Prevalence, Antibiotic Susceptibility and Plasmid Profile of Bacteria from Door Knobs of Female Restrooms in BRAC University, Dhaka

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Declaration

I hereby declare that the thesis project titled **"Prevalence, Antibiotic Susceptibility and Plasmid Profile of Bacteria from Door Knobs of Female Restrooms in BRAC University, Dhaka"** has been written and submitted by me, Innas Sultana 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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DEDICATED

TO

MY

BELOVED FATHER AND MOTHER

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Abstract

Non-porous environmental surfaces such as door knobs of restrooms are routinely touched with hands and can serve as vehicles for transmitting infectious diseases. This study aimed at isolating, identifying the bacterial contaminants, determining the antibiotic susceptibility pattern of the isolates to some commonly used antibiotics and investigating plasmid profile of the bacteria isolated from door knobs of female restrooms in BRAC University, Dhaka, Bangladesh. Fifteen (15) samples collected through sterile swabs were kept for enrichment into nutrient broth at 37ºC for 24 hours and were cultured on various selective media the next day. Identification of bacteria was done through conventional biochemical tests according to Bergey's Manual of Systematic Bacteriology. Antibiotic susceptibility test was done by Kirby-Bauer method and plasmid extraction was done according to modified hot alkaline method. Out of the 15 samples processed, 15 (100%) of them showed bacterial growth. A total of about 37 bacterial isolates were obtained with *Staphylococcus* having the highest prevalence 12 (32.43%), followed by *Bacillus* species 11 (29.73%), *E.coli* 7 (18.92%), and *Micrococcus* species 2 (5.41%). Presence of fecal coliform (13.51%) was also observed in this study. Antibiotic susceptibility pattern of the bacterial isolates showed that almost all of the isolates were resistant to at least two antibiotics. Among the 37 isolates, 78.38% were found resistant to more than two antibiotics and 21.62% were found resistant to at least two antibiotics. Highest resistance percentage of the isolates was observed to penicillin (100%) followed by amoxicillin and rifampicin (75.68%), SXT (48.65%), tetracycline (13.51%), ciprofloxacin (10.81%), chloramphenicol (8.11%), gentamycin and streptomycin (5.41%). Plasmid profiling revealed 29.37% (11 out of 37) bacterial isolates contained 1 or more plasmids with 6 different profiles. A significant proportion of the bacterial isolates (21.63%; 8 out of 37) carried large plasmids with high molecular weight (> 20 MDa). Six isolates (of 37; 16.22%) carried large plasmids of approximately 85 MDa size. Findings of this study indicate the presence of multi-drug resistant bacterial strains in door knobs of restrooms which can serve as potential source of diseases. Regular cleaning of restrooms, use of hand sanitizers, and public awareness on personal hygiene can help minimize the spread of diseases from door knobs. Among the multi-drug resistant isolates, three of the isolates contained multiple number of plasmids and eight of the isolates contained single number of plasmid. Further study on the correlation between antibiotic resistance and the presence of plasmid would be an interesting line of inquiry.

Contents

List of figures

List of tables

List of abbreviations

MSA	Mannitol Salt Aagar
MR	Methyl Red
VP	Voges-proskauer
TSI	Triple Sugar Iron
MDR	Multi-drug resistant
MHA	Muller Hinton Agar
MIU	Motility Indole Urease
µL	Microliter
Ml	Milliliter
spp.	Species

Chapter 1

Introduction

Introduction:

Microorganisms are living things which are found everywhere including the environment and the human body. They are present in major part of the ecosystem. In these environments they live either freely or as parasites (Sleigh and timbury, 1998)

Human body harbor a number of microbes including several species of bacteria, viruses, fungi and protozoa. The sites where bacteria are found include: skin (staphylococci and bacteroides), Oropharynx (streptococci, anaerobes), large intestine (Enteric bacilli) and vagina (lactobacilli) (Beaugerie and Petit, 2004).

1.1 Normal flora of human body:

Normal flora refers to the population of microorganisms that reside in the skin and mucus membranes of a healthy normal person without causing any disease (Jawetz *et al*, 2007). They protect us from disease by competing with invaders for space and nutrients, producing bateriocins which kill harmful bacteria and lowering the pH so that other bacteria can't grow.

Table 1.1: Classification of Normal flora of the human body (Eckburg *et al*, 2005)

Human body	**Normal flora**
Skin	*Sthaphylococci, micrococci*, diptheroids
Oral and upper respiratory tarct	*Neisseria*, *Bordetella*, *Corynebacterium*, and *Streptococcus* spp
Conjunctiva	*Haemophilus* and *Staphylococcus*
Gastrointestinal tract	*Enterococci*, non-haemolytic *streptococcus*, *E.coli*, lactobacillus
Genital tract	*Corynebacterium*, *Lactobacillus* spp, non-pathogenic *Neisseria* spp,

1.2 Factors associated in microbial flora infection:

Normal microbial flora usually don't cause infection in body but can cause infection if the following factors are involved:

Individual susceptibility: Important factors influencing acquisition of the infection by microbial flora include; immune status, age, underlying disease and therapeutic interventions (Ducel *et al*,

2002). If the host immunity is impaired then normal flora can cause disease (Ann M O'Hara, Fergus Shanahan, 2006). Malnutrition, irradiation, indiscriminate use of antibiotics can lower the patient's immunity thereby making them moves vulnerable to the infection (Ducel *et al*, 2002). **Environmental Factors:** Microbial flora may contaminate objects and materials and subsequent contact by a susceptible individual to these objects may come down with an infection. These contaminated objects can easily be picked by mere contact and transferred by many people who fail to follow the basic infection control such as washing of hands (Ducel *et al*, 2002).

1.3 Sources and Mode of transmission of infection:

Microorganisms can be transferred to the host, either directly from the environment or indirectly through an intermediate agent. Reservoir is a site or natural environment in which the pathogen is normally found living and from which infection of the host can occur (Prescott *et al*, 1999). Transmission of infection can be referred to as the movement of pathogens from a source to appropriate portal of entry. The sources and modes of transmission can be of different ways which include:

a. **Direct contact**: Person-to-person transmission is a form of direct contact transmission. Here the agent is transmitted by physical contact between two individuals. For example shaking hands. Direct contact can be categorized as vertical, horizontal, or droplet transmission.
b. **Indirect contact**: Indirect contact transmission involves inanimate objects called fomites that become contaminated by pathogens from an infected individual or reservoir. For example, an individual with the common cold may sneeze, causing droplets to land on a fomite such as a tablecloth or carpet, or the individual may wipe her nose and then transfer mucus to a fomite such as a doorknob or towel. Transmission occurs indirectly when a new susceptible host later touches the fomite and transfers the contaminated material to a susceptible portal of entry. Fomites can also include objects used in clinical settings that are not properly sterilized, such as syringes, needles, catheters, and surgical equipment. Pathogens transmitted indirectly via such fomites are a major cause of healthcare-associated infections.

The United State (US) center for disease control (CDC) and prevention stated that contaminated public surfaces most of which are of microorganisms are perhaps the most wide spread problems

in contemporary world and is responsible for about one third of death world-wide through infections, with adverse effects which can reduce economic productivity (WHO, 2002).

1.4 Transmission of pathogens by hands:

Human hands serve as vectors for the transmission of microorganisms from place to place and from person to person. Human hands usually constitute microorganisms both as part of the body normal flora as well as transient microbes contracted from the environment (Dodrill *et al*., 2011). Although it is nearly impossible for the hand to be free of microorganisms but the presence of pathogenic bacteria on hands may lead to chronic or acute illness. Curtis *et al.* (2003) and Lorna *et al.* (2005) reported that hands often act as medium that carry disease-causing pathogens including bacteria and viruses from person to person either through direct contact or indirectly via surfaces. Al- Ghamdi *et al*., (2011) stated that 80% of infections are spread through hands contact with hands or other objects. *Staphylococcus epiderdimis* is present on almost every hand (Larson *et al*., 1992). Some members of Enterobacteriaceae family also found on hands (Leyden *et al*., 1991; Scott and Bloomfield, 1990). Pathogens that may be present on the hand include *Escherichia coli*, *Salmonella typhi*, *Shigella* spp, *Clostridium perfringes*, *Giardia lamblia*, Norwalk virus and Hepatitis A virus, *Pseudomonas aeruginosa*, *S. aureus*, *Proteus mirabilis*, *Citrobacter freundii*, *Enterobacter spp*; *Streptococcus spp*, *Klebsiella spp*. (Orskov *et al*., 1997).

1.5 Fomites:

Fomites refers to the porous or non-porous surfaces or nonliving objects that when contaminated with pathogenic organisms can transfer them to the new host and act as medium in transmitting infection (Greene 2009; Cramer, 2013). The fomites include door handles of conveniences, showers, toilet, hand lockers especially those found in public offices, hospitals, hotels, restaurants and restrooms (Bright *et al*., 2010). These surfaces constitute a major source of spread of infectious diseases. Presscott *et al*., 1993 stated that the major source of spread of community acquired infections are fomites.

1.5.1 Transfer rate of bacteria from fomites to hands:

Transfer rates of microbes to hands are more significant from hard, nonporous surfaces such as stainless steel (Rheinbaben, Schunermann, Gross, & Wolff, 2000; Rusin et al., 2002). 40% transfer rate was evaluated for *Escherichia coli* from a nonporous surface to hands in one study (Scott & Bloomfield, 1990). Rusin *et al.* (2002) observed bacterial transfer rates of 38.5% to 41.8% from

the telephone and rates of 27.6% to 40.0% from a sink faucet handle to a person's hand with minimal contact times.

1.5.2 Factors associated in bacterial transfer between environmental surfaces:

The factors involved in bacterial transfer between 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

1.6 Toilet door knobs/handles:

Toilets especially when not cleaned routinely can act as a major source of microbial transmission and act as a hidden source for infections. Toilets can provide a perfect condition for spread of pathogens from gut, respiratory tract also, skin by means of hands and surfaces from one individual to another (Gerhardts *et al*., 2012). If toilet handles are contaminated then organisms that are not resident in the hand can be easily picked up by contact with surfaces. Due to the unhygienic use of the toilets pathogens such as fecal coliform may cause disease and in adverse conditions it may bring outbreaks of infection (Maori *et al*., 2013). The ability of the pathogen deposited to survive on the different surfaces in the toilets poses a great risk of infection to the toilet users (Boon and Gerba, 2007). Non-porous surfaces, such as door handles, have the highest bacterial transfer rates to hands (Rusin *et al*., 2002).

1.7 Diseases transmitted by environmental surfaces:

Diseases commonly spread by means of environmental surfaces such as computers, classroom walls, toilets, chairs, and so on include the common cold, cold sores, conjunctivitis, giardiasis, impetigo, meningitis, pin worm disease, diarrhea and pneumonia (WHO, 1980). Bacteria such as *Escherichia coli*, *Shigella dysenteriae*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Staphylococcus aureus* as well as *Corynebacterium diphtheriae* cause diarrhoea, dysentery, pneumonia, skin infections, food poisoning and intoxication as well as whooping cough respectively (FAO, 1989; WHO, 1980).The organism and the diseases that can be transmitted

through the use of restrooms include boil and food borne diseases (*Staphylococcus aureus* and *Escherichia coli),* Urinary tract Infections (UTI) and diarhoea *(Escherichia coli, Pseudomonas aeruginosa)* and sore throat (*Streptococcus pyogenes)* (Peleg and Hooper 2010; Schmidt and Brubaker 2004).

1.8 Antibiotic resistance:

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. Multidrug resistance refers to antimicrobial resistance shown by the organisms to multiple antimicrobial drugs usually at least two or more than two antibiotics.

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, Acinetobacter baumannii, Pseudomonas aeruginosa*

1.8.1 Antibiotic resistance in bacteria:

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.

1.8.2 Mechanisms of multi-drug resistance:

Antibiotic resistance genes might be transferred to pathogenic bacteria infecting humans, particularly under the selection pressure of antibiotics as well as via the "SOS" response (Beaber *et al.*, 2002; Ubeda *et al.*, 2005).

Microorganisms employ several mechanisms to acquire multidrug resistance:

- No longer depending on a glycoprotein cell wall
- Enzymatic destruction or inactivation of antibiotics
- Decreased cell wall permeability to antibiotics
- Altered target sites of antibiotic
- Rapid efflux mechanisms to remove antibiotics
- Increased mutation rate as a stress response

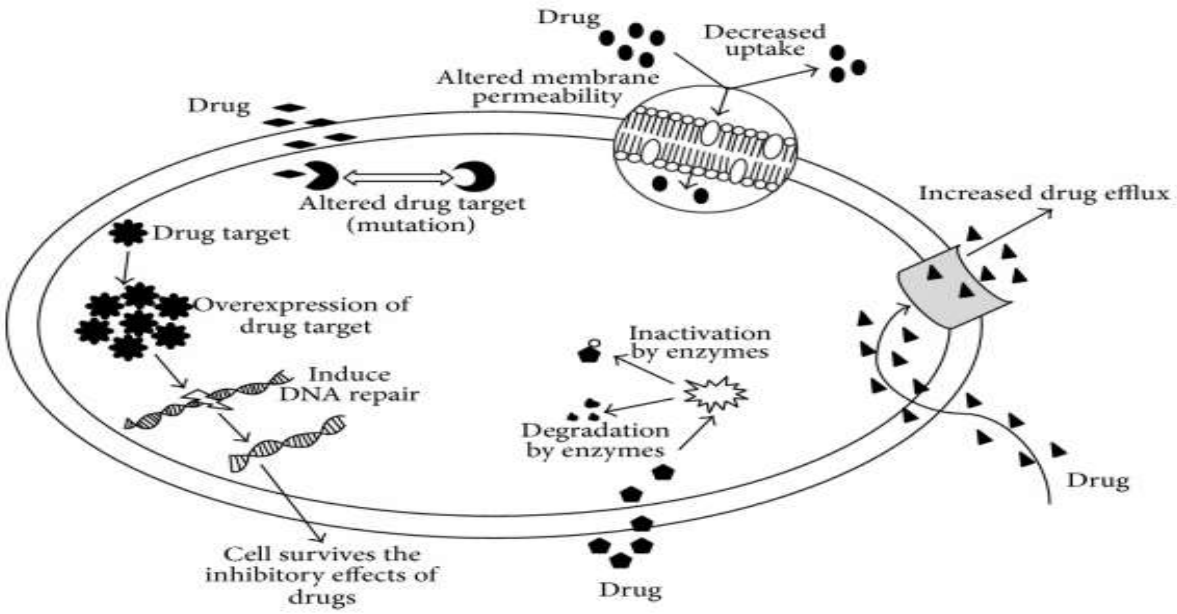


Figure 1.1: Mechanisms of multi-drug resistance (Jyoti *et al,* 2014)

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

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)

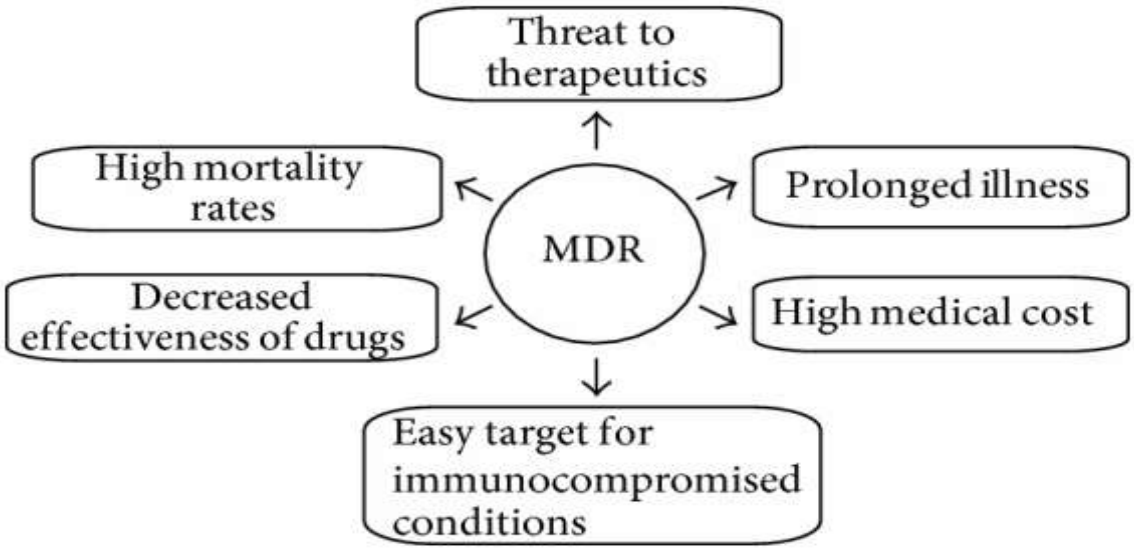


Figure 1.2: Problems associated with multi-drug resistance (Jyoti *et al,* 2014)

1.9 Plasmids:

Plasmids are self-replicating extra chromosomal DNA molecules found in Gram-negative and Gram-positive bacteria as well as in some yeast and other fungi. Plasmids are not essential for the survival of bacteria, but they encode a wide variety of genetic determinants, which permit their bacterial hosts to survive better in an adverse environment or to compete better with other microorganisms occupying the same ecological niche.

