Determination of prevalence and antibiotic susceptibility pattern of bacteria isolated from kitchen utensils of BRAC university of 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 bacteria isolated from kitchen utensils of BRAC university of Dhaka, Bangladesh."** has been submitted by me, Tasnim Tamanna 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

Kitchen utensils become contaminated by people, food, pet or other environmental sources and cross contamination occurs. This study aimed at isolating, identifying, determining antibiotic susceptibility pattern and investigating antimicrobial activity of dishwashing liquid against the bacterial isolates collected from kitchen utensils (air, knife, spoon and cutting board) of BRAC University, Dhaka, Bangladesh. Eight samples collected through sterile swabs were kept for enrichment into nutrient broth at 37ºC for 24 hours and then cultured on various selective media. 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 ten commercial antibiotic discs [Amoxicillin (10 µg), Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Gentamycin (10 µg), Cefepime (30 µg), Penicillin-G (10 µg), Rifampicin (5 µg), Cefixime (5 µg), Trimethoprim-sulphamethoxazole (5 µg), Tetracycline (30 µg)] by using Kirby-Bauer disc diffusion method. Minimum Inhibitory Concentration (MIC) of commercially used dishwasher was also performed using Broth Microdilution method against six selected isolates. A total of about 43 bacterial isolates were identified where *Staphylococcus* sp. showed the highest prevalence 14 (32.56%), followed by *Enterobacter* sp. 7 (16.28%), *Bacillus* sp. 6 (13.95%), *Vibrio* sp. 5 (11.63%), *Salmonella* sp. 4 (9.30%), *Klebsiella* sp. 4 (9.30%), *E.coli* 2 (4.65%) and *Shigella* sp. 1 (2.33%). Antibiotic susceptibility pattern of the bacterial isolates showed that almost all of the isolates were resistant to at least two antibiotics and 74.42% were found resistant to more than two antibiotics. Broth Microdilution assay revealed that the highest MIC value (400 µl of stock solution) was showed by *Salmonella* spp. and *Vibrio* spp. showed the lowest MIC value (190 µl of stock solution). Besides, *Bacillus* spp., *Staphylococcus* spp., *Shigella* spp. and *E. coli* had shown the values 250 µl, 250 µl, 250 µl and 200 µl of stock solution respectively. These results indicate that kitchen utensils can be an important source of potential pathogens and food spoilage bacteria causing foodborne diseases. The spread of antibiotic resistant bacteria from kitchen utensils can be minimized by regular cleaning of kitchen utensils, use of dishwashing liquid and public awareness on personal hygiene.

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Table of contents:

List of tables:

List of figures:

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
MIC	Minimum Inhibitory Concentration
WHO	World Health Organization

Chapter 1: Introduction

1.1 Introduction

The kitchen is probably the most crucial area that harbors and transmits infection. Germs are prevalent everywhere in the kitchen in sink sponges, countertops, cutting boards, kitchen utensils, refrigerators, sinks, towels, and even stove tops. Growth of undesirable contaminating bacteria not only causes deterioration in the sensory and organoleptic properties of food but can also cause illnesses. Most pathogenic microorganisms in food products are intestinal in origin; however, some are found in nasal passages, in the throat, on hair, and on skin. Thus, food handlers are often a main source of contamination and cross-contamination. The ability of bacteria to adhere to food contact surfaces compromises the hygiene of those surfaces. Surface physicochemical properties of the bacterial cell as well as of the materials, such as hydrophobicity and roughness, are determinants during the initial attachment phase. It has also been demonstrated that, even after adhering to typical and specific hygienic procedures, pathogenic microorganisms can survive in kitchens, often for hours. The main sites in the kitchen responsible for cross-contamination are chopping boards, sinks, taps, dish cloths, knives, and other working surfaces. Various types of surface such as plastic, stainless steel, glass and wood are used in the kitchen. These surfaces are subject to contamination by microorganisms, some of which are able to form biofilms. Contamination of surfaces depends on their characteristics, such as smooth, rough, porous, or irregular, and their state, for example before or after the cleaning process, new or old, dry or wet. Deliberate or undesirable contamination, a method of recovering microorganisms from the different solid surfaces is necessary as a tool for protecting family member's health. (Othman, 2015) The contamination of surfaces can be a public health problem, and in fact some disease outbreaks were subsequently found to be due to surface contamination. For example, Gill *et al.* showed that inadequately cleaned equipment contributed to the contamination of meat by *Escherichia coli* and the unambiguous role of the equipment surface in contact with meat. Allen *et al.* described poultry contamination by equipment in the slaughterhouse when poultry meat from broiler flocks, otherwise negative for *Campylobacter*, might have been contaminated if the previously slaughtered flock had been positive and the bacteria had remained on the equipment surfaces. (Ismail *et al.,* 2013)

1.2 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. Bacterial transfer studies can be divided into two groups based on the degree of experimental control. The first category includes studies with a great degree of control and is typically conducted in a laboratory. Control of some factors while varying others allows the determination of certain environmental factors on bacterial transfer rate. For example, longer contact time leads to greater coalescence and more interactions on recipient surfaces and that leads to higher transfer of bacteria (Dawson et al., 2006). A second type of experiments is studies performed outside the laboratory in food environments. Bacterial transfer studies are useful in indentifying contamination routes in processing environment such as factories, food service operations, and domestic, kitchen, etc. (Perz-Rodriguez, 2008). Factors affecting bacterial transfer can be divided into two groups: environmental and intrinsic factors. The first group includes surface material properties, presence of bio-fouling layers, moisture availability, contact pressure and contact time. The second group includes factors unique to bacterial species such as exopolysaccharide layers, biofilm forming ability, clump formation and the presence of extracellular structures. Environmental factors include adherence of bacteria enhanced by surface structural hydrophilic and hydrophobic properties. Bacterial attachment depends upon the degree of surface roughness with rough surfaces having a lower level of bacterial transfer initially but a greater degree of adhesion. 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). The concentration of bacteria on the surface or in an inoculum can also affect bacterial transfer (Montville and Schaffner, 2003). Montville and Schaffner (2003) reported that the higher the inoculum size on the source surfaces the lower the transfer rate. 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 microflora 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 corneum. They are not typically considered pathogens but may cause infections in body cavities such as the eyes. Resident

bacteria can survive longer on intact skin than do gram negative transient species. The transient skin flora consists of bacteria, fungi and viruses that may sometimes be found on skin. Usually they do not multiply on the skin but they can survive and cause disease. 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 microflora 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 kitchen environment play an important role in sporadic and epidemic food borne illnesses. Hands are potentially a critical control point for reducing or preventing bacterial cross contamination from ill and asymptomatic food workers who might shed high levels of pathogens particularly those originating from the nasal cavity. Sharing of food may also be responsible for cross contamination and may lead to a higher number of food borne illness outbreaks. Sharing food from the same utensils is traditional in many Asian cultures (India, Japan, China, and Pakistan) and utensils used for the ethnic cuisine may also support cross contamination. The present study focuses on the bacterial transfer from utensils to food. (Purohit, 2009)

1.3 Bacteria commonly found in kitchen environment:

Foods may also be contaminated with bacteria during food preparation in a restaurant or home kitchen. If food preparers do not thoroughly wash their hands, kitchen utensils, cutting boards, and other kitchen surfaces that come into contact with raw foods, cross-contamination.

Many types of bacteria cause foodborne illnesses. 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.

- ***Shigella,*** a bacterium spread from person to person. These bacteria are present in the stools of people who are infected. If people who are infected do not wash their hands thoroughly after using the bathroom, they can contaminate food that they handle or prepare. Water contaminated with infected stools can also contaminate produce in the field.
- ***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)

1.4 Antibiotic resistance

Antibiotics are medicines used to prevent and treat bacterial infections. Antibiotic resistance occurs when bacteria change in response to the use of these medicines. Bacteria, not humans or animals, become antibiotic-resistant. These bacteria may infect humans and animals, and the infections they cause are harder to treat than those caused by non-resistant bacteria. A growing number of infections – such as pneumonia, tuberculosis, gonorrhoea, and salmonellosis – are becoming harder to treat as the antibiotics used to treat them become less effective. The world urgently needs to change the way it prescribes and uses antibiotics. Even if new medicines are developed, without behaviour change, antibiotic resistance will remain a major threat. Behaviour changes must also include actions to reduce the spread of infections through vaccination, hand washing, practising safer sex, and good food hygiene. (WHO, 2017)

The rapid emergence of resistant bacteria is occurring worldwide, endangering the efficacy of antibiotics, which have transformed medicine and saved millions of lives. Many decades after the first patients were treated with antibiotics; bacterial infections have again become a threat. The antibiotic resistance crisis has been attributed to the overuse and misuse of these medications, as well as a lack of new drug development by the pharmaceutical industry due to reduced economic incentives and challenging regulatory requirements. The Centers for Disease Control and Prevention (CDC) has classified a number of bacteria as presenting urgent, serious, and concerning threats, many of which are already responsible for placing a substantial clinical and financial burden on the U.S. health care system, patients, and their families. Coordinated efforts to implement new policies, renew research efforts, and pursue steps to manage the crisis are greatly needed. (Ventola, 2015)

1.5 Safety from cross contamination:

Kitchens are breeding grounds of bacteria (sinks, drains). It contain objects for spreading the bacteria (sponges, cloths, tea towels), which are used regularly by all the family. The surfaces of kitchen are in contact with hands and food and they contain a whole range of germs, for example chopping boards, work surfaces, fridges and utensils. Bacteria that are found in kitchen: *E.Coli, Pseudomonas aeruginosa, Staphylococus aureus, Salmonella, Enterobacter, Listeria***.** So, kitchen utensils should be kept cleaned so that bacteria do not spread to food. They should be washed thoroughly with dishwashing liquid after using them with raw food.

1.6 Literarture review

Gerba et al., (2014) investigated the occurrence of bacteria in kitchen towels often used to dry dishes, hands and other surfaces in the domestic kitchen. A total of 82 kitchen hand towels were collected from households in five major cities in the United States and Canada. The numbers of heterotrophic bacteria, coliform bacteria, and *Escherichia coli* in each towel were determined. In addition, identification of the enteric bacteria was performed on selected towels. Coliform bacteria were detected in 89.0% and *E. coli* in 25.6% of towels.

