ISOLATION AND IDENTIFICATION OF BACTERIA FROM TOUCH SURFACES OF PUBLIC TRANSPORT IN DINAJPUR TOWN

A THESIS

BY

ABDISALAN ABDULLAHI MOHAMED

REGISTRATION NO. 1705191

SEMESTER: JANUARY-JUNE, 2018

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MASTER OF SCIENCE (MS)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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Approved as to style and content by

_____	_____
(Prof. Dr. Md. Khaled Hossain)	**(Dr. Nazmi Ara Rumi)**
Research Supervisor	Research Co- Supervisor

_____

(Prof. Dr. Md. Mostafizer Rahman)

Chairman

Department of Microbiology

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

JUNE, 2018

DEDICATED

TO

MY BELOVED PARENTS, SISTERS AND BROTHERS

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ABSTRACT

The goal of this study was to investigate bacterial contamination on touch surfaces in the public transport system and buses of HSTU, Dinajpur, Bangladesh. For this study, a total number of 45 samples were collected from 15 buses in different places of Dinajpur. The total viable counts (TVC) in distinct locations of the bus (Grab rail, Armrest and Vinyl seat) samples were ranged from $9.7x10^4$ CFU/g to $5.2x10^6$ CFU/ml. A total of 60 bacterial isolates belong to seven genera (*staphylococcus spp., Bacillus, spp., Escherichia coli, Klebsiella spp, Salmonella spp, Pseudomonas spp and Shigella spp*.) were identified. Swab samples were examined using microbiological methods and different microorganisms were determined. The prevalence of *Escherichia coli* was 30%, *staphylococcus spp. was* 25%, *Salmonella spp*. was 15% *Klebsiella spp*. was 10%, *Shigella spp* was 10%, *Pseudomonas spp.* was 5%, and *Bacillus spp.* was 5%. Antibiotic sensitivity test showed that *Staphylococcus spp, Salmonella spp* and *Bacillus spp* and *Shigella spp* were resistant to Amoxicillin. *Shigella spp* and *Pseudomonas spp* were resistant to Erythromycin and Cloxacillin. *Salmonella spp* were resistant to Chloramphenicol. *Klebsiella spp, E. coli* and *Pseudomonas spp* were resistant to Cephalexin. *Salmonella spp* and *Shigella spp* were resistant to Cefaclor. *Staphylococcus spp* were resistant to Streptomycin. *Klebsiella spp* were resistant to Penicillin and Levofloxacin. *E. coli* was resistant to Amikacin and Ampicillin. *Salmonella spp* was resistant to Chloramphenicol.

Frequent skin contact due to popularity and over-crowdedness of the buses, absence of routine cleaning of public buses, poor public sanitation practice and lack of consciousness among passengers can be attributed for the abundance of bacteria in the sampling locations. Hence, it is recommended that regular washing of hands with suitable disinfectants after travelling in public buses to avoid chance of transmission of these pathogens. In addition to that it is very important to make inspection regularly of these vehicles to ensure hygienic public transport.

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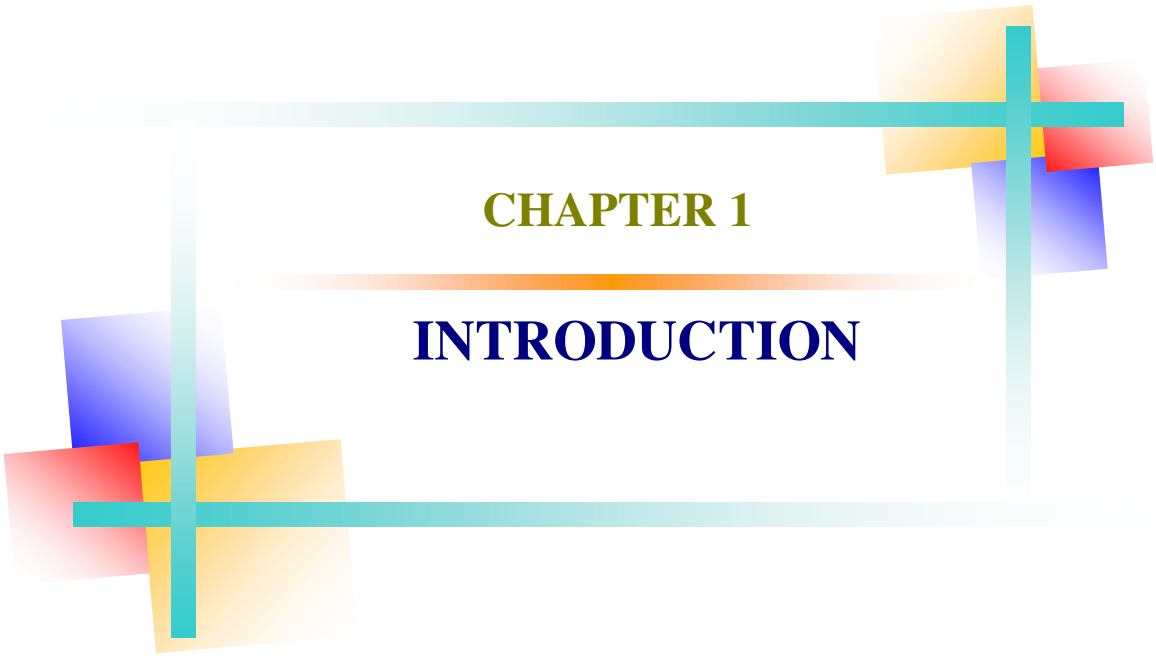

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

Transportation is the movement of humans, animals and goods from one location to another. Transportation networking system is continually expanding to meet up the need of very large load of passengers and goods carried. At the same time this networking has drawn great attention from public health scientists as pathogenic microbes are now succeeded or gained a better way of amplification that is faster and in extensive number than before (Mendes *et al., 2015;* Kassem, 2009; Stepanovic *et al*., 2008; Otter and French, 2009; Yeh *et al.,* 2011). The past 500 years have provided more examples of how the establishment and contraction of worldwide transport networks has facilitated internalization pandemics of communicable diseases (Tatem *et al., 2006).* The efficiency, speed and reach of modern transport networks puts people at risk from the emergence of new strain of familiar diseases or from completely new diseases (Tatem *et al., 2006;* Guimera *et al*., 2005). Current news of WHO cautioned us about the pandemic status of numerous diseases which is the reflection of the faster movement of pathogenic microorganisms. This issue would be a severe matter of concern if the pathogens are drug resistant. Several studies have already been conducted throughout the world focusing on presence and large quantity of microbial contamination on public hand touch surfaces of buses. Among the pathogens such as *Escherichia coli*, *Vibrio cholerae*, multi drug resistant *Staphylococcus aureus* and *Mycobacterium tuberculosis* those isolated from hand touch surfaces are frequently being reported (Gavaldà *et al*., 2015; McMullen *et al*., 2009; Markley *et al*., 2012; Shapiro *et al.,* 2009; Gerba *et al*., 2016).

As part of living in society, many common spaces are shared with other people. This makes it possible to spread diverse microorganisms that can lead to infections. People who use public transport can pass bacteriological, virological, or fungal infections to other people (Rusin et al. 2002). The greatest risk for infectious diseases in these vehicles is that people sit close together in a closed environment and breathe the same air. These vehicles can become a significant source of microorganisms when passengers do not close their mouths when coughing and sneezing. Handles, seats, anchors, floors, and windows are areas that can host infectious microorganisms (Yatagan 1991; Furuya 2007; Edelson and Phypers 2011).

A route of transmission of cold, flu, diarrhea and other common infections is through contact with surfaces contaminated with infectious microorganism (Boone and Gerba, 2007). Contamination occurs by settling of droplets from coughs and sneezes onto surfaces, and by touching of surfaces with hands contaminated with pathogens. The pathogens then contaminate the hands of the next person who touches the same surface and when they bring their hand to their eyes, nose, or mouth infection can result. Mass transportation systems create an environment in which large numbers of persons on a daily basis share space and interact with surfaces found within system vehicles (Troko *et al*., 2011).

Microorganisms are found everywhere and compose a major part of every ecosystem, which lives either freely or as parasites (Sleigh and Timbury, 1998). The live as transient contaminants in fomites or hands where they constitute a major health hazards as sources of community (Pittet, D *et a*l., 1999).

Microorganisms in public transport can be an important issue in public health, because of the ease of transfer of pathogens from individual to individual; this is a particular cause for concern when microbes are drug resistant and pathogenic (Ehrenkranz, 1964; Scott and Bloomfield, 1990; Brook J and Brook I, 1994; Rusin *et al*., 2002; Kassem *et al*., 2007).

Public transportation may also be used by "cloud" *S. aureus* carriers (colonized individuals who may disperse high concentrations of the bacteria; also called "supershedders"), who can disperse up to a 40-fold increase in airborne MRSA over "non-cloud" carriers (individuals colonized with bacteria, but not enhanced shedders). This could increase the likelihood that MRSA strains, particularly HA-MRSA strains (because "cloud" carriers are frequently healthcare workers, who are more likely to carry healthcare-associated strains), may be isolated on public transportation vehicles (Belani et al., 1986; Sherertz et al., 1996; Tanner et al., 1980). These "cloud" carriers can transmit MRSA through skin-to-surface contact and the release of skin squames, but may also transmit significant amounts of airborne MRSA, particularly when experiencing an upper respiratory infection (Boyce et al., 1993; Hare & Thomas, 1956). These individuals also transmit higher *S. aureus* concentrations when moving or where there is skin friction, which is rather unavoidable on public transportation (Cimolai, 2008; Hare & Thomas, 1956).

The major healthcare associated (HA-MRSA) in human pathogen is methicillin-resistant *staphylococcus aureus* that is responsible for moderate to severe life-threatening infections worldwide (de Lencastre and Tomasz, 2011; European Centre for Disease Prevention and Control, 2012).

Since the middle-1990 s, methicillin resistance *staphylococcus aureus* (MRSA) has also been determined as the etiological agent in the community acquired infections {CA-MRSA} (Graves *et al*., 2010; Naimi *et al*., 2003; Patel, 2009).

Considering the importance of public transportation in people and also considering the public health importance the present study was undertaken with the following objectives:

1. To isolate and identify some important bacteria from touch surfaces in public transport.
2. To determine the antibiotic sensitivity pattern of the isolated bacteria from hand and touch surfaces in public transport.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2

REVIEW OF LITERATURE

Tasneem Chowdhury et al. **(2016)** observed the Microbiological investigation into public hand touch surfaces has been gaining significant attention from researchers because contaminated surfaces may act as potential reservoirs of pathogens. The current study was designed to get information on bacterial contamination level on three predominant hand touch surfaces of public buses from densely crowded Chittagong city of Bangladesh. 45 swab samples from three hand touch surfaces: grab rail, armrest and vinyl seat of 15 buses were collected and analyzed. All samples were contaminated with numerous bacteria and grab rails were found to harbor significantly more bacteria than the other two surfaces. Besides magnitude of contamination, the study focused on recovery of three enteric pathogenic bacteria and methicillin resistant *Staphylococcus aureus* (MRSA). Among the enteric bacterial isolates 33 were identified as *E. coli*, 17 as *Salmonella typhi* and 11 as *Shigella*. Sensitivity patterns of enteric bacterial isolates were upsetting enough as almost all isolates exhibited resistance against ampicillin, amoxicillin, ceftriaxone and chloramphenicol. 12 MRSA isolates were recovered and the isolates did not show resistance against any test antibiotics except ceftazidime. The results indicate poor hygienic condition of the buses under study. Moreover presence of enteric bacteria and MRSA portent onset of community-acquired diseases. Though the study dealt with small number of samples and the results are representatives of only a minute fraction of overall transportation system of the city; our findings are auspicating enough in terms of public health concern which will spur concern from the nation's public health management system.

Gerba, (2007) studied that public transport may serve as a vehicle for the transmission of infectious disease. The goal of this study was to assess bacterial loads on high touch areas within municipal buses and assess the use of a new coating comprising silicon--- oxide bonds and titanium--- oxide bonds provide by Allied Bioscience, Inc. on the long term suppression of bacterial numbers on high touch areas within buses. Public buses were tested on selected sites for heterotrophic bacteria. The most contaminated sites were the driver's compartment and the fire box. One group of buses

was then treated with the disinfectant and another was not. After 30 days statistically significantly fewer bacteria where present on the treated buses.

Birteksoz and Erdogdu, (2017) studied to determine the role of public transport in the transmission of microorganisms. A total of 60 swab samples were collected in the morning and evening from handles in public transport trams, metrobuses, and buses. Swab samples were examined using microbiological methods and the number and types of microorganisms were determined. Total aerobic bacterial and fungal counts in samples collected in the evening were higher than those in samples collected in the morning from trams and metrobuses. However, the total bacterial and fungal counts were very high in samples collected in the morning and evening from buses. *Staphylococcus aureus*, coagulase-negative *staphylococcus*, and *Enterococcus spp*. were isolated from these samples. The results of our study show that public transportation can be a significant reservoir for spreading pathogenic microorganisms. For this reason, it is very important to regularly follow cleaning and hygiene rules and to inspect these vehicles.

Otter *et al.,* (2009) investigated the bacterial contamination on hand-touch surfaces in the public transport system and in public areas of a hospital in central London. Dipslides were used to sample 118 hand-touch surfaces in buses, trains, stations, hotels and public areas of a hospital in central London. Total aerobic counts were determined, and *Staphylococcus aureus* isolates were identified and characterized. Bacteria were cultured from 112 (95%) of sites at a median concentration of 12 CFU cm. Methicillin-susceptible *Staphylococcus Aureus* (MSSA) was cultured from nine (8%) of sites; no sites grew methicillin-resistant *Staph. aureus* (MRSA). Hand-touch sites in London are frequently contaminated with bacteria and can harbour MSSA, but none of the sites tested were contaminated with MRSA. Significance and Impact of the Study: Hand-touch sites can become contaminated with *staphylococci* and may be fomites for the transmission of bacteria between humans. Such sites could provide a reservoir for community-associated MRSA (CA-MRSA) in high prevalence areas but were not present in London, a geographical area with a low incidence of CA-MRSA.

Teresa Conceicao *et al.,* (2013) discovered in a previous study we have shown that public buses in Oporto, the second largest city in Portugal, were highly contaminated with MRSA. Here we describe the results of a similar study performed in another urban area of Portugal– Lisbon, the capital. Between May 2011 and May 2012, hand touched

surfaces of 199 public buses in Lisbon were screened for MRSA contamination. Subsequently, the hands of 575 passengers who frequently use these bus lines were also screened. All hand carriers of MRSA were further screened for nasal carriage. The isolates were characterized by PFGE, *staphylococcal cassette chromosome* mec typing, spa typing, MLST and were tested for the presence of mecA, Panton-Valentine leukocidin and arginine catabolic mobile element genes. MRSA contamination was shown in 72 buses (36.2%). The majority of the isolates belonged to three major clones: Clone A was identified as EMRSA-15 defined by pattern PFGE A, spa types t2357/t747/t025/t379/t910, ST22, and SCCmec IVh (n=21; 29%). Clone B was the New York/Japan clone characterized by PFGE B-t002/t10682-ST5-II (n=15; 21%). Clone C included isolates with characteristics of the international community acquired USA300 or related clones, PFGE C-t008-ST8-IVa/IVc/IVg/IVnt/VI (n=19; 26%). The first two clones are currently the two major lineages circulating in Portuguese hospitals. The hands of 15 individuals were contaminated with MRSA belonging to the nosocomial clones A or B. Eleven of these individuals were not nasal carriers of MRSA and all but one had travelled by public transportation, namely by bus, prior to sampling. Public buses in two major cities in Portugal are often contaminated with MRSA representing clones dominant in hospitals in the particular geographic area. MRSA contamination of public transport and the transfer of the bacteria to the hands of passengers may represent a route through which hospital-acquired MRSA clones may spread to the community.

Lorrane *et al.,* (2016) identified *Staphylococcus aureus* is an important agent of health care and community infections. Environmental surfaces, especially those in frequent contact with the hands, can serve as reservoirs of microorganisms, working as fomites in microbial spread. In the public transportation system, contact surfaces with hands can act as transmission vehicles, assisting the dispersion of *S. aureus* in the community. The aim of this study was to determine the prevalence of *S. aureus* and methicillin resistant *S. aureus* (MRSA) in buses, terminals and platforms of a larger route of the public transportation system in the city of Goiânia-GO, to determine the susceptibility and virulence profiles and assess the genetic similarity among *S. aureus* isolates. We collected 852 swabs from handrails of buses and ratchets of platforms and terminals that compose the East-West line. The isolation of *staphylococci* was done by standard techniques and species identification by detection of the femA gene. *S. aureus* identified were submitted to antimicrobial susceptibility testing, conventional PCR for detection of

mecA and lukS-Fgenes, multiplex PCR to detect virulence factors genes, SCCmec typing and PFGE to assess the genetic similarity among strains. The contamination prevalence of handrails and ratchets by *S. aureus* was 17.7% (151/852). MRSA was detected in 0.5% of samples. The phenotype known as iMLSB was detected in 41.0% (62/151) of the isolates. Several virulence profiles were detected in 43 (28.5%) isolates, PFGE analysis revealed extensive genetic diversity of circulating strains, including MRSA strains similar to USA300 and USA800. The results suggest that *S. aureus* and MRSA strains, showing different susceptibility and virulence profiles are circulating in the community. They may be transmitted between individuals through fomites, putting the user population of the service at risk.

Pamela *et al*., (2011) evaluated to determine the diversity and abundance of *Staphylococcus* bacteria on different components of a public transportation system in a mid-sized US city (Portland, Oregon) and to examine the level of drug resistance in these bacteria. We collected 70 samples from 2 cm × 4 cm sections from seven different areas on buses and trains in Portland, USA, taking 10 samples from each area. We isolated a subset of 14 suspected *Staphylococcus* spp. colonies based on phenotype, and constructed a phylogeny from16S rRNA sequences to assist in identification. We used the Kirbye–Bauer disk diffusion method to determine resistance levels to six common antibiotics. We found a range of pathogenic *Staphylococcus* species. The mean bacterial colony counts were 97.1 on bus and train floors, 80.1 in cloth seats, 9.5 on handrails, 8.6 on seats and armrests at bus stops, 3.8 on the underside of seats, 2.2 on windows, and 1.8 on vinyl seats per 8 cm^2 sample area. These differences were significant ($p < 0.001$). Of the 14 isolates sequenced, 11 were *staphylococci*, and of these, five were resistant to penicillin and ampicillin, while only two displayed intermediate resistance to bacitracin. All 11 isolates were sensitive to trimethoprim-sulfamethoxazole, vancomycin, and tetracycline. We found six different strains of *Staphylococcus*, and while there were varying levels of drug resistance, we did not find extensive levels of multidrug-resistant bacteria, and no *S. aureus* was found. We found floors and cloth seats to be areas on buses and trains that showed particularly high levels of bacteria.

Scott *et al.,* (1990) observed the Survival and transfer of bacteria from laminated surfaces and cleaning cloths were investigated under laboratory conditions. Drying produced substantial reductions in numbers of recoverable organisms and achieved satisfactory decontamination of clean laminate surfaces. On soiled surfaces and on clean and soiled cloths, Gram-positive and some Gram-negative species survived for up to 4 h, and in some cases up to 24 h. Where contaminated surfaces or cloths came into contact with the fingers, a stainless steel bowl, or a clean laminate surface, organisms were transferred in sufficient numbers to represent a potential hazard if in contact with food.

Rusin *et al.,* (2002) studied to determine the transfer efficiency of micro-organisms from fomites to hands and the subsequent transfer from the fingertip to the lip. Volunteers hands were sampled after the normal usage of fomites seeded with a pooled culture of a Gram-positive bacterium (*Micrococcus luteus*), a Gram-negative bacterium (*Serratia rubidea*) and phage PRD-1 (Period A). Activities included wringing out a dishcloth/sponge, turning on/off a kitchen faucet, cutting up a carrot, making hamburger patties, holding a phone receiver, and removing laundry from the washing machine. Transfer efficiencies were 38.47% to 65.80% and 27.59% to 40.03% for the phone receiver and faucet, respectively. Transfer efficiencies from porous fomites were <0.01%. In most cases, *M. luteus* was transferred most efficiently, followed by phage PRD-1 and *S. rubidea*. When the volunteers' fingertips were inoculated with the pooled organisms and held to the lip area (Period B), transfer rates of 40.99%, 33.97%, and 33.90% occurred with *M. luteus, S. rubidea*, and PRD-1, respectively. The highest bacterial transfer rates from fomites to the hands were seen with the hard, non-porous surfaces. Even with low transfer rates, the numbers of bacteria transferred to the hands were still high (up to 10(6) cells). Transfer of bacteria from the fingertip to the lip is similar to that observed from hard surfaces to hands. Infectious doses of pathogens may be transferred to the mouth after handling an everyday contaminated household object.

