Prevalence, Antibiotic Susceptibility and Plasmid Profile of Bacteria Isolated from Door Handles of Washrooms of a Hospital in Dhaka

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

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Dedication

To My Beloved Parents
&
My Thesis Supervisor

Declaration

I hereby declare that the thesis project titled **"Prevalence, Antibiotic Susceptibility and Plasmid Profile of Bacteria Isolated From Door Handles of Washrooms of a Hospital in Dhaka"** submitted by me 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 the research work presented here is based on actual and original work carried out by me. Any references to work done by any other person or institution or any material obtained from other sources have been duly cited and referenced.

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ABSTRACT:

Background: The fomites can serve as vehicles in transmission of pathogens. This study was conducted to determine the bacterial contamination in door handles of washrooms of a hospital, to determine the prevalence and antibiotic susceptibility pattern of the isolates. Plasmid profile was done to observe the presence or absence of plasmid among isolated bacteria from door handles of washrooms of a hospital.

Methods: Washrooms of four different wards of a hospital were included in this study. Sixteen (16) swabs were taken from sixteen (16) door handles. Bacterial identification was carried out by different biochemical tests according to Bergey's Manual of Systematic Bacteriology and antibiotic susceptibility test was done by Kirby-Bauer method. Plasmid extraction was done according to modified hot alkaline method.

Results: Out of the 16 samples processed, 16 (100%) of them showed bacterial growth. A total of forty three (43) isolates were obtained. The bacteria isolated were *Staphylococcus* spp. (37.21%), *Bacillus* spp.(18.6%), *E. coli*.(16.28%), Fecal Coliform (13.95%), *Micrococcus* spp.(6.98%), *Pseudomonas* spp.(4.65%), *Klebsiella* spp.(2.33%).Plasmid profiling revealed (11 out of 43) bacterial isolates contained 1 or more plasmids with different profiles.

The isolated bacteria showed varying susceptibility pattern to the antibiotics used and were all resistant to at least two antibiotics. Among the 43 isolates, 83.72% were found resistant to more than two antibiotics. Highest resistance percentage of the isolates was observed against Penicillin G (95.35%) followed by SXT (74.42%) and amoxicillin (65.12%), rifampicin (55.81%), tetracycline (18.60%), ciprofloxacin (23.26%), chloramphenicol (4.65%), gentamycin (2.33%) and streptomycin (6.98%).

Conclusion: Findings of this study indicate the presence of bacterial strains resistant to more than two antibiotics in door handles of washrooms of a hospital which can serve as potential source of diseases.

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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	Microlitre
ml	Millilitre
spp.	Species

CHAPTER ONE

Introduction

INTRODUCTION:

1.1: Background of the study:

Hospitals are regarded a place for attaining treatment against medical diseases and maladies. However, sometimes a person can be infected with severe diseases while staying in the hospital. These infections are called hospital acquired infections (HAI). One of the major causes of HAIs that is associated with patient morbidity and mortality is fomites (Weber et al., 2010; Nwankiti et al., 2012).Fomites are inanimate objects or materials e.g. utensils, pen, door knobs, tables, towels, money, clothing's, dishes, books, toys, lockers, chairs etc., that act as intermediate carriers of microbial contamination. If pathogenic organisms are growing on the fomites and human are in constant contact with these fomites, the pathogenic organism has a way to infect human via those (Osterholm et al., 1995).

In hospitals, fomites can serve as a reservoir of pathogens being spread from the inanimate environment to an animate (patient) environment via the hands of health care workers (HCW) (Bhalla et al., 2004; Kramer et al., 2006; Ikeh and Isamade, 2011; Nwankiti et al., 2012). To reduce morbidity and mortality in hospitals, identification of common fomites associated pathogens in any hospital settings is important. Because, the most important factor in prevention of a disease is to simply identify what has been transferring the disease.

Among the vast range of fomites, door handles is one of the most common one, which serves as route for contamination (Reynolds, 2005). Hard and nonporous surface allows more adhesion of bacteria to it. Due to having such a surface, door handles provide the highest rate of bacterial transfer to the hands (Rusin et al., 2002). As not much attention is paid in the cleaning of the door handles, the growth of microbes on those are not diminished. Moreover, with time it starts increasing and evolving into more pathogenic form.

1.1.1: Door handles pathogens:

Bacterial pathogens that have been isolated from door handles in previous studies includes *S. aureus*, *K.pneumonia*, *E. coli*, *Enterobacter* spp, *Citrobacter* spp, *P. aeruginosa*, *Proteus* spp, *Streptococcus* spp, *Salmonella* spp, *Shigella* spp, *Campylobacter* spp (Nworie et al.,2012; Itah and Ben, 2004). These organisms have been known to cause one or more diseases that are mild and could be sometimes serious. The examples of such diseases range from simple skin diseases like pimple, impetigo, scalded skin syndrome to respiratory diseases like, pneumonia to even

severe meningitis, osteomyelitis, rhinoscleroma, kidney failure, septicemia and so on (Clauditz *et al*., 2006).

1.1.2: Spread of toilet door pathogens:

Toilets act as a vehicle for the transmission of pathogens from gut, respiratory tract and skin via hands and surfaces from one person to another (Gerhardts et al., 2012). Toilet handles contamination is one of the common ways by which organisms that are not resident in the hand are picked up by contact with surfaces. Due to the unhygienic use of the toilet facilities, fecal matter remains a major reservoir source of human pathogens. When hands containing fecal remnant uses a door knob, the bacteria pass on to it. As previously mentioned, the bacteria can adhere to solid surfaces like door handles and propagate. Later, when another person holds that door handle, the bacteria can pass on. In adverse situation, such a transfer may even bring about outbreaks of infection (Maori et al., 2013).

The hands serve as a medium for the propagation of microorganisms from place to place and from person to person. Although, it is nearly impossible for the hand to be free of microorganisms, the presence of pathogenic bacteria may lead to chronic or acute illness. Human hands usually harbor microorganisms both as part of the body normal flora as well as transient microbes collected from the environment (Dodrill et al., 2011). Bacteria like, *S. aureus* including methicillin resistant *S. aureus* (MRSA), from the skin, or gut microbes removed from the body during bathing or hand-washing, can survive on the surfaces of the doorknobs.

Enteric 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).

The environment we live and work also plays major roles in human-microbe contamination relationship. A relationship that could sometimes lead to the transmission and spread of pathogens as is seen through community acquired infections.

Many dangerous outbreaks like shigellosis are caused due to fecal contamination. The fecal remaining on hand could lead to such a breakout (Francesco Zinzaro, 2010). The occurrence of this may be attributed to the unhygienic use of the toilet facilities, which results to the filthy

contamination of the place including door-handle, which individuals are less likely to see as contaminated (Francesco Zinzaro, 2010). Improper use of the toilets, inadequate cleanliness of the toilets facilitates transmission of bacteria from the toilets to even household living rooms. Contaminated hands of toilet users can transmit the bacteria from their hands to the flushing handles, door handles and faucets of the toilets as well as household door handles and equipment. Toilet flushing results in a large quantity of flush aerosols, which can reach the seats and leads, surrounding floors and nearby surfaces (Barker and Jones 2005). 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 (Boone and Gerba 2007). The time of survival depends on the type of pathogen, majority including *Shigella* species, *Escherichia* species, *Clostridium* species, severe acute respiratory syndrome (SARS) coronavirus, and norovirus which can survive on surfaces for weeks or even months (Kramer et al. 2006).

1.2: LITERATURE REVIEW

Nwori et al. (2012) investigated 180 door handles/knobs of public conveniences ofselected public offices, motor parks and markets in Abuja Metropolis, Nigeria. They found that 156 (86.7%) of the handles/knobs were positive for bacterial growth. The most prevalent bacteria were *Staphylococcus aureus* (30.1%), *Klebsiella pneumonia* (25.7%), *Escherichia coli*(15.6%), *Enterobacter* spp.(11.2%), *Citrobacter* spp., (7.1%),*Pseudomonas aeruginosa* (5.9%), while *Proteus* species had the least prevalence,(4.5%).

Kamiya el al., (2002) investigated the contamination of room door handles by methicillin-sensitive/ methicillin-resistant *Staphylococcus aureus* in wards of a university hospital. They reported that 53 (27%) of 196 rooms were contaminated by methicillin-sensitive *Staphylococcus aureus* and/or methicillin-resistant *Staphylococcus aureus.*

In Taif, Kingdom of Saudi Arabia, a determination of the level of contamination and bacterial load in public female restrooms was done by Sabra (2013). The result showed that among the 260 specimens, 187 (71.9%) of those had positive bacterial growth. Most growth was seen in toilet hands with having 91.3% of those showing positive growth. The other results were, room handles 59 (73.8%), and followed by room sink (63.3%). The most prevalent bacteria were *Staphylococcus aureus*(40.6%), *Escherichia. coli*(22.5%),*Bacillus* spp.and *Klebsiella. Pneumonia* (21.4%), *Enterococcus faecalis* 13.4%, *Citrobacter* spp. (9.6%), *Pseudomonas aeruginosa* (8.6%) and *Proteus mirabilis* (7%).

Moayad and his colleagues (2011), hypothesized that door handles may aid in the spread of microbes between individuals and that they may be areservoir of microbial contamination. They found that a larger percentage of the bacteriasampled from the door handles were Gram negative. However, among the Gram positive ones, S*taphylococcus aureus* was the most prevalent. This could attribute to presence on skin and its diseases.

Aminu et al. (2014) conducted a study to determine the antibiotic sensitivity of bacteria isolated from fomites in teaching hospital in Nigeria. Thirty five samples were used for that study. The number of isolates was 23 (65.7%). Additionally, the ratio of Gram-positive toGram-negative organisms was 1.2 to 1.1. 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 isolation of pathogenic bacteria from fomites indicates that they can be vehicles for pathogens transfer.

Watutantrige et al. (2012) indicated that harmful microorganisms can be transferred to hands from contaminated surfaces people come into contact in daily life and can transfer disease to one self as well as to others. According to this hypothesis they conducted a study to determine the extent to which hand hygiene practices and toilet door handles contribute to the bacterial load of hands of toilet users in a medical school. They investigated 60 swabs taken from medical students for bacterial count from both hands before and after toilet use and from door handles of six toilets. They reported that bacterial load in the hands of both males and females showed an increase after toilet use. The increase was significant among male students.

Bacteria that are often found in a healthcare environment include coagulase-negative *Staphylococcus*, *Bacillus* species, *Corynebacterium* species, *Streptococci*, and *Clostridium perfringens*, *Enterococcus* species, and *Staphylococcus aureus*. The samples also included antibiotic sensitive strains of microbes that have significant importance in healthcare environments. These were *Staphylococcus aureus*, Vancomycin-resistant *enterococci*, and methicillin-resistant *Staphylococcus aureus* (MRSA). Additionally, these bacteria could survive for more than 24 hours. Therefore, their ability to contaminate other places such as door handles increases (Rutala,et al. 2006).

