Determination of prevalence and antibiotic susceptibility pattern of bacteria isolated from household and restaurant kitchen utensils 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 household and restaurant kitchen utensils of Dhaka, Bangladesh"** has been written and submitted by me, Tashfia Anwar and has been carried out under the supervision of Namista Islam, 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. All explanations that have been adopted literally or analogously are marked as such.

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TO

MY

BELOVED FATHER, MOTHER, and SISTER

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Abstract

The kitchen is one of the most crucial areas that harbors and transmits infection. This study aimed at isolating, identifying, determining antibiotic susceptibility pattern and investigating antimicrobial activity of dishwashing liquid against the bacterial isolates collected from household and restaurant kitchen utensils (air, knife, spoon and cutting board) of Dhaka, Bangladesh. Sixteen (16) samples collected through sterile swabs were kept for enrichment into nutrient broth at 37ºC for 24 hours and were 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 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 61 bacterial isolates were identified where *Bacillus* spp. showed the highest prevalence 18 (29.51%) followed by *Vibrio* spp. 17 (27.87%), *Staphylococcus* spp. 13 (21.31%), *Klebsiella* spp. 6 (9.84%), *Shigella* spp. 4 (6.56%), *Salmonella* spp. 2 (3.28%), and *E.coli* 1 (1.63%). Antibiotic susceptibility pattern of the bacterial isolates showed that almost all of the isolates were resistant to at least two antibiotics. Among the 61 isolates, 72.13% were found resistant to more than two antibiotics and 27.87% were found resistant to two antibiotics. Highest resistance percentage of the isolates were observed to penicillin (83.61%) followed by cefixime (60.65%), amoxicillin and SXT (50.82%), rifampicin (49.18%), cefepime (40.98%), chloramphenicol (18.03%), tetracycline (14.71%), ciprofloxacin (6.56%) and gentamycin (1.64%). Broth Microdilution assay revealed that *Shigella* spp. showed the highest MIC value (400µl of stock solution) and *Staphylococcus* spp. showed the lowest MIC value (190µl of stock solution). Besides, *Escherichia coli*, *Salmonella* spp., *Vibrio* spp. and *Bacillus* spp. showed the MIC values 250µl, 300µl, 300µl and 250µl of stock solution respectively. These results indicate that, household and restaurant kitchens can easily be contaminated with a variety of bacterial contaminants. Findings of this study also indicated the presence of multi-drug resistant bacterial strains in kitchen utensils which can serve as potential source of food-borne diseases. Regular cleaning of kitchen utensils, use of dishwashing liquid and public awareness on personal hygiene can help minimize the spread of food-borne diseases from kitchen utensils.

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Abbreviation	***Elaboration***
MSA	Mannitol Salt Aagar
MR	Methyl Red
VP	Voges-proskauer
TSI	Triple Sugar Iron
MDR	Multi-drug resistant
MIC	Minimum inhibitory concentration
MIU	Motility Indole Urease
µL	Microliter
ml	Milliliter
spp.	Species
WHO	World Health Organization
USA	United States of America
FDA	Food and Drug Administration

Chapter 1

Introduction

1.1 Introduction:

Microbial populations in indoor environments, where we live and eat, are important for public health. Various bacterial species reside in the kitchen, and refrigerators, the major means of food storage within kitchens, can be a direct source of food borne illness (Jeon, Chun and Kim, 2013). Growth of undesirable contaminating bacteria not only causes deterioration in the sensory and organoleptic properties of food but can also cause illnesses (Othman, 2016). Presumably, food-borne diseases sometimes acquired in hotels and restaurants are through dishes, plates and other kitchen equipments (Fawole and Oso, 1988). The reputation of many hotels often rest on the quality of dishes, spoons, drinking cup and cutlery (Cracknel and Nobis, 1989). Vanderzant and Splittstoesser (1992) mentioned in their study that contamination of food by specific types or species of microorganisms is due to poor sanitation during the handling and processing of the food.

In market there are many types of products that are specifically manufactured for the reduction of bacterial contaminants in cleaning kitchen utensils. These products include bleach solutions, detergents, and dishwashing liquids. Limited studies have addressed the effectiveness of these products in inactivating microorganisms on kitchen utensils.

Apart from isolating and identifying different bacterial contaminants from kitchen utensils, this study also assessed the prevalence of those bacterial contaminants. 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. Besides, in this study, the effects of a particular dishwashing liquid on food borne pathogens were investigated in broth micro-dilution method.

1.2 Factors affecting bacterial transfer:

The factors involved in bacterial transfer between environmental surfaces include:

- The relative humidity or moisture levels
- Bacterial species involved
- The temperature
- The surface materials and properties
- Pressure and friction between the contact surfaces

- Inoculums size on surfaces

1.3 Cross contamination of bacteria from kitchen utensils to food:

Cross contamination is the transfer of bacteria from raw food, unclean utensils or unclean surfaces to ready to eat food, clean utensils or clean surfaces. Microbiological cross contamination from improper personal hygiene is a significant factor in food borne illness incidence. Factors that cause food borne illness events include an agent (microorganisms), source (surface), mode of transmission (contact) and host (human). Pathogenic microorganisms can contaminate food from food prepared by an infected person (person to person spread), through the air, or by insects, pests, rodent or pets. Cross contamination during preparation of raw chicken has a major contribution towards outbreaks of salmonellosis and infections related to *Campylobacter* and *Staphylococcus aureus.* Improper handling and sanitation practices lead to person-to-person, person to food and utensils to food cross contamination that ultimately results into 27% of reported outbreaks and infection from food borne pathogens (WHO, 2001). After preparation of artificially contaminated chicken, target organisms were found on utensils and surfaces in contact with the contaminate food (Wit *et al.,* 1979). Surfaces play very important role in the transmission of bacteria from hand or cloth to other surfaces (Scott and Bloomfield, 1990). Campylobacter and Salmonella were recovered from hands and contact surfaces following food preparation of meat and chicken (Cogan *et al.,* 1999).

In the home, a major concern is the transmission of food borne pathogens by cross contamination of food via food contact surfaces. These surfaces include cutting boards which play an important role in food cross contamination and are considered to be one of the top five sites most contaminated with heterotrophic bacteria in the kitchen (Messina, 2008). Large numbers of bacteria can be transferred to cutting boards after use with raw chicken. These bacteria can survive for more than four hours on cutting boards and can be transferred to other foods if the cutting board is not properly cleaned (Zhao *et al.,* 1998).

1.4 Humans as a source of food cross contamination:

Humans are a major source of food cross contamination. Hands, breath, hair and perspiration contaminate food. Coughing and sneezing are also responsible for the transmission of bacteria. Illnesses such as respiratory tract infections, the common cold, fever, sore throat, intestinal disorders also occur due to cross contamination. The mouth plays an important role in the

transfer of bacteria. During sneezing, bacteria are transferred to the air and may land on hands or on food. Spitting may also be a mode of bacterial transmission and product contamination. In some cases the disease causing microorganisms can remain with the person after recovery. A person with this condition is known as carrier. Fingers can transfer bacteria through touching equipment, contaminated food, clothing or other areas of the body. Handling of food (person to person spread) by a colonized person is a frequently identified factor that contributing to typhoid fever, shigellosis and staphylococcal food poisoning. Twenty percent of reported salmonellosis and 22% of *Vibrio parahaemolyticus* gastroenteritis are due to cross contamination (WHO, 2001).

1.5 Dishwashing liquid:

Dishwashing liquid is also known as dishwashing soap, dish detergent and dish soap. It is a detergent that is used to assist in dishwashing. It is usually a highly-foaming mixture of surfactants with low skin irritation, and is primarily used for hand washing of glasses, plates, cutlery, and other kitchen utensils. Any dishwashing liquid may contain bleach, enzymes, or rinsing aids. The main ingredient of dishwashing liquid is water, other main active ingredients are detergents. There are also thickening and stabilizing agents present in any dishwashing liquid. Other ingredients may include surfactants, hydrotrope, salts, preservatives, fragrances, and dyes. (Zoller, 2008).

Some dishwashing products contain phosphates in detergent. Phosphate helps to make dishes cleaner but can also cause harmful algal bloom as the wastewater goes back to the natural environment. That is why it is banned as a component of dishwashing detergent in many places. In 2010, the United States FDA raised health concerns over triclosan, an antibacterial substance used in some dish liquids (Layton and Lyndsey, 2010). Elsewhere, triclosan has been found to create problems at wastewater treatment plants, whereby it can "sabotage some sludge-processing microbes and promote drug resistance in others" as per reported by The Inquisitr News in 2015. The United States FDA has found that triclosan provides no health benefits over soap and water (Boone and Lisa, 2014). As of 2014, some of the states within the United States have banned triclosan in dishwashing liquids.

1.6 Effect of dishwashing liquid on bacterial contaminants:

A kitchen can look perfectly clean, yet be contaminated with a lot of organisms that cause diseases. Cleaning and disinfecting are two different processes. Cleaning removes grease, food residues, and dirt, as well as a large number of bacteria. But cleaning may also spread other bacteria around. Disinfecting kills organisms (bacteria, virus, and parasites). Generally in market two types of dishwashing liquids are available: Antibacterial and non-antibacterial. Antibacterial soaps and cleansers have become the dominant products in their category. Today, more than three-quarters of soaps contain an antibacterial ingredient. Nielsen (2002) did a study on the use of liquid dishwashing compounds to control bacteria in kitchen sponges. Nielsen found that some antibacterial soaps and sponges can be very effective, while others are not as effective. The experiment demonstrated that not all the products are 100% effective at reducing bacterial growth. Bacteria such as *E. coil, S. aureus* and *Salmonella* survive on hands, sponges/cloths, utensils, and currency for hours or days after initial contact with microorganisms (Erdogrul and Erbilir, 2003). Erdogurl and Erbilir tested the microorganisms in kitchen sponges by evaluating the growth conditions of bacteria, liquid held in sponges that cause bacteria, and the effect that dishwashing liquid has when using sponges. The results indicated the use of dishwashing liquid daily had no effect on fungal growth, *Pseudomonas* growth or *E. coli* growth, but the number of *Salmonella* decreased. Burr (2006) interpreted his research on the effectiveness of antibacterial dish detergent and non-antibacterial dish detergent on kitchen sponge that not all antibacterial products prevent all bacterial growth and that some may cause more bacteria to grow depending on its application.

1.7 Rate of food-borne illness:

Food borne illness is the ultimate outcome of failure in the management of good food safety practices. Each year a remarkable number of people are suffered from food borne illnesses. Food borne illness is not only due to the presence of pathogenic microorganisms but also the presence of toxic and hazardous chemical present in the food. Lack of personal hygiene and sanitation is mainly responsible for food borne illnesses caused by the contamination of microorganisms (Masud, 2017). About one in every 10 people around the world is sickened by foodborne disease each year. Of those 600 million people, 420,000 die as a result. Diarrheal diseases were responsible for most of the global burden, causing 550 million illnesses and 230,000 deaths,

WHO reported. And children younger than 5 years old carried 40 percent of the food borne disease burden, despite representing only 9 percent of the global population (Zuraw, 2015). These numbers are the first global estimates of food borne illnesses and were calculated by the World Health Organization (WHO).