Gerardo *et al.,* (2013) identified the Fomites are known to play a role in the transmission of bacterial and viral pathogens. Advances in quantitative microbial risk assessment have allowed for the development of models, which can be used to determine the probability of infection via the hand and the likely success of interventions. Critical to these models is information on the effectiveness of organism transfer from fomites to hands and vice versa. Numerous factors influence the transfer efficiency of microorganisms, with environmental humidity being the most important factor with

increased transfer. Other factors influencing transfer include drying time, contact time, pressure, friction type of material, and nature of the fomite. The greater the pressure applied to a surface and longer the contact time the greater the amount of transfer that can be expected. Much greater transfer occurs with hard surfaces (stainless steel) than porous surfaces (paper, cloth). Overall trends can be observed but transfer is organism dependent. Both Gram negative and positive bacteria have a greater transfer from hand-to-fomite than virus. Fomite-to-hand transfer is similar for both virus and bacteria. Hand-to-hand, hand-to-fomite-to-hand and the fomite-to hand-to-fomite transfer direction showed an overall lower transfer than hand-to-fomite and fomite-to-hand transfer. Hand-to-fomite-to-hand showed the least transfer.

Vinod *et al*., (2018) studied the role of public transport as reservoirs of antibiotic-resistant *staphylococci* were determined. 200 swabs were collected from 50 public buses (urban and rural) circulating in Davangere, Karnataka. Swabs collected were inoculated on Blood agar, Mannitol salt agar and MacConkey agar plates. After incubation for 24-48 hours, plates were examined for the growth of *Staphylococcus aureus*. Anti-microbial susceptibility test was performed using oxacillin 1ug disc to detect methicillin resistance as per CSLI guidelines. Out of 40 *Staphylococcus aureus* isolated 35 isolates were resistant to more than two classes of antibiotics, hence multidrug resistant *Staphylococcus aureus*. Out of 35 MDR isolates, 18 were resistant to oxacillin and cefoxitin. Minimum inhibitory concentration test revealed that out of 35 MDR isolates, 18 isolates had MIC value of $\geq$ 4µg/ml. The recovery methicillin-resistant *Staphylococcus aureus* from public transport system implies a potential risk for transmission of these bacteria in community.

Gaymard *et al*., (2016) stated methicillin-resistant *Staphylococcus aureus* (MRSA) is involved in community-acquired and nosocomial diseases. The means of MRSA transmission and dissemination in the community remain uncertain. Studies have shown that Public transport systems could be a source of MRSA and may serve as a potential source for community acquired MRSA infections. This study aimed to investigate MRSA contamination on Lyon's metropolitan network (Métro) in France. Hand-touched surfaces were sampled with sterile swabs (Transystem) during a 1-day transversal study by collecting 50 samples in seven hub stations and two trains for each of the four Métro lines. Then, during a longitudinal study, one sample was collected twice daily for 30 consecutive days in the busiest and most congested hub station. All swabs were

incubated in enrichment medium for 24 hours and then each suspension was plated on to a chromogenic selective medium for MRSA. After 24h at 36°C, all presumptive MRSA colonies were tested using VITEK MS to confirm identification as *Staphylococcus aureus* as well as by Alere™ PBP2a Culture Colony Test and mecA/mecC PCR to check methicillin resistance. Of the 110 swabs tested, 24 presumptive MRSA colonies were isolated, of which 2 were confirmed as *S. aureus* by VITEK MS. These two isolates were tested negative using the PBP2a Culture Colony Test and PCR. Unlike other foreign cities such as Lisbon, the current data suggest a low level of MRSA contamination of hand-touched surfaces on Lyon's Métro. This should be put in perspective with the low level of MRSA colonization in the French community.

Guzman *et al*., (2009) observed that methicillin resistant *staphylococcus aureus* has become a serious threat to public health worldwide. Ongoing surveillance is essential to support infection control committees and clinicians in the prevention and treatment of infection. However, in Latin America, resources for monitoring the changing epidemiology of MRSA remain limited. In this article, we review the current situation of MRSA in Latin America in order to highlight the need for a more harmonized effort to improve its management. MRSA is already the leading cause of nosocomial infection in Latin America region, and the number of reports of community-acquired MRSA infection is also rising. However, the extent of the problem is not fully understood, especially since data tend to come from large hospitals whereas much of the population is served by small community healthcare centers that do not have extensive facilities for performing microbiological surveillance. Wider-reaching and co-ordinated programmes to provide regular MRSA surveillance reports are required across the Latin America region.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

The present research work was conducted during the period from January to June 2018, in the Bacteriology Laboratory of the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh science & Technology University (HSTU), Dinajpur. The detailed outline of materials and methods are given below:

3.1 Materials

The materials used in this study comprise sample materials and laboratory materials

3.1.1 Sample Collection Site

A total of 45 samples were collected from the public buses of Dinajpur Town and from the HSTU student buses.

Plate 1: Collection of samples from different buses

3.1.2 Study area and Period

This study was carried out in the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh science & Technology University (HSTU), Dinajpur. Study was period from January to June 2018.

3.1.3 Sample materials

Include PBS, centrifuge tubes, cotton swabs, hand gloves, and ice box.

3.1.4 Laboratory materials

Laboratory materials used for isolation, identification and antibiogram study of bacteria from public transport buses.

3.1.5 Media used for culture

Different bacteriological culture media and biochemical reagents were used for isolation, identification and propagation of bacteria are mentioned below:

3.1.5.1 Solid culture media

1) **Plate Count Agar (HI-MEDIA, India)**

Plate Count Agar (PCA), also called Standard Methods Agar (SMA), is a microbiological growth medium commonly used to assess or to monitor "total" or viable bacterial growth of a sample. PCA is not a selective medium (Cheesbrough, 1985)

2) **Nutrient Agar (HI-MEDIA, India)**

Nutrient agar (NA) medium was used to grow the bacteria from the collected samples. (Cheesbrough, 1985)

3) **MacConkey agar (Difco)**

MacConkey Agar (MAC) medium was used for the identification of organisms under the family Enterobacteriaceae through studying fermentation characteristics. (Cheesbrough, 1985).

4) **Cetrimide Agar Base (Difco)**

Cetrimide agar base promotes the production of pyocyanin a water-soluble pigment as well as fluorescence, under ultraviolet light, of *Pseudomonas* spp which constitutes a presumptive identification. Cetrimide is the selective agent as it inhibits the growth of the accompanying microbial flora. Typical *Pseudomonas* spp colonies are greenish or yellowish green in color (Cheesbrough, 1985).

5) **Blood Agar Medium, (HI-MEDIA, India)**

For the isolation, cultivation and detection of hemolytic reaction of fastidious microorganisms. Blood Agar Base is suitable to isolate and cultivate a wide range of microorganisms with difficult growth. Upon adding blood, it can be utilized for determining hemolytic reactions *Staphylococcus aureus* gives beta hemolysis. *Streptococcus pneumoniae* gives alpha hemolysis. *Streptococcus pyogenes* gives beta hemolysis (Clin. Path, 1951).

6) **Eosin Methylene Blue (HI-MEDIA, India)**

Eosin methyl blue agar (EMB) medium was used for the purpose of selective growth of *Escherichia coli*. (Cheesbrough, 1985)

7) **Salmonella-Shigella Agar (Difco)**

Salmonella-Shigella agar medium was used as a selective medium for *Salmonella* organism which causes enhancement of the growth of *Salmonella* while inhibiting the growth of contaminating organisms and shows typical colony characters. (Cheesbrough, 1985)

8) **Mannitol Salt Agar (HI-MEDIA, India)**

Each isolate was streaked on MSA agar plate and incubated at 37°C for overnight. Next demonstrated morphological characteristics of the bacterial colonies. (Cheesbrough, 1985)

3.1.5.2 Liquid culture media

1. **Nutrient Broth**

Nutrient broth (NB) was used to preliminary growth and also propagation of the microorganisms from collected the samples. (Cheesbrough, 1985).

3.1.5.3 Media Used for Biochemical Test

In order to identify bacteria the following media were used for biochemical tests:

1. Catalase test
2. Indole test
3. Triple sugar iron (TSI) agar slant.
4. Methyl- Red- Voges Proskauer medium base (MR-VP medium base).
5. Simmon's Citrate Agar.

3.1.6 Reagents

The chemicals and reagents used during the study were:

- Gram's staining reagents (Crystal violet, Gram's iodine, Acetone alcohol and Safranin)
- Potassium-di-hydrogen phosphate
- Dehydrated sodium citrate
- Phosphate Buffered Saline (PBS)
- Physiological Saline Solution (PSS)
- Methylene Blue stain
- Di-sodium hydrogen phosphate
- Sugar media (Lactose and Mannitol) and other chemicals and reagents as when required during the experiment.
- Distilled water
- Phenol red solution

3.1.7 Glasswares and appliances

The different kinds of glasswares and appliances used during the course of the experiment were as follows:

1) Conical flask,
2) Pipette
3) Clean free grease slide
4) Cover slips
5) Stop Watch
6) Test tube stand
7) Inoculating loop
8) Water bath, Detergent power
9) Aluminum foil roll
10) Sterile cotton, Immersion Oil,
11) Bacteriological incubator
12) Autoclave
13) Refrigerator
14) Hot air oven
15) Compound microscope
16) Spirit lamp
17) Durham's tube
18) Petri dishes
19) ice-box

3.1.8 Materials required for Antibiotic susceptibility test

Antibiotic sensitivity testing materials:

- Test tube rack
- Bunsen burner

Inoculating loop or needle

- Forceps
- Sterile swabs
- Mueller-Hinton or Nutrient agar plates
- Antibiotic disks
- Stock broth cultures of experimental bacteria

3.1.9 Antimicrobial sensitivity discs

- To determine the drug sensitivity pattern of different bacterial isolate with different types of commercially available antimicrobial discs (Oxoid Ltd, UK) were used. The followings are antibiotics that were tested against, the selected organism with their disc concentration.

Table 1: Antibacterial Sensitivity Discs

Sl. No	Name of the antibiotics	Disc concentration (µg/disc)
1	Amoxicillin (AMX)	30 µg
2	Penicillin (P)	5 µg
3	Chloramphenicol (C)	5 µg
4	Cephalexin (CN)	30 µg
5	Streptomycin (S)	10 µg
6	Norphalaxin (NX)	10 µg
7	Azithromycin (AZM)	30 µg
8	Gentamycin (GEN)	10 µg
9	Erythromycin (E)	15 µg
10	Co-trimoxazole (COT)	15 µg
11	Ofloxacin (OFX)	5µg
12	Levofloxacin (LE)	5µg
13	Colaxicillin (COX)	1µg
14	Cefaclor (CEC)	30µg
15	Vancomycin (VA)	30µg
16	Amikacin (AK)	30 µg
17	Ampicillin (AMP)	25 µg
18	Neomycin (N)	5 µg
19	Ciprofloxacin (CIP)	5 µg

Note: Sl. No. = Serial Number, µg = Microgram

3.2 Methods

The following methods were used for the isolation and identification of bacteria.

3.2.1 Experimental Layout

The experimental layout is schematically presented in figure 1. The entire study is divided into two steps. The first step includes isolation and identification of the bacteria from the sample by cultural, morphological and biochemical test. The second step includes evaluation of antibiotics sensitivity against the isolated bacteria.

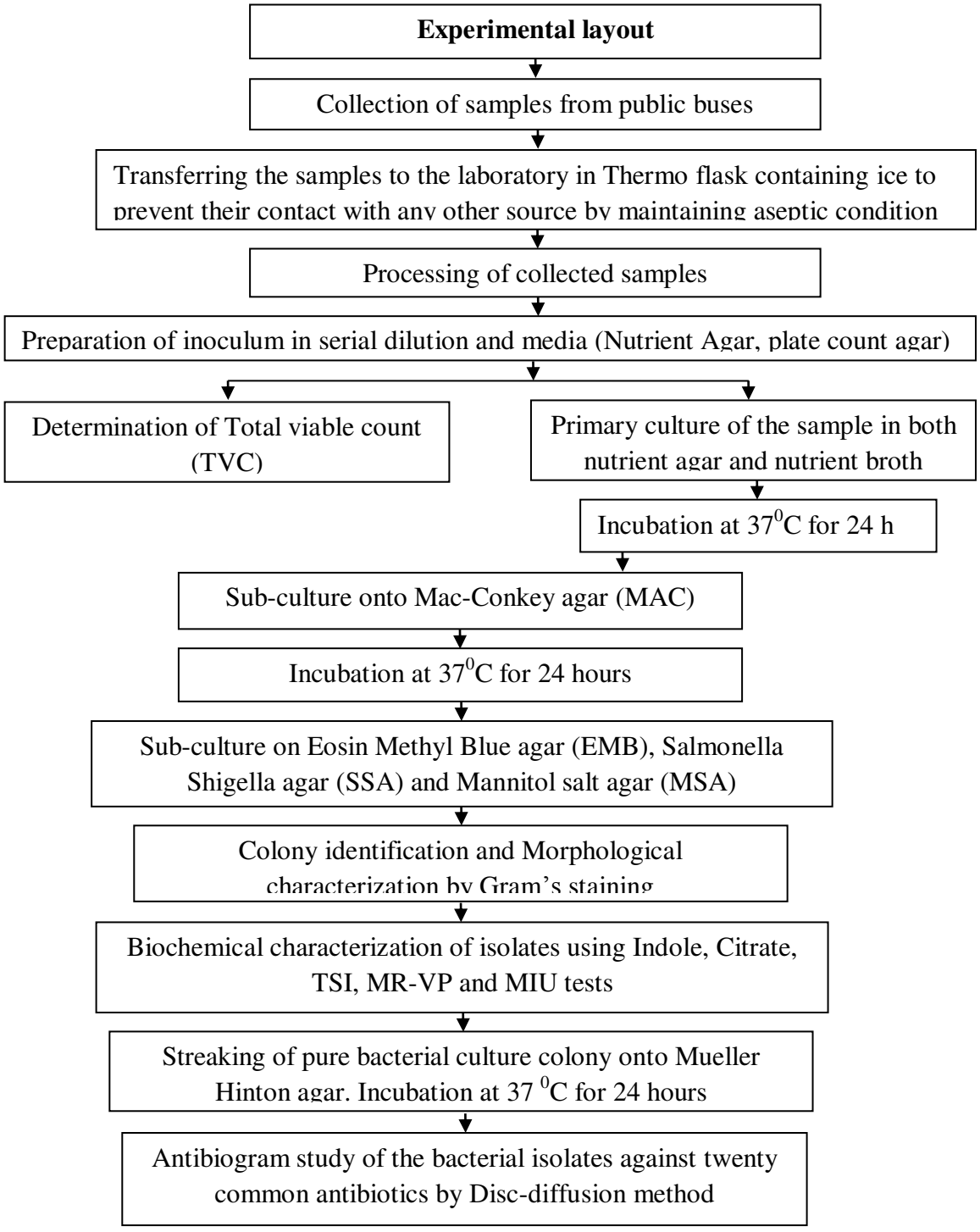

Figure 1: The schematic illustration of the experimental layout

3.2.2 Laboratory preparations

All items of glassware including test tubes, pipettes, cylinder, flasks, conical flasks, and others necessary instruments cleaned by detergent powder. The glassware were then cleaned by brushing, washed thoroughly and finally sterilized by autoclaving for 15 minutes at 12 1°C under 15 lbs pressure per square inch. Autoclaved items were dried in a Hot air Oven over 50°C. All the glass wares kept in oven at 50°C for future use.

3.2.3 Preparation of Culture Media

All the media, broth and reagents used in this experiment were prepared according to instruction of the manufacturer.

3.2.3.1 Nutrient broth media

Thirteen grams of dehydrated nutrient broth was suspended into 1000 ml of distilled water and boil to dissolve it completely. The solution was then distributed in tubes, stopper with cotton plugs and sterilized in autoclaving at 121° C and 1.2 kg/cm2 pressure for 15 minutes. The sterility of the medium was checked by incubating at 37° C for overnight and stored at 4° C in aerator for further use (Cater 1979).

3.2.3.2 Plate Count Agar (PCA)

17.5g of PCA was added to 1 liter of distilled water. Dissolve by bringing to the boil with frequent stirring, mix and distribute into final containers. Sterilize by autoclaving at 121°C for 15 minutes. After autoclaving, the medium was poured into each sterile petri dish and allowed to solidify.

After solidification of the medium in the petri dishes, these were incubated at 37°C for overnight to check their sterility and used for cultural characterization or stored at 4°C refrigerator for future use (Cater 1979).

3.2.3.3 Nutrient agar (NA) media

28 grams of nutrient agar powder was dissolved in 1000 ml of cold distilled water in a flask. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile petri dish and allowed to solidify. After solidification of the medium in the petri dishes, these were incubated at 37°C for overnight to check their

sterility and used for cultural characterization or stored at 4°C refrigerator for future use (Cater 1979).

3.2.3.4 Eosin Methylene Blue (EMB) agar

Thirty six grams of EMB agar base was added to 1000 ml of water in a flask and boil to dissolve the medium completely. The medium was sterilized by autoclaving at 1.2 kg/cm^{2}pressure and 121°C for 15 minutes and I to 50° C and shake the medium in order to oxidize the methylene blue (i.e. to restore its blue colour). Then 10 ml of medium was poured into each sterile Petri dish sized and allowed to solidify. After solidification of the medium in the Petridishes, these were incubated at 37° C for overnight to check their sterility and petri dishes without contamination were used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.3.5 MacConkey agar

51.5 grams MacConkey agar base powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 45- 50^{0}C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass Petri dishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the Petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.3.6 Cetrimide Agar Medium

46.7 grams of Cetrimide agar base powder was added in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely (necessary calculation was done for required number of plates). The medium was then sterilized by autoclaving for 15 minutes at 121°c maintaining a pressure of 1.2 kg/. Then 20/10 ml of medium was poured into each sterilized petri dishes and allowed to cool and to solidify.

After solidification all petri dishes was incubated at 37°c for overnight to check their sterility. The sterile medium was then used for differential cultural characterization.

3.2.3.7 Blood agar media

Forty grams of Blood agar base (HI-MEDIA, India) powder was dissolved in 1000 ml of distilled water (necessary calculation was done for required number of plates). The medium was sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121° C for 15 minutes and 45° C. Then 5-10 % sterile defibrinated blood was added to the medium and distributed to sterile petri dishes and allowed to solidify. After solidification of the medium in the petri dishes, these were incubated at 37°C for overnight to check their sterility and used for cultural characterization or stored at 4°C refrigerator for future use. (Cater 1979).

3.2.3.8 Salmonella Shigella (SS) Agar

Suspend 50g in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by boiling for 5 minutes. After boiling the medium was put into water bath at 45- 50^{0}C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass petri dishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.3.9 Mannitol Salt Agar (MSA)

111 grams Mannitol Salt Agar base powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121^{0}C for 15 minutes. After autoclaving the medium was put into water bath at 45- 50^{0}C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass Petri dishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about

2 hours with the covers of the Petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.3 Preparation of reagents

3.3.1 Methyl- Red solution

The indicator MR solution was prepared by dissolving 0.1 grams of Bacto methyl- red in 300 ml of 95% alcohol and diluted to 500 ml with the addition of distilled water.

3.3.2 Methyl Red

A quantity of 17 grams of MR-VP medium was dissolved in 1000 ml of distilled water, dispensed in 2 ml amount in each tube and the tubes were autoclaved. After autoclaving, the tubes containing medium were incubated at 37oC for overnight o check their sterility and then in refrigerator for future use.

3.3.3 Alpha- naphthol solution

Alpha- naphthol solution was prepared by dissolving 5 grams of Alpha- naphthol in 100 ml of 95% ethyl alcohol.

3.3.4 Potassium hydroxide solution

Potassium hydroxide (KOH) solution was prepared by adding 40 grams of Potassium hydroxide crystals in100 ml of cooled water.

