Characterization and Antibiotic Resistance Profile of Urinary Tract Infectious Bacteria Isolated from Urine Samples of Pregnant Women

A THESIS

BY

MD. AOULAD HOSEN

REGISTRATION NO. 1605457

SEMESTER: JULY-DECEMBER, 2017

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MASTER OF SCIENCE (MS)
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DECEMBER, 2017


Dedicated
To
My Beloved Parents

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ABSTRACT

Urinary tract infection is a commonly encountered disease during pregnancy that can result in critical and costly complications for both mother and fetus which urge the need for timely detection and prevention. The study aimed to determine the frequency of uropathogens and their antibiotic susceptibility pattern of urine sample from pregnant women in different hospital at Dinajpur district. The urine samples were randomly collected from 100 patients of different Hospitals and Diagnostic Center in Dinajpur district. A total of 75 bacteria isolated from urine samples of 33 patients and identified by conventional methods. 80% of total isolated organisms were found to be gram negative while remaining 20% were gram positive. Among gram negative *E. coli* was the most frequent bacteria. The percentages of gram negative isolates were *E. coli* (40%) followed by *Klebsiella spp* (21.33%), *Proteus spp* (12%) and *Pseudomonas spp* (6.67%). The percentage of gram positive isolates includes, *Staphylococcus spp* (16%) and *Streptococcus spp* (4%). Molecular techniques used for the characterization of *E. coli* include; Pulsed-field gel electrophoresis (PFGE), phylogenetic typing, amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), comparative genomic hybridization, single-nucleotide polymorphisms (SNPs**),** optical mapping, and whole genome sequencing (Sabat *et al*., 2013). The antibiotic resistances of identified organisms were carried out by disc-diffusion method with commercially available 20 discs of antibiotics having different mode of actions such as cell wall synthesis inhibitors, membrane permeability alternatives, protein synthesis inhibitors and DNA synthesis inhibitors. Gram negative showed more resistance to these antibiotics as compared to gram positive organisms. The sensitivity rates of isolated gram negative bacteria were sensitive to Ciprofloxacin (100%), Chloramphenicol (100%), and Levofloxacin (100%), Gentamycin (80%), Neomycin and Colistin (70%), Amikacin (40%), Kanamycin (20%). The isolates were resistant to Penicillin (100%), Amoxicillin (100%) and Ampicillin (100%), Vankomycin (90%), Cephalexin (80%). The gram positive isolates were highly sensitive to Levofloxacin (100%), Ofloxacin (100%), Chloramphenicol (100%) and Gentamycin (100%), Novobiocin, Kanamycin and Tobramycin (80%). The isolates were resistant to Cephalexin, Cloxacillin, Amoxicillin and Ampicillin (100%), Colistin and Norofloxacin (60%).This study showed that the frequency of *E. coli* increases the probability of urinary tract infection. It is now very necessary to develop new antimicrobials and therapeutic agents having high effectiveness with no side effects, easy availability and less expensive.

CONTENT

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LIST OF ABBREVIATIONS AND SYMBOLS

-	:	Negative
#	:	Identifying number
%	:	Percentage
@	:	At the rate of
+	:	Positive
µg	:	Microgram
µl	:	Microlitre
^{0}C	:	Degree of celcius
Ag	:	Antigen
Assist	:	Assistant
BA	:	Blood Agar
BD	:	Bangladesh
BGA	:	Brilliant Green Agar
EMB	:	Eosin Methylene Blue
ER	:	Erythromycin
et al.	:	Associated
etc	:	Etcetera
FAO	:	Food and Agricultural Organization
Gm	:	Gram
H.S	:	Haemorrhagic septicemia
H_2O_2	:	Hydrogen peroxide
H_2S	:	Hydrogen sulphide
HSTU	:	Hajee Mohammad Danesh Science and Technology University
i.e.	:	That is
Ltd	:	Limited
M.S	:	Master of Science
MC	:	Mac-Conkey Agar
MSA	:	Mannitol Salt Agar

LIST OF ABBREVIATIONS AND SYMBOLS (Contd.)

MI	:	Milliliter
MIU	:	Motility Indole Urease
MR	:	Methyl Red
NA	:	Nutrient Agar
NB	:	Nutrient Broth
No.	:	Number
PBS	:	Phosphate Buffer Saline
Prof.	:	Professor
PSS	:	Physiological Saline Solution
RPM	:	Rotation Per Minute
SC	:	Subcutaneous
SE	:	Standard Error
SL	:	Serial number
spp	:	Species
SSA	:	Salmonella- Shigella Agar
v/v	:	Volume by volume
vp	:	Voges-Proskauer
w/v	:	Weight by volume
UTI	:	Urinary Tract Infection
PCR	:	Polymerase Chain Reaction
kb	:	killo base
bp	:	base pair
fig	:	Figure

CHAPTER-1

INTRODUCTION

Urinary tract infection (UTI) is an infection in any part of urinary system including kidneys, ureters, bladder and urethra. UTIs are mainly caused by the presence and growth of microorganisms in the urinary tract, which are the single commonest bacterial infections of all age groups and especially in pregnancy. It may involve the lower urinary tract or the bladder (Erica *et al*., 2013); After anemia, UTIs are the second common complications in pregnant women, which if untreated can adversely affect the health of infant or the pregnant mother (Laura *et al.*, 1994). UTI is a common health problem among pregnant women (Mittal and Wing, 2005; Saidi *et al*., 2005). Pregnancy causes numerous hormonal and mechanical changes in the body (Schnarr and Smaill, 2008). This usually begins in week 6 and peaks during weeks 22 to 24 of pregnancy due to a number of factors including uretheral dilatation, increased bladder volume and decreased bladder tone, along with decreased uretheral tone which contributes to increased urinary stasis and ureterovesical reflux (Chaliha *et al.*, 2002). Up to 70% of pregnant women develop glycosuria, which encourages bacterial growth in the urine (Al-Issa, 2009). These changes along with already short urethra and difficulty with hygiene due to the distended pregnant belly increase the frequency of UTI in pregnant women. UTIs are bacterial infections with a global annual incidence of approximately 150 million cases. The estimated economic burden is more than 6 billion U.S. dollars (Patton, 1991). About 40% of women and 12% of men experience at least one symptomatic UTI during their lifetime, and as many as 40% of affected women show recurrent UTI (Foxman, 2010). The prevalence of asymptomatic UTI has been previously reported to be 2% to 13% in pregnant women (Delzell *et al.,* 2000; Christensen, 2000; Kutlay *et al*., 2003; McIsaac, 2005; Masinde *et al*., 2009) compared with that of symptomatic UTI which occurs in 1–18% during pregnancy (Dwyer and Reilly, 2002; Masinde *et al*., 2009). Incidence of UTI is higher in women than men, 40% to 50% of whom will suffer at least one clinical episode during their lifetime (Leigh *et al.*, 1990). The increase risk factor for UTI in women may be due to short urethra, absence of prostatic secretions, pregnancy and easy contamination of urinary tract with faecal flora (Awaness *et al.*, 2000). Approximately 90% of pregnant women develop urethral dilation, which will persist until delivery (Delzell and Lefevre, 2000) and it may contribute to increased urinary stasis and ureterovesical reflux. Additionally, the physiological increase in plasma volume during pregnancy decreases urine concentration and up to 70% of pregnant women develop glycosuria, which is considered to encourage bacterial

growth in the urine (Patterson and Andriole, 1987). The pathogenesis of UTIs involves complex interaction between an organism, the environment and the potential host. The symptoms of a person with urinary tract infections depend on the age and location. The signs and symptoms associated with UTI are pain or burning (discomfort) when urinating, frequent urination, feeling of urgency, blood or mucus in the urine, cramps or pain in the lower abdomen, pain during sexual intercourse, pain, pressure or tenderness in the area of the bladder. When bacteria spread to the kidneys patient may experience: back pain, chills, fever, nausea and vomiting. (Franklin and Monif, 2000). The increased risk of having UTI during pregnancy is mainly due to past history of UTIs and other risk factors includes- lower socio economic status, individual hygiene, chronic urinary retention, sickle cell trait and anemia, increased parity or age, number of child births, number of inter-courses per week, and lack of prenatal care. (Smaill *et al*., 2007). The functional urinary tract abnormalities and diabetes mellitus can also increase susceptibility to UTIs during pregnancy.(Valentina *et al*., *2016)* . Other risk factors include, previous urinary tract infections history, young age, neuromuscular dysfunction bladder, structural disorders of urinary tract, renal stones, and catheterization (Mundy, 2010). Using a diaphragm also causes UTI as it pushes against the urethra and makes the urethra unable to empty the bladder completely and the small concentration of urine left in the bladder leads to the growth of bacteria which ultimately causes UTI (Okonko *et al*., 2009). These changes alongside with an already short urethra (3-4cm in females) increase the frequency of urinary tract infection during pregnancy. (Rane and Dasgupta, 2013). More than 95% of urinary tract infections are caused by a single bacterial species. *E. coli* is the most frequent infecting organism in acute infection (Ronald, 2002). *Enterobacter, Staphylococci, Klebsiella, Proteus, Pseudomonas,* and *Enterococci species* are more often isolated from inpatients, whereas there is a greater preponderance of *E. coli* in an outpatient population. (Bronsema *et al*., 1993)

The most common organism implicated in UTIs (80–85%) is *E. coli*, (Nicolle, 2008) while *Staphylococcus saprophyticus* is the cause in 5–10%. It is notable that, in women, the colonization of the vaginal and periurethral mucous can precede UTI, the infection can ascend, causing cystitis and, if not treated, pyelonephritis, kidney damage, high blood pressure and results in death.. Sepsis is now the most common cause of direct maternal death (Cantwell *et al*., 2011) with 1.1 maternal deaths for 100 000 pregnancies in the UK between 2006 and 2008. The incidence of bacteraemia during pregnancy varies from 3 to 7.5 for 1000 pregnancies. (Blanco *et al.*, 1981). Bacteraemia during pregnancy is also life-

threatening for fetuses, with 10% of foetal death in a retrospective study (Surgers *et al.*, 2013). The virulence factors and clinical picture presented by Uropathogenic *E.coli* infections indicate that these pathogens are extra-intestinal pathogenic *E. coli* (*ExPEC*) strains (Johnson and Russo, 2005). There is a tendency of recurrence of UTIs in about 25-30% of women after the initial infection due to either re-infection or recrudescence (Bower *et al*., 2005) *E. coli* can be broadly classified into three groups: commensal *E. coli* which constitute the normal floral of the intestine; intestinal pathogenic *E. coli* which 146 causes various infections in the intestine and *ExPEC* which elicits infections in various parts of the body excluding the intestine (Diard *et al*., 2010).

Extra-intestinal uropathogenic *E. coli* strains are defined as *E. coli* with enhanced ability to cause infections outside the intestinal tract, such as in the bloodstream, cerebrospinal fluid or urinary tract of the host (Diard *et al*., 2010). Traditional typing of *E. coli* is based on phenotypes, serotype, biotype, phage-typing or antibiotype. Molecular techniques used for the characterization of *E. coli* include; Pulsed-field gel electrophoresis (PFGE) which is considered a gold standard among molecular typing methods for a variety of clinically important bacteria, other molecular methods include: phylogenetic typing, amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), Variable-Number Tandem Repeat (VNTR) typing, Multi-locus sequence typing (MLST), comparative genomic hybridization, single-nucleotide polymorphisms (SNPs**),** optical mapping, and whole genome sequencing (Sabat *et al*., 2013). Urinary tract infections can usually be easily treated with a short course of antibiotics with no significant difference between the classes of antibiotics commonly used (Zalmanovici *et al*., 2010).

Increasing multidrug resistance in bacterial uropathogens is an important and emerging public health problem. Increasing drug resistance in UTI needs regular monitoring of the antibiotic susceptibility of uropathogens in a particular area. Various factors such as the type of UTI (complicated or uncomplicated), gender, age, and previous history of antibiotic therapy of each UTI patient should also be considered to find out the correct global data on susceptibility (Alos, 2005). The distribution of antimicrobial susceptibility data of UTI-causing microorganisms changes from time to time and from place to place (Okonko *et al*., 2009). Urinary tract infected patients use several types of antibiotic without microbiological test in laboratories and bacteria are resistance to that antibiotics because the antibiotics are not examined and the patients can not remove UTI. Bacterial identification and susceptibility tests are important for selecting the appropriate antimicrobial agent affecting bacterial

diseases (Gentilini *et al*., 2002). Thus, the accurate diagnosis of UTI and determination of antibiotic sensitivity pattern is important for selection of appropriate drug for effective treatment. Antibiotics are the main treatment for all UTIs. A prolonged course (six months to a year) of low-dose antibiotics (usually nitrofurantoin or TMP/SMX) is effective in reducing the frequency of UTIs in those with recurrent UTIs (Nicolle, 2008). Cranberry (juice or capsules) may decrease the incidence of UTI in those with frequent infections. Long-term tolerance, however, is an issue (Brown *et al*., 2011) Subsequent research has questioned these findings (Raz *et al*., 1993). For post-menopausal women intravaginal application of topical estrogen cream can prevent recurrent cystitis. Breastfeeding can reduce the risk of UTIs in infants (Warren, *et al*., 1999).

With a great consideration given to the above facts in view, the aims of the present study were

- Microbial assessment of urine samples of pregnant women by total viable count method.
- Isolation and identification of bacteria cause UTI in pregnant women.
- Molecular characterization of highly pathogenic bacteria.
- To determine antibiotic sensitivity patterns of isolated bacteria.

CHAPTER -2

REVIEW OF LITERATURE

The objective of this chapter is to collect selective reviews of the research works accomplished in relation to the present study. Literatures on Urinary Tract Infection related to this study are reviewed:

Nora *et al.*, (2017) studied to determine the prevalence and risk factors of urinary tract infection among pregnant women in Ismailia city, Egypt. Descriptive cross-sectional design was utilized by them for their experiment. They collected urine sample from 330 pregnant women in Ismailia city and the sample was cultured on media. They found 29% of the studied women had urinary tract infection and factors associated with UTI during pregnancy were previous UTI history; maternal anemia; unhealthy urination habits; increase of sexual intercourse frequency; poor hygienic practices; child spacing less than two years; inadequate socioeconomic conditions; suboptimal nutritional habits; constipation; and family history. They suggested to Providing a health educational program about preventive measures of UTI for pregnant women and periodical screening to assure timely detection and proper treatment of infected pregnant women.

Shashi *et al.*, (2017) experimented the Urinary Tract Infection among Pregnant Women at a Secondary Level Hospital in Northern India. Their main objective was to estimated the proportion of pregnant women with UTI among antenatal clinic attendees in rural Haryana because their w no regular screening for UTI, and facility for diagnosis of UTI is not available at peripheral government health centers. They collected urine sample from pregnant women attending antenatal clinic of secondary care center of rural Haryana from March to May 2015 and 1253 pregnant women were included in the study. The proportion of women with symptoms of UTI on the basis of history was 33.3% (95% confidence interval [CI] - 30.7, 35.9), and UTI by colony count was 3.3% (95% CI - 2.4, 4.5). The presence of UTI was found to be significantly associated with the presence of any symptom of UTI on multivariate analysis (odds ratio [95% CI] - 7.35 [1.95, 27.77] The study suggested that considering the burden of UTI and its complications, diagnosis of UTI at a resource constrained setting like a secondary care hospital can be done after screening women for symptom suggestive of UTI.

Amit *et al.,* (2017) **reported that** the Prevalence of UTI among pregnant women and complications in their newborns. Their observational study was carried out over a period of 6 months and total of 120 pregnant women were enrolled . Out of 120 pregnant women, they confirmed 35% of them had urinary tract infection. It was mostly observed high in age group of <25yrs, Primigravida, winter season and during third trimester of pregnancy. The commonest causative organism was found to be *E.coli* (50%). They observed the prevalence rate of urinary tract infection (UTI) during pregnancy was high. They suggested to routine screening of all pregnant women for significant bacteriuria to reduced the complications on both maternal and fetal health.

Natasha *et al.*, (2017) investigated that the bacteriological profile of urinary tract infection in pregnant women at a tertiary health care center. This was a cross sectional study carried out 280 urine samples from suspected UTI in pregnant women during March 2013 to March 2014. They found 56 positive results and the percentage of isolates was *E. coli* (39.29%), *Staphylococcus aureus* (19.64%), coagulase negative *Staphylococuss* (16.07%), *Klebsiella sp. (*12.50%), *Enterococcus sp.* (8.93%) and *Acinetobacter sp.* (3.57%). They observed that *E. coli* was sensitive to Nitofurantoin (77.27%), *Klebsiella* to Norfloxacin (71.43%) and Acinetobacter was 100% sensitive to Amoxiclav, Amikacin, Tetracyclines, Norfloxacin and gram positive *Staphylococcus aureus* was mostly sensitive to Contrimoxazole and Erythromycin. *E. coli* and *Staphylococcus aureus* was the most responsible pathogen for UTI that found on their study.

Ayogu *et al*., (2017) designed to determine the antibiotic resistance patterns of *Escherichia coli* and *Staphylococcus aureus* isolated from UTI Positive pregnant women from Ezza South L.G.A of Ebonyi State and to determine the location of the resistant genes through plasmid curing. They collected 106 urine samples from pregnant women and isolated *Escherichia coli* and *Staphylococcus aureus.* They observed that pregnant women in the age range of 23–28 had the highest (54.4%) cases of UTI while pregnant women in the age range of 17–22 had the least (34.4%) cases of UTI. Pregnant women in their first trimester have the highest prevalence of *S. aureus* 14 (82.3%) while pregnant women in their third trimester had the highest prevalence of *E. coli* 11 (73.3%). They reported the most effective antibiotics against *E. coli* were gentamicin, imipenem, meropenem, ciprofloxacin, ofloxacin and ertapenem and *Staphylococcus aureus* was susceptible to ciprofloxacin, gentamicin, erythromycin, ceftazidime and ofloxacin. The result obtained from multi antibiotics resistant index (MARI) ranged from 0.69 and 0.41 for *Staphylococcus aureus* and *Escherichia coli.*

Ifeanyichukwu *et al.*, (2017) stated that 41 (34.2%) *E. coli* was isolated from the urine samples. The isolated *E. coli* was resistant to amoxicillin, sulphamethoxazole-trimethoprim, cefazidime, amoxicillin-clavulanic acid, cefriaxone, ofloxacin, nitrofurantoin, aztreonam and nalidixic acid; and they were found to be multiply resistant to the tested antibiotics.

Mahmood *et al.*, (2016) investigated that the antibiotic susceptibility pattern of different bacteria that was isolated from urinary tract infection to different antibiotics. 83 uropathogen bacteria were isolated from 300 urine samples taken from patients who were admitted to Tikrit Teaching Hospital from March, 2011 through February, 2012. The bacterial pathogens were isolated, identified and purified by morphological, microscopic, and biochemical characteristics. The antibiotic sensitivity test was done by using some antibiotics on disk diffusion method. On that study they were observed a clear zone of inhibition that indicated the bacteria were sensitive to that organisms. 55% *Escherichia coli* was isolated on that study. On their study majority of the isolated bacteria were pathogenic. The study also determined that Ampicillin, Rifampicin, and Erythromycin showed no effect on all 83 isolates.

Amany *et al.*, (2016) studied to detection of bacterial pathogens causing urinary tract infection and study their susceptibility to antibiotics at asuqalshukh hospital in the province of dhi –qar. They found highest number of *Esherichia coli* which was isolated from 14 patients with percentage of 31.1% . E.coli was the most prevalent followed by *Proteus mirabilis* 22.2%(10), *Pseudomonas aeruginosa* 15.5%(7), *Staphylococcus aureus* 11.1%(5), *Klebsiella pneumonia* 13.3%(4), *Staphylococcus saprophyticus* 4.4%(2) and *Serratia marcescenes* 2.2%(1). They used 8 antibiotics for antibiotics susceptibility test here, Amikacin was more antibiotic that effect on all kinds of isolate, the sensitivity of isolates to this antibiotic was registered (95.7%) followed by Ciprofloxacin (80.7%), while the highest resistant of all isolates was to Amoxicillin, and was registered (9.2%) .

Shadi *et al.*, (2016) **reported that the** Detection of Urinary Tract Infection (UTI) and Asymptomatic Bacteriuria using Urinalysis Parameters, from pregnant women. The main objective of their study was to detecting UTIs and Asymptomatic Bacteriuria during pregnancy. Their study demonstrated that the urine dipstick test alone seems to be useful in to exclude the presence of an infection if the results of both nitrites and leukocyte-esterase are negative. Different studies reported broad ranges of sensitivities and specificities for urinalysis parameters. Combination of nitrite and leukocyte esterase when both parameters

are positive, seem to have the highest specificity in detecting bacteriuria in asymptomatic pregnant women. They used gold standard method for diagnosis of urinary tract infection and asymptomatic bacteruria.