1.9.1 Plasmid architecture:

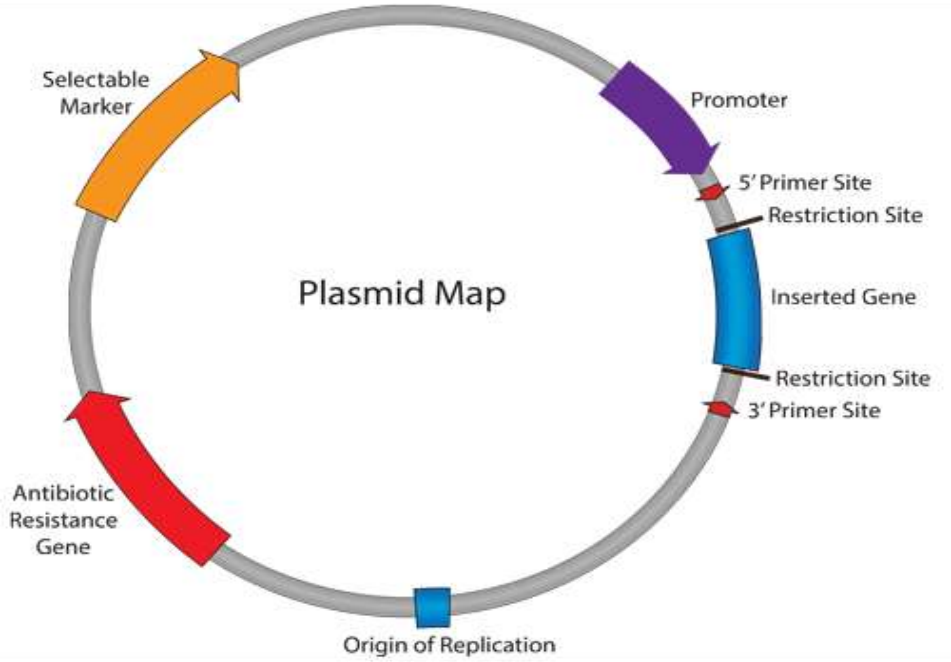


Figure 1.3: Plasmid Map (Millan *et al*, 2016)

Table 1.2: Vector elements of plasmid (Monroe.R.M., 2014)

Vector elements	Description
Origin of replication (ORI)	DNA sequence which allows initiation of replication within a plasmid by recruiting transcriptional machinery proteins
Antibiotic resistance gene	Allows for selection of plasmid-containing bacteria.
Multiple cloning site (MCS)	Short segment of DNA which contains several restriction sites allowing for the easy insertion of DNA. In expression plasmids, the MCS is often downstream from a promoter.
Promoter region	Drives transcription of the target gene. Vital component for expression vectors: determines which cell types the gene is expressed in and amount of recombinant protein obtained.
Selctive marker	Selective marker is required for suceessful cloning of plasmid into other bacterial cells.
Primer binding site	A short single-stranded DNA sequence used as an initiation point for PCR amplification or sequencing. Primers can be exploited for sequence verification of plasmids.

1.9.2 Size and copy number:

Plasmids range from about 1.0 kb for the smallest to over 250 kb for the largest plasmids.

The copy number refers to the number of molecules of an individual plasmid that are normally found in a single bacterial cell.

1.9.3 Classification and types of plasmids:

By their ability to be transferred to other bacteria:

1. **Conjugative:**

   The sexual transfer of plasmids to another bacterium through a pilus. Those plasmids possess the 25 genes required for transfer.

2. **Non-conjugative**

   Non-conjugative plasmids do not initiate conjugation. They can only be transferred with the help of conjugative plasmids.

3. **Mobilizable**

   An intermediate class of plasmids are mobilizable. They carry a subset of the genes required for transfer. These plasmids can 'parasitise' another plasmid, transferring at high frequency in the presence of a conjugative plasmid

By function:

Plasmids can be further classified by their function:

1. **Fertility-F-plasmids**: Facilitate bacterial conjugation
2. **Resistance-(R) plasmids**: contain genes that can build a resistance against antibiotics or poisons.
3. **Col-plasmids**: contain genes that code for bacteriocins, proteins that can kill other bacteria.
4. **Degradative plasmids**: enable the digestion of unusual substances, e.g., toluene or salicylic acid.
5. **Virulence plasmids**: turn the bacterium into a pathogen.

1.9.4 Function of plasmids:

- Antibiotic resistance
- Antibiotic production
- Degradation of aromatic compounds
- Haemolysin production
- Sugar fermentation
- Enterotoxin production
- Heavy metal resistance
- Bacteriocin production
- Induction of plant tumors
- Hydrogen sulfide production
- Host controlled restriction and modification

1.9.5 Plasmid mediated antibiotic resistance:

Antibiotic sensitivity and resistance are often under the control of the bacterial chromosome. Frequently, however, an organism may exhibit resistance to one or several antibiotics as a dominant character determined by genes located on a plasmid, a relatively small, circular DNA molecule which replicates, with some degree of autonomy, in the bacterial cytoplasm. A new study conducted by the scientists at the University of Oxford has observed that plasmids play key role in spreading the major global health threat of antibiotic resistance (Millan *et al*, 2016). The international team of researchers have found that plasmids which are known to be a vehicle for transferring antibiotic resistance genes, can accelerate the evolution of new forms of resistance and making them more significant to the process than previously thought. Various important resistance genes are present on plasmids. Plasmids are capable of moving between bacteria and act as a medium to transfer the resistance genes between bacteria. Acquisition of resistance genes through horizontal transfer facilitated by plasmid has been found to be ubiquitous in clinical pathogens (Mendal *et al.,* 2003, 2004). Plasmids can also act as evolutionary catalysts that accelerate the evolution of new forms of resistance. This occurs because bacteria usually carry more than one copy of a plasmid. These multiple copies of plasmids encoding resistance genes allow the host to rapidly evolve new functions such as the ability to degrade an antibiotic (Millan *et al*, 2016). Moreover plasmids automatically amplify the number of copies of these new and improved resistance genes. During antibiotic treatment, bacteria with resistance genes have a higher reproductive rate than sensitive bacteria, and, as a result, the use of antibiotics causes the spread of resistance genes. Plasmids can be transformed into neighboring bacterial cells through conjugation and can produce a whole resistant colony or even resistant strain. Most of the resistance plasmids are conjugative which means they encode the functions necessary to promote cell-to-cell DNA transfer, particularly their own transfer. Plasmid-encoded resistance has been observed for virtually all classes of antibiotics and in a wide variety of Gram-positive and Gram-negative organisms; many antibiotics are no longer effective due to such plasmid-encoded resistance (Bennett, P.M. 2008).

1.9.6 R (Resistance)-plasmids:

Resistance plasmids or R factors are the plasmids that carry genes conferring antibiotic resistance. R factors carry mainly two groups of genes:

- **Resistance transfer factors (RTF):** includes genes for plasmid replication and conjugation.
- **R-determinant**: has the resistance genes which codes for the production of enzymes that inactivate certain drugs or toxic substances.

R plasmids resist various antibiotics. When a bacterial population is exposed to an antibiotic, individuals with the R plasmid will survive and increase in the overall population.

R-Factors specify the formation of sex pilus which is a filamentous appendages on the cell surface. These promote bacterial conjugation and permit the transfer of a copy of the plasmid from the resistant organism to one which may previously have been drug-sensitive. Each ex-conjugant is then capable of acting as a plasmid donor during subsequent pairings. R factors are commonly responsible for the epidemic spread of multiple drug-resistance throughout an entire bacterial population. This can present serious problems in antibiotic therapy, particularly as plasmids are often transmissible between organisms of different species, and even different genera.

In some cases, acquisition of resistance genes within single plasmid is quite remarkable. For example, resistance plasmid R100 carry resistance genes for sulfonamides, streptomycin, chloramphenicol, and tetracycline. This particular plasmid can be transferred between a number of enteric species including *E.coli, Klebsiella* and *Salmonella.*

Transfer of resistance genes between the plasmids within the bacterium:

- By transposons
- By integrons

1.10. Hand hygiene programs:

Hand Hygiene applies to hand washing, antiseptic hand wash, antiseptic hand rub, or surgical hand antisepsis (Jasmine, A. and Iyer. H. R., 2013). Hand washing is fundamental cautionary measure to protect against the transmission of diseases and is one of the primary practices to reduce the transfer of bacteria from person to person, or from person to food contact surfaces (Chinakwe *et al*., 2012). It is established that unwashed hands can transmit pathogens, especially fecal pathogens, to food product after visit to the toilet. Investigation of food borne illness showed that poor personal hygiene, primarily ineffective hand washing is an important contributor to foodborne illness (Lambrechts *et al*., 2014).

Contamination by hands or environmental objects due to human involvement harbor microorganisms that increase the risk of illness among students. In order to reduce the risk of bacterial infection form the toilets, regular hand washing and cleaning of toilets with disinfectants is particularly recommended for infection control programs. Closing the toilet seats can also reduce the number of microorganisms released into the air (Schmidt and Brubaker, 2004). In view of the problems associated with the level of hygiene in most of the universities in Dhaka city there was a need to determine the type of microorganisms that are associated with the contact surfaces of the universities. This study is expected to highlight the problem of toilet door handles contamination and to raise awareness about hand washing programs among the students of BRAC University.

1.11 Literature review:

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

Maori *et al*., (2011) carried out an investigation on the prevalence of bacterial organisms on toilet door handles in secondary schools in Bokkos L.G.A., Jos, Plateau State, Nigeria. A total of about 120 samples were collected and cultured for bacterial isolates, 40 from each of the secondary schools (Government Secondary School Bokkos, All nation academy and Government secondary School Mushere). Out of the 120 samples 60 (50%) showed growth and 60 showed no growth at all. The organisms that were isolated in this investigation include *Staphylococcus* species (43.3%), *Candida specie*s (10%), *Escherichia coli* (16.7%), *Citrobacter* species (1.7%), *Klebsiella* species (20%), *Proteus* species (6.7%) and *Salmonella species* (1.7%). The result showed that G. S.S. B

has the highest contamination (48.3%) followed by All Nations Academy (30%) and the lowest contamination was found in G. S. S. M (21%).

Badhaim *et al*., (2011) conducted a research on distribution and prevalence of bacteria found on the door handles of Olin Hall, Drake University. They indicated that the door handles may assist in the spread of microbes between individuals and that they may be a reservoir of microbial contamination. In their experiments, they evaluated the prevalence of Gram negative bacteria that were found on door handles of Olin Hall. It was hypothesized that during times where the building was near its peak usage, a larger percentage of the bacteria sampled from the door handles of Olin Hall would be Gram negative. According to the result 49% Gram negative bacteria was found among the total microbial colonies isolated.

Nworie *et al*., (2012) investigated bacterial contamination of door handles/ knobs in selected public conveniences in Abuja Metropolis, Nigeria. Door handles/ knobs of public conveniences such as public offices, motor parks, and markets in Abuja metropolis were investigated for bacterial contamination. Out of the 180 samples, 156 (86.7%) were found positive. More positive samples were found in female toilet handles/knobs (41.7%) and bathroom door handles/knobs (11.5%) than males. In this study most of the bacterial contaminants were found coliforms. The isolated organisms were *Staphylococcus aureus* (30.1%), *Klebsiella pneumoniae* (25.7%), *Escherichia coli (*1%), *Enterobacter* species (11.2%), *Citrobacter* species (7.1%), *Pseudomonas aeruginosa* (5.9%), and *Proteus* species (4.5%). The study found higher rate of contamination in toilet door knobs/handles of markets, motor parks and restaurants compared to government offices and banks.

Lorina *et al*., (2015) carried out a research on potential pathogenic bacterial contaminants of shared utility devices in a university setting at Al-Hofuf, Saudi Arabia. Samples were collected from office and toilet doors handles/knobs, washroom tap heads, elevator buttons and compuer keyboards. Antibiotic susceptibility test as well as minimum inhibitory concentrations (MICS) were also determined. The bacterial contaminants found were *Staphylococcus aureus* (4.02%), *Staphylococcus haemolyticus* (18.59%), *Staphylococcus epidermidis*(21.10%), other *Staphylococcus* species (51.76%), *Enterococcus faecalis* (2.01%), *Enterococcus* species (1.51%), *Klebsiella pneumoniae* (0.50%), *Streptococcus sanguins* (0.50%), *Pseudomonas aeruginosa* (14.03%), *Pseudomonas stutzeri* (3.5%), *Pseudomonas luteola* (10.53%), and *Pantoea* spp. (72%).

The study also found multidrug resistant organisms among the isolates which indicates the source of infection in the university community.

Augustino *et al*., (2014) determined the bacterial load and antibiotic susceptibility of bacteria isolated from students toilets at Sokoine University of Agriculture, Morogoro, Tanzania. About 60 samples were collected from 30 different toilets in different surfaces including toilet seats, toilet bowls, door handles in and out of the restrooms, faucet handles, toilet flush handles and the restroom floors. The following bacteria species were isolated from this research; *Staphylococcus aureus* (25%), *E.coli* (36.7%), *Pseudomonas aeruginosa* (13.3%), *Streptocoocus pyogenes* (6.7%), *Proteus mirabilis* (6.7%) and *Klebsiella pneumoniae* (11.6%). The results indicated that surfaces routinely touched with hands had the highest bacterial contamination compared to restroom floor and toilet seats. Results of antibiotic susceptibility testing indicated that all bacterial isolates were resistant and intermediate resistant to at least one antibiotic.

Opere *et al* (2013) conducted a study on antibiotic susceptibility and plasmid profile analysis of pathogenic bacteria isolated from environmental surfaces in public toilets. Samples were collected from door handles, tap handles and flush handles of a public toilet. A total of eight (8) organisms were isolated which include *Bacillus spp (4.35%), Staphylococcus aureus (34.72%), Staphylococcus epidermidis (34.72%), Micrococcus spp. (4.35%), Pseudomonas spp. (8.70%), Enterococcus faecalis (4.35%), Salmonella typhi (4.35%)* and *Shigella dysenteriae (4.35%).* Results of antibiotic susceptibility testing revealed that *Salmonella typhi* had the highest level of multidrug resistance, showing resistance to all eight antibiotics tested. *Bacillus spp.* and *Pseudomonas spp*. Showed susceptibility to five of the eight antibiotics. Plasmid profile analysis of the isolates revealed the absence of plasmids.

Omar B Ahmed and Bashir Sirag (2016) conducted a research on microbial contamination of door knobs in public toilets during Hajj. Samples were taken from door knob surfaces of 224 randomly selected toilets in Arafat, Muzdalifah and Mina places. Bacterial Contamination was found in (78.3%) of doorknobs. The highest number of contamination was found in Muzdalifah (100%) followed by Arafat (73.3%). The study revealed the presence of both Gram positive (49.2%) and Gram negative bacteria (35.0%). The isolates that were found include *Staphylococcus aureus* (22%), Coagulase negative Staphylococci (CoNS) (17.3%) and *Acinetobacter* (10%). Out of 42 *S. aureus* isolates (16.7%) were found to be MRSA (positive for

mecA genes) and (31 %) were positive for PVL. The mecA and PVL genes of *Staphylococcus* isolates were detected by PCR.

Sabra (2013) investigated bacterial public health hazard in the public female restrooms at Taif, KSA. Total 260 specimens were collected from each restrooms (RR) differentiated as follow: RR Door (No. =20), RR Handle (No. =20), RR Sink (No. =20X3=60), RR Toilet Door (No. =20X4=80) and RR Toilet Handle (No. =20X4=80). Positive results were found in 187 samples including RR Toilet Handle in 73/80 (91.3%), RR Toilet Door in 59/80 (73.8%), RR Sink in 38/60 (63.3%), RR Handle in 10/20 (50%), finally lower positive were found from RR Door in 7/20 (35%). The isolated bacteria arranged according to their percentage as *Staphylococcus aureus* 76/187 (40.6%), *Escherichia coli* 42/187 (22.5%), *Bacillus* spp. 40/187 (21.4%), *Klebsiella pneumoniae* 25/187 (13.4%), *Enterococcus faecalis* 18/187 (9.6%),*Citrobacter* spp. 16/187 (8.6%), *Pseudomonas aeruginosa* 13/187 (7%) and *Proteus mirablilis* 10 /187 (5.3%).

Maryam *et al*., (2014) aimed at characterization and determination of antibiotic susceptibility pattern of bacteria isolated from some fomites (tables, chairs, pens, stethoscopes, uniforms, doorknobs and IVF stands) in a teaching hospital in northern Nigeria. 35 samples were used for this study among which 23 (65.7%) isolates were obtained. The ratio of Gram positive to Gram negative bacteria was 12:11. The bacteria isolated were *Staphylococcus aureus* (21.7%), *Staphylococcus epidermidis* (8.7%), *Streptococcus* spp. (8.7%), *Bacillus* spp. (13.0%), *Escherichia coli* (26.1%), *Pseudomonas* spp. (8.7%) and *Klebsiella* spp. (13.0%). The isolated bacteria were found susceptible to the antibiotics used and all were susceptible to erythromycin and streptomycin.

J.A. Otter and G.L French (2009) carried out a study to investigate bacterial contamination on hand-touched surfaces in the public transport system and in public areas of a hospital in central London. Total 118 samples were collected from hand-touched surfaces in buses, trains, stations, hotels and public areas of a hospital. Total aerobic counts were determined, and *Staphylococcus aureus* isolates were identified and characterized. The study revealed that hand-touch sites in London are frequently contaminated with bacteria and can harbor MSSA (8%) but none of the sites tested were contaminated with MRSA.

Adewoyin *et al*., (2013) evaluated antibiotic resistance profile of microbial isolates of toilet bowl of some students' hostels in Ogbomoso, Nigeria. A total of fifteen bacterial isolates were identified

from toilet bowl of six prominent student hostels. Out of the fifteen bacteria isolates, the genus *Streptococcus* was highly dominant with highest prevalence (19.36%) followed by *Streptococcus faecium* and least (3.23%) by *S. pyogenes* and *S. zymogenes*. *Pseudomonas aeruginosa* showed highest resistance (90%) and *Streptococcus zymogenes* showed lowest resistance (20%) to antibiotics tested. About 80% of all the isolates were found resistant to augmentin while 26.67% resisted pefloxacin and chloramphenicol. The highest and lowest total bacterial counts (TBC) were 33.90±4.23 x 10 and 9.00±1.80 x10CFU/ml, respectively.

1.12 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 toilet door knobs/handles 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. As plasmid carries many antibiotic resistance genes so the study also aimed at investigating the presence of plasmid in multi-drug resistant organisms and to establish plasmid mediated antibiotic resistance. Besides the purpose of this study was also to raise awareness about hand hygiene and hand washing programs among the students of BRAC University.

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

- Isolating the bacterial contaminants present in the door knobs of female restrooms of BRAC University.
- 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.
- Plasmid profile analysis of the bacterial isolates to determine whether there is any correlation between plasmid and antibiotic resistance phenomenon.

Chapter 2

Materials and Methods

Materials and Methods:

2.1 Study area:

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.

2.2 Study duration:

The study was conducted during the period January-September, 2017.

2.3 Sample size:

A total of about 15 toilet door knobs samples were collected from the female toilet door knobs of BRAC University.