3.3.5 Phosphate Buffered Saline solution

Eight grams of sodium chloride (NaCl), 2.89 grams of di-sodium hydrogen phosphate Na_2HPO_4, $12H_2O$), 0.2 gram of potassium chloride (KC1) and 0.2 gram of potassium hydrogen phosphate (KH_2PO_4) were suspended in 1000 ml of distilled for the preparation of phosphate buffered saline solution. The solution was heated to dissolve completely. Then the solution was sterilized by autoclaving at 1.2 kg / cm^2 pressure and 121° C for 15 minutes and stored for future use.

3.3.6 Indole reagent (Kovac's reagent)

This solution was prepared by dissolving 25 ml of concentrated Hydrochloride acid in 75 ml of amyl alcohol and to the mixture 5 grams of paradimethyl–amino- benzyldehyde crystals were added. This was then kept in a flask equipped with rubber cork for future use.

3.4 Samples Collection

A total of 45 samples were collected for bacteriological examination. The bus stations of Dinajpur town were chosen for sample collection. The samples were collected from Hanif, Shaymol and Nabil. The samples were congregated during the months of January to June 2018. All the collected samples were kept on an ice-box during transportation to the laboratory and stored at 4°C until testing. They were analyzed within 24 hours of sampling.

3.4.1 Serial dilution

Serial dilutions are made by making the same dilution step over and over, using the previous dilution as the input to the next dilution in each step. The result obtained in the form of reduces number of bacterial colonies in order to get pure colonies by (James G. Cappuccino. 7Edition).

Procedure:

- Test tubes were placed into the test tube rack with appropriate label (10^{-1},10^{-2},10^{-3}, 10^{-4})
- 9 ml distil water was taken in each tube.
- I ml sample was added into first tube.
- Gently shake.
- 100µl samples were spread on nutrient agar plate.
- Incubated at 37°C for 24 hours.
- Plates were observed for result.

3.4.2 Total viable count (TVC)

50µl of each fivefold dilution was transferred and spread onto *Plate Count Agar* using a micropipette for each dilution for the determination of total bacterial count. The diluted

samples were spread as quickly as possible on the surface of the plate. The plates were kept in an incubator at 37^0C for 24 hrs. After incubation, plates exhibiting 30-300 colonies were counted. The average number of colonies in particular dilution was multiplied by the dilution factor to obtain the total viable count. The total viable count was calculated according to ISO (1995). The results of the total bacterial count were expressed as the number of colony forming units (CFU) per ml of bus samples.

Number of cells per ml=number of colonies × Dilution factor.

3.5 Characterization of bacteria

Individually isolated colonies of the same morphology were selected from appropriate agar plates after 24 hrs of incubation at 37 C, such as shape, size, surface texture, edge and elevation, color and opacity. Characterization into respective genera and species were done on the basis of morphological, cultural and biochemical and serological reactions. The classification and specification of organisms was based on the scheme presented in Bergey's Manual of Systematic Bacteriology (Holt, 1985) Stock culture was prepared and maintained for subsequent studies. Strict aseptic measures were maintained throughout the period of study.

3.6 Identification of bacteria

The identification of bacteria was performed on the basis of colony morphology Gram's staining reaction and biochemical test.

3.6.1 Gram's staining:

The most widely used staining procedure in microbiology is the gram stain ,discovered by the Danish scientist and physician Hans Christian Joachim Gram in 1884,Gram staining is a differential staining technique that differentiates bacteria into two groups: gram- positives and gram-negatives. The procedure is based on the ability of microorganisms to retain color of the stains used during the gram stain reaction. Gram negative bacteria are decolorized by the alcohol, losing the color of the primary stain, purple. Gram-positive bacteria are not decolorized by alcohol and will remain as purple. After decolorization step and a counterstain is used to impart a pink color to the decolorized gram- negative organisms.

3.6.2 Preparation of Gram's staining solution

The four solutions needed for the Gram staining procedure;

- ✓ Crystal violet
- ✓ Gram's iodine
- ✓ 95% alcohol
- ✓ Safranin

3.6.3 Gram staining procedure

1. Clean glass slides were taken.

2. A sterile technique was used; a smear of each sample was prepared. Smear made of a drop of water on the slide was placed then each organism separately to the drop water with a sterile was transferred. A circular motion of the inoculating loop of organism was mixed and spreads.

3. Air –dry the smears were allowed then heat fixed in the usual manner.

4. Smears were flooded with crystal violet and let stood for 2 min gently washed with tap water.

5. Smears were flooded with Grams iodine mordant and let stood for 1 min .Gently washed with tap water.

6. Smears were decolorized with 95% ethyl alcohol and waited it for 15 second

7. Gently washed with tap water.

8. Smears were counter stain with safranin for 1 minute.

9. Slides ware washed with tap water.

10. Slides ware examine under oil immersion (James G. Cuppuccion, Natalie Sherman, 1996).

3.7 Biochemical tests

Biochemical tests are the tests used for the identification of bacteria species based on the differences in the biochemical properties of different bacteria. Bacterial physiology differs from one species to the other.

3.7.1 Catalase Test

The present of catalase is determined by its ability to break down peroxide into water and oxygen, releasing bubbles of oxygen. This test is used to differentiate those bacteria that produce the enzyme catalase, such as staphylococci, from non-catalase producing bacteria such as streptococci by (Cheesbrough, 1985).

Procedure:

a) A colony was picked up of the bacteria from a plate and transferred the colony on a glass slide in a drop of water.
b) Introduce a few drops of 3% H_2O_2 (dilute 30% commercial solution (1: 10) over the culture.
c) Production of gas bubbles (released oxygen) indicated a positive reaction.

3.7.2 Methyl red (MR) Test

This test was performed to differentiate *Enterobacter*iaceae family. Some *enterobacter*iaceae family when cultured in buffered glucose peptone water, ferment glucose to produce sufficient acidity, which gives red color with methyl red indicator (PH range: 4.4-6.2, Color change: red yellow) by (Cheesbrough, 1985).

Procedure

(a) Sterile MR-VP broth was inoculated with the test organism and following incubation at 370 c for 24 hours.
(b) Few drops of methyl red solution were added.
(c) A distinct red color indicated MR positive test while yellow or orange color indicated a negative result.

3.7.3 Voges Proskauer (VP) Test

The test organisms were cultured in glucose phosphate peptone water for 24 hours. Acetone formed was converted to diacetyl. It was converted to a pink compound by the action of creatine. This test was used to assist in the differentiation of *Enterobacteri*aceae family such as *Vibriocholerae*, *Klebsiella* spp and some strains of *Enterobacter* by (Cheesbrough, 1985).

Procedure

- Sterile MR-VP broth was inoculated with the test organism and following incubation at 37° C for 24 hours.
- After incubation, 5 drops of naphthol solution and 5 drops of KOH solution were added.
- The development of a bright red or pink-red color was recorded as a positive result.

3.7.4 Triple Sugar Iron (TSI) agar Test

TSI slants are useful in the identification of *enterobacter*iaceae by their specific reaction on the slants Alkaline reaction (red color) was shown by the organisms, who fail to ferment any one of the sugar. Fermentation of the sugars was indicated by yellow color since pH range of phenol red is 6.8 and color change from yellow to red. Since the glucose (Dextrose) present on the surface of the medium was used up and since the surface of the slant was exposed to atmosphere, under aerobic conditions, the acid reaction on the surface reverts to alkaline (red color) in 18 to 24 hours. (This is a critical duration for this observation). In the butt, since anaerobic condition exists, the color of the butt remains yellow. Gas production (carbon dioxide) was indicated by splitting of the agar. Production of hydrogen sulfide imparts black shade to slant by reacting with ferrous ions. It was an indication of H2S producing organisms. TSI slants are useful in the identification of *Enterobacter*iaceae by their specific reaction on the slants by (Cheesbrough, 1985).

Procedure

(a) A loop of bacteria was spread across the surface of the agar.
(b) A needle of bacteria was inserted (stabbed) into the bottom (butt) of the tube.

(c) Kept the tubes at 37^0C for 24 hours for incubation.

(d) Examined the tubes for the result.

Result(Slant/butt	**Symbol**	**Interoperation**
Red/yellow	K/A	Glucose fermentation only
Yellow/Yellow	A/A	Glucose and lactose and/or sucrose fermentation
Red/Red	K/K	No fermentation
Yellow/Yellow with bubbles	A/A,G	Glucose and lactose and/or sucrose fermentation; Gas produced
Red/Yellow with bubbles	K/A,G	Glucose fermentation only; Gas produced
Red/Yellow with bubbles and black precipitation	K/A,G H_2S	Glucose fermentation only; Gas produced H2S Produced
Red/Yellow with black precipitation	K/A, H_2S	Glucose fermentation only; H_2S Produced
Yellow/Yellow with black precipitation	A/A, H_2S	Glucose and lactose and/or sucrose fermentation; H_2S Produced
No Change/ No Change	NC/NC	No fermentation

Note: A=acid production: K= alkaline reaction: G=gas production: H_2S= Hydrogen sulfide production.

3.7.5 Citrate Utilization Test

The test organism was cultured in a medium containing sodium citrate, an ammonium salt and bromothymol blue indicator. The organisms use citrate (the only source of nitrogen). The citrate utilization is followed by alkaline reaction (change of the color form light to blue) and growth in the medium was indicated by appearance of turbidity. This test was performed in the identification of *Enterobacter*ia by (Cheesbrough, 1985).

Procedure

(a) A loop of bacteria was spread across the surface of the agar.

(b) Kept the tubes at 37^0C for 24 hours for incubation.

(c) Examined the tubes for the result.

(d) (Positive: - produce blue color,

(e) Negative: - no color).

3.7.6 Indole Test

The test organism was cultured in a medium containing tryptophan. The organisms break down tryptophan and Indole is released. It was detected by the action of Kovac's reagent or Ehrlich reagent (formation of red colored compound). This test was important for the identification of *Enterobacter*ia such as *Escherichia coli* and *Pseudomonas vulgaris* etc. by (Cheesbrough, 1985).

Procedure

(a) Tryptophan containing broth was inoculated with bacteria.
(b) The tube was incubated at 37° C for 24 hours.
(c) Added 0.5 ml of the Kovac's reagent after the bacterial growth.
(d) If Indole positive within a 30 second a red color ring appeared at the junction of medium in the tube
(e) Negative: No color development or slightly pink color.

3.8 Antibiotic Sensitivity Assay of Test Organisms

The standard disk diffusion method known as Kirby-Bauer was used to determine the antimicrobial susceptibility profiles of the isolates (Bauer, 1999). Labeled the covers of each of the agar plates with name of the test organisms was inoculated.

(a) Using sterile technique, inoculated all agar plates with their respective test organisms as follow:
    1) A sterile cotton swab was dipped into a well-mixed saline test culture and removed excess inoculums by pressing the saturated swab against the inner wall of the culture tube.
    2) Using the swab streaked the entire agar surface horizontally, vertically, and around the outer edge of the plate to ensure a heavy growth over the entire surface.
(b) All culture plates were allowed to dry for about 5 minutes.
(c) Distributed the individual antibiotic discs at equal distance with forceps dipped in alcohol and flamed.
(d) Gently pressed each disc down with the wooden end of the cotton swab or sterile forceps to ensure that the discs adhered to the surface of the agar.
(e) The plates were then inverted and incubated at 370 C for 24 hours.

(f) After incubation, the plates were examined and the diameter of the zones of complete inhibition was measured in mm.

(g) The zone diameter for individual anti-microbial agents was used to determine susceptible, intermediate, and resistant categories by referring to an interpreting table.

(h) Clinical and Laboratory Standards Institute (CLSI, 2007).

3.8.1 Recording and Interpreting Results

The zones of growth inhibition was compared with the zone-size interpretative table standard for *Shigella spp, Staphylococcus spp, Salmonella spp, Klebsiella spp, Escherichia coli, Bacillus spp,* and *Pseudomonas spp* (Table2) provided by Clinical and Laboratory Standards Institute (CLSI, 2007).

Table 2: The list of commercially available antimicrobial disc used in this study against *Shigella spp, Staphylococcus spp, Salmonella spp, Klebsiella spp, Escherichia coli, Bacillus spp* and *Pseudomonas spp* and zone diameter imperative standards for isolated bacteria

Sl. No	Name of the antibiotics	*Sh* spp (mm)	Staph. spp (mm)	*Kleb* spp (mm)	*Sal spp* (mm)	E. Coli (mm)	*Pse spp* (mm)	*B spp* (mm)
1	Penicillin-G (P)	I: 0 R:≤14 S≥ 15	I: 0 R:≤14 S≥ 15	I: 0 R:≤14 S≥ 15	I: 0 R:≤ 14 S≥ 15	I: 0 R:≤ 14 S≥15	I: 0 R:≤ 14 S≥15	I: 0 R:≤ 14 S≥15
2	Ciprofloxacin (CIP)	I:16-20 R:≤15 S:≥21	I:16-20 R:≤15 S:≥21	I:16-20 R:≤15 S:≥21	I:16-20 R:≤15 S:≥21	I:16-20 R:≤15 S:≥21	I:16-20 R:≤15 S:≥21	I:16-20 R:≤15 S:≥21
3	Gentamycin (GEN)	I:13-14 R:≤12 S:≥15	I:13-14 R:≤12 S:≥15	I:13-14 R:≤12 S:≥15	I:13-14 R:≤12 S:≥15	I:13-14 R:≤12 S:≥15	I:13-14 R:≤12 S:≥15	I:13-14 R:≤12 S:≥15
4	Cephalexin(CN)	I:15-17 R:≤14 S:≥18	I:15-17 R:≤14 S:≥18	I:15-17 R:≤14 S:≥18	I:15-17 R:≤14 S:≥18	I:15-17 R:≤14 S:≥18	I:15-17 R:≤14 S:≥18	I:15-17 R:≤14 S:≥18
5	Kanamycin (K)	I:14-17	I:14-17	I:14-17	I:14-17	I:14-17	I:14-17	I:14-17

		R:≤13 S:≥18	R:≤13 S:≥18	R:≤13 S:≥18	R:≤13 S:≥18	R:≤13 S:≥18	R:≤13 S:≥18	R:≤13 S:≥18
6	Neomycin (N)	I:14-15 R:≤13 S:≥16	I:14-15 R:≤13 S:≥16	I:14-15 R:≤13 S:≥16	I:14-15 R:≤13 S:≥16	I:14-15 R:≤13 S:≥16	I:14-15 R:≤13 S:≥16	I:14-15 R:≤13 S:≥16
7	Amikacin (AK)	I:15-16 R:≤14 S:≥17	I:15-16 R:≤14 S:≥17	I:15-16 R:≤14 S:≥17	I:15-16 R:≤14 S:≥17	I:15-16 R:≤14 S:≥17	I:15-16 R:≤14 S:≥17	I:15-16 R:≤14 S:≥17
8	Ampicillin (AMP)	I:14-16 R:≤13 S:≥17	I:14-16 R:≤13 S:≥17	I:14-16 R:≤13 S:≥17	I:14-16 R:≤13 S:≥17	I:14-16 R:≤13 S:≥17	I:14-16 R:≤13 S:≥17	I:14-16 R:≤13 S:≥17
9	Erythromycin (E)	I:14-22 R:≤ 13 S≥ 23	I:14-22 R:≤ 13 S≥ 23	I:14-22 R:≤ 13 S≥ 23	I:14-22 R:≤ 13 S≥ 23	I:14-22 R:≤ 13 S≥ 23	I:14-22 R:≤ 13 S≥ 23	I:14-22 R:≤ 13 S≥ 23
10	Chloramphenicol (C)	I:13-17 R: ≤12 S: ≥18	I:13-17 R: ≤12 S: ≥18	I:13-17 R: ≤12 S: ≥18	I:13-17 R: ≤12 S: ≥18	I:13-17 R: ≤12 S: ≥18	I:13-17 R: ≤12 S: ≥18	I:13-17 R:≤12 S: ≥18
11	Amoxicillin (AMX)	I:14-17 R: ≤13 S: ≥18	I: 14-17 R: ≤13 S: ≥18	I:14-17 R: ≤13 S: ≥18	I:14-17 R: ≤13 S: ≥18	I:14-17 R: ≤13 S: ≥18	I:14-17 R: ≤13 S: ≥18	I:14-17 R: ≤13 S: ≥18
12	Vancomycin (V)	I:15-16 R: ≤14 S: ≥17	I:15-16 R: ≤14 S: ≥17	I:15-16 R: ≤14 S: ≥17	I:15-16 R: ≤14 S: ≥17	I:15-16 R: ≤14 S: ≥17	I:15-16 R: ≤14 S: ≥17	I:15-16 R: ≤14 S: ≥17
13	Cefaclor (CEC)	I:15-17 R: ≤14 S: ≥18	I:15-17 R: ≤14 S: ≥18	I:15-17 R: ≤14 S: ≥18	I:15-17 R: ≤14 S: ≥18	I:15-17 R: ≤14 S: ≥18	I:15-17 R: ≤14 S: ≥18	I:15-17 R: ≤14 S: ≥18
14	Levofloxacin (LE)	I:24-30 R: ≤23 S: ≥31	I:23-29 R: ≤22 S: ≥30	I:24-30 R: ≤13 S: ≥31	I:24-30 R: ≤13 S: ≥31	I:24-30 R: ≤13 S: ≥31	I:24-30 R: ≤13 S: ≥31	I:24-30 R: ≤13 S: ≥31

Clinical and Laboratory Standards Institute (CLSI, 2007). [Note: Sh=*Shigella*, Staph=*Staphylococcus*, Kleb=*Klebsiella*, Sal=*Salmonella*, E=*Escherichia*, Pse=*Pseudomonas*, B=*Bacillus*.]

3.9 Maintenance of stock culture

Stock culture is a culture of a microorganism maintained solely for the purpose of keeping the microorganism in a viable condition by subculture, as necessary, into fresh medium.

3.9.1 Agar slant method

The stock culture was maintained the following procedures, Isolated and identified bacteria were inoculated into nutrient agar slants and incubated at 37°C for 24 hours and then examine for growth. One slant was used for an individual isolate. Then the sterile mineral oil was poured into the tube until the colonies were covered completely. The tube were sealed off with paraffin and kept at room temperature for future use as seed. By this method bacteria can be preserved with no deviation of their original characters for few months (Buxton and Fraser, 1977).

3.9.2 Sterile buffered glycerine method

Sterile buffered glycerine (20%) was prepared by mixing 20 parts of pure glycerin and 80 parts of PBS. Then a loopful of thick bacterial culture was mixed with 20%, sterile buffered glycerin in smear vials and was preserved at -20°C This method is more appropriate for preserving bacteria with no deviation of their original characters for several years (Buxton and Fraser, 1977).

CHAPTER 4

RESULTS

CHAPTER 4

RESULTS

Result of total viable count, Bacteriological investigation, culture test, staining characteristics, biochemical test and antibiotic sensitivity test, including percentage of isolated bacteria are presented in different tables and described below under the following heading:-

4.1 Result of Total Viable Count on the sampling surfaces

The Total Viable Counts (in the form of CFU/ml) of swab samples collected from 3 distinct locations (Hand rail, Armrest and Vinyl seat) of each of the 15 buses are presented in Table- 3.

Table 3: Results of total viable count of samples from each sampling point (dilution 10^{-4})

Place of Sample taken	Dilution	Average of total Viable Counts
Hand rail	10^{-1}	TNTC
	10^{-2}	9.7×10^{4}
	10^{-3}	7.0×10^{5}
	10^{-4}	6.2×10^{6}
Armrest	10^{-1}	TNTC
	10^{-2}	9.5×10^{4}
	10^{-3}	6.9×10^{5}
	10^{-4}	5.8×10^{6}
Vinyl seat	10^{-1}	TNTC
	10^{-2}	8.5×10^{4}
	10^{-3}	6.6×10^{5}
	10^{-4}	5.2×10^{6}

Legends: TNTC= Too numerous to count

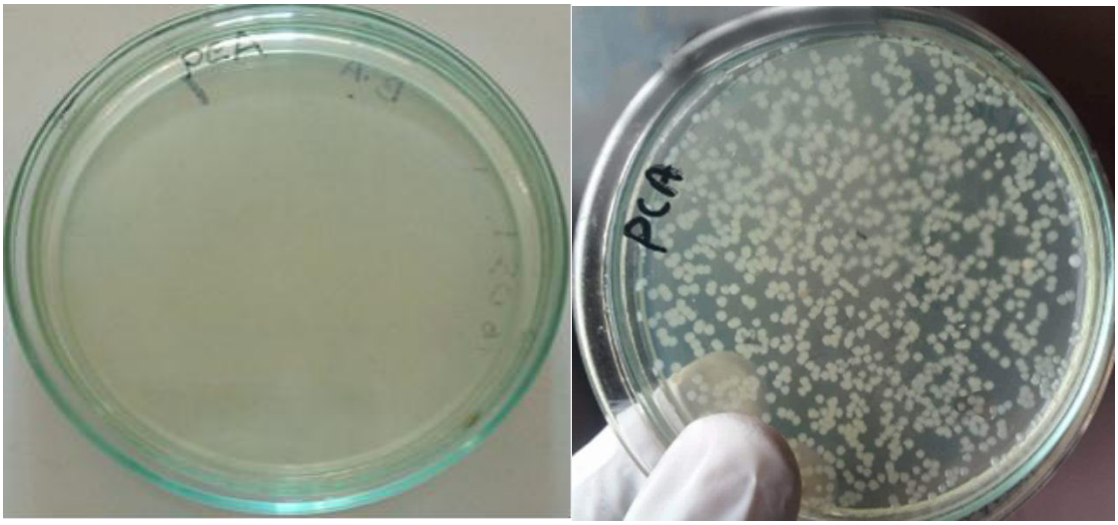

Plate 2: Colony on Plate Count Agar (Right) and Uninoculated control of Plate Count Agar (Left)

4.2 Bacteriological investigation

A total of 45 swab samples were collected for this study. From those samples a total of 60 bacterial were isolated which is described in table 4.