Wolde and Bacha, (2016) evaluated the microbiological safety of sponges as it has been used in selected food establishments of Jimma town. A total of 201 kitchen sponges randomly collected from food establishments were evaluated against the total counts of aerobic mesophilic bacteria (AMB), Enterobacteriaceae, coliforms, and yeast and molds. The mean counts of aerobic mesophilic bacteria ranged from 7.43 to 12.44 log CFU/mm^3. The isolated genera were dominated by *Pseudomonas* (16.9%), *Bacillus* (11.1%), *Micrococcus* (10.6%), *Streptococcus* (7.8%), and *Lactobacillus* (6%) excluding the unidentified Gram positive rods (4.9%) and Gram negative rods (9.9%). The high microbial counts (aerobic mesophilic bacteria, coliforms, Enterobacteriaceae, and yeast and molds) reveal the existence of poor kitchen sponge sanitization practice.

Wolde et al., (2015) investigated the prevalence and antibiotic resistance patterns of *Staphylococcus aureus* isolated from kitchen sponges used in food establishments of Jimma town, between October, 2010 and June, 2011. A total of 201 kitchen sponge 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%) kitchen sponges 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 kitchen sponges 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

This research work was focused on isolation, identification and determination of the prevalence of bacterial contaminants present in the kitchen utensils and air. 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. So, the purpose of this study is mentioned below:

- Isolating the bacterial contaminants present in kitchen utensils and air.
- 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.
- Finding out the effects of liquid dishwasher on selected bacterial isolates.

Chapter 2: Materials & Methods

Materials and Methods:

2.1 Study area and duration:

The laboratory processing, analysis of data and the overall experimental work were done in Microbiology Research Laboratory of the Department of Mathematics and Natural Sciences of BRAC University. The study was conducted during the period February- December, 2017.

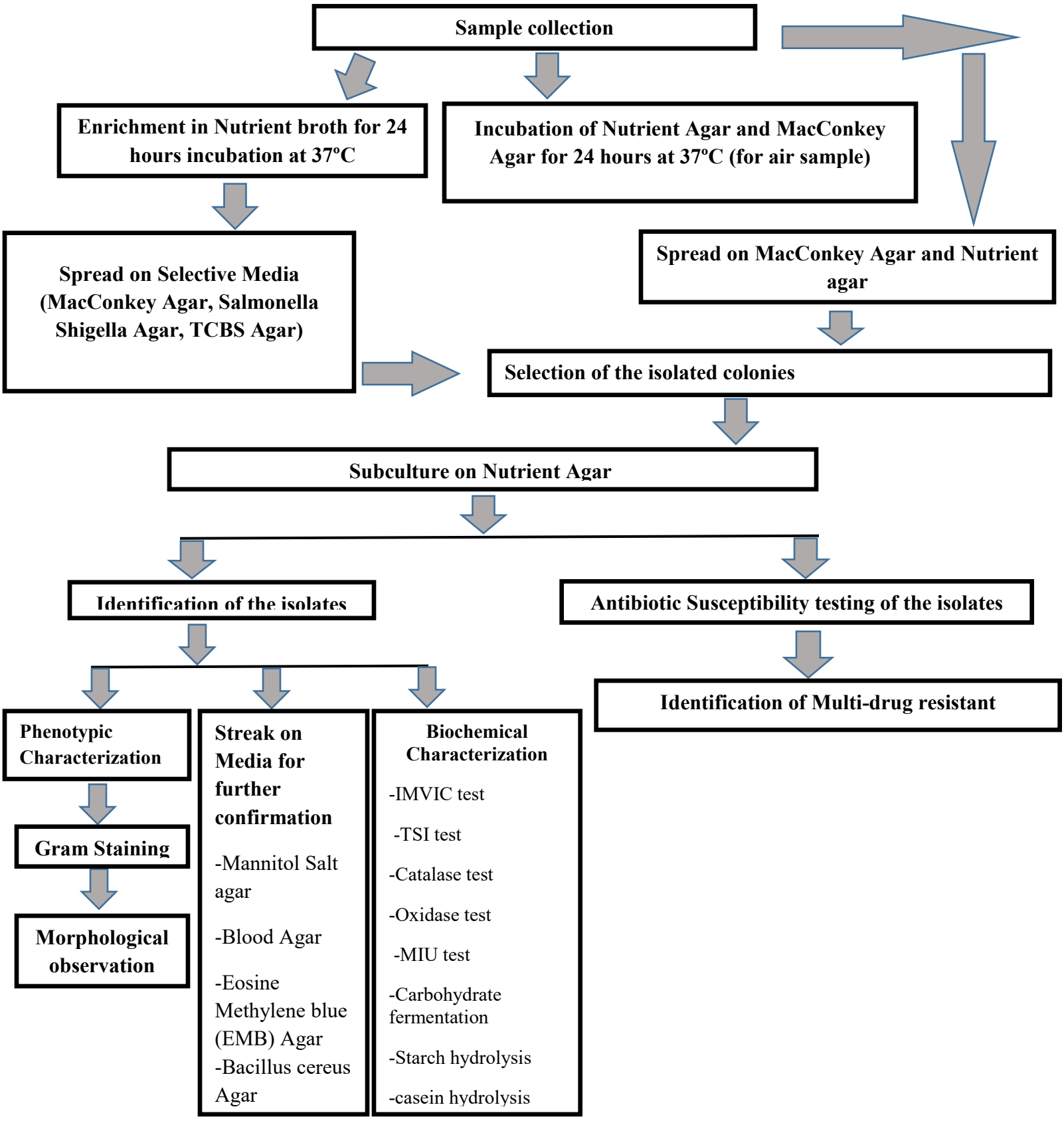
2.2 Sample size:

A total of about 8 kitchen utensils samples (air, knife, spoon cutting board) were collected from the two kitchens of BRAC University.

2.3 Sample collection and processing:

A total of 8 samples were collected from kitchen utensils (spoon, knife and cutting board) using the swab-rinse method. Microbial load of the kitchen air is determined by settle plate method. All samples were collected at noon when people were about to use the utensils to check the prevalence of microorganisms. Kitchen utensils were swabbed with sterile cotton swabs moistened with sterile normal saline. The swab was wiped firmly on the entire surface of the utensils. It was then introduced into a test-tube containing sterile nutrient broth and properly labeled. The test-tube was shaken, loosely capped. Then it was immediately transported to the Microbiology Research Laboratory of BRAC University for further processing and analysis.

2.4 Experimental design:



2.5 Isolation, purification and storage of sample:

Sources of 8 samples collected from air, spoon, knife and cutting board 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	Kitchen 1 (Air)	07.02.2017	11.00 pm	6	k1A1, k1A2, k1A3, k1A4, k1A5, k1A6
2	Kitchen 1 (Knife)	07.02.2017	11.00 pm	4	k1K1, k1K3, k1K4, kiK5
3	Kitchen 1 (Cutting board)	07.02.2017	11.00 pm	4	k1C1, k1C2, k1C3, k1C4
4	Kitchen 1 (Spoon)	07.02.2017	11.00 pm	4	k1S1, k1S2, k1S3, k1S4
5	Kitchen 2 (Air)	08.03.2017	11.00 pm	5	k2A1, k2A3, k2A4, k2A5, k2A6
6	Kitchen2 (Knife)	08.03.2017	11.00 pm	6	k2K1, k2K3, k2K4, k2K6, k2K10, k2K11
7	Kitchen 2 (Spoon)	08.03.2017	11.00 pm	5	k2S3, k2S4, k2S5, k2S7, k2S8
8	Kitchen2 (cutting board)	08.03.2017	11.00 pm	9	k2C1, k2C2, k2C3, k2C4, k2C6, k2C7, k2C9, k2C10, k2C11

After sample collection, samples were purified by using some selective media. Some samples were spreaded on Nutrient Agar and they were kept for biochemical tests. Other samples were directly spreaded on some selective media plates (Mannitol Salt agar, Blood Agar, Eosine Methylene blue Agar, Bacillus cereus Agar, MacConkey Agar, Salmonella Shigella Agar, TCBS Agar) from nutrient broth. Then the plates were incubated for 24 hours at 37ºC. After that isolates from the selective media plates were streaked on nutrient agar plates to get pure cultures for storage.

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.

2.6 Biochemical identification:

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

2.6.1 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.

2.6.2 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

2.6.3 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.

2.6.4 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 wasinoculated 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.

2.6.5 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 wasinoculated 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

2.6. 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 asterile inoculating loop, a small amount of bacteria from 24-hour pure culture was placed ontothe reagent drops of the microscopic slide

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

2.6.7 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, smallamount of the experimental bacteria from fresh culture was inoculated into the tubesby means of stab inoculation method with an inoculating needle
- The tubes were thenincubated for 24 hours at 37ºC.
- The growth of the organism would spread throughout the test tube from downward to theupward of the test tube, if the organism is motile.
- The colour of the media will turn to deeppink 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 overnightincubation. 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.

2.6.8 Carbohydrate (Maltose) fermentation test:

Carbohydrate (Maltose) fermentation test was done to determine the ability of microorganisms to degrade and ferment carbohydrates with the production of acid and gas. Phenol Red Maltose broth was used in this test.

- In Phenol Red Maltose broth containing test tubes, Durham tubes were inserted upside down to detect gas production.
- After autoclave and cooling process, each test tube was inoculated aseptically with the test microorganism using an inoculating loop.
- The test tubes were incubated at 37°C for 24 hours.
- After incubation if the liquid in the tubes turn yellow, it indicates the fermentation of Maltose present in the media and it is a positive result. If the tube containing medium remains red, it indicates negative result.
- A bubble will be seen in the inverted Durham tube if the test organism produces gas from the fermentation of Maltose. There will not be any bubble in the inverted Durham tube if the organism does not produce gas.

2.6.9 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.

2.6.10 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)

2.7Antimicrobial 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	**Amoxicillin**	AML	10
2	**Ciprofloxacin**	CIP	5
3	**Chloramphenicol**	C	30
4	**Gentamycin**	CN	10
5	**Cefepime**	FEP	30
6	**Penicillin-G**	P	10
7	**Rifampicin**	RD	5
8	**Cefixime**	CFM	5
9	**Trimethoprim-sulphamethoxazole**	SXT	5
10	**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: 2.3).