1.8 List of common food-borne pathogens:

The bacterium that specifically causes disease and food-borne illnesses are called 'pathogens.' There are over two hundred different types of bacteria, parasites and viruses that can cause food borne diseases. However it is estimated that the vast majority of food poisoning (over 90 per cent) is caused by just a handful of bacteria which is listed below:

- *Staphylococcus aureus*
- Salmonella
- *Clostridium perfringens*
- Campylobacter
- *Listeria monocytogenes*
- *Vibrio parahaemolyticus*
- *Bacillus cereus*
- Entero-pathogenic *Escherichia coli*.

The onset of foodborne disease is generally acute, with resolution of an uncomplicated illness in 72 hours for most episodes. Proper food handling and preparation, personal hygiene, and improved methods of decontamination of consumer products could significantly reduce the extent of morbidity and mortality of this common problem (Taege, 2010).

1.9 Antibiotic resistance in bacteria:

Antibiotics are type of antimicrobial drugs which are used in the treatment and prevention of bacterial infections caused by bacterial pathogens. Antibiotic resistance is one of the biggest threats to global health, food security, and development today. The WHO list is divided into three categories according to the urgency of need for new antibiotics: critical, high and medium priority. (WHO, 2017)

Critical group:

Organisms	**Resistant to antibiotics**
Enterobacteriaceae,	carbapenem-resistant, cephalosporin-resistant
Pseudomonas aeruginosa,	carbapenem-resistant
Acinetobacter baumannii,	carbapenem-resistant, ESBL-producing

High group:

Organisms	**Resistant to antibiotics**
Enterococcus faecium,	vancomycin-resistant
Staphylococcus aureus	methicillin-resistant, vancomycin-intermediate and resistant
Salmonellae,	fluoroquinolone-resistant
Helicobacter pylori,	clarithromycin-resistant
Campylobacter spp.	fluoroquinolone-resistant
Neisseria gonorrhoeae	cephalosporin-resistant, fluoroquinolone-resistant

Medium group:

Organisms	**Resistant to antibiotics**
Streptococcus pneumoniae,	penicillin-non-susceptible
Shigella spp.	fluoroquinolone-resistant
Haemophilus influenzae,	ampicillin-resistant

The high prevalence of multidrug resistant bacteria encoding various multidrug resistance genes has now become a major threat to public health. Without effective antimicrobials, medical procedures such as organ transplantation, cancer chemotherapy, diabetes management and major surgery (for example, caesarean sections or hip replacements) become very high risk. Globally, 480,000 people develop multi-drug resistance each year, and drug resistance is starting to complicate the fight against HIV and malaria, as well (Tanwar *et al.,* 2014). An influential report from the O'Neill Commission predicts that antibiotic resistance

will lead to 10 million deaths per year by 2050, surpassing cancer as a source of human mortality.

1.10 Factors associated in developing multi-drug resistance (MDR):

Antibiotic resistance is one of the biggest threats to global health, food security, and development today. Multidrug resistance refers to antimicrobial resistance shown by the organisms to multiple antimicrobial drugs usually at least two or more than two antibiotics. Although, the development of MDR is a natural phenomenon, following factors play key role in developing multidrug resistance (Tanwar *et al.,* 2014).

- Inappropriate use of antimicrobial drugs
- Inadequate sanitary conditions
- Inappropriate food-handling
- Poor infection prevention and control practices contribute to emergence of and encourage the further spread of MDR.

Besides, long term exposure of microorganisms to high concentration of antibiotics also giving rise to the multi-drug resistant organisms (Li *et al.,* 2002). Researchers have observed that there has been a "sigmoidal rise in resistance over time in the presence of a constant rate of antibiotic consumption" and a threshold level of antibiotic usage needed to "trigger the emergence of resistance to significant levels (Austin *et al.,* 1999).

Common multidrug-resistant organisms include:

- Vancomycin-Resistant Enterococci (VRE)
- Methicillin-Resistant *Staphylococcus aureus* (MRSA)
- Extended-spectrum β-lactamase (ESBLs) producing Gram-negative bacteria
- *Klebsiella pneumoniae* carbapenemase (KPC) producing Gram-negatives
- MultiDrug-Resistant Gram negative rods (MDR GNR) MDRGN bacteria such as *Enterobacter species*, *E.coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa.*

1.11 Food hygiene program:

Diseases that people get from eating contaminated food are an important cause of illness, disability and deaths around the world, as revealed by the first ever WHO Estimates of the Global Burden of Foodborne diseases published in December 2015. Foodborne diseases-especially those caused by bacteria, viruses, parasites and fungi-are preventable, and education in safe food handling is a key measure for prevention, including to contain antimicrobial resistance.

WHO built the Five Keys to Safer Food Programme to assist Member States in promoting safe food handling behaviors and educate all food handlers, including consumers, with tools easy to adopt and adapt. The Five Keys to Safer Food explain the basic principles that each individual should know all over the world to prevent foodborne diseases. Over 100 countries have reported using the Five Keys to Safer Food. As a result, billions of food handlers, including consumers, are empowered to prevent foodborne diseases, make safe and informed choices and have a voice to push for a safer food supply. The core messages of the Five Keys to Safer Food are:

- keep clean;
- separate raw and cooked;
- cook thoroughly;
- keep food at safe temperatures; and
- use safe water and raw materials.

The poster has been translated into more than 87 languages and is being used to spread WHO's food hygiene message throughout the world.

1.12 Literature review:

Previous studies on bacteriological profile and antibiotic susceptibility pattern of the bacteria isolated from kitchen utensils and various environmental surfaces are given below:

Maori and Nandita conducted a research in 2009 to determine bacteriological quality of crockery and cutlery of seven restaurants in Federal University of Technology, Yola (FUTY) kiosk. Samples (147) were collected and cultured in appropriate media and the bacterial isolates were

identified according to their morphological and biochemical characteristics. The result revealed a profile of seven (7) different bacterial species including *Staphylococcus aureus*, *Proteus vulgaris*, *Salmonella typhi*, *Escherichia coli* and the species of *Shigella, Klebseilla* and *Bacillus*. The total values of bacterial count (TBC per ml) of thesamples were in the range of 1.1×10^4 - 3.0×10^5 for cups, 2.2×10^4 - 1.6×10^5 for forks, 1.0×10^4 - 3.3×10^5 for knives, 1.2×10^4 – $2.5 \times 10^5$for plates and 1.5×10^4 - 4.7×10^5 for spoons cfu/ml.

Kitchen sponges are among the possible sources of contaminants in food establishments. Wolde and Bacha in 2016 carried out a research on kitchen sponges. The main purpose of the current study was to assess the microbiological safety of sponges as it has been used in selected food establishments of Jimma town. Accordingly, the microbiological safety of a total of 201 kitchen sponges randomly collected from food establishments was 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.

Josephson, Rubino and Pepper conducted a research in 1997. This two year study evaluated the prevalence of indicator bacteria and specific pathogens in 10 'normal' kitchens in the United States. In Phase I, none of the kitchens was cleaned with an antimicrobial cleaner or disinfectant. Eight locations within the kitchens were monitored for: total heterotrophs, *staphylococci*, *Pseudomonas*, total coliforms and faecal coliforms. Almost all locations at all households exhibited contamination, with the sink and sponge samples exhibiting large bacterial concentrations. The faecal coliform concentrations in sink and sponge samples were very high, with 63 and 67% of all samples being positive, respectively. *Escherichia coli* was detected in 16.7% of all sink surfaces and 33.3% of all sponges. *Salmonella* was detected once and *Campylobacter*, on two occasions. In a second phase, households were provided with an antimicrobial disinfectant cleaner which families were encouraged to use but not forced to do so; in some cases, the product was used infrequently or not at all. This regimen did not demonstrate

any consistent reduction in the incidence of bacterial contamination. By contrast, in the final phase of the study where disinfectant use was targeted for surfaces soon after contamination with foods or hands, the incidence of contamination decreased dramatically. These data show that normal kitchens can easily be contaminated with a variety of bacterial contaminants including faecal coliforms, *E. coli*, *Salmonella* and *Campylobacter.* Irregular use, or not using antimicrobial agents, is unlikely to reduce the risk of these infectious agents. By contrast, targeted use is likely to reduce the incidence of bacterial contaminants.

In response to increasing concern about home hygiene, the use of antibacterial products to reduce microorganisms in kitchen sponges and cleaning cloths is strongly promoted by some producers of detergent for domestic use. Kusumaningrum, Putten, Rombouts and Beumer carried out a study on Effects of Antibacterial Dishwashing Liquid on Foodborne Pathogens and Competitive Microorganisms in Kitchen Sponges in 2001. The effects of an antibacterial dishwashing liquid on *Escherichia coli*, *Salmonella enteritidis*, *Staphylococcus aureus*, and *Bacillus cereus* were investigated in a modified suspension test and in used sponges with and without food residues under laboratory conditions. A limited study was conducted in households to assess the efficacy of antibacterial dishwashing liquid as used by the consumer. In the suspension tests, *S. aureus* and *B. cereus* were shown to be susceptible to low concentrations of antibacterial dishwashing liquid (0.5%), whereas *E. coli* and *Salmonella enteritidis* maintained their initial numbers for at least 24 h at 25ºC. At higher concentrations (2 to 4%), all test organisms decreased to 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 for sponges involved in daily household use. The results of this study demonstrate that the antibacterial dishwashing liquid was effective in reducing pathogens in the suspension test but not in the used sponges. This finding indicates that to determine the efficacy of antibacterial products, their use in a household setting must be considered.

Othman carried out a study in Egypt (2016), in that study a total of 90 samples were collected; 85 samples were taken from different sites from five home kitchens and five samples were collected from different types of food. Samples were obtained (before and after disinfection) from kitchen towels, cooking gas stove knobs, refrigerator handles, water taps, and kitchen

sponges used for washing utensils by using sterile cotton swabs. Bacteria were identified according to the conventional biochemical methods. DNA fragmentation was done to show the effect of disinfectants on the most common bacteria. *Escherichia coli*, *Klebsiella* spp., and *Staphylococcus aureus* were the most abundant bacteria in the isolates. After disinfection using disinfectants containing sodium perborate and sodium silicate (detergent), sodium hypochlorite (Clorox), 5% amphoteric surfactant and chlorine (dishwashing powder), and Dettol, the samples were free of bacterial contamination. There was also a correlation between food contamination and bacteria isolated from the kitchens. As *E. coli* was the most highly abundant pathogen in the kitchen and was removed by the tested disinfectants, it was chosen for DNA fragmentation assay to examine the effect of the disinfectants on the bacterial DNA.

The common occurrence of enteric bacteria in kitchen sponges and dishcloths suggests that they can play a role in the cross-contamination of foods, fomites and hands by food borne pathogens. Gerba *et al*., (2014) conducted a study on kitchen hand towels. This study 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 and 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. The presence of *E. coli* was related to the frequency of washing.