Lynn et al. (2013) conducted a study about the prevalence of bacterial organisms on toilet door handles in secondary schools. They investigated a total 120 samples for bacterial isolates; among those, 60(50%) yielded growth and 60(50%) showed no growth at all. The following organisms were isolated: *Staphylococcus* spp., 26(43.3%), *Candida* spp., 6(10%), *Escherichia coli* (16.7%), *Citrobacter* spp., 1(1.7%), *Klebsiella* spp., 12(20%), *Proteus* spp., 4(6.7%) and *Salmonella* spp., 1(1.7%).

1.3: Aims and objectives:

The objectives of this research work were to identify and evaluate the occurrence of bacterial contaminations form the door handles of washrooms of a hospital and their harmful consequence to public health. Because of evolving incidence of multi-drug resistant organisms, this study was also aimed at determining the antibiotic resistance profile and detecting the multi-drug resistant organisms from the isolated bacterial pollutants. Moreover, as plasmid carries many antibiotic resistance genes, the study also aimed to observe the presence of different sized plasmids in multi-drug resistant organism. Furthermore, the purpose of the study was also to raise awareness about hand hygiene and hand washing programs. Overall the main objectives were:

- To determine the presence of bacterial contamination on the door handles of washrooms of a hospital.
- To isolate and identify both Gram-positive and Gram-negative bacteria from the door handles of washrooms of a hospital.
- To determine the prevalence and antibiotic susceptibility pattern of bacteria isolated from washroom door handles of hospital.
- To carry out the plasmid profile of the isolated bacteria from the door handles of washrooms of hospital.

CHAPTERTWO

Materials and Method

2. Materials and methods

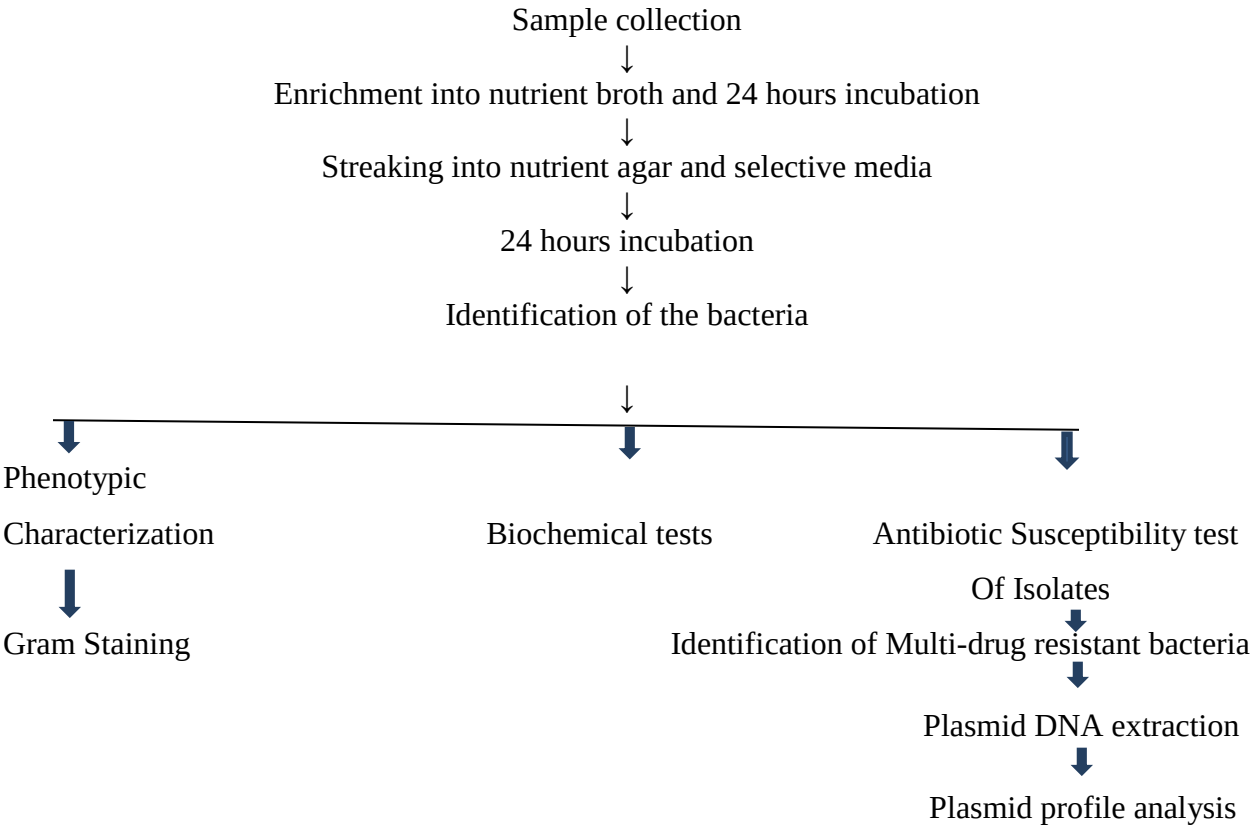
2.1 Study Area:

The study was carried out in the Microbiology, Biotechnology and Molecular Biology Laboratory of the Department of Mathematics and Natural Sciences of BRAC University in Dhaka, Bangladesh.

2.2 Sample size:

A total of 16 door handles of washrooms of Shaheed Suhrawardy Medical College Hospital were included in this study.

2.3 Flow Diagram of the Study Design



2.4 Materials:

2.4.1 Equipments:

Equipments that were used in this study include:

- Laminar airflow cabinet
- Incubator
- Vortex machine
- Autoclave machine
- 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 are**:**

2.4.2.1 Nutrient Agar (NA):

Nutrient Agar is used for the growth of a wide range of non-fastidious organisms.

2.4.2.2 MacConkey Agar:

MacConkey agar is a selective and differential medium 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 and non-fermenting bacteria. After 24-48 hours incubation , *E.coli* and *Klebsiella* produced pink colonies. Bacteria which can not ferment lactose like *Pseudomonas aeruginosa, Salmonella* spp., and *Proteus* spp., will appear colorless on the medium and the medium surrounding the bacteria remains relatively transparent.

2.4.2.3 Membrane 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.

2.4.2.4 Cetrimide Agar: This media is known as cationic detergent which is selective media for *Pseudomonas aeruginosa*.

2.4.2.5 Mannitol salt Agar (MSA):

Mannitol Salt Agar is used as a selective media for the isolation of *Staphylococci*. *S.aureus* ferment mannitol and produce yellow colored colonies. Non mannitol fermenters such as *S.epidermidis* will give colorless colonies and the media will remain red.

2.4.2.6 Eosine Methylene Blue Agar (EMB):

This medium can differentiate among lactose fermenters and lactose non fermenters bacteria. In case of lactose fermenters such as *E.coli*, the colonies will give metallic green sheen and for lactose non fermenters colorless and transparent colonies will be obtained.

2.4.2.7 HiCrome UTI 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*, *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 colonies and *Pseudomonas* give colorless colonies on Hi-Crome agar after 24-48 hours of incubation.

2.4.2.8 Bacillus cereus Agar (BC Agar):

BC agar is selective for *Bacillus* spp. It will give blue zone surrounding the growth of *Bacillus cereus*. Other species of *Bacillu*s are also able to grow in BC agar and will produce green colonies.

2.4.2.9 T1N1 Agar:

Effective maintenance of stock cultures is essential for quality control, method validation and research purposes. T1N1 Agar is used to stock bacteria which are found from the samples.

2.4.2.10 Blood agar (BA):

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 isdetermined by observing the clear zones around the bacterial growth.

2.4.2.11. Mueller-Hinton Agar:

Mueller Hinton agar is used for the antibiotic susceptibility test of bacteria.

2.4.3 Biochemical test media:

Media used for biochemical tests are:

- 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

2.5 Methods:

2.5.1 Sample collection:

The samples were collected from toilet door handles using the swab-rinse method. Door knobs/ handles were swabbed with sterile cotton swabs moistened with sterile normal saline. The swab was wiped firmly on the entire surface of the door handles/ knobs. It was then introduced into a test-tube containing sterile nutrient broth. Then it was immediately transported to the Microbiology Research Laboratory of BRAC University for further processing and analysis. The test tube containing the sample incubated at 37ºC overnight.

2.5.2 Sample Analysis:

For sample analysis following processing techniques were applied:

1. Culture
2. Gram staining
3. Biochemical tests

2.5.2.1 Culture technique:

After 24 hours, each sample was streaked onto Nutrient agar, MacConkey agar, Mannitol salt agar and Membrane fecal coliform agar plates. Four-quadrant streak plate technique was performed. All the plates were incubated for 24 hours at 37ºC. After the overnight incubation, the plates were 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.

2.5.2.1.1 Streak plate method:

Streak plate technique is used for the isolation of pure culture of the organisms.

Materials needed for streak plate method:

- A source of bacteria (stock culture, previously streaked agar plate or any other inoculums)
- 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 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.

2.5.2.2 Gram staining:

Gram staining was done for differentiating Gram positive and Gram negative bacteria.

- 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 washed off the slide with the tap water.
- Few drops of 70% ethanol was added and 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, and then it was observed under the microscope.

2.5.2.3 Spore staining:

Spore staining was done to determine the endospore forming bacteria.

- 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 containing flask and malachite green was added continuously.
- The slide was heated for 2 to 3 minutes.
- After heating, the slide was cooled and rinsed with tap water.
- Then the smear was stained with safranin for 30 seconds, washed with tap water and blot dried with bibulous paper.
- Then bacterial observation was done under the oil immersion lens (1000X) for the presence of endospores.

2.5.2.4 Biochemical tests:

2.5.2.4.1 Indole test:

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

- In test tube 6ml of indole broth 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
- The tubes were then incubated for 24 hours at 37ºC.
- To detect the indole production, 10 drops of Kovacs reagent was added to all the tubes.
- If red 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)

2.5.2.4.2 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 at37ºC.
- After 48 hours, 5 drops of methyl red indicator were 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).

2.5.2.4.3 Voges-Proskauer (VP) test:

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

- For Voges-Proskauer test each VP broth containing dipeptone, dextrose and potassium phosphate was taken.
- Using sterile technique, each tube was inoculated by fresh culture of experimental bacteria by means of loop inoculation method.
- The tubes were then incubated for 48 hours at37ºC.
- After 48 hours, 10 drops of Barritt's reagent A was added to each tube and the tubes were shaken. Then immediately 10 drops of Barritt's reagent B was added and the tubes were shaken.
- The colour was observed after 15-30 minutes of the reagent addition.

If red colour developed then it indicates that the organism was capable of fermenting glucose with ultimate production of acetyl methyl carbinol and it indicates positive result

- If no colour developed then it indicates voges- proskauer negative result. (Cappuccino &Sherman, 2005)

2.5.2.4.4 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.
- 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.
- If there was no colour change then it indicates citrate negative result. (Cappuccino &Sherman, 2005)

2.5.2.4.5 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).