Hanna and Marcin, (2016) investigated that urinary tract infections during pregnancy. They observed urinary tract infections (UTIs) are the most common type of infection during pregnancy, affecting up to 10% of pregnant women. They were also recognized as the second most common ailment of pregnancy, after anemia. Three clinical types of pregnancy-related UTI are distinguished: asymptomatic bacteriuria (ASB), cystitis, and pyelonephritis. They identified a particular form of ASB is the presence of Group B streptococci in the urinary tract of the pregnant woman and also confirmed UTI may lead to serious maternal and fetal complications.

Mohammed and Taslima, (2016) conducted that the Prevalence of Urinary Tract Infection among Pregnant Women at Ibrahim Iqbal Memorial Hospital, Chandanaish, Bangladesh. This cross-sectional study was carried out from 247 pregnant women and 78 (31.5%) were symptomatic and 169 (68.4%) asymptomatic. The prevalence of bacteriuria among symptomatic and asymptomatic pregnant women were 17.9% and 13.0%. The sensitivity and specificity results of urine dipstick was 38.9% and 86.7% . They found *Escherichia coli* (47.2%) and *Enterococcus spp* (22.2%) . They observed that the rate of resistance of Escherichia coli to Cefuroxime, nitrofurantoin, ceftriaxone, and imipenem were 64.7%, 5.9%, 29.4% and 0%. They suggested to all pregnant women for routine urine culture and susceptibility test.

Bukola *et al.*, (2016) assessed to determine the prevalence of urinary tract infections among pregnant women receiving antenatal care in two primary health care centres in Karu Nasarawa State. The incidence of UTI was 62.67% and 94 bacterial isolates were identified based on colonial morphology, microscopic characteristics, and biochemical tests. *Escherichia coli* (22.97%), *Klebsiella spp* (18.08%), *Staphylococcus spp* (15.95%), *Proteus spp* (13.82%), coagulase negative *Staphylococcu*s (10.63%) and *Enterococcus spp* (8.51%) were isolated. The prevalence of urinary tract infection from this study was significant value, and they suggested the screening of routine urine culture of pregnant women.

Ordaz *et al.*, (2016) determined the most frequent pathogen and its antibiotic resistance in pregnant patients with urinary tract infection in Chihuahua, Mexico. 101 patients were UTI positive and found *Escherichia coli* 82 (81.2%), *Klebsiella* 13 (12.8%), *Proteus* 5 (4.9%), *Staphylococcus* coagulase negative 1 (0.9%). The most widely used drug was Ampicillin (79%) as well as the one with the highest rate resistance (100%). They recommended Nitrofurantoin and amoxicillin antibiotic for the treatment of UTI during pregnancy.

Lessandra *et al.*, (2016) analyzed Urinary Tract Infection in Pregnancy. They observed that Pregnant women are higher risk for UTI because of physiological adaptations, a relationship between adverse outcomes and maternal UTI. They found on their study acute cystitis was suspected in pregnant women who complain about dysuria, while most cases of pyelonephritis occur during the second and third trimesters, and complications include septic shock syndrome, anemia, bacteremia, respiratory insuffciency, and renal dysfunction. Their review summarized epidemiology, clinical features and clinical management of this important infection in pregnancy.

Rodrigo *et al.*, (2015) analyzed that the study was determined the in vitro susceptibility to fosfomycin of bacteria that were isolated from urine samples of pregnant women with urinary tract infection. The urine samples of pregnant women were collected from clinical laboratories in Tubarao, state of Santa Catarina, Brazil, between September 2012 and May 2013. 134 samples were collected from the study area and the antibiotics sensitivity test was performed by using Karby- Bauer method. *Escherichia coli* and *Staphylococcus aureus* were most commonly identified species and 89% of cases, the microorganisms were sensitive to fosfomycin. In the study area the urinary tract infection was caused by *Escherichia coli* and *Staphylococcus aureus* in pregnant women. They observed that these two species are mainly responsible for urinary tract infection in pregnant women.

Khan *et al.*, (2015) focused the main objectives of their study, In developing countries, bacteriuria is associated with significant maternal and foetal risks. Their study was included 1358 pregnant women who attended the antenatal clinic at the Department of Obstetrics and Gynecology between July 2013 and August 2014. They processed urine specimens for isolation and identification of bacterial species following standard microbiological methods. Three hundred and seventeen isolates were positive for significant bacteriuria. *E. coli, K. pneumonia, P. aeruginosa, E. faecalis, S. aureus, P. mirabilis, CoNS,* and *P. vulgaris* accounted for 61.5%, 17%, 7.5%, 5.3%, 2.8%, 2.5%, 1.8%, and 1.2% of bacterial isolates.

Resistance to erythromycin (58.6%), co-trimoxazole (57.4%) and ciprofloxacin (50.1%) was observed. Among the 317 positive isolates, 203(64%) were from women in the 21- 30 years old age group, and the rate of bacteriuria in this group was statistically significantly more than those for the other age groups ($P < 0.05$).They suggested that Erythromycin, co-trimoxazole and ciprofloxacin should not be empirically used as first-line drugs in the treatment of UTIs. Continuous local monitoring of resistance patterns is necessary to determined the appropriate empirical antimicrobial therapy.

Angoti *et al.*, (2015) studied to determine the prevalence of bacteria isolated from urinary tract infection (UTI) in patients and determination of the antibiotic susceptibility patterns of the gram negative bacteria. Their study was performed in Imam Reza hospital, Tabriz(north west of Iran) during March 2012 to February 2013. They surveyed 8153 patients, who had clinical manifestations of UTI. They used Disk diffusion susceptibility test for identification of bacterial pathogens. They found *E. coli* (55.38%) was the most common isolated pathogen, followed by *Enterobacter spp.* (29.61%), *Pseudomonas spp.* (4.9%), *S. aureus* (3.21%), *Enterococcus spp.* (2.3%), *fungi* (1.5%) and *Klebsiell*a (0.48%). They confirmed the sensitivity rates of isolated gram negative bacteria were for Amikacin (95.7%), Nitrofurantoin(91.5%), Gentamicin(64.1%), Ceftizoxim (56.8%), Ciprofloxacin (37.6%), Cotrimoxazole (31.4%)and Nalidixic acid(23.5%). They suggested to improved the effectiveness of integrated infection control programs to control and manage nosocomial infections caused by highly resistant organisms.

Abdullahi *et al.*, (2015) studied to Asymptomatic Bacteriuria among Pregnant Women Attending Antenatal: Evaluation of Screening Test. They observed the physiological changes of pregnant women during pregnancy and found sensitivity and specificity of urine culture. They performed prospective, cross sectional and hospital based study for their findings. The efficacy of microscopy method was evaluated as evidence by the presence of pus cells and positive Gram's stain (positive or negative Gram organism) and compared with gold standard culture, sensitivity (true positive) of 81.3%, a specificity of 94.5% was obtained. They confirmed Microscopy method has appreciable sensitivity and specificity, biochemical methods have low sensitivities but high specificities when compared to the gold standard.

Choi and Chang, (2015) established that Molecular Defense Mechanisms during Urinary Tract Infection. The complete mechanisms of urinary tract infection (UTI) are still unknown. They reported that the strategies of the uropathogenic *Escherichia coli* are adherence, motility, iron acquisition, toxin, and evasion of host immunity. Host immune responses play a significant part in defense of UTI. Various antimicrobial peptides (AMPs) including defensins, cathelicidin, hepcidin, ribonuclease 7, lactoferrin, lipocalin,Tamm-Horsfall protein, and secretory leukocyte proteinase inhibitor help to prevent UTI by modulation of innate and adaptive immunity. Toll-like receptors (TLRs) play an important role of microorganism identification in innate immunity. Stimulation of TLRs on the cell membrane by ligand of bacteria triggers production of inflammatory chemokines, cytokines, and AMPs. These mechanisms are an attempt to defend the urinary tract against UTI.

Surgers *et al.*, (2014) performed molecular characterization of strains for 29 E. coli bacteraemia occurring in pregnant women. Bacteraemia mostly occurred in the third trimester of pregnancy (45%) and was community-acquired (79%). Portals of entry were urinary (55%) and genital (45%). E. coli strains belonged mainly to phylogroups B2 (72%) and D (17%). Four clonal lineages (i.e. sequence type complex (STc) 73, STc95, STc12 and STc69) represented 65% of the strains. The strains exhibited a high number of virulence factor coding genes (10 (3–16)). Six foetuses died (27%), five of them due to bacteraemia of genital origin (83%). Foetal deaths occurred despite adequate antibiotic regimens. Strains associated with foetal mortality had fewer virulence factors (8 (6–10)) than strains involved in no foetal mortality (11 (4–12)) (p 0.02. These results show that E. coli bacteraemia in pregnant women involve few highly virulent clones but that severity, represented by foetal death, is mainly related to bacteraemia of genital origin.

Abiodun *et al.*, (2014) reported that the characterization of uropathogenic *E. coli* (UPEC) in urine samples of pregnant women with confirmed urinary tract infections (UTIs) in Ondo and Ekiti States, Nigeria. Phylogenetic typing of the isolates was by multiplex polymerase chain reaction (PCR). **The** prevalence of UTIs with positive cultures was 66.0%. *Escherichia coli* only was 56.5%, mixed-infection (9.5%), non-*E. coli* infection (12.5%) and no growth (21.5%). Presence of *chuA* gene in most of the isolates shows the significance of iron acquisition in the pathogenesis and urovirulence of UPEC.

Bekir *et al.*, (2014) aimed to investigate the bacterial profile and the adequacy of antimicrobial treatment in pregnant women with urinary tract infection. This retrospective observational study was conducted with 753 pregnant women who needed hospitalization because of UTI in each of the three trimesters. Midstream urine culture and antimicrobial susceptibility tests were evaluated. They isolated isolates of *E.Coli* (82.2%), followed by *Klebsiella spp.* (11.2%). In each of the three trimesters, E.Coli remained the most frequently isolated bacterium (86%, 82.2%, 79.5%, respectively), followed by *Klebsiella spp.* (9%, 11.6%, 12.2%). *Enterococcus spp.* were isolated as a third microbial agent, with 43 patients (5.7%) in the three trimesters. The bacteria were found to be highly sensitive to fosfomycin, with 98-99% sensitivity for *E.Coli* and 88-89% for *Klebsiella spp.* and for *Enterococcus spp.* 93-100% nitrofurantoin sensitivity for each of the three trimesters. They demonstrated that *E.Coli* and *Klebsiella spp.* are the most common bacterial agents isolated from urine culture of pregnant women with UTI in each of the three trimesters. They considered fosfomycin to be the most adequate first-line treatment regimen due to high sensitivity to the drug, ease of use and safety for use in pregnancy.

Hani *et al.*, (2013) aimed to study the prevalence of UTI among pregnant women in Makkah, KSA and to investigate the most frequent causative agents and drug resistance profiles associated with such infections. 20% of investigated pregnant women were positive for UTI (12% with symptomatic UTI and 8% were asymptomatic).The most common isolate was *Escherichia coli* (25%) from both symptomatic and asymptomatic bacteriuria. Amoxicillin, cefoxitin, celtaxidime, fusidic acid, norfloxacin, ofloxzcin and penicillin showed the least resistance frequency (2.6%). The highest rate of UTI infection (20%) was recorded among pregnant women with *E. coli*. They suggested antibiotics such as amoxicillin, cefoxitin, celtaxidime, norflaoacin, penicillin and fusidic acid for treatment of UTI.

Devanand *et al*., (2013) studied to Distribution and Antimicrobial Susceptibility Pattern of Bacterial Pathogens Causing Urinary Tract Infection in Urban Community of Meerut City, India. Their study was conducted todetermined the distribution and antimicrobial susceptibility of uropathogens in the Indian community as well as to determined the effect of gender and age on the etiology of bacterial uropathogens. They collected Clean catch midstream urine samples from 288 patients of the age ranging from 15 to ≥48 years and performed antimicrobial susceptibility on all isolated bacteria by Kirby Bauer's disc diffusion method. They used multiple antibiotic for susceptibility test and they observed UTI prevalence was 53.82% in patients and significantly higher in females (73.57%).

Dr. Rajshekhar *et al.*, (2013) investigated that the Prevalence of Asymptomatic Bacteriuria among Pregnant women in a tertiary care hospital. The main objective of their study was determined the prevalence of Asymptomatic bacteriuria (ASB) in pregnant women & to isolated, identified th causative organisms; and to test the antimicrobial susceptibility of isolated pathogens. Midstream urine samples from 300 pregnant women were collected and studied over a period of six months at I.P.G.M.E.R & S.S.K.M hospital, Kolkata, a tertiary health care centre. They found significant bacteriuria in 33 patients (11%) and 4% patients had insignificant bacteriuria from out of 300 patients and *E.Coli* was the most common etiological agent(72%), followed by *Staphylococcus aureus.* They suggested all pregnant women should be screened by urine culture to detected asymptomatic bacteriuria at their first visit to prevent over UTI & other complications in both mother & fetus.

Geoffrey *et al.*, (2013) research works was conducted to Isolation, Identification and Characterization of Urinary Tract Infectious Bacteria and the Effect of Different Antibiotics. Their study focused on the frequency of uropathogens and their antibiotic susceptibility in different gender in Madurai District. **They ovserved both gram positive and gram negative bacteria and** to ensure appropriate therapy, current knowledge of the organisms that cause UTI and their antibiotic was susceptibility was mandatory. *E. coli* was the predominant isolate isolated from the urine specimen followed by *Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, Proteus mirabilis* and *Enterococcus faecalis on their study.* Chloraphenicol and ciprofloxacin (100%) were found to be effective for empirical treatment of UTI and has covered the majority of urinary pathogens followed by tetracycline, gentamycin and kanamycin (83%), Ampicillin (67). Streptomycin, Rifampicin and amoxicillin were less effective (50%).

Poonam *et al.*, (2013) studied to determine Isolation and identification of bacteria causing urinary tract infections in pregnant women in vidarbha and their drug susceptibility patterns in them. Retrospective analysis was carried out for 280 mid-stream urine specimens processed for culture and antimicrobial drug susceptibility testing between July and October 2012. 280 urine specimens were collected from women contacting Hospitals for children and & Delivery in various cities of Vidarbha that suffering from urinary tract infection .Isolated and diagnosed bacteria from pregnant women were : *Escherichia coli* (43.47%), *Staphylococcus aureus* (23.91%), *Proteus vulgaris* (19.56%), *Klebsiella* species (5.43%), Coagulase negative *Staphylococci* (7.6%).The isolated uropathogens showed resistant to ampicillin, co-trimoxazole, ciprofloxacin, ceftazidime and sensitive to nitrofurantoin and

cephotaxime. *E coli* were found to be the common cause of UTI among the pregnant women on their study. They suggested all pregnant women to regular screening of urine culture.

Tamalli *et al.*, (2013) analyzed that the Urinary tract infection during pregnancy at Al-khoms, Libya. Their study was to determined the prevalence and identify the etiological agents associated with asymptomatic bacteriurea in antenatal mothers in two private hospitals (Gynecology and obstetrics department) at Alkhoms City, Libya. They identified the bacterial pathogens from pregnant women and performed antibiotic sensitivity test. They found the prevalence of bacteriuia from pregnant women was about 30%.From the results they mentioned that there was an association of gestational age, education, age, sexual activity, previous history of UTI and socio-economic conditions with bacteriuria. They identified *Escherichia coli* (64.4%), *Proteus spps.* (16.7%), *Klebsiella spps.* (13.3%) and *Pseudomonas aeruginosae* (5.6). and *E. coli, Proteus spps.* and *Klebsiella spps. were* highly sensitive to nitrofurantoin, ofloxacin, cefotaxim, ciprofloxacin, norfloxacin and amikacin and resistant to ampicillin and cotrimoxazole. *Pseudomonas aeruginosae* isolates were resistant to all tested antimicrobial agents except amikacin and nitrofurantoin. They suggested all pregnant women to screened for UTI with a urine culture, treated with antibiotics if the culture is positive and then retested for cure to prevent complications with all the added benefits to the mother and the fetus.

Schneeberger *et al*., (2012) studied to assess the effects of interventions for preventing recurrent urinary tract infections in pregnant women. They observed on their experiment to primary maternal outcomes were RUTI before birth (variously defined) and preterm birth (before 37 weeks). The primary infant outcomes were small-for-gestational age and total mortality.

They found Recurrent urinary tract infections (RUTI) was common in women who was pregnant and may cause serious adverse pregnancy outcomes for both mother and child including preterm birth and small-for-gestational-age babies. Interventions used to prevent RUTI in women who was pregnant could be pharmacological (antibiotics) or non-pharmacological (cranberry products, acupuncture, probiotics andbehavioural modifications).No significant differences were found for the primary outcomes: recurrent pyelonephritis (risk ratio (RR) 0.89, 95% confidence interval (CI) 0.31 to 2.53, one study, 167 women), recurrent urinary tract infection before birth (RR 0.30, 95% CI 0.06 to 1.38; one study 167 women) and preterm birth (before 37 weeks) (RR 1.18, 95% CI 0.42 to 3.35; one

study 147 women). The incidence of asymptomatic bacteriuria (ASB) (at least 103 colonies per mL) (secondary outcome), only reported in women with a clinic attendance rate of more than 90% (RR 0.55, 95% CI 0.34 to 0.89; one study, 102 women), was significantly reduced in women who received nitrofurantoin and close surveillance. They suggested **to** randomised controlled trials comparing different pharmacological and non-pharmacological interventions are necessary to investigate potentially effective interventions to prevent RUTI in women who are pregnant.

Alex *et al.*, (2012) investigated the incidence of urinary tract infections among pregnant women attending antenatal clinics in the Cape Coast Metropolis of the Central Region of Ghana. They performed Physical, chemical, microscopic, and microbial analysis for isolation and identification of bacterial pathogens from urine samples obtained from 200 pregnant women aged 15 - 45 years attending the University of Cape Coast Hospital, Cape Coast Metropolitan Hospital and Ewim Urban Health Centre. Three trimesters was determined together with sensitivity testing of the bacteria isolates to antimicrobial drugs. They showed on their study overall prevalence stood at 56.5 %, comparatively high in pregnant women in the second trimester (50.4 %). They isolated *Escherichia coli* (48.7 %) from the pregnant women aged between 15 – 32 years and most were affected and gentamycin was the most effective antimicrobial against the bacteria isolates. Their results indicated that the incidence of urinary tract infections was high among pregnant women on the study area and they suggested routine antenatal checkups for pregnant women to detect the asymptomatic infections to reduced its risk to pregnancies.

Ziad and Claude, (2011) investigated the bacterial etiology of urinary tract infections in one of the busiest hospitals of Lebanon and to examine the epidemiologic and microbiologic properties of *Escherichia coli* isolated from urinary tract infections of Lebanese patients over a 10-year period. They analyzed the data generated between 2000 and 2009 (10,013 Gram-positive and Gram-negative bacteria). Bacterial identification was based on standard culture and biochemical characteristics of isolates on their study. They used antimicrobial susceptibility test that was performed by the disk diffusion method, and ESBL production was detected by synergy with third-generation cephalosporins and amoxiclav. *They found highest number of E. coli 60.64%) on their study and also identified Klebsiella pneumoniae* and *Proteus sp., Pseudomonas aeruginosa, Enterococcus sp.*, and *Streptococcus agalactiae*. The lowest percentage of susceptibility of *E. coli* was manifested against penicillin and ampicillin.

Jean *et al.*, (2011) compared the virulence properties of a collection of asymptomatic bacteriuria (ABU) *Escherichia coli* strains to urinary tract infection (UTI) strains isolated from pregnant women in a university hospital over 1 year. The *in vitro* and *in vivo* studies suggested that ABU strains presented a virulence behavior similar to that of strains isolated from cases of cystitis.

Manaal *et al.*, (2011) studied to determine the Isolation, Identification and Characterization of *Escherichia Coli* from Urine Samples and their Antibiotic Sensitivity Pattern. The main objective of their study was to identified *E. Coli* from urine sample of women.Urinary tract infection cause serious complications when is spread to kidney. They showed on their study to used antibiotics such as Norfloxacin, Tetracycline, and Ampicillin etc for the treatment of UTI. Their main focus was antibiotic sensitivity pattern.

Sabrina *et al.*, (2010) isolated bacterial isolates and drug susceptibility patterns of urinary tract infection among pregnant women at Muhimbili National Hospital in Tanzania. Retrospective analysis was performed on 200 mid-stream urine specimens processed for culture and antimicrobial drug susceptibility testing between January 2007 and December 2009. Signifcant bacteriuria (> 105 colony forming units/ml of urine) was found in 42/200 (21%) specimens. Their isolated bacteria were *Escherichia coli* 14 (33.3*%), Klebsiella spp* 9 (21.4%) coagulase negative *Staphylococcus* 7 (16.7%), *Staphylococcus aureus* 6 (14.3%), *Proteus species* 3 (7.1%) *and Enterococcus species* 3 (7.1%) from 42 samples. They recommended to monitor the levels of resistance for nitrofurantoin, fluoroquinolone and cefotaxime and to screen for Extended Spectrum Beta Lactamase production among cefotaxime resistant *E. coli and Klebsiella spp.*

Danielle *et al.*, (2009) concluted to identify the accuracy of the simple urine test for UTI diagnosis in low-risk pregnant women. Diagnostic test performance was conducted in Botucatu, SP, involving 230 pregnant women, between 2006 and 2008. They showed on their results 10% UTI prevalence. Sensitivity, specificity and accuracy of the simple urine test were 95.6%, 63.3% and 66.5%, The analysis of positive (PPV) and negative (NPV) predictive values showed that, when a regular simple urine test was performed, the chance of UTI occurrence was small (NPV 99.2%).Their study was concluded that the accuracy of the simple urine test as a diagnostic means for UTI was low, and that performing a urine culture is essential for appropriate diagnosis.