1.13 Aims and objectives:

The aims of this research work carried out at BRAC University were to isolate, identify and evaluating the prevalence of bacterial contaminants from the household and restaurant kitchen utensils and their harmful implication to public health. 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. The worldwide occurrence of multi drug resistant bacterial strains is a noteworthy concern today. Dishwashing liquid is an important detergent to clean kitchen utensils and helps us to protect from infection &food borne pathogens. Therefore, it is significant to find out the effects of dishwashing liquid on isolated bacterial contaminants and its relation with antibiotic resistance.

Besides, the purpose of this study was also to raise awareness about kitchen and food hygiene and food hygiene programs among the general people.

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

- Isolating the bacterial contaminants present in the household and restaurant kitchen utensils.
- 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.
- Determining the effect of dishwashing liquid on selected multi-drug resistant pathogenic isolates.

Chapter 2

Materials and Methods

2.1 Study area and duration:

The study was conducted at the BRAC University in Dhaka, Bangladesh. 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 May-December, 2017.

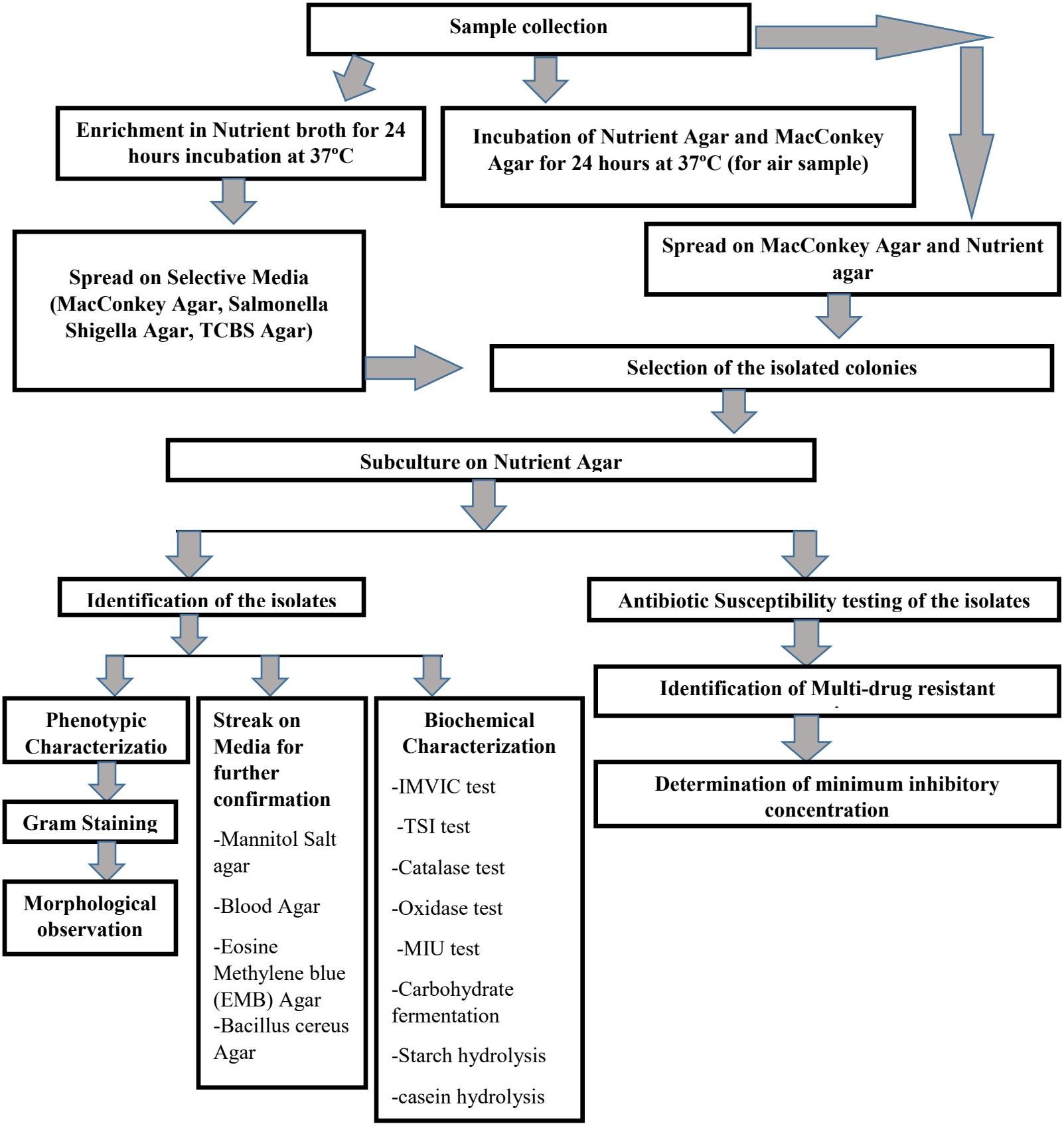
2.2 Sample size:

A total of about 16 kitchen samples were collected from two household kitchens and two restaurant kitchens.

2.3 Sample collection and processing:

A total of 16 samples were collected from household and restaurant kitchen utensils (spoon, knife and cutting board) using the swab-rinse method and from air by exposing the media plates in the air. The 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. The test tube containing the sample was incubated at 37ºC overnight for enrichment.

2.4 Experimental design:



2.5 Isolation, purification and storage:

A total of 16 samples were collected from household and restaurant kitchen utensils. Their source, 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 3 (Air)	03.05.2017	11.00 am	5	K3A1,K3A2,K3A3,K3A4, K3A5
2	Kitchen 3 (Knife)	03.05.2017	11.00 am	6	K3K1,K3K2,K3K3,K3K4, K3K5,K3K6
3	Kitchen 3 (Cutting board)	03.05.2017	11.00 am	1	K3C1
4	Kitchen 3 (Spoon)	03.05.2017	11.00 am	4	K3S1, K3S2, K3S3, K3S4
5	Kitchen 4 (Air)	09.06.2017	11.00 am	5	K4A1, K4A2, K4A3, K4A4,K4A5
6	Kitchen 4 (Knife)	09.06.2017	11.00 am	3	K4K1, K4K2, K4K3
7	Kitchen 4 (Spoon)	09.06.2017	11.00 am	4	K4S1, K4S2, K4S3, K4S4
8	Kitchen 4 (cutting board)	09.06.2017	11.00 am	4	K4C1, K4C2, K4C3, K4C4
9	Kitchen 5 (Air)	01.07.2017	12.00 pm	2	K5A1,K5A2
10	Kitchen 5 (Knife)	01.07.2017	12.00 pm	5	K5K1,K5K2,K5K3,K5K4, K5K5
11	Kitchen 5 (cutting board)	01.07.2017	12.00 pm	2	K5C3,K5C4
12	Kitchen 5 (Spoon)	01.07.2017	12.00 pm	4	K5S1,K5S3,K5S4,K5S5

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
13	Kitchen 6 (Air)	08.08.2017	12.30 pm	4	K6A1,K6A2,K6A3,K6A4
14	Kitchen 6 (Knife)	08.08.2017	12.30 pm	6	K6K1,K6K2,K6K3,K6K4, K6K5,K6K6
15	Kitchen 6 (Cutting board)	08.08.2017	12.30 pm	3	K6C1,K6C2,K6C3
16	Kitchen 6 (Spoon)	08.08.2017	12.30 pm	3	K6S1, K6S2, K6S3

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 -20ºC.

2.6 Biochemical identification:

Biochemical identification of the isolates was done through conventional biochemical tests according to 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 acid tryptophan 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)

2.6.2 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 (Cappuccino &Sherman, 2005).

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. (Cappuccino &Sherman, 2005)

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 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. (Cappuccino &Sherman, 2005)

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 was inoculated into the tubes by means of stab inoculation method with an inoculating needle.
- The tubes were then incubated at 37˚C for 24-48 hours.
- After 24-48 hours the color of both the butt and slant of agar slant cultures were observed.
- The results were recorded based on the following observation (Cappuccino &Sherman, 2005).

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 a sterile inoculating loop, a small amount of bacteria from 24-hour pure culture was placed onto the reagent drops of the microscopic slide
- An immediate bubble formation indicated a positive result and no bubble formation indicated catalase negative result (Reiner, 2010).

2.6.7 Oxidase test:

Oxidase test was done to determine the presence of the enzyme cytochrome oxidase in the bacteria.

- Filter papers were taken, and two drops of oxidase reagent (p-Amino dimethyl aniline oxalate) were added onto the filter papers (Whatman, 1MM).
- The filter papers were labeled according to the sample being tested.
- Using an inoculating loop, a well isolated colony from pure 24-hour culture was picked and rubbed onto filter paper (Whatman, 1MM) and observed for color change.
- A positive reaction would turn the paper from violet to purple within 1 to 30 seconds.
- Delayed reactions should be ignored as that might give false positive result (Shields & Cathcart, 2010).

2.6.8 MIU (Motility-indole-urease) test:

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

- Using sterile technique, small amount of the experimental bacteria from fresh culture was inoculated into the tubes by means of stab inoculation method with an inoculating needle
- The tubes were then incubated for 24 hours at 37ºC.
- The growth of the organism would spread throughout the test tube from downward to the upward of the test tube, if the organism is motile.
- The 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 over night 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).

2.6.9 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)

2.6.10 Carbohydrate fermentation test:

The purpose was to observe if the microbe can ferment the carbohydrate (sugar) maltose as a carbon source.

- Trypticase, Sodium chloride, and Phenol red was weighed and dissolved in distilled water and transferred into conical flasks.
- Desired amount of carbohydrate (Maltose) was added into all flasks.
- Inverted Durham tubes were inserted into all tubes, the Durham tubes were fully filled with broth and was autoclaved.
- Each labeled carbohydrate broths were aseptically inoculated with bacterial culture.
- The tubes were incubated at 18-24 hours at 37^{o}C.
- The reaction was observed.

Expected Result:

1. **Acid production**: Changes the medium into yellow color- organism ferments the given carbohydrate and produce organic acids there by reducing the ph of the medium into acidic.
2. **Acid and Gas production**: Changes the medium into yellow color-organism ferments the given Carbohydrate and produce organic acids and gas. Gas production can be detected by the presence of small bubbles in the inverted durham tubes.
3. **Absence of fermentation**: The broth retains the red color. The organism cannot utilize the carbohydrate but the organism continues to grow in the medium using other energy sources in the medium.(Cappuccino &Sherman, 2005)

2.6.11 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.

2.7 Antimicrobial assay using antibiotic disc:

Kirby-Bauer disc diffusion method was used to determine the susceptibility of bacterial isolates from household and restaurant kitchen utensils. Ten different antibiotic discs were used for identifying antibiotic sensitive and resistant bacteria. Antibiotics those were used in the study are given in table: 2.3.