2.4 Materials:

2.4.1 Equipment:

Equipment that were used in this study include:

- Laminar airflow cabinet (Model-SLF-V, vertical, SAARC group Bangladesh)
- Incubator (Model-0SI-500D, Digi system Laboratory Instruments Inc. Taiwan)
- Vortex machine (Digi system Taiwan, VM-2000)
- Autoclave machine (Model: WIS 20R Daihan Scientific Co. ltd, Korea)
- Centrifuge machine
- Gel apparatus
- Glass wares, Laboratory distillation apparatus- fractional distillatory set up, Microscope, Petri-dishes, Test-tubes, Micro-pipettes, Bunsen burner, Electric balance, etc.

2.4.2 Culture Media:

Culture media used for bacterial isolation and identification include**:**

2.4.2.1 MacConkey Agar:

MacConkey agar is a selective and differential media used for the isolation and differentiation of non-fastidious gram-negative rods, particularly members of the family Enterobacteriaceae. It also can distinguish between lactose fermenting from non-fermenting bacteria. After 24-48 hours at

37ºC of incubation period, *E.coli* and *Klebsiella* will produce pink colonies. Bacteria which can't ferment lactose like *Pseudomonas aeruginosa*, *Salmonella* species, and *Proteus* species will appear colorless on the medium and the agar surrounding the bacteria remains relatively transparent.

2.4.2.2 Mannitol salt Agar (MSA):

Mannitol Salt Agar is used as a selective media for the isolation of pathogenic *Staphylococci*. *S.aureus* ferment mannitol and produce yellow colored colonies surrounded by yellow zones. Non mannitol fermenters such as *S.epidermidis* will give colorless colonies and the media will remain red. MSA is also used to differentiate between *S.aureus* and *S.epidermidis*.

2.4.2.3Membrane fecal coliform agar (MFC):

M-FC Agar Base is used for the detection and enumeration of fecal coliforms at higher temperature (44.5°C). After 24-48 hours incubation Fecal Coliforms will form blue colored colonies whereas non fecal-coliforms will form gray colored colonies on M-FC Agar Base.

2.4.2.4 Nutrient Agar (NA):

Nutrient Agar is used for the cultivation of microbes supporting growth of a wide range of non-fastidious organisms. Nutrient agar is popular because it can grow a variety of types of bacteria and fungi, and contains many nutrients needed for the bacterial growth.

2.4.2.5 Eosine Methylene Blue Agar (EMB):

This media can differentiate among lactose fermenters and lactose non fermenters bacteria. In case of lactose fermenters such as *E.coli*, the colonies will be blue/black in color with a metallic green sheen and for lactose non fermenters colorless, transparent colonies will be obtained. Other coliform such as *Enterobacter aerogenes* can also ferment lactose and grow on EMB media. They will give thick mucoid pink colored colonies.

2.4.2.6 Hi-Crome agar:

This agar media is selective for urine infection causing microorganisms such as *Klebsiella pneumonia, Enterococcus fecalis, Staphylococcus aureus, Proteus mirabilis, E.coli, Pseudomonas aeruginosa* and they produce distinctive different colors on media. *E.coli* gives pink-purple colonies, *Staphylococcus aureus* gives golden yellow colonies, *Proteus, Morganella and Providencia* give brown colonies, *Enterococcus faecalis* produce blue colonies, *Klebsiella*

pneumoniae produce blue, mucoid colonies and *Pseudomonas* give colorless colonies on Hi-Crome agar after 24-48 hours of incubation.

2.4.2.7 Bacillus cereus Agar (BC Agar):

Bacillus Cereus Agar Base with added supplements is used as a selective medium for the isolation and enumeration of *Bacillus cereus*. Colonies of *Bacillus cereus* give turquoise to peacock blue colour surrounded by a good egg yolk precipitate of the same color. Other species of *Bacillus* are also able to grow in BC agar but they will produce green colonies.

2.4.2.8 Blood agar (BA):

Blood Agar (BA) is an enriched medium used to culture those bacteria or microbes that do not grow easily. Such bacteria are called fastidious as they demand a special, enriched nutritional environment compared to the routine bacteria. Blood Agar is used to grow a wide range of pathogens particularly those that are more difficult to grow such as *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Neisseria* species. It is also a differential media in allowing the detection of hemolysis (destroying the RBC) by cytolytic toxins secreted by some bacteria, such as certain strains of *Bacillus, Streptococcus, Enterococcus, Staphylococcus, and Aerococcus*. It is used to see the lysis of red blood cells by the organisms. Usually three types of hemolysis are found including alpha hemolysis, beta hemolysis and gamma hemolysis. Hemolysis is determined by observing the clear zones around the bacterial growth.

2.4.3 Biochemical test media:

Table 2.1: Media used for biochemical tests

Media used for biochemical tests
✓ **Indole broth**
✓ **Methyl Red (MR) broth**
✓ **Voges-Proskauer (VP) broth**
✓ **Simmons citrate agar**
✓ **Triple Sugar Iron (TSI) agar**
✓ **Motility Indole Urease (MIU) agar**
✓ **Nitrate reduction broth**

2.4.4 Stock culture media:

a. Tryptone soya broth glucose glycerol (TGG):

This is a broth for stock culture of microorganisms. One liter broth requires 30gm tryptone soya broth, 5gm glucose and 100ml glycerol.

b. T1N1 agar:

T1N1 media were used for short term preservation of bacterial culture. One hundred milliliter of T1N1 media contains 1gm tryptone, 1gm sodium chloride and 0.7gm agar powder.

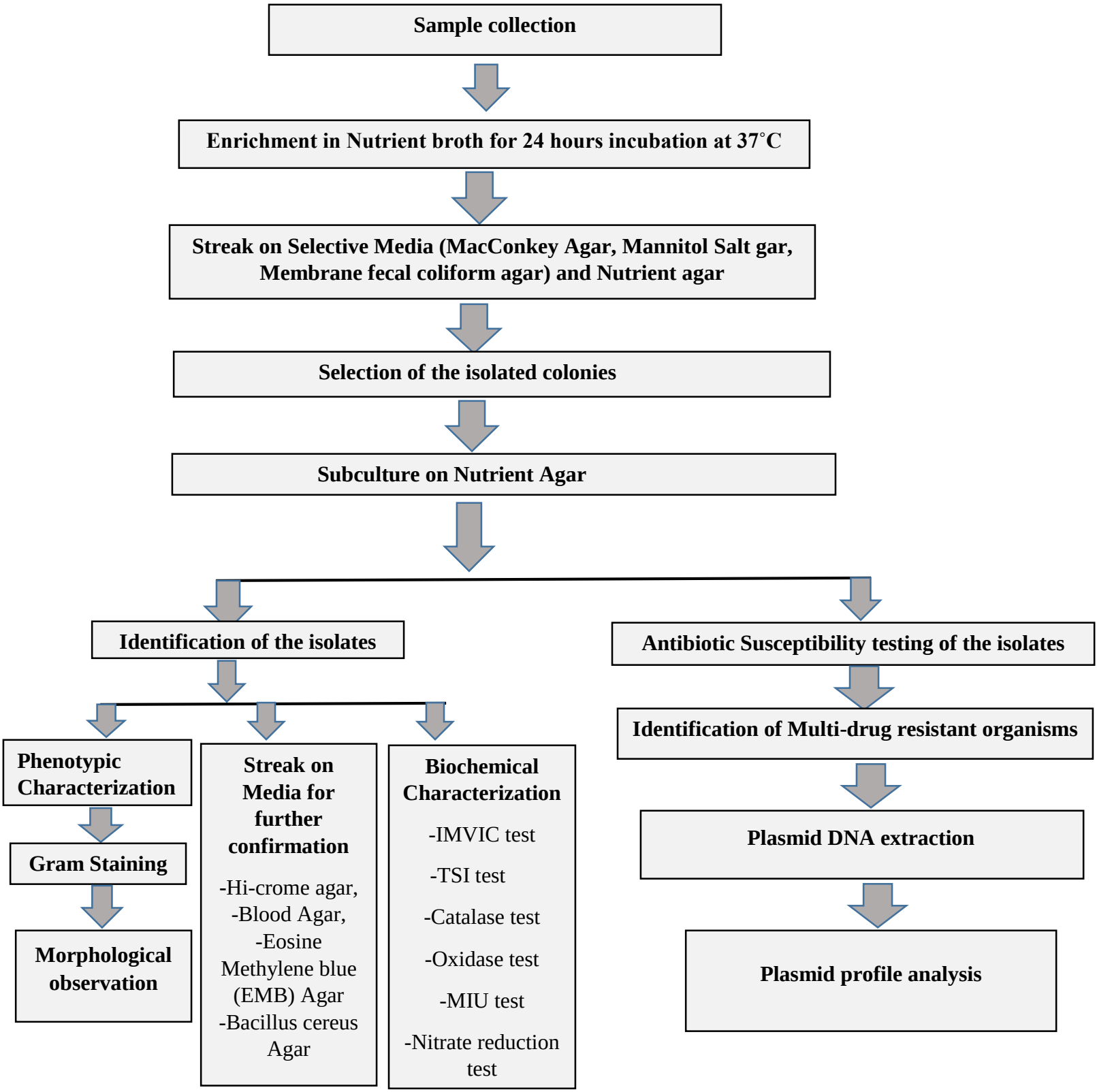
2.4.5 Antibiotics:

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 2.2: List of antibiotics and their zone ranges

Serial no	Antibiotic	Disc code	Disc potency (µg)	Inhibition Zone diameter (in mm)		
				Resistant	Intermediate	Susceptible
1	**Amoxicillin**	AML	10	≤13 / ≤19	14-17	≥18 / ≥20
2	**Ciprofloxacin**	CIP	5	≤15 / ≤20	16-20/ 21-30	≥21 / ≤31
3	**Chloramphenicol**	C	30	≤12	13-17	≥18
4	**Gentamycin**	CN	10	≤12	13-14	≥15
5	**Methicillin**	MET	5	≤9	10-13	≥14
6	**Penicillin-G**	P	10	≤23	-	≥29
7	**Rifampicin**	RD	5	≤16	17-19	≥20
8	**Streptomycin**	S	10	≤11	12-14	≥15
9	**Trimethoprim-sulphamethoxazole**	SXT	5	≤10	11-15	≥16
10	**Tetracycline**	TE	30	≤11	12-14	≥15

2.5 Flow chart of the overall study design



2.6 Methods:

2.6.1 Sample collection:

The samples were collected from toilet door knobs/handles using the swab-rinse method of the American Public Health Association as described by Reynols *et al*., 2005. The samples were collected at noon when people made use of the toilets to maximize the chances of isolation (Amala *et al*., 2015). Door knobs were swabbed with sterile cotton swabs moistened with sterile normal saline. The swab was wiped firmly on the entire surface of the door knob. 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. All the samples were collected in the same procedure.

Table 2.3: Sample Collection: Source, Time, Temperature, Number of the isolates found and their given name in the study

Sample No	Source	Date	Time	Temperature	Number of the isolates found	Isolates ID
1	**University Building 2 (First floor)**	9.01.2017	3:00 pm	21ºC	4	1(a), 1(b), 1(c), 1(d)
2	**University Building 2 (Second floor)**	9.01.2017	3:15 pm	21ºC	2	2(a), 2(b)
3	**University Building 2 (Fourth floor)**	9.01.2017	3:30 pm	21ºC	1	4
4	**University Building 2 (Fifth floor)**	21.03.2017	3:30 pm	25ºC	3	5(a), 5(b), 5(c)
5	**University Building 2 (Seventh floor)**	21.03.2017	3:45 pm	25ºC	3	7(a), 7(b), 7(c)

Table 2.3: Sample Collection: Source, Time, Temperature, Number of the isolates found and their given name in the study

Sample No	Source	Date	Time	Temperature	Number of the isolates found	Isolates ID
6	**University Building 2 (Eighth floor)**	21.03.2017	4:00 pm	25ºC	2	8(a), 8(b)
7	**University Building 2 (Ninth floor)**	21.03.2017	4:15 pm	25ºC	3	9(a), 9(b), 9(c)
8	**University Building 2 (Eleventh floor)**	21.03.2017	4:30 pm	25ºC	2	11(a), 11(b)
9	**University Building 4 (Tenth floor)**	23.05.2017	3:30 pm	29ºC	2	12(a), 12(b)
10	**University Building 4 (Seventh floor)**	23.05.2017	3:50 pm	29ºC	1	13(a)
11	**University Building 4 (First floor)**	23.05.2017	4:10 pm	29ºC	3	14(a),14(b), 14(c)
12	**University Building 4 (Third floor)**	23.05.2017	3:30 pm	29ºC	2	15(a), 15(b)
13	**University Building 1 (First floor)**	10.07.2017	3:46 pm	32ºC	3	16(a),16(b), 16(c)
14	**University Building 1 (Second floor)**	10.07.2017	4:10pm	32ºC	2	17(a), 17(b)
15	**University Building 1 (Forth floor)**	10.07.2017	4:20 pm	32ºC	4	18(a),18(b), 18(c),18(d)

2.6.2 Sample Analysis:

The collected samples were processed to identify the bacteria in sample. The following processing techniques were applied:

1. Culture
2. Gram staining
3. Biochemical tests

2.6.2.1 Culture technique:

After 24 hours, each sample was streaked onto Nutrient agar, MacConkey agar, Mannitol salt agar and Membrane fecal coliform agar plates. Here, using the swab stick, a primary streak was made while secondary and tertiary streaks were made from the primary streak in parallel pattern with the aid of a sterilized wire loop to make a four-quadrant streak plate technique. All the plates were incubated for 24 hours at 37ºC. After the overnight incubation, the plates were removed from the incubator and presumptively observed for colony characteristics. Isolated colonies were then sub-cultured onto fresh nutrient agar. Single isolated colonies from nutrient agar plates were subjected to Gram staining, Spore staining and Standard Biochemical tests to identify the organism.

2.6.2.1.1 Streak plate method:

Streak plate technique is used for the isolation of pure culture of the organisms from mixed population. It is necessary to study the colony morphology of an organism to perform the biochemical tests needed to identify the organism.

Materials needed for streak plate method:

- A source of bacteria (stock culture, previously streaked agar plate or any other inoculum)
- Inoculating loop
- Bunsen burner
- Agar plate (Nutrient agar or any other agar medium)

Procedure:

Four quadrant streaking:

1. The inoculating loop is sterilized in the Bunsen burner by putting the loop into the flame until it is red hot. Then the loop is allowed to cool.
2. The inoculating loop is inserted into the test-tube containing bacterial culture and some of the inoculum is taken with the help of the loop.
3. The inoculating loop is streaked immediately very gently over a quarter of the plate using a back and forth motion.
4. The loop is flamed again and is allowed to cool
5. By going back to the edge of the area one which has been just streaked, the streaks are extended into the second quarter of the plate
6. The loop is flamed again and is allowed to cool
7. Going back to the edge of the area two which has been just streaked, the streaks are extended into the third quarter of the plate
8. The loop is not burned after streaking the third quadrant of the plate
9. The loop is touched over the surface of the third quadrant and zigzag line is drawn from the third quadrant
10. The loop is flamed and cooled.

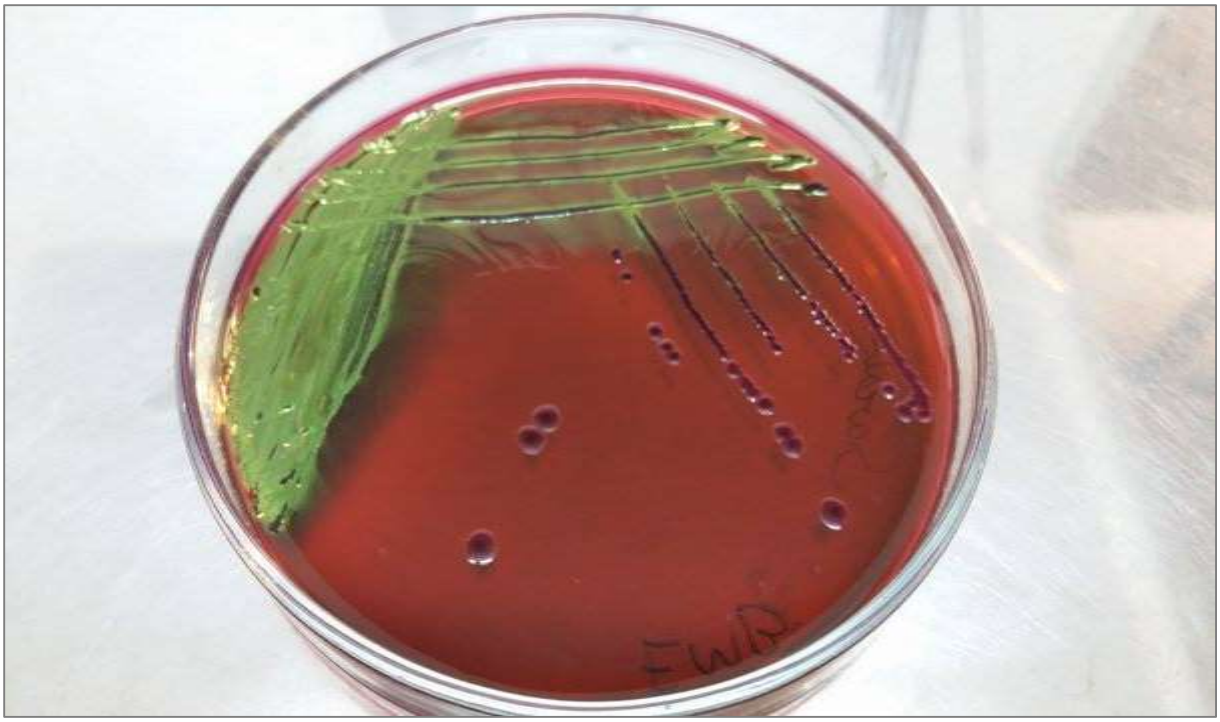


Figure 2.1: Four quadrant streaking on EMB agar plate

2.6.2.2 Gram staining:

Gram staining was done for differentiating between two principal groups of bacteria: Gram positive and Gram negative.

- A sterile microscopic glass slide was taken.
- A drop of saline was taken by the loop and added to the slide.
- A colony from fresh culture of the experimented bacteria was taken and was smeared on the glass slide with the saline. Then the smear was heat fixed and was allowed to dry for few minutes.
- One drop of crystal violet was added to the smear and after one minute, the crystal violet was gently washed off the glass slide with the tap water.
- Then one drop of Grams iodine was added and then after one minute the Grams iodine was gently washed off the slide with the tap water.
- Few drops of 70% ethanol was added and was washed immediately.
- One drop of Safranin was added and after 45 seconds it was washed off the glass slide.
- The slide was allowed to dry off completely, after which it was observed under the microscope.