2.5.2.4.6 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 (Cappuccino &Sherman, 2005).

2.5.2.4.7 MIU (Motility-indole-urease) test:

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

- Using sterile technique, 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 may have a yellow or brown layer (Cappuccino &Sherman, 2005).

2.5.2.4.8 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.
- If 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)

2.5.3 Preparation of Stock Sample:

2.5.3.1 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 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.

2.5.3.2 Long term preservation:

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.

2.6 Antibiotic susceptibility testing (AST):

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

2.6.1 Disk diffusion method:

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

2.6.1.1 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.

2.6.1.2 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 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.

2.6.1.3 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 sterilized forceps.
- Once all the discs were properly placed, the MHA plates were inverted and incubated at 37^0C for 24 hours.

2.6.1.4 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.

2.7 Plasmid profiling:

2.7.1 Plasmid DNA extraction:

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

2.7.1.1 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.

2.7.2 Plasmid profile analysis: Agarose gel electrophoresis:

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.

For running twenty samples 75 ml 0.7% agarose gel was prepared by TBE buffer. After a while 4ul EtBr from 10mg/ml stock was added. The gel was left undisturbed at room temperature for about 20 minutes to allow for uniform solidification.

Afterwards the comb was gently removed and the gel tray with the gel was placed in the electrophoresis chamber and covered (until all wells were submerged) with electrophoresis buffer (TBE buffer). To prepare samples for electrophoresis, 3µl of gel loading dye was added for every 12µl of plasmid DNA solution. The gel was run at seventy volts and it took approximately one and a half hour for the run to be completed. The gel was distained in distilled water for 20 minutes for band visualization under short wave UV light.

Chapter THREE

Result

RESULTS

3.1: Bacterial Identification:

A total of 16 samples were collected from the toilet doorknobs of different wards of Shaheed Suhrawardy Medical College and Hospital. These samples were streaked onto various selective, differential and nutrient agar media to identify organisms present in each sample. Gram positive and Gram negative bacteria were found in the samples. Results were recorded according to their colony morphology and biochemical characteristics of the isolates in different agar media. Cultural, morphological and biochemical characteristics of the isolates are shown in Table 1 and Table 2.

3.1.1: Cultural and morphological characteristics of the bacterial isolates:

In Table 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 given.

Table 1: Cultural and Morphological characteristics of bacterial colonies isolated from door handles of washrooms of a hospital

Bacterial isolates	**Agar medium**	**Size**	**Form**	**Pigmentation**	**Margin**	**Elevation**	**Suspected organism**
1a	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus spp.*
1b	Nutrient agar	Pin point	Circular	Yellow	Entire	Convex	*Micrococcus spp.*
1d	Nutrient agar	Large	Irregular	White, dull	Undulate	Umbonate	*Bacillus spp.*
2a	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus spp.*
2b	MacConkey agar	Large	Circular	Pink translucent	Undulate	Umbonate	*Klebsiella spp.*
2d	Nutrient agar	Large	Irregular	White, dull	Undulate	Umbonate	*Bacillus spp.*
4a	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus spp.*
4b	EMB agar	Small	Circular	Metallic green sheen	Entire	Raised	*E.coli*
4c	EMB agar	Small	Circular	Metallic green sheen	Entire	Raised	*E.coli*
4d	Nutrient agar	Large	Irregular	White, dull	Undulate	Umbonate	*Bacillus spp.*
5a	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus spp.*
5b	Nutrient agar	Pin point	Circular	Yellow	Entire	Convex	*Micrococcus spp.*
5c	Nutrient agar	Pin point	Circular	Yellow	Entire	Convex	*Micrococcus spp.*

Table 1: Cultural and Morphological characteristics of bacterial colonies isolated from door handles of washrooms of a hospital

Bacterial isolates	**Agar medium**	**Size**	**Form**	**Pigmentation**	**Margin**	**Elevation**	**Suspected organism**
5d	Nutrient agar	Large	Irregular	White, dull	Undulate	Umbonate	*Bacillus spp.*
6a	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus spp.*
6b	EMB agar	Small	Circular	Metallic green sheen	Entire	Raised	*E.coli*
7a	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus spp*
7b	EMB agar	Small	Circular	Metallic green sheen	Entire	Raised	*E.coli*
7d	Nutrient agar	Large	Irregular	White, dull	Undulate	Umbonate	*Bacillus spp.*
8a	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus spp*
8d	Nutrient agar	Large	Irregular	White, dull	Undulate	Umbonate	*Bacillus spp.*
9a	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus spp*
9b	EMB agar	Small	Circular	Metallic green sheen	Entire	Raised	*E.coli*

Table 1: Cultural and Morphological characteristics of bacterial colonies isolated from door handles of washrooms of a hospital

Bacterial isolates	**Agar medium**	**Size**	**Form**	**Pigmentation**	**Margin**	**Elevation**	**Suspected organism**
9c(F)	MFC agar	Small, moderate	Circular	Blue	Entire	Raised	Fecal coliform
9d	Nutrient agar	Large	Irregular	White, dull	Undulate	Umbonate	*Bacillus spp.*
10d	Nutrient agar	Large	Irregular	White, dull	Undulate	Umbonate	*Bacillus spp.*
11a	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus* spp
11b(F)	MFC agar	Small, moderate	Circular	Blue	Entire	Raised	Fecal coliform
12a	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus*
12b	MacConkey agar	Small	Circular	Pink	Entire	Raised	*E.coli*
12c(F)	MFC agar	Small, moderate	Circular	Blue	Entire	Raised	Fecal coliform
13a	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus*s pp

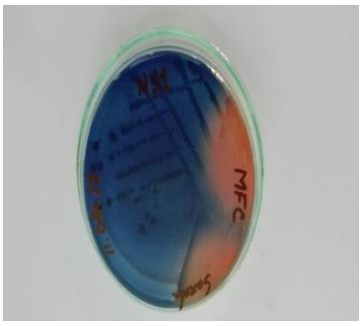
Table 1: Cultural and Morphological characteristics of bacterial colonies isolated from door handles of washrooms of a hospital

Bacterial isolates	**Agar medium**	**Size**	**Form**	**Pigmentation**	**Margin**	**Elevation**	**Suspected organism**
14a	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus* spp
14b(F)	MFCagar	Small, moderate	Circular	Blue	Entire	Raised	Fecal coliform
14c	Nutrient agar	Medium	Oval	Diffusible Green	Wavy	Umbonate	*Pseudomonas spp.*
15a	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus* spp
15b	MacConkey agar	Small	Circular	Pink	Entire	Raised	*E.coli*
15c	Nutrient agar	Medium	Oval	Diffusible Green	Wavy	Umbonate	*Pseudomonas* spp.
16a	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus* spp
16b(F)	MFC agar	Small, moderate	Circular	Blue	Entire	Raised	Fecal coliform
17a	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus* spp

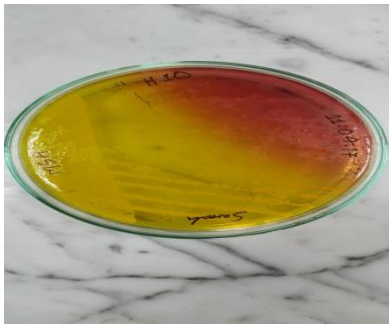
Table 1: Cultural and Morphological characteristics of bacterial colonies isolated from door handles of washrooms of a hospital

Bacterial isolates	**Agar medium**	**Size**	**Form**	**Pigmentation**	**Margin**	**Elevation**	**Suspected organism**
10a	MSA agar	Small	Circular	Yellow	Entire	Convex	*Staphylococcus spp*
17b(F)	MFC agar	Small	Circular	Blue	Entire	Raised	Fecal coliform

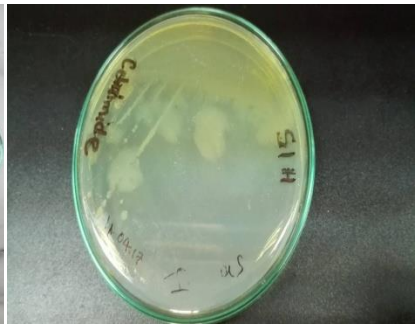
MFC Agar



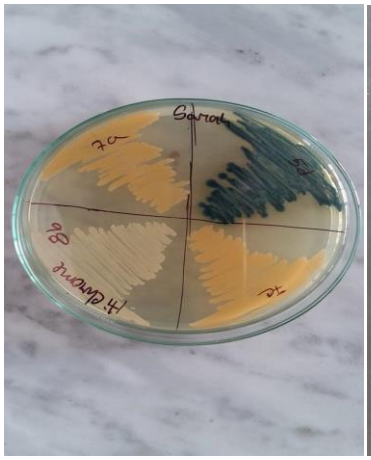
MSA agar



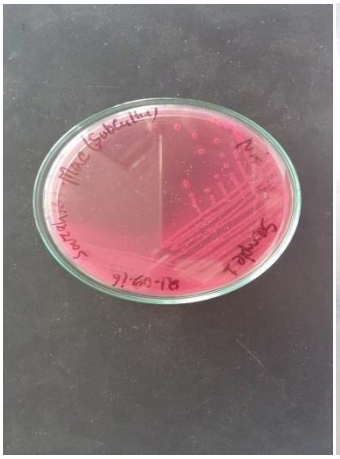
Cetrimide agar



Hichrome agar



MacConkey agar



EMB agar

BC agar

Nutrient agar

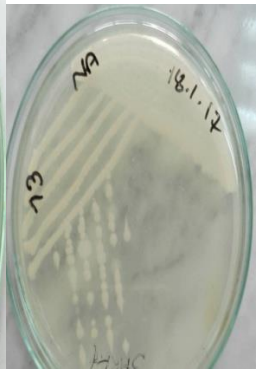


Figure 01: Bacterial growth on selective and nutrient media

3.1.2: Biochemical characteristics of the isolated bacteria:

All the isolated bacteria were tested by different biochemical tests for the confirmation of unknown organisms. After streaking on different agar plates organisms were isolated and sub-cultured for biochemical tests. For performing every biochemical test fresh cultures of the isolates were taken. The isolated organisms were identified with the help of reference books including Microbiology: A Laboratory Manual by Cappuccino and Sherman and Bergey's manual of systematic bacteriology. The biochemical test results of the isolates are given in Table 2:

Table 2: Biochemical characteristics of the bacterial isolates of different washroom door handles of hospital