Obiogbolu *et al.*, (2009) designed to determine the Incidence of Urinary Tract Infections (UTIs) among pregnant women in Akwa metropolis, Southeastern Nigeria. They collected 100 urine samples from pregnant women and cultured on culture media for isolation and identification of bacterial pathogens, 54 showed significant bacterial growth while 46 showed no significant bacterial growth. Bacterial agents were isolated from 54 pregnant women and were identified as: *Escherichia coli*; *Klebsiella* spp., *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. They observed *Escherichia coli* [20(37%)], *Klebsiella spp.,* [11(20.4%)], *Proteus mirabilis* [9(16.7%)], *Pseudomonas aeruginosa* [7(13%)], *Staphylococcusaureus* [4(7.4%)] and *Staphylococcus epidermidis* [3(5.6%)]. Their study indicated a high incidence of UTIs (54%) though most of the pregnant women showed no clinical manifestation.

Timothy *et al.*, (2008) studied to determine the Urinary tract infections in pregnancy. The main objective of their study was clinical presentation of the organisms that cause UTI in pregnant women. **They determined the** incidence of UTI was approximately 8%.The incidence of asymptomatic bacteriuria in pregnant women as determined in UK studies is 2–5%.They surveyed one study over 6-year period and observer 1.3% incidence rate. The incidence of pyelonephritis during pregnancy was 2%, with up to 23% of women experiencing a recurrence in the same pregnancy.

Asad and Zaman, (2006) investigated the multiple drug resistance pattern in Urinary Tract Infection patients in Aligarh. The aim of their study was to explained the drug resistance pattern in E. coli isolated from community acquired Urinary tract infection (UTIs). 168 urine samples were collected from the UTI patients followed by isolation and identification of E. coli strains by the experts. Antibiotic sensitivity and resistance analysis was performed by the disc diffusion method employing multiple antibiotic discs. The sensitivity was monitored by zone of inhibition around the disc. They found on their study young pregnant women were more infected. Antibiotic susceptibility data revealed that majority of the isolates were resistant against 4 or more antibiotics. Their study concluded that E. coli is one of the important causative agents of urinary tract infection in young women especially during the state of pregnancy.

Warren *et al.*, (2005) studied to screening for asymptomatic bacteriuria in Pregnancy. They used 4 screening strategies for detecting asymptomatic bacteriuria (ABU) in pregnancy. 1050 number of ABU cases that would be detected by each of the 4 strategies (LEN dipstick testing only, a single urine culture, 2 cultures, and 3 cultures) was determined and compared. There were 49 cases of ABU among 1050 women (4.7%). LEN testing at each prenatal visit identified 7 case strategies for detecting asymptomatic bacteriuria (ABU) in pregnancy: (14.3%), compared with 20 cases (40.8%) with 1 urine culture, 31 (63.3%) with 2 urine cultures, and 43 (87.8%) with 3 urine cultures.

Nadia *et al.*, (2004) designed to determine the isolation, identification and antibiotic resistance profile of indigenous bacterial isolates from urinary tract infection patients. They identified 65 bacterial strains from urine samples of patients who were suffering from urinary tract infection.80% of gram negative and 20% of gram positive bacteria were isolated. They found *E. coli* (47.6%), *Pseudomonas aeroginosa* (9.2%), *Klebsiella pneumonia* (7.6%), *Enterobacter aerogenes* (6.1%), *Protius mirabilis* and *Serratia marcescens* (4.6%), *Staphylococcus aureus* and *Streptococcus pyogenes* (4.6%) *Enterococcus faecalis, Staphylococcus epidermidis* and *Bacillus subtilis* (3%) *and Staphylococcus saprophyticus*(1.5%). Antibiotic sensitivity test was done by disc-diffusion method. They observed on their study to gram negative bacteria was more sensitive than gram positive bacteria.

John *et al.*, (2000) investigated to Urinary tract infections during pregnancy and isolated the most common causative organism *Escherichia coli*. They suggested all pregnant women to screened for bacteriuria and subsequently treated with antibiotics such as nitrofurantoin, sulfisoxazole or cephalexin. They decleared pyelonephritis can be life threatening illness, with increased risk of perinatal and neonatal morbidity. Pregnant women with urinary group B streptococcal infection should be treated and should receive intrapartum prophylactic therapy. **They found** in pregnant women the incidence of UTI was 8%. They focused on the pathogenesis and bacteriology of UTIs during pregnancy.

Asscher, (1975) designed to determine the urinary tract infection and to isolation, identified the bacterial pathogens that causes it. The prevalence of bacteriuria in adults has been studied among defined populations in South Wales, Jamaica and Japan. About 4% of females between the ages of 16 and 65 yr showed significant bacteriuria as compared with 0.5% of males. Spontaneous remissions and new infections occur at the rate of about 1% of the total female population per annum. They suggested that the regular screening of urine culture could help the pregnant women to prevent the infections.

Nathaniel *et al.*, (1965) studied to determine the Detection of Urinary Tract Infections in Pregnant Women. They used several methods for the detection of bacterial pathogens from urine samples. They used streak plate, poured plate method for their experiment. They collected 1156 urine samples from 986 women and cultured on media and observed several bacterial pathogens. 173 (4.6 percent) were falsely negative. The positive correlation was 95.4 percent. Of the 964 urine specimens which showed less than 100,000 organisms per milliliter, 192 had 1 or more organisms per oil-immersion field on the gram stain, giving 19.9 percent falsely positive gram stains.

CHAPTER-3

MATERIALS AND METHODS

The present research work was conducted between July to December, 2017 in the Microbiology Laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh for the identification of bacteria by different microbiological methods. The detailed outline of Materials and Methods are given below.

3.1 Materials

3.1.1 Selection of study area

Urine samples were collected from pregnant women of different hospital under Dinajpur District of Bangladesh. These includes M *Abdur Rahim Medical College* and Hospital, Saint Vincent Hospital and Sheba Diagnostic and consultancy center.

3.1.2 Collection of Sample

The urine samples were collected from pregnant women of different age (17-35) and brought to the Microbiology laboratory of the department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur. Sterile container were used during collection of urine. A total of 100 urine samples were collected for the present research work.

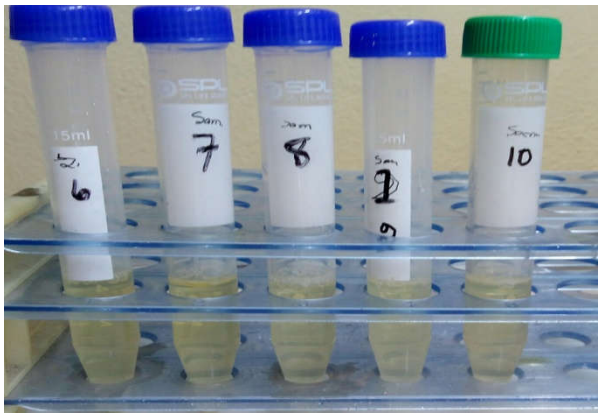


Plate no 1: Collected urine sample

3.1.3 Transportation and Preservation of Samples

The samples were carried out to the laboratory in an ice box contained ice and processed for the isolation, identification and characterization of bacteria subsequently. And the remaining samples were stored at 4 ºC for future use.

Plate no 2: Sample transferred to laboratory with ice box

3.1.4 Media for culture

Different bacteriological culture media and reagents were used for isolation and identification and also propagation of bacteria from urine samples. The culture media and reagents used in this experiment are mentioned below:

3.1.4.1 Liquid Media

1. Nutrient broth (Difco)

2. 1% Pepton Water (Difco)

3. Pepton broth (Difco)

1. Nutrient broth

Nutrient broth was used to grow the organisms from the samples collected from the study areas before performing biochemical test and antibiotic sensitivity test (Cheesebrough. 1984)

3.1.4.2 Solid Media for culture

1. Plate Count Agar
2. Nutrient Agar Medium, (HI-MEDIA, India)
3. MacConkey Agar medium, (Difco)
4. Eosin Methylene Blue, (EMB) (HI-MEDIA, India)
5. Manitol Salt Agar, (HI-MEDIA, India)
6. *Staphylococcus* Agar No. 110, (HI-MEDIA, India)
7. Salmonella-Shigela Agar (Difco)
8. Blood Agar Medium, (HI-MEDIA, India)
9. Cetrimide Agar Base (Difco)
10. Tryptic Soy Agar, (HI-MEDIA, India)
11. Baird Parker Agar, (HI-MEDIA, India)
12. Mitis Salivarius Agar Base, (HI-MEDIA, India)
13. Mueller-Hinton agar (Difco)

Solid Media

1. Plate Count Agar

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 Medium, (HI-MEDIA, India)

Nutrient agar is used for cultivating of non-fastidious microorganisms (Cheesbrough, 1985).

3. Eosin Methylene Blue, (EMB) (HI-MEDIA, India)

EMB contains dyes that are toxic for Gram positive bacteria and bile salt which is toxic for Gram negative bacteria other than coliforms. EMB is the selective and differential medium for coliforms. *Escherichia coli*: Blue-black bulls eye; may have green metallic sheen. *Escherichia coli* colonies grow with a metallic sheen with a dark center, *Aerobacter aerogenes* colonies have a brown center, and nonlactose-fermenting gram-negative and *Klebshella spp* bacteria appear pink (Cheesbrough,1985).

4. MacConkey Agar medium, (Difco)

A differential medium for the isolation of coliforms and intestinal pathogens in water and biological specimens. Mac-Conkey agar is a culture medium designed to selectively grow Gram-negative bacteria and differentiate them for lactose fermentation. Lactose-fermenting organisms grow as pink to brick red colonies with or without a zone of precipitated bile. Non-lactose fermenting organisms grow as colorless or clear colonies (Cheesbrough, 1985).

5. Manitol Salt Agar (HI-MEDIA, India)

Mannitol salt agar or MSA is a differential medium and commonly used growth medium in microbiology. Gram positive *Staphylococcus*: Fermenting mannitol: Media turns yellow. Gram positive *Staphylococci*: Not fermenting mannitol. Media does not change color (Cheesbrough, 1985).

6. *Staphylococcus* Agar No. 110 (HI-MEDIA, India)

Staphylococcus Agar No. 110 is used to isolate *Staphylococci* and formation of golden yellow colonial pigments. (Cheesbrough, 1985).

7. Salmonella-Shigela Agar (Difco)

Salmonella Shigella Agar (SS Agar) is a selective and differential medium widely used in sanitary bacteriology to isolate *Salmonella* and *Shigella* from feces, urine, and fresh and canned foods. *Shigella* and the major part of *Salmonella*: Clear, colorless and transparent (Cheesbrough, 1985).

8. 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 alfa hemolysis. *Streptococcus pyogenes* gives beta hemolysis (Clin. Path 1951).

9. 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).

10. Tryptic Soy Agar Base (HI-MEDIA, India)

Trypticase Soy Agar is general purpose media that is not selective or differential, their purpose is simply to grow many types of bacteria cheaply and to compare the growth on the selective and differential media. *Staphylococcus spp* colonies are yellow, *E. coli* colonies are creamy colour, *Pseudomonas spp* are creamy colonies with yellow colour mixed. (Cheesbrough, 1985).

11. Baird Parker Agar (HI-MEDIA, India)

The presence of black, convex, shiny colonies 1-1.5mm in diameter is a presumptive positive test for the presence of *S. aureus*. Absence of *S. aureus* colonies do not form the black pigmentation (Baird-Parker. 1962).

12. Mitis Salivarius Agar Base (HI-MEDIA, India)

Enzymatic Digest of Casein and Enzymatic Digest of Animal Tissue provide carbon, nitrogen, and amino acids used for general growth requirements in Mitis Salivarius Agar. Sucrose and Dextrose are carbohydrate sources. Dipotassium Phosphate is the buffering agent. Trypan Blue is absorbed by the colonies, producing a blue color. Crystal Violet and Potassium Tellurite inhibit most Gram-negative bacilli and Gram-positive bacteria except *streptococci. S. mitis* produces small blue colonies. These colonies may become easier to distinguish with longer incubation. *S.salivarius* produces blue, smooth or rough “gum drop” colonies, 1 - 5 mm in diameter depending on the number of colonies on the plate. *Enterococcus spp* form dark blue or black, shiny, slightly raised, 1 - 2 mm colonies. (Facklam and Washington, 1991)

16. Mueller-Hinton agar (Difco)

Mueller Hinton Agar is used for the determination of susceptibility of microorganisms to antimicrobial agents. It has become the standard medium for the Bauer Kirby method and its performance is specified by the NCCLS.

3.1.4.3 Media for Biochemical tests

1) Sugar Fermentation Broth
2) Oxidase test
3) Catalase test
4) Indole Broth
5) Methyl Red Broth
6) Voges-proskauer Broth
7) Simmon's citrate Agar (HI-MEDIA, India)
8) Triple sugar iron agar (HI-MEDIA, India)
9) Motility Indole Urease (HI-MEDIA, India)

3.1.5 Reagent

1. Crystal violet dye
2. Gram's iodine
3. Alcohol
4. Safranin
5. Saline
6. Iodine solution
7. Kovac's reagent
8. Methyl- red solution
9. 3% H2O2
10. P – Amino dimethylanilin oxalate
11. Phenol red
12. Phosphate buffered saline (PBS) solution
13. Potassium tellurite (1%)

3.1.6. Glassware and Appliances

The different types of important equipment used for this work are listed as follow down-

1. Distilled water

2. Sterile bent glass or plastic spreader rods.

3. Micropipette (1-5µl; 5-50µl; 10-100µl; 50-500µl; 100-1000µl)

4. Freeze (-20°C)

5. Refrigerator (40C)

6. Spirit lamp

7. Water bath

8. Vortex Mixture

9. Labeling tape

10. Experimental test tube

11. Stopper

12. Petri dish

13. Conical flask.

14. Durham's tube

15. Slide

16. Microscope

17. Cotton

18. Immersion Oil

19. Toothpick

20. Autoclave

21. Thermometer

22. Incubator

23. Jar

24. Beaker

25. Cylinder

26. Electric Balance

27. Filter paper

28. Spirit lamp and

29. Bacteriological loop etc.

3.1.7 Materials used for bacterial genomic DNA isolation

- TE buffer
- 10% (w\v) Sodium dodecyl sulfate (SDS)
- 20 mg\ml protinase k (stored in small single-use aliquots at -20^{0}C)
- 3 M Sodium Acetate, pH 5.2
- 25:24:1 Phenol/Chloroform/Isoamyl alcohol
- Isopropanol
- 70% Ethanol
- 95% Ethanol
- 1.5 ml microcentrifuge tubes

3.1.8 Materials used for Polymerase Chain Reaction

Table 1: PCR Reaction Mixture for 16s rRNA.

Buffer	2.5 µl
dNTP	2.5 µl
$MgCl_2$	2.5 µl
Forward Primer (27F)	1.0 µl
Reverse Primer (1492R)	1.0 µl
Nano Pure Water	12.5 µl
DNA	2.0 µl
Taq DNA Polymerase	1.0 µl
Final Volume	25 µl

- Primers used for PCR:

16S rRNA gene region was amplified with the universal primers.

  - Forward primer- 27F (5'AGAGTTTGATCCTGGCTCAG 3')
  - Reverse primer- 1492R (5' TACCTTGTTACGACTT 3')

- Product size: 1500bp
- Thermal Cycler (Thermo cycler, ASTEC, Japan)
- 2% agarose gel
- Gel casting tray with gel comb
- TAE buffer
- Microwave oven
- Conical flask
- Electrophoresis apparatus (Biometra standard power pack P 2T)

- 100 bp DNA size marker
- Bromphenicol blue of loading bufter.
- Ethidium bromide (0.5 µg/ml)
- Distilled water
- UV trans-illuminator

3.1.9 Antimicrobial sensitivity discs

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

Table 2: Antimicrobial agents with their discs concentration.

S/N.	Name of antimicrobial agents	Disc concentration (µg /disc)
1.	Ampicillin(AMP)	25µg
2.	Amoxicillin(AMX)	30µg
3.	Amikacin(AK)	30µg
4.	Cephalesin (CN)	30µg
5.	Ciprofloxacin(CIP)	5µg
6.	Gentamycin(GEN)	10µg
7.	Kanamycin(K)	30µg
8.	Penicillin (P)	10 units
9.	Erythromycin(E)	15µg
10.	Vancomycin (VA)	30µg
11.	Chloramphenicol (C)	30µg
12.	Cloxacillin (COX)	1µg
13.	Tobramycin (TOB)	30µg
14.	Colistin (CL)	10µg
15.	Levofloxacin(LEV)	5µg
16.	Neomycin(N)	30µg
17.	Norofloxacin(NX)	10µg
18.	Novobiocin (NOV)	30µg
19.	Cefotaxime (CTM)	30µg
20.	Ofloxacin(OFX)	2µg

Note: µg = Microgram

3.2 Methods

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

3.2.1 Experimental layout

The experimental work was divided into three steps: The first step was performed for the isolation and identification of the organisms from the collected sample using cultural, staining and biochemical characteristics. The second step was performed for molecular characterization of selective isolates by 16s rRNA gene sequencing. The third step was conducted for the determination of antibiotic sensitivity and resistant pattern of isolated organisms of various samples by using different antibiotic discs available in the market. The layout of the diagrammatic illustration of the present study is shown in figure 1.

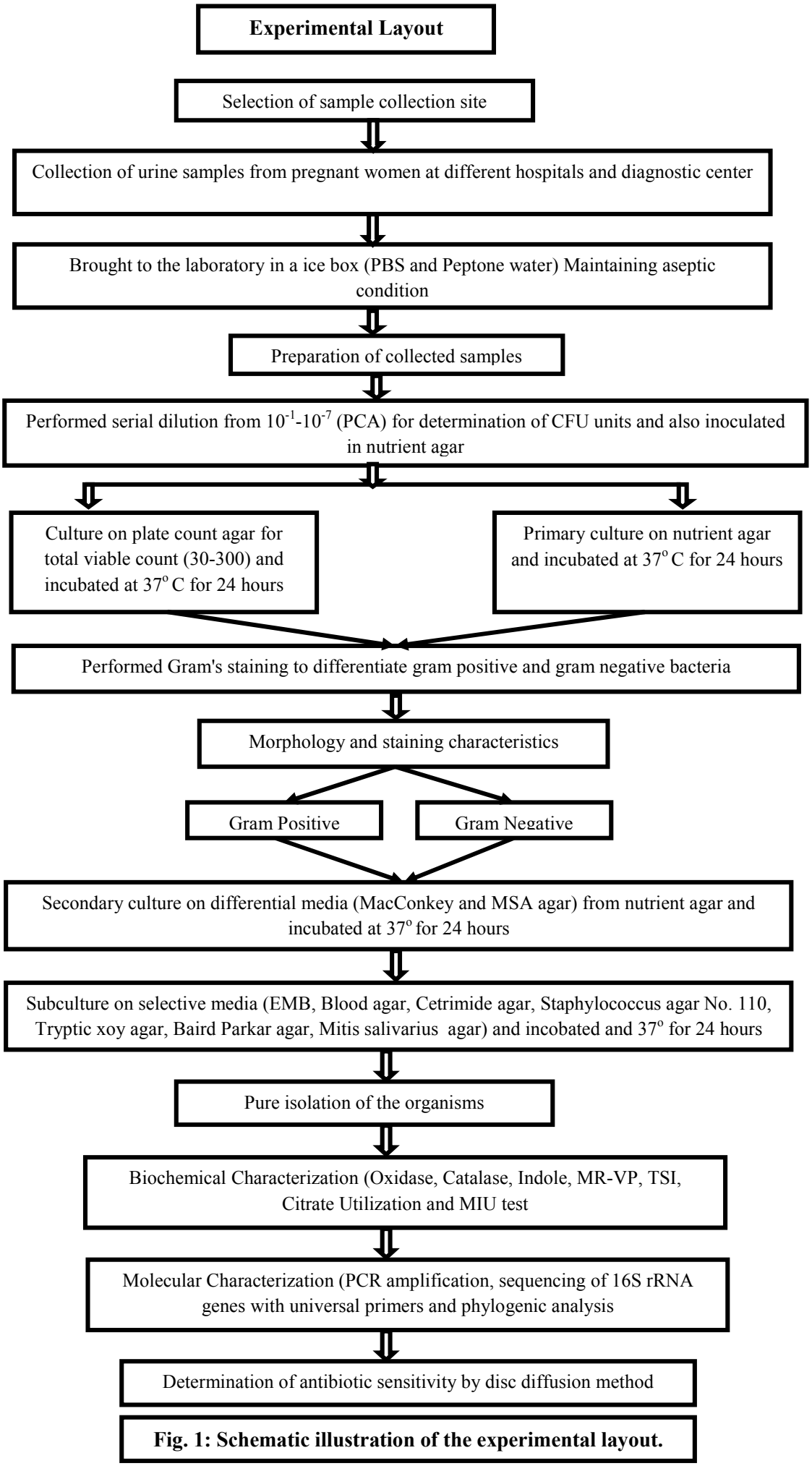


Fig. 1: Schematic illustration of the experimental layout.