2.6.2.3 Spore staining:

Spore staining was done to determine whether the bacteria was endospore forming or not.

- A sterile microscopic glass slide was taken.
- A drop of saline was taken by the loop and added to the slide.
- A colony from fresh culture of the experimented bacteria was taken and was smeared on the glass slide with the saline. Then the smear was heat fixed and was allowed to dry for few minutes.
- The slide was placed over a water bath and malachite green was added continuously so that the dye did not dry out.
- The slide was heated for 2 to 3 minutes.
- After heating, the slide was cooled and rinsed thoroughly with tap water.
- Then the smear was stained with safranin for 30 seconds, washed with tap water and blot dried with bibulous paper.
- Finally, bacterial observation was made under the oil immersion lens (1000X) for the presence of endospores.

2.6.2.4 Biochemical tests:

2.6.2.4.1 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 at 37ºC.
- 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)

2.6.2.4.2 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 at 37ºC.
- 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).

2.6.2.4.3 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 at 37ºC.
- 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)

2.6.2.4.4 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)

2.6.2.4.5 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 2.4: 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

2.6.2.4.6 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 reagent 3% 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).

2.6.2.4.7 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).

2.6.2.4.8 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 overnight incubation. Then the colour of the media were 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).

2.6.2.4.9 Nitrate reduction test:

Nitrate reduction test was done to determine the ability or inability of the bacteria to reduce nitrate to nitrite or beyond the nitrite stage using anaerobic respiration by the enzyme nitrate reductase.

- 5 ml of nitrate broth containing peptone, beef extract, potassium nitrate was prepared.
- 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 at 37ºC for 24-48 hours.
- After 48 hours, five drops of nitrate reagent A and five drops of nitrate reagent B were added to all nitrate broth cultures.
- If red colour develops then it indicates nitrate positive result which means nitrate has been reduced to nitrite.

- If there was no red colour development, a small amount of zinc was added to each broth. If red colour develops after addition of zinc powder then it indicates nitrate negative result. (Cappuccino & Sherman, 2005)

2.6.2.5 Preparation of Stock Sample:

2.6.2.5.1 Short term preservation:

Three ml of T1N1 media were prepared into sterile vials. Colonies from the cultures to be preserved were touched by a needle from nutrient agar plates and stabbed onto the butt of the vials. Then the vials were incubated at 37^0C (SAARC) for 6 hours. After the incubation period was over, 200µl of paraffin oil was added into the surface of the medium contained in each of the vials. All the vials were carefully labeled and stored at room temperature.

2.6.2.5.2 Long term preservation:

Trypticase Soya broth media were prepared in a sterile cryovial. For long-term preservation, 500µl of bacterial culture was grown in Trypticase Soy Broth at 37^0C for 6 hours. After the incubation period, 500µl of sterile glycerol was added to the broth culture and the cryovial was stored at -20^0C.

2.7 Antibiotic susceptibility testing (AST):

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

2.7.1 Disk diffusion method:

Various methods can be used for antibiotic susceptibility testing but among them disk diffusion method is most common. Because of convenience, efficiency and cost, the disk diffusion method is probably the most widely used method for determining antimicrobial resistance. In this research work the antibiotic susceptibility testing of the organisms were performed by agar disc diffusion method and interpreted according to CLSI standards and guidelines (Wayne, 2009).

2.7.1.1 Preparation of McFarland Solution:

Preparation of McFarland standard solution is one of the important steps in antibiotic susceptibility testing. This solution is usually used as a reference in order to calibrate the turbidity of both the test and the control microorganisms. The turbidity of tested organism is compared to the turbidity of McFarland solution. One of the advantages of this technique is that it allows the tests to be

carried out with an equal concentration of the test and control microorganisms and avoids the possibilities of working with a high amount of the bacterial culture. Therefore it ensures that the number of bacteria is within a given range.

- 97% H_2SO_4 was diluted to a concentration of 1%
- $BaCl_2.2H_2O$ was diluted to a concentration of 1.175%
- To make 5 ml McFarland solution, 4975µl H_2SO_4 was mixed with 25µl $BaCl_2.2H_2O$.

2.7.1.2 Preparation of inoculum:

- Pure culture plate of one of the organisms to be tested was selected.
- Using a sterile loop a colony from the plate was aseptically emulsified in the tube containing sterile saline solution and it was mixed thoroughly to ensure that no solid material from the colony is visible in the saline solution.
- The tube was vortexed properly so that the suspension becomes homogenous.

2.7.1.3 Comparison with McFarland solution:

- Using the Colourimeter (Labtronics; ISO 9001: 2008 Certified) provided in the Microbiology and Biotechnology laboratory, the OD (optical density) of the McFarland solution was measured to be 11.
- The Colourimeter was also used to measure the OD of all the inoculums that were prepared. This was done to balance out the OD of these inoculums in the same range as the OD of the McFarland solution.
- The inoculums having the same OD to that of the McFarland solution were taken. The inoculums having a higher OD were diluted to bring the OD back into the reference range.

2.7.1.4 Inoculation of the Muller Hinton Agar (MHA) plates:

- Muller Hinton agar plates were prepared.
- A sterile cotton swab was taken and was dipped into the broth culture of the organism.
- The swab was squeezed against the inside of the tube in order to remove excess fluid in the swab.

- The swab was later streaked at least four to six times onto the dried surface of the MHA plate to make a lawn culture and to ensure that the cotton swab is touched entirely on the agar surface.
- After the streaking is complete the plate is allowed to dry for 5 minutes.

2.7.1.5 Placing the antibiotic discs on MHA plates:

- Sterilized forceps were used to place the antibiotic discs.
- After taking the discs, the discs were gently pressed onto the surface of the agar using flame sterilized forceps.
- Once all the discs were properly placed, the MHA plates were inverted and incubated at 37^0C for 24 hours.

2.7.1.6 Measuring zone size:

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

2.8 Plasmid profiling:

2.8.1 Plasmid DNA extraction:

Plasmid extraction of the isolates were done according to the modified hot alkaline method by Kado and Liu.

2.8.1.1 Modified hot alkaline method by Kado and Liu (Kado and Liu, 1981):

- 1.5 ml of fresh shaking bacterial culture was taken into micro centrifuge tubes.
- The tubes containing bacterial suspension were centrifuged at 14000 rpm for 5 minutes.

- After centrifugation, the supernatant was discarded as much as possible and the pellet was taken.
- 40µL Kado-I buffer was added to the pellet and was mixed properly by pipetting
- Then 80µL Kado-II buffer was added and was mixed by inverting the tubes (rolling 3 or 4 times).
- The tubes were placed in hot water bath at 55ºC for 1 hour.
- After 1 hour 250µL phenol chloroform mixture (1:1) was added to the tubes and was mixed well by upside down the tubes for 30 minutes.
- After 30 minutes the tubes were centrifuged at 14000 rpm for 5 minutes.
- After centrifugation, 3 layers will be observed in the tubes. Top layer containing plasmid DNA, middle layer containing the protein debris and the bottom layer containing the phenol
- Plasmid DNA is carefully removed from the top layer by micropipette and is transferred to a new tube.
- The new tube containing plasmid DNA is then stored at -20ºC for using it further in gel electrophoresis.

2.8.2 Plasmid profile analysis:

After performing the plasmid extraction of the bacterial isolates, the isolates were subjected to agarose gel electrophoresis in order to reveal the presence or absence of plasmids and also to study the molecular weight of the plasmids.

2.8.2.1 Agarose gel electrophoresis:

Agarose gel electrophoresis is the most widely used method for preparation and analysis of nucleic acids. It is the most effective way of separating and analyzing DNA molecules ranging from 100bp to 25kb. It separates the DNA based on their size and as DNA is negatively charged so it moves towards the anode.

1. Making agarose gel:

0.7% Agarose gel was made by combining agarose powder and TBE buffer solution. The solution was heated in micro oven so that agarose completely melts in the buffer solution. The final solution appeared clear without any undissolved particles.

2. Staining the gel:

Agarose solution was stained with staining agent ethidium bromide and was kept for 10-15 minutes so that the ethidium bromide is properly mixed with the solution.

3. Preparation of casting tray:

It was prepared by sealing the edges of the casting tray and putting the combs into it. The gel combs should not touch the surface of the tray.

4. Pouring the gel into casting tray:

The melted agarose solution was poured in the casting tray. Each of the gel combs should be submerged in the melted agarose solution. Then it was allowed to cool down so that the gel becomes solidified. When it was cooled completely, a flexible agarose gel was formed in the tray.

5. Placing the agarose gel in electrophoresis chamber:

The solidified agarose gel was placed carefully in the electrophoresis chamber. Enough electrophoresis buffer was added in the chamber to cover the gel to a depth of at least 1 mm. It was made sure that each well of the chamber was filed with buffer

6. Sample preparation and gel loading:

The micropipette was set to the appropriate volume and 10µL sample was taken. The sample was then mixed with 3µL tracking dye bromophenol blue.

7. Inclusion of Reference strain:

In the first and last wells the reference strain (plasmid of known sizes) was loaded. *E.coli* K12 strain V-517 was used as reference strain. This strain contained 8 plasmids of various sizes including 85 MDa, 35.6 MDa, 7 MDa, 4.8 MDa, 3.7 Mda, 3.4 MDa, 2 MDa, 1.8 MDa and 1.4 MDa. The rest of the wells were loaded with samples. Before loading every sample the tips of the pipette were changed.

8. Running the gel:

After the samples were loaded, the cover was placed down carefully. Then it was connected with the electrode terminals correctly. The plug of the black wire was inserted into the black input of the power source (negative input) and the plug of the red wire was inserted into the red input of the power source (positive input). The power source was set at 70V and the electrophoresis was conducted for approximately 1 hour.

9. Visualizing the plasmid DNA:

The staining gel containing the plasmid DNA sample was placed under UV trans-illuminator for visualization.

Chapter 3

Results

3.1 Bacterial isolation and identification:

A total of about 15 samples were collected from the door knobs of female restrooms of BRAC University. These samples were streaked on various selective, differential and nutrient media for identifying the organisms present in toilet door knobs. 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.

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

Isolates ID	Growth on Selective, Differential and Enriched Media							Colony morphology on Nutrient Agar					Suspected organism
	Mac-Conkey Agar	Mannitol Salt Agar (MSA)	Membrane Fecal Coliform Agar (MFC)	Eosine Methylene Blue Agar (EMB)	Bacillus Cereus Agar (BC Agar)	Hi-Chrome Agar	Blood Agar Hemo-Lysis	Size	Color	Form	Margin	Elevation	
1. 1(a)	-	Small, Yellow coloured colonies	-	-	-	Golden Yellow colour colonies	Beta Hemo-Lysis	Small	Yellow	Circular	Entire	Convex	***Staphylococcus* spp.**
2. 1(b)	Medium Pink coloured colonies			Metallic Green sheen colonies	-	Purple Colour colonies	Gamma Hemo-Lysis	Small	White	Circular	Entire	Raised	***E.coli***
3. 1(c)	Medium Pink coloured colonies			Metallic Green sheen colonies		Purple Colour colonies	Gamma Hemo-Lysis	Medium	Creamy	Circular	Entire	Raised	***E.coli***
4. 1(d)	-		Blue coloured Colonies				Gamma Hemo-Lysis	Medium	White	Circular	Entire	Raised	**Fecal coliform**
5. 2(a)		Small, yellow coloured colonies				Golden Yellow colour colonies	Beta Hemo-Lysis	Small	Yellow	circular	Entire	Convex	***Staphylococcus* spp.**
6. 2(b)					Blue Coloured colonies	Green Colour colonies	Beta-Hemo-Lysis	Large	White	circular	Entire	Convex	***Bacillus* spp.**

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

Isolates ID	Growth on Selective, Differential and Enriched Media							Colony morphology on Nutrient Agar					Suspected organism
	Mac-Conkey Agar	Mannitol Salt Agar (MSA)	Membrane Fecal Coliform Agar (MFC)	Eosine Methylene Blue Agar (EMB)	Bacillus Cereus Agar (BC Agar)	Hi-Chrome Agar	Blood Agar Hemo-Lysis	Size	Color	Form	Margin	Elevation	
7. 4	-		-	-	Blue Colour colonies	Green Colour colonies	Alpha Hemo-Lysis	Large	White	Circular	Entire	Convex	***Bacillus* spp.**
8. 5(a)	Medium, Pink Colour colonies			Metallic Green Sheen colonies		Purple Colour colonies	Gamma Hemo-Lysis	Medium	White	Circular	Entire	Convex	***E.coli***
9. 5(b)	Small, Pink Colour colonies			Metallic Green Sheen colonies		Purple Colour colonies	Gamma Hemo-Lysis	Small	Creamy	Circular	Entire	Raised	***E.coli***
10. 5(c)			Blue colour colonies				Gamma Hemo-Lysis	Medium	White	Circular	Entire	Raised	**Fecal coliform**
11. 7(a)		Small, yellow coloured colonies				Golden Yellow Colour colonies	Alpha Hemo-Lysis	Small	Yellow	Circular	Entire	Convex	***Staphylococcus* spp.**
12. 7(b)					Blue Colour colonies	Green Colour colonies	Alpha Hemo-Lysis	Large	White	Circular	Entire	Convex	***Bacillus* spp.**

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

Isolates ID	Growth on Selective, Differential and Enriched Media							Colony morphology on Nutrient Agar					Suspected organism
	Mac-Conkey Agar	**Mannitol Salt Agar (MSA)**	**Membrane Fecal Colform Agar (MFC)**	**Eosine Methylene Blue Agar (EMB)**	**Bacillus Cereus Agar (BC Agar)**	**Hi-Chrome Agar**	**Blood Agar Hemo-Lysis**	**Size**	**Color**	**Form**	**Margin**	**Elevation**	
13. 7(c)	-	Pinpoint, Yellow Colonies	-	-		Golden Yellow Colour colonies	Beta Hemo-Lysis	Pinpoint	Yellow	Circular	Entire	Raised	***Staphylococcus* spp.**
14. 8(a)		Small, Yellow colonies				Off-White colonies	Beta Hemo-Lysis	Small	Off White	Circular	Entire	Convex	***Staphylococcus* spp.**
15. 8(b)			Blue Colour Colonies				Gamma Hemo-Lysis	Medium	White	Circular	Entire	Raised	**Fecal coliform**
16. 9(a)					Blue Colour colonies	Green Colour colonies	Beta Hemo-Lysis	Large	White	Circular	Entire	Convex	***Bacillus* spp.**
17. 9(b)	Small, Pink Colour colonies			Metallic Green Sheen colonies		Purple Colour colonies	Gamma Hemo-Lysis	Small	White	Circular	Entire	Raised	***E.coli***
18. 9(c)			Blue Colour Colonies				Gamma Hemo-Lysis	Medium	White	Circular	Entire	Convex	**Fecal coliform**

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

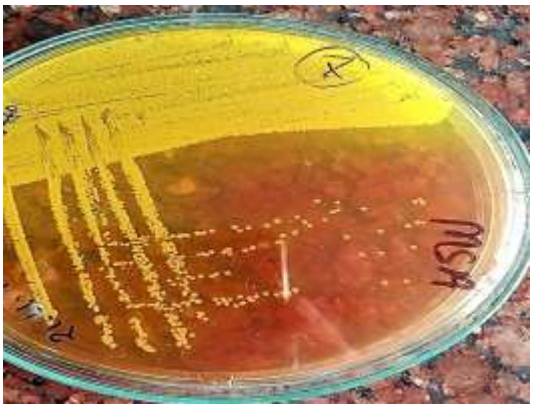
Isolates ID	Growth on Selective, Differential and Enriched Media							Colony morphology on Nutrient Agar					Suspected organism
	Mac-Conkey Agar	Mannitol Salt Agar (MSA)	Membrane Fecal Colform Agar (MFC)	Eosine Methylene Blue Agar (EMB)	Bacillus Cereus Agar (BC Agar)	Hi-Chrome Agar	Blood Agar Hemo-Lysis	Size	Color	Form	Margin	Elevation	
19. 11(a)	-		-	-	Blue Colour Colonies	Green Colour colonies	Beta Hemo-Lysis	Large	White	Circular	Entire	Convex	***Bacillus* spp.**
20. 11(b)		Medium, Yellow colonies			Beta Hemo Lysis	Golden Yellow	Beta Hemo-Lysis	Medium	Yellow	Circular	Entire	Convex	***Staphylococcus* spp.**
21. 12(a)		Pinpoint, Yellow colonies				Golden Yellow colonies	Beta Hemo-Lysis	Pinpoint	Yellow	Circular	Entire	Convex	***Staphylococcus* spp.**
22. 12(b)					Blue Colour colonies	Green Colour colonies	Beta Hemo-Lysis	Large	White	Circular	Entire	Convex	***Bacillus* spp.**
23. 13(a)		Medium, Light Yellow colonies				Cream Colour colonies	Beta Hemo-Lysis	Medium	Creamy	Circular	Entire	Convex	***Staphylococcus* spp.**
24. 14(a)		Pinpoint, White colonies				Golden Yellow colonies	Beta Hemo-Lysis	Pinpoint	White	Circular	Entire	Convex	***Staphylococcus* spp.**