Table 4: Distribution of Target pathogenic bacteria over the sampling locations.

Sl. No	Bacterial isolates	Hand rail	Armrest	Vinyl seat	Total No. (%)
1	***E. coli***	10	6	2	18 (30%)
2	***Pseudomonas spp***	2	1	0	3 (5%)
3	***Klebsiella spp***	3	2	1	6 (10%)
4	***Salmonella spp***	5	3	1	9 (15%)
5	***Staphylococcus spp***	8	5	2	15 (25%)
6	***Shigella spp***	4	1	1	6 (10%)
7	***Bacillus spp***	1	1	1	3 (5%)
	Total	**33**	**19**	**8**	**60 (100)**

Table 5: Distribution of samples taken from 3 distinct locations (Hand rail, Armrest and Vinyl seat) of each of the 15 buses.

Sample sites	Number of Total Samples	No. of Positives (%)	No. of Negatives (%)
Hand rail	15	15 (100%)	0
Armrest	15	14 (93.33%)	1 (6.67%0
Vinyl seat	15	13 (86.67)	2 (13.33%)

4.3 Result of cultural characteristics

The cultural characteristics of *E. coli spp, Klebsiella spp, Salmonella* spp, *Bacillus spp, Shigella spp, Pseudomonas spp, and Staphylococcus spp* on various media are presented in table 6.

Table 6: Result of cultural characteristics of bacteria isolated from public bus and HSTU Campus

Name of bacteria	Staining characteristic	Name of media	Cultural characteristics
Staphylococcus spp	Gram positive cocci arranged in grape like cluster	Nutrient Agar	Small Yellow Colonies.
		MS Agar	Small whitish colony or yellowish colony. Large colony.
Klebsiella spp	Gram-negative, large rod shaped organism pink-colored.	Nutrient agar	Large colony.
		Mac-Conkey agar	Pink colored colonies.
		EMB agar	Smooth pink color, mucoid colonies.

***E. coli* spp**	Gram negative long rod shaped pink colour.	Nutrient Agar	Large mucoid, white colony.
		Mac-Conkey Agar	Rose pink colored Colonies
		EMB agar	Metallic-sheen color colonies
***Salmonella* spp.**	Gram negative small rod shaped pink colour.	Nutrient agar	Circular, smooth, opaque and translucent colonies.
		Mac-Conkey agar	Smooth and circular white/transparent colony
		S.S agar	Small non-lactose fermented with black center colony.
Pseudomonas spp.	Gram negative, rod shaped organism	Nutrient Agar	Smooth, Raised, irregular and semi-translucent colony.
		Mac-Conkey Agar	Pale colour flat non lactose fermenting colonies.
		Cetrimide Agar	Colonies are greenish or yellow in color.
Bacillus spp.	Gram-positive, rod-shaped organism	Nutrient Agar	Thick, grayish white or, cream colored colonies
		Blood Agar	Produce non hemolytic colonies
Shigella spp.	Gram-negative, rod shaped organism	Nutrient Agar	Small, circular, grayish or translucent colonies.
		Mac-Conkey Agar	Pale and yellowish colonies due to the non-lactose fermented
		SS Agar	Small non-lactose fermented colony

Legends: MS=Mannitol Salt, EMB=Eosin Methylene Blue, SS=Salmonella and Shigella.

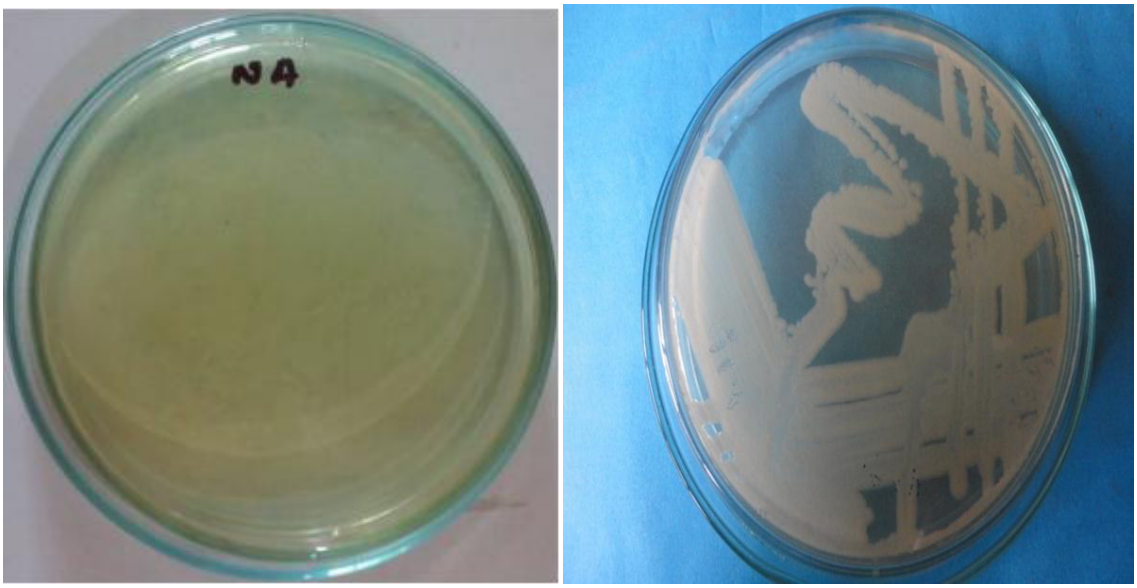

Plate 3: *Staphylococcus spp* produce small yellow colonies on Nutrient Agar (Right) and uninoculated control (Left)

Plate 4: *Staphylococcus spp* produce β-hemolytic reaction on Blood Agar (Right) and uninoculated control (Left)

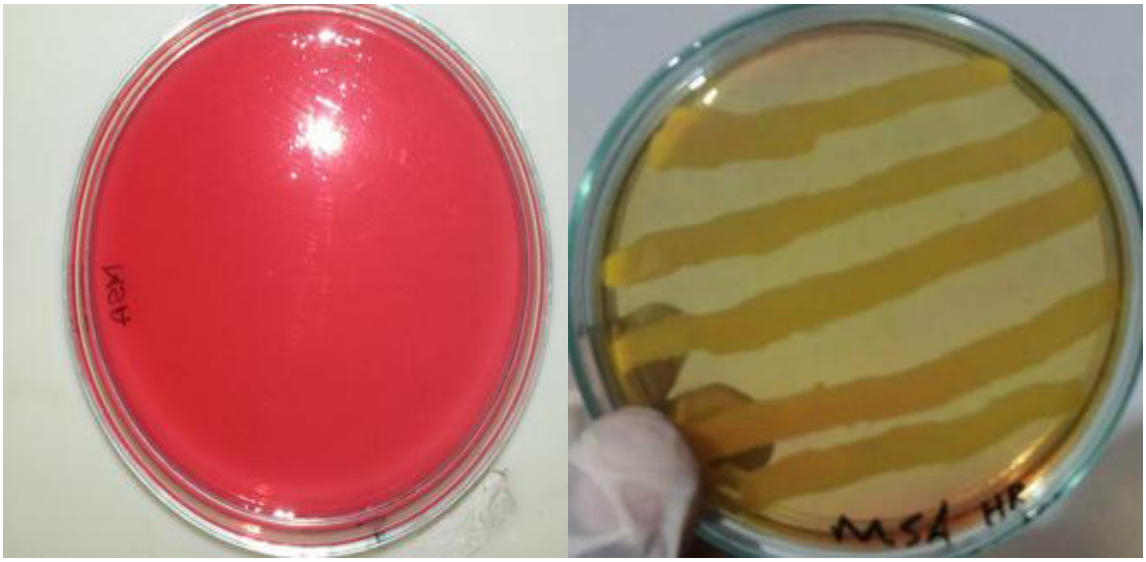

Plate 5: *Staphylococcus spp* produce yellowish colonies on MS Agar (Right) and uninoculated control (Left)

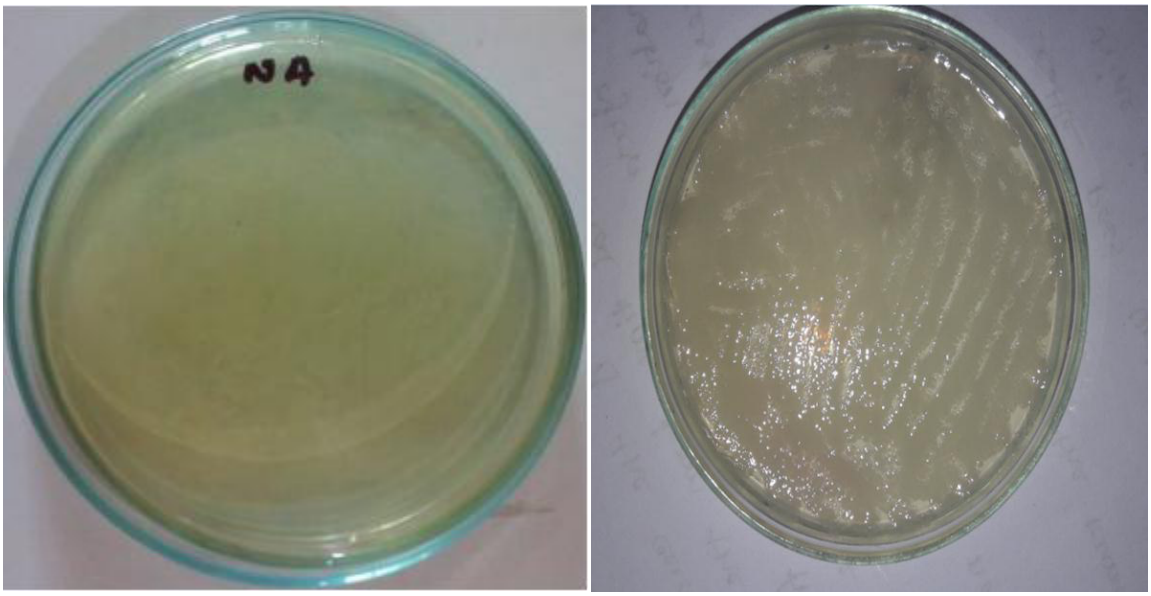

Plate 6: *Klebsiella spp* produce large, mucoid, white colonies on Nutrient Agar (Right) and uninoculated control (Left)

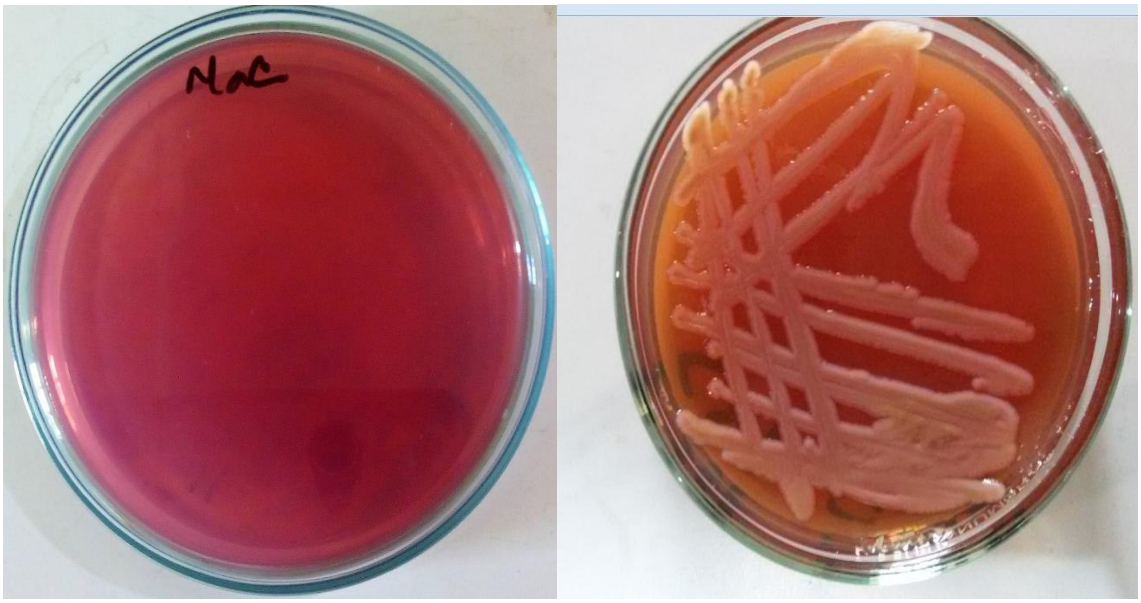

Plate 7: *Klebsiella spp* produce large, mucoid, bright pink lactose fermented colony on Mac-Conkey agar (Right) and uninoculated control (Left)

Plate 8: *Klebsiella spp* produce smooth pink color, mucoid colonies on EMB Agar (Right) and uninoculated control (Left)

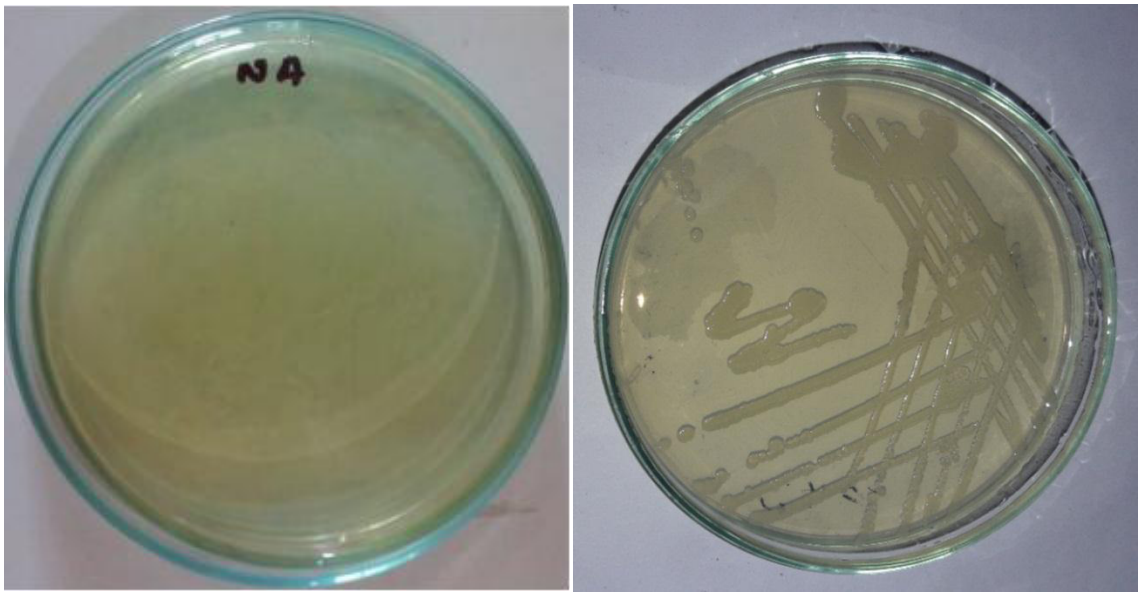

Plate 9: *Escherichia coli spp* produce large colorless colonies on Nutrient Agar (Right) and uninoculated control (Left)

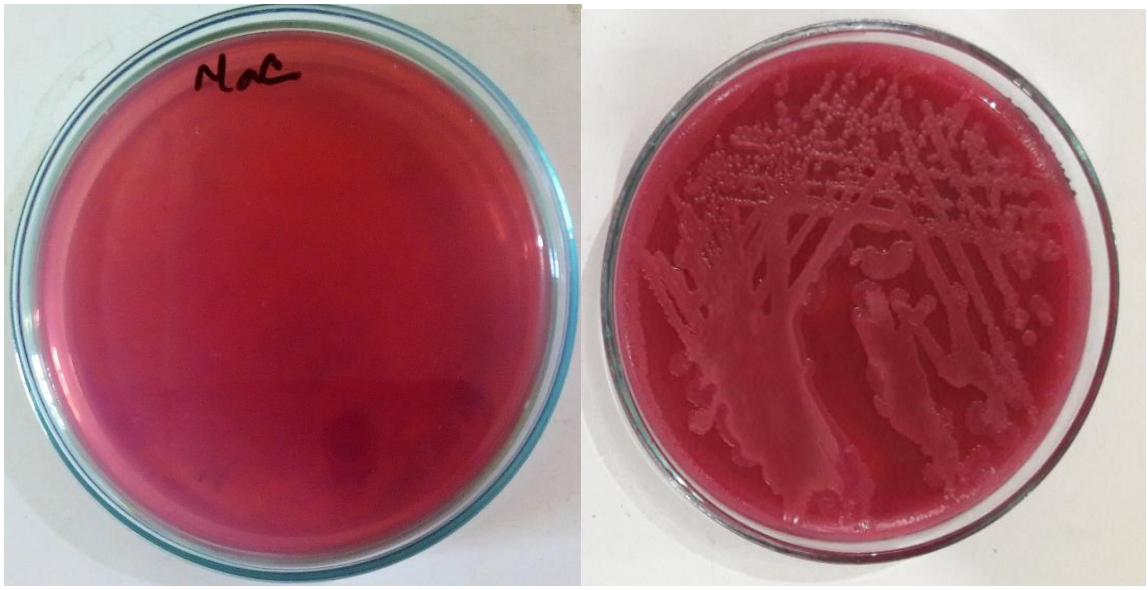

Plate 10: *Escherichia coli spp* produce pink colour colonies on Mac-Conkey Agar (Right) and uninoculated control (Left)

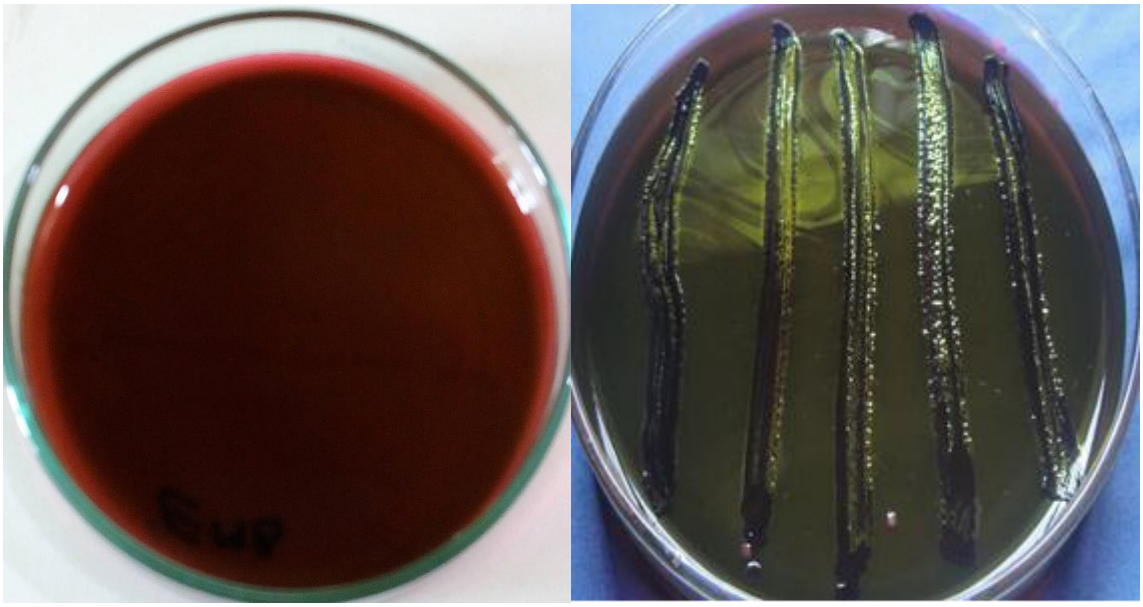

Plate 11: *Escherichia coli* produce metallic sheen (greenish black) colony on Eosin Methylene Blue Agar (Right) and uninoculated control (Left)