3.2.2 Laboratory preparations

All items of required glassware including test tubes, pipettes plate, slides, cylinder, flasks, conical flasks, glass and vials soaked in a household dishwashing detergent solution ('Trix' Recket and Colman Bangladesh Ltd.) overnight. Contaminated glassware was disinfected with 2% sodium hypochlorite solution prior to cleaning. The glassware were then cleaned by brushing, washed thoroughly in running tape water, rinsed within distilled water and finally sterilized either by dry heat at 160˚C for 2 hours or by autoclaving for 15 minutes at 121˚C under 15 lbs pressure per sq inch. Autoclaved items were dried in a hot air oven over at 50˚C. Disposable plastic (items e.g. micropipette tips) was sterilized by autoclaving. All the glassware was kept in oven at 50˚C for future use.

3.2.3 Sampling and Processing of Samples

Proper care was taken during the sampling procedure to prevent contamination of sample. The samples tubes were completely tied at the time of sampling that prevent contamination. After came to the laboratory 1ml urine sample was taken from each sample and mixed with 9ml PBS (Phosphate Buffer Solution) in each test tube for serial dilution (10-1 to 10-7) and 50 µl samples were seeded on nutrient agar, plate count agar, using spread plate method. The plating was done in the laminar flow to maintain aseptic conditions and the medium were then incubated at 37˚C for 24 h.

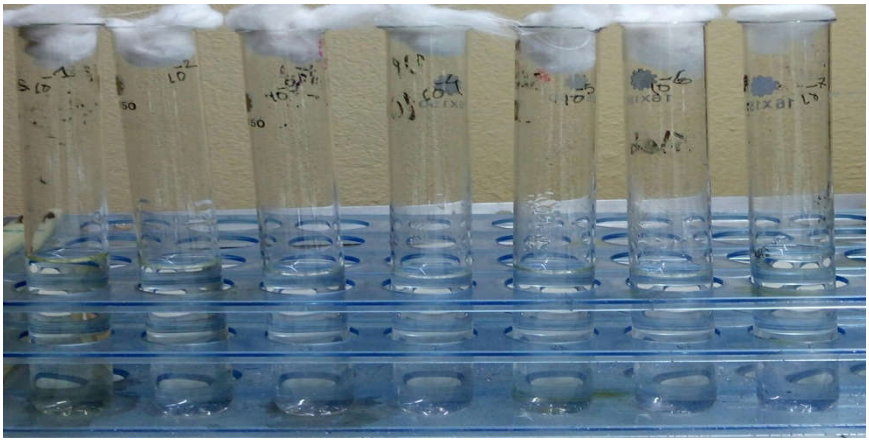


Plate no 3: Serial dilution from sample

3.2.4 Microbial assessment of the collected samples by total viable count

Samples were collected and each of the samples were diluted with distilled water 10-1, 10-2, 10-3, 10-4, 10-5, 10-6 10-7. Then 50µl (0.05 ml) samples were taken and spread in plate count agar (PCA) plate following the spread-plate method and incubated at 37ºC for 24 h. The number of organisms per ml or per gram of original culture was calculated by multiplying the number of colonies counted by the dilution factor:

Number of cells per ml or per gram = number of colonies × Dilution factor/Volume of dilution.

Colonies per plate=43

Dilution factor=1:1×10^7 (1:100, 00000)

Volume of dilution added to plate=50µl (0.05 ml)

So,

43 x 10^7 /0.05 = 8 x 10^9 CFUs/ml ; CFU= (Colony-forming units)

[**Note-** statistically valid plate counts are only obtained from bacterial cell dilutions that yield between 30 and 300 colonies. Plates with more than 300 colonies cannot be counted and are designated as too numerous to count- **TNTC**; plates with fewer than 30 colonies are designated as too few to count-**TFTC**]. Record the observations and calculated bacterial counts per ml of samples. (James G. Cappuccino, 1996).

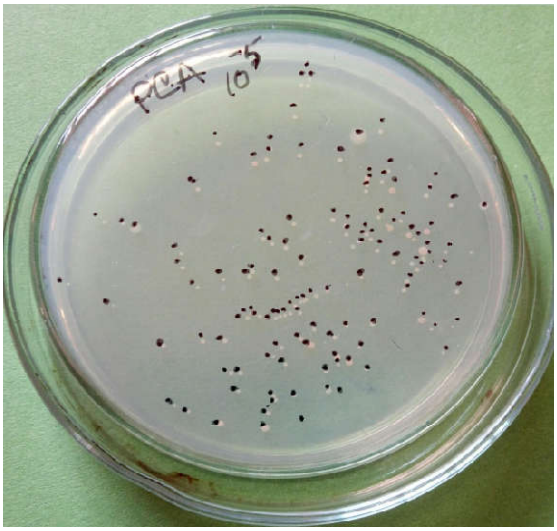


Plate no 4: Colony count by conventional method (PCA)

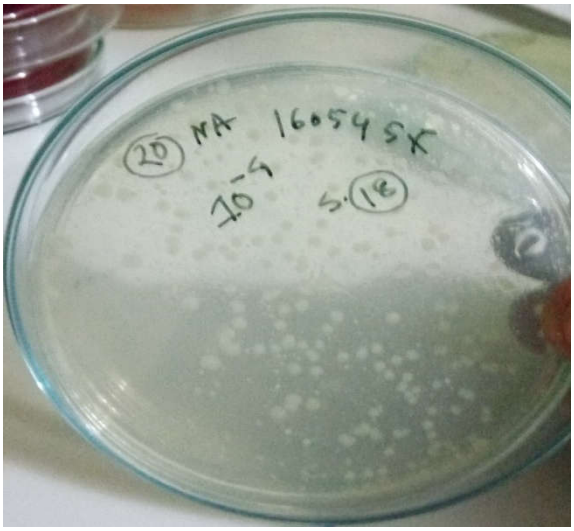


Plate no 5: Colony count by conventional method (NA)

3.2.5 Plan of the experiment work at a glance

All of those samples were collected from different hospitals of Dinajpur District with a ice containing ice box. Then all of the samples were transferred to the microbiological laboratory of department of Microbiology, HSTU, Dinajpur, Bangladesh. 50 µl (0.05 ml) of urine samples were primarily inoculated into Nutrient agar and plate count agar (PCA) for determining the density of bacterial total viable count (TVC). Subsequently Nutrient agar, Blood agar, EMB agar, SS agar, MacConkey agar, Cetrimide agar base, Tryptic soy agar, Baird parker agar, Mitis salivarius agar, MSA agar, *Staphylococcus* media no. 110 were employed and specific biochemical tests were done for isolation and identification of bacteria. Molecular characterization also performed for genome sequencing. At last performed antibiotic sensitivity test with the pure isolated bacteria.

3.2.6 Preparation of culture media

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

3.2.6.1 Nutrient broth media

Thirteen grams of dehydrated nutrient broth (HI-MEDIA, India) 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).

3.2.6.2 Plate Count Agar (PCA)

Add 17.5g 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).

3.2.6.3 Nutrient agar media

Twenty eight grams of nutrient agar powder (HI-MEDIA) 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 Petridish 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 used for cultural characterization or stored at 4°C refrigerator for future use. (Cater 1979).

3.2.6.4 Blood agar media

Forty grams of Blood agar base (HI-MEDIA, India) powder was dissolved in 1000 ml of distilled water. 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 petridishes 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 used for cultural characterization or stored at 4°C refrigerator for future use. (Cater 1979).

3.2.6.5 Eosin Methylene Blue agar

Thirty six grams of EMB agar base (HI-MEDIA, India) 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 Petridish 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 petridishes without contamination were used for cultural characterization or stored at 4°C in refrigerator for future use. (Cater 1979).

3.2.6.6 MacConkey agar

51.5 grams MacConkey agar base (HI- MEDIA, India) 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 45^0- 50^{0}C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petridishes

(medium sized) and in 15 ml quantities in sterile glass Petridishes (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 Petridishes 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. Petridishes, 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)

3.2.6.7 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/cm2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 450- 500C 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).

3.2.6.8 *Staphylococcus* Agar No. 110

149.5 grams Staphylococcus Agar No. 110 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/cm2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 450- 500C 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).

3.2.6.9 *Salmonella Shigela* (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 450- 500C 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).

3.2.6.10 Trypticase soy agar

Suspended 40 g of the medium in one liter of purified water. Heated with frequent agitation and boil for one minute to completely dissolve the medium. Then autoclaved at 121°C for 15 minutes. Prepared 5 to 10% blood agar by adding appropriate volume of sterile defibrinated blood to melted sterile agar medium, cooled to 45 – 50°C and distributed to sterile petridishes 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 used for cultural characterization or stored at 4°C refrigerator for future use. (Leavitt *et al*., 1955)

3.2.6.11 Baird parker agar base

Suspend 63 grams in 950 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 50°C and aseptically add 50 ml concentrated Egg Yolk Emulsion (FD045) and 3 ml sterile 3.5% Potassium Tellurite solution (FD047) or 50 ml Egg Yolk Tellurite Emulsion (FD046). If desired add rehydrated contents of 1 vial of BP Sulpha Supplement (FD069). Alternatively 1 vial of Fibrinogen Plasma Trypsin Inhibitor Supplement (FD195) may be used per 90 ml medium in place of Egg yolk Tellurite Emulsion (FD046) for identification of coagulase, positive *Stapylococci.* Mix well and pour into sterile Petri plates. (Baird-Parker AC., 1962).

3.2.6.12 Mitis Salivarius Agar Base

Suspend 90.07 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 50-55°C and add 1 ml of sterile 1% Potassium Tellurite Solution (FD052). Do not reheat the medium after the addition of tellurite solution. Mix well and pour into sterile Petri plates. (Chapman G. H., 1944)

3.2.6.13 Mueller-Hinton agar (Difco)

Suspended 38 gm of the medium in one liter of distilled water. Then heated with frequent agitation and boil for one minute to completely dissolve the medium. Then autoclaved at 121°C for 15 minutes. Pour cooled Mueller Hinton Agar into sterile petri dishes on a level, horizontal surface to give uniform depth.

3.2.6.14 Sugar media

The medium consists of peptone water of which fermentable sugar was added to the proportion of 1%. One gram of Bacto peptone (HI-MEDIA) and 0.5 gram of sodium chloride were added in 100 ml of distilled water. The medium was boiled for 5 adjusted to P^H 7.0, cooled and then filtered through filter paper. Phenol red, indicator at the strength of 0.2% solution was added to peptone water and then in 5 ml into cotton plugged test tubes containing Durham's fermentation and placed invertedly. These were then sterilized by autoclaving at 1.2 kg /cm^2 121°C for 15 minutes. The sugars used for fermentation were prepared separately 10% solution in distilled water (10 grams sugar was dissolved in 100 ml of distilled water). A mild heat was necessary to dissolve the sugar. The sugar solutions were sterilized in Arnold steam sterilizer at 100°C for 30 minutes for 3 consecutive days. An amount of 0.5 ml of sterile sugar solution was added aseptically in each culture containing 4.5 ml sterile peptone water, indicator and Durham's fermentation tube. Before use, the sterility of the sugar media was examined by incubating it at for 24 hours (Cater 1979).

3.2.7 Preparation of Reagents

3.2.7.1 Methyl-Red Solution

The indicator MR solution was prepared by dissolving 0.1 gram of Bacto methyl-red in 300 ml of 95% alcohol and diluted to 500 ml with the addition of distilled water. (Merchant and Parker 1967).

3.2.7.2 Methyl Red

A quantity of 17 gms of MR-VP medium (HI-MEDIA) 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 37^{o}C for overnight o check their sterility and then in refrigerator for future use. (Merchant and Parker, 1967).

3.2.7. 3 Alpha-Naphthol Solution

Alpha-naphthol solution was prepared by dissolving 5 gram of alpha-naphthol in 100 ml of 95% ethyl alcohol (Merchant and Parker, 1967).

3.2.7.4 Potassium hydroxide solution(H_2O_2)

Potassium hydroxide (KOH) solution was prepared by adding 40 grams of Potassium hydroxide crystals in100 ml of cooled water. (Merchant and Parker, 1967).

3.2.7.5 Kovac's Reagent

The solution was prepared by mixing 25 ml of concentrated Hydrochloric acid in 5 ml of Amyl alcohol and 5 gram of paradmethyl-aminobenzyldehide crystals were added to this mixture. This was then kept in a flask equipped with rubber cork for future use (Merchant and Parker, 1967).

3.2.7.6 Phosphate Buffered Saline Solution

For preparation of Phosphate buffered saline (PBS) solution, 8 gram of sodium chloride, 2.89 gram of disodium phosphate, 0.2 gram of potassium chloride and 0.2 gram of potassium hydrogen phosphate were suspended in 1000 ml of distilled water. The solution was heated to dissolve completely. The solution was then sterilized by autoclave at 121c maintaining a pressure of 15 pounds per square inch for 15 minutes and stored at refrigerator until use. The pH of the solution was measured by a pH meter and maintained at 7.0-7.2 pH. (Cheesbrough, 1984)

3.2.7.7 Crystal Violet Solution (0.5%)

Dissolve 500 mg Crystal Violet mixed 75 ml distilled water then added 25 ml Methanol and store at room temperature which acts as a primary coloring agent. (Isenberg HD.,1995).

3.2.7.8 Gram's Iodine

Dissolve 6.7 gram of potassium iodide in 100mL of demonized water, add 3.3 g of iodine; stir to dissolve, then dilute to 1 L. Store in a bottle which acts as a mordant. (Isenberg HD.; 1995)

3.2.7.9 Safranin

Dissolve 0.1 g safranin in 75 mL of distilled water, then dilute to 100 ml. Filter before use which acts as a counter stain. (Isenberg HD.; 1995)

3.2.8 Isolation of bacteria

3.2.8.1 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.

Procedure:

- Test tubes were placed into the test tube rack with appropriate label (10^{-1},10^{-2},10^{-3},10^{-4}, 10^{-5}, 10^{-6} and 10-7)
- 9 ml distil water was taken in each tube.
- 1 ml sample was added into first tube.
- Gently shake.
- Dilution up to 1:10^{7}.
- 50 µl samples were spread on nutrient agar plate.
- Incubated at 37˚C for 24 hours.
- Plate were observed for result.

3.2.8.2 Isolation of bacteria in pure culture

For isolation of bacteria in pure culture, the mixed culture was inoculated into Nutrient agar media by streak plate technique to obtain isolated colonies as per suggested by Poindexter (1971).

3.2.8.2.1 Procedure for isolation and identification of bacterial pathogens

Primary culture on nutrient agar

With the help of sterile inoculating loop the collected samples were directly inoculated into nutrient agar and incubated at 37^{o}C for 24 hours. The incubated media were then examined for growth of bacteria.

Inspection: Growth of microorganisms and their colony characteristics were recorded according to procedures described by (Carter, GR. 1979)

Secondary culture on differential media

The organisms were inoculated into MacConkey agar, EMB agar and incubated at 37^{o}C for 24 hours. The incubated media were then examined for growth of bacteria.

Step-1. A inoculum was picked up with a sterile inoculating loop and was spread on a area of the medium in the petridish.

Step -2. The loop was sterilized by being heated as red hot in a flame.

Step -3. The inoculums was spread over the remainder of the plate by drawing the cooled, sterilized loop across the part of the inoculated area, then streaking in a single direction in each parallel line. This method was repeated as many times as necessary to obtain a culture containing only one type of colony and usually at least two more times to ensure purity.

Culture on selective media

1. EMB agar for *E. coli* and *Klebsiella sp.*
2. MSA, TSA, BPA, Staphylococcus media-110 for *Staphylococcus sp.*
3. CTM agar for *Pseudomonas sp.*
4. Blood agar base for *streptococcus sp.*
5. Mitis salivarius agar for *Enterococcus faecalis*

3.2.8.3 Isolation of bacteria by colony morphological characteristics

Each of the colonies was seen for size, shape, pigment, diameter, form, margin and elevation.

3.2.8.4 Gram's staining

Gram's staining method was followed to study the morphological and staining characteristics bacteria and to provide information about the presumptive bacterial identification as per recommendation of Merchant and Packer (1967). A pure colony of each isolates was picked and gram staining was performed. Then the shape, arrangement and gram reaction of the isolates were observed under microscopic field.

Gram's staining method

- Clean glass slides were obtained.
- Using sterile technique, smear of each of the organisms were prepared by placing a drop of normal saline on the slide, and then transferring each organism separately on the slide with a sterile, cooled loop and mixing organism by means of circular of the inoculating loop.
- Smears were allowed to air-dry and then heat fixed in the usual manner.
- Smears were flooded with crystal violet and let stood for one minute then washed with tap water.
- Smear were gently flooded with gram's iodine mordant and let stood one minute. Gently washed with tap water.
- 95% alcohol-acetone was added for 10 seconds, gently washed with tap water.
- Safranin was added as counter stain for 1 minute.
- Gently washed with tap water and dry by air.
- Then examined under microscope with high power objects (100 X) using immersion oil.

Gram's staining observation –

- Gram positive: Dark purple
- Gram Negative: pale to dark red
- Cocci: Round shape
- Bacilli: Rod shape

3.2.9 Biochemical tests

Isolated organism with supporting growth characteristics of suspected identified by biochemical test. Several types of biochemical tests were performed in this study to confirm the specific bacteria that's as follow down:

- 1) Sugar fermentation test
  2) Oxidase test
  3) Catalase test
- 4) Indole test
  5) Methyl Red I(MR) Test
  6) Voges-proskauer (VP) test
  7) Simmon's citrate
  8) Triple Sugar Iron (TSI) agar
  9) Mortility Indole Urease (MIU) test

3.2.9.1 Sugar fermentation test

- The sugar fermentation test was performed by inoculating a loop full of NB culture of the organisms into each tube containing five basic sugars (e.g. dextrose, sucrose, lactose, maltose and mannitol) separately and incubated for 24 hours at 37°C acid production was indicated by the color change from reddish to yellow in the medium and the gas production was noted by the appearance of gas Bubbles in the inverted Durham's tube (Cheesbrough, 1985).

3.2.9.2 Oxidase test

- The oxidase test uses Kovac's reagent (a 1% [wt/vol] solution of N, N, N', N' – tetramethyl-ρ-phenylenediamine dihydrochloride) to detect the presence of cytochrome c in a bacterial organism's respiratory chain; if the oxidase reagent is catalyzed, it turns purple. The oxidase test can be performed on filter paper or on a swab (Cheesbrough, 1985).

3.2.9.3 Catalase test

- This test was used to differentiate bacteria which produce the enzyme catalase. To perform this test, a small colony of good growth pure culture of test organism was smeared on a slide. Then one drop of catalase reagent (3%H2O2) was added on the

smear. The slide was observed for bubbles formation. Formation of bubble within few seconds was the indication of positive test while the absence of bubble formation indicated negative result (Cheesbrough, 1985).

3.2.9.4 Indole test

- Two milliliter of peptone water was inoculated with the 5 ml of bacterial culture and incubated at 37°c for 48 hours. Kovac's reagents (0.5ml) were added, shake well and examined after 1 minute. A red color in the reagent layer indicated indole test positive. In negative case there is no development of red color (Cheesbrough, 1985).

3.2.9.5 Methyl Red test (MR)

- Sterile MR-VP broth was inoculated with the test organism and following incubation at 37°c for 24 hours. if the organism would ferment glucose via the mixed acid fermentation pathway like lactic, acetic, which decreases the PH, hence upon the addition of the indicator methyl red the broth becomes red in color and yellow color indicated a negative result (Cheesbrough, 1985).

3.2.9.6 Voges-Proskauer test (VP)

- Voges Proskauer Test – If the organism would ferment glucose via the butylenes glycol pathway, an intermediate product, acetyl methyl carbinol or acetone which is neutral is converted to diacetyl upon the addition of the VP – Reagent –B (40% KOH with 0.3% creatine) in the presence of VP – Reagent – A (5% alpha-naphthol in methyl alcohol). Diacetyl is red in color. Negative is yellow color (Cheesbrough, 1985).

3.2.9.7 Simmon's Citrate Agar (SCA)

- This tube medium is used to identify Gram negative enteric bacilli based on the ability of the organisms to utilize citrate s the sole source of carbon. (Citrate utilization test).The organism which utilizes citrate as its source of carbon degrades it to ammonia and subsequently converts it to ammonium hydroxide. The pH of the medium is then increased and this is indicated by a change in color from green to blue (Cheesbrough, 1985).