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

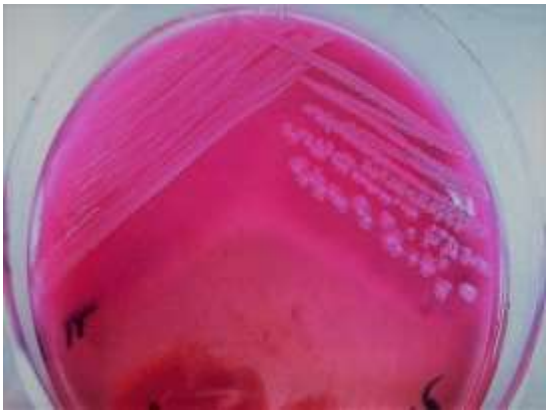
Isolates ID	Growth on Selective, Differential and Enriched Media							Colony morphology on Nutrient Agar					Suspected organism
	Mac-Conkey Agar	**Mannitol Salt Agar (MSA)**	**Membrane Fecal Colform Agar (MFC)**	**Eosine Methylene Blue Agar (EMB)**	**Bacillus Cereus Agar (BC Agar)**	**Hi-Chrome Agar**	**Blood Agar Hemo-Lysis**	**Size**	**Color**	**Form**	**Margin**	**Elevation**	
25. 14(b)	Medium, Pink Colonies		-	Metallic Green Sheen colonies		Purple Colour colonies	Gamma Hemo-Lysis	Medium	White	Circular	Entire	Raised	***E.coli***
26. 14(c)		Small, White Colonies				Yellow Colour colonies	Gamma Hemo-Lysis	Small	Yellow	Circular	Entire	Convex	***Micrococcus* spp.**
27. 15(a)					Blue Colour colonies	Green Colour colonies	Beta Hemo-Lysis	Large	White	Circular	Entire	Convex	***Bacillus* spp.**
28. 15(b)		Small, Yellow Colonies				Golden Yellow colonies	Beta Hemo-Lysis	Small	Yellow	Circular	Entire	Convex	***Staphylococcus* spp.**
29. 16(a)		Pinpoint, White Colonies				Cream Colour colonies	Alpha Hemo-Lysis	Pinpoint	White	Circular	Entire	Convex	***Staphylococcus* spp.**
30. 16(b)					Blue Colour colonies	Green Colour colonies	Beta Hemo-Lysis	Large	White	Circular	Entire	Convex	***Bacillus* spp.**

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

Isolates ID	Growth on Selective, Differential and Enriched Media							Colony morphology on Nutrient Agar					Suspected organism
	Mac-Conkey Agar	Mannitol Salt Agar (MSA)	Membrane Fecal Colform Agar (MFC)	Eosine Methylene Blue Agar (EMB)	Bacillus Cereus Agar (BC Agar)	Hi-Chrome Agar	Blood Agar Hemo-Lysis	Size	Color	Form	Margin	Elevation	
31. 16(c)			Blue Colour Colonies				Gamma Hemo-Lysis	Medium	White	Circular	Entire	Raised	**Fecal coliform**
32. 17(a)		Small, Yellow colonies				Cream Colour Colonies	Alpha Hemo-Lysis	Small	Creamy	Circular	Entire	Convex	***Staphylococcus* spp.**
33. 17(b)					Blue Colour colonies	Green Colour colonies	Beta Hemo-Lysis	Large	White	Circular	Entire	Convex	***Bacillus* spp.**
34. 18(a)		Small, White colonies				Cream Colour colonies	Alpha Hemo-Lysis	Small	White	Circular	Entire	Convex	***Staphylococcus* spp.**
35. 18(b)	Small, pink colonies			Metallic GreeSheen colonies		Purple Colour Colonies	Gamma Hemo-Lysis	Small	White	Circular	Entire	Raised	***E.coli***
36. 18(c)						Yellow Colour colonies	Gamma Hemo-lysis	Small	Yellow	Circular	Entire	Convex	***Micrococcus* spp.**
37. 18(d)					Blue colonies	Green colonies	Beta Hemo-Lysis	Large	White	Circular	Entire	Convex	***Bacillus* spp.**

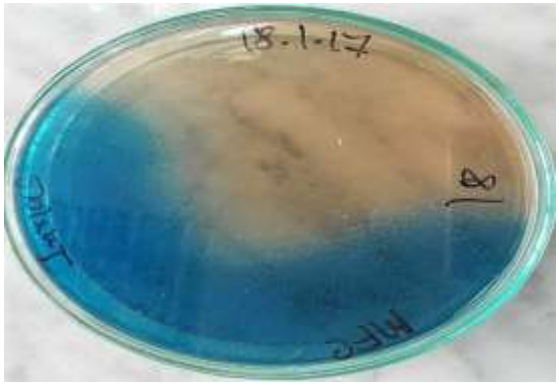


Growth of *Staphylococcus* species on MSA



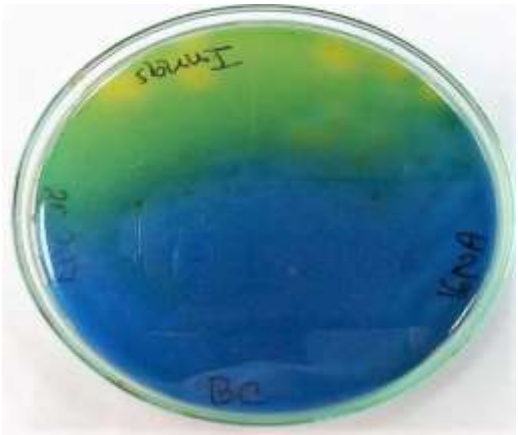
Growth of *E.coli* on MacConkey agar

Growth of *Bacillus* species on Nutrient Agar



Growth of Fecal coliform on MFC

Growth of *E.coli* on EMB Agar



Growth of *Bacillus* on BC Agar

Figure 3.1: Bacterial growth on various selective media

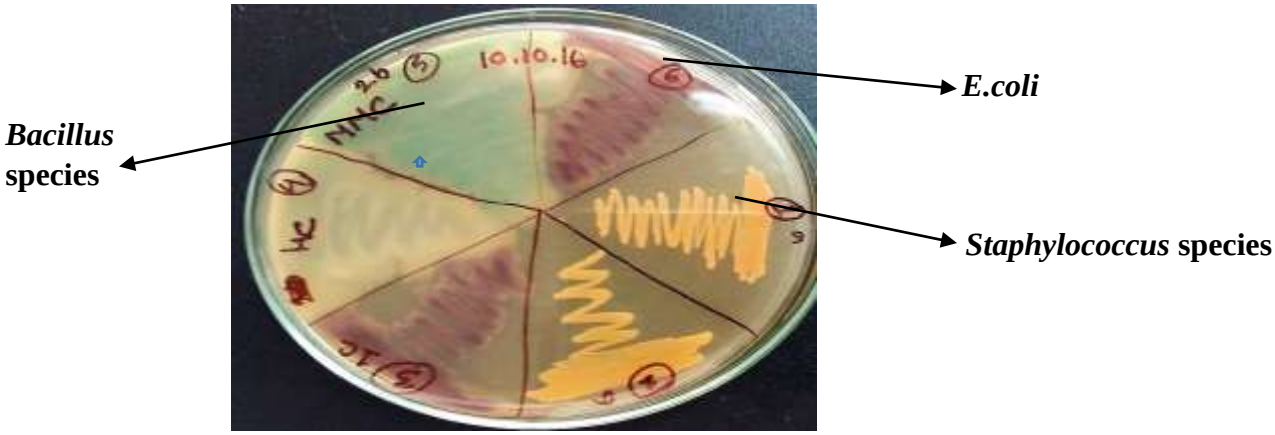
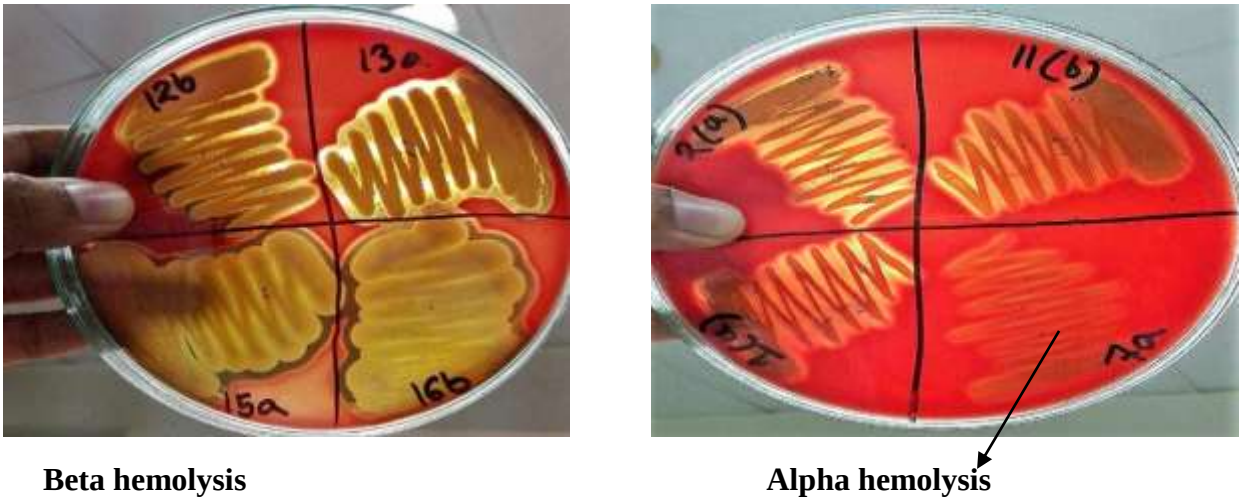


Figure 3.2: Growth of various organisms on Hi-Chrome agar



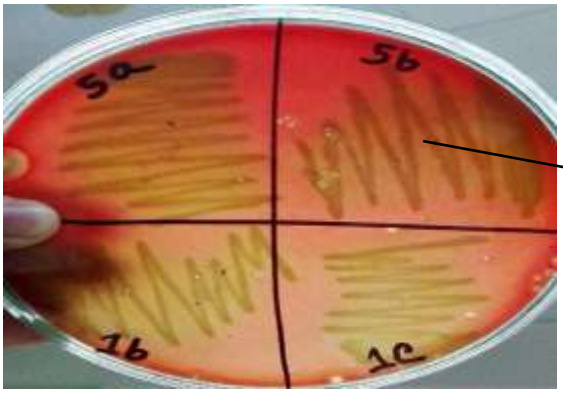


Figure 3.3: Bacterial growth on Blood agar

3.1.2 Biochemical characteristics of the bacterial isolates:

Bacteria those were isolated from female toilet door knobs 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. After subculture, some specific biochemical tests were done and recorded. 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.

Table 3.2: Biochemical characteristics of the bacteria isolated from female toilet door knobs of BRAC University

Isolates No	Isolates ID	Gram Staining		Indole test	Methyl Red (MR) Test	Voges- Proskauer (VP) Test	Citrate Test	Triple Sugar Iron test (TSI)						MIU Test			Nitrate Reduction Test	Catalase test	Oxidase Test	Suspected organism
		Gram Reaction	Shape					Slant/butt	Glucose	Lactose	Sucrose	H_2S production	Gas production	Motility	Indole	Urease				
1.	1(a)	+	Cocci in cluster	-	+	+	+	Y/Y	+	+	+	-	-	-	-	+	+	+	-	*Staphylococcus* spp.
2.	1(b)	-	Short Rods	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	+	-	*E.coli*
3	1(c)	-	Short Rods	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	+	-	*E.coli*
4	2(a)	+	Cocci in cluster	-	+	+	+	Y/Y	+	+	+	-	-	-	-	+	+	+	-	*Staphylococcus* spp.
5	2(b)	+	Long Rods	-	-	+	-	R/Y	+	-	-	-	-	-	-	+	+	+	-	*Bacillus* spp.
6	4	+	Long Rods	-	-	+	-	R/Y	+	-	-	-	-	-	-	+	+	+	-	*Bacillus* spp.
7	5(a)	-	Short rods	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	+	-	*E.coli*
8	5(b)	-	Short rods	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	+	-	*E.coli*
9	7(a)	+	Cocci in cluster	-	+	+	-	Y/Y	+	+	+	-	-	-	-	+	+	+	-	*Staphylococcus* spp.
10	7(b)	+	Long rods	+	+	+	-	R/Y	+	-	-	-	-	-	-	+	+	+	-	*Bacillus* spp.
11	7(c)	+	Cocci in cluster	-	+	+	-	Y/Y	+	+	+	-	-	-	-	+	+	+	-	*Staphylococcus* spp.
12	8(a)	+	Cocci in cluster	-	+	+	+	Y/Y	+	+	+	-	-	-	-	+	+	+	-	*Staphylococcus* spp.
13	9(a)	+	Long rods	-	+	+	+	R/Y	+	-	-	-	-	-	-	+	+	+	-	*Bacillus* spp.
14	9(b)	-	Short rods	+	+	-	+	Y/Y	+	+	+	-	+	+	+	-	+	+	-	*E.coli*
15	11(a)	+	Long rods	-	-	+	-	R/Y	+	-	-	-	-	-	-	+	+	+	-	*Bacillus* spp.
16	11(b)	+	Cocci in cluster	-	+	-	-	Y/Y	+	+	+	-	-	-	-	+	+	+	-	*Staphylococcus* spp.
'+'= Positive, '-'= Negative, Y= Yellow(Acidic), R=Red (Alkaline)																				

Table 3.2: Biochemical characteristics of the bacteria isolated from female toilet door knobs of BRAC University

Isolates No	Isolates ID	Gram Staining		Indole test	Methyl Red (MR) Test	Voges- Proskauer (VP) Test	Citrate Test	Triple Sugar Iron test (TSI)						MIU Test			Nitrate Reduction Test	Catalase test	Oxidase Test	Suspected organism
		Gram Reaction	Shape					Slant/butt	Glucose	Lactose	Sucrose	H_2S production	Gas production	Motility	Indole	Urease				
17	12(a)	+	Cocci in cluster	-	+	+	+	Y/Y	+	+	+	-	-	-	-	+	+	+	-	*Staphylococcus* spp.
18	12(b)	+	Long rods	-	+	+	-	R/Y	+	-	-	-	-	-	-	+	+	+	-	*Bacillus* spp.
19	13(a)	+	Long rods	-	+	-	-	R/Y	+	-	-	-	-	-	-	+	+	+	-	*Bacillus* spp.
20	14(a)	+	Cocci in cluster	-	+	+	-	Y/Y	+	+	+	-	-	-	-	+	+	+	-	*Staphylococcus* spp.
21	14(b)	-	Short rods	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	+	-	*E.coli*
22	14(c)	+	Cocci	-	-	-	-	R/R	-	-	-	-	-	-	-	-	+	+	-	*Micrococcus* spp.
23	15(a)	+	Long rods	-	+	+	-	R/Y	+	-	-	-		-	-	+	+	+	-	*Bacillus* spp.
24	15(b)	+	Cocci in Cluster	-	+	+	+	Y/Y	+	+	+	-	-	-	-	+	+	+	-	*Staphylococcus* spp.
25	16(a)	+	Cocci in Cluster	-	+	+	-	Y/Y	+	+	+	-	-	-	-	+	+	+	-	*Staphylococcus* spp.
26	16(b)	+	Long rods	+	-	+	-	R/Y	+	-	-	-	-	-	-	+	+	+	-	*Bacillus* spp.
27	17(a)	+	Cocci in Cluster	+	-	+	-	Y/Y	+	+	+	-	-	-	-	+	+	+	-	*Staphylococcus* spp
28	17(b)	+	Long rods	+	+	+		R/Y	+	-	-	-	-	-	-	+	+	+	-	*Bacillus* spp.
29	18(a)	+	Cocci in Cluster	+	+	-	+	Y/Y	+	+	+	-	-	-	-	+	+	+	-	*Staphylococcus* spp.
30	18(b)	-	Short rods	+	+	-	-	Y/Y	+	+	+	-	+	+	+	-	+	+	-	*E.coli*
31	18(c)	+	Cocci	-	-	-	-	R/R	-	-	-	-	-	-	-	-	+	+	-	*Micrococcus* spp.
32	18(d)	+	Long rods	-	-	+	-	R/Y	+	-	-	+	-	-	-	+	+	+	-	*Bacillus* spp.
'+'= Positive, '-'= Negative, Y= Yellow(Acidic), R=Red (Alkaline)																				