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	**Amoxicillin**	≤13 / ≤19	14-17	≥18 / ≥20
2	**Ciprofloxacin**	≤15 / ≤20	16-20/ 21-30	≥21 / ≥31
3	**Chloramphenicol**	≤12	13-17	≥18
4	**Gentamycin**	≤12	13-14	≥15
5	**Cefepime**	≤14 / ≤21	15-17/ 22-23	≥18/ ≥26/ ≥31/ ≥24
6	**Penicillin-G**	≤23	-	≥29
7	**Rifampicin**	≤16	17-19	≥20
8	**Cefixime**	≤15	16-18	≥19/ ≥21/ ≥31
9	**Trimethoprim-sulphamethoxazole**	≤10	11-15	≥16
10	**Tetracycline**	≤11	12-14	≥15

2.8 Determination of MIC:

In broth micro-dilution method (Lee and Mary, 2013) a mixture of nutrient broth, stock solution and microorganisms of 10 ml volume was prepared in test-tubes. Here, the broth and stock solution constitute 9 ml and the rest 1 ml was for the microorganisms cultured in nutrient broth. Different concentration of stock solution that are used in MIC determination are described in Table 2.5.

Table 2.5: Mixture composition in the test-tubes.

Vim liquid (ml)	Nutrient-Broth(ml)	Sample clinical isolates (ml)
0.06 ml	8.94 ml	1ml
0.09 ml	8.91 ml	
0.1 ml	8.90 ml	
0.13 ml	8.87 ml	
0.16ml	8.84 ml	
0.19 ml	8.81 ml	
0.2 ml	8.80 ml	
0.25 ml	8.75 ml	
0.3 ml	8.70 ml	
0.4 ml	8.60 ml	

2.8.1 Steps in MIC determination:

1. A test-tube containing nutrient broth with desired microorganism and a test-tube with liquid stock solution were taken and treated as control to see the growth.
2. At first the liquid vim and the broth were pipetted into test-tubes in the laminar air-flow cabinet and then 1 ml of desired microorganisms were pipetted from the cultured broth into the test-tubes. Then all the test-tubes were incubated in 37°C in the incubator for 24 hrs. After 24hrs the test-tubes were observed and compared with the control and MIC (Minimum Inhibitory Concentration) value was obtained and noted.
3. All the steps were performed in the Laminar Air Flow Cabinet.

Chapter 3: Result

3.1 Bacterial isolation and identification:

3.1.1 Cultural and morphological characteristics of the bacterial isolates:

In Table 3.1 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 from Kitchen utensils on various Selective, Differential, Enriched media and Nutrient Agar

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)**	***Bacillus cereus* Agar (BC Agar)**	***Salmonella -Shigella* Agar (SS Agar)**	**TCBS Agar**	**Blood Agar Hemo-Lysis**	**Size**	**Color**	**Form**	**Margin**	**Elevation**	
k1A1	-	-	-	Blue coloured colonies	-	-	Alpha Hemo-Lysis	Large	White	Circular	Entire	Convex	***Bacillus* spp.**
k1A2				Blue coloured colonies	-		Beta Hemo-Lysis	Large	White	Circular	Entire	Convex	***Bacillus* spp.**
k1A3	Pink coloured mucoid colonies		Pink coloured mucoid colonies				Alpha Hemo-Lysis	Large	Creamy	Circular	Entire	Flat	***Klebsiella* spp.**
k1A4					Colourless colonies		Alpha Hemo-Lysis	Small	Colour less	Circular	Entire	Convex	***Shigella* spp.**
k1A5		Small, pink coloured colonies					Alpha Hemo-Lysis	Small	Orange	Circular	Entire	Flat	***Staphylococcus* spp.**
k1A6		Small, yellow coloured colonies					Alpha Hemo-Lysis	Small	Yellow	Circular	Entire	Convex	***Staphylococcus* spp.**

Table 3.1: Cultural and Morphological Characteristics of the Bacterial Isolates from Kitchen utensils on various Selective, Differential, Enriched media and Nutrient Agar

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)**	***Bacillus cereus* Agar (BC Agar)**	***Salmonella -Shigella* Agar (SS Agar)**	**TCBS Agar**	**Blood Agar Hemo-Lysis**	**Size**	**Color**	**Form**	**Margin**	**Elevation**	
k1K1	Pink coloured mucoid colonies	-	Pink coloured mucoid colonies	-	-	-	Beta Hemo-Lysis	Large	Creamy	Circular	Entire	Flat	***Klebsiella* spp.**
k1K3	Pink coloured mucoid colonies		Pink coloured mucoid colonies		-		Alpha Hemo-Lysis	Large	Creamy	Circular	Entire	Flat	***Klebsiella* spp.**
k1K4						Green coloured colonies	Beta Hemo-Lysis	Small	White	Circular	Entire	Flat	***Vibrio* spp.**
k1K5	Pink coloured colonies		Pink coloured colonies				Alpha Hemo-Lysis	Small	Creamy	Circular	Entire	Convex	***Enterobacter* spp.**
k1C1				Blue coloured colonies			Alpha Hemo-Lysis	Large	White	Circular	Entire	Convex	***Bacillus* spp.**
k1C2		Small, yellow coloured colonies					Beta-Hemo-Lysis	Small	Yellow	Circular	Entire	Convex	***Staphylococcus* spp.**

Table 3.1: Cultural and Morphological Characteristics of the Bacterial Isolates from Kitchen utensils on various Selective, Differential, Enriched media and Nutrient Agar

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)**	***Bacillus cereus* Agar (BC Agar)**	***Salmonella -Shigella* Agar (SS Agar)**	**TCBS Agar**	**Blood Agar Hemo-Lysis**	**Size**	**Color**	**Form**	**Margin**	**Elevation**	
k1C3	-	Small, yellow coloured colonies	-	-	-	-	Beta Hemo-Lysis	Small	Yellow	Circular	Entire	Convex	***Staphylococcus* spp.**
k1C4		Small, yellow coloured colonies			-		Beta Hemo-Lysis	Small	Yellow	Circular	Entire	Convex	***Staphylococcus* spp.**
k1S1		Small, pink coloured colonies					Alpha Hemo-Lysis	Small	Orange	Circular	Entire	Flat	***Staphylococcus* spp.**
k1S2				Blue coloured colonies			Alpha Hemo-Lysis	Large	White	Circular	Entire	Convex	***Bacillus* spp.**
k1S3		Small, pink coloured colonies					Alpha Hemo-Lysis	Small	Orange	Circular	Entire	Flat	***Staphylococcus* spp.**

Table 3.1: Cultural and Morphological Characteristics of the Bacterial Isolates from Kitchen utensils on various Selective, Differential, Enriched Media and Nutrient Agar

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)**	***Bacillus cereus* Agar (BC Agar)**	***Salmonella -Shigella* Agar (SS Agar)**	**TCBS Agar**	**Blood Agar Hemo-Lysis**	**Size**	**Color**	**Form**	**Margin**	**Elevation**	
k1S4		Small, pink coloured colonies					Alpha Hemo-Lysis	Small	Orange	Circular	Entire	Flat	***Staphylococcus* spp.**
k2A1	-	-	-	Green coloured colonies	-	-	Alpha Hemo-Lysis	Medium	Offwhite	Circular	Entire	Flat	***Bacillus* spp.**
k2A3		Small, yellow coloured colonies			-		Beta Hemo-Lysis	Small	Entire	Circular	Entire	Convex	***Staphylococcus* spp.**
k2A4		Small, pink coloured colonies					Alpha Hemo-Lysis	Small	Orange	Circular	Entire	Flat	***Staphylococcus* spp.**
k2A5		Small, pink coloured colonies					Alpha Hemo-Lysis	Small	Orange	Circular	Entire	Flat	***Staphylococcus* spp.**

Table 3.1: Cultural and Morphological Characteristics of the Bacterial Isolates from Kitchen utensils on various Selective, Differential, Enriched Media and Nutrient Agar

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)**	***Bacillus cereus* Agar (BC Agar)**	***Salmonella -Shigella* Agar (SS Agar)**	**TCBS Agar**	**Blood Agar Hemo-Lysis**	**Size**	**Color**	**Form**	**Margin**	**Elevation**	
k2A6		Small, pink coloured colonies					Alpha Hemo-Lysis	Small	Orange	Circular	Entire	Flat	***Staphylococcus* spp.**
k2K1		Small, pink coloured colonies					Alpha Hemo-Lysis	Small	Orange	Circular	Entire	Flat	***Staphylococcus* spp.**
k2K3	-	-	-	Blue coloured colonies	-	-	Beta Hemo-Lysis	Large	White	Circular	Entire	Convex	***Bacillus* spp.**
k2K4					-	Yellow coloured colonies	Alpha Hemo-Lysis	Small	White	Circular	Entire	Flat	***Vibrio* spp.**
k2K6	pink coloured colonies		pink coloured colonies				Alpha Hemo-Lysis	Small	Creamy	Circular	Entire	Convex	***Enterobacter* spp.**
k2K10					Black centered colourless colonies		Alpha Hemo-Lysis	Medium	White	Circular	Entire	Convex	***Salmonella* spp.**

Table 3.1: Cultural and Morphological Characteristics of the Bacterial Isolates from Kitchen utensils on various Selective, Differential, Enriched Media and Nutrient Agar