Table 2.3: List of antibiotics and their disc potency ranges

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

2.7.1 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 the organisms.
2. Using sterile cotton swabs, each of the test bacterial isolates were lawn cultured on properly labeled Mueller Hinton Agar plates.
3. Sterile forceps were used to carefully pick up antibiotic disks from the stacks and were placed very carefully on the lawn culture.
4. Care was taken to ensure that the disks are well-spaced in order to prevent overlapping of inhibition zones.
5. Then the plates were incubated at 37°C for 24 hours.
6. After incubation antibiotic susceptibility was determined by measuring the diameter of inhibition zone in millimeter.

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

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

Serial no	Antibiotic	Inhibition Zone diameter (in mm)		
		Resistant	Intermediate	Susceptible
1	**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 minimum inhibitory concentration (MIC):

2.8.1 Sample collection:

The liquid dishwashing sample of renowned manufacturer used for the study was purchased from a standard departmental store in Dhaka city. The batch numbers, expiry dates and the presence or absences of the manufacturers seal were also noticed and recorded.

2.8.2 Preparation of stock solution of dishwashing liquid:

The dishwashing liquid sample contain Sodium LAS, Disodium EDTA, Sodium laureth sulfate (SLES), concentrated lime juice, CI 19140, CI 42051, water. Firstly, 50ml dishwashing liquid was poured in a sterile beaker and then sterile 50ml distilled water was added. So, it can be said that a stock solution of 2^{-1} was prepared.

2.8.3 Microbial cultures used in the study:

Six bacterial isolates collected from different kitchen utensils were taken from the laboratory. They were *Escherichia coli, Salmonella* spp., *Shigella* spp., *Bacillus* spp., *Staphylococcus* spp. and *Vibrio* spp.

2.8.4 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 concentrations of stock solution that are used in MIC determination are described in Table: 2.5.

Table 2.5: Mixture composition in the test-tubes

Dishwashing liquid (ml)	Nutrient-Broth(ml)	Sample clinical isolates (ml)
60 µl (0.06 ml)	8.94 ml	1ml
90 µl (0.09 ml)	8.91 ml	
100 µl (0.1 ml)	8.9 ml	
130 µl (0.13 ml)	8.87 ml	
160 µl (0.16 ml)	8.84 ml	
190 µl (0.19 ml)	8.81 ml	
200 µl (0.2 ml)	8.8 ml	
250 µl (0.25 ml)	8.75 ml	
300 µl (0.3 ml)	8.7 ml	
400 µl (0.4 ml)	8.6 ml	

2.8.5 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 dishwashing liquid and the broth were pipetted into test-tubes. After that 1 ml of desired culture were pipetted from the cultured broth into the test-tubes.
3. Then all the test-tubes were incubated in 37°C in the incubator for 24 hours and after 24 hours the test-tubes were observed by comparing with the control tubes.
4. All the MIC (Minimum Inhibitory Concentration) values were obtained and noted.

Chapter 3

Results

3.1 Bacterial isolation and identification:

A total of about 16 samples were collected from the household and restaurant kitchen utensils. These samples were streaked onto various selective, differential and nutrient media for identifying the organisms present in kitchen utensils. The results showed that all samples (100%) had bacterial contamination. Both the Gram positive and Gram negative organisms were found from the samples. All the isolates were identified based on Cultural, Morphological and Biochemical characteristics of the isolates on various selective, differential, enriched and nutrient media. Physical and Biochemical characteristics of the isolates obtained from the study are shown in Table 3.1 and Table 3.2.

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.

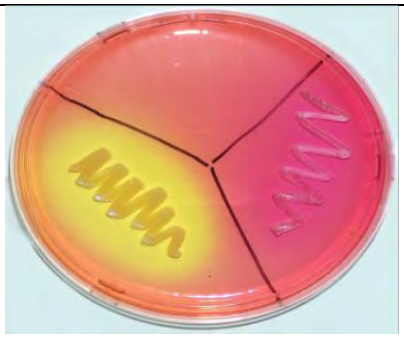


Figure: Growth of *Staphylococcus* spp.on MSA.

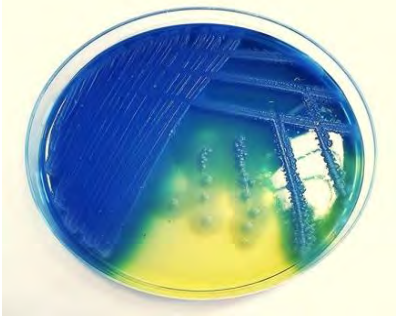


Figure: Growth of *Bacillus* spp. on BC agar.

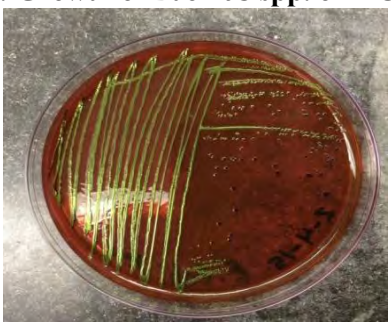


Figure: Growth of *E.coli* on EMB agar.

Figure: Bacterial growth on blood agar (Alpha and Beta hemolysis).

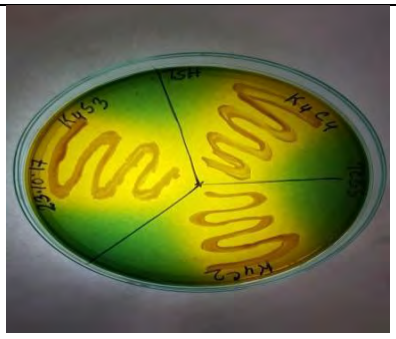


Figure: Growth of *Vibrio* spp. on TCBS agar.

Figure: Growth of *Klebsiella* spp. on MacConkey agar.

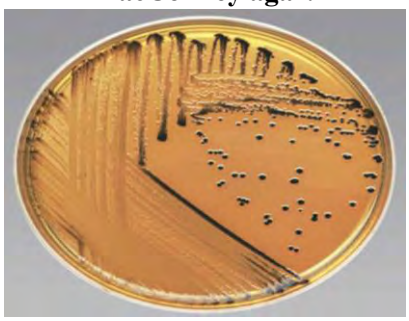


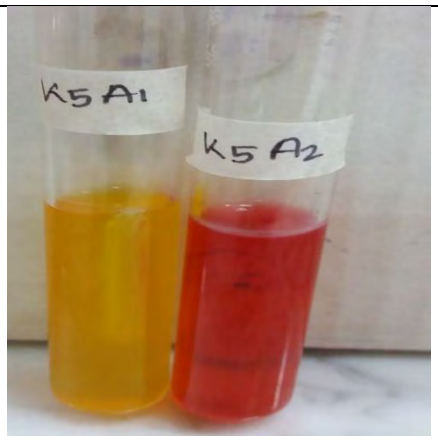
Figure: Growth of *Salmonella* spp. on SS agar.

Figure: Growth of *E.coli* on nutrient agar.

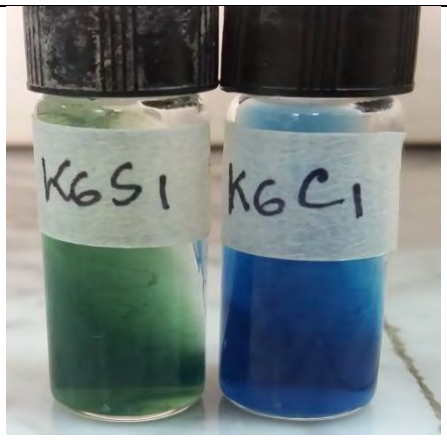
Figure 3.1: Bacterial growth on various selective, differential and enriched media.

3.1.2 Biochemical characteristics of the bacterial isolates:

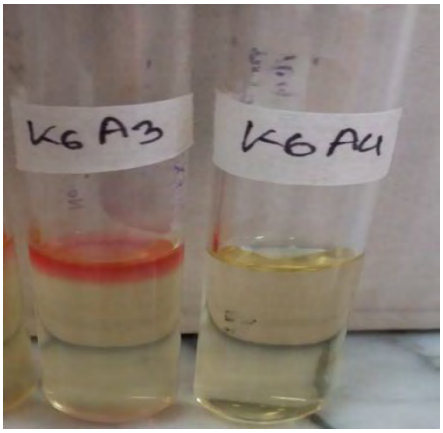
Bacteria isolated from household and restaurant kitchen utensils were tested by different types of biochemical tests. Biochemical tests are important for identification and confirmation of the unknown organisms. After spreading and streaking on the agar plates, microorganisms were isolated and sub-cultured for biochemical tests. These tests were done with 24 hours fresh culture of the isolates. Then organisms were analyzed and identified with the help of reference books including Bergey's manual of Systematic Bacteriology and Cappuccino and Sherman. The biochemical tests that were performed are described precisely in materials and method chapter 2 and the biochemical test results of the isolates are given below in Table 3.2.



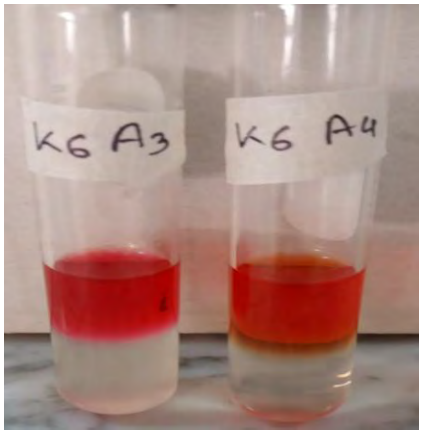
(Positive) (Negative)
Figure: Carbohydrate fermentation test.



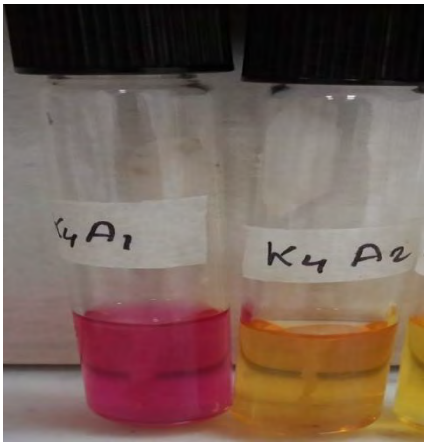
(Negative) (Positive)
Figure: Citrate utilization test.



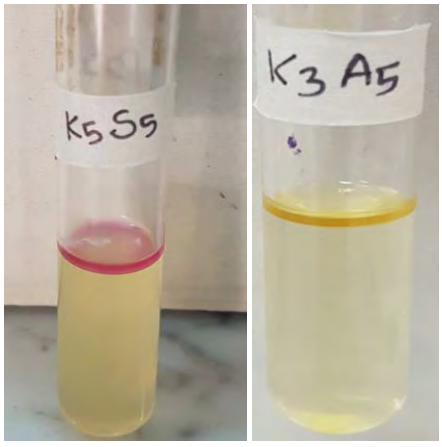
(Positive) (Negative)
Figure: Voges-Proskauer test.



(Positive) (Negative)
Figure: Methyl red test.