Plate 12: *Salmonella* spp produce small non-lactose fermented colony of black color on *Salmonella Shigella* Agar (Right) and uninoculated control (Left)

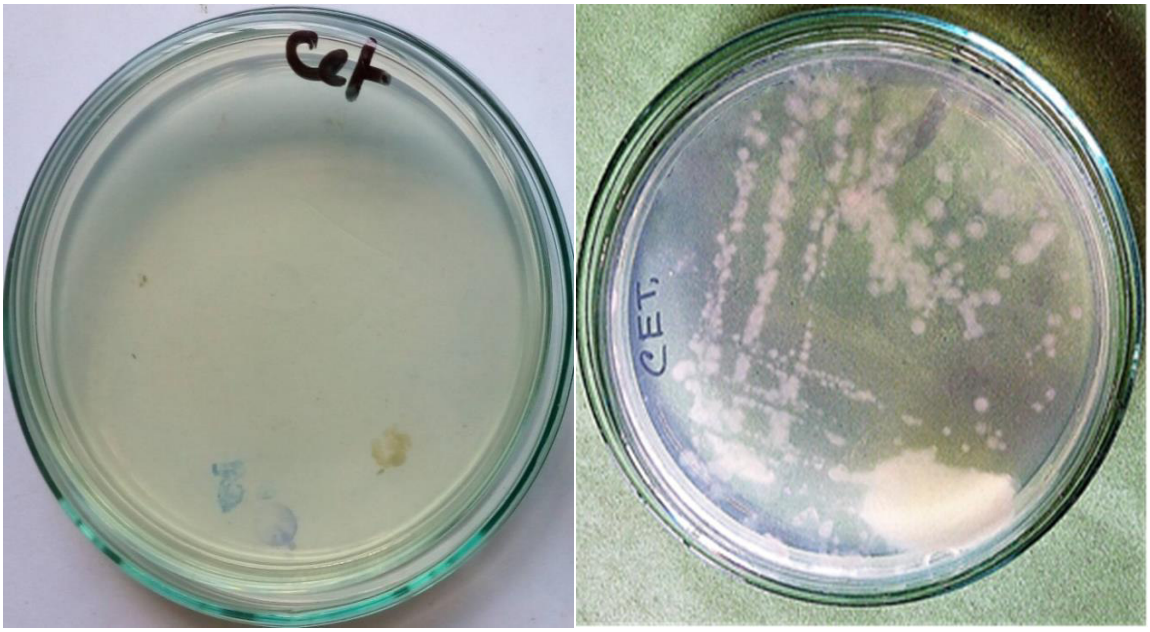

Plate 13: *Pseudomonas spp* produce greenish or yellow colonies on Cetrimide Agar (Right) and uninoculated control (Left)

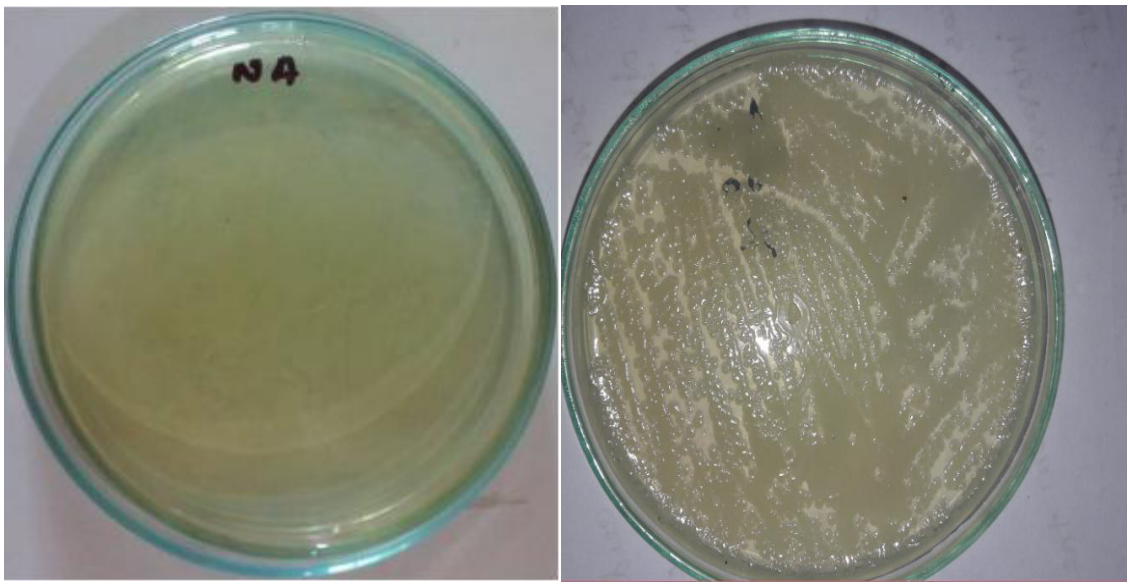

Plate 14: *Bacillus spp* produce grayish white or cream colored colonies on Nutrient Agar (Right) and uninoculated control (Left)

Plate 15: *Bacillus spp* produce non hemolytic colonies on Blood Agar (Right) and uninoculated control (Left)

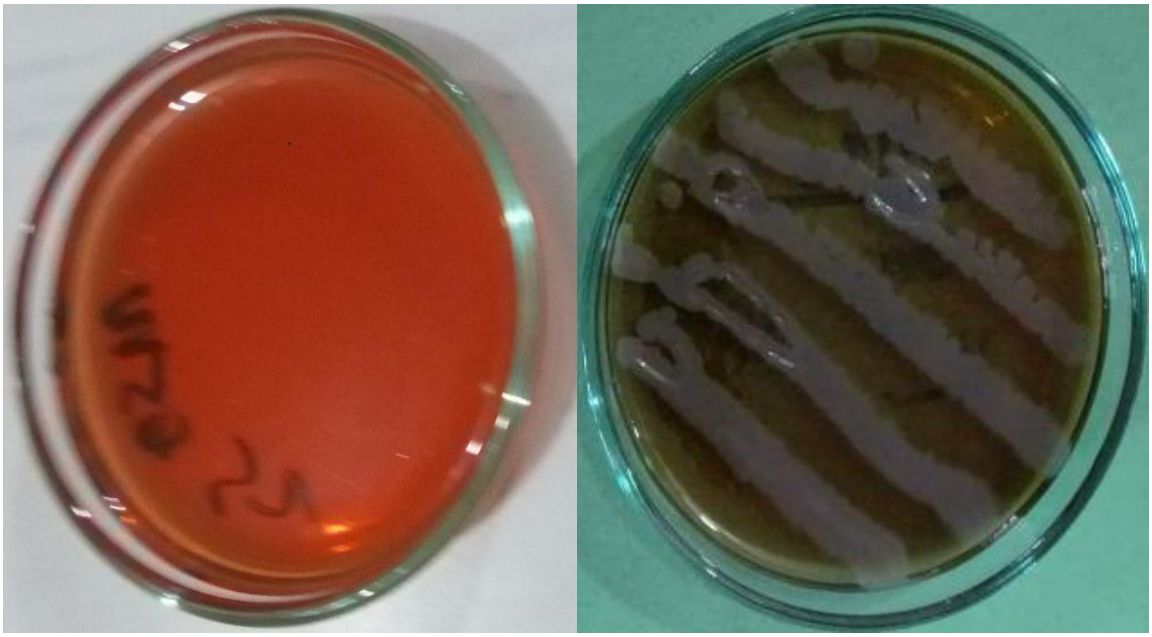

Plate 16: *Shigella* spp produce small non-lactose fermented colony on *Salmonella Shigella* Agar (Right) and uninoculated control (Left)

4.4 Result of staining characteristics of Bacterial isolates

The staining characteristics of the isolated organisms were determined according to Gram's staining technique. Morphological and staining characteristics of bacteria recorded from the swab samples by Gram's staining are presented in Table-7 and Plate-17, 18, 19, 20, 21, 22, 23.

Table 7: Morphological and staining properties of isolated bacteria by Gram's staining

Sl. No	Bacterial isolates	Shape	Arrangement	Gram's Staining character
1.	**Staphylococcus spp**	Cocci in shape	Arranged in cluster	Gram (+)
2.	**Klebsiella spp**	Rod in shape	Single, pairs or cluster	Gram (-)
3.	**Escherichia coli spp**	Rod in shape	Single, pair or in short chain	Gram (-)
4.	**Salmonella spp**	Rod in shape	Single or pair	Gram (-)
5.	**Pseudomonas spp**	Rod in shape	Arranged in single	Gram (-)
6.	**Bacillus spp**	Rod in shape	Arranged in single	Gram (+)
7.	**Shigella spp**	Rod in shape	Single or pair	Gram (-)

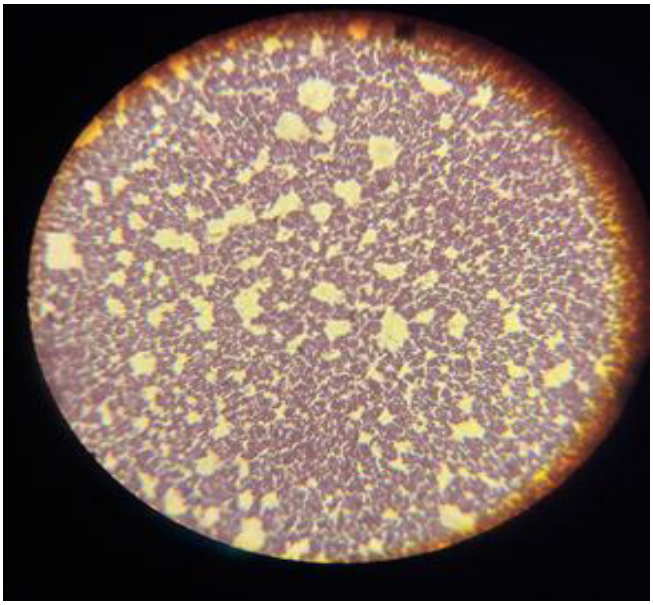

Plate 17: Gram positive spherical shaped purple color *Staphylococcus spp* 100x microscopy.

Plate 18: Gram negative rod shaped pink color *Klebsiella spp* 100x microscopy.

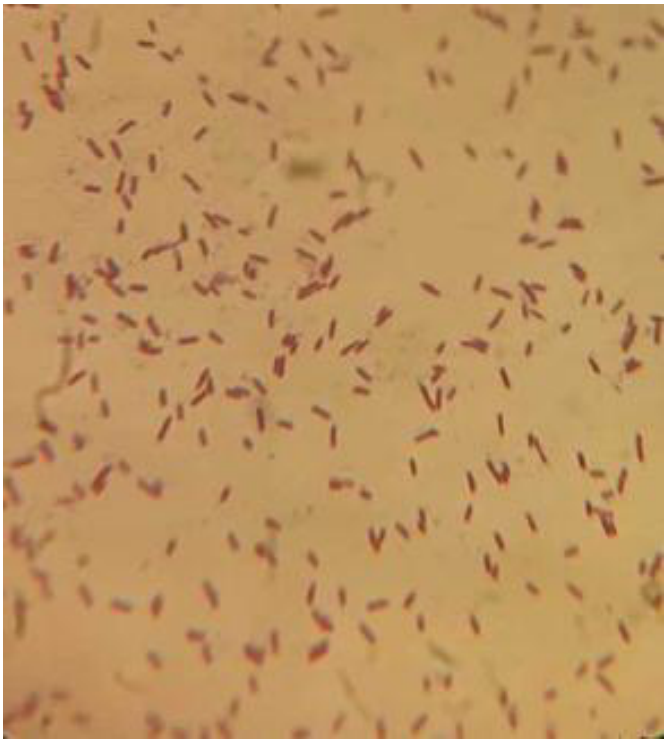

Plate 19: Gram negative rod shaped pink color *Escherichia coli spp* 100x microscopy.

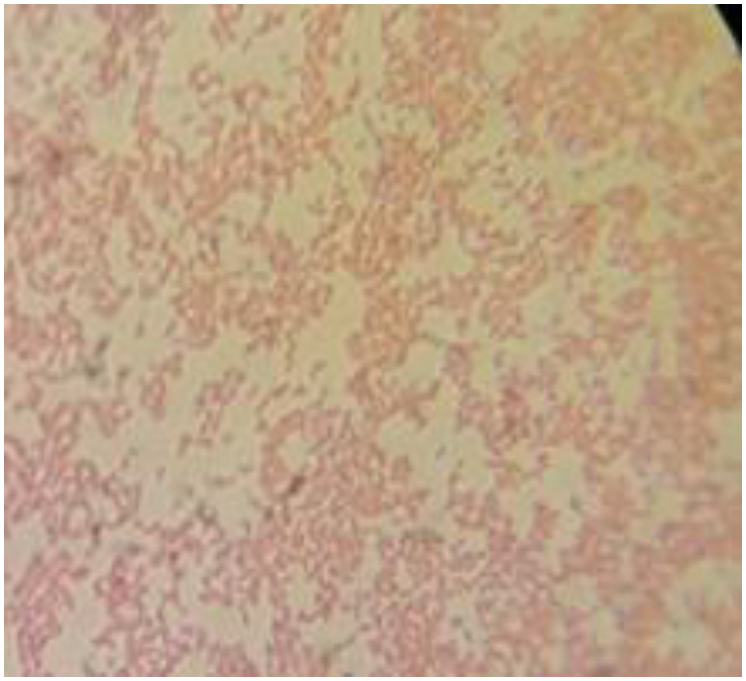

Plate 20: Gram negative small rod shaped pink colour *Salmonella spp* 100x microscopy.

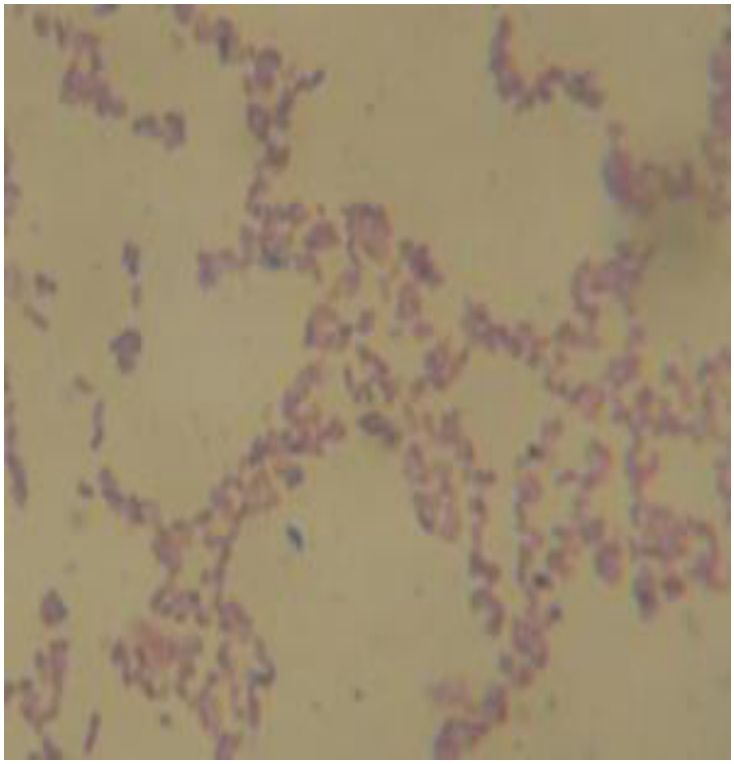

Plate 21: Gram negative small rod shaped pink colour *Pseudomonas spp* 100x microscopy.

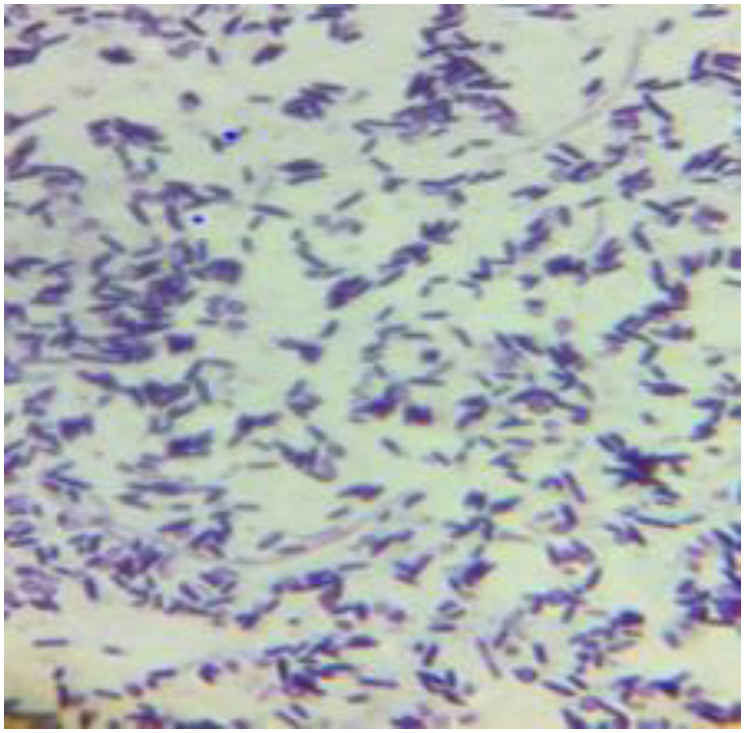

Plate 22: Gram positive rod shaped purple color *Bacillus spp* 100x microscopy.

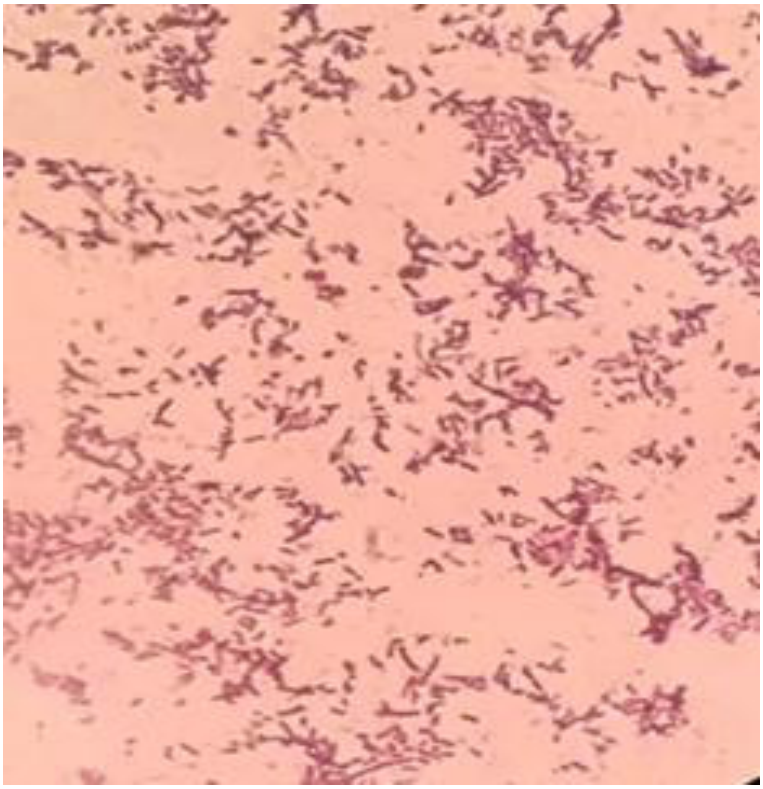

Plate 23: Gram negative rod shaped *Shigella spp* 100x microscopy.

4.5 Result of Biochemical characteristics of Bacterial isolates

Table 8: Result of Biochemical tests of isolated Bacteria

Biochemical test	Isolated Bacteria													
	Staphylococcus spp		Klebsiella spp		Escherichia coli spp		Salmonella spp		Pseudomonas spp		Bacillus spp		Shigella spp	
Catalase	+		+		+		+		+		+		+	
MR	+		-		+		+		-		-		+	
VP	+		+		+		+		-		-		-	
Indole	-		-		+		-		-		-		-	
SC	+		+		-		+		+		-		+	
TSI	Slant	Butt	Slant	Butt	Slant	Butt	Slant	Butt	Slant	Butt	Slant	Butt	Slant	Butt
	Y	Y	Y	Y	Y	Y	Y	R	R	R	Y	R	R	Y

Note: MR=Methyl Red, VP=Voges Proskauer, SC=Simon's Citrate, TSI=Triple Sugar Iron, Y=Yellow, R=Red, (+)=Positive, (-)=Negative.