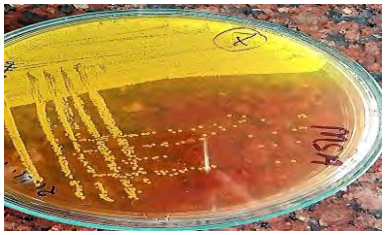
Isolates ID	Growth on Selective, Differential and Enriched Media							Colony morphology on Nutrient Agar					Suspected Organism
	Mac-Conkey Agar	**Mannitol Salt Agar (MSA)**	**Eosine Methylene Blue Agar (EMB)**	***Bacillus cereus* Agar (BC Agar)**	***Salmonella -Shigella* Agar (SS Agar)**	**TCBS Agar**	**Blood Agar Hemo-Lysis**	**Size**	**Color**	**Form**	**Margin**	**Elevation**	
k2K11						Yellow coloured colonies	Alpha Hemo-Lysis	Small	White	Circular	Entire	Flat	***Vibrio* spp.**
k2S3	Medium Pink coloured colonies		Metallic Green sheen colonies				Alpha Hemo-Lysis	Medium	Creamy	Circular	Entire	Raised	***E.coli***
k2S4	Medium Pink coloured colonies		Metallic Green sheen colonies	-	-	-	Alpha Hemo-Lysis	Medium	Creamy	Circular	Entire	Raised	***E.coli***
k2S5	Pink coloured mucoid colonies		Pink coloured mucoid colonies		-		Alpha Hemo-Lysis	Large	Creamy	Circular	Entire	Flat	***Klebsiella* spp.**
k2S7	pink coloured colonies		pink coloured colonies				Alpha Hemo-Lysis	Small	Creamy	Circular	Entire	Convex	***Enterobacter* spp.**

Table 3.1: Cultural and Morphological Characteristics of the Bacterial Isolates from Kitchen utensils on various Selective, Differential, Enriched Media and Nutrient Agar

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)**	***Bacillus cereus* Agar (BC Agar)**	***Salmonella -Shigella* Agar (SS Agar)**	**TCBS Agar**	**Blood Agar Hemo-Lysis**	**Size**	**Color**	**Form**	**Margin**	**Elevation**	
k2S8					Black centered colourless colonies		Alpha Hemo-Lysis	Medium	White	Circular	Entire	Convex	***Salmonella* spp.**
k2C1	pink coloured colonies		pink coloured colonies				Alpha Hemo-Lysis	Small	Creamy	Circular	Entire	Convex	***Enterobacter* spp.**
k2C2	pink coloured colonies		pink coloured colonies				Alpha Hemo-Lysis	Small	Creamy	Circular	Entire	Convex	***Enterobacter* spp.**
k2C3	pink coloured colonies		pink coloured colonies	-	-	-	Alpha Hemo-Lysis	Small	Creamy	Circular	Entire	Convex	***Enterobacter* spp.**
k2C4	pink coloured colonies		pink coloured colonies		-		Alpha Hemo-Lysis	Small	Creamy	Circular	Entire	Convex	***Enterobacter* spp.**
k2C6		Small, pink coloured colonies					Alpha Hemo-Lysis	Small	Orange	Circular	Entire	Flat	***Staphylococcus* spp.**

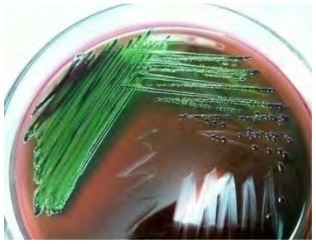
Table 3.1: Cultural and Morphological Characteristics of the Bacterial Isolates from Kitchen utensils on various Selective, Differential, Enriched Media and Nutrient Agar

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)	*Bacillus cereus* Agar (BC Agar)	*Salmonella -Shigella* Agar (SS Agar)	TCBS Agar	Blood Agar Hemo-Lysis	Size	Color	Form	Margin	Elevation	
k2C7						Yellow coloured colonies	Alpha Hemo-Lysis	Small	White	Circular	Entire	Flat	***Vibrio* spp.**
k2C9						Yellow coloured colonies	Alpha Hemo-Lysis	Small	White	Circular	Entire	Flat	***Vibrio* spp.**
k2C10					Black centered colourless colonies		Alpha Hemo-Lysis	Medium	White	Circular	Entire	Convex	***Salmonella* spp.**
k2C11					Black centered colourless colonies		Alpha Hemo-Lysis	Medium	White	Circular	Entire	Convex	***Salmonella* spp.**



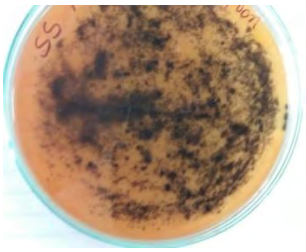
Growth of *Staphylococcus* spp. on MSA

Growth of *Shigella* spp. on SS Agar

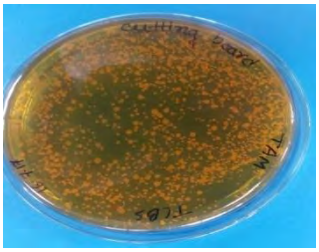


Growth of *E.coli* on EMB Agar

Growth of *Bacillus* on BC Agar

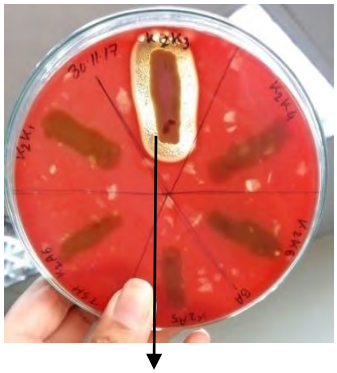


Growth of *Salmonella* spp. on SS Agar

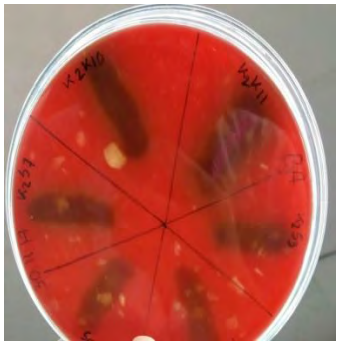


Growth of *Vibrio* spp. on TCBS Agar

Figure 3.1: Bacterial growth on various selective media



Beta hemolysis



Alpha hemolysis

Figure 3.2: Bacterial growth on Blood agar

3.1.2 Biochemical characteristics of the bacterial isolates:

Table 3.2: Biochemical characteristics of the bacteria isolated from kitchen utensils of BRAC University

Isolates ID	Gram staining		Indole Test	Methyl red Test	VP Test	Citrate test	Triple Sugar Iron Test						Carbohydrate fermentation		MIU Test			Catalase Test	Starch Hydrolysis	Casein hydrolysis	Suspected Organism
	Gram reaction	Shape					Slant/Butt	Glucose	Lactose	Sucrose	H_2S	Gas	Maltose	Gas	Motility	Indole	Urease				
k1A1	+	rod	-	-	-	+	R/R	-	-	-	-	-	-	-	+	-	-	+	-	-	***Bacillus* spp.**
k1A2	+	rod	-	-	-	+	R/R	-	-	-	-	-	-	-	+	-	-	+	-	-	***Bacillus* spp.**
k1A3	-	rod	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	-	+	***Klebsiella* spp.**
k1A4	-	rod	-	+	-	-	R/Y	+	-	-	-	+	+	+	-	-	-	+	-	+	***Shigella* spp.**
k1A5	+	cocci	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	-	-	***Staphylococcus* spp.**
k1A6	+	cocci	-	+	-	-	Y/Y	+	+	+	-	-	+	-	-	-	+	+	-	+	***Staphylococcus* spp.**
k1K1	-	rod	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	-	+	***Klebsiella* spp.**
k1K3	-	rod	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	-	+	***Klebsiella* spp.**
k1K4	-	rod	+	-	-	+	R/R	-	-	-	-	-	+	-	+	+	-	+	+	+	***Vibrio* spp.**
k1K5	-	rod	-	-	+	+	Y/Y	+	+	+	-	+	+	+	+	-	-	+	-	-	***Enterobacter* spp.**
k1C1	+	Rod	-	-	-	-	Y/Y	+	+	+	-	-	+	-	-	-	-	+	-	-	***Bacillus* spp.**
k1C2	+	cocci	-	+	+	-	Y/Y	+	+	+	-	-	+	-	-	-	+	+	-	+	***Staphylococcus* spp.**
k1C3	+	cocci	-	+	-	-	Y/Y	+	+	+	-	-	+	-	-	-	+	+	-	+	***Staphylococcus* spp.**
k1C4	+	cocci	-	+	+	-	Y/Y	+	+	+	-	-	+	-	-	-	+	+	-	+	***Staphylococcus* spp.**
k1S1	+	cocci	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	-	-	***Staphylococcus* spp.**
k1S2	+	cocci	-	-	-	+	R/R	-	-	-	-	-	-	-	-	-	-	+	-	-	***Bacillus* spp.**
k1S3	+	cocci	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	-	-	***Staphylococcus* spp.**
k1S4	+	cocci	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	-	-	***Staphylococcus* spp.**

Table 3.2: Biochemical characteristics of the bacteria isolated from kitchen utensils of educational institution