3.2.9.8 Triple Sugar Iron Agar (TSI)

- This tube medium is used to identify Gram negative enteric bacilli based on the following biochemical characteristics (Cheesbrough, 1985)
- Glucose fermentation – indicated by yellow butt
- Lactose fermentation – indicated by yellow slant

- Hydrogen sulfide production – indicated by blackening of the medium
- Gas production – indicated by presence of a crack, bubble or gas space
- pH indicator – phenol red
- Hydrogen sulfide indicator – ferric ammonium citrate with sodium thiosulfate.(H2S)

3.2.9.9 Mortility Indole Urease (MIU) test

MIU medium is a semisolid medium used in the qualitative determination of motility, production of indole and urease decarboxylase. MIU medium is used for the differentiation of the family Enterobacteriaceae. The organisms tested must ferment glucose for proper performance of the medium. The medium contains a small amount of agar allowing for detection of motility (Cheesbrough, 1985).

3.2.10 PCR Amplification, Sequencing of 16S rRNA Genes with Universal Primers and Phylogenetic Analysis of *Escherichia coli*

3.2.10.1 Basic protocol of bacterial genomic DNA isolation

Bacteria from saturated liquid culture are lysed and proteins are removed by digestion with protinase-k. Cell wall debris, polysaccharides and remaining proteins are removed by Phenol-chloroform extraction and high-molecular-weight DNA is recovered from the resulting supernatant by isopropanol precipitation.

Procedure

- Inoculate a 25 ml of liquid culture with *Pseudomonas spp.* Grow in conditions appropriate for *E. coli.* until the culture is saturated.
- Spin 1.0 ml of the overnight culture in a micro centrifuge tube for 5 minutes at 10000 rpm.
- Discard the supernatant.
- Repeat this step. Drain well onto a kimwipe.
- Re-suspend the pellet in 467 µl TE buffer by repeated pipetting. Add 30 µl of 10% SDS and 3 µl of 20 mg/ ml Proteinase k to give a final concentration of 100 µg/mg Proteinase k in 0.5% SDS. Mix thoroughly and incubate 30 min for 1 hr at 37^0C.

- Add an approximately equal volume (500 µl) of Phenol/Chloroform/Isoamyl alcohol. Mix thoroughly but very gently to avoid shearing the DNA, by inverting the tube until the phase are completely mixed.
- Then centrifuge the tubes at 12000 rpm for 10 minutes.
- Remove aqueous, viscous supernatant (≈ 400 µl) to a fresh microcentrifuge tube , leaving the interface behind. Add an equal volume of Phenol/Chloroform/Isoamyl alcohol extract thoroughly and spin in a microcentrifuge at 10000 rpm for 5 min.
- Transfer the supernatant to a fresh tube (≈400 µl).
- Add 1/10th volume of 3 M sodium acetate and mix.
- Add 0.6 volumes of isopropanol to precipitate the nucleic acids, keep on ice for 10 minutes.
- Centrifuge at 13500 rpm for 15 minutes.
- Discard the supernatant.
- Wash the obtained pellet with 1 ml of 95% ethanol for 5 minutes. Then centrifuge at 12000 rpm for 10 minutes.
- Discard the supernatant.
- Dry the pellets as there is no alcohol.
- Resuspend the pellet in 50 µl of TE and then 7.5 µl of RNase. Store DNA at 4^{0}C for short term and at -20^{0}C for long term.

3.2.10.2 PCR amplification and sequencing of 16S rRNA

PCR Condition:

Table 3: Condition of PCR.

Step	Temperature	Duration	Cycles
1. Initial denaturation	95ºC	5 min	01
2. Denaturation	95ºC	30 Sec	35
3. Annealing	56ºC	30 Sec	
4. Extension	72ºC	1.5 min	
5. Final extension	72ºC	10 min	01
6. Holding	4ºC	hold	-

3.2.10.3 Electrophoresis

Process of Electrophoresis:

- Preparation of gel.
- Sample application in the gel.
- Adjustment of voltage or current (gel-electrophoresis about 70-100 volts).
- Set up run time about 30-60 minute
- When DNA migrated sufficiently, as judged from the migration of bromphenicol blue of loading buffer, the power supply was switched off.
- The gel stained in ethidium bromide (0.5µg/ml) for 10 minutes, in a dark place.
- The gel was distained in distilled water for 10 minutes. The distained gel was placed on the imaging system in the dark chamber of the image documentation system.
- The UV light of the system was switched on; the image was viewed on the monitor, focused, acquired and saved in an USB flash drive.

3.2.10.4 Nucleotide sequence accession number and BLAST analysis

The nucleotide sequence 16S rRNA gene region data was submitted to NCBI nucleotide sequence database. Using BLAST tool, phylogenic tree, primer pairs were designed from NCBI database search tool

3.2.10.5 Chain-termination methods (Sanger sequencing)

Steps of Sanger sequencing using ABI 3130 Genetic analyzer

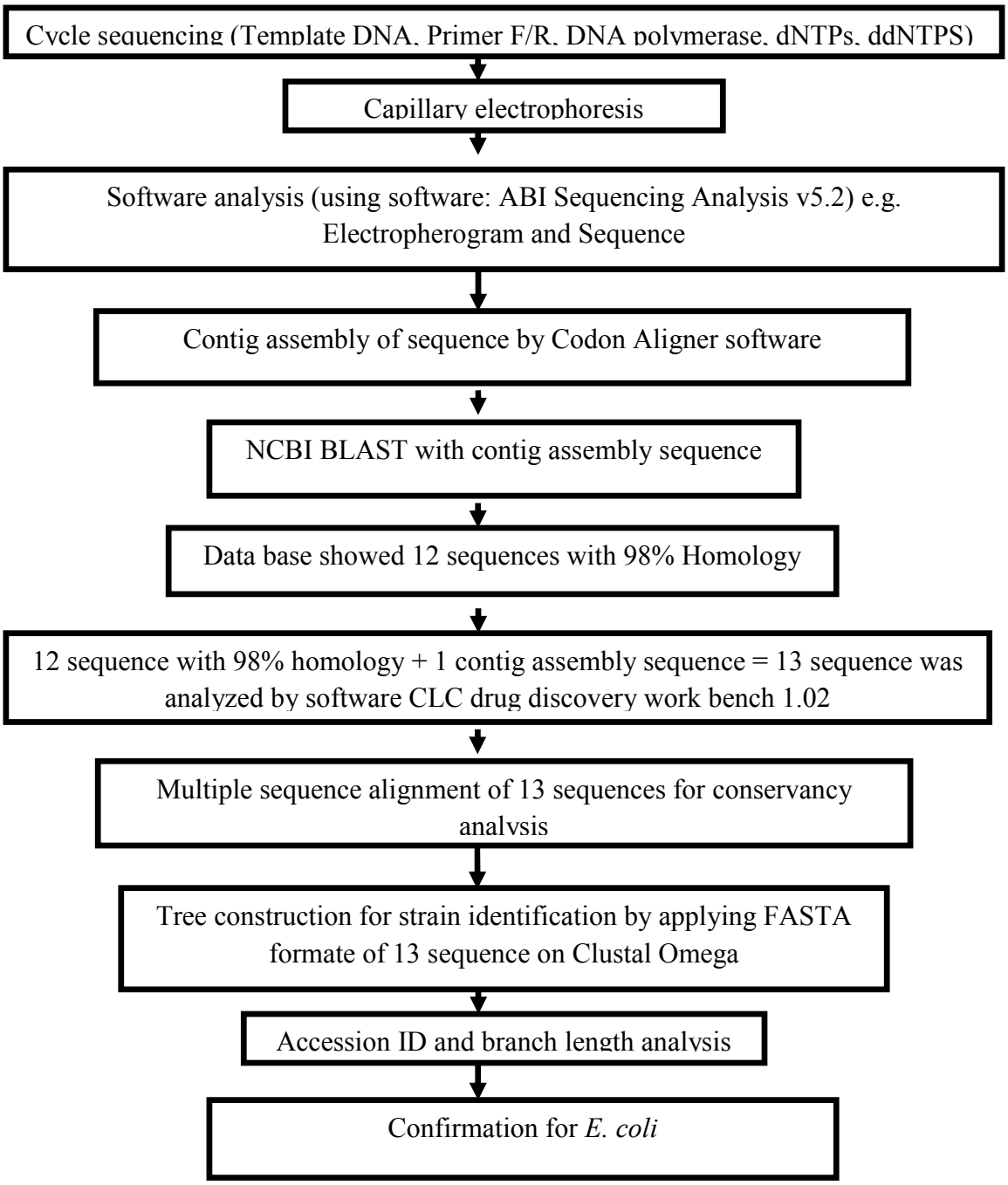


Figure 2: Steps of Sanger sequencing using ABI 3130 Genetic analyzer

3.2.11 Antibiotic sensitivity tests

Kirby-bauer (K-B) 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
- 35°C to 37°C non-CO_2 incubator

Antimicrobial sensitivity test was performed according to the procedure Kirby-bauer disk diffusion susceptibility test protocol suggested by Jan Hudzicki (2009). First developed in the 1950s, it was refined and by W. Kirby and A. Bauer, then standardized by the World Health Organization in 1961. The Kirby-Bauer (K-B) disk diffusion test is the most common method for antibiotic resistance/susceptibility testing. Sensitivity to antibiotics was studied on Mueller-Hinton agar plate used the different types of commercial antibiotic discs. After the discs were placed on the plates, the plates were inverted and incubated at 37°C for 16 to 18 hours. After incubation, the diameter of the zones of complete inhibition (including the diameter of the disc) was measured and recorded in millimeters. The measurements were made with a ruler on the undersurface of the plate without opening the lid. The value was compared with the zone-size table. The zones of growth inhibition were provided by Clinical and Laboratory Standards Institute (CLSI, 2013). Isolates were classified as susceptible, intermediate and resistant categories based on the standard interpretation tables updated according to the Clinical and Laboratory Standards Institution (CLSI, 2013).

Table No 4: Antimicrobial agents with their dis-concentration

Sl. No	Antimicrobial Agents	Symbol	Disc concentration (µg/disc)	Diameter of zone of inhibition (ZOI)		
				Resistant	Intermediate	Susceptible
1	Ampicillin	AMP	25	≤13	14-16	≥17
2	Amoxicillin	AM	30	≤13	14-17	≥18
3	Amikacin	AK	30	≤14	15-16	≥17
4	Cephalexin	CN	30	≤14	-	≥14
5	Ciprofloxacin	CIP	5	≤20	21-30	≥31
6	Gentamycin	GEN	10	≤12	13-14	≥15
7	Kanamycin	K	30	≤13	14-17	≥18
8	Penicillin	P	10	≤11	12-21	≥22
9	Erythromycin	E	15	≤13	14-17	≥18
10	Vancomycin	VA	30	≤9	10-11	≥12
11	Chloramphenicol	C	30	≤12	13-17	≥18
12	Cloxacillin	COX	1	≤15	16-18	≥19
13	Tobramycin	TOB	3O	≤10	-	≥12
14	Cliostin	CL	10	≤10	-	≥11
15	Levofloxacin	LE	5	≤13	14-16	≥17
16	Neomycin	N	30	≤12	13-16	≥17
16	Cloxacillin	COX	1	≤10	11-12	≥13
17	Norofloxacin	NX	10	≤10	-	≥11
18	Novobiocin	NOV	30	≤13	14-16	≥17
19	Cefotaxime	CTM	30	≤10	-	≥11
20	Ofloxacin	OFX	2	≤12	13-5	16

Source: CLSI- 2013 [Note: S=Sensitive, R=Resistant, I=Intermediate]

3.2.12 Maintenance of stock culture

After completion of characterization of bacterial pathogens it was necessary to preserve the isolated organisms for longer periods. For this purpose, pure culture of the isolated *E. coli, Klebsiella spp., Proteus spp, Staphylococcus aureus, Pseudomonas spp, Streptococcus spp., Enterococcuss spp* were stored in 10% glycerin and used as stock culture. The equal volume of 10% glycerin and bacterial culture were mixed and sealed with paraffin wax and stored at 37^{o}C. The isolated organisms were given code name for convenience. (Buxton and Fraser, 1977).

3.2.13 Statistical analysis

Data were analyzed using SPSS version 21. The chi-square (χ^2) test was used to assess statistical differences between the groups. A *p*-value less than 0.05 were statistically considered significant.

CHATER-4

RESULTS

The present research was designed for the isolation, identification, characterization and antibiogram study of bacterial pathogens isolated from urine samples of pregnant women under different hospitals at Dinajpur district of Bangladesh. The collected samples were subjected to various bacteriological examination such as cultural, biochemical techniques and antibiotic sensitivity pattern in the Laboratory of the Department of Microbiology, HSTU, Dinajpur; for isolation and identification of bacteria. A total of 100 urine samples were collected from pregnant women (3-9 month) for this study. Out of 100 urine samples, 33 were found to be positive for UTI and total 75 isolates were isolated. Result of morphological, staining, cultural, biochemical, antibiotic sensitivity pattern and percentage of incidence of isolated bacteria are presented in different tables.

4.1 Results of microbial assessment of collected urine samples by total viable counts

Serial dilution was performed to reduce the number of bacteria and also to obtained total viable counts. Samples (50 µl) were spread on PCA, EMB, MAC and MSA plate up to dilution 10^{-7}. For the growth of bacteria after 24 hours of incubation at 37^{0}C aerobically and were indicated by the growth of circular, small smooth, convex, and gray white or yellowish colonies. Then the number of colonies were observed and recorded. The numbers of total viable count are shown in table 5, 6, 7 and 8. Incase of PCA the highest total viable count 8.6 $\times 10^9$ cfu/ml was found in 10^{-7} dilution and lowest 1.6×10^9 cfu/ml in 10^{-6} dilution. In EMB agar the highest total viable count 5.8 $\times 10^7$ cfu/ml was found in 10^{-4} dilution and lowest 1.0×10^9 cfu/ml in 10^{-7} dilution. In MAC agar the highest total viable count 2.4×10^9cfu/ml was found in 10^{-7} dilution and lowest was 1.0×10^9 cfu/ml in 10^{-6} dilution. In MSA agar the highest total viable count 8.2 $\times 10^8$ cfu/ml was found in 10^{-6} dilution and lowest was 1.5 $\times 10^8$ cfu/ml in 10^{-5} dilution.

Table 5: Results of total viable counts in PCA agar (18)

Dilution	Number of colony	Result
10^{-1}	More than 300	TNTC
10^{-2}	More than 300	TNTC
10^{-3}	More than 300	TNTC
10^{-4}	280	5.6×10^{7} cfu/ml
10^{-5}	115	2.3×10^{8} cfu/ml
10^{-6}	80	1.6×10^{9} cfu/ml
10^{-7}	43	8.6×10^{9} cfu/ml

Table 6: Results of total viable counts in EMB agar (18)

Dilution	Number of colony	Result
10^{-4}	290	5.8×10^{7} cfu/ml
10^{-5}	130	2.6×10^{8} cfu/ml
10^{-6}	24	4.8×10^{8} cfu/ml
10^{-7}	5	1.0×10^{9} cfu/ml

Table 7: Results of total viable counts in MAC agar (18)

Dilution	Number of colony	Result
10^{-4}	113	2.26×10^{7} cfu/ml
10^{-5}	90	1.8×10 cfu/ml
10^{-6}	50	$1.\times 10^{9}$ cfu/ml
10^{-7}	12	2.4×10^{9}cfu/ml

Table 8: Results of total viable counts in MSA agar

Dilution	Number of colony	Result
10^{-4}	101	2.02×10^{7} cfu/ml
10^{-5}	75	1.5×10^{8} cfu/ml
10^{-6}	41	8.2×10^{8} cfu/ml
10^{-7}	18	3.6×10^{9} cfu/ml

Legends: PCA=Plate count agar, EMB= Eosin-methylene blue, MAC= MacConkey agar, MSA= Manitol salts agar and cfu= Colony forming unit

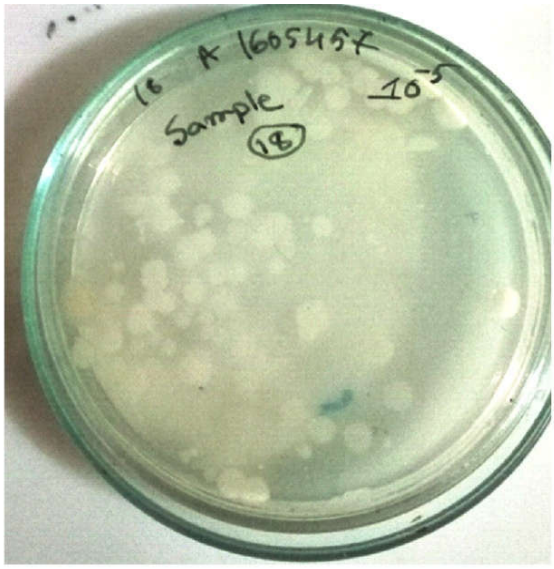


Plate no 6: Colony count on Plate Count Agar plate

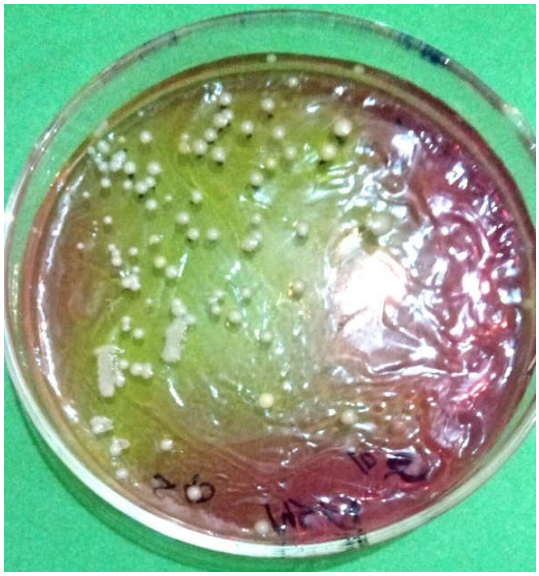


Plate no 7: Colony count on MSA agar plate

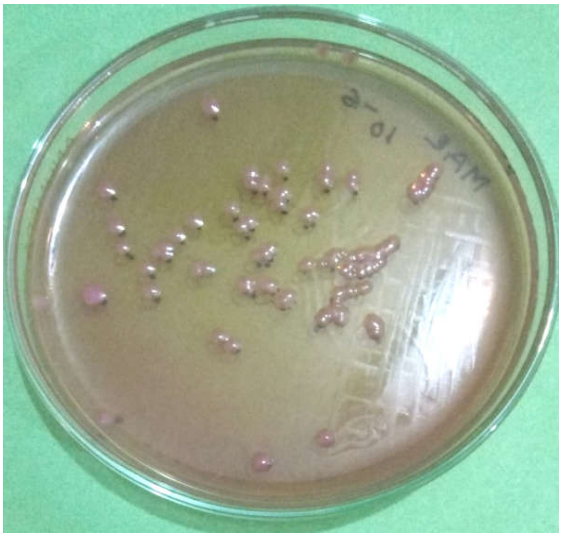


Plate no 8: Colony count on MacConkey agar plate

Plate no 9: Colony count on EMB agar plate

4.1.1 Results of urine sample inoculation on Nutrient agar and Nutrient broth

After inoculation of urine sample on Nutrient agar and Nutrient broth, it was observed that mat growth was found in Nutrient agar and turbidity in Nutrient broth.

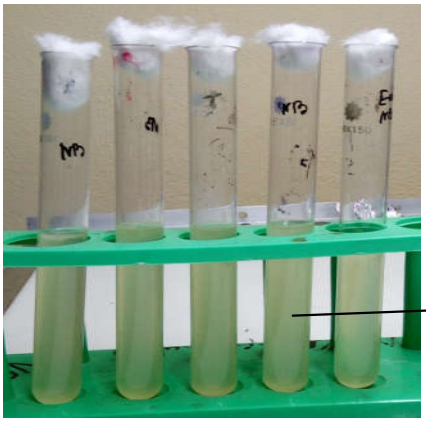


Plate no 10: Organisms show turbidity on nutrient broth

Plate no 11: Organisms on Nutrient agar plate with convex, circular, white colony

4.2 Isolation and identification of bacteria by different bacteriological methods

4.2.1 Results of Cultural Examination

The cultural characteristics of *E. coli, Klebsiella spp, Proteus spp, Pseudomonas spp, Staphylococcus spp*, and *Streptococcus spp* on various media are presented in Table 9.

Table 9. The results of cultural characteristics of the bacteria which were isolated from urine samples of pregnant women

Name of bacteria	Staining characteristic	Name of Media	Colony characteristics
1.E. coli	Gram negative large rod shaped pink color.	Nutrient Agar	Large, mucoid, white colony.
		Mac-Conkey's Agar	Produce large mucoid rose pink colony.
		EMB agar	Transmitted light blue black center with a narrow, clear edge. Blue-green metallic sheen with reflected light.
		Tryptic soy agar	Small white colony.