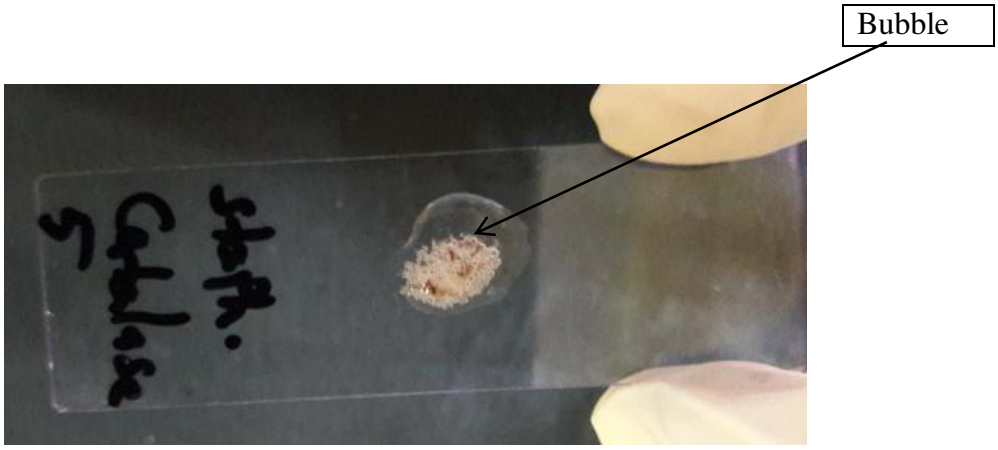

Plate 24: *Staphylococcus spp* showing positive result on Catalase test

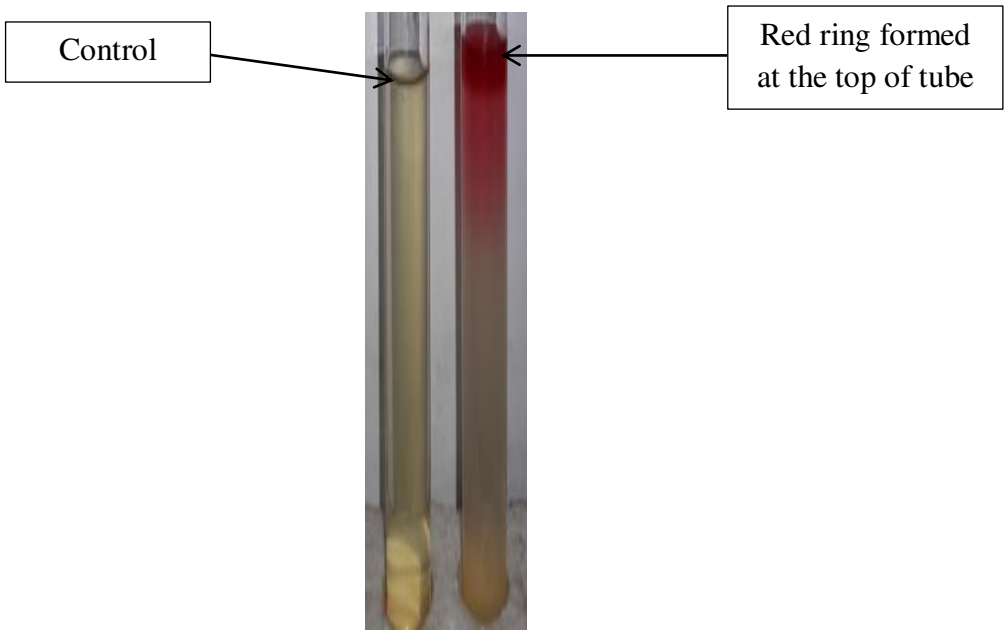

Plate 25: *Staphylococcus spp* showing positive result on MR test (Right) with control (Left)

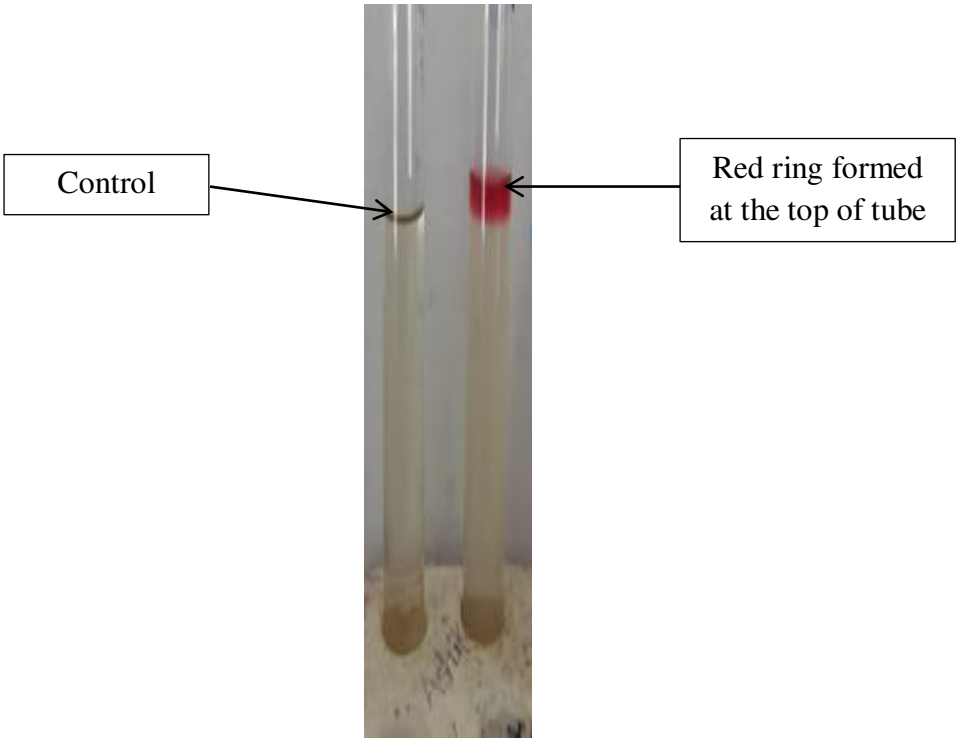

Plate 26: *Staphylococcus spp* showing positive result on VP test (Right) with control (Left)

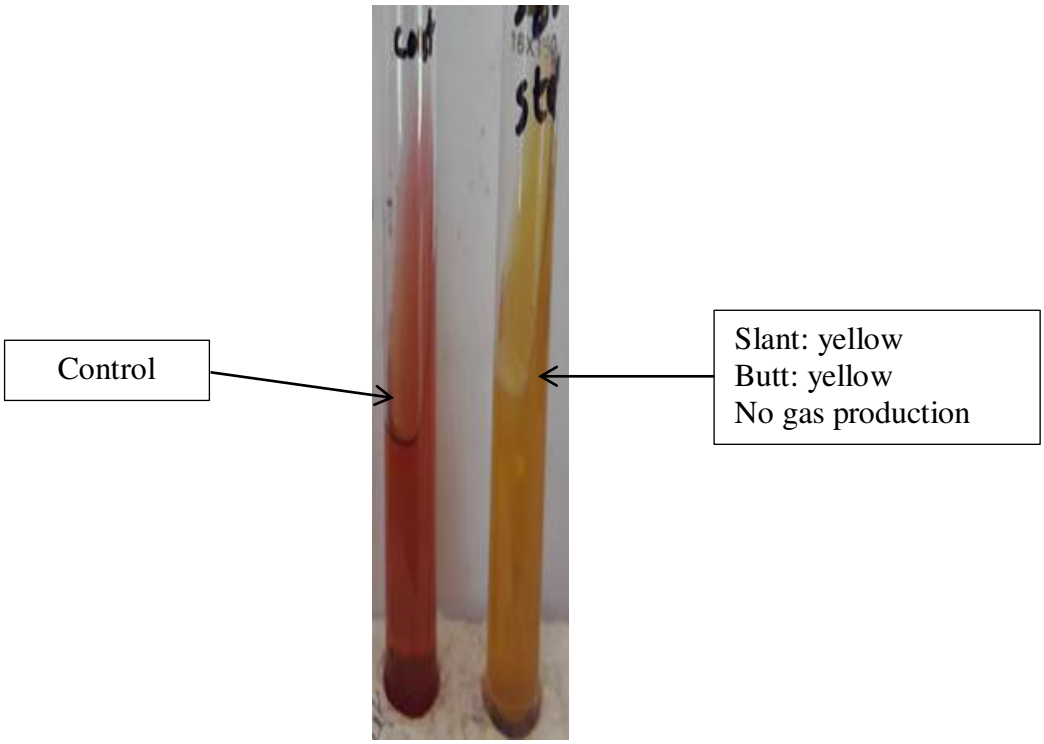

Plate 27: *Staphylococcus spp* showing positive result on TSI test (Right) with control (Left)

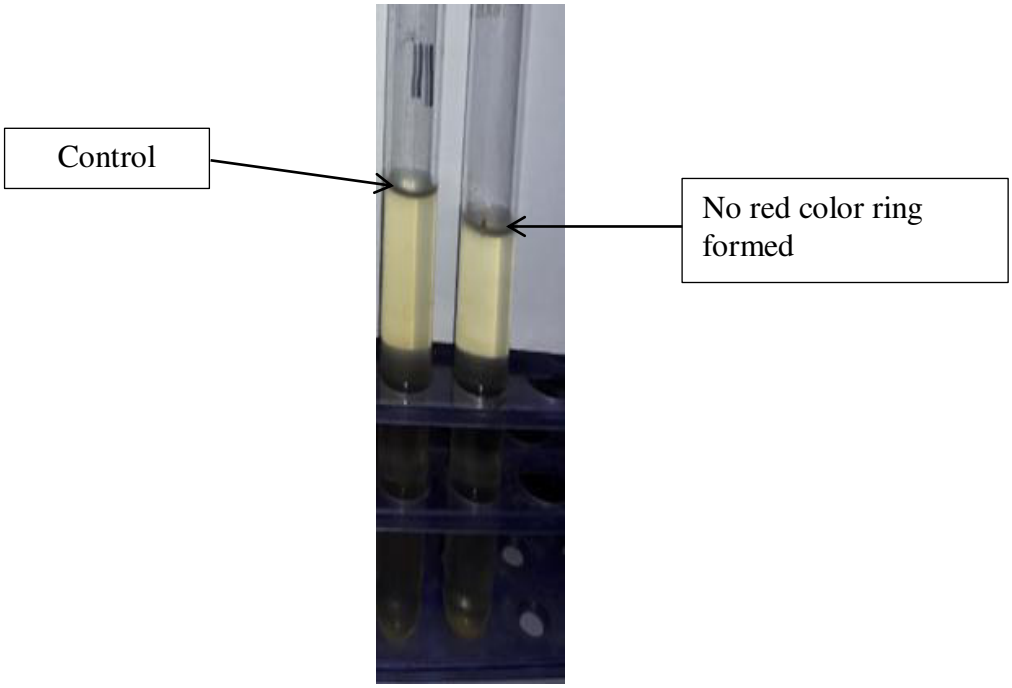

Plate 28: *Klebsiella spp* showing negative result on Indole test (Right) with control (Left)

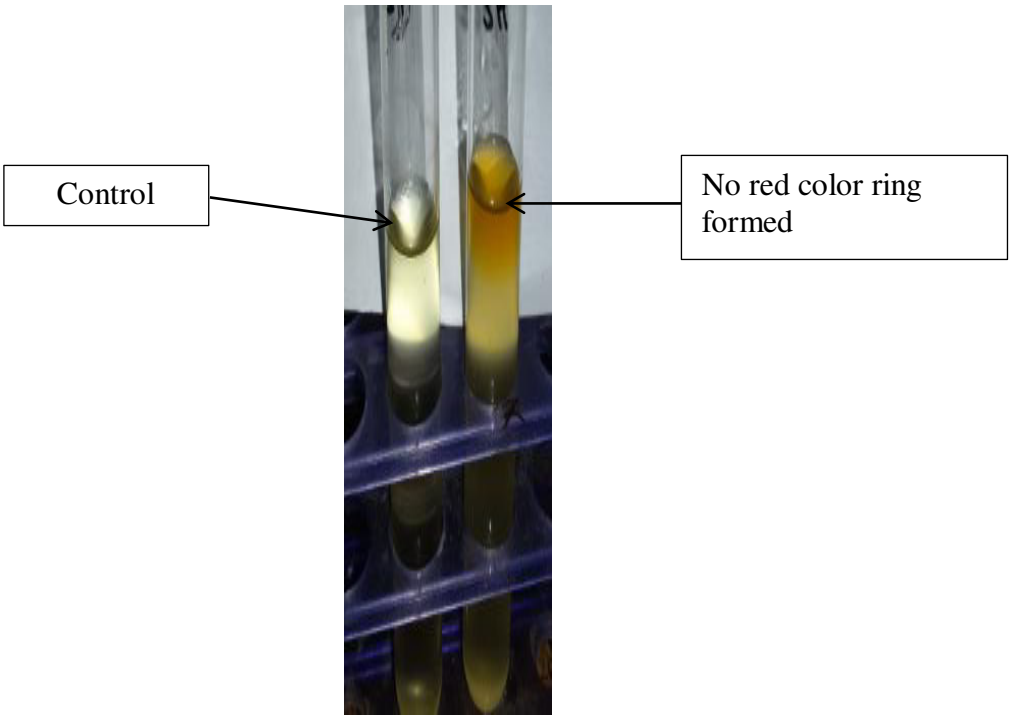

Plate 29: *Klebsiella spp* showing negative result on MR test (Right) with control (Left)

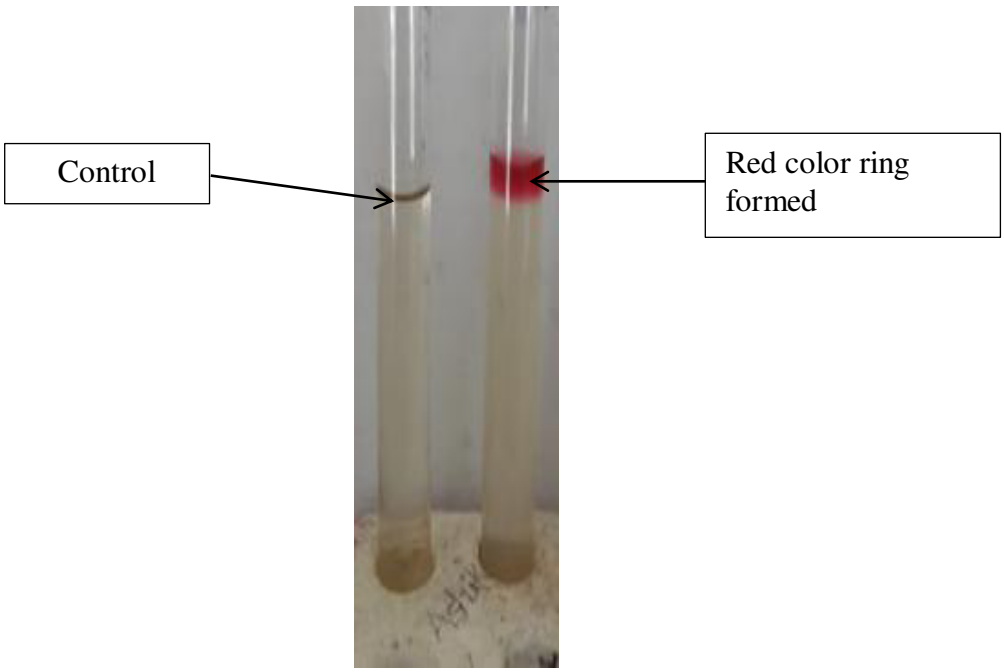

Plate 30: *Klebsiella spp* showing positive result on VP test (Right) with control (Left)

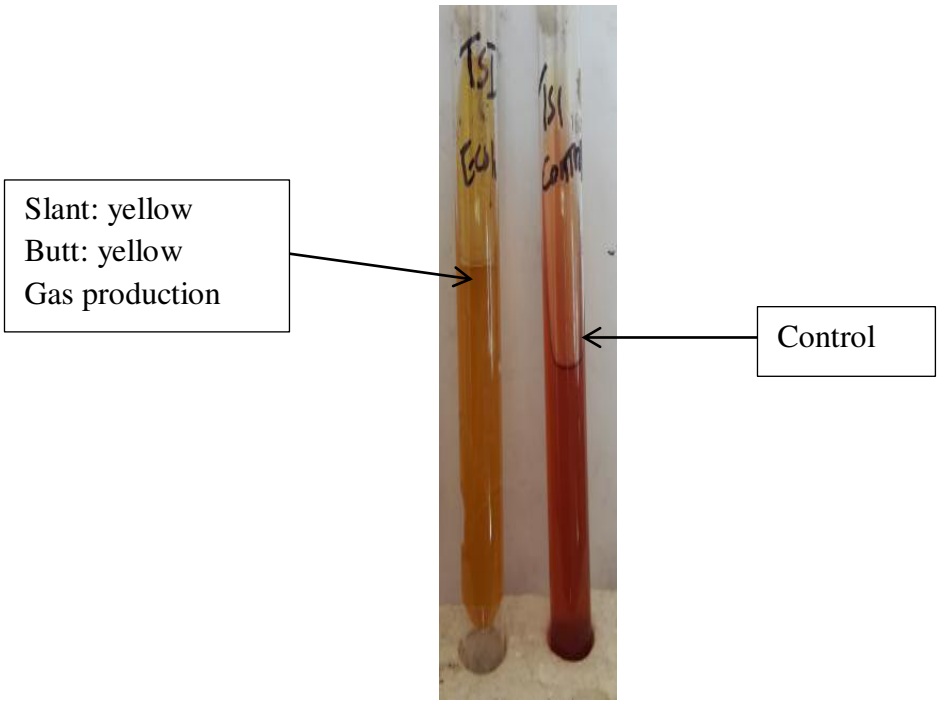

Plate 31: *Escherichia coli spp* showing positive result on TSI (Left) with control (Right)

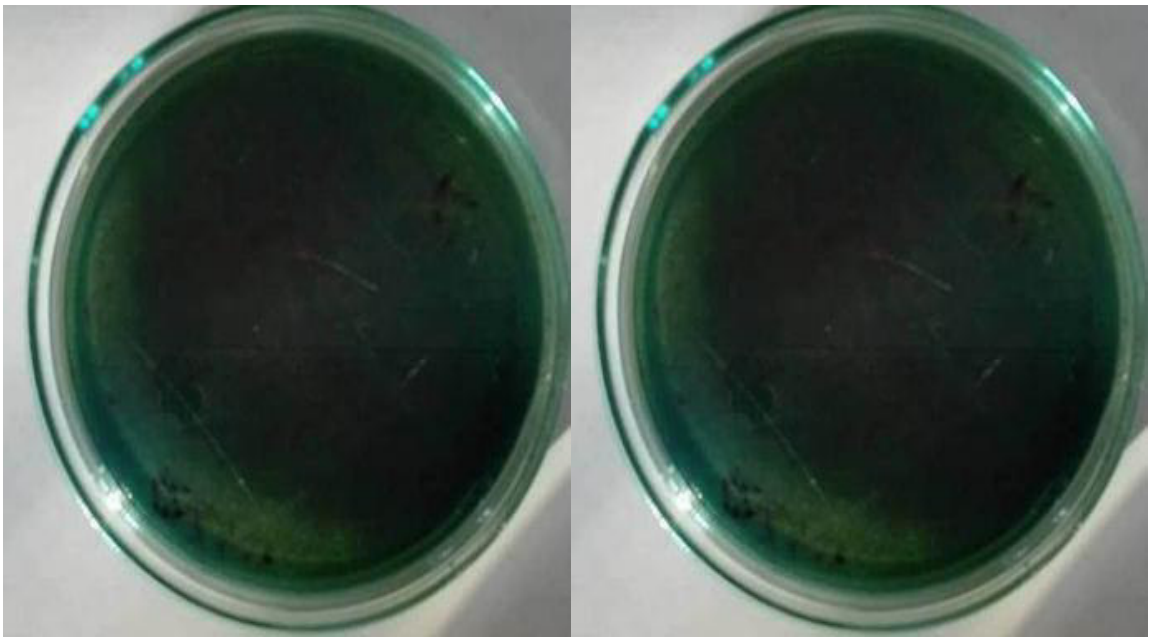

Plate 32: *Escherichia coli spp* showing negative result on Simon's Citrate test (Left) with control (Right)