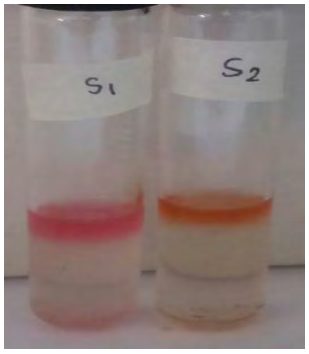
Isolates ID	Gram staining		Indole Test	Methyl red Test	VP Test	Citrate test	Triple Sugar Iron Test						Carbohydrate fermentation		MIU Test			Catalase Test	Starch Hydrolysis	Casein hydrolysis	Suspected Organism
	Gram reaction	Shape					Slant/Butt	Glucose	Lactose	Sucrose	H_2S	Gas	Maltose	Gas	Motility	Indole	Urease				
k2A1	+	rod	-	-	+	+	R/Y	+	-	-	-	-	+	-	+	-	-	+	+	+	***Bacillus* spp.**
k2A3	+	cocci	-	+	+	-	Y/Y	+	+	+	-	-	+	-	-	-	+	+	-	+	***Staphylococcus* spp.**
k2A4	+	cocci	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	-	-	***Staphylococcus* spp.**
k2A5	+	cocci	-	+	-	-	Y/Y	+	+	+	-	-	+	-	-	-	+	+	-	+	***Staphylococcus spp.***
k2A6	+	cocci	-	+	-	-	Y/Y	+	+	+	-	-	+	-	-	-	+	+	-	+	***Staphylococcus spp.***
k2K1	+	cocci	-	+	-	-	Y/Y	+	+	+	-	-	+	-	-	-	+	+	-	+	***Staphylococcus spp.***
k2K3	+	rod	-	-	-	-	R/Y	+	-	-	-	-	+	-	-	-	-	+	-	-	***Bacillus* spp.**
k2K4	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	+	+	-	-	+	-	+	***Vibrio spp.***
k2K6	-	rod	-	-	+	+	Y/Y	+	+	+	-	+	+	+	+	-	-	+	-	-	***Enterobacter spp.***
k2k10	-	rod	-	+	-	+	Y/B	+	+	+	+	+	+	-	+	-	-	+	-	-	***Salmonella spp.***
k2k11	-	rod	-	+	-	+	Y/Y	+	+	+	-	-	+	-	+	-	-	+	-	+	***Vibrio* spp.**
k2C1	-	rod	-	-	+	+	Y/Y	+	+	+	-	+	+	+	+	-	-	+	-	-	***Enterobacter* spp.**
k2C2	-	rod	-	-	+	+	Y/Y	+	+	+	-	+	+	+	+	-	-	+	-	-	***Enterobacter* spp.**
k2C3	-	rod	-	-	+	+	Y/Y	+	+	+	-	+	+	+	+	-	-	+	-	-	***Enterobacter* spp.**
k2C4	-	rod	-	-	+	+	Y/Y	+	+	+	-	+	+	+	+	-	-	+	-	-	***Enterobacter* spp.**
k2C6	+	cocci	-	+	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	-	-	***Staphylococcus* spp.**
k2C7	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	-	+	-	-	+	-	+	***Vibrio* spp.**
k2C9	-	rod	-	-	-	-	Y/Y	+	+	+	-	-	+	+	+	-	-	+	-	+	***Vibrio* spp.**
k2C10	-	rod	-	+	-	+	B/B	+	+	+	+	+	+	-	+	-	-	+	-	-	***Salmonella* spp.**
k2C11	-	rod	-	+	-	+	Y/B	+	+	+	+	+	+	+	+	-	-	+	-	-	***Salmonella* spp.**

Table 3.2: Biochemical characteristics of the bacteria isolated from kitchen utensils of educational institution

Isolates ID	Gram staining		Indole Test	Methyl red Test	VP Test	Citrate test	Triple Sugar Iron Test						Carbohydrate fermentation		MIU Test			Catalase Test	Starch Hydrolysis	Casein hydrolysis	Suspected Organism
	Gram reaction	Shape					Slant/Butt	Glucose	Lactose	Sucrose	H_2S	Gas	Maltose	Gas	Motility	Indole	Urease				
k2S3	-	rod	+	+	-	-	Y/Y	+	+	+	-	+	+	+	+	+	-	+	-	-	***E. coli***
k2S4	-	rod	+	+	-	-	Y/Y	+	+	+	-	+	+	+	+	+	-	+	-	-	***E. coli***
k2S5	-	rod	-	-	+	+	Y/Y	+	+	+	-	+	+	+	-	-	+	+	-	+	***Klebsiella* spp.**
k2S7	-	rod	-	-	+	+	Y/Y	+	+	+	-	+	+	+	+	-	-	+	-	-	***Enterobacter* spp.**
k2S8	-	rod	-	+	-	+	Y/Y	+	+	+	+	-	+	+	+	-	-	+	-	-	***Salmonella* spp.**

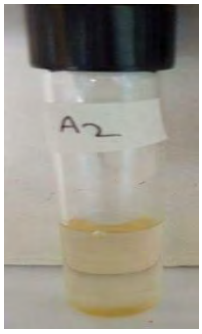
Indole test (positive)

Indole test (negative)



Methyl red test positive (left) and negative (right)

Voges-Proskauer test (positive)



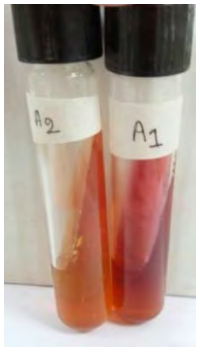
Voges-Proskauer test (negative)

Citrate test (positive)

Citrate test (negative)

Yellow slant, yellow butt (gas produced)

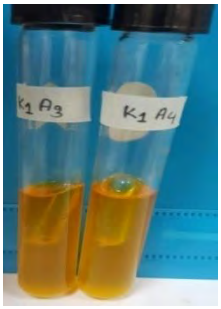
Yellow slant, black butt (gas produced) (left) and black slant, black butt(right)(gas produced)



Red slant, red butt

Figure 3.3: Biochemical test results of bacterial isolates

Maltose fermentation (positive)



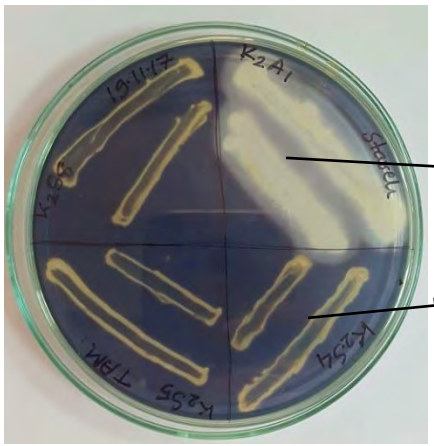
Maltose fermentation (positive) (gas produced)

Maltose fermentation (negative)

MIU test (Urease +ve, Non-motile)

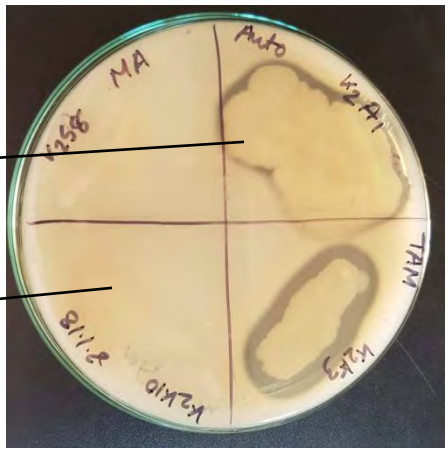
MIU test (Urease -ve, motile)

Catalase test (positive)



Positive

Negative



Starch hydrolysis (positive), clear zone around bacterial growth and (negative), no clear zone around bacterial growth

Casein hydrolysis (positive), clear zone around bacterial growth and (negative), clear zone around bacterial growth

Figure 3.3: Biochemical test results of bacterial isolates

After observing the cultural and morphological characteristics of bacterial isolates and performing the biochemical tests, 43 isolates could have been identified from 8 different samples collected from kitchen utensils of BRAC University. The isolates that have been confirmed include *Staphylococcus* sp., *Enterobacter* spp., *Bacillus* spp., *E.coli*, *Vibrio* spp., *Salmonella* spp., *Klebsiella* spp. and *Shigella* spp. The total number and the percentage of the isolates obtained from the samples are shown in table 3.3 and figure 3.4.

Table 3.3: Prevalence of bacteria species isolated from kitchen utensils

Bacterial isolates	**Number of the isolates**	**Total bacterial isolates**	**% Prevalence**
Staphylococcus spp.	14	43	32.56
Enterobacter spp.	7		16.28
Bacillus spp.	6		13.95
Vibrio spp.	5		11.63
Salmonella spp.	4		9.30
Klebsiella spp.	4		9.30
E.coli	2		4.65
Shigella spp.	1		2.33

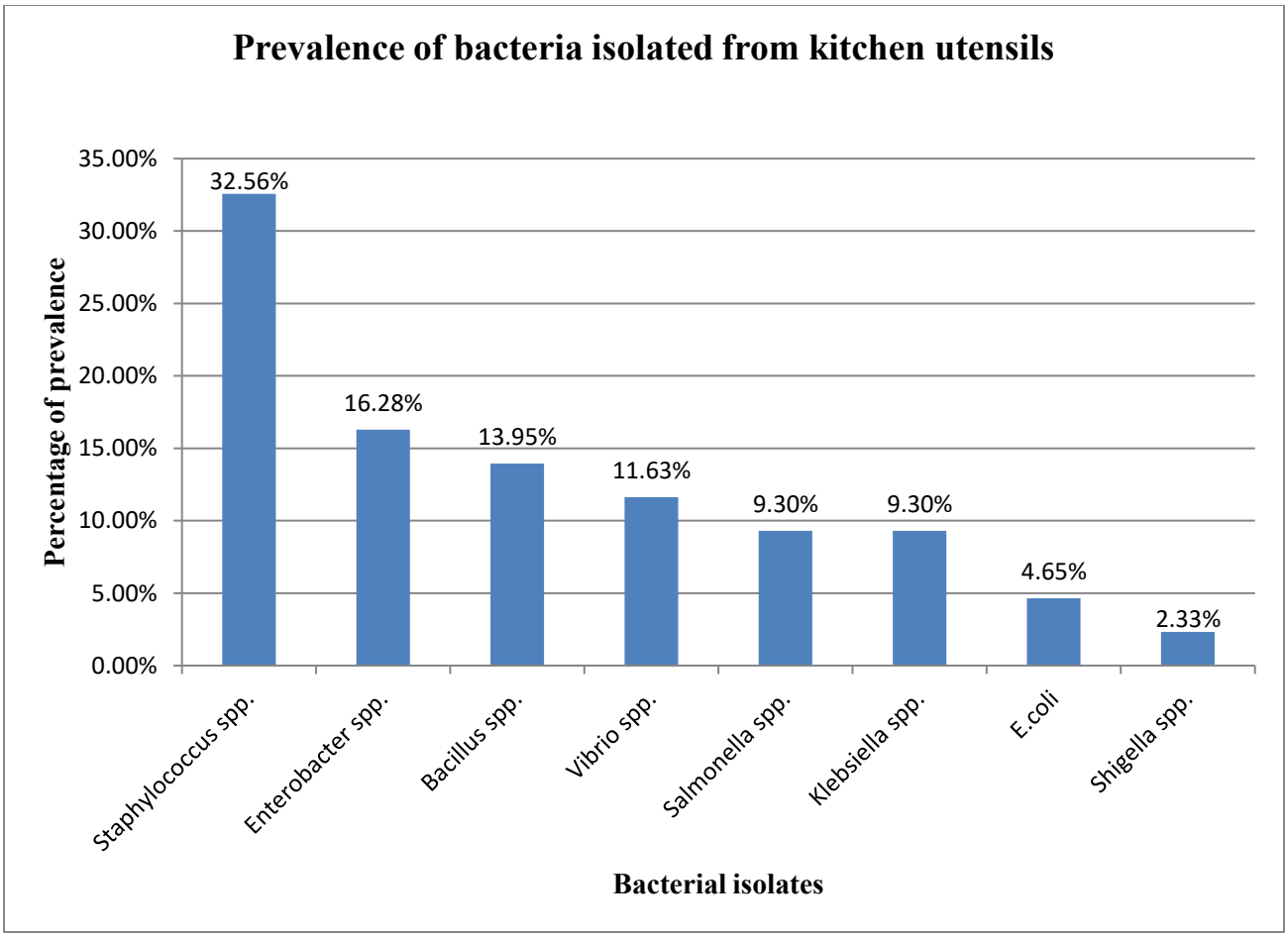


Figure 3.4: Percentage of prevalence of isolated bacteria from kitchen utensils

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, *Shigella* spp, *Vibrio* spp, *Enterobacter* 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 3.4: Distribution of the isolates according to Gram's Reaction