2. Pseudomonas spp.	Gram negative small rod shaped pink colour	Nutrient agar	Large, smooth, low convex and greenish pigment with fruity odor.
		Mac-Conkey agar	Pale colour flat non lactose fermenting Colonies.
		Cetrimide agar	Colonies are greenish in color.
		Tryptic soy agar	Colonies are large white colour.
3.*Klebsiella spp*	Gram negative rod shaped pink colour.	Nutrient Agar	Large colony.
		Mac-Conkey's Agar	Large, red, mucoid
		EMB agar	Mucoid, no metallic sheen. With transmitted light, gray brown centers and pink color with clear edges.
4.*Proteus spp*	Gram negative small rod shaped pink colour.	Nutrient agar	Smooth. Opaque, translucent white colonies.
		Mac-Conkey agar	Colourless and translucent dewdrop like colonies.
		EMB agar	Pale colour small colonies.
5.*Staphylococcus spp.*	Gram positive cluster liked violet colour.	Nutrient Agar	Black colour/ non-colour smooth, glistening colonies.
		Manitol Salt Agar	Yellow colonies.
		Baired parker agar	Large white colony with yellowish background
		Staphylococcus Agar No.110	Yellow colonies.
6.*Streptococcus spp*	Gram positive short chain formed liked violet colour.	Nutrient agar	Uniform turbidity
		Nutrient broth	Moderate turbidity
		Manitol salt agar	Pink colony
		Blood agar	Bête hemolytic colony
		Mitis salivarius agar	Dark bluish colour colony

4.2.1.1 Nutrient Agar

Nutrient agar plates spread with the samples revealed the growth of bacteria after 24 hours of incubation at 37^{o}C aerobically and were indicated by the growth of circular, small smooth, convex, and gray white or yellowish colonies.

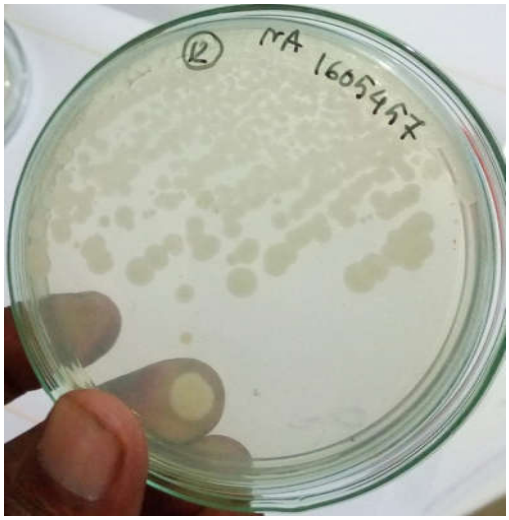


Plate no 12: Culture of organism on Nutrient Agar plate with circular, small smooth, gray white colonies

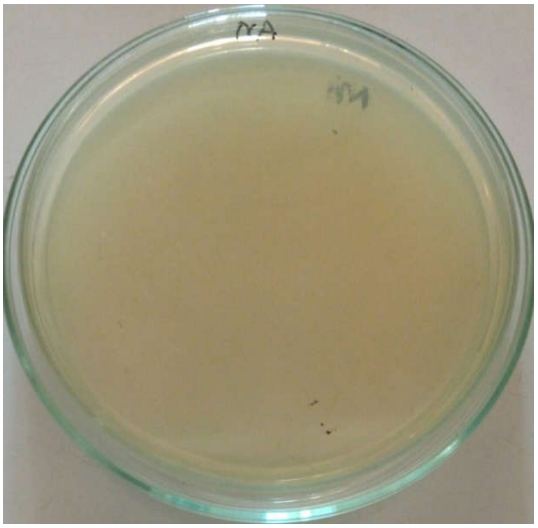


Plate no 13: Fresh Nutrient agar plate

4.2.1.2 Mac-Conkey Agar

Mac-conkey agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37° C aerobically and were indicated the colorless colonies after prolonged incubation pink color and colorless colonies.

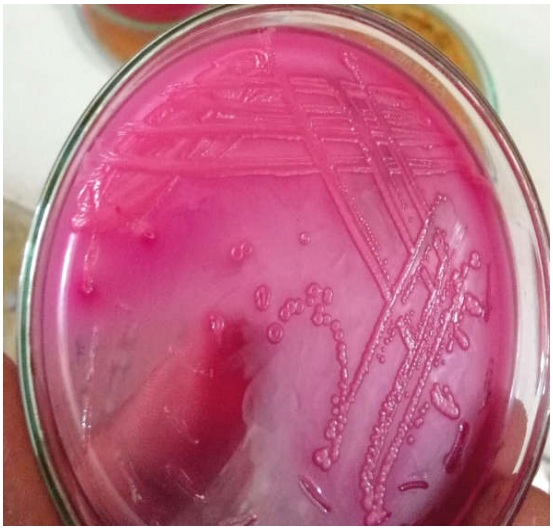


Plate no 14: *E. coli* on Mac-Conkey agar plate with pink color colony

Plate no 15: *Klebsiella spp* on Mac-Conkey agar plate with bright pink colony

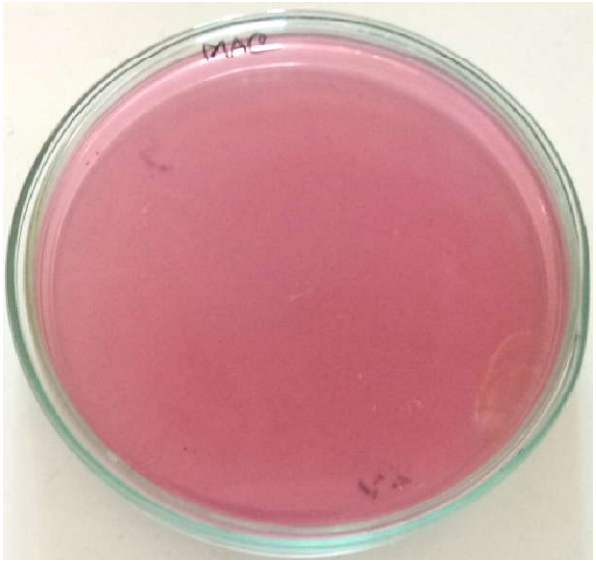


Plate no 16: Fresh MacConkey agar plate

4.2.1.3 Eosin Methylene Blue agar

Eosin methylene blue agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37^{0}C aerobically and were indicated by the growth of smooth, circular , black color center with blue-green metallic sheen and gray-brown center with pink color colonies.

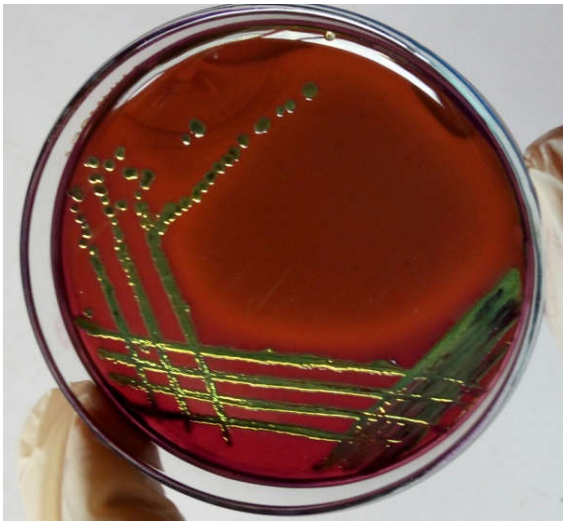


Plate no 17 *E. coli* on EMB agar plate with metallic sheen colony

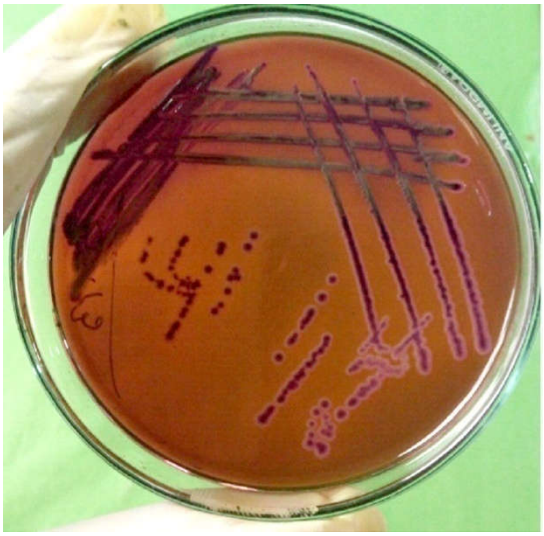


Plate no 18: *Klebsiella spp* on EMB agar plate with gray-brown center with pink color colonies.

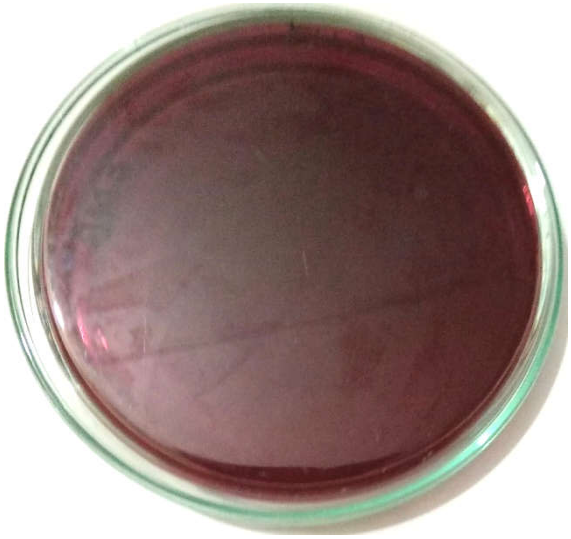


Plate no 19: Fresh Eosin Methylene Blue agar plate

4.2.1.4 Salmonella-Shigella Agar

Salmonella-shigella agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37^{0}C aerobically and were indicated by transparent colonies, pink color colony.

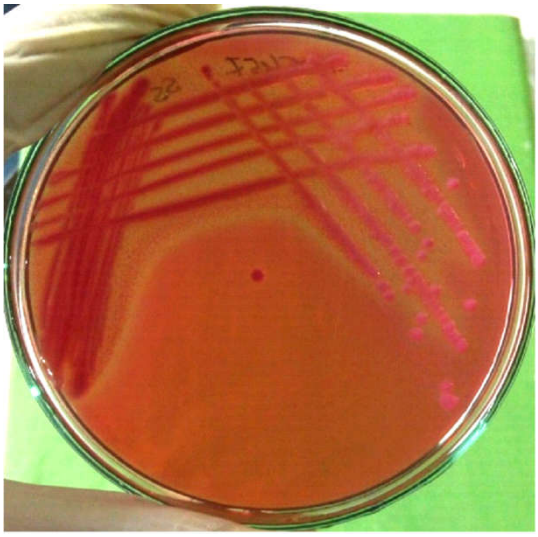


Plate no 20: *E. coli* on Salmonella-Shigella Agar plate with rose pink colony

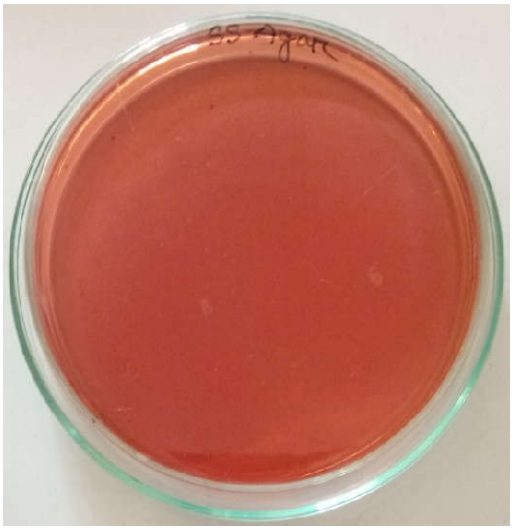


Plate no 21: Fresh Salmonella-Shigella Agar plate

4.2.1.5 Cetrimide Agar

Cetrimide agar streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37^{0}C aerobically and observed greenish color colonies.

Plate no 22: *Pseudomonas spp* on Cetrimide Agar plate with greenish colony

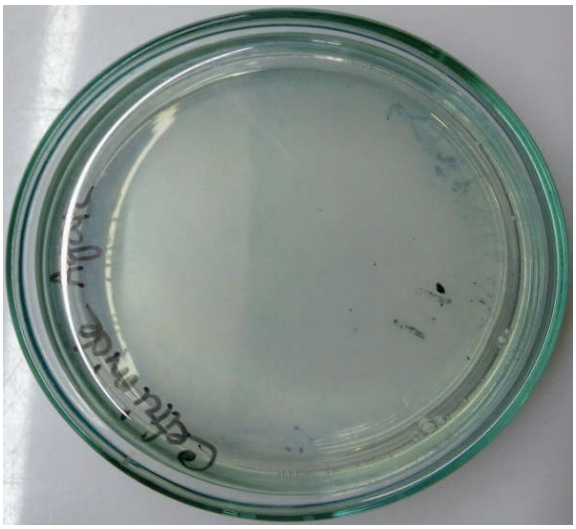


Plate no 23: Fresh Cetrimide Agar plate

4.2.1.6 Manitol Salt Agar

Manitol salt agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37^{o}C aerobically and were indicated by the pink and yellow color colonies.

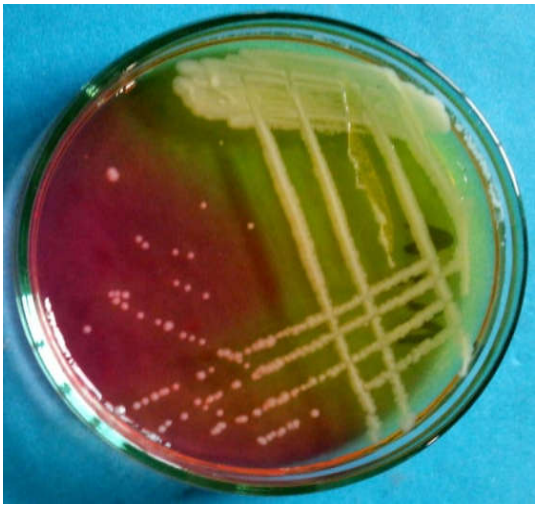


Plate no 24: *Staphylococcus spp* on Manitol Salt Agar plate with yellow colony

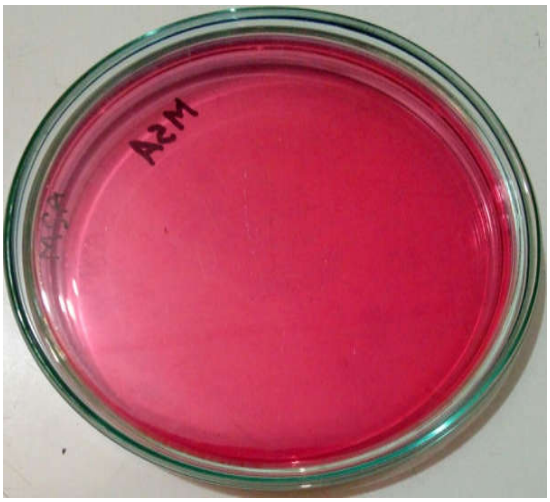


Plate no 25: Fresh Manitol Salt Agar plate

4.2.1.7 Mitis salivarius agar

Mitis salivarius agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37^{o}C aerobically and were indicated by the purple or dark bluish color colonies.

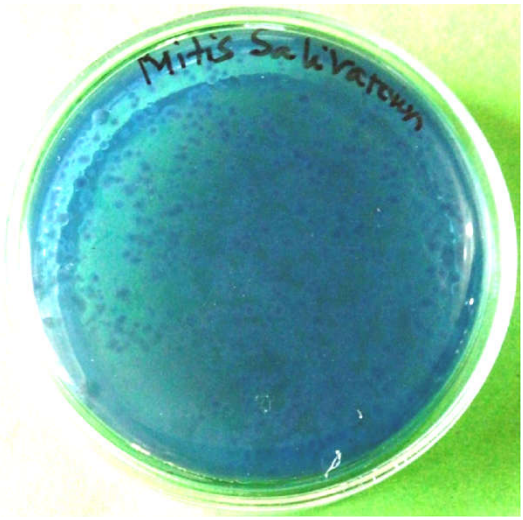


Plate no 26: *Enterococcus spp on Mitis salivarius* agar plate with bluish colony

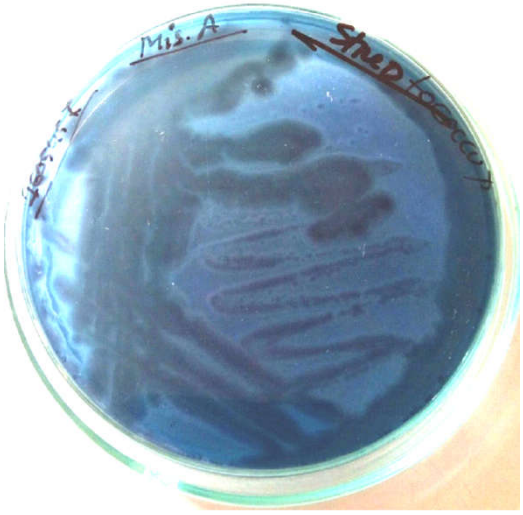


Plate no 27: *Streptococcus spp Mitis salivarius* agar plate with dark blue colony

4.2.1.8 Blood agar

Blood agar plates streaked separately with the *Staphylococcus spp* & *Streptococcus spp* and incubated at 37^{o}C aerobically for 24 hours and observed β-hemolytic colonies.

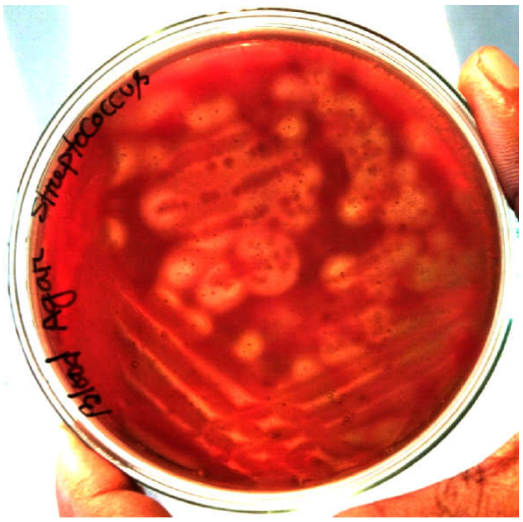


Plate no 28: *Streptococcus spp* on Blood agar plate with β-hemolytic colonies.

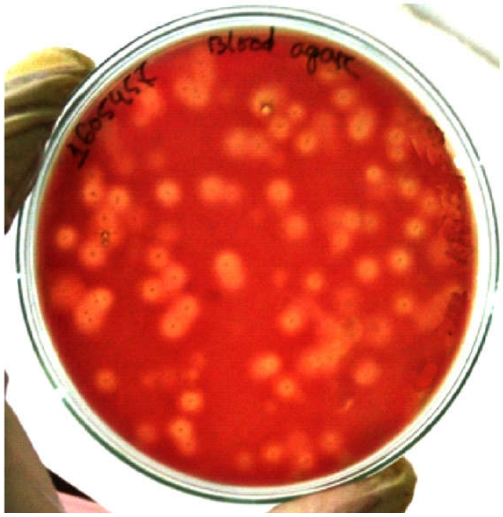


Plate no 29: *Staphylococcus spp* on Blood agar plate with β-hemolytic colonies.

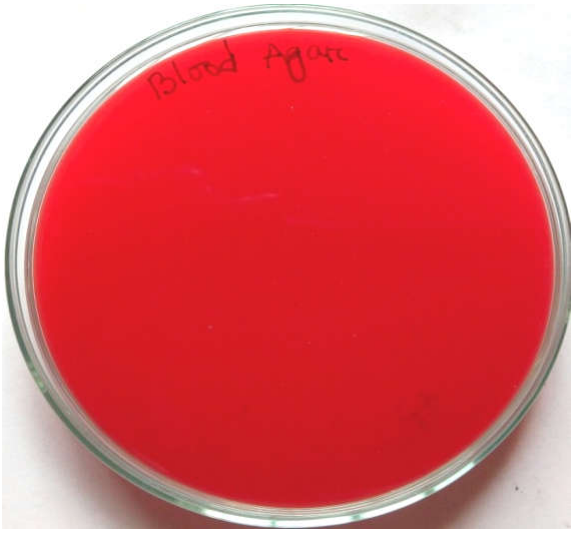


Plate no 30: Fresh Blood agar plate

4.2.1.9 *E. coli* on Nutrient agar, MacConkey agar, EMB agar and TSA agar.