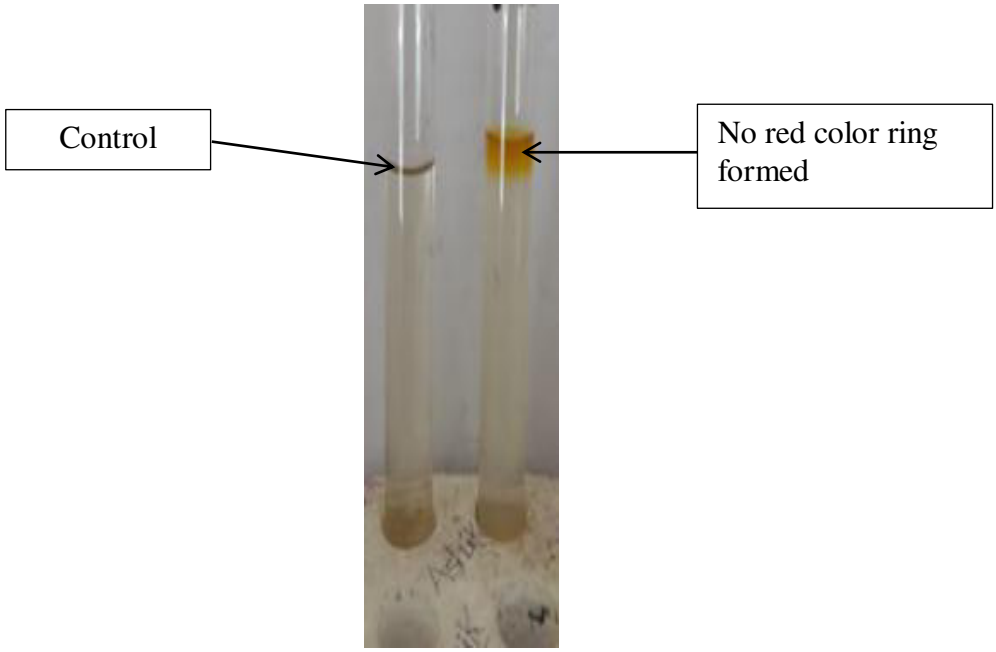

Plate 33: *Escherichia coli spp* showing negative result on VP test (Right) with control (Left)

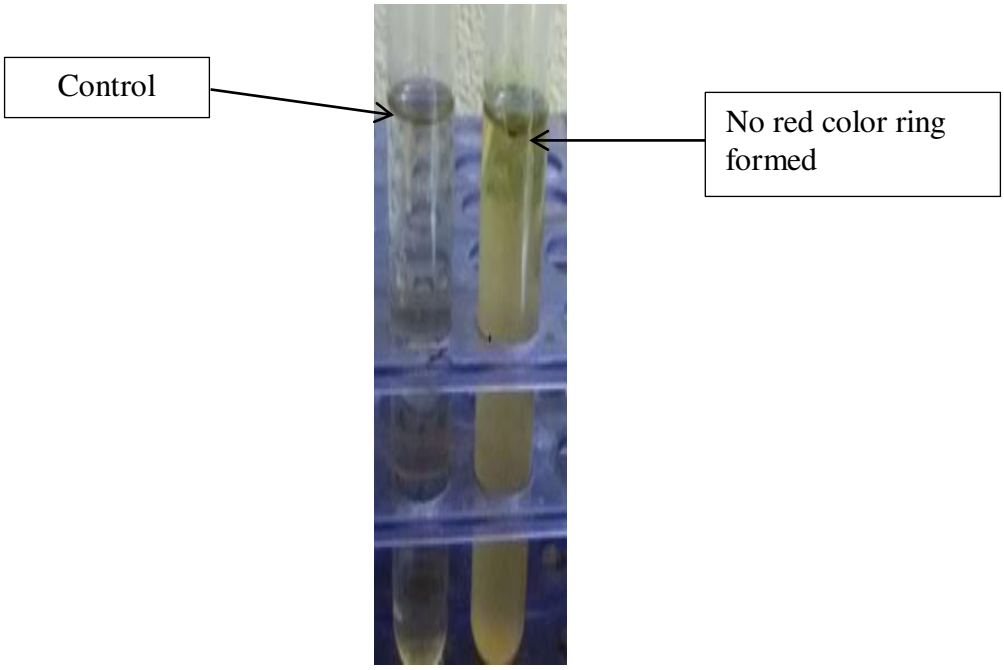

Plate 34: *Salmonella spp* showing negative result on Indole test (Right) with control (Left)

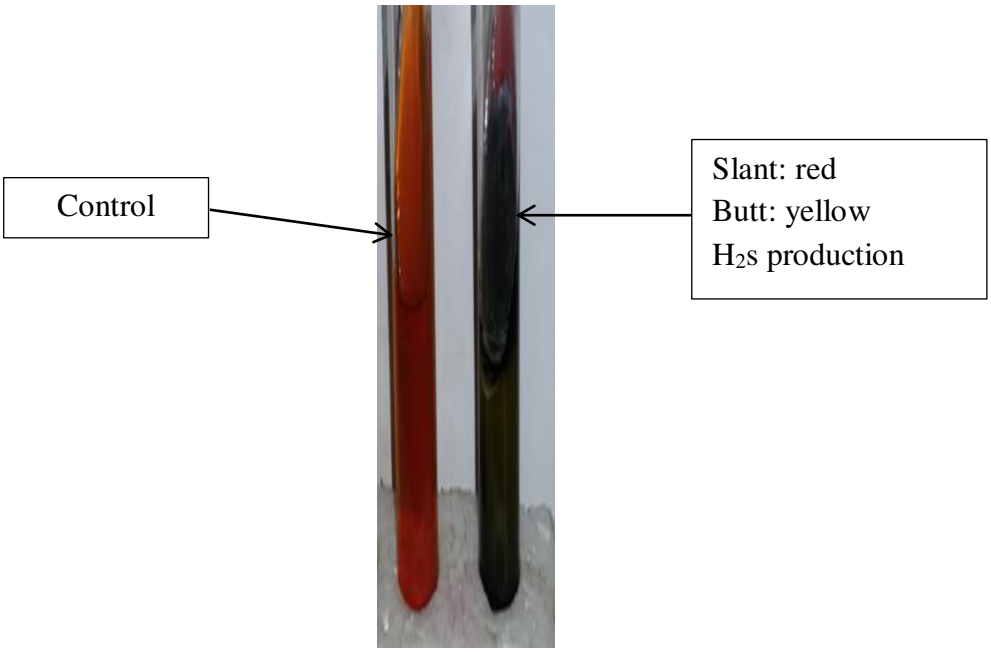

Plate 35: *Salmonella spp* showing positive result on TSI test (Right) with control (Left)

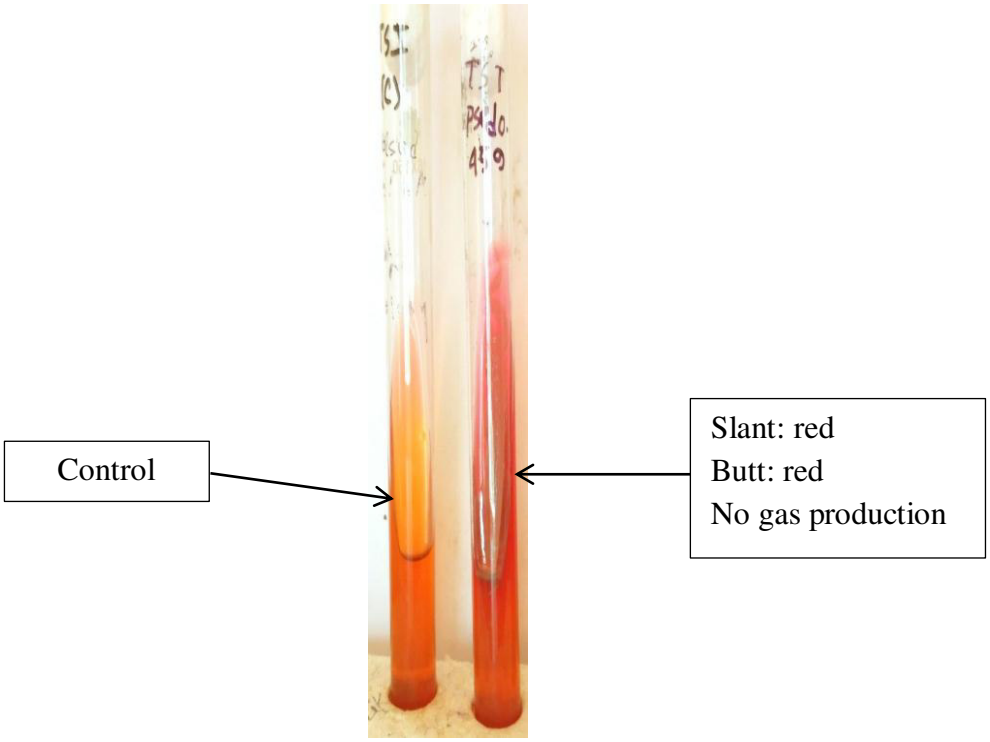

Plate 36: *Pseudomonas spp* showing negative result on TSI test (Right) with control (Left)

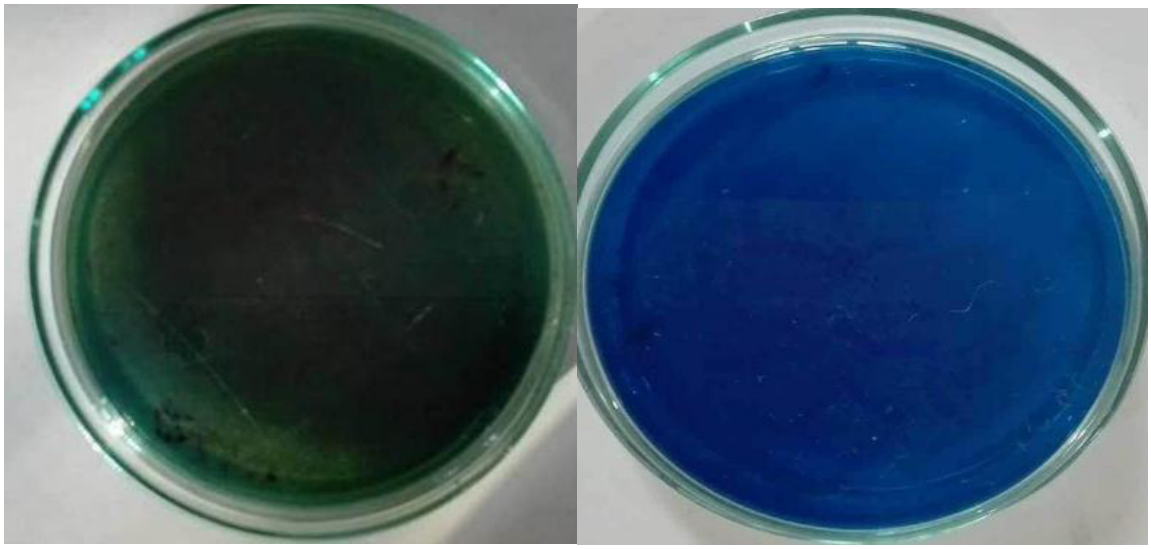

Plate 37: *Bacillus spp* showing positive result on Simon's Citrate test (Left) with control (Right)

4.6 Results of antibiotic sensitivity test

A total of 60 bacterial isolates belonging to seven genera such as *Bacillus spp, Staphylococcus spp, Pseudomonas spp, Salmonella spp, Klebsiella spp, Shigella spp and E. coli* were subjected to antibiotic sensitivity assay. The results of antibiotic sensitivity assay are presented in Table 9, and Plate- 38, 39, 40, 41, 42, 43, 44.

Table 9: Antibiogram test of isolated bacteria

Isolated Bacteria	Name of antibiotic	Disc concentration	Zone of inhibition	Interpretation
Staphylococcus spp	Amoxicillin	30 µg	-	R
	Chloramphenicol	30µg	16	I
	Gentamycin	10µg	28	S
	Vancomycin	30µg	20	S
	Streptomycin	10µg	-	R
Klebsiella spp	Cephalexin	30µg	-	R
	Penicillin	10µg	-	R
	Norfloxacin	10µg	23	S
	Azithromycin	30µg	20	S
	Levofloxacin	5µg	22	R
E. coli spp	Cephalexin	30µg	04	R
	Chloramphenicol	30µg	18	S
	Amikacin	30µg	10	R
	Ampicillin	25µg	-	R
	Gentamycin	10µg	16	S
Pseudomonas spp	Erythromycin	15µg	-	R
	Streptomycin	10µg	18	S
	Cloxacillin	1µg	0	R

	Gentamycin	10µg	20	S
	Cephalexin	30µg	-	R
Salmonella spp	Amoxicillin	30µg	-	R
	Chloramphenicol	30µg	-	R
	Gentamycin	10µg	19	S
	Ofloxacin	5µg	21	S
	Cefaclor	30µg	-	R
Bacillus spp	Ciprofloxacin	5µg	26	S
	Neomycin	30µg	14	I
	Co-trimoxazole	15µg	14	I
	Amoxicillin	30µg	5	R
	Erythromycin	15µg	20	I
Shigella spp	Gentamycin	30µg	21	S
	Cloxacillin	10µg	-	R
	Erythromycin	5µg	-	R
	Cefaclor	15µg	4	R
	Amoxicillin	30µg	-	S

Note: S=Sensitive, R=Resistant, I=Intermediate

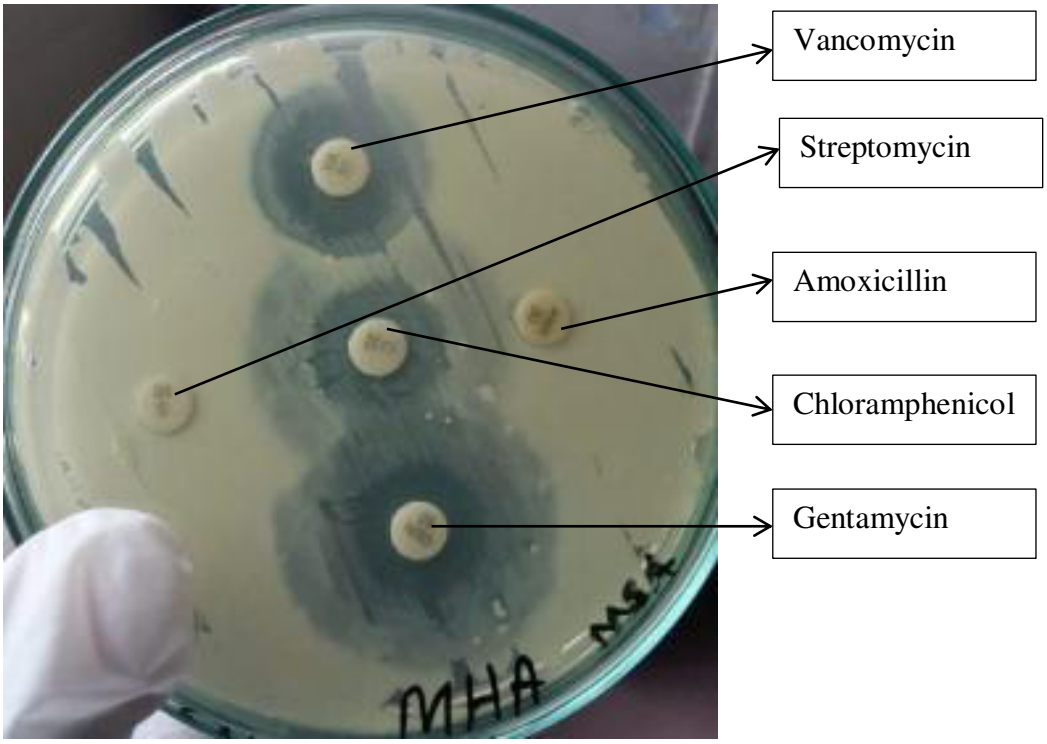

Plate 38: Antibiotic sensitivity test result of *Staphylococcus spp.* on Muller Hinton Agar

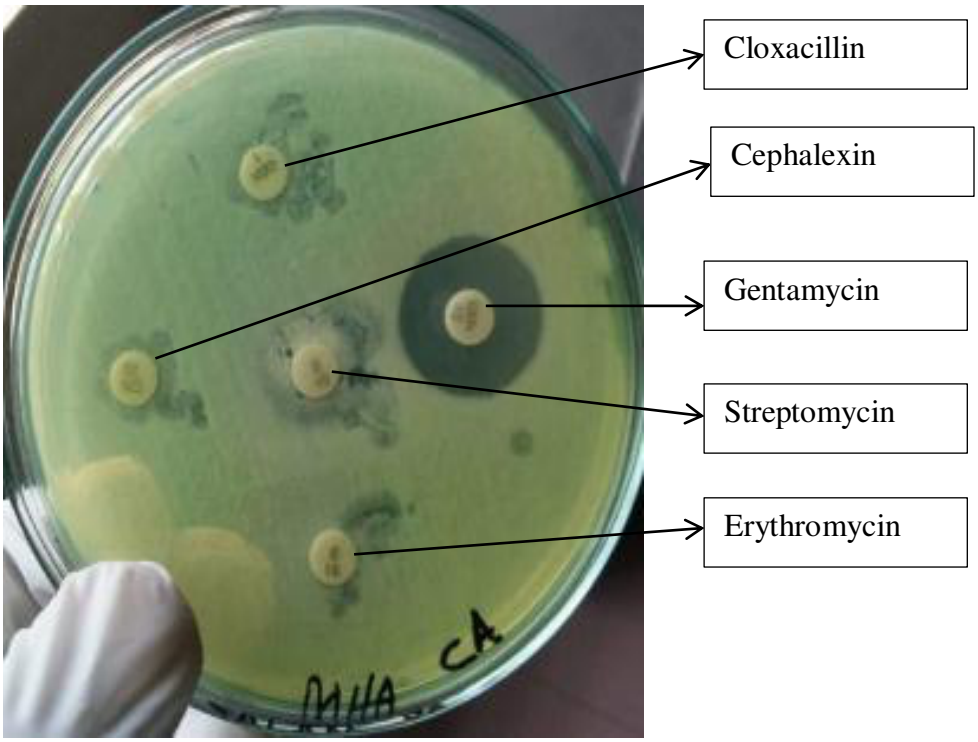

Plate 39: Antibiotic sensitivity test result of *Pseudomonas spp.* on Muller Hinton Agar

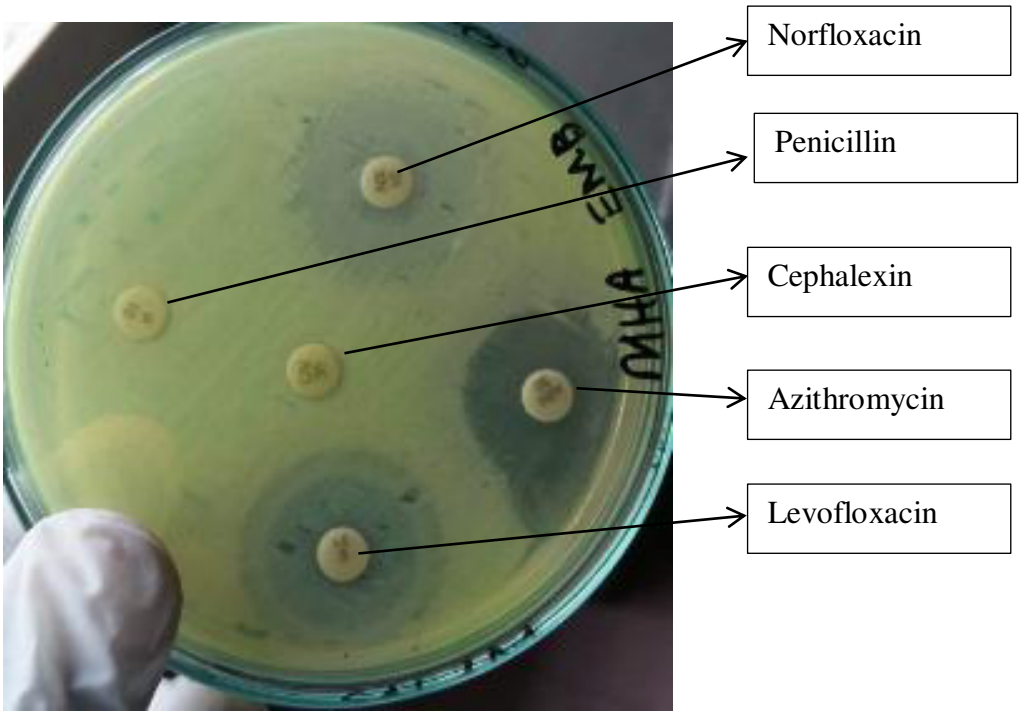

Plate 40: Antibiotic sensitivity test result of *Klebsiella spp.* on Muller Hinton Agar