(Positive) (Negative)
Figure: MIU test



(Positive) (Negative)
Figure: Indole test

Figure: Casein hydrolysis test.

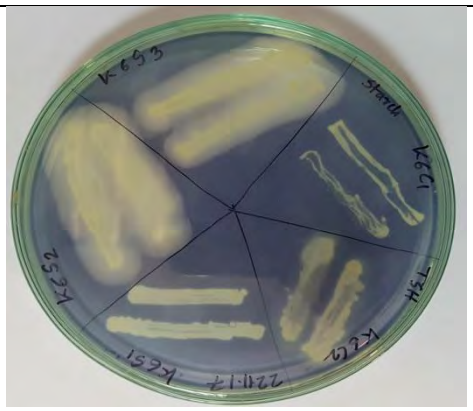


Figure: Starch hydrolysis test.

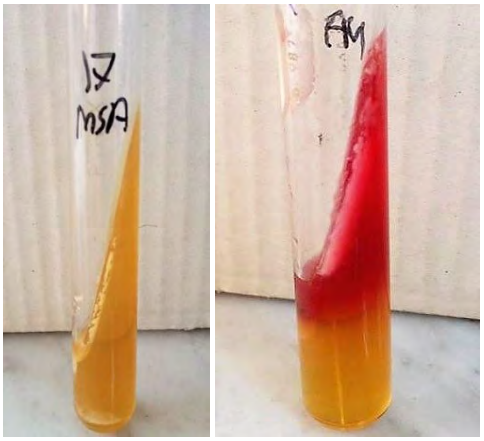


Figure: TSI test. (Yellow slant, yellow butt and red slant, yellow butt)

Figure: TSI test. (Yellow slant, butt with gas production and red slant, red butt)

(Positive)
Figure: Catalase test.

Figure 3.2: Biochemical test results of different bacterial isolates.

After observing the cultural and morphological characteristics of bacterial isolates and performing the biochemical tests, 61 isolates could have been identified from 16 different samples collected from household and restaurant kitchen utensils. The isolates that have been

confirmed include *Staphylococcus* spp., *Bacillus* spp., *Vibro* spp., *Klebsiella* spp., *Shigella* spp., *Salmonella* spp. and *E.coli*. The total number and the percentage of the isolates obtained from the samples are shown in table 3.3 and figure 3.3

Table 3.3: Prevalence of bacteria species isolated from household and kitchen utensils.

Bacterial isolates	Number of the isolates	Total bacterial isolates	% Prevalence
***Bacillus* spp.**	18	61	29.51
***Vibrio* spp.**	17		27.87
***Staphylococcus* spp.**	13		21.31
***Klebsiella*spp.**	6		9.84
***Shigella* spp.**	4		6.56
***Salmonella* spp.**	2		3.28
E.coli	1		1.63

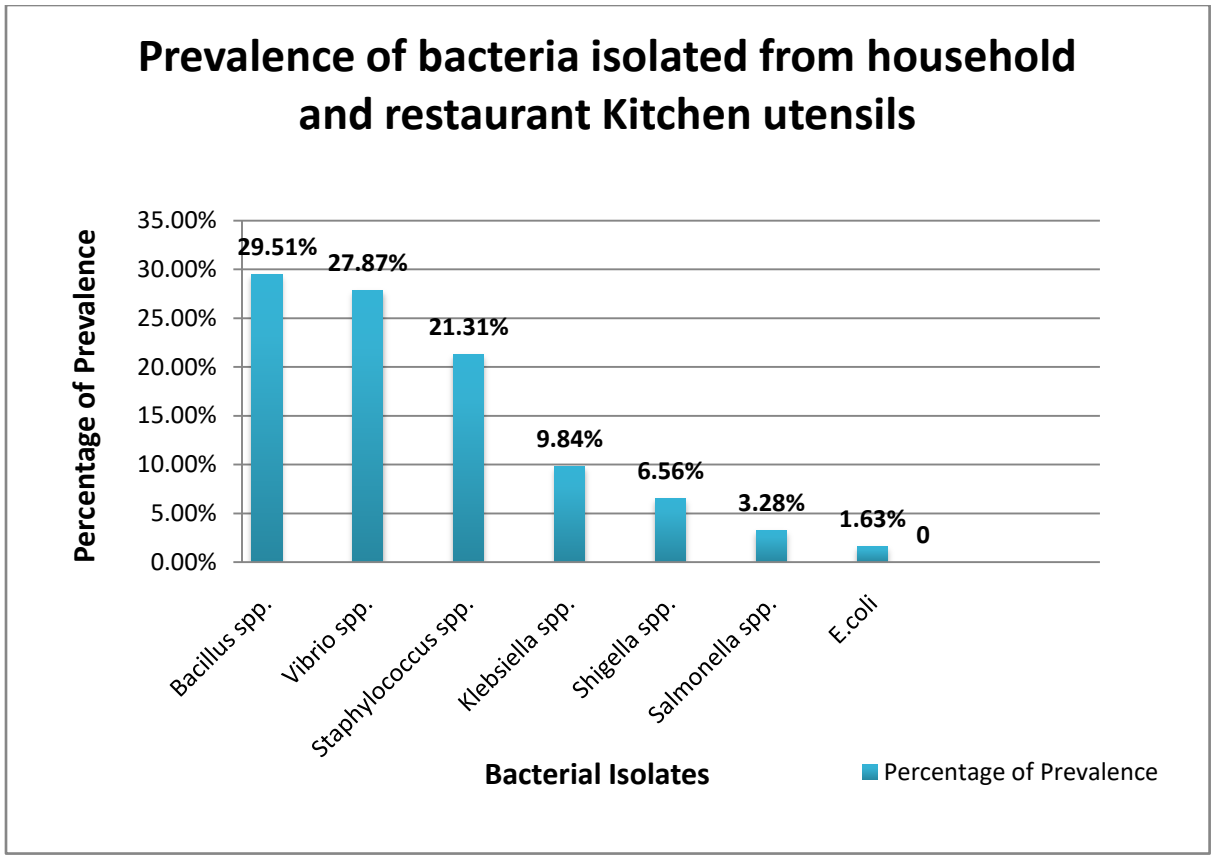


Figure 3.3: Percentage of prevalence of isolated bacteria from household and restaurant kitchen utensils.

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 *Vibrio* spp., *Klebsiella* spp., *Shigella* spp., *Salmonella* spp. and *E.coli*. The differentiation, number and the percentage of the identified bacterial isolates based on Gram reaction are shown in Table 3.4 and Figure 3.4.

Table 3.4: Distribution of the isolates according to Gram's Reaction

Gram's Reaction	Number of isolates found	Percentage (%)
Gram positive	31 (out of 61)	50.82
Gram negative	30 (out of 61)	49.18

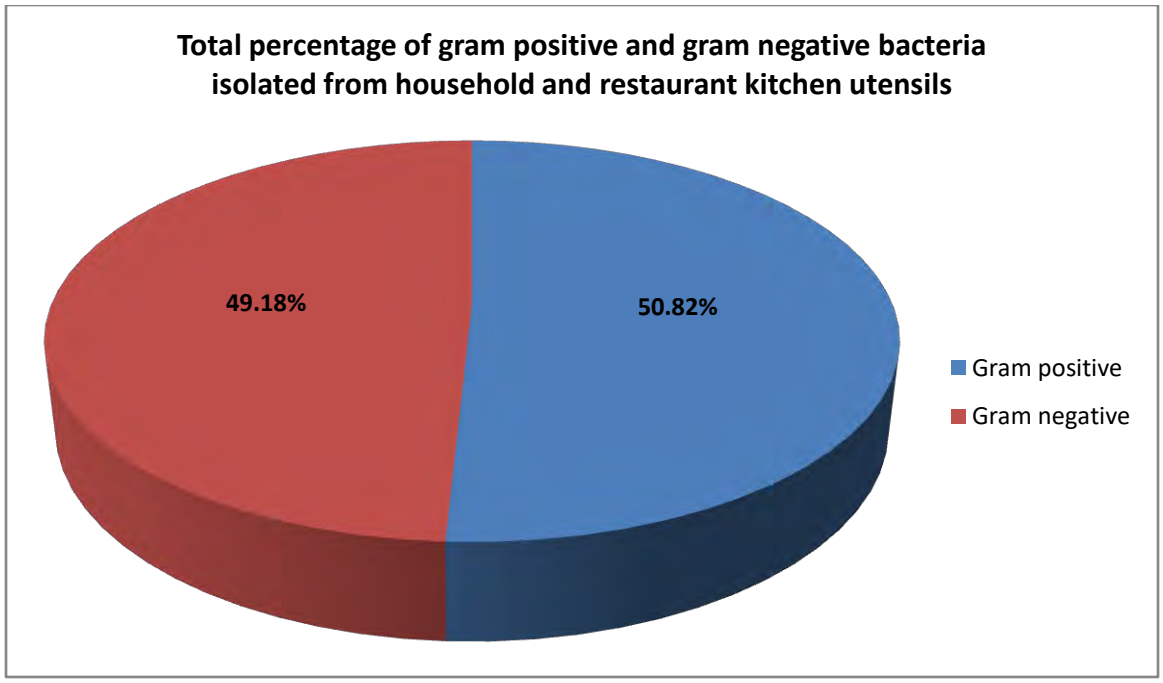


Figure 3.4: Total percentage of gram positive and gram negative bacteria isolated from household and restaurant kitchen utensils.

3.2 Antibiotic susceptibility test:

After identifying and confirming the organisms, the isolates were selected for antibiotic susceptibility test. Ten antibiotics were used for each of the 61 isolates isolated from household and kitchen utensils. The sensitive and resistance pattern of the isolates to these antibiotics were determined.

In table 2.3, the zone of inhibition of the isolates according to the zone range for resistance, intermediate and sensitivity to different antibiotics are shown. The zone of inhibition is measured in millimeter. In some cases no zone of inhibition was observed which means that that particular antibiotic failed to kill the bacterium and the bacterium is resistant to that antibiotic. The interpretation of each bacterium either resistant or susceptible to antibiotic is shown in Table 3.5.