Gram's Reaction	Number of isolates found	Percentage (%)
Gram positive	20 (out of 43)	46.51
Gram negative	23 (out of 43)	53.49

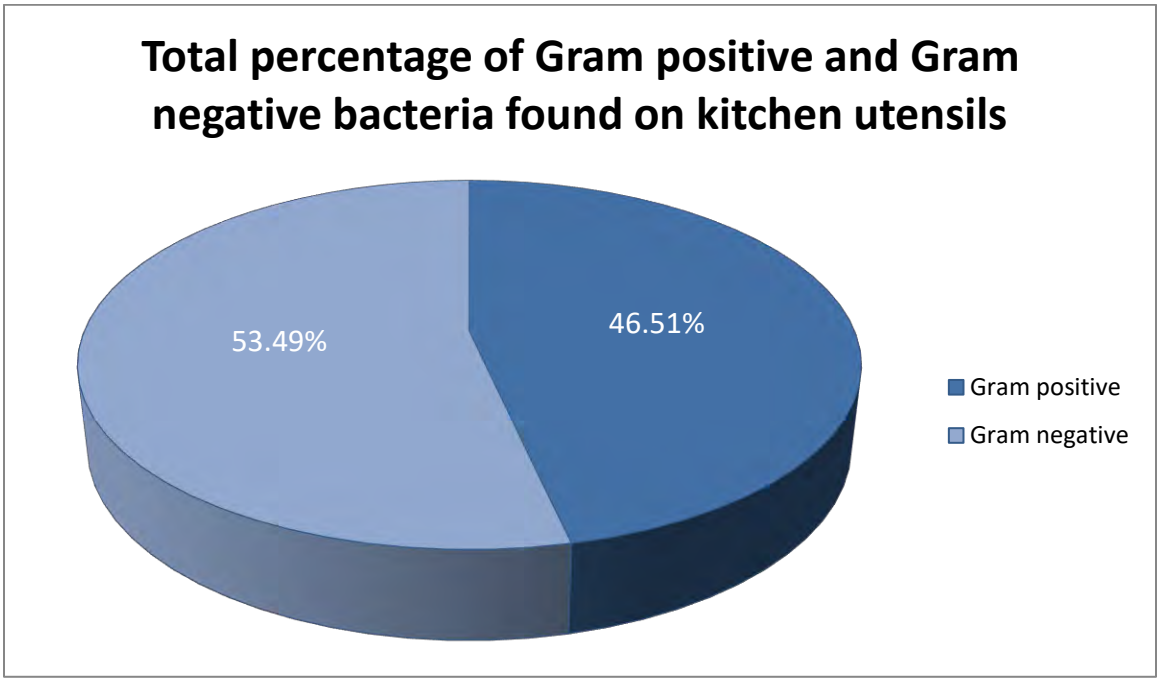


Figure 3.5: Total percentage of Gram positive and Gram negative bacteria identified from kitchen utensils

3.2 Antibiotic susceptibility test:

After identifying and confirming the organisms, the isolates were selected for antibiotic susceptibility test. About nine to ten antibiotics were used for each of the forty three isolates isolated from kitchen utensils of educational institution. The sensitive and resistance pattern of the isolates to these antibiotics were determined. The interpretation of each bacterium either resistant or susceptible to antibiotic is shown in Table 3.5.

Table 3.5: Antibiotic susceptibility pattern of various organisms isolated from kitchen utensils

Isolates Id	Suspected organism	Penicillin	Ciprofloxacin	Chloramphenicol	Amoxicillin	Gentamycin	Rifampicin	Tetracycline	SXT	Cefixime	Cefepime
		INP	INP	INP	INP	INP	INP	INP	INP	INP	INP
k1A1	***Bacillus* spp.**	R	S	R	R	S	R	S	S	R	R
k1A2	***Bacillus* spp.**	R	S	S	R	S	R	S	I	S	R
k1A3	***Klebsiella* spp.**	R	S	S	R	S	R	S	S	R	R
k1A4	***Shigella* spp.**	R	S	S	R	S	S	S	S	I	R
k1A5	***Staphylococcus* spp.**	R	S	S	R	S	R	S	S	I	R
k1A6	***Staphylococcus* spp.**	S	S	S	S	S	S	S	S	R	R
k1K1	***Klebsiella* spp.**	R	S	S	R	S	R	S	R	S	S
k1K3	***Klebsiella* spp.**	R	S	S	R	S	R	I	I	S	R
k1K4	***Vibrio* spp.**	I	S	I	S	R	I	S	S	R	R
k1K5	***Enterobacter* spp.**	I	S	S	R	S	R	R	S	R	R
k1C1	***Bacillus* spp.**	R	S	S	S	S	S	R	I	R	S
k1C2	***Staphylococcus* spp.**	S	S	S	S	S	I	S	S	R	R
k1C3	***Staphylococcus* spp.**	R	S	I	S	S	I	S	S	R	R
k1C4	***Staphylococcus* spp.**	R	S	S	R	S	R	S	I	R	R
k1S1	***Staphylococcus* spp.**	R	S	S	I	S	S	S	R	R	R
k1S2	***Bacillus* spp.**	R	S	I	R	S	S	S	S	R	R
k1S3	***Staphylococcus* spp.**	I	S	S	R	S	R	S	S	I	R
k1S4	***Staphylococcus* spp.**	R	S	R	R	S	S	S	S	R	R
INP= Interpretation, S= Sensitive, I= Intermediate, R=Resistant											

Table 3.5: Antibiotic susceptibility pattern of various organisms isolated from kitchen utensils

Isolates Id	Suspected organism	Penicillin	Ciprofloxacin	Chloramphenicol	Amoxicillin	Gentamycin	Rifampicin	Tetracycline	SXT	Cefixime	Cefepime
		INP	INP	INP	INP	INP	INP	INP	INP	INP	INP
k2A1	***Bacillus* spp.**	R	S	S	R	S	R	S	S	R	S
k2A3	***Staphylococcus* spp.**	S	S	S	S	S	S	S	S	R	R
k2A4	***Staphylococcus* spp.**	R	S	S	S	S	S	S	S	S	S
k2A5	***Staphylococcus* spp.**	R	S	S	S	S	S	S	S	R	S
k2A6	***Staphylococcus* spp.**	R	S	S	S	S	S	S	S	S	S
k2K1	***Staphylococcus* spp.**	S	S	S	S	S	S	S	S	R	S
k2K3	***Bacillus* spp.**	R	R	S	R	S	R	S	R	R	R
k2K4	***Vibrio* spp.**	I	S	S	S	S	S	I	S	R	S
k2K6	***Enterobacter* spp.**	R	I	S	R	S	R	S	S	S	S
k2k10	***Salmonella* spp.**	R	R	I	R	S	R	R	R	R	S
k2k11	***Vibrio* spp.**	R	R	S	R	R	R	S	S	R	R
k2C1	***Enterobacter* spp.**	R	S	S	R	S	R	S	S	S	S
k2C2	***Enterobacter* spp.**	R	S	S	R	S	R	S	S	S	S
k2C3	***Enterobacter* spp.**	R	S	I	R	S	R	S	S	S	S
k2C4	***Enterobacter* spp.**	R	S	S	R	S	R	S	S	S	S
k2C6	***Staphylococcus* spp.**	S	S	S	S	S	S	S	S	R	S
k2C7	***Vibrio* spp.**	R	S	S	S	S	S	S	S	R	S
k2C9	***Vibrio* spp.**	I	S	S	S	S	S	S	S	R	S
k2C10	***Salmonella* spp.**	R	S	S	S	S	R	S	R	R	S
k2C11	***Salmonella* spp.**	R	S	R	R	S	R	R	R	S	R
k2S3	***E. coli***	R	S	S	R	S	R	S	R	R	S
k2S4	***E. coli***	R	S	S	R	S	R	S	R	R	S
k2S5	***Klebsiella* spp.**	R	S	S	R	S	R	S	R	R	S
k2S7	***Enterobacter* spp.**	R	S	S	R	R	R	S	R	R	R
k2S8	***Salmonella* spp.**	R	S	S	R	S	R	I	R	S	S
INP= Interpretation, S= Sensitive, I= Intermediate, R=Resistant											

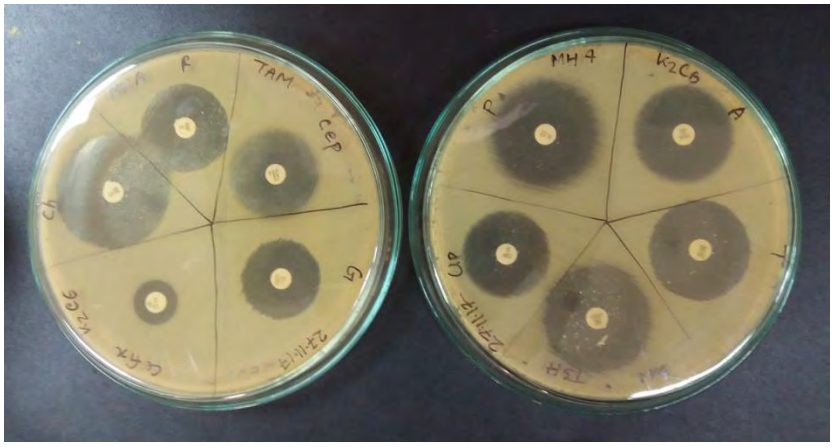


Figure 3.6: Antibiotic susceptibility test of *Staphylococcus* spp.