Isolates no.	Isolates ID	Indole	Methyl red	VogesProskauer	Simmon's	MIU		Catalase	TSI				Gram staining	Blood agar hemolysis	Suspected Organism
						Motility	Urease		Slant/ Butt	H_2S production	Gas Productio	Nitrate reduction			
1.	1a	-	+	+	-	+	+	+	Y/Y	-	-	+	+cocci	-	*Staphylococcus spp.*
2.	1b	-	-	-	-	-	+	+	R/R	-	-	-	+cocci	-	*Micrococcus spp.*
3.	1d	-	+	+	-	-	+/-	+	R/Y	+	+	+	+ Rod	+	*Bacillus spp.*
4.	2a	-	+	+	-	+	+	+	Y/Y	-	-	+	+cocci	+	*Staphylococcus spp.*
5.	2b	-	+	+	+	+	+	+	Y/Y	-	+	+	-Rod	-	*Klebsiella spp.*
6.	2d	-	+	-	-	-	-	+	R/Y	-	-	+	+Rod	+	*Bacillus spp.*
7.	4a	-	+	+	-	-	+	+	Y/Y	-	-	+	+cocci	-	*Staphylococcus spp.*
8.	4b	+	+	-	-	+	+/-	+	Y/Y	-	-	+	-Rod	-	*Escherichia coli*
9.	4c	+	+	-	-	+	+/-	+	R/Y	-	-	+	-Rod	-	*Escherichia coli*
10.	4d	-	-	-	-	-	+/-	+	R/Y	-	-	+	+Rod	+	*Bacillus spp.*
11.	5a	-	+	-	-	+	+	+	Y/Y	-	-	+	+cocci	-	*Staphylococcus spp.*
12.	5b	-	-	-	-	-	+	+	R/R	-	-	-	+cocci	-	*Micrococcus spp.*
13.	5c	-	-	-	-	-	+	+	R/R	-	-	-	+cocci	-	*Micrococcus spp.*
14.	5d	-	-	+	+	-	+/-	+	R/Y	+	+	+	+Rod	-	*Bacillus spp.*
15.	6a	-	+	+	-	+	+	+	Y/Y	-	-	+	+cocci	+	*Staphylococcus spp.*
16.	6b	+	+	-	-	+	-	+	R/Y	-	+	+	-Rod	-	*Escherichia coli*
17.	7a	-	+	-	-	+	+	+	Y/Y	-	-	+	+cocci	+	*Staphylococcus spp.*

Table 2: Biochemical characteristics of the bacterial isolates of different washroom door handles of hospital

Isolates no.	IsolatesID	Indole	Methyl Red	Vogsporskaue	Citrate	Motility	Urease	Catalase	Stant/Butt	H_2S production	Gas Productio	NO_3 reduction	Gram Staining	Blood Agar hemolysis	Suspected Organism
18.	7b	+	+	-	-	-	-	+	R/Y	-	+	+	-Rod	-	*Escherichia coli*
19.	7d	-	+	-	+	-	+/-	+	R/Y	+	-	+	+Rod	+	*Bacillus spp.*
20.	8a	-	+	+	-	+	+	+	Y/Y	-	-	+	+cocci	+	*Staphylococcus spp.*
21.	8d	-	-	-	-	-	+/-	+	R/Y	-	-	+	+Rod	+	*Bacillus spp.*
22.	9a	-	+	+	-	-	+	+	Y/Y	-	-	+	+cocci	+	*Staphylococcus spp.*
23.	9b	+	+	-	-	+	-	+	Y/Y	-	-	+	-Rod	-	*Escherichia coli*
25.	9d	-	+	+	-	-	+/-	+	R/Y	-	-	+	+Rod	+	*Bacillus spp.*
26.	10a	-	+	+	-	+	+	+	Y/Y	-	-	+	+cocci	+	*Staphylococcus spp.*
27.	10d	-	+	+	-	+	+/-	+	Y/Y	-	-	+	+Rod	+	*Bacillus spp.*
28.	11a	-	+	+	-	+	+	+	Y/Y	-	-	+	+cocci	-	*Staphylococcus spp.*
30.	12a	-	+	+	-	+	+	+	Y/Y	-	-	+	+cocci	+	*Staphylococcus spp.*
31.	12b	+	+	-	-	+	-	+	Y/Y	-	+	+	-Rod	-	*Escherichia coli*
33.	13a	-	+	+	-	+	+	+	Y/Y	-	-	+	+cocci	+	*Staphylococcus spp.*
34.	14a	-	+	+	-	+	+	+	Y/Y	-	-	+	+cocci	+	*Staphylococcus spp.*
36.	14c	-	-	-	+	-	-	+	R/R	-	-	+	-Rod	-	*Pseudomonas spp.*
37.	15a	-	+	+	-	+	+	+	Y/Y	-	-	+	+cocci	+	*Staphylococcus spp.*
38.	15b	+	+	-	-	+	-	+	Y/Y	-	+	+	-Rod	-	*Escherichia coli*

Table 2: Biochemical characteristics of the bacterial isolates of different washroom door handles of hospital

Isolates no.	**IsolatesID**	**Indole**	**Methyl Red**	**Vogsporskaue**	**Citrate**	**Motility**	**Urease**	**Catalase**	**Stant/Butt**	**H_2S production**	**Gas Production**	**NO_3 reduction**	**Gram Staining**	**Blood Agar hemolysis**	**Suspected Organism**
39.	15c	-	-	-	+	+	+/-	+	R/R	-	-	+	-Rod	-	*Pseudomonas spp.*
40.	16a	-	+	+	-	+	+	+	Y/Y	-	-	+	+cocci	+	*Staphylococcus spp.*
42.	17a	-	+	+	-	+	+	+	Y/Y	-	-	+	+cocci	+	*Staphylococcus spp.*
"+"=Positive, "-''= Negative, Y=Yellow(Acidic), R=Red(Alkaline)															

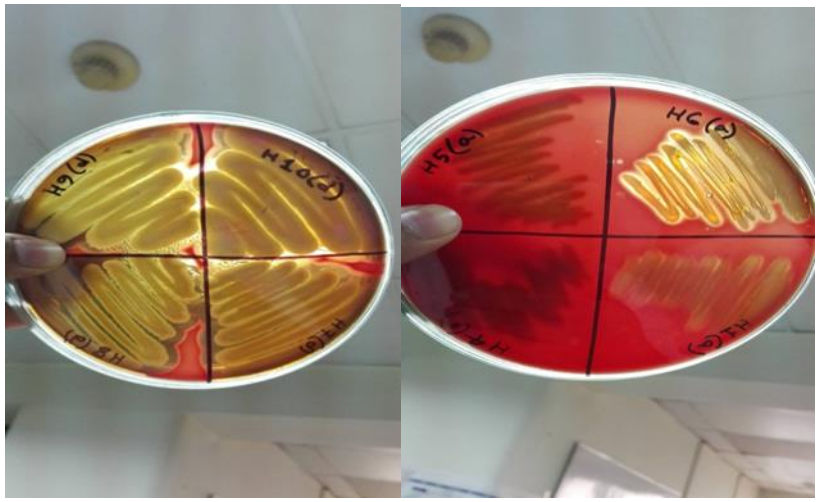
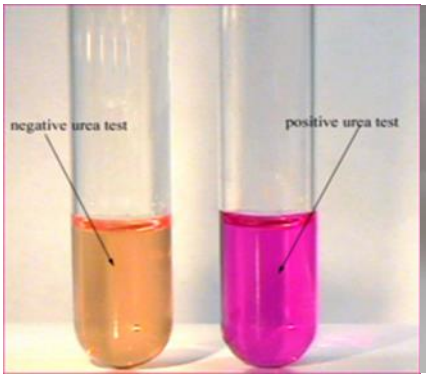
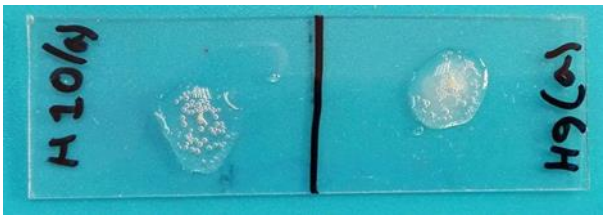


Figure 02: Blood Agar hemolysis

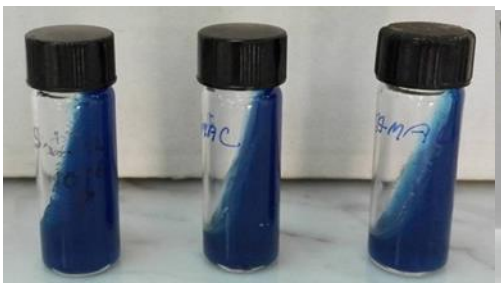


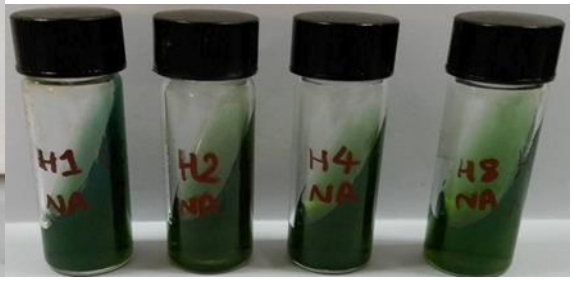
MIU test

Nitrate reduction test



Catalase test





Citrate test

Yellow slant, yellow butt	Red slant, Red butt	Red slant, yellow butt	Yellow slant, Yellow butt

VP test MR Test

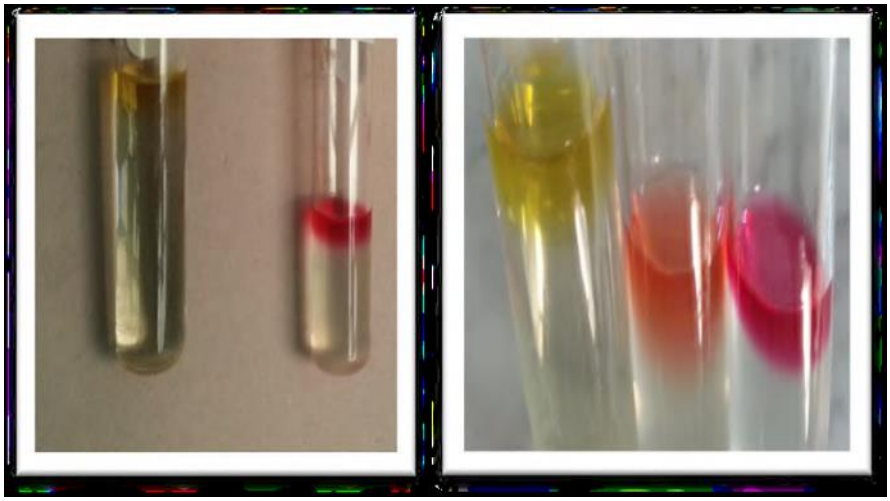


Figure 03: Biochemical test results of bacterial isolates

After performing the biochemical tests and observing cultural and morphological characteristics 43 isolates were identified from 16 different samples collected from hospital toilet doorknobs. The isolates include *Staphylococcus* spp., (found in 16 samples), *Bacillus* spp., (found in 8 samples), *E.coli* (found in 7 samples), Fecal coliform (found in 6 samples), *Micrococcus* spp., (found in 3 samples), *Pseudomonas* spp, (found in 2 samples), *Klebsiella* spp. (found in 1 sample).