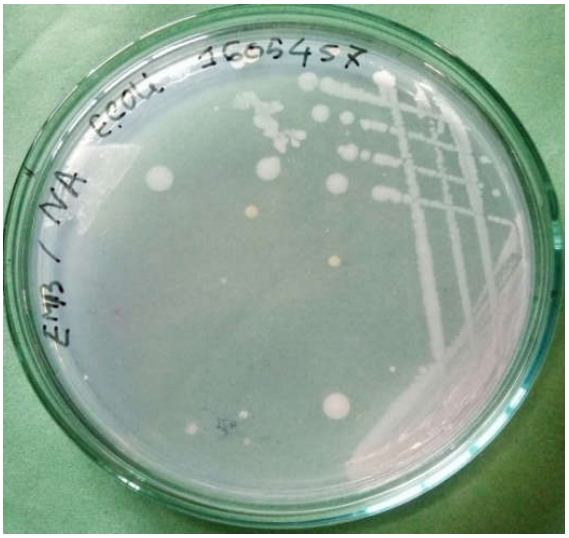


Plate no 31: *E. coli* on Nutrient agar plate with white colony.

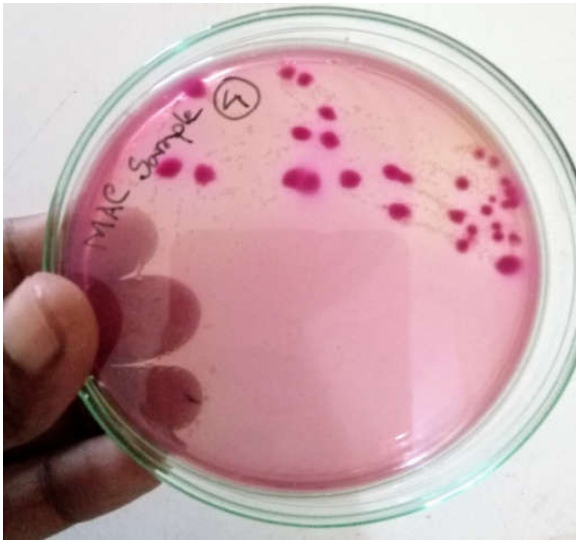


Plate no 32: *E. coli* on MacConkey agar plate with pink colony.

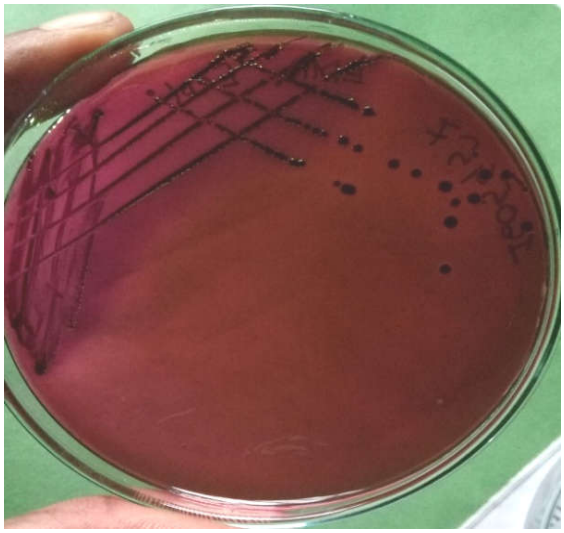


Plate no 33: *E. coli* on EMB agar plate with metallic seen.

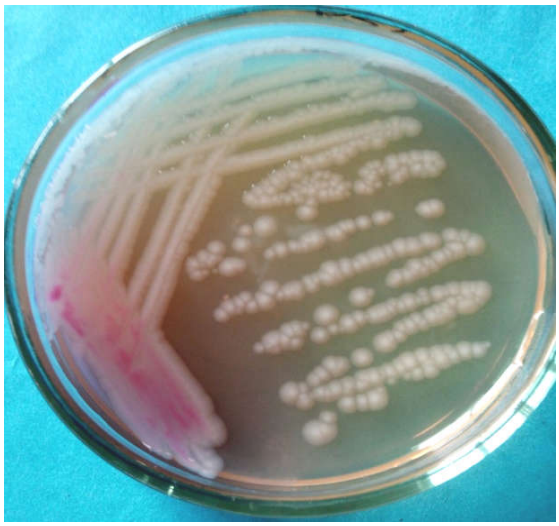


Plate no 34: *E. coli* on TSA agar plate with white colony.

4.2.1.10 *Klebsiella spp* on Nutrient agar, MacConkey agar, EMB agar and Blood agar.

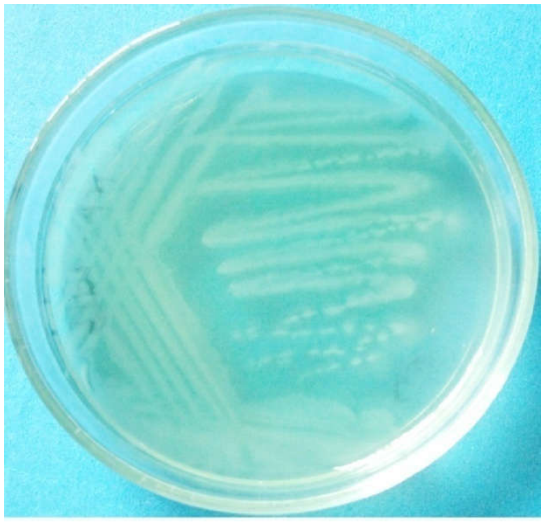


Plate no 35: *Klebsiella spp* on Nutrient agar plate with white colony

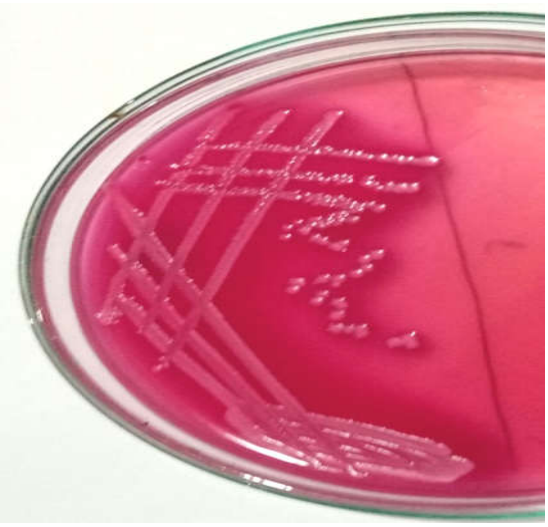


Plate no 36: *Klebsiella spp* on MacConkey agar plate with red, mucoid colony

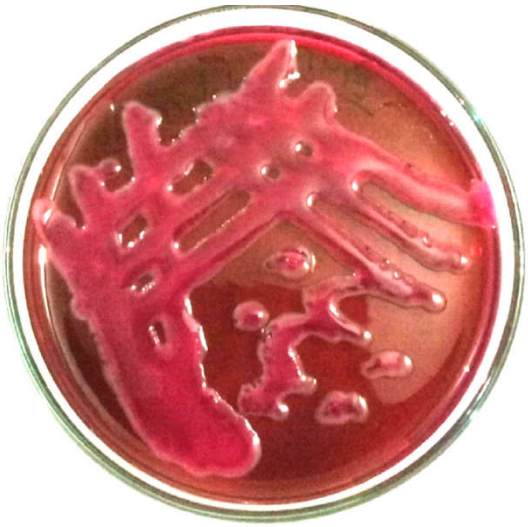


Plate no 37: *Klebsiella ssp* on EMB agar plate with Mucoid, no metallic sheen

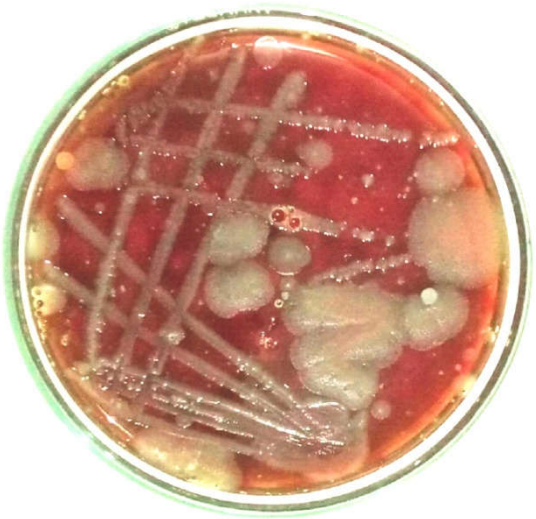


Plate no 38: *Klebsiella spp* on Blood agar plate with non hemolytic colony

4.2.1.11 *Pseudomonas spp* on Nutrient agar, MacConkey agar, Cetrimide agar and Tryptic soy agar.

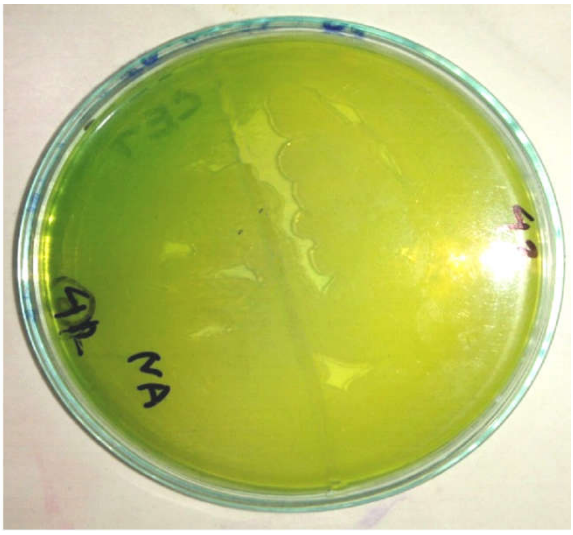


Plate no 39: *Pseudomonas spp* on Nutrient agar plate with large smooth greenish colony

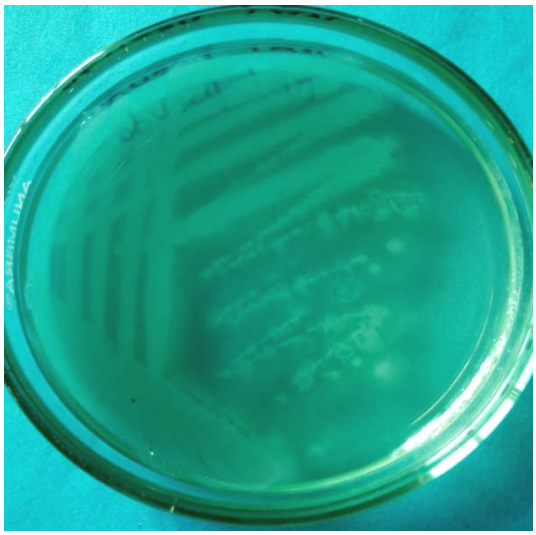


Plate no 40: *Pseudomonas spp* on Cetrimide agar plate with greenish colony

Plate no 41: *Pseudomonas spp* on Tryptic soy agar plate with large white colony

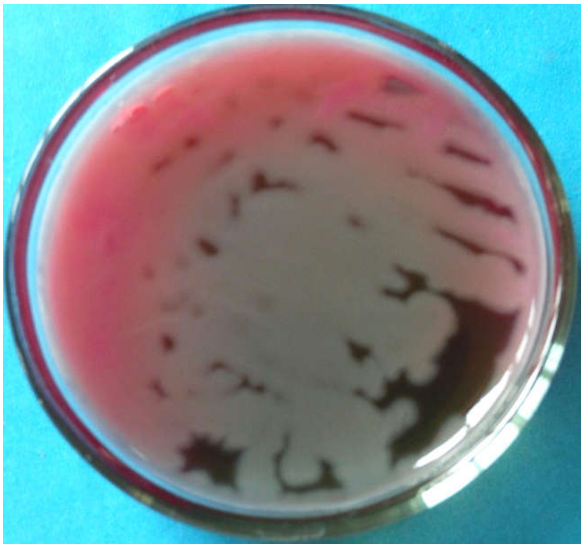


Plate no 42: *Pseudomonas spp* on MacConkey agar plate with pale color colony

4.2.1.12 *Staphylococcus spp* on Nutrient agar, MSA agar, Tryptic soy agar, Baired parker agar

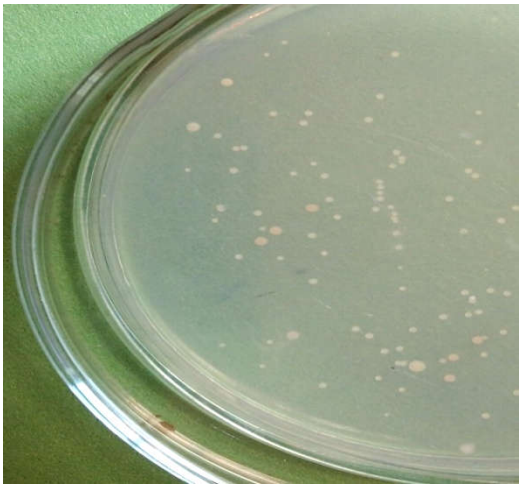


Plate no 43: *Staphylococcus spp* on Nutrient agar plate with white & yellow colony

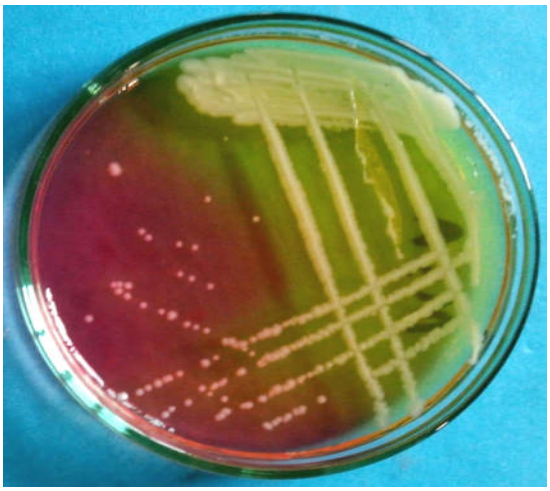


Plate no 44: *Staphylococcus spp* on MSA agar plate with yellow colony

Plate no 45: *Staphylococcus spp* on Tryptic soy agar plate with yellow colony

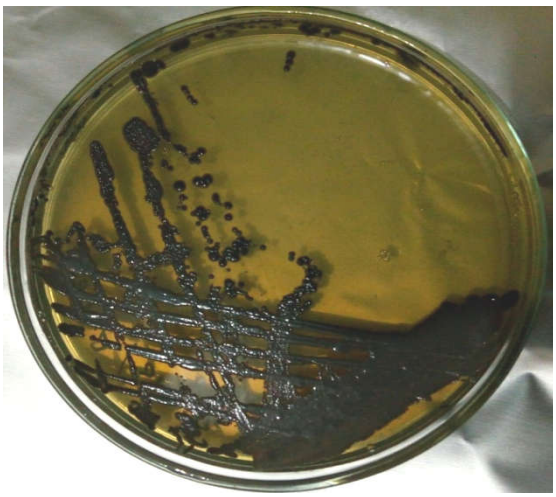


Plate no 46: *Staphylococcus spp* on Baird parker agar plate with black colony

4.2.1.13 *Staphylococcus spp* on Nutrient agar, MSA agar, Tryptic soy agar and Blood agar.

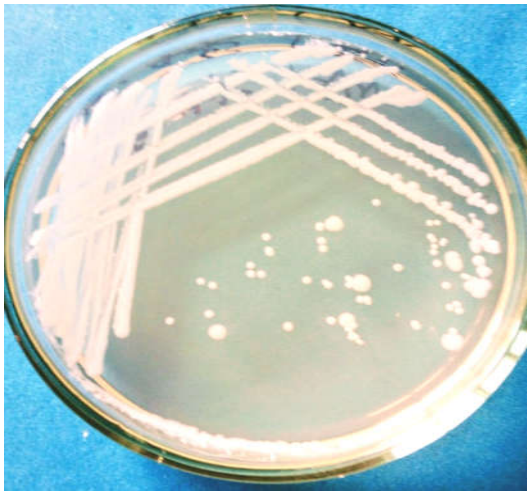


Plate no 47: *Staphylococcus spp on* Nutrient agar plate with white colony

Plate no 48: *Staphylococcus spp* on MSA agar plate with pink colony

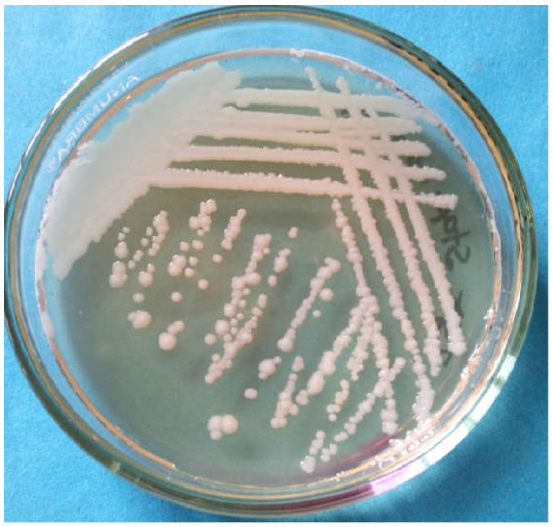


Plate no 49: *Staphylococcus spp* on Tryptic soy agar plate with translucent colony

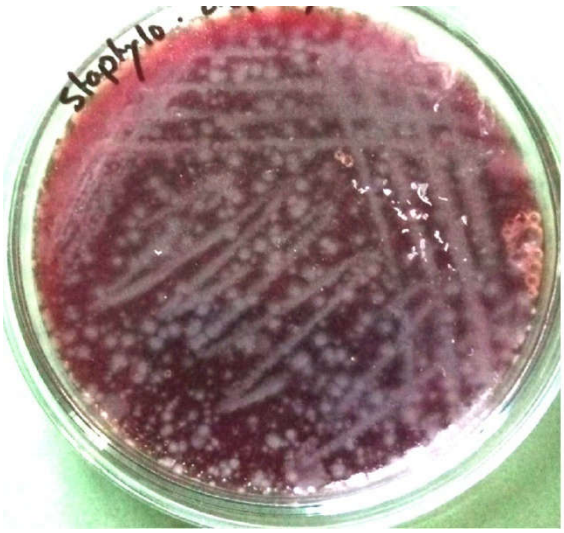


Plate no 50: *Staphylococcus spp* on Blood agar plate with alpha hemolytic colony

4.2.1.14 *Proteus spp* on Nutrient agar, MacConkey agar and EMB agar

Plate no 51: *Proteus spp* on MacConkey agar plate with Colourless colonies

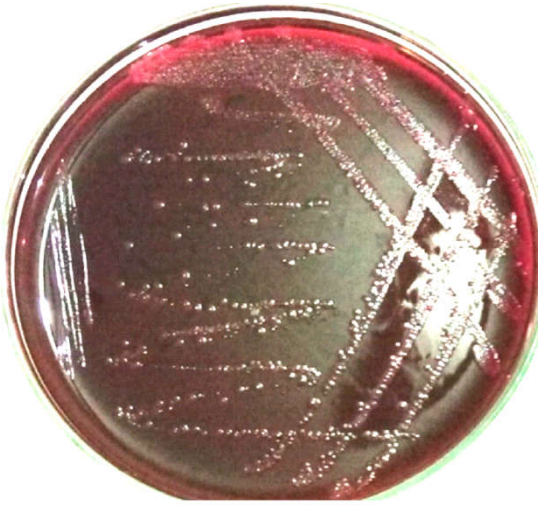


Plate no 52: *Proteus spp* on EMB agar plate with pale color single colony

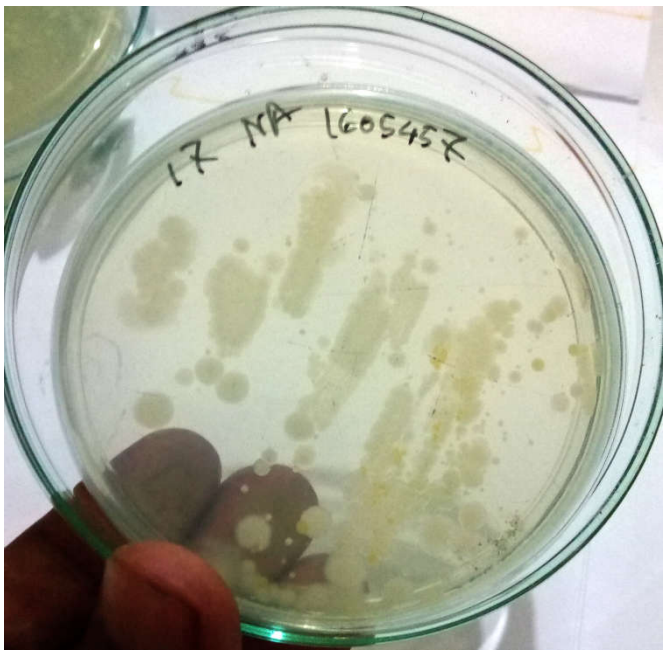


Plate no 53: *Proteus spp* on Nutrient agar plate with white colonies

4.2.1.15 *E. coli* and *Klebsiella spp* on EMB agar; *Klebsiella spp* and *Pseudomonas spp* on MacConkey agar, *Staphylococcus spp* on MSA agar and *Pseudomonas spp* and *Staphylococcus spp* on Nutrient agar.

