators makes them consistently reliable elements of the chilled food chain, and less likely to act as significant sources of human food borne diseases.

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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