The total number and the percentage of the isolates obtained from the samples are shown in Table 3

Table 3: Prevalence of bacteria isolated from door handles of washrooms of a hospital

Name of the Bacteria	**Number of isolates**	**Percentage %**
Staphylococcus spp.	16	37.21
Bacillus spp.	8	18.6
E. coli.	7	16.28
Fecal Coliform	6	13.95
Micrococcus spp.	3	6.98
Pseudomonas spp.	2	4.65
Klebsiella spp.	1	2.33
	Total= 43	100

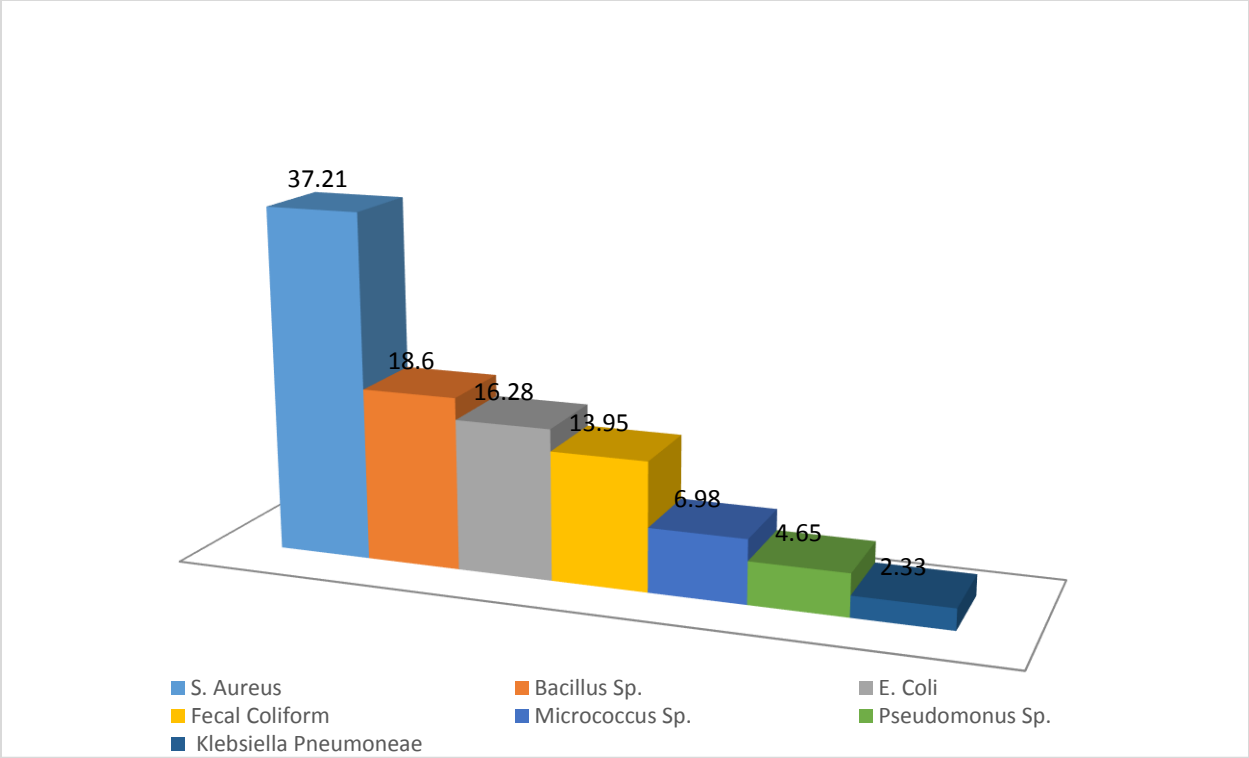


Figure 04: Prevalence of the isolated Gram positive and Gram negative bacteria from door handles of washrooms of a hospital

Both Gram positive and Gram negative organisms were found among the identified isolates. The Gram positive organisms include *Staphylococcus* spp., *Bacilllus* spp., and *Micrococcus* spp. The Gram negative organisms include *E.coli*, Fecal coliform, *Pseudomonas* spp., and*Klebsiella* spp. The number and the percentage of the identified Gram positive and Gram negative bacteria are shown in Table 4 and Figure 5

Table 4: Distribution of the isolates according to Gram's Reaction

Isolates	**Number of isolates**	**Percentage (%)**
Gram Positive	27 (out of 43)	62.8%
Gram Negative	16 (out of 43)	37.2%

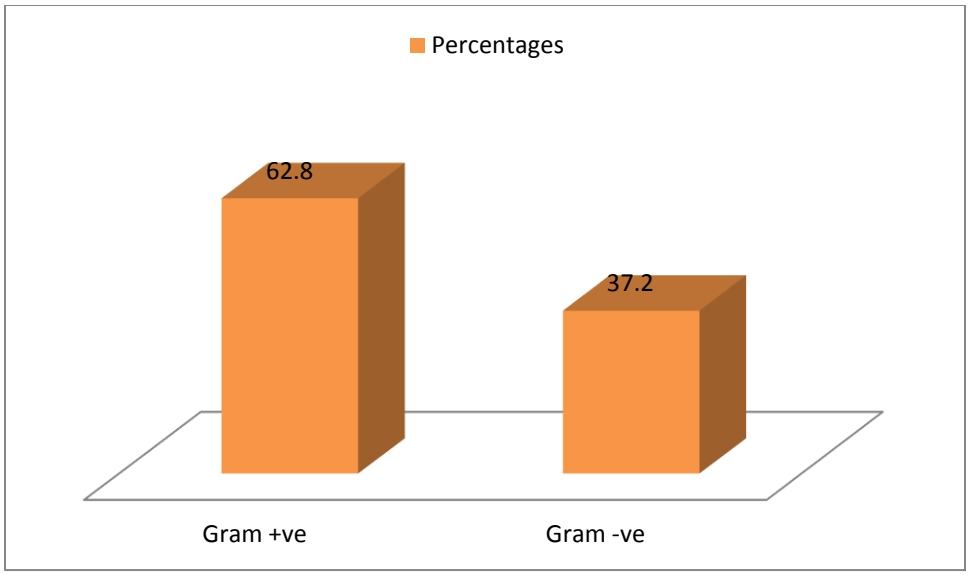


Figure 05: Total percentage of Gram positive and Gram negative bacteria isolated from door handles of washrooms of a hospital

3.2: Antibiotic susceptibility test

From forty three isolates, all isolates were selected for antibiotic susceptibility test. 9 antibiotic discs were used to see the sensitivity and resistance pattern of the isolates.

Table 5 was prepared showing resistance, intermediate and sensitivity pattern of isolates to different antibiotics. Some bacteria were resistant to more than two antibiotics and some were resistant to at least two antibiotics. Some bacteria were intermediate to some antibiotics that means the specific bacteria was neither susceptible nor resistant to that particular antibiotic. The interpretation of each bacterium either resistant or susceptible to antibiotic is shown in Table 5.

Table 5: Antibiotic susceptibility test of various organisms isolated from different door handles of washrooms of a hospital.

Isolates	Suspected organism	amoxicillin	chloramphenicol	ciproflox acin	SXT	rifampicin	gentamicin	tetracyclin	streptomycin	Penicillin G
1(a)	*Staphylococcus* spp.	S	S	S	R	S	S	S	S	S
1(b)	*Micrococcus* spp.	S	S	S	I	I	S	S	S	S
2(a)	*Staphylococcus* spp.	R	S	R	R	R	R	S	S	R
2(b)	*Klebsiella*spp.	R	S	S	R	R	S	S	S	R
4(a)	*Staphylococcus* spp.	S	S	R	R	S	S	S	S	R
4(b)	*E.coli*	R	I	S	R	I	S	S	S	R
4(c)	*E.coli*	R	I	R	R	R	S	R	R	R
5(a)	*Staphylococcus* spp.	S	S	S	R	S	S	S	S	S
5(b)	*Micro-coccus*sp.	R	S	S	R	I	S	S	S	R
5(c)	*Micro-.Coccus spp.*	S	S	S	R	R	S	S	S	R
6(a)	*Staphylococcus*spp.	S	S	S	I	S	S	S	S	R
7(a)	*Staphylococcus*spp.	R	S	R	S	S	S	S	S	R
8(a)	*Staphylococcus* spp.	R	S	R	R	S	S	S	S	R

Table 5: Antibiotic susceptibility test of various organisms isolated from different door handles of washrooms of a hospital.

isolates	Suspected organism	tetracycline	Amoxi-cillin	Genta-micin	SXT	Rifam-picin	Chloram-phenicol	Cipro-floxacin	Strepto-mycn	Peni-cillin
1(d)	*Bacillus*spp.	S	R	S	R	R	S	S	S	R
2(d)	*Bacillus*spp.	S	R	S	R	R	S	S	S	R
4(d)	*Bacillus*spp.	R	R	S	R	R	S	S	S	R
5(d)	*Bacillus*spp.	R	S	S	R	S	S	S	S	R
7(d)	*Bacillus*spp.	S	R	S	R	R	S	S	S	R
8(d)	*Bacillus*spp.	S	R	S	R	R	S	S	S	R
9(d)	*Bacillus*spp.	S	R	S	R	R	S	S	S	R
11(a)	*Staphylococcus*spp.	S	R	I	R	R	I	R	S	R
9(a)	*Staphylococcus*spp.	S	S	S	S	S	S	R	S	R
10(a)	*Staphylococcus*spp.	S	S	S	S	S	S	I	S	R
10(d)	*Bacillus*spp.	S	R	S	R	R	S	S	S	R
12(a)	*Staphylococcus*spp.	R	S	S	R	S	S	I	R	R
13(a)	*Staphylococcus*spp.	R	R	R	R	S	S	R	R	R

Table 5: Antibiotic susceptibility test of various organisms isolated from differentdoorhandles of washrooms of a hospital.

isolates	Suspected organism	tetracycline	amoxicillin	gentamicin	ciprofloxacin	SXT	Chloramphenicol	rifampicin	streptomycin	Penicillin G
15(a)	*Staphylococcus* spp.	S	S	S	R	R	S	S	S	R
15(c)	*Pseudomonass pp.*	R	R	S	S	R	S	R	S	R
16(a)	*Staphylococcus* spp.	S	S	S	S	I	S	S	S	R
17(a)	*Staphylococcus* spp.	S	S	S	S	I	S	S	S	R
12(b)	*E.coli*	S	R	S	S	I	S	R	S	R
14(a)	*Staphylococcus spp.*	S	S	S	R	R	I	S	S	R
14(c)	*Pseudomonass* pp.	R	R	S	S	R	S	R	S	R

Table 5: Antibiotic susceptibility test of various organisms isolated from different door handles of washrooms of a hospital.