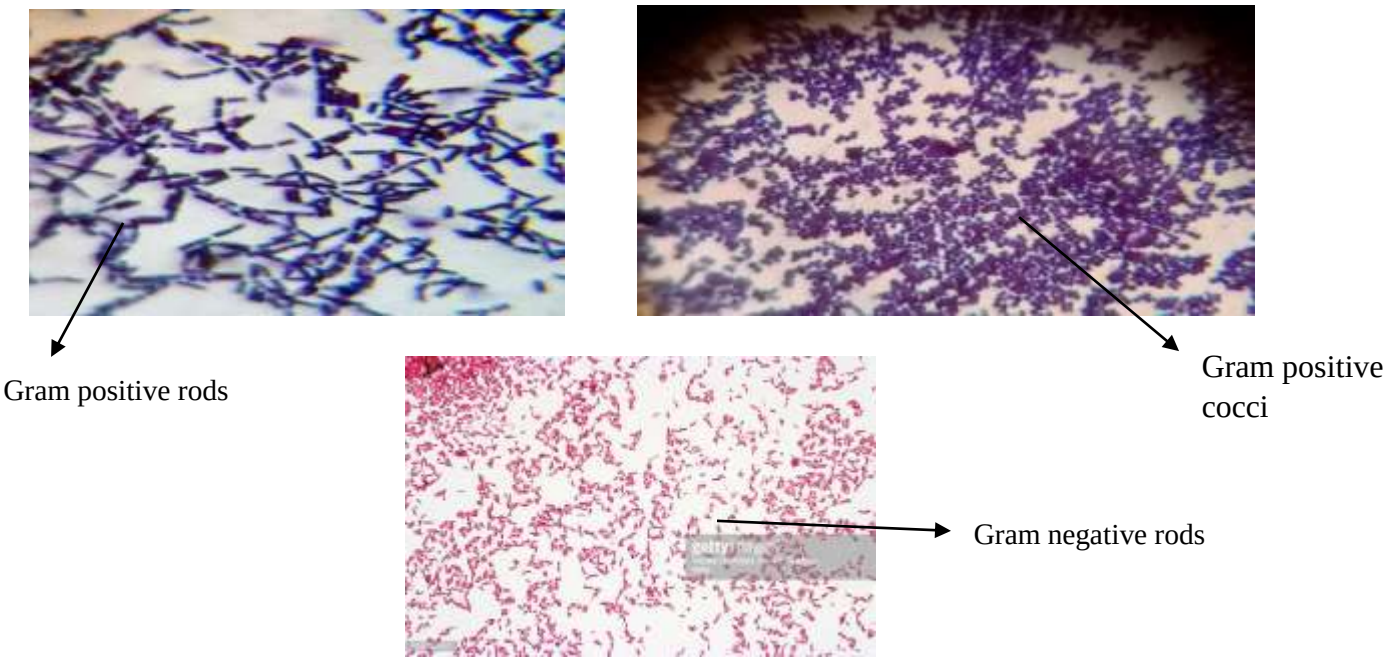
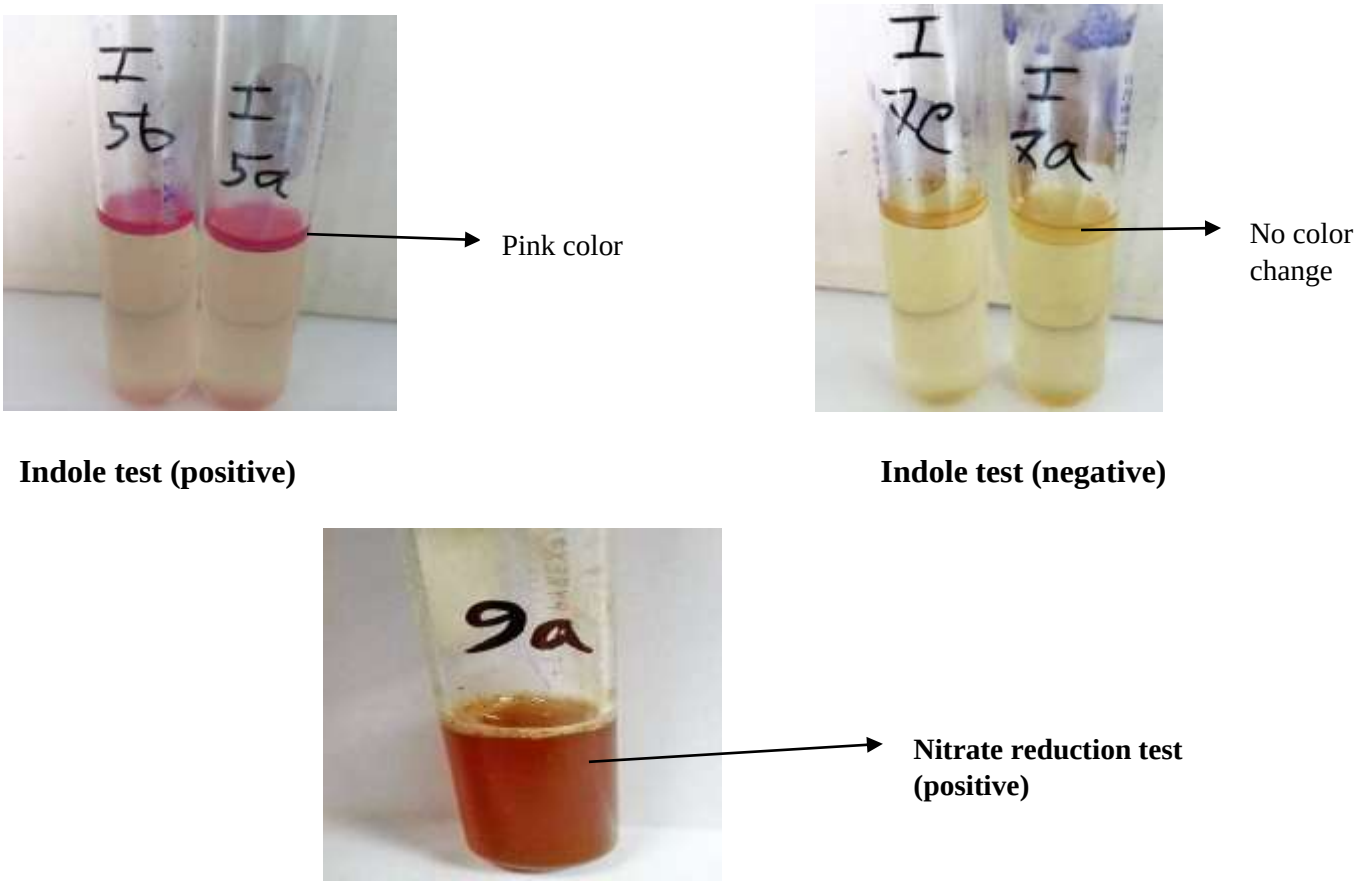


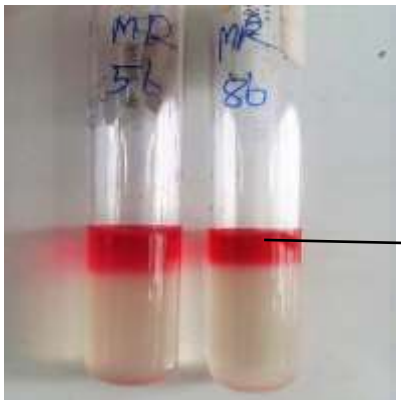
Figure: Gram staining of bacterial isolates



Indole test (positive)

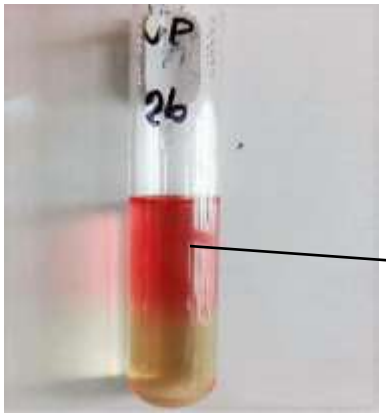
Indole test (negative)

Figure 3.4: Biochemical test results of bacterial isolates

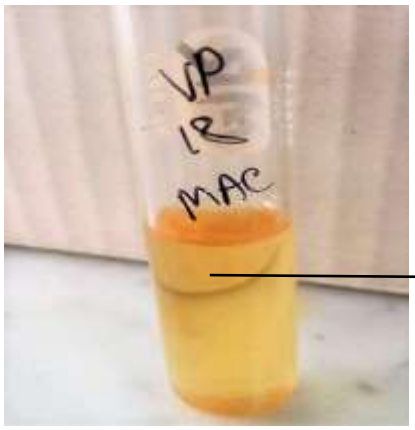


Methyl red test (positive)

Methyl red test (negative)



Voges-Proskauer test (positive)



Voges-Proskauer test (negative)

Yellow slant, yellow butt

Red slant, yellow butt

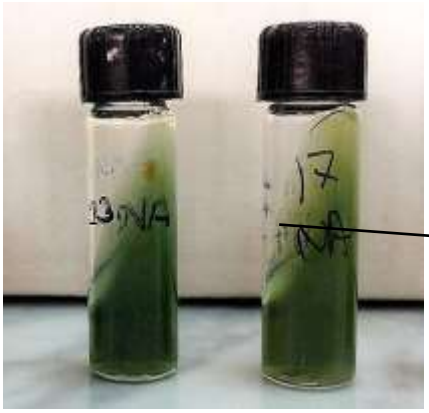
Yellow slant, yellow butt (gas produced)

Red slant, red butt

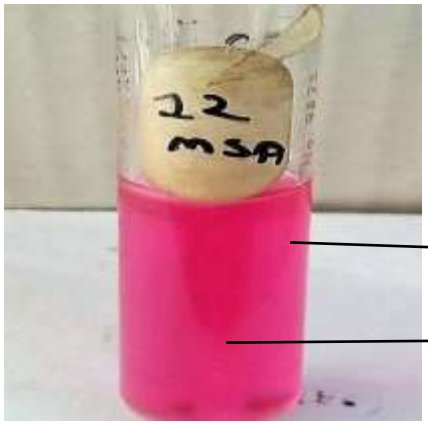
TSI test

Figure 3.4.: Biochemical test results of bacterial isolates

Citrate test (positive)

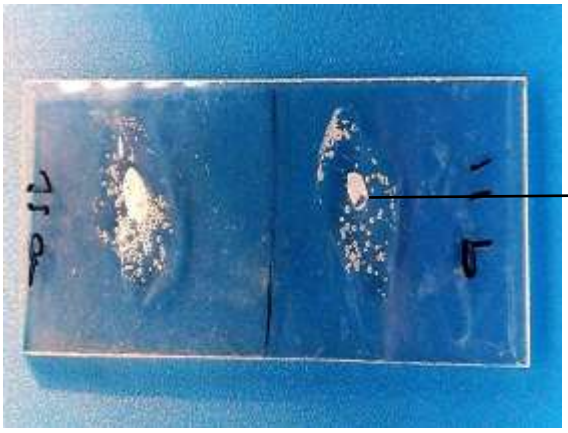


Citrate test (negative)

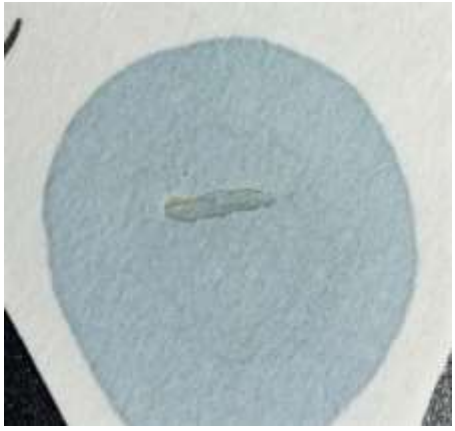


MIU test (Urease +ve, Non-motile)

MIU test (Urease -ve, Non-motile)



Catalase test (positive)



Oxidase test (negative)

Figure 3.4.: Biochemical test results of bacterial isolates

After observing the cultural and morphological characteristics of bacterial isolates and performing the biochemical tests, 37 isolates could have been identified from 15 different samples collected from toilet door knobs. The isolates that have been confirmed include *Staphylococcus* species (found in 14 samples), *Bacillus* species (found in 15 samples), *E.coli* (found in 10 samples), Fecal coliform (found in 5 samples) and *Micrococcus* species (found in 2 samples). The total number and the percentage of the isolates obtained from the samples are shown in table 3.3 and figure 3.5

Table 3.3: Prevalence of bacteria species isolated from toilet door knobs

Bacterial isolates	Number of the isolates	Total bacterial isolates	% Prevalence
***Staphylococcus* species**	12	37	32.43
***Bacillus* species**	11		29.73
E.coli	7		18.92
Fecal coliform	5		13.51
***Micrococcus* species**	2		5.41

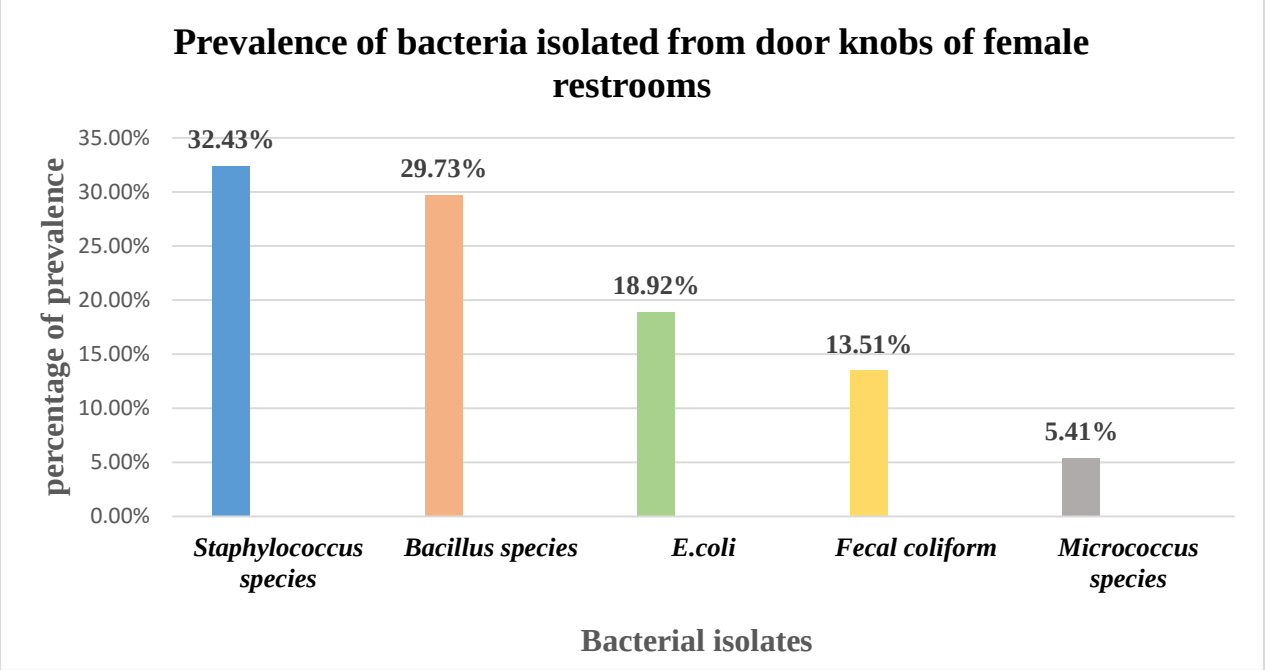


Figure 3.5: Percentage of prevalence of isolated bacteria from door knobs of female restrooms

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, *Bacillus* spp and *Micrococcus* spp. The Gram negative organisms that have been identified include *E.coli* and fecal coliform. The differentiation, number and the percentage of the identified bacterial isolates based on Gram reaction are shown in Table 3.4 and Figure 3.6.

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

Gram's Reaction	Number of isolates found	Percentage (%)
Gram positive	25 (out of 37)	67.57
Gram negative	12 (out of 37)	32.43

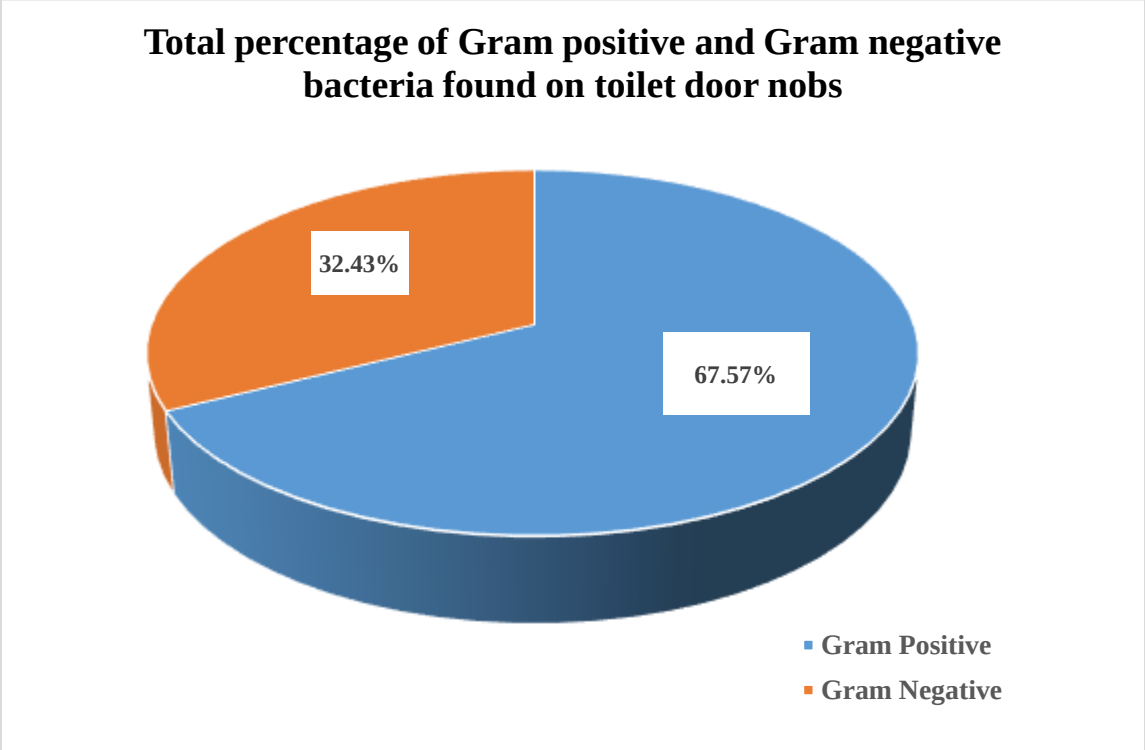


Figure 3.6: Total percentage of Gram positive and Gram negative bacteria identified from toilet door knobs

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 thirty seven isolates isolated from toilet door knobs of BRAC University. The sensitive and resistance pattern of the isolates to these antibiotics were determined.