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 3.7.

Table 3.6: Antibiotic resistance pattern of total 43 bacterial isolates

Antibiotics	**Penicillin**	**Ciprofloxacin**	**Chloramphenicol**	**Amoxicillin**	**Gentamycin**	**Rifampicin**	**Tetracycline**	**SXT**	**Cefixime**	**Cefepime**
No of isolates resistant to tested antibiotics	33	2	3	27	3	26	4	11	28	21
Percentage of isolates resistant to antibiotics	76.74	4.65	6.98	62.79	6.98	60.47	9.30	25.58	65.12	48.84

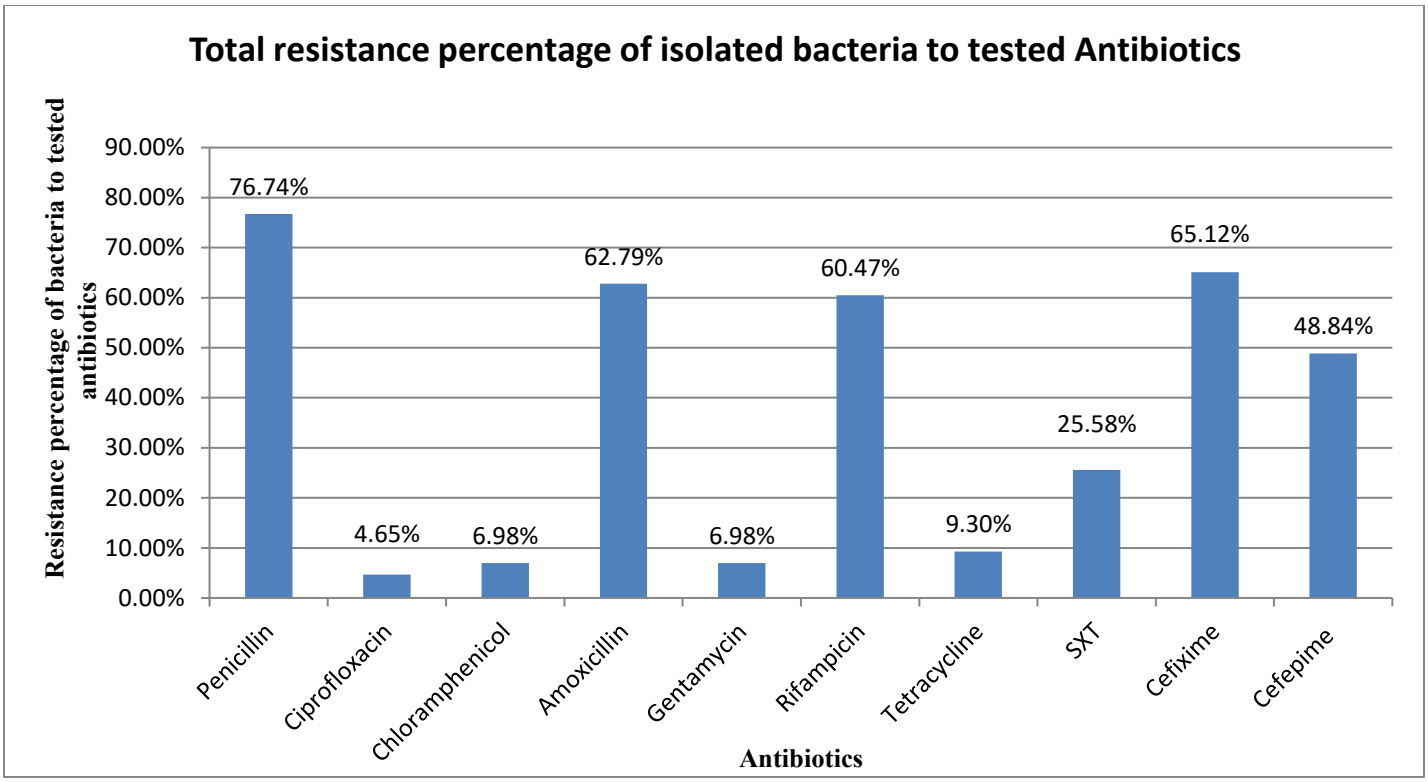


Figure 3.7: Resistance percentage of the isolated bacteria to tested antibiotics

3.2.2: Prevalence of Multiple drug resistant organisms:

After observing the antibiotic resistance pattern of the organisms, it was found that all organisms were resistant to two or more antibiotics. Their total number and percentage are given below in Table 3.7 and Figure 3.8.

Table 3.7: Total number and percentage of the isolates resistant to more than two antibiotics and the isolates resistant to two antibiotics

Total bacterial isolates	Number Of isolates Resistant to more than two antibiotics	Percentage of isolates Resistant to more than two antibiotics	Number of Isolates Resistant to two antibiotics	Percentage Of isolates Resistant to two antibiotics
43	32	74.42	5	11.63

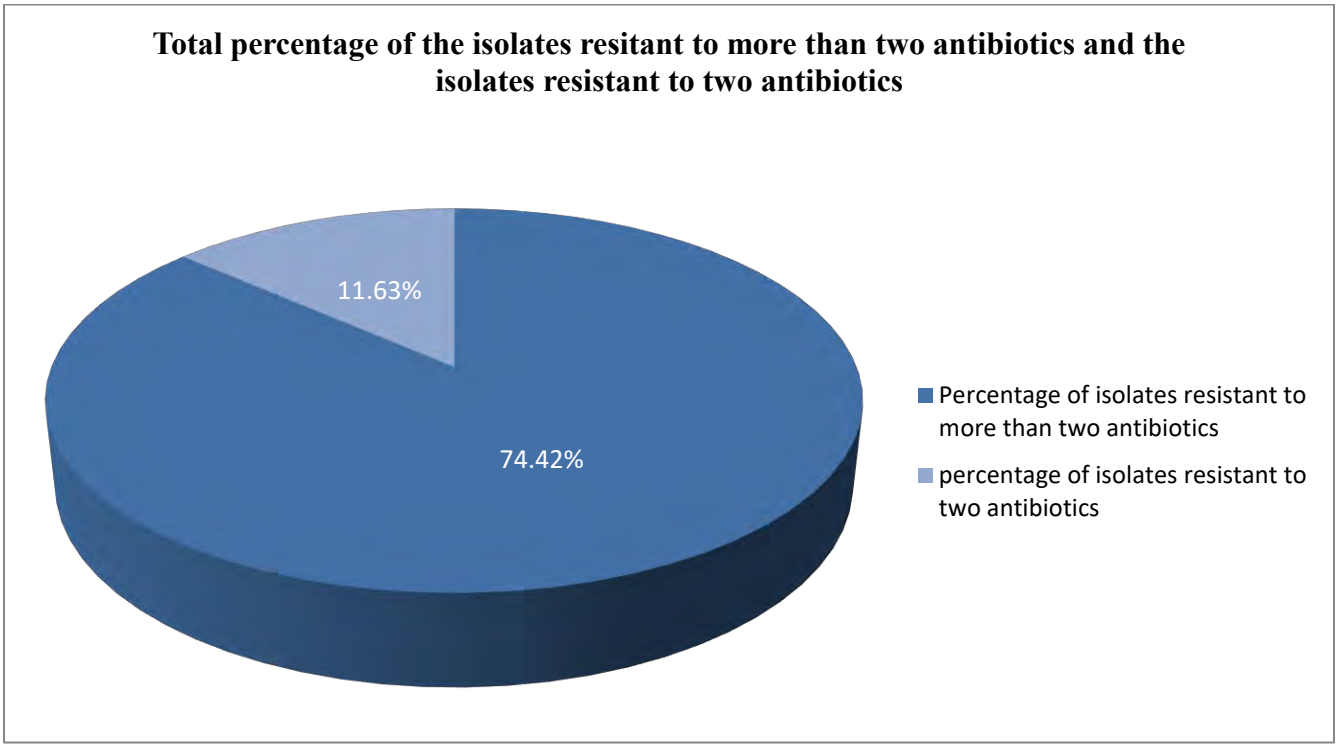


Figure 3.8: Total percentage of the isolates resistant to more than two antibiotics and the isolates resistant to two antibiotics.

3.3 Minimum Inhibitory Concentration (MIC) Test Result:

Minimum Inhibitory Concentration of commercially used dishwasher was determined by broth micro dilution method (Lee and Mary, 2013) against six selected organisms and their MIC value was obtained.

Table 3.8: Minimum Inhibitory Concentration of bacterial isolates:

Isolates ID	**MIC value**
k2K3 (*Bacillus* spp.)	250 µl of stock solution
k2C7 (*Vibrio* spp.)	190 µl of stock solution
k2K10 (*Salmonella* spp.)	400 µl of stock solution
k2S4 (*E. coli*)	200 µl of stock solution
k1C3 (*Staphylococcus* spp.)	250 µl of stock solution
k1A4 (*Shigella* spp.)	250 µl of stock solution

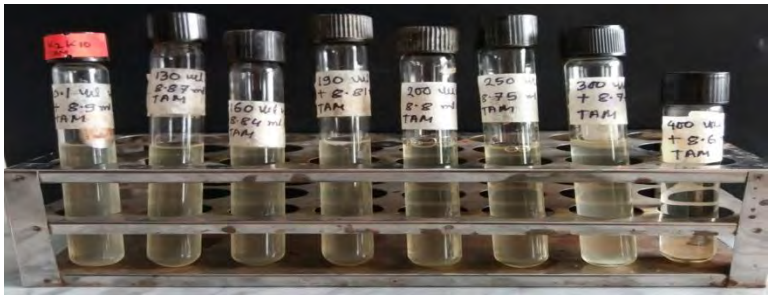


Figure 3.9: MIC test of *Salmonella* spp.