Isolates	Suspected organism	Tetra cycline	Amoxi cillin	Gentami cin	Ciproflo xacin	SXT	Chloram phenicol	Rifam picin	Strepto mycin	Penicillin G
6b	*E.coli*	S	R	S	S	R	S	R	S	R
7b	*E.coli*	S	R	S	S	S	S	R	S	R
9b	*E.coli*	S	R	S	S	S	S	R	S	R
15b	*E.coli*	S	R	S	S	R	I	R	S	R
9c(F)	Fecal coliform	S	R	S	S	R	S	R	S	R
11b(F)	Fecal coliform	S	R	S	S	R	S	R	S	R
12c(F)	Fecal coliform	S	R	S	S	S	S	R	S	R
14b(F)	Fecal coliform	R	R	S	S	R	S	R	S	R
16b(F)	Fecal coliform	S	R	S	S	R	R	R	S	R
17b(F)	Fecal coliform	S	R	S	S	R	R	R	S	R

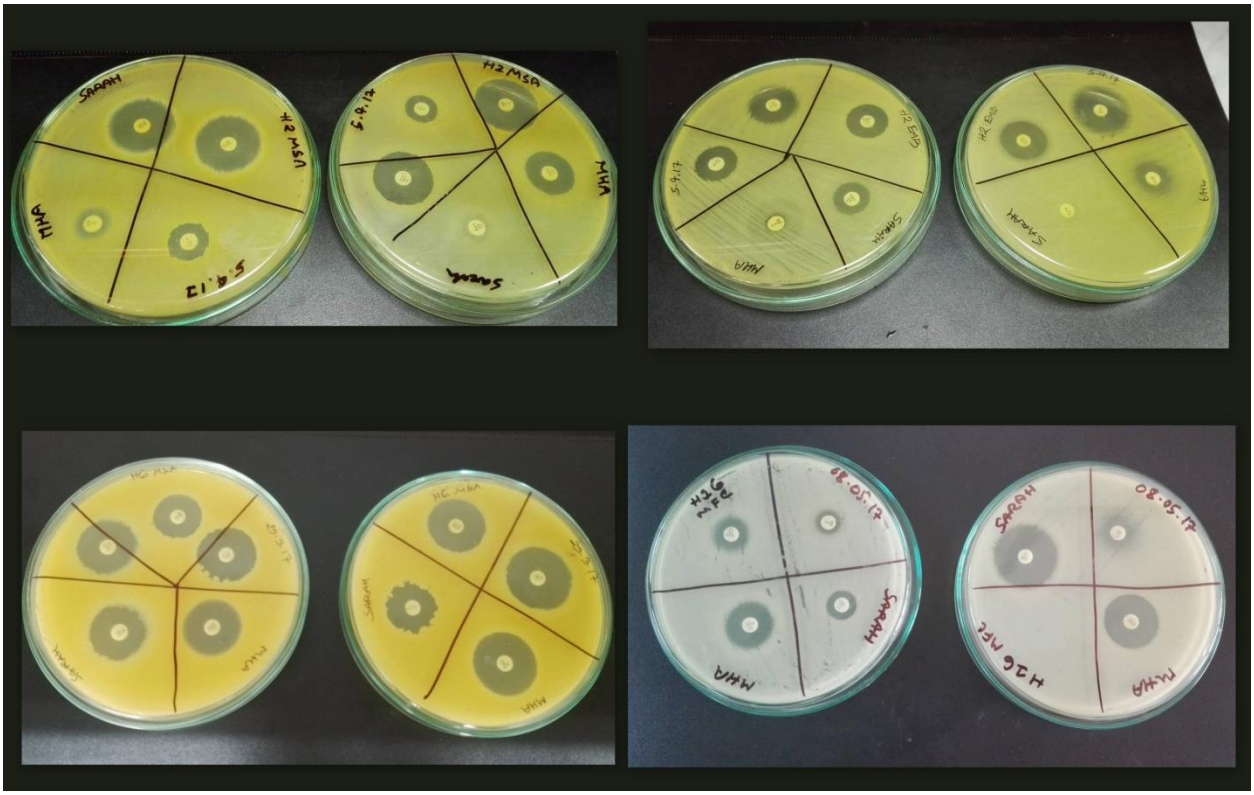


Figure 06: Antibiotic Susceptibility Test of isolates (disc diffusion method)

3.2.2: Resistance pattern of the organisms to the tested antibiotics:

After determining the antibiotic resistant bacteria isolated from hospital toilet doorknobs, the percentage of the resistance to the antibiotics tested was also determined. The results are shown in Table 6

Table 6: Percentage of isolates resistant to antibiotics

Serial number	Antibiotics	Percentage of isolates resistant to antibiotics	No. of isolates resistant to antibiotics
1	Amoxicillin	65.12	28
2	Chloramphenicol	4.65	2
3	Ciprofloxacin	23.26	10
4	SXT	74.42	32
5	Rifampicin	55.81	24
6	Gentamicin	2.33	1
7	Tetracycline	18.60	8
8	Streptomycin	6.98	3
9	Penicillin G	95.35	41

The most resistance was seen against penicillin G, with a number of 41(95.35%) isolates being resistant against it. Next to penicillin G, 32 isolates were resistant to SXT, giving a percentage of 65.12. The third highest resistance was seen against, amoxicillin where 32 (65.12) isolates were resistant to it. Whereas, the isolates were most sensitive toward gentamicin (2.33%) followed by resistance to chloramphenicol (4.65%) and streptomycin (6.98%).

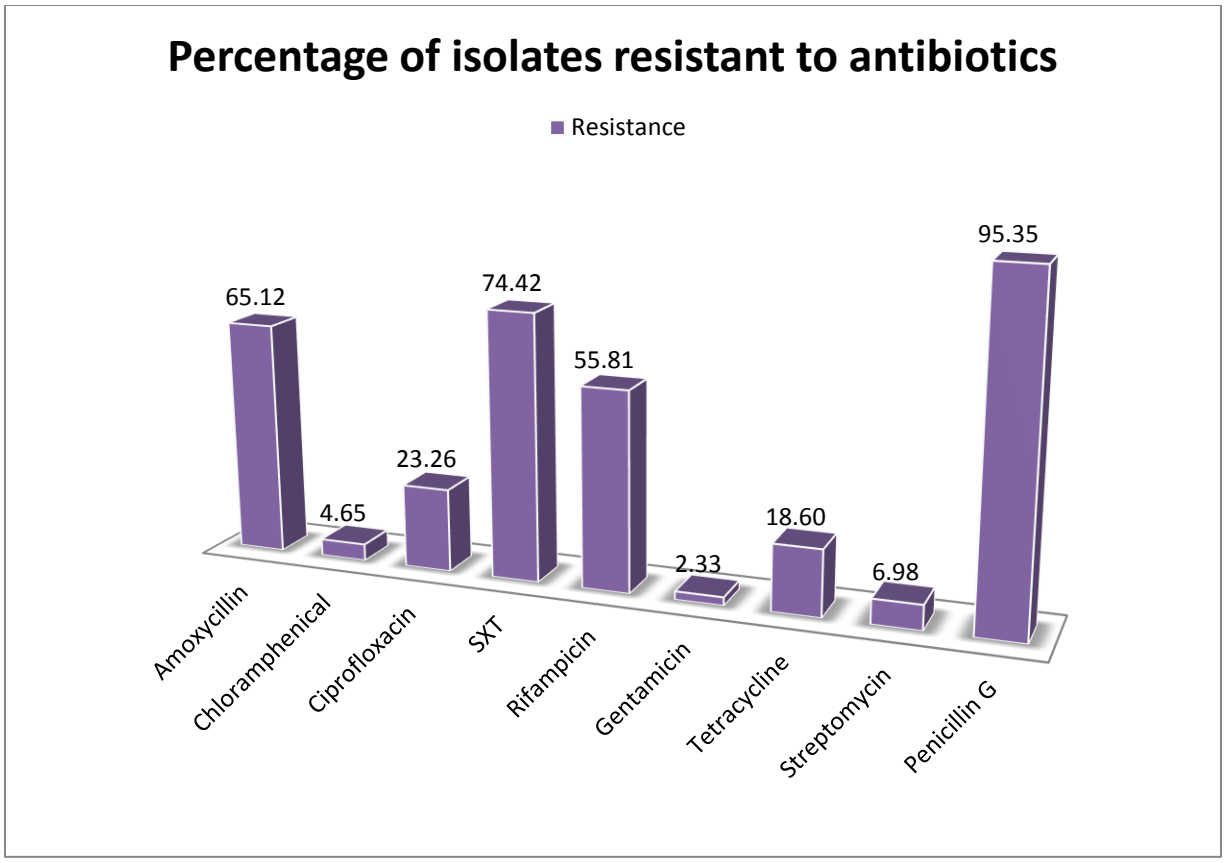


Figure 07: Percentage of isolates resistant to antibiotics

3.2.3: Prevalence of isolates resistant to more than two antibiotics and resistant to at least two antibiotics

After observing antibiotic resistance pattern of the isolated organisms, the percentage of organisms resistant to more than two antibiotics and resistant to at least two antibiotics was investigated and the percentage is given in table 7

Table 7: Prevalence of isolates resistant to more than two antibiotics and resistant to at least two antibiotics

Total bacterial isolate	Number of isolates resistant to more than two antibiotics	Percentage of isolateresistant to more than two antibiotics (%)	Percentage of isolate resistant to two antibiotics (%)
43	36	83.72%	16.28%