In table 8, 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. If the zone diameter is smaller than the sensitive diameter scale and within the range of resistant diameter scale then it also indicates that the organism is resistant to the antibiotic. If the zone diameter is larger than resistant diameter scale and less than sensitive diameter scale, then this result is called intermediate and it means the specific bacteria is neither susceptible nor resistant to that particular antibiotic. If the zone diameter is larger than the resistant diameter scale or within the range of sensitive diameter scale then the antibiotic is effective against that bacterium which means the bacterium is sensitive to the antibiotic. 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 female toilet door knobs of BRAC University

Isolates Id	Suspected organism	Penicillin		Ciprofloxacin		Chloramphenicol		Amoxicillin		Gentamycin		Rifampicin		Tetracycline		SXT		Streptomycin		Methicillin	
		ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP
1(a)	***Staphylococcus* spp.**	Nil	R	28	S	25	S	14	R	23	S	34	S	31	S	24	S	17	S	Nil	R
1(b)	***E.coli***	Nil	R	33	S	29	S	Nil	R	Nil	R	9	R	26	S	Nil	R	20	S	-	-
1(c)	***E.coli***	Nil	R	31	S	26	S	Nil	R	20	S	10	R	25	S	Nil	R	20	S	-	-
1(d)	**Fecal Coliform**	Nil	R	24	S	28	S	Nil	R	21	S	8	R	10	R	Nil	R			-	-
2(a)	***Staphylococcus* spp.**	19	R	25	S	27	S	21	S	18	S	30	S	17	S	28	S	19	S	Nil	R
2(b)	***Bacillus* spp.**	Nil	R	28	S	26	S	Nil	R	23	S	18	R	20	S	Nil	R	22	S	-	-
4	***Bacillus* spp.**	13	R	30	S	25	S	Nil	R	22	S	19	R	25	S	Nil	R	24	S	-	-
5(a)	***E.coli***	Nil	R	40	S	28	S	20	S	17	S	Nil	R	25	S	26	S	18	S	-	-
5(b)	***E.coli***	Nil	R	34	S	24	S	17	I	20	S	Nil	R	23	S	23	S	17	S	-	-
5(c)	**Fecal coliform**	Nil	R	29	S	12	R	13	R	19	S	10	R	24	S	14	I	-	-	-	
7(a)	***Staphylococcus* spp.**	16	R	Nil	R	20	S	Nil	R	26	S	33	S	29	S	24	S	18	S	22	S
7(b)	***Bacillus* spp.**	18	R	27	S	30	S	16	R	20	S	18	R	31	S	Nil	R	23	S	-	-
7(c)	***Staphylococcus* spp.**	15	R	Nil	R	25	S	10	R	25	S	33	S	30	S	27	S	20	S	21	S

ZI= Zone of Inhibition, INP= Interpretation, S= Sensitive, I= Intermediate, R=Resistant, '-'= Not done

Table 3.5: Antibiotic susceptibility pattern of various organisms isolated from female toilet door knobs of BRAC University

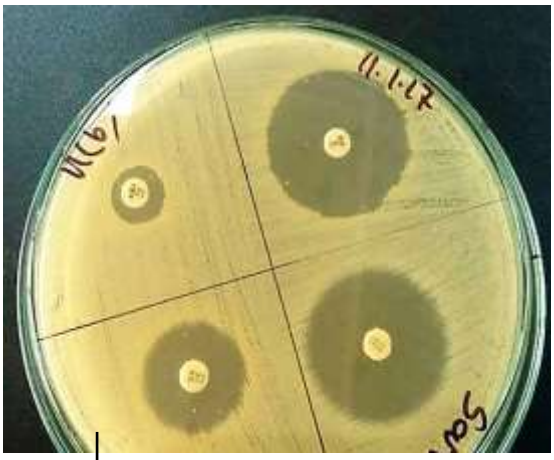
Isolates Id	Suspected organism	Penicillin		Ciprofloxacin		Chloramphenicol		Amoxicillin		Gentamycin		Rifampicin		Tetracycline		SXT		Streptomycin		Methicillin	
		ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP
8(a)	***Staphylococcus* spp.**	Nil	R	Nil	R	34	S	8	R	11	R	Nil	R	12	R	Nil	R	Nil	R	Nil	R
8(b)	**Fecal Coliform**	Nil	R	27	S	11	R	15	I	20	S	10	R	21	S	15	I	-		-	-
9(a)	***Bacillus* spp.**	Nil	R	31	S	25	S	11	R	19	S	13	R	30	S	Nil	R	20	S	-	
9(b)	***E.coli***	Nil	R	39	S	27	S	Nil	R	21	S	13	R	24	S	27	S	22	S	-	
9(c)	**Fecal coliform**	Nil	R	26	S	19	S	16	I	22	S	15	R	26	S	17	S	-		-	-
11(a)	***Bacillus* spp.**	Nil	R	27	S	29	S	11	R	23	S	10	R	26	S	Nil	R	18	S	-	-
11(b)	***Staphylococcus* spp.**	17	R	16	I	25	S	13	R	23	S	33	S	32	S	26	S	18	S	22	S
12(a)	***Staphylococcus* spp.**	14	R	34	S	30	S	14	R	24	S	36	S	14	R	35	S	27	S	10	I
12(b)	***Bacillus* spp.**	Nil	R	29	S	32	S	9	R	21	S	Nil	R	28	S	Nil	R	21	S	-	-
13(a)	***Bacillus* spp.**	Nil	R	26	S	25	S	15	R	21	R	13	R	22	S	Nil	R	19	S	-	-
14(a)	***Staphylocccus* spp.**	Nil	R	Nil	R	14	I	Nil	R	14	I	Nil	R	23	S	Nil	R	22	S	Nil	R
14(b)	***E.coli***	Nil	R	33	S	26	S	19	S	19	S	8	R	24	S	23	S	20	S	-	-
14(c)	***Micrococccus* spp.**	13	R	31	S	33	S	16	R	21	S	17	R	25	S	21	S	20	S	-	-

ZI= Zone of Inhibition, INP= Interpretation, S= Sensitive, I= Intermediate, R=Resistant, '-'= Not done

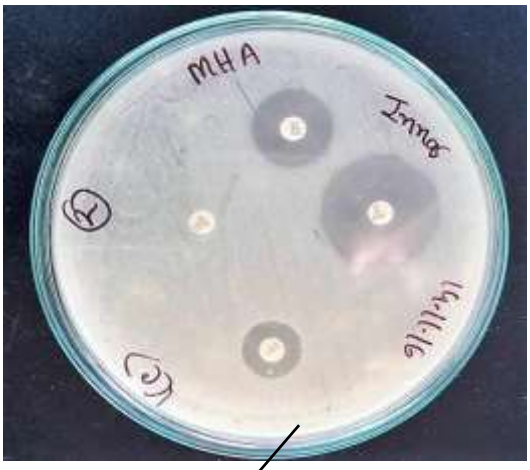
Table 3.5: Antibiotic susceptibility pattern of various organisms isolated from female toilet door knobs of BRAC University

Isolates Id	Suspected organism	Penicillin		Ciprofloxacin		Chloramphenicol		Amoxicillin		Gentamycin		Rifampicin		Tetracycline		SXT		Streptomycin		Methicillin	
		ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP	ZI	INP
15(a)	***Bacillus* spp.**	Nil	R	27	S	25	S	Nil	R	20	S	12	R	25	S	Nil	R	19	S	-	-
15(b)	***Staphylococcus* spp.**	Nil	R	16	I	32	S	23	S	17	S	Nil	R	8	R	Nil	R	Nil	R	21	S
16(a)	***Staphylococcus* spp.**	12	R	32	S	31	S	14	R	27	S	35	S	22	S	Nil	R	24	S	20	S
16(b)	***Bacillus* spp.**	Nil	R	31	S	28	S	10	R	21	S	11	R	27	S	Nil	R	19	S	-	-
16(c)	**Fecal coliform**	Nil	R	31	S	32	S	Nil	R	20	S	Nil	R	24	S	18	S			-	-
17(a)	***Staphylococcus* spp.**	12	R	33	S	29	S	14	R	24	S	34	S	14	R	24	S	23	S	20	S
17(b)	***Bacillus* spp**	Nil	R	30	S	22	S	10	R	23	S	13	R	24	S	Nil	R	-	-	-	-
18(a)	***Staphylococcus* spp.**	12	R	Nil	R	Nil	R	16	R	26	S	35	S	28	S	24	S	25	S	25	S
18(b)	***E.coli***	Nil	R	35	S	28	S	19	S	20	S	Nil	R	24	S	19	S	23	S	-	-
18(c)	***Micrococccus* spp.**	19	R	32	S	35	S	23	S	18	S	15	R	24	S	18	S	19	S	-	-
18(d)	***Bacillus* spp.**	Nil	R	28	S	22	S	13	R	21	S	16	R	25	S	Nil	R	20	S	-	-

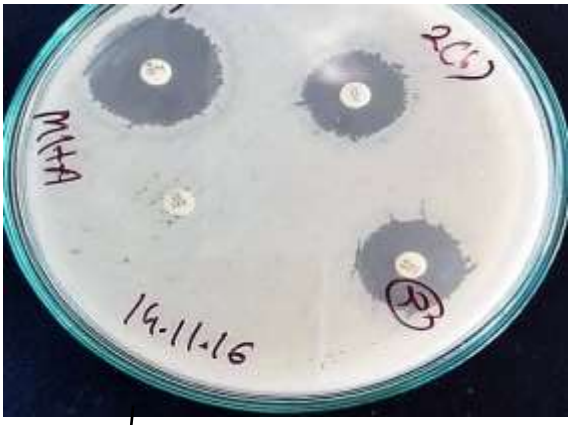
ZI= Zone of Inhibition, INP= Interpretation, S= Sensitive, I= Intermediate, R=Resistant, '-'= Not done



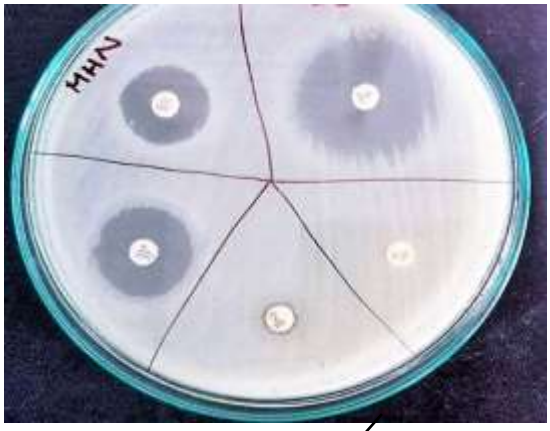
***Staphylococcus* species**



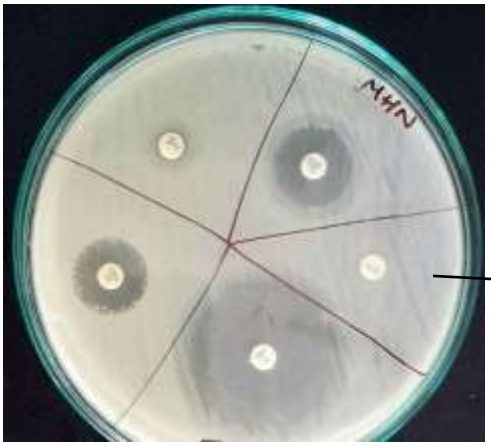
E.coli



***Bacillus* species**



Fecal coliform



***Micrococcus* species**

Figure 3.7: Antibiotic susceptibility test of 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 3.6 and in the following figure.

Table 3.6: Antibiotic resistance pattern of total 37 bacterial isolates

Antibiotics	Penicillin	Ciprofloxacin	Chloramphenicol	Amoxicillin	Gentamycin	Rifampicin	Tetracycline	SXT	Streptomycin
No of isolates resistant to tested antibiotics	37	4	3	28	2	28	5	18	2
Percentage of isolates resistant to antibiotics	100	10.81	8.11	75.68	5.41	75.68	13.51	48.65	5.41

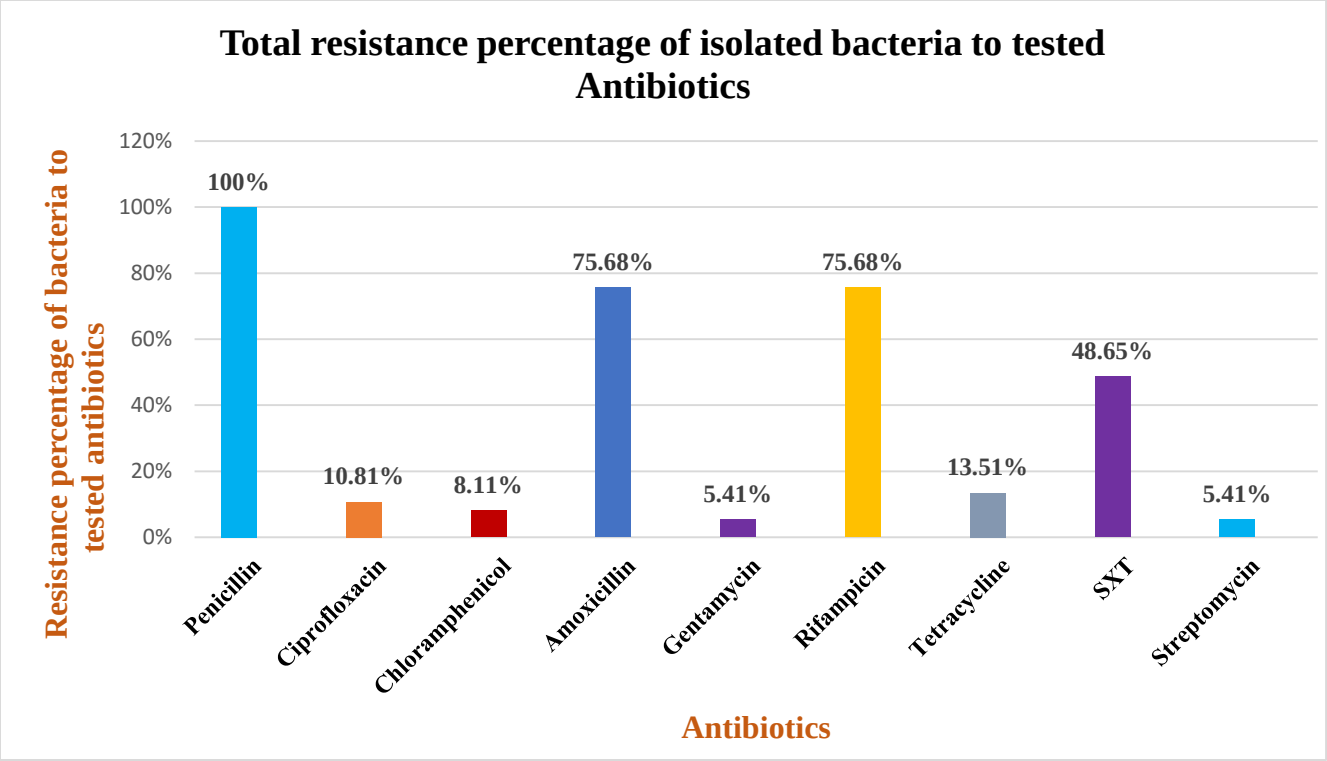


Figure 3.8: Resistance percentage of the isolated bacteria to tested antibiotics

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 at least 2 or more 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. So the bacterial isolates obtained from this study belong to Multi-drug resistant (MDR) organisms. Furthermore the Multi-drug resistant (MDR) bacterial isolates are divided into two categories in this study: one that are resistant to at least 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 3.9.

Table 3.7: Total number and percentage of the isolates resistant to more than two antibiotics and the isolates resistant to at least 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 at least two antibiotics	Percentage Of isolates Resistant to at least two antibiotics
37	29	78.38	8	21.62

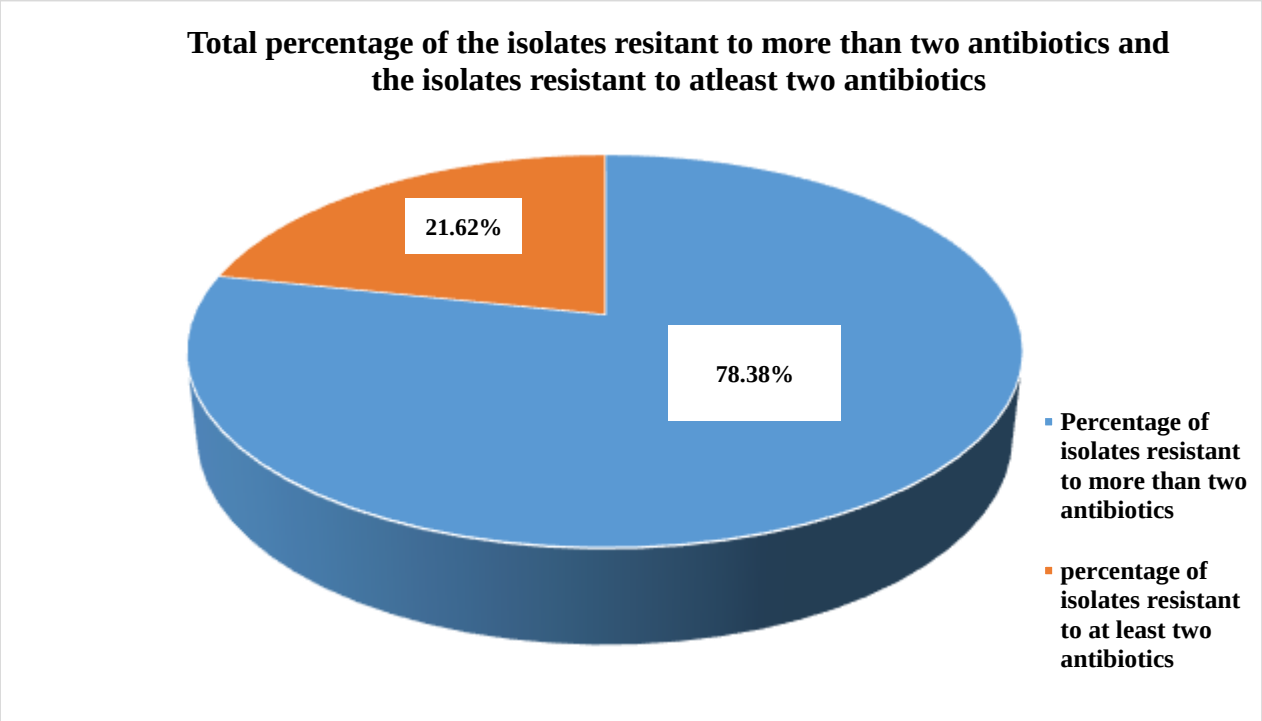


Figure 3.9: Total percentage of the isolates resistant to more than two antibiotics and the isolates resistant to at least two antibiotics.

3.3 Plasmid profile of the bacteria isolated from students' toilet door knobs:

Plasmid extraction revealed that 29.73% (11 out of 37) of the bacterial isolates were found to contain plasmid with 6 different profiles. Each DNA band in the gel was considered to represent one plasmid. Number of plasmid bands varied from one to five. The size of the bands were determined by comparing with the plasmid size of the reference strain *E.coli* V-517 used in this study. A significant proportion of the bacterial isolates (21.63%; 8 out of 37) carried large plasmids with high molecular weight (> 20 MDa). Six isolates (of 37; 16.22%) carried large plasmid of approximately 85 MDa size. Three isolates contained multiple copies (more than 1) of plasmid and remaining eight isolates contained only one copy of plasmid. The Table 3.8 shows plasmid patterns, plasmid contents of each pattern with their approximate molecular weight as shown in following figure:

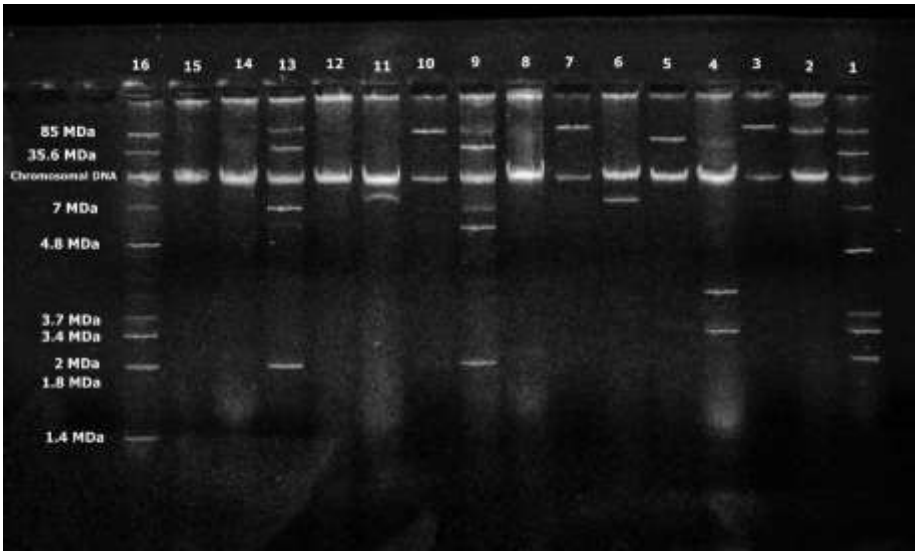


Figure 3.10: Plasmid profile of bacteria isolated from students' toilet door knobs of BRAC University (Lane 1 and 16 represent plasmid DNA marker obtained from the organism *E.coli* K-12 strain V-517. Lane 2-13 except 12 represents 6 different plasmid profile exhibited by 37 bacterial isolates obtained from students' toilet door knobs. Lane 12, 14, 15 represent 26 plasmid less bacterial isolates shared by the isolates resistant to both two and more than two antibiotics).