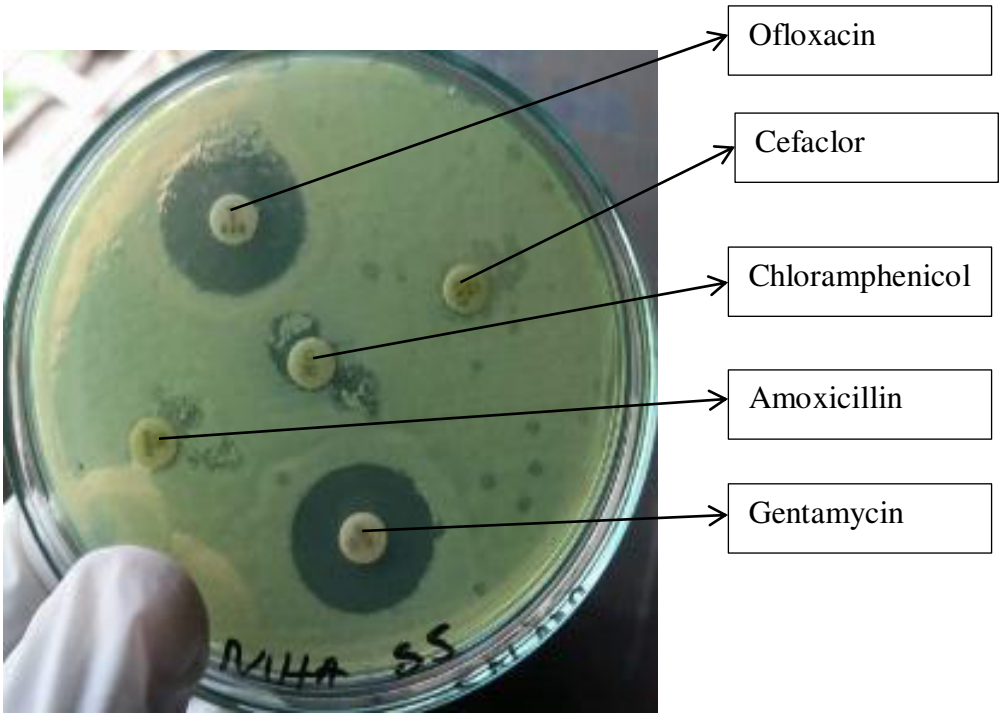

Plate 41: Antibiotic sensitivity test result of *Salmonella spp.* on Muller Hinton Agar

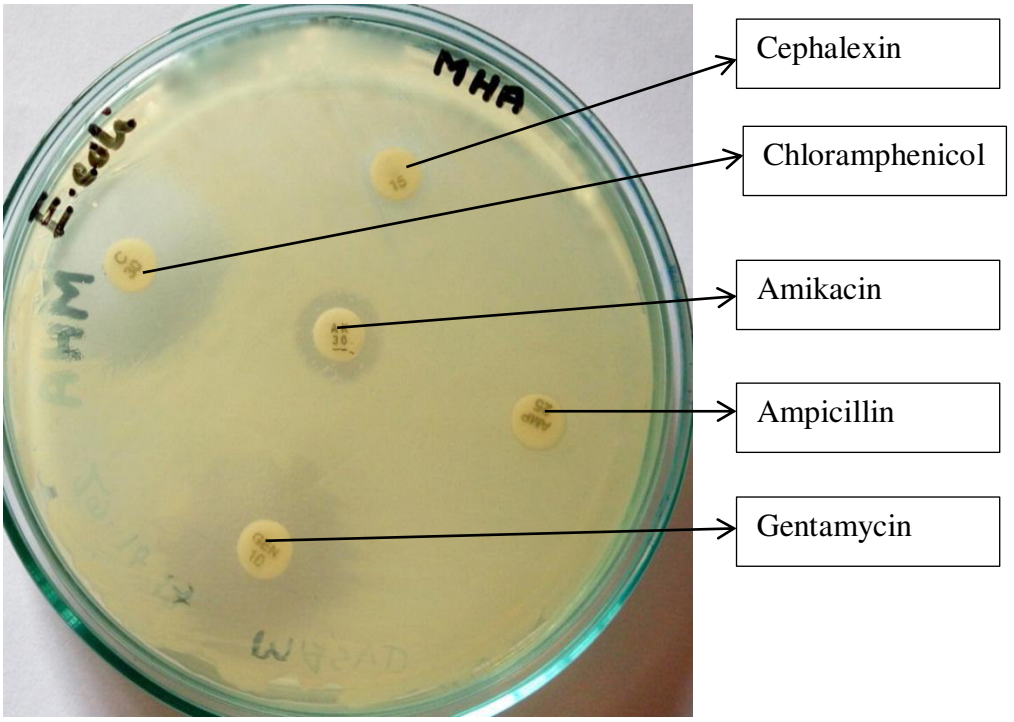

Plate 42: Antibiotic sensitivity test result of *Escherichia coli spp.* on Muller Hinton Agar

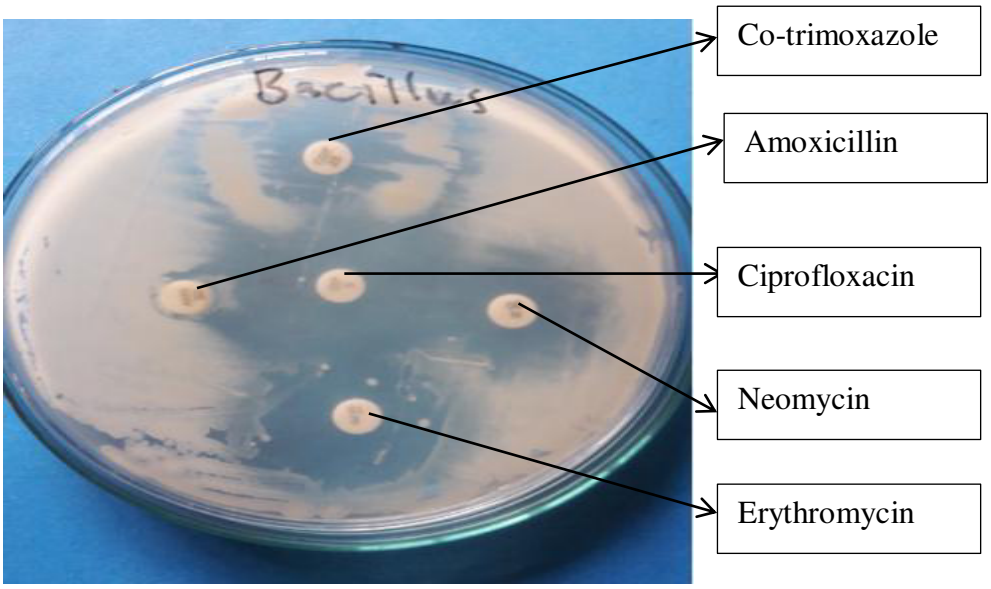

Plate 43: Antibiotic sensitivity test result of *Bacillus spp.* on Muller Hinton Agar

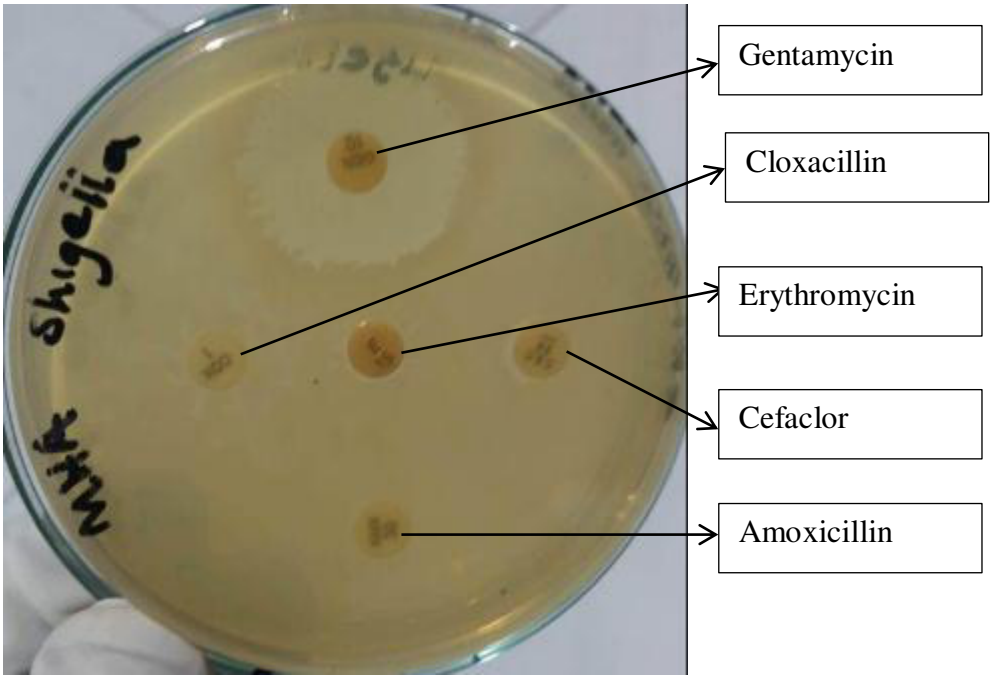

Plate 44: Antibiotic sensitivity test result of *Shigella spp.* on Muller Hinton Agar

CHAPTER 5

DISCUSSION

CHAPTER 5

DISCUSSION

Public transportation vehicles facilitate the spread of various pathogens that can cause frequent infections in the community. These vehicles are vectors of colds, flu, and bronchitis in winter months. Many public transport vehicles carry passengers well above their capacity. This leads to the spread of disease among people using these vehicles (Birteksoz and Erdogdu, 2017). According to the information provided by the İETT General Directorate, vehicles that serve millions of people every day have a detailed interior and exterior cleaning to provide a healthier environment for the passengers. It is stated that these routine cleanings are carried out every day before the vehicles are readied for morning service. It is also stated that the vehicles are subjected to detailed disinfection treatment once a week, thus making the vehicles safe from microbiological contamination (İETT 2015). Present research work was conducted to measure TVC and common bacteria in different locations of the bus (Hand rail, Armrest and Vinyl seat) taken at different places in Dinajpur. This study was carried out to investigate the microbiological quality of bacterial contamination on hand-touch surfaces in the public transport system and buses of HSTU, Dinajpur, Bangladesh. For this study, a total number of 45 of samples were collected from 15 buses in different places of Dinajpur. A total of 60 bacterial isolates belong to seven genera (*staphylococcus spp., Bacillus, spp., Escherichia coli, Klebsiella spp, Salmonella spp, Pseudomonas spp and Shigella spp*.) were identified. The prevalence of *Escherichia coli* were 30%, *staphylococcus spp. were* 25%, *Salmonella spp*. were 15% *Klebsiella spp*. were 10%, *Shigella spp* were 10%, *Pseudomonas spp.* were 5%, and *Bacillus spp.* were 5%.

These findings were nearly similar to the findings made by Tasneem *et al.* (2016), in which they reported the enteric bacteria isolates 33 were identified as *Escherichia coli, 17 Salmonella spp. 12 Staphylococcus spp*. and 11 *Shigella spp.* in buses samples were respectively.

Bilgehan, 1992; Gurler, 2008; Winston and Chambers, 2009 also identified the presence of *Staphylococcus spp* in the buses is of great importance for both hospital-acquired and community acquired infections.

The in vitro antibiotic sensitivity test of isolated bacteria with 19 different antibiotics such as Gentamycin, Ciprofloxacin, Chloramphenicol, Streptomycin, Cephalexin, Penicillin, Norphalaxin, Co-trimoxazole, Kanamycin, Neomycin, Erythromycin, Amoxicillin, Vancomycin, Azithromycin, Levofloxacin, Amikacin, Cloxacillin, Ofloxacin, Cefaclor, and Ampicillin were used. Antimicrobial sensitivity test was performed according to the procedure Kirby-Bauer disk diffusion susceptibility test protocol suggested by Janet Hudzicki (2009). Antibiotic sensitivity test showed that *Staphylococcus spp, Salmonella spp* and *Bacillus spp* and *Shigella spp* were resistant to Amoxicillin. *Shigella spp* and *Pseudomonas spp* were resistant to Erythromycin and Cloxacillin. *Salmonella spp* were resistant to Chloramphenicol. *Klebsiella spp, E. coli* and *Pseudomonas spp* were resistant to Cephalexin. *Salmonella spp* and *Shigella spp* were resistant to Cefaclor. *Staphylococcus spp* were resistant to Streptomycin. *Klebsiella spp* were resistant to Penicillin and Levofloxacin. *E. coli* was resistant to Amikacin and Ampicillin. *Salmonella spp* was resistant to Chloramphenicol.

Frequent skin contact due to popularity and over-crowdedness of the buses, absence of routine cleaning of public buses, poor public sanitation practice and lack of consciousness among passengers can be attributed for the abundance of bacteria in the sampling locations. Hence, it is recommended that a washing hands with suitable disinfectants after travelling in public buses to avoid chance of transmission of these pathogens. For this reason it is very important to make inspection regularly of these vehicles to ensure hygienic public transport.

CHAPTER 6

CONCLUSION

CHAPTER 6

CONCLUSION

The present study was conducted for the investigation on the microbiological parameters on various parts of the bus that show the hand-touch surfaces in the public buses are frequently contaminated with bacteria and can harbour different organisms. In the context of this study, it may be concluded that *Staphylococcus spp*, *Bacillus spp*, *E. coli spp*, *Klebsiella spp*, *Pseudomonas spp*, *Salmonella spp* and *Shigella spp* were the major etiological agents isolated from the samples of the buses. Maintaining good hygienic conditions require attention from researchers, governments, and owners of the buses as well as increased personal hygienic practices from users to prevent possible health hazards.

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APPENDICES

APPENDICES

APPENDIX-1

Composition of the media used:

Nutrient Agar	**Grams/Liter**
Peptone	5.0
Bacto beef extract	3.0
NaCl	5.0
Agar	15.0
Distilled water	1000 ml
P^H	7.2

Sterilized at 121^0C under 151b/in^2 pressure for 15 minutes.

Nutrient Broth	**Grams/Liter**
Peptone	5.0
NaCl	5.0
Beef Extract	1.5
Yeast Extract	1.5
P^H	7.4
Distilled water	1000 ml

Sterilized at 121^0C under 151b/in^2 pressure for 15 minutes.

MacConkey agar	**Grams/Liter**
Bacto Peptone	17.0
Proteas Peptone	3.0
Lactose	10.0
Bile Salt	1.5

Agar	15.0
Neutral red	0.03
Crystal violet	0.001
Distilled water	1000 ml
P^H	7.1

Sterilized at 121^0C under 151b/in^2 pressure for 15 minutes.

Eosin methylene blue (EMB) agar	**Gram/Liter**
Peptone	10.0
Lactose	10.0
K_2HpO_4	2.0
Eosin	0.4
Methylene blue	0.065
Agar	20.0
Distilled water	1000 ml
P^H	6.8

Sterilized at 121^0C under 151b/in^2 pressure for 15 minutes.

Blood Agar	**Gram/Liter**
Blood Agar	60 gm.
Distilled Water	1000 ml
Bovine blood	5.00 ml
Or Nutrient agar	500 ml
Sterile Defibrinated blood	25 ml

Sterilized at 121^0C under 151b/in^2 pressure for 15 minutes.

Mueller Hinton Agar	**Gram/Liter**
Beef infusion	2.0
Bacto casamino acid (technical)	17.5
Starch	1.5
Bacto agar	17.5
Distilled water	1000 ml
P^H	7.3

Sterilized at 121^0C under 151b/in^2 pressure for 15 minutes.

Mannitol Salt Agar	**Gram/Liter**
Proteas peptone	10.0
Beef extract	1.0
D-Mannitol	10.0
NaCl	75.0
Phenol red	0.025
Agar	20
Distilled water	1000ml

Sterilized at 121^0C under 151b/in^2 pressure for 15 minutes.

***Salmonella Shigella* Agar**	**Gram/Liter**
Lactose	10.0
Bile salt No.3	8.5
Sodium citrate	8.5
Sodium Thiosulfate	8.5
Beef extract	5.0
Proteas peptone	5.0
Ferric citrate	1.0
Brilliant Green	0.33

Neutral Red	0.025
Agar	13.5
Distilled water	1000ml

Sterilized at 121°C under 15 lb/in^2 pressure for 15 minutes

Normal Saline	**Gram/Liter**
NaCl	0.85
Distilled water	1000ml

Autoclaved at 121^{0}C for 15 minutes.

APPENDIX-II

Composition of the media used in biochemical test

MR-VP broth	**Gram/Liter**
Peptone	7.0
Dextrose	5.0
Di-potassium phosphate	5.0
Distilled water	1000 ml
P^H	6.9

Sterilized at 121^0C under 151b/in^2 pressure for 15 minutes.

Triple Sugar Iron (TSI) Agar	**Gram /Liter**
Peptone	10.0
Tryptone	10.0
Yeast Extract	3.0
Lactose	10.0
Saccharose	10.0
Dextrose	1.0
Ferrous Sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Phenol Red	0.024
Agar	12.0
P^H	7.4

Sterilized at 121^0C under 151b/in^2 pressure for 15 minutes.

Simmons citrate Agar	**Gram/Liter**
Magnesium Sulphate	0.2
Manoammonium phosphate	1.0

Di-potassium phosphate	1.0
Sodium citrate	2.0
Sodium Chloride	5.0
Agar	15.0
Brom-Thymol Blue	0.08
P^H	6.8

Sterilized at 121^0C under 151b/in^2 pressure for 15 minutes.

Urea broth medium	**Gram/Liter**
Urea	20.0
Yeast extract	0.1
KH_2PO_4	9.0
K_2HPO_4	9.5
Phenol red	0.01
Distilled water	1000 ml
P^H	6.8

Sterilized at 121^0C under 151b/in^2 pressure for 15 minutes.

Indole tryptopon broth medium	**Gram/Liter**
Tryptone	10.0
Distilled water	1000 ml

Sterilized at 121^0C under 151b/in^2 pressure for 15 minutes.

Nitrate broth	**Gram/Liter**
Peptone	5.0
Beef extract	3.0
NaCl	5.0
Potassium nitrate	1.0
Agar	1.0
Distilled water	1000 ml
P^H	7.2

Sterilized at 121^0C under 151b/in^2 pressure for 15 minutes.

APPENDIX-III

Composition of chemicals and reagents

Crystal violet

Solution-A

Crystal violet (90% dye content)	2.0 g
Ethyl alcohol (95%)	20.0 ml

Solution-B

Ammonium oxalate	0.8
Distilled water	80.0 ml

Note-Mix the solution A and B

Gram's iodine

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300.0 ml
Ethyl alcohol (95%)	
Ethyl alcohol (100%)	95.5 ml
Distilled water	5.0 ml

Safranin

Safranin O	0.25 ml
Ethyl alcohol (95%)	10.0 ml
Distilled water	100. 0 ml

Kovac's reagent (for detection of Indole)

P-Dimethylaminobenzaldehyde	5.0 g
Amyl alcohol	75.0 ml
Hydrochloric acid (concentrated)	25.0 ml

Concentrated P-Dimethylaminobenzaldehyde was dissolved in the amyl alcohol and HCl was added slowly.

Methyl red solution

Methyl red	0.04 g
Ethanol	40.0 g
Distilled water	100 .0 ml

Methyl red dissolved in ethanol and diluted water.

Barrit's reagent

Solution-A

α- naptho	l5.0 g
Ethanol (Absolut)	95.0 g

α- naptho was dissolved in ethanol with constant stirring.

Solution-B

KOH	40.0 g
Creatine	0.3 g
Distilled water	100.0 ml

Hydrogen peroxide

3% aqueous solution of H_2O_2 was prepared from the H_2O_2 absolute solution.