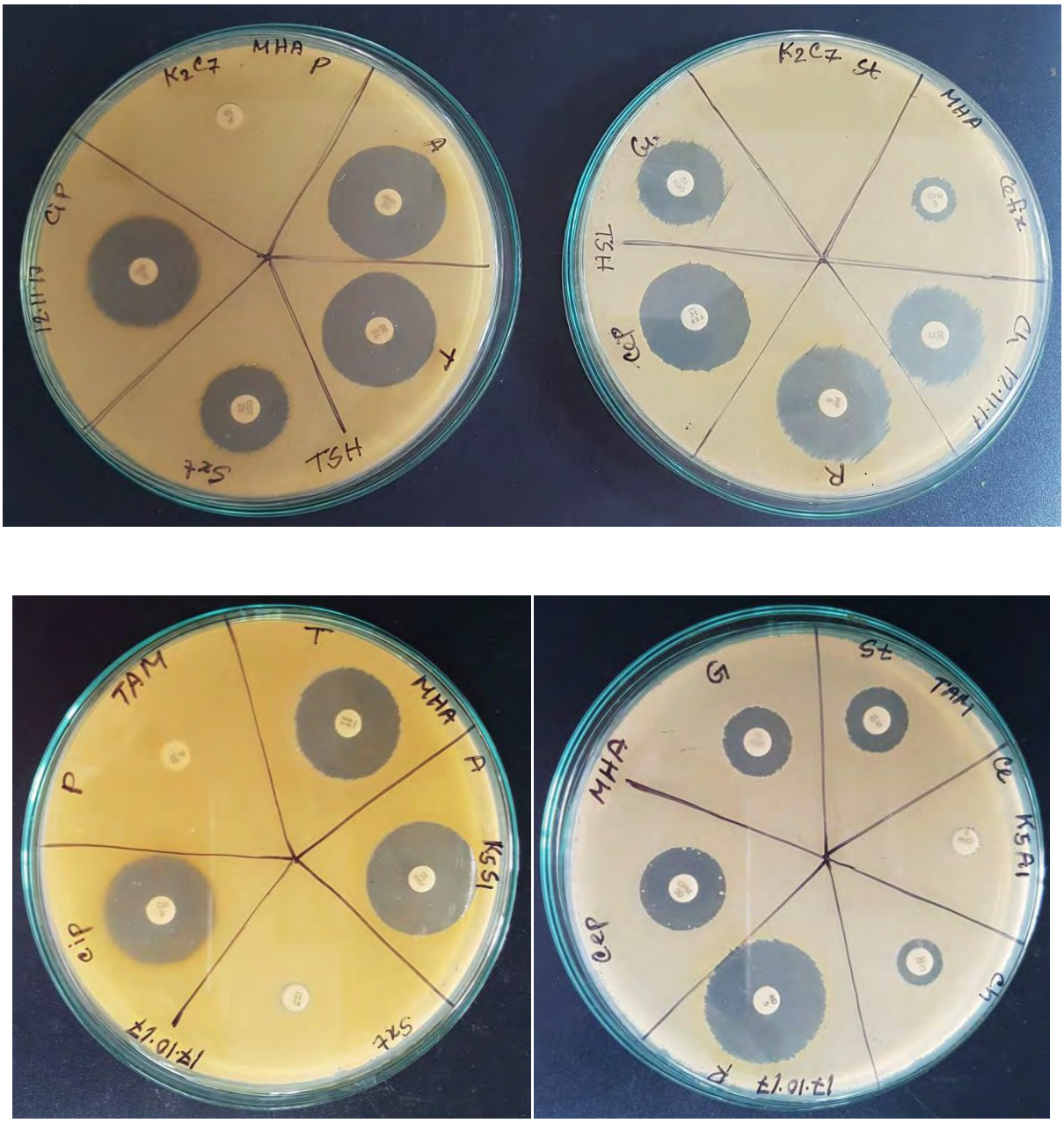


Figure 3.5: Antibiotic susceptibility test of different bacterial isolates.

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 3.6and in the following figure 3.6.

Table 3.6: Antibiotic resistance pattern of total 61 bacterial isolates

Antibiotics	Penicillin	Ciprofloxacin	Chloramphenicol	Amoxicillin	Gentamycin	Rifampicin	Tetracycline	SXT	Cefepime	Cefixime
No of isolates resistant to tested antibiotics	51	4	11	31	1	30	9	31	25	37
Percentage of isolates resistant to antibiotics	83.61	6.56	18.03	50.82	1.64	49.18	14.71	50.82	40.98	60.65

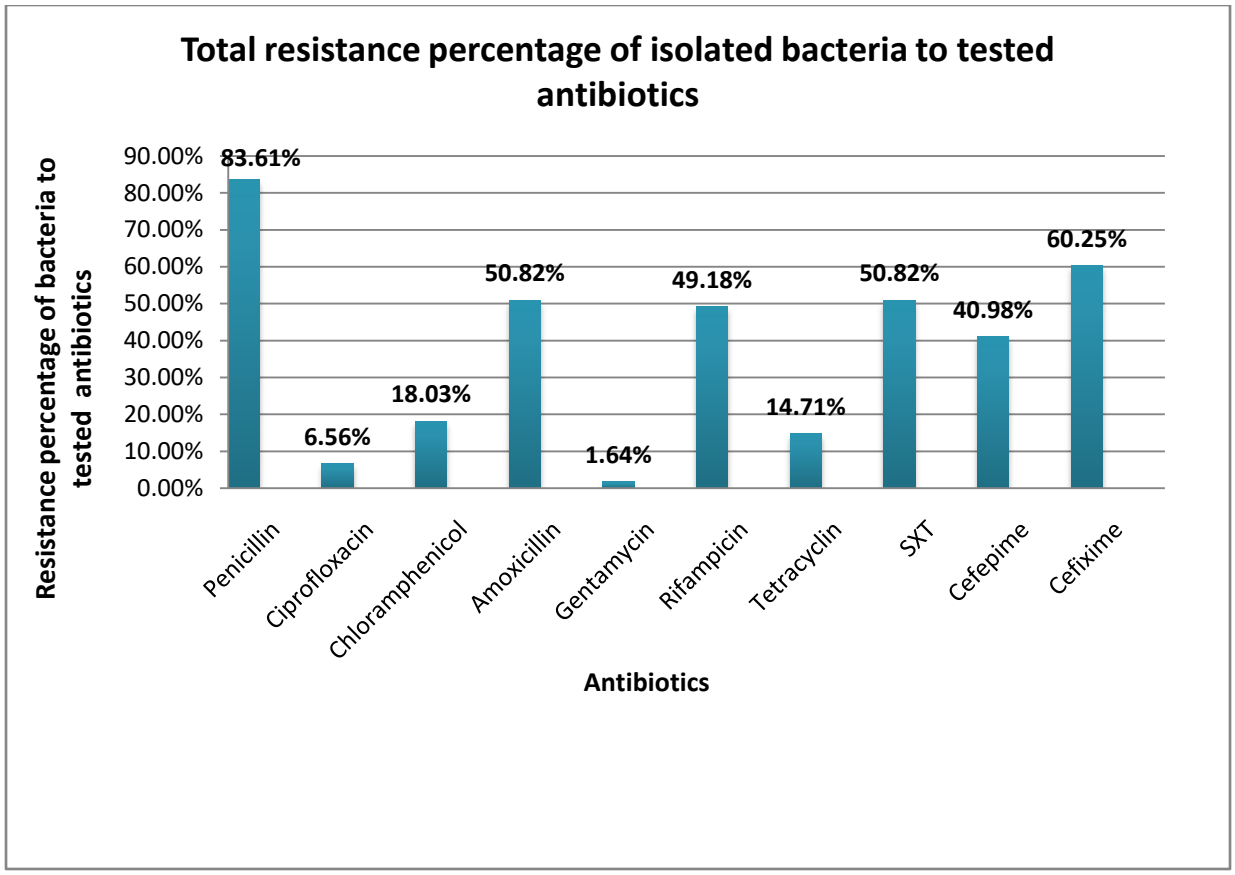


Figure 3.6: Resistance percentage of the isolated bacteria to tested antibiotics

3.2.2: Prevalence of Multi-drug resistant (MDR) organisms:

After observing the antibiotic resistance pattern of the organisms, it was found that all organisms were resistant to two or more than two antibiotics. According to Magiorakos *et al.,* (2011), organisms that are susceptible to at least one agent in three or more antimicrobial categories are considered as Multi-drug resistant organisms. In this study all the bacterial isolates were found resistant to at least one agent in more than three antibiotic categories. Furthermore the Multi-drug resistant (MDR) bacterial isolates are divided into two categories in this study: one that are resistant to two antibiotics and other that are resistant to more than two antibiotics. Their total number and percentage are given below in Table 3.7 and Figure 3.7.

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
61	44	72.13	17	27.87

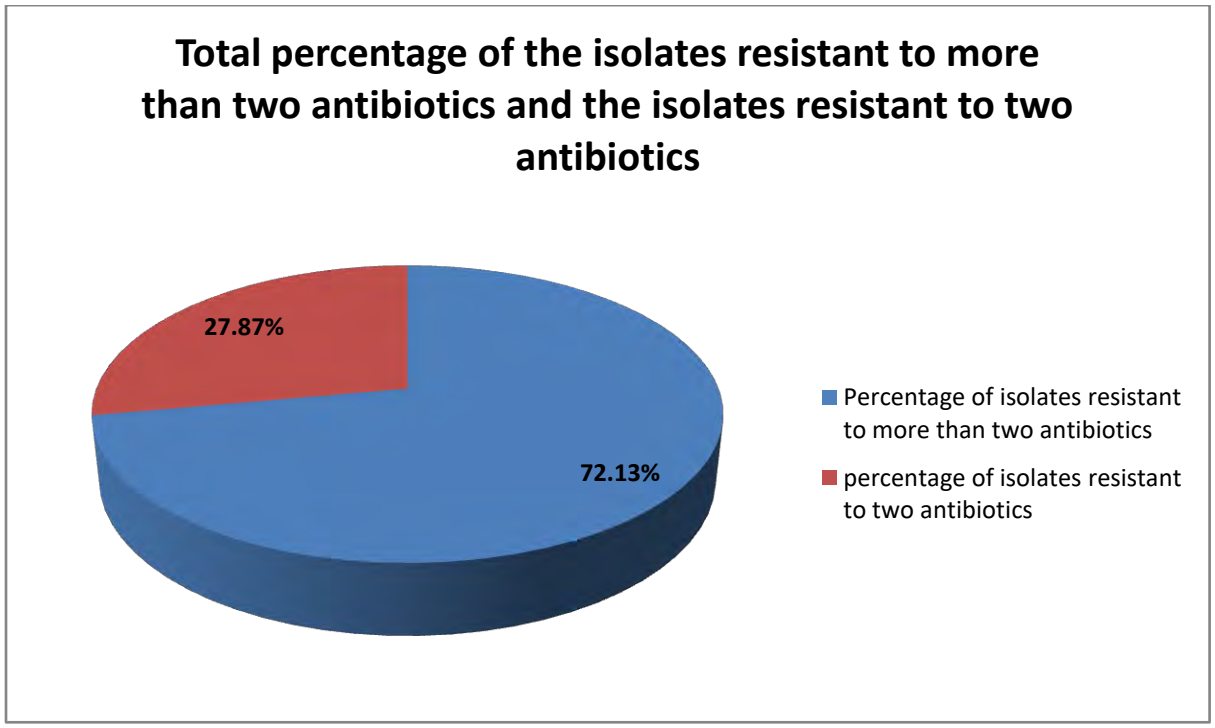


Figure 3.7: Total percentage of the isolates resistant to more than two antibiotics and the isolates resistant to two antibiotics.