Graph of MIC Value

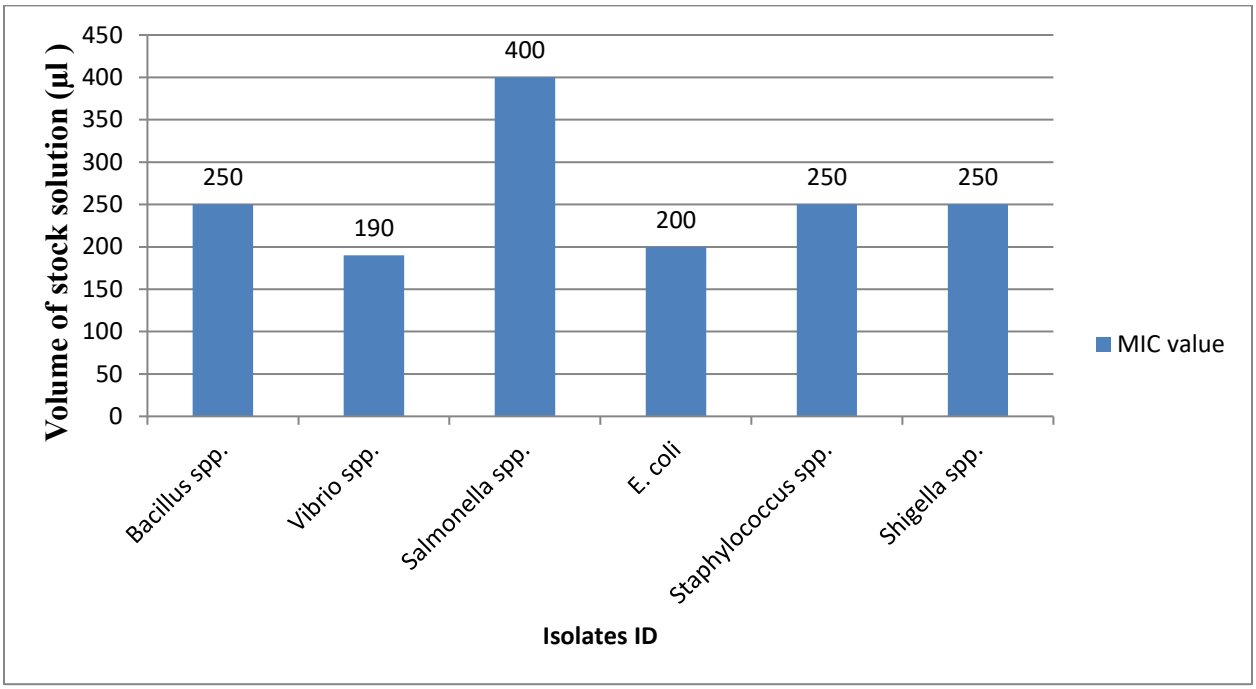


Figure 3.10: Graph of MIC Value of bacterial isolates from kitchen utensils and air

Chapter 4: Discussion and Conclusion

Discussion

It is now accepted that the prevalence of foodborne illnesses originating from home kitchens could not be neglected (Biranjia-Hurdoyal and Latouche, 2016). Items in the kitchen become contaminated by contact with contaminated people, foods, pets or other contaminated sources. In terms of food poisoning occur at home, with the kitchen being the area with the most contamination (Rusin et al., 1998). Foods that prepared from raw meat, poultry, fruits and vegetables can serve as vehicles of transmission for numerous infectious diseases caused by microorganisms. Some of these pathogenic microorganisms include *Salmonella*, *Escherichia coli* and *Campylobacter*. These pathogenic microorganisms have also been known to contribute to food poisoning, specifically gastroenteritis. (Hernandez, 2014)

In this research, a total of 43 isolates had been identified from 8 different samples collected from kitchen utensils (knife, spoon and cutting board) and air of BRAC University. 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 43 bacterial isolates, *Staphylococcus* spp. showed the highest prevalence 14 (32.56%), followed by *Enterobacter* spp. 7 (16.28%), *Bacillus* spp. 6 (13.95%), *Vibrio* spp. 5 (11.63%), *Salmonella* spp. 4 (9.30%), *Klebsiella* spp. 4 (9.30%), *E.coli* 2 (4.65%) and *Shigella* spp. 1 (2.33%).

Some studies have reported on the transfer of bacteria from surfaces to food while other studies can be found on transfer of bacteria from food to other surfaces (Purohit, 2009). Biranjia-Hurdoyal and Latouche (2016) studied the bacterial load and food spoilage bacteria from kitchen tablesincluding preparation tables and dining tables. Coliforms, *Enterococcus* spp., *Pseudomonas* spp., *Proteus* spp., and *S. aureus* were detected from both dining and preparation tables. Another study was conducted by Wolde and Bacha (2016) to assess the microbiological safety of sponges as it has been used in selected food establishments of Jimma town. The isolated genera were dominated by *Pseudomonas*, *Bacillus*, *Micrococcus*, *Streptococcus* and *Lactobacillus*.The term cross contamination is used to describe the transfer of pathogens from a contaminated food or surface (usually raw items such as meat, poultry and vegetables) to other foods whether it occurs directly or indirectly. Direct contamination describes when a contaminated source touches food while indirect contamination

occurs when transfer requires an intermediate surface. Indirect contaminations would also occur when raw meat juices are left on a knife which is later be used for slicing ham (Scott and Bloomfield 1990; Zhao et al.1998; Chen et al. 2001; Montville et al. 2001).Bacterial transfer from stainless steel to cucumber were reported by Kusumaningrum et al., (2003) and Chen et al., (2001) while Moore et al., (2003) studied bacterial transfer to lettuce from cutting board and stainless steel.

Recently there has been an increase in public awareness regarding antibiotic resistant microorganisms. As a result, antibacterial dishwashing detergents are prevalent in many households. Many of these antibacterial products contain chemicals such as triclosan, tricolocarban, pine oils, or quarternary ammonium compounds. When used, the antibacterial chemicals leave residues that may affect the microenvironment of the application site; the remaining microorganisms that are exposed to these residues acquire resistance to the antibacterial chemical, which may also induce cross resistance to antibiotics (Wegener, 2012).

In this study, antibiotic susceptibility pattern of bacteria isolated from kitchen utensils was determined against 10 antibiotics. 76.74% isolates showed resistance against penicillin. The percentage of resistance to other antibiotics was ciprofloxacin (4.65%),chloramphenicol (6.98%), amoxicillin (62.79%), gentamycin (6.98%), rifampicin (60.47%), tetracycline (9.30%), SXT (25.58%), cefixime (65.12%), cefepime (48.84%). Out of 43 bacterial isolates, 74.42% (32 out of 43) showed resistant to more than two antibiotics. Results showed that, most of the isolates showed resistance against penicillin and ciprofloxacin resistance is less frequent.

Wolde and Bacha, (2015) investigated the prevalence and antibiotic resistance patterns of *Staphylococcus aureus* isolated from kitchen sponges used in food establishments of Jimma town. Ampicillin and Streptomycin were the most resisted drugs. Norfloxacin, Amikacin and Ciprofloxacin showed maximum sensitivity. 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.

Many antibacterial products are specially manufactured for the reduction of bacteria. These products include bleach solutions, detergents and dishwashing liquids. In this study, the effects of a dishwashing liquid without antibacterial product on six selected organismswere investigated and their MIC value was obtained by broth dilution method. In results, *Salmonella* spp. showed the highest MIC value (400 µl of stock solution) and *Vibrio* spp. showed the lowest MIC value (190 µl of stock solution). Besides, *Bacillus* spp., *Staphylococcus* spp., *Shigella* spp. and *E. coli* had shown the values 250 µl, 250 µl, 250 µl and 200 µl of stock solution respectively.

Kusumaningrum et al. (2002) studied the effect of an antibacterial dishwashing liquid on *E. coli, S. enteritidis, S. aureus* and *B. cereus* in a modified suspension test and in used sponges with and without food residues under laboratory conditions. In the suspension tests *S. aureus* and *B. cereus* were susceptible to low concentrations of antibacterial dishwashing liquid (0.5%), whereas *E. coli* and *S. enteritidis* maintained their initial numbers for at least 24 h at 25º C. With higher concentrations (2-4%), all test organisms decreased below the detection limit after 24 h. Over a 24 h period, the antibacterial dishwashing liquid did not significantly reduce these organisms in used sponges in which food residues were present. The antibacterial product did not reduce the competitive microorganisms either. Similar results were found in sponges involved in daily household use. The presence of food residues strongly reduces the product efficacy. This indicates that to determine the efficacy of an antibacterial product and other similar products, treatment under use conditions must be included.

Conclusion

The present study indicated that kitchen utensils of educational institution can be very important sources of potential pathogens which cause various foodborne illnesses.The presence of *Staphylococcus* spp., *Enterobacter* spp., *Bacillus* spp., *Vibrio* spp., *Salmonella* spp., *Klebsiella* spp., *E.coli* and *Shigella* spp. indicates poor personal hygiene. Besides this, multidrug resistant bacteria that can be easily spread during food preparation, via cutting boards, knife etc. are a serious threat to human health. The characterization of kitchen associated microbial communities, in combination with future experimental studies to show specific treatment affect on survivability, growth and transmission of bacteria on particular kitchen surfaces, with improve our understanding of the microbial ecology of kitchen.

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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

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

Blood Agar Base

Component	Amount (g/L)
Beef heart infusion from (beef extract)	500.0
Tryptose	10.0
Sodium chloride	5.0
Agar	15.0
Final pH	6.8 ± 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

Bacillus cereus Agar (BC Agar):

Component	Amount (g/L)
Peptic digest of animal tissue	1.0
Mannitol	10.0
Sodium chloride	2.0
Magnesium sulphate	0.1
Disodium phosphate	2.5
Monopotassium phosphate	0.25
Sodium pyruvate	10.0
Bromo thymol blue	0.12
Agar	15.0
Final pH	7.12± 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

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

Sabouraud Dextrose Agar

Component	Amount (g/L)
Dextrose	40.0
Mycological, peptone	10.0
Agar	15.0
Final pH	5.6 ± 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