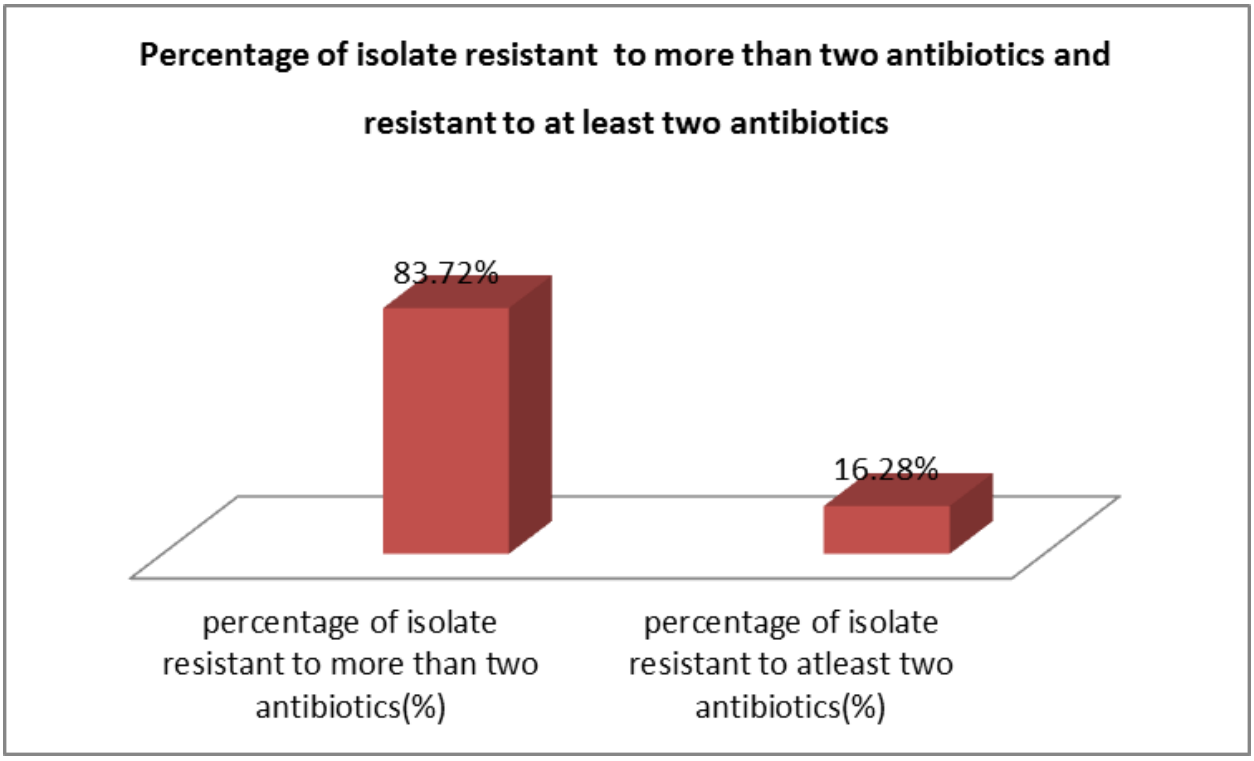


Figure 08: Total percentage of isolates resistant to more than two antibiotics and resistant to at least two antibiotics

3.3: Plasmid profile analysis of the isolated bacteria:

Plasmid was present among 25.58% of the bacterial isolates. Three organisms carried large plasmid with approximately 85 MDal size. The plasmid patterns, their approximate molecular weight are given below in the following figure:

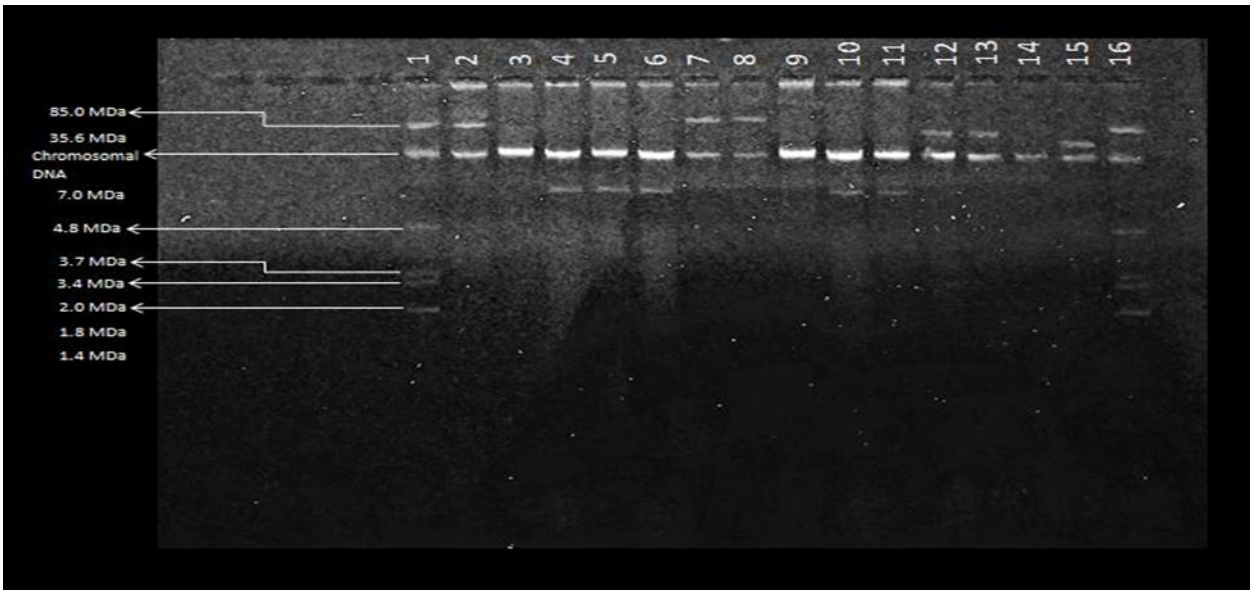


Fig 09: Plasmid profile of isolates obtained from hospital washroom door handles

Table 8: Plasmid pattern and number of isolates hosting the plasmid

Pattern	Lane in Fig.	Approximate size of plasmid (MDa)	Number of isolates hosting the pattern
1	2,7,8	~85	3
2	4,5,6,10,11	~8	5
3	12,13	~50	2
4	15	~35.6	1
5	1,16	~85.0, ~4.8, ~3.7, ~3.4, ~2.0	1 (v517)control strain of *E.coli*

Presence of Plasmid in isolates:

Among 43 isolates plasmid was found in organisms which were resistant to more than two antibiotics and total number and percentage of organism that harboring plasmids are following:

Table 9: Total number and percentage of organism that harboring plasmids and also resistant to more than two antibiotics are given below:

Total isolates resistant to more than two antibiotics	Number of isolates harboring plasmids which are resistant to more than two antibiotics	Percentage of isolates harboring plasmids which are resistant to more than two antibiotics
36	11	30.56%

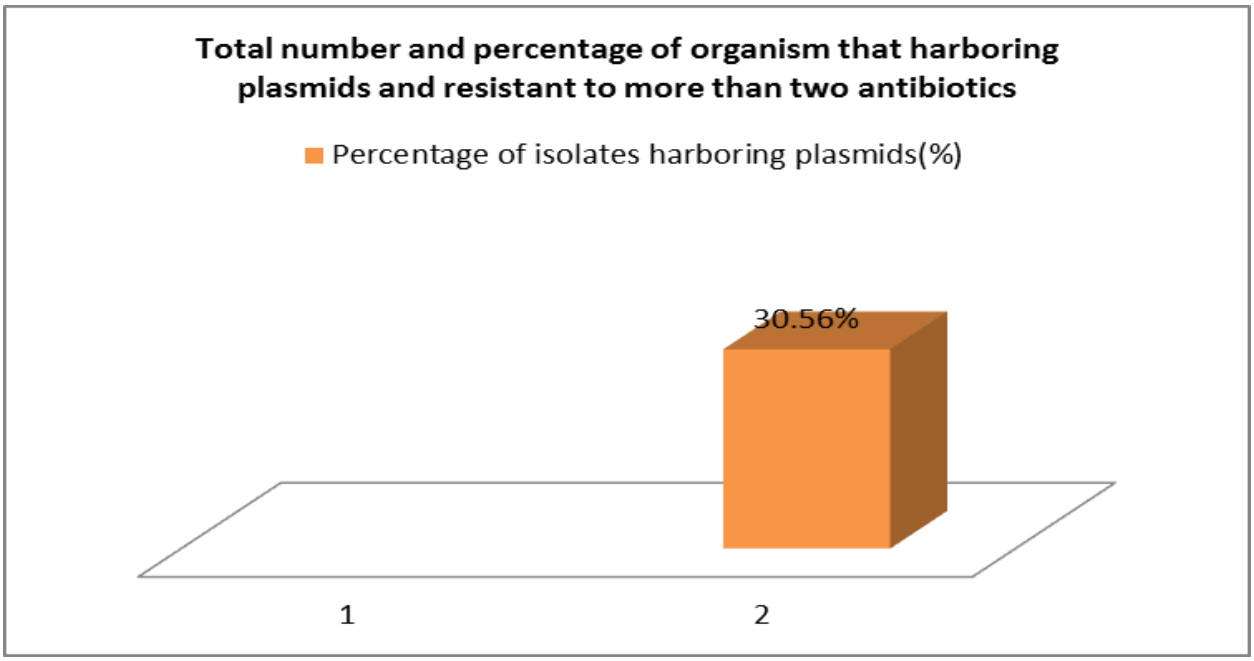


Fig 10: Percentage of organisms harboring plasmids which are resistant to more than two antibiotics

Chapter Four

Discussion

Discussion:

The result obtained from this study was that out of 16 samples 16 of them showed bacterial contamination. After conducting the biochemical tests, the isolates were confirmed as the following organisms: *E. coli*, *Klebsiella* spp., *Micrococcus* spp., *Staphylococcus* spp., *Bacillus* spp., *Pseudomonas* spp., and some other fecal organisms.

In this study, among the isolates, the most predominant bacteria were *Staphylococcus* spp., with a percentage of 37. This is anticipated as it is a major component of the normal flora of the skin and nostrils. The findings of other researchers (Nworie *et al*., 2012; Ducel *et al.,* 2002; Brooks *et al.,* 2007), is in accordance with this finding.

Similar in selected hospitals in Akoko, Ondo State Southwest Nigeria (Alabi et al., 2013) showed that the frequency of Gram positive bacteria was higher than the Gram negative bacteria. This also corroborates the findings of this study and agrees with the statement that Gram-positive bacteria have overtaken the Gram-negative as the major bacteria isolated from fomites (Inweregbu et al., 2005).

The result of this study is also consistent with Jalalpoor et al.,(2009) who reported that *Staphylococcus species* (54.7%) was the most frequent bacteria isolated in hospital environment. In contrast, the result of this study did not agree with the work of Orji et al *(2005)* which showed that *Staphylococcus aureus* was the least isolated bacteria.

The surfaces of the hospital environment can serve as an important secondary reservoir for multidrug resistant microorganisms, such as the MRSA as reported by Carvalho et al. (2007). Because of the apparent ability of these pathogens to survive on dry surfaces, these can grow well in the hospital environment. Therefore, the spread of multidrug resistant *Staphylococcus aureus* in hospital may pose a great threat to the people coming and staying there.

As mentioned before, Gram-positive bacteria are found more in the hospital fomites than Gram Negative one. This can become dangerous as Gram positive bacteria are causing more infections than ever before in surgical patients, who are increasingly aged, ill and debilitated (Barie, 1998).

Isolation of more Gram positive bacteria than Gram negative canbe explained, as they are members of the body flora of both asymptomatic carriers and sick persons. These organisms can be spread by the hand, expelled from the respiratory tract or transmitted by animate or inanimate

objects (Chikere et al., 2008). Their main source(s) of colonization on the fomites might likely be nasal carriage by hospital personnel (Graham et al., 2006), likely facilitated by hand-to-mouth or hand-to-nose contact while using these fomites, and/or by improper hand washing (ASM, 2005).

Isolation of *Staphylococcus aureus* from almost all the fomites indicates their ubiquitous nature. Additionally, they can be sources of infection to patients as previously noted (Hartmann et al., 2004; Inweregbuet al., 2005; Ikeh and Isamade, 2011).