3.3 Minimum Inhibitory Concentration (MIC) Test Result:

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

Table 3.8: Minimum Inhibitory Concentration of kitchen isolates:

Isolates ID	MIC value
K5C3 *(Escherichia coli)*	250µlof stock solution
K5S1 (*Staphylococcus* spp.)	190µlof stock solution
K5K5 (*Shigella* spp.)	400µlof stock solution
K6K5 (*Salmonella* spp.)	300µl of stock solution
K6S3 (*Vibrio* spp.)	300µl of stock solution
K6A2 (*Bacillus* spp.)	250µlof stock solution

Here, K5K5 (*Shigella* spp.) showed the highest MIC value i.e. in 400µl of stock solution, whereas K5S1 (*Staphylococcus* spp.) had shown the lowest MIC value i.e. in 190µl of stock solution. Besides, K5C3 *(Escherichia coli)*, K6K5 (*Salmonella* spp.), K6S3 (*Vibrio* spp.), K6A2 (*Bacillus* spp.) had shown the values 250µl, 300µl, 300µl, 250µl respectively. The MIC value was observed by their turbidity in the test-tube which was compared with the turbidity of the control.

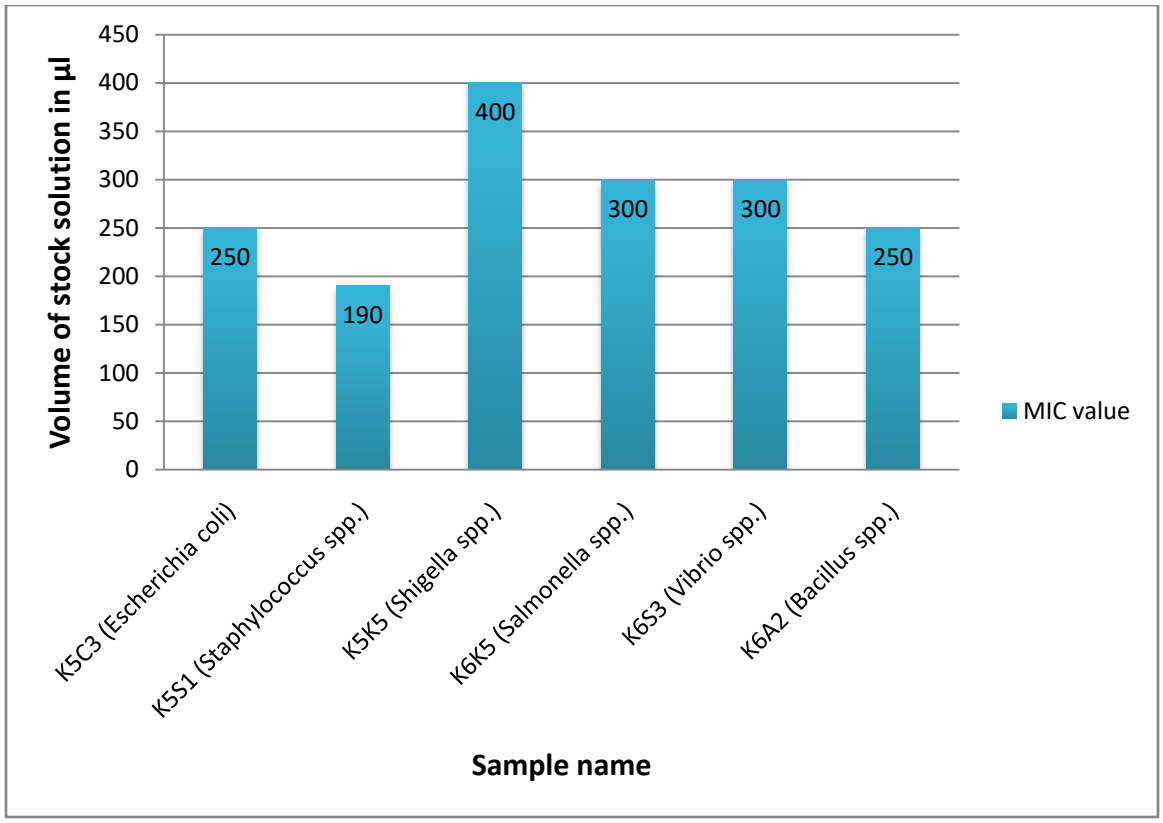


Figure 3.8: Graph of MIC value.

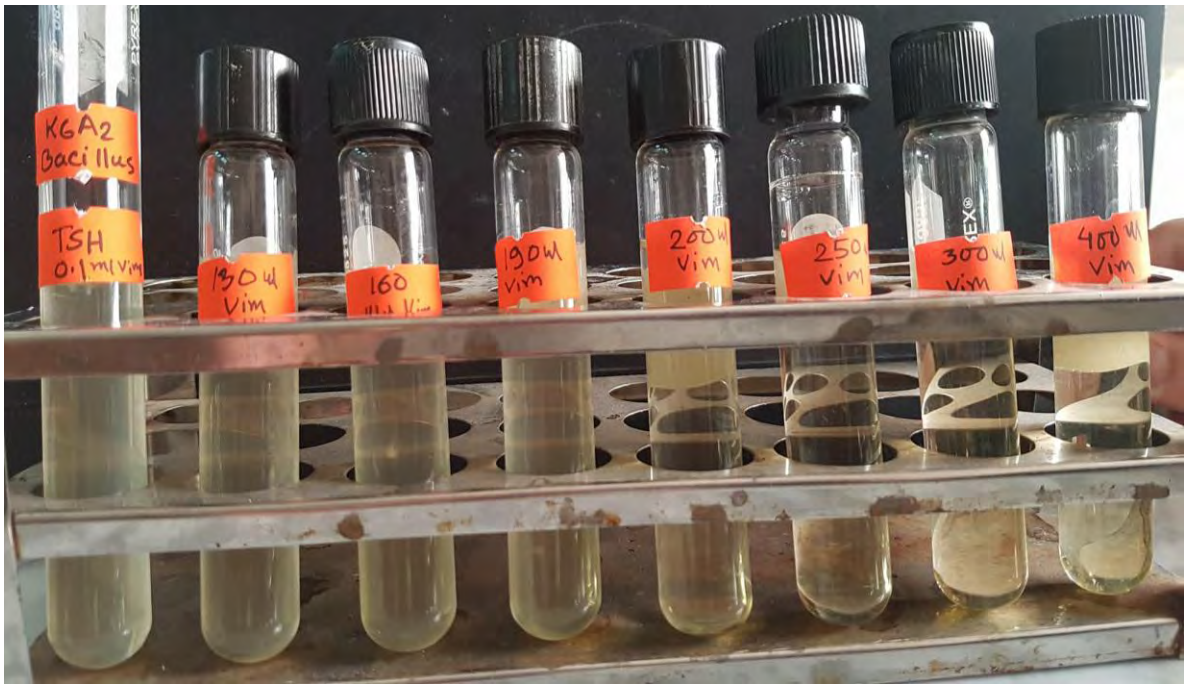


Figure 3.9: MIC test of *Bacillus* spp. using broth micro dilution method.

Chapter 4

Discussion and Conclusion

The kitchen is one of the most crucial areas 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 (Othman, 2016).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 (Purohit, 2009).

This study aimed at isolating, identifying, determining antibiotic susceptibility pattern and investigating antimicrobial activity of dishwashing liquid against the bacterial isolates collected from household and restaurant kitchen utensils. This study also revealed high level of bacterial contaminants on kitchen air and utensils which were contaminated with considerable number of Gram positive bacteria and Gram negative bacteria. However, Gram positive bacteria were found to occur more than Gram negative bacteria. The study showed a statistically significant difference in this regard.

Exposure to pathogens may occur by either indirect contact with contaminated objects or indirectly through airborne particles. Some bacterial species, such as *E. coli*, *S. aureus*, and *Salmonella* spp., could survive on hands, sponges, and other objects for up to several days after contact (Kusumaningrum *et al*., 2002). In this study 16 samples were collected from 4 kitchens and out of 16 samples processed, all of them showed bacterial contamination. A similar study (Adiga *et al*., 2012) was conducted, which revealed that out of 50 samples collected; that is, five each from 10 kitchens; 32 samples (64% of sample collected) were found to harbor pathogenic microorganisms. Another similar study was conducted by Othman in 2016, in that study, 21 of 25 samples that were collected from the five (84%) kitchens were contaminated with pathogenic microorganisms. The significantly high count could be accounted to failure to use sanitizing chemicals to clean kitchen utensils, extended use of the same utensils without cleaning, and underprivileged hygienic conditions of the food serving establishments as observed during data collection.

After 24 hours incubation of various selective and differential media, some different morphological characteristic showing colonies were isolated to perform biochemical tests. After performing the biochemical characteristics, the isolates were finally confirmed as *Vibrio* spp., *Klebsiella* spp., *Shigella* spp., *Salmonella* spp., *E.coli*, *Staphylococcus* spp. and *Bacillus* spp.

Similar findings were observed in another study (Othman, 2016). The study investigated that kitchens were contaminated with pathogenic microorganisms such as *E. coli*, *Klebsiella* spp., *S. aureus*, *S. epidermidis*, *Salmonella* spp., *Shigella* spp., and *Micrococcus* spp. Another similar study was conducted by Maori and Nandita in 2009, the result revealed a profile of seven (7) different bacterial species including *Staphylococcus aureus*, *Proteus vulgaris*, *Salmonella typhi*, *Escherichia coli* and the species of *Shigella*, *Klebseilla* and *Bacillus* isolated and identified from kitchen crockery and cutlery.

The presence of these isolates from kitchen utensils could create health hazard when they are ingested, or they come in contact with the human skin. The most prevalent bacterial contaminant found in this study was *Bacillus* spp. (29.50%). Its high prevalence could be demonstrated by the fact that *Bacillus* spp. is ubiquitous in nature and also they are spore forming bacteria. These spores are able to resist environmental changes, withstand dry heat and certain chemical disinfectants for prolonged periods. Second most prevalent bacterial contaminant found was *Vibrio* spp. (27.86%). Pathogenic *Vibrio* spp. can cause food borne illness (infection), usually associated with eating undercooked seafood or through handling raw fish or even by washing kitchen utensils with contaminated water. This human pathogen causes acute gastroenteritis characterized by diarrhea, vomiting and abdominal cramps through consumption of contaminated raw fish or shellfish (Rippey, 1994). Another most prevalent bacterial contaminant found was *Staphylococcus* spp. (21.13%). This may be due to the fact that it is a major component of the normal flora of the skin and nostrils and it can be easily be discharged by several human activities. This particular species of bacteria does not form spores but can cause contamination of food products during food preparation and processing. They can grow in a wide range of temperatures (7° to 48.5° C; optimum 30 to 37°C), pH (4.2 to 9.3; optimum 7 to 7.5), and sodium chloride concentration up to 15% NaCl (Kadariya *et al*., 2014). It is a desiccation tolerant organism with the ability to survive in potentially dry and stressful environments, such as the human nose and on skin and inanimate surfaces such as clothing and kitchen surfaces. Although *E. coli* itself is not harmful, its presence in any numbers can be regarded as evidence that eating utensils and cutlery were contaminated with fecal discharges, if not of human origin then at least is an important cause of food intoxication (Berdgoll, 1989). *Salmonella* spp. causes several diseases such as gastroenteritis, septicemia typhoid etc. which is transmitted via food or

water (Michael et al., 2004). Presence of *Shigella* spp. also indicated potential source of food borne illness such as shigellosis.