Table 3.8: Plasmid pattern and number of isolates hosting the plasmid

Pattern	Lane in Figure 3.10	Approximate size of the plasmid (MDa)	Number of Isolates hosting the pattern	Isolates	Total isolates
1	2	~85	4	1(d), 8(b), 9(b), 16(c)	37
2	3				
3	7				
4	10				
5	4	~50, ~3.4, ~4.8	1	5(c)	
6	5	~35.6	1	9(c)	
7	6	~20	2	5(a), 14(b)	
8	11				
9	8	~2.2	1	5(b)	
10	9	~85, ~35.6, ~18, ~16, ~2	2	1(c), 1(b)	
11	13				
12	12	No plasmid	26	12(a),15(a), 18(b) representing other plasmid less isolates	
13	14				
14	15				

3.3.1 Existence of plasmids in the isolates resistant to two antibiotics and in the isolates resistant to more than two antibiotics:

After plasmid profiling of 37 bacterial isolates it was found that plasmid was present in both of the isolates resistant to more than two antibiotics and resistant to at least two antibiotics**.** The Total number and percentage of the isolates (resistant to more than 2 antibiotics) and the percentage of the isolates (resistant to at least two antibiotics) are given in Table 3.9 and Figure 3.11

Table 3.9: Total number and percentage of the isolates (resistant to more than 2 antibiotics) and the percentage of the isolates (resistant to at least 2 antibiotics) harboring plasmids

Total isolates (Resistant to more than two antibiotics)	**Number of the isolates (Resistant to more than two antibiotics) harboring plasmid**	**Percentage of the isolates (Resistant to more than two antibiotics) harboring plasmid**	**Total isolates (Resistant to at least two antibiotics)**	**Number of the isolates (Resistant to at least two antibiotics) harboring plasmid**	**Percentage of the isolates (Resistant to at least two antibiotics) harboring plasmid**
29	7	24.14	8	4	50

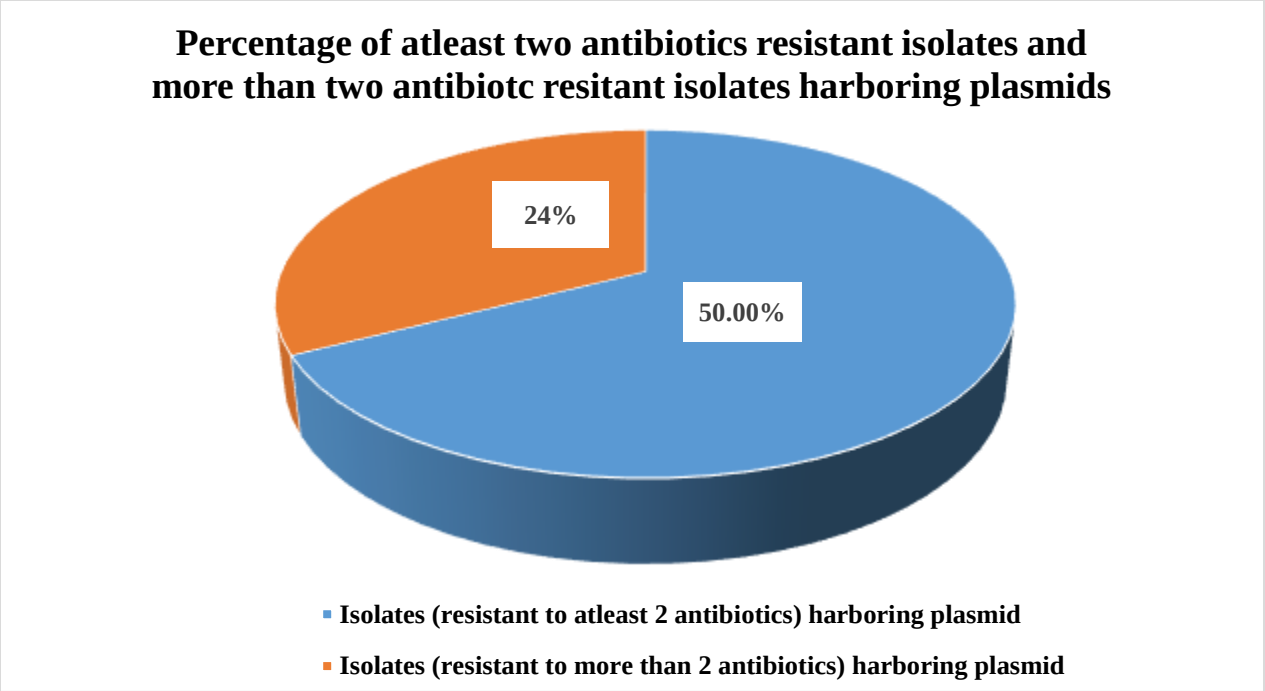


Figure 3.11: Percentage of the isolates (resistant to more than 2 antibiotics) and the isolates (resistant to at least 2 antibiotics) harboring plasmids.

Chapter 4

Discussion and Conclusion

Door knobs/handles of restrooms are important reservoir of microorganisms. It has been reported that some people have a habit of not washing their hands after using the toilets and those who wash hands use short time with or without soap (Toshima *et al*. 2001). This has an implication in transmitting microorganisms from one area to another and from one individual to another. Toilet door knobs can become automatically reservoir of infection when contaminated since the toilet user must have to touch the site both at entrance and exit from the toilet (Maori *et al,* 2013). This study aimed at isolating, identifying the bacterial contaminants, determining the antibiotic resistance pattern and plasmid profiling of the bacteria isolated from the door knobs of female restrooms in BRAC University. It revealed high level of bacterial contaminants on door handles 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.

Out of 15 samples processed, 15 (100%) of them showed bacterial contamination which is in accordance with the reports of some researchers (Otter and French, 2009) who observed 95% positive cultures on touch surfaces in the public transport system and in a public areas of a hospital in London. It is also similar with the reports of some other researchers (Nworie *et al*, 2012 and Bashir *et al*, 2016) who observed 86.7% and 84.7% positive bacterial cultures from toilet door knobs of public conveniences and public toilets respectively. However this finding is less similar with the reports of some researchers (Maryam *et al,* 2016) who found 65.7% bacterial growth from some fomites in a teaching hospital in Nigeria and (Maori *et al*, 2013) who found only 50% bacterial growth from toilet door knobs in secondary schools in Nigeria. This variation in the number of positive samples from one place to the other may be connected with differences in hygiene and sanitary conditions in the environment.

After 24 hours incubation of various selective and differential medium, some different morphological characteristic showing colonies from nutrient agar, pink colonies from MacConkey Agar (considered as coliforms), blue colonies from Membrane fecal coliform (MFC) agar (considered as fecal coliform) and yellow colonies from Mannitol Salt Agar (considered as *Staphylococcus spp.),* extra-large colonies from Nutrient agar and blue colonies from Bacillus cereus Agar (considered as *Bacillius spp.)* were initially isolated. Isolates from MacConkey and MFC agar media were observed as Gram negative, single, short rods, compared to the

characteristic of coliforms whereas isolates from Mannitol salt agar (MSA) were Gram positive in a cluster arrangement which were typical for *Staphylococcus spp.* After performing the biochemical characteristics, the isolates were finally confirmed as *E. coli, Staphylococcus* spp., *Micrococcus* spp, *Bacillus* spp.

In this study, the most prevalent bacterial contaminant found was *Staphylococcus* species (32.43%). 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. Besides the toilet door knobs are routinely touched with hands and *Staphylococcus* belong to the normal flora of hands which also explains its high prevalence in these surfaces. This observation is consistent with the findings of other researchers (Nworie *et al*., 2012; Ducel *et al.,* 2002; Brooks *et al.,* 2007).

A high percentage of *Bacillus* species (29.73%) was also investigated in this study and its high prevalence could be demonstrated by the fact that *Bacillus* species are 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. This is also in agreement with the research carried out by Brooks *et al.,* (2007) and Onwubiko *et al.,* (2015) who reported that *Bacillus* species was found to be among the predominant organism that was isolated from door handles.

Prevalence of Gram-negative rods including *E.coli* (18.92%) investigated in this study is in favor with the work of Augustino *et al* (2014) who found 36.7% *E.coli* isolates from students' toilets in Tanzania. However this finding is also in contrary to the work of other researchers (Bashir *et al*, 2016; Nwankwo *et al*, 2015; Mensah-Agyei *et al*, 2016) who found only 13.9%, 3% and 7.1% of the *E.coli* isolates respectively.

Occurrence of Fecal coliform (13.51%) revealed in this study indicates the possibility of the presence of fecal contamination on the door handles. This might be due to the fact that most people go to toilet and end up contaminating their hands with fecal and urinal material and fail to wash their hand because they take the issue of hygiene lightly and they also lack the concept of hand washing as a simple means of stopping this spread of infectious agents. This finding is in compliance with the work of Zhad *et al.* (1998), who investigated that the high rate of isolation of these organisms is only achieved during epidemics in which human hands serve as the vehicle of transmission.

The isolation of *Micrococcus* species (5.41%) from this study is relative to the work of Opera *et al*., (2013) who also reported the isolation of *Micrococcus* species from public toilets.

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). Their main sources of colonization on the fomites might likely be nasal carriage (Graham *et al*., 2006) which is likely to be transmitted by hand-to-mouth or hand-to-nose contact while using these fomites, or by poor hand-washing habits (ASM, 2005).

Possible diseases that can be caused by the isolated bacteria include Foodborne diseases (*S. aureus, E.coli, Bacillus* species), Urinary tract infections (*E. coli*), Skin infections (*Staphylococcus* species), Waterborne diseases including dysentery, diarrhea, (Fecal coliform) and Diarrhea (*E. coli*) (Agbagwa and Nwechem 2010). *E.coli* has been reported to be the most common cause of UTIs with some clones that may also be associated with gastrointestinal infections (Schönning et al. 2004). So it can be said that the organisms isolated in this study belong to disease causing organisms and it can cause certain diseases in human if it is transmitted from one individual to another.

Determination of antibiotic susceptibility pattern revealed that all bacterial isolates tested were resistant to at least two antibiotics. Among the 37 bacterial isolates, 29 (78.38%) of them were found to be resistant to more than two antibiotics and 8(21.62%) of them were found to be resistant to at least two antibiotics. Out of the nine antibiotics tested penicillin was found to be least effective because all bacterial isolates showed resistance to penicillin. Amoxicillin and rifampicin can also be considered less effective because 75.68% of the bacterial isolates showed resistance to these antibiotics. The resistance percentage of the isolates to other antibiotics include ciprofloxacin (10.81%), chloramphenicol (8.11%), SXT (48.65%), tetracycline (13.51%), both streptomycin and gentamycin (5.41%). This finding is contradictory to the work of Adewoyin *et al* (2013) who found 40.02% resistance to ciprofloxacin, 26.67% to chloramphenicol, 46.69% to gentamycin and streptomycin in the bacteria isolated from Toilet-Bowl of some Students' Hostels. 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).

Plasmid profile analysis of the bacterial isolates revealed that 29.73% (11 out of 37) of the bacterial isolates were found to contain plasmid with 6 different profiles. This finding is in disagreement with the work of Opere Bolanle O. and Ojo, James O. (2013) who discovered no plasmid in pathogenic bacteria isolated from environmental surfaces in public toilets. The molecular size of the plasmids varied from ~2 to ~85 MDa. A notable proportion of the bacterial isolates (21.63%; 8 out of 37) carried large plasmid (> 20 MDa). In this study plasmids were found to be present on only gram negative bacteria including *E.coli* and Fecal coliform. Absence of plasmids were found in gram positive bacterial isolates. This might may be due to the fact that antibiotic resistance in Gram negative bacterial isolates is often plasmid mediated (Xiao-Zhe *et al*, 2012). Six isolates (of 37; 16.22%) carried large plasmid of approximately 85 MDa size. These isolates were resistant to more than two antibiotics. Large plasmids present in the members of the family Enterobacteriaceae are found to encode diverse virulence factor determinants and multiple antibiotic resistance genes. Several large plasmids are transferable and shown to mobilize resistance gene among the other enteric members (Jahan *et al*, 2016). Occurrence of large plasmids including plasmids of 85 MDa in gram negative bacteria indicates that they may contain equivalence plasmids with pathogenic genes and may function as reservoir for virulence genes in normal flora. Screening of the plasmids for the presence of virulence genes and also plasmid curing is required to prove this assumption. In this study plasmid was found to be present on 24.14% (7 out of 29) of the isolates resistant to more than two antibiotics and 50% (4 out of 8) of the isolates resistant to at least two antibiotics. Although plasmids have been directly implicated in the acquisition of resistance to many antibiotics, several other mechanisms, such as blocking of antibiotic entry, efflux mechanism, enzymatic inactivation of antibiotics, target site alteration etc. may also be responsible for antibiotic resistance phenomenon (Mcdermott *et al*, 2003). Among the 37 Multi-drug resistant bacterial isolates, multiple plasmids (more than 1) arranged in different patterns were observed in only 3 of the bacterial isolates that were resistant to 4 antibiotics tested.

Conclusion:

The findings of this research work indicate that hand contact surfaces such as door knobs of restrooms are often colonized by several bacteria and may serve as a potential source of infections if they are transmitted from one individual to another through hands. Contaminated and improperly washed hands contaminate door handles and it is important to be noted that there is a high level of bacterial contamination as well as high level of prevalence of the bacterial infectious disease due to contaminants. On the basis of the above findings, it is therefore recommended that the university management should provide more hand sanitizers to the users or spray disinfectants with regular cleaning of the toilets to ensure reduction in microbial load and to minimize the spread of diseases, more cleaners should also be employed to ensure proper cleaning of restrooms. Individuals should adopt the habit of hand washing practice after using the restrooms and routine surface disinfection of the door handles can prevent cross contamination. Besides public awareness and campaigns on proper environmental and personal hygiene should be intensified within the university environments. In this study plasmid was found on the 29.73% (11 out of 37) of Multi-drug resistant bacterial isolates. Further study on the significance of these plasmids and determining the presence of virulence genes would be an interesting line of enquiry to determine the pathogenic evolution of the gram negative bacterial isolates.

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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

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

HiCrome UTI Agar:

Component	Amount (g/L)
Peptic digest of animal tissue	15.0
Chromogenic mixture	26.80
Agar	15.0
Final pH	7.1 ± 0.2 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 ± at 25°C

Nitrate Reduction Broth

Component	Amount (g/L)
Beef extract	3.0
Gelatin peptone	5.0
Potassium nitrate	1.0

Indole broth

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

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.

Malachite green (100 ml)

To 20 ml distilled water, 5 g malachite green was dissolved in a beaker. The solution was transferred to a reagent bottle. The beaker was washed two times with 10 ml distilled water separately and a third time with 50 ml distilled water and the solution was transferred to the reagent bottle. The remaining malachite green in the beaker was washed a final time with 10 ml distilled water and added to the reagent bottle. The stain was stored 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.

Oxidase Reagent (100 ml)

To 100 ml distilled water, 1% tetra-methyl-*p*-phenylenediamine dihydrochloride was added and stored in a reagent bottle covered with aluminum foil at 4°C to prevent exposure to light.

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.

Nitrate Reagent A (100 ml)

5N acetic acid was prepared by adding 287 ml of glacial acetic acid (17.4N) to 713 ml of deionized water. In a reagent bottle, 0.6 g of N,N-Dimethyl-α-naphthylamine was added along with 100 ml of acetic acid (5N) and mixed until the colour of the solution turned light yellow. The reagent was stored at 4°C.

Nitrate Reagent B (100 ml)

In a reagent bottle, 0.8 g of sulfalinic acid was added along with 100 ml acetic acid (5N)[a] to form a colorless solution and stored at 4°C.

1M Tris HCl:

In a McCartney bottle, 1.576g Tris HCl was added. Then 10 ml distilled water was added to prepare 10 ml 1M Tris HCl. After that pH was adjusted to 8. Then it was stored at 4ºC.

0.5M EDTA:

In a McCartney bottle, 1.861 g EDTA was added. Then 10 ml distilled water was added to prepare 10 ml 0.5M EDTA. After that pH was adjusted to 8. Then it was stored at room temperature.

2N NaOH:

In a small Durham bottle 4 g NaOH was added. Then 50 ml distilled water was added to prepare 50 ml of 2N NaOH. Then it was stored at room temperature.

Kado-I Buffer:

In a Durham bottle, 4 ml of 1M Tris Hcl and 400µL of 0.5M EDTA were added. Then 96 ml distilled water was added to prepare 100ml Kado-I Buffer. Then it was stored at room temperature.

Kado-II Buffer:

In a Durham bottle, 0.6 g of Tris base, 3 g of SDS, 6.4 ml of 2N NaOH were added. Then 94ml distilled water was added to prepare 100ml Kado-II Buffer. Then it was stored at room temperature.

1X TBE Buffer:

In a Durham bottle, 5.4 g of Tris base, 2.75 g of Boric Acid, 2ml of 0.5M EDTA were added. Then 500 ml distilled water was added to prepare 500 ml 1X TBE Buffer. After that pH of the buffer was adjusted to 8. Then it was autoclaved at 15psi 121ºC. After autoclave, it was stored at room temperature.