A high percentage of *Bacillus* spp. was isolated from hospital washroom door handles. This is also in agreement with the research carried out by Brooks *et al.,* (2007) who reported that *Bacillus* spp. was found to be the predominant organism among all the organisms that were isolated from door handles.

Bacillus spp., the only Gram positive bacilli encountered in this study, has been isolated with the highest frequency in some studies in Nigeria (Nwankitiet al., 2012) and (Ikeh and Isamade, 2011). This organism forms endospores, which, allows them to settle well on the surface from fomites from air.

Although, Gram positive organisms were more frequently isolated in this study, the Gram negative bacterium *E. coli* was also isolated from toilet doorknobs. This indicates improper hand washing after the use of toilet.

Pseudomonas spp., and *Klebsiella* spp., and some fecal coliform bacteria were also isolated which are Gram negative. *Micrococcus* spp., which is Gram positive bacteria, was also isolated from hospital toilet door knobs.

From the findings in this study, it was observed that most of the isolates obtained were resistant to most commonly used antibiotics. These antibiotics are Amoxicillin, SXT and Penicillin G. The resistance to these antibiotics which is in accord with the research carried out by Adewoyin*et al.*(2013), who reported that antibiotic resistant microorganism contaminates environmental surfaces such as toilet. Moreover, reported that most of the isolates obtained in their study were resistant to commonly used antibiotics such as Amoxicillin and Ampicillin.

Among 43 isolates 16 isolates were *Staphylococcus* spp. and 7 of them were multidrug resistant. These MDR *Staphylococcus* isolates were mostly resistant to Penicillin G, Ciprofloxacin, SXT,

Amoxicillin and Rifampicin. The number of *Bacillus* spp. isolates was 8 and all of them were resistant to Amoxicillin, SXT, Rifampicin, Tetracycline and Penicillin G. All the *E.coli* isolates were mostly resistant to Amoxicillin, Penicillin G, SXT and Rifampicin. Similarly, *Klebsiella* spp. isolates showed resistance to Amoxicillin, Penicillin G, SXT and Rifampicin. Three isolates were *Micrococcus* spp. and they were resistant to Rifampicin and Penicillin G. The *Pseudomonas* spp. isolates were resistant to Tetracycline, Amoxicillin, SXT, Rifampicin and Penicillin G. Other fecal coliforms were resistant to Amoxicillin, Rifampicin, SXT, and Penicillin G. High percentage drug resistance was observed for Penicillin G (95.35%), SXT (74.42%), Amoxicillin (65.12%) and Rifampicin (55.81%) (Table 5). The result of susceptibility of antibiotics presented different degree of resistance to the different drugs used against different organisms. From the result of antibiotic susceptibility test, all isolates were resistant to at least one of the nine antibiotics tested and all the isolates were mostly sensitive to Chloramphenicol, Gentamycin and Streptomycin.

The plasmid profile showed the absence of plasmids for maximum isolates. The occurrence of plasmids in Gram negative bacteria was found. Eleven isolates including *E.coli, Klebsiella* spp., and fecal coliforms contained plasmid. Findings also indicated that isolates that were resistant to more than two antibiotics may harbor plasmid. Isolation of plasmids using agarose gel electrophoresis and observation under UV trans-illuminator showed the bands for the *E.coli*, *Klebsiella* spp., and other fecal coliforms with the molecular weights of plasmids ranging from approximately 8 to 85 MDal.

Conclusion:

Recently, nosocomial infections are rising at an alarming rate. The causes of these infections in hospitals can be connected to increased microbial load of fomites of these places. The data from this study indicates that there is a high level of bacterial contamination on door handles of hospital washrooms. This is of tremendous clinical significance, because of its potential to cause epidemics in hospital. Moreover, the antibiotic susceptibility of isolates showed resistance to at least two antibiotics. Furthermore, it indicated a plausible similar scenario in other hospitals or places. The rise of antibiotic resistance in microbes, especially pathogenic organisms can lead to lethal outcomes. Therefore, it should be tackled with high importance. However, this problem is not limited to this area of study alone. Thus, this will require combined effort of governmental, private organizations and individuals to educate the population on personal and environmental hygiene.

Regular disinfection of door handles as well as frequent washing of hands could also go long way. Hand washing practice after using toilet should be adopted by everyone to prevent the spread of microorganisms. The hospital management should give more attention to the distribution of hand sanitizers to the users. More trained cleaner should be employed for maintaining proper cleaning of hospital washrooms. The patients, visitors, employees, nurses, doctors, even cleaners should maintain personal hygiene. Otherwise, it will be difficult to control microbial contamination of door handles of washrooms of hospital. To minimize microbial load and to prevent cross contamination, regular cleaning of washroom surfaces with spray disinfectants might be useful. In this study most of the isolates were resistant to more than two antibiotics and plasmids were found in some multi-drug resistant organisms. Further study can be done to find out any correlation between multidrug resistance of bacteria and presence of plasmids. So, further research upon plasmid will also be very significant.

Chapter Five

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Appendices

Appendices

APPENDIX-I

Media composition:

The composition of the media used in this study has been given below. Unless otherwise mentioned, all the media were autoclaved at 121 ^{0} C for 15 min.

1). Nutrient agar (Himedia, India)

Ingredients	Amount (g/L)
Peptic digest of animal tissue	5.0
Beef extract	1.50
Sodium chloride	5.0
Yeast extract	1.50
Agar	15.0
Final pH(at 25˚C)	7.4±0.2

2). MacConkey agar (Oxoid, England)

Ingredients	Amount (g/L)
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0
pH	7.4±0.2

3). Mannitol Salt agar (Himedia)

Ingredients	Amount (g/L)
Peptic digest of animal tissue	5.00
D-Mannitol	10.00
Pancreatic digest of casein	5.00
Beef extract	1.00
Sodium chloride	75.00
Phenol red	0.025
Agar	15.00
pH after sterilization (at 25˚C)	7.4±0.2

4). Eosine methylene blue agar (Oxoid, England)

Ingredients	Amount (g/L)
Peptone	10.0
Lactose	10.0
Di-potassium hydrogen phosphate	2.0
Eosin Y	0.4
Methylene blue	0.06
Agar	15.0
Final pH	6.8±0.2

5). MFC Agar

Ingredients	Amount (g/L)
Biosate peptone	10.0
Polypeptone peptone	5.0
Yeast extract	3.0
Sodium chloride	5.0
Lactose	12.5
Bile salts	1.5
Aniline blue	0.1
Rosolic acid	10 ml
Final pH (at 25˚C)	7.4±0.2

6). Cetrimide Agar (Himedia)

Ingredients	Amount (g/L)
Cetrimide	0.3
Gelatine peptone	20.0
Magnesium chloride	1.4
Potassium sulfate	10.0
Agar	15.0

7). Blood Agar

Ingredients	Amount (g/L)
Beef heart infusion from (beef extract)	500.0
Tryptose	10.0
Sodium chloride	5.0
Agar	15.0

8). T_1N_1 Agar

Ingredients	Amount (g/L)
Tryptone	1.0
Sodium chloride	1.0
Agar	0.6-0.75

9). Mueller-Hinton Agar (Himedia)

Ingredients	Amount (g/L)
Beef, infusion	300.0
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17.0

10). HiCrome UTI Agar (Himedia)

Ingredients	Amount (g/L)
Peptic digest of animal tissue	15.00
Chromogenic mixture	26.80
Agar	15.00
Final pH (at 25˚ C)	6.8±0.2

11). Bacillus Cereus Agar Base (Himedia)

Ingredients	Amount(g/L)
Peptic digest of animal tissue	1.000
Mannitol	10.000
Sodium chloride	2.000
Magnesium sulphate	0.100
Disodium phosphate	2.500
Monopotassium phosphate	0.250
Sodium pyruvate	10.000
Bromothymol blue	0.120
Agar	15.000
Final pH (at 25˚C)	7.2±0.2

12). Simmon's Citrate Agar

Ingredients	**Amount(g/L)**
Magnesium sulphate	0.2
Ammoniundihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bactobromothymol blue	0.08

13). Methyl red VogusPrekaure (MRVP) Media

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

14). Triple Sugar Iron Agar

Ingredients	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

15). Motility Indole Urease (MIU) Agar

Ingredients	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

Appendix –II

Reagents

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.

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.

Kovac's Reagent (150 ml)

To a reagent bottle, 150 ml of reagent grade isoamyl alcohol, 10 g of pdimethylaminobenzaldehyde (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.64

Barrit's Reagent A (100 ml)

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

Barrit's Reagent B (100 ml)

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

Catalase Reagent (20 ml 3% hydrogen peroxide)

From a stock solution of 35 % hydrogen peroxide, 583 µlsolution 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) to form a colourless solution and stored at 4°C.

MacFarlane turbidity standard no. 5

- Sulfuric acid 0.18 M

- Barium chloride 0.048 M
- Distilled water 1000 ml

KADO-I composition (pH 7.4):

4ml of 1M TrisHcl and 400µl of 0.5M EDTA was added into 100ml of distilled water.

KADO-II composition:

0.6 gm of Tris base, 3 gm of SDS and 6.4 ml of 2N NaoH (0.8 gm in 10 ml) was added into the 100 ml of distilled water.

Preparation of 0.5M EDTA:

1.861 gm of EDTA was mixed into 10ml of distilled water and then pH level was adjusted at 8.0.

Preparation of 1M TrisHcl:

1.576 gm of TrisHcl was mixed into 10ml of distilled water and the pH level was adjusted at 8.0.

Preparation of 1* TBE Buffer (500ml):

5.4 gm of Tris base, 2.75 gm of Boric Acid and 2ml of 0.5M EDTA was added into 500ml of distilled water and the pH level was adjusted at 8.0 and then the buffer was autoclaved.

Appendix – III

Gadgets

List of gadgets that were used in the study

Instruments	**Manufacturer**
Weighing Machine	Adam equipment, UK
Incubator	SAARC
Laminar Flow Hood	SAARC
Autoclave Machine	SAARC
Sterilizer	Labtech, Singapore
Shaking Incubator, Model: WIS-20R	Daihan Scientific Companies, Korea
Microscope	A. Krüssoptronic, Germany
UV Transilluminator, Model: MD-20	Wealtec Corp, USA
-20°C Freezer	Siemens, Germany
Vortex Machine	VWR International
Microwave Oven, Model:MH6548SR	LG, China
pH Meter: pHep Tester	Hanna Instruments, Romania
Micropipette	Eppendorf, Germany
Disposable Micropipette tips	Eppendorf, Ireland
Refrigerator (4˚C) Model: 0636	Samsung
Water Bath	Daihan Scientific Companies, Korea
Agarose Gel Electrophoresis Apparatus	Cleaver Scientific Ltd, Denmark
Microcentrifuge Machine : Minispin Plus	Eppendorf, Germany