Prevalence of more Gram positive organisms compared to Gram negative organisms correspond with previous studies (Chikere *et al.,* 2008) and in favor with the statement that Gram positive bacteria have overtaken the Gram-negative as the predominant bacteria isolated from fomites (Inweregbu *et al.*, 2005). It is probably because Gram positive organisms are the members of the body flora of both asymptomatic carriers and sick persons. These organisms can be transmitted by the hand, expelled from the respiratory tract or spread by animate or inanimate objects (Chikere *et al*., 2008).

Determination of antibiotic susceptibility pattern revealed that all bacterial isolates tested were resistant to two antibiotics. Among the 61 bacterial isolates, 44 (72.13%) of them were found to be resistant to more than two antibiotics and 17(27.87%) of them were found to be resistant to two antibiotics. Out of the ten antibiotics tested penicillin was found to be least effective because 83.61% bacterial isolates showed resistance to penicillin. Cefixime can also be considered less effective because 60.65% of the bacterial isolates showed resistance to this antibiotic. The resistance percentage of the isolates to other antibiotics include, amoxicillin and SXT (50.82%), rifampicin (49.18%), cefepime (40.98%), chloramphenicol (18.03%), tetracycline (14.71%), ciprofloxacin (6.56%) and gentamycin (1.64%).The resistance phenomenon of the bacteria to antibiotics may be due to the inappropriate use of the antibiotics which accelerates the evolution of resistant strains of bacteria (Maryam *et al.*, 2014). The improper use of antibiotics in human and livestock, wrong and substandard prescriptions by unqualified medical personnel along with poor diagnosis or lack of it all have been reported to be among the main factors contributing to the development of resistant microbes (Kimang'a, 2012).

Previous studies have caused increasing concern about food borne disease outbreaks in the households as well as restaurants. These outbreaks have frequently occurred as a result of improper food preparation and cross-contamination in combination with inadequate storage or cooking has been implicated in many instances (Kusumaningrum *et al*., 2001). These previous studies have suggested that although raw material is probably the main source of contamination in the kitchen, the areas surrounding the kitchen and the kitchen utensils could also act as sources of free living populations of bacteria. Many commercial products are specifically manufactured

for the reduction of bacteria in cleaning dishcloths, sponges and different kitchen utensils. These products include bleach solutions, detergents, and dishwashing liquids. Limited studies have addressed the effectiveness of these products in inactivating microorganisms on kitchen utensils. However, in this study Minimum inhibitory concentration (MIC) was determined against commercial liquid dishwasher through Broth Micro dilution method. In results, *Shigella* spp. showed the highest MIC value i.e. in 400µl of stock solution, whereas *Staphylococcus* spp. had shown the lowest MIC value i.e. in 190µl of stock solution. Besides, *Escherichia coli*, *Salmonella* spp., *Vibrio* spp., *Bacillus* spp. had shown the values 250µl, 300µl, 300µl, 250µl respectively. It is less similar with the reports of some other researchers (Ikawa *et al.,* 1999 and Scott *et al.,* 1990) who reported that soaking of dishcloths or sponges for 5 min in bleach solution resulted in a >99.9% reduction of microorganisms. It was contradictory with the findings of another study which was conducted by Erdogrul and Erbilir in 2003, and the results indicated the use of dishwashing liquid daily had no effect on fungal growth, *Pseudomonas* growth or *E. coli* growth, but the number of *Salmonella* decreased (Burr, 2006).

Conclusion:

The present study revealed that kitchen utensils used at home and restaurants could be very important sources of potential pathogens which have been reported to cause food borne illnesses. However, to prevent cross-contamination, surfaces and utensils that are used to make and prepare food, particularly poultry and meat, should be thoroughly cleaned with an antibacterial cleanser or disinfectant after each use.

Food contamination and consumers exposure to food hazards have major implication on the food security and consumers health. Lack of the execution of existing Food laws and regulation and low level of awareness are also contributing to aggravating the country's food safety situation. A comprehensive risk analysis and risk management approach of food safety from production to consumption is required to protect public health from such hazards. Raising awareness, ensuring safe water, sanitation and improved basic food hygiene practice will play an important role in reducing food borne illness.

References

1. Scott, E., Bloomfield, S., & Barlow, C. (1982). An investigation of microbial contamination in the home. *Journal of Hygiene, 89*(2), 279-293. doi:10.1017/S0022172400070819
2. Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck. (1966). Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 36:493-496.
3. Maryam, A., Hadiza, U. S., and Aminu, U. M. (2014). Characterization and determination of antibiotic susceptibility pattern of bacteria isolated from some fomites in a teaching hospital in northern Nigeria. *African Journal of Microbiology Research,* 8(8): 814-818
4. Erdogrul, O. and Erbilir, F. 2003. Microorganisms in Kitchen Sponges. *Journal of Food Safety*. 6:17-22.
5. Scott, E.A., and Bloomfield, S.F. (1990). The survival and transfer of microbial contamination via clothes, hands and utensils. *Journal of Applied Bacteriology*, 68: 271-278.
6. Jeon YS, Chun J, Kim BS. (2013). Identification of household bacterial community and analysis of species shared with human microbiome. *Curr Microbiol*. 2013 Nov;67(5):557-63.
7. Othman, A. S. (2015). Isolation and microbiological identification of bacterial contaminants in food and household surfaces: How to deal safely. *Egyptian Pharmaceutical Journal*, *14*(1), 50.
8. Nielsen, P. (2002). Evaluation of the use of liquid dishwashing compounds to control bacteria in kitchen sponges. *Journal of AOAG International*. 85(1):107-12.
9. Wolde, T., & Bacha, K. (2016). Microbiological Safety of Kitchen Sponges Used in Food Establishments. *International journal of food science*, *2016*.
10. Josephson, K. L., Rubino, J. R., & Pepper, I. L. (1997). Characterization and quantification of bacterial pathogens and indicator organisms in household kitchens with and without the use of a disinfectant cleaner. *Journal of Applied Microbiology*, *83*(6), 737-750.
11. Koenig, D. W. (2014). Occurrence in Kitchen Hand Towels. *Food Protection Trends*, *34*(5), 312-317.

12. Cracknel W, Nobis CF .(1989). Food Microbiology, 4th Edition Tata McGraw-Hill Publishing Company limited, New Delhi p. 56.
13. Fawole MO, Oso BA .(1988). Laboratory Manual of Microbiology Ibadan. Spectrum Books Limited p. 127.
14. Vanderzant C, Splittstoesser DF .(1992). Compendium of methods for the microbiological examination of foods, 3rdedition American public Health Association, Washington DC.
15. Cogan T., A., S.F.Bloomfield, and T.J.Humphery .(1999). The effectiveness of hygiene procedures for prevention of cross contamination from chicken carcass in the domestic kitchen. Letters in Applied Microbiology. 29:354-358.
16. De Wit, J.C., G. Broekhuizen, and E.H.Kampelmacher .(1979). Cross contamination during the preparation of frozen chickens in the kitchen. *Journal of Hygiene Cambridge*. 83:27-32.
17. Scott, E.; Bloomfield S.F. (1990).The survival and transfer of microbial contamination via cloths, hands and utensils. *Journal of Applied Bacteriology* 68(3): 271- 278.
18. WHO 2001 WHO Surveillance program for control of food borne infections and intoxications in Europe, Seventh report 1993-1998.Berlin, Germany
19. Messina M. ,MD, Lindesey 2008. Hand Hygiene in the dermatologist's office: To wash or to rub?. *Journal of American Acad Dermatol*.59:1043-9.
20. Zhao,P., Zhao, T., Doyle, M.P., Rubino,J.R., Meng,J. 1998. Developmentof a model for evaluation of microbial cross-contamination in the kitchen. *Journal of Food Protection*. 61 (8): 960-963.
21. Uri Zoller *(29 October 2008).* Handbook of Detergents, Part E: Applications. *CRC Press*. pp. 39–65. *ISBN 978-1-57444-757-6*.
22. Layton, Lyndsey (8 April 2010). "FDA says studies on triclosan, used in sanitizers and soaps, raise concerns". *Washington Post*. Retrieved 10 January 2015.
23. Boone, Lisa (21 May 2014). "Minnesota bans triclosan, an ingredient in antibacterial soaps". *latimes.com*. Retrieved 10 January 2015.
24. Burr L. (2006). The Effectiveness of Antibacterial Dish Detergent and Non-antibacterial Dish Detergent when Used on Kitchen Sponges. *Saint Martin's University Biology Journal*. Volume 1

25. Li, D., Yu, T., Zhang, Y., *et al*. (2010). Antibiotic resistance characteristics of environmental bacteria from an oxytetracycline production wastewater treatment plant and the receiving river. *Applied Environmental Microbiology,* 3444-3451.
26. Lynn, M., Vivian O.A., and Wasa, A.A. (2013). Prevalence of bacterial organism on toilet door handles. *IOSRJournal of Pharmacy and Biological Sciences*, 8:85-91
27. Maori, L., Agbor, V. O., and Ahmed, W. A. (2013). The prevalence of bacterial organisms on toilet door handles in Secondary Schools in Bokkos L.G.A., Jos, Plateau State, Nigeria. *IOSR Journal of Pharmacy and Biological sciences*, 8 (4): 85-91.
28. Chikere, CB, Omoni VT, Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. *African Journal of Biotechnology*, 7(20): 3535-3538.
29. Tanwar, J., Das. S., Fatima, Z., and Hameed, S. (2014). Multidrug Resistance: An Emerging Crisis. *Interdisciplinary Perspectives on Infectious Diseases*, 2014. Article ID 541340.
30. Maori, L. (2010). Bacterial contamination of crockery and cutlery within the kiosks restaurants of the Federal University of Technology, Yola. *African Journal of Microbiology Research*, *4*(3), 147-153.
31. Koenig, D. W. (2014). Occurrence in Kitchen Hand Towels. *Food Protection Trends*, *34*(5), 312-317.
32. Purohit, C. (2009). Bacterial transfer from mouth to different utensils and from utensils to food.
33. Sneath, P. H., Mair, N. S., Sharpe, M. E., & Holt, J. G. (1986). *Bergey's manual of systematic bacteriology. Volume 2*. Williams & Wilkins.
34. James, C., & Natalie, S. (2014). *Microbiology. A laboratory manual*. Pearson Education.
35. Kimang'a, A.N. (2012). Review article situational analysis of antimicrobial drug resistance in Africa: Are we losing Battle? *Ethiopian Journal of Health Sciences*, 22:135-140
36. Kadariya, J., Smith, T. C., & Thapaliya, D. (2014). Staphylococcus aureus and staphylococcal food-borne disease: an ongoing challenge in public health. *BioMed research international*, *2014*.

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