Plate no 54: *E. coli* (up) and *Klebsiella spp (down)* on EMB agar plate

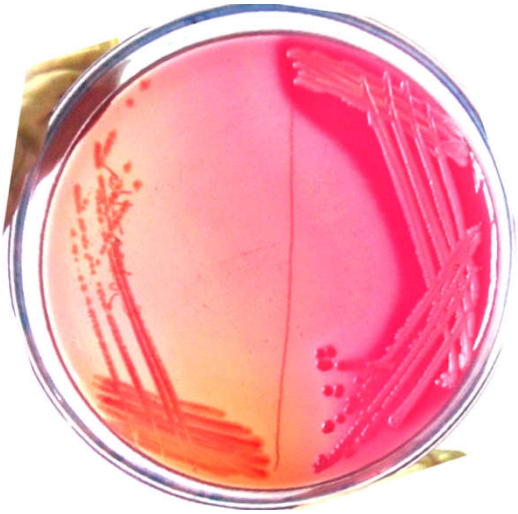


Plate no 55: *Pseudomonas spp* (left) & *Klebsiella spp* (right) on MacConkey agar plate

Plate no 56:*Stapylococcus spp* on MSA agar Plate

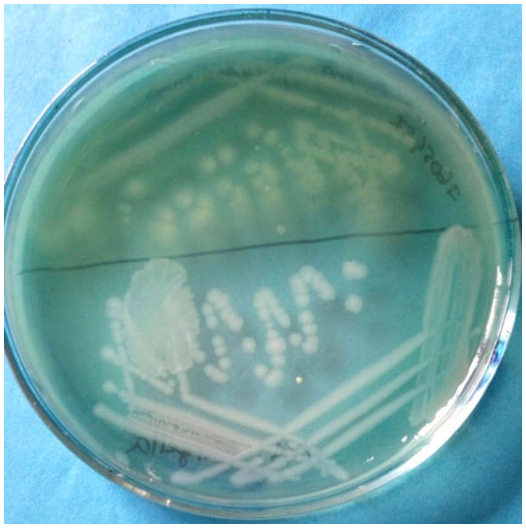


Plate no 57: *Pseudomonas spp* and *Staphylococcus spp* on Nutrient agar plate

4.2.2 Results of Microscopic Examination

Microscopic observation was performed to observe the morphology of the isolates. Under microscopy both gram positive and negative isolates were found. Gram negative isolates were curved, slender, rod shape and also showed pink colour colonies. Gram positive isolates were grape like cluster and spherical chain shape and showed purple coloured colonies under 100X microscopy.

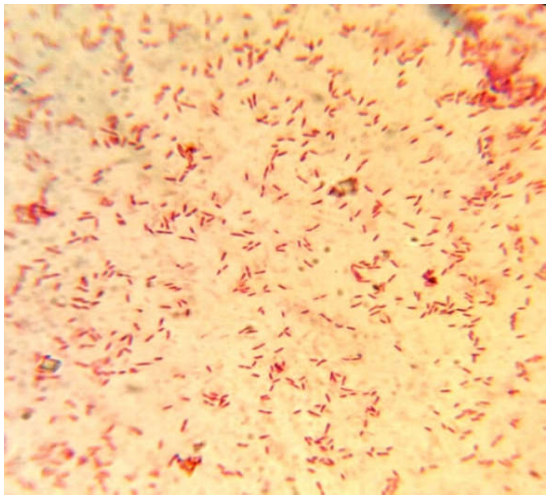


Plate no 58: *E.coli* showing Gram negative (pink colour) rod shape under 100X microscopy

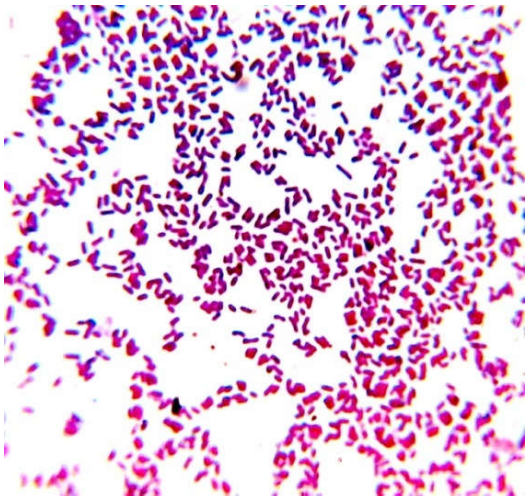


Plate no 59: *Klebsiella spp* showing Gram negative (pink colour) rod shape under 100X microscopy

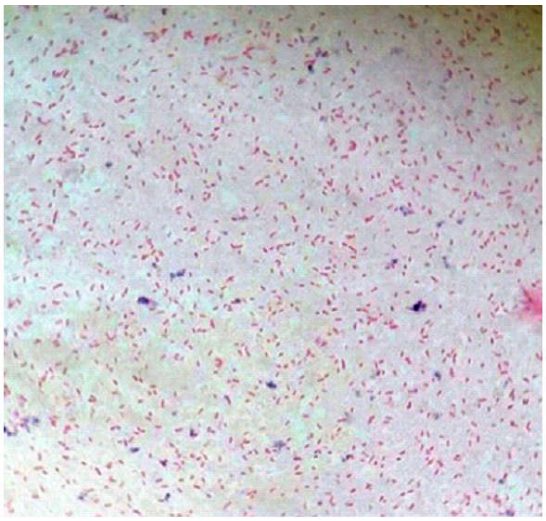


Plate no 60: *Pseudomonas spp* showing Gram negative (pink colour) small rod shape under 100X microscopy

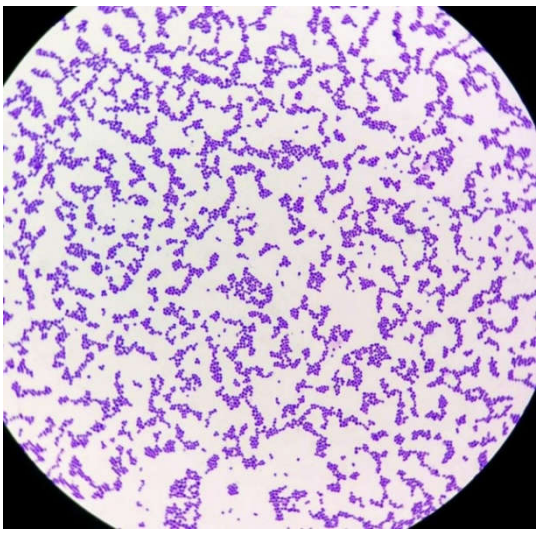


Plate no 61: *Staphylococcus spp* showing Gram positive (violet colour) cocci arranged in group or grape like clusters at 100X microscopy

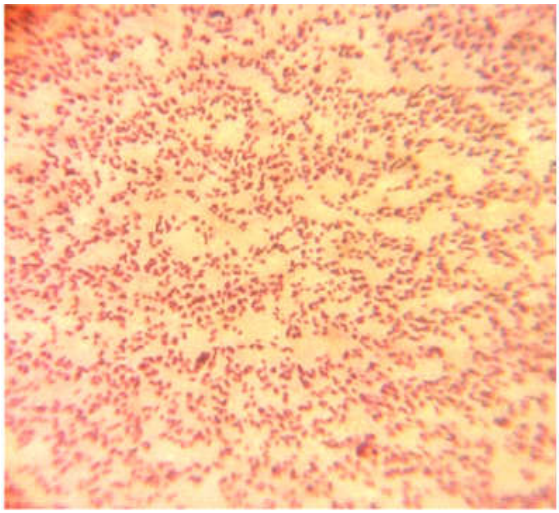


Plate no 62: *Proteus spp* showing Gram negative Pink colour small rod shape under 100X microscopy

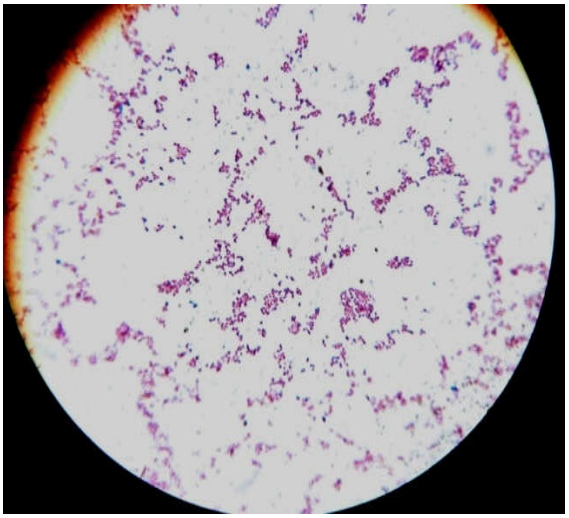


Plate no 63: *Streptococcus spp* showing Gram positive purple colour short chain under 100Xmicroscopy

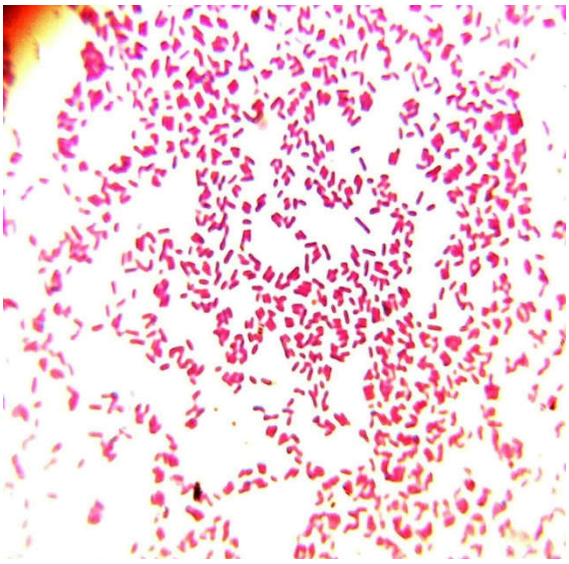


Plate no 64: *E. coli* showing large rod shape Under 100X microscopy

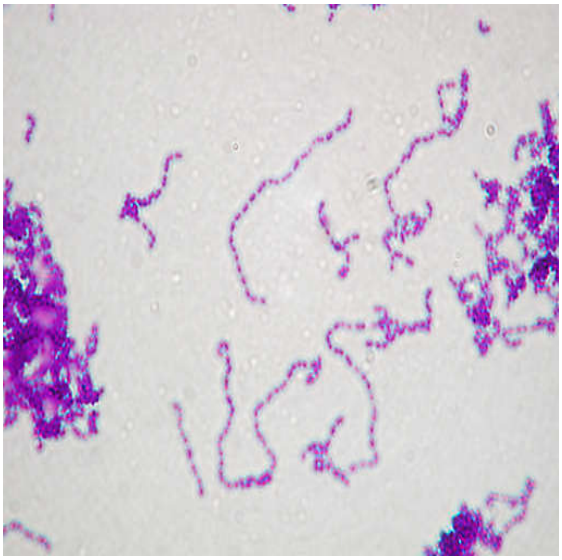


Plate no 65: *Streptococcus spp* showing long chain under 100X microscopy

4.2.3 Results of Biochemical Tests

Biochemical test was performed to confirm the bacteria that cause Urinary Tract Infection (UTI) in pregnant women. After seen the colony characters on different media and also gram stain I selected 6 identify bacteria for biochemical test. Following table showed the biochemical test results.

Table no 10: Results of Biochemical Tests

Name of isolates	OX	CT	IN	MR	VP	SC	TSI		MIU		
							slant	Butt	M	I	U
E. coli	-	+	+	+	-	-	A(yellow)	A(yellow)	+	+	+
Klebsiella spp	-	+	+	-	+	+	K(red)	A(yellow)	+	-	+
Pseudomonas spp	+	+	+	-	-	+	K(red)	K(red)	+	+	+
Proteus spp	-	-	+	+	-	-	A(yellow)	A(yellow)	+	+	+
Staphylococcus spp	-	+	+	+	+	+	A(yellow)	AG	-	-	-
Streptococcus spp	-	-	-	-	+	+	A(yellow)	NC	+	-	+

Legends:+ = positive, - = negative, A=acid, K= alkaline, G= gas, NC= no color change

OX= oxidase, CT=catalase, IN= indole, MR= methyl-red, VP= voges-proskauer, SC= simmon's citrate, TSI= triple sugar iron, MIU= motility indole urease

4.2.3.1Oxidase Test

E.coli; Klebsiella spp; Proteus spp; Staphylococcus spp & Streptococcus spp were negative and *Pseudomonas* spp was positive result for oxidase test.

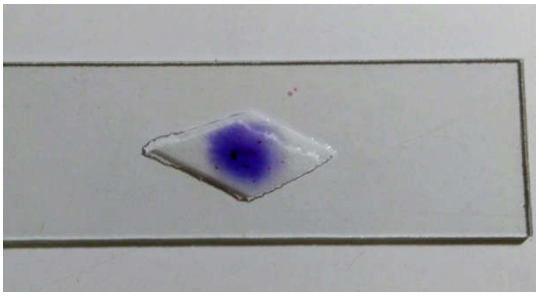


Plate no 66: Oxidase positive *Pseudomonas spp*

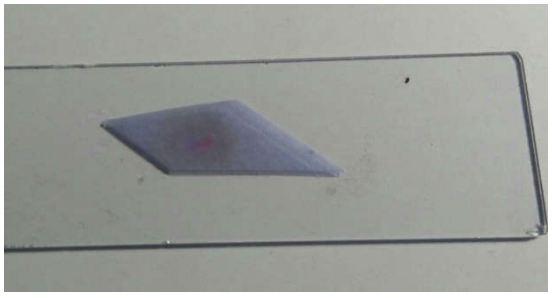


Plate no 67: Oxidase negative *E. coli*

4.2.3.2 Catalase Test

E.coli; Klebsiella spp; Proteus spp; Pseudomonas spp & *Staphylococcus spp* were positive and *Streptococcus spp* is negative result for Catalase test.

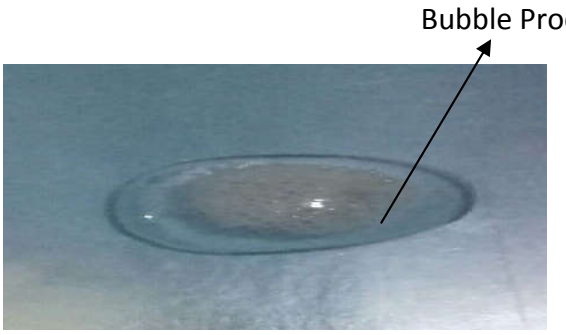


Plate no 68: Catalase positive *Staphylococcus spp*

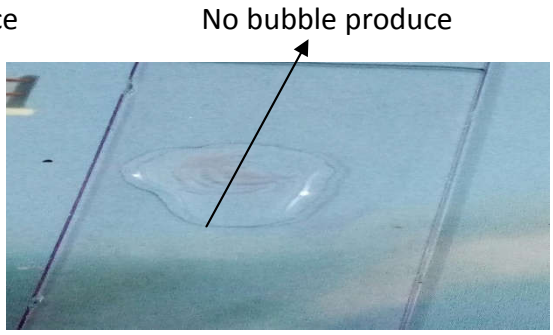


Plate no 69: Catalase negative *Streptococcus spp*

4.2.3.3 Methyl- Red Test

E.coli; Proteus spp & *Staphylococcus spp* were positive results by indicated red coloration and *Klebsiella spp; Pseudomonas* spp *& Streptococcus spp* were negatives result by indicated no red coloration for Methyl- Red test.

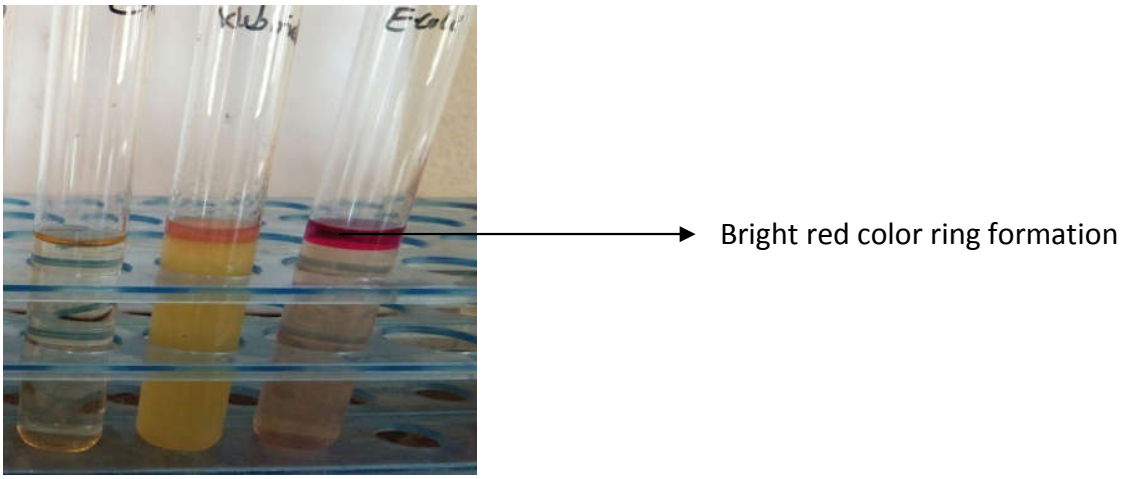


Plate no 70: Methyl-Red test was positive result indicated by the changed of medium to bright red colour inoculated (right), negative result indicated by the no change bright red colour (middle) and left is control.

4.2.3.4 Voges-Proskaur Test

Klebsiella spp; Proteus spp; Staphylococcus spp & *Streptococcus spp* were positive results by indicated rose coloration and *E.coli; Pseudomonas* spp were negative results by indicated no rose coloration for Voges-Proskaur test.

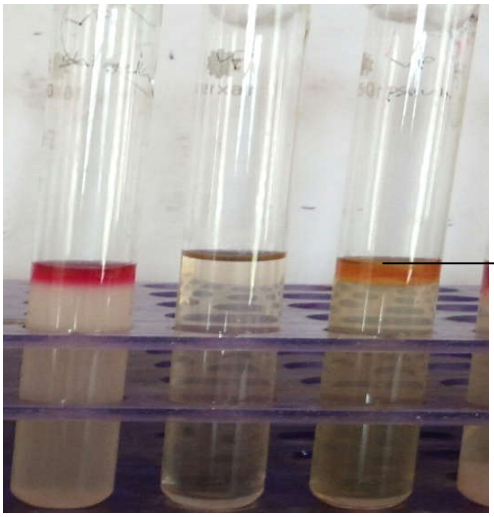


Plate no 71: Voges - Proskauer test indicated positive result by the changed of medium to rose red colour inoculated (left); control (middle) and negative result indicated by the no change to rose red colour (right).

4.2.3.5 Indole Test

E.coli & Proteus spp were positive results by indicated cherry red coloration and *Klebsiella spp; Pseudomonas* spp; *Staphylococcus spp & Streptococcus spp* were negative results by indicated no cherry red coloration for indole test.

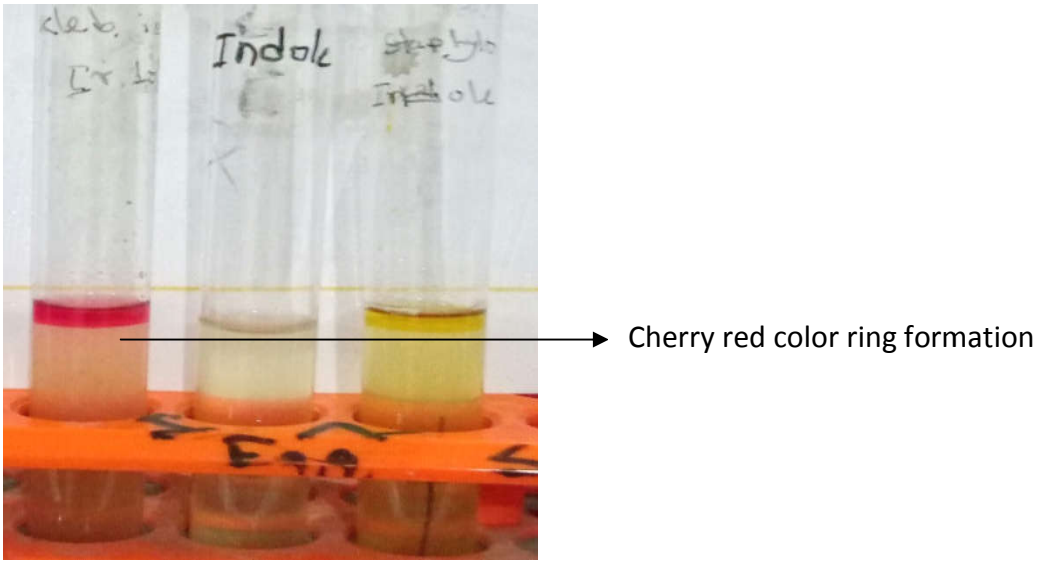


Plate no 72: Indole test showing positive result by red coloration of the medium colour inoculated (left); control (middle) and negative result indicated by the no red coloration of the medium (right).

4.2.3.6 Simmons Citrate Test

Klebsiella spp; Salmonella spp; Proteus spp & Pseudomonas spp were positive results by changed medium green to blue coloration and *E.coli; Staphylococcus spp & Streptococcus spp* were negative results by no changed medium green to blue coloration for simmons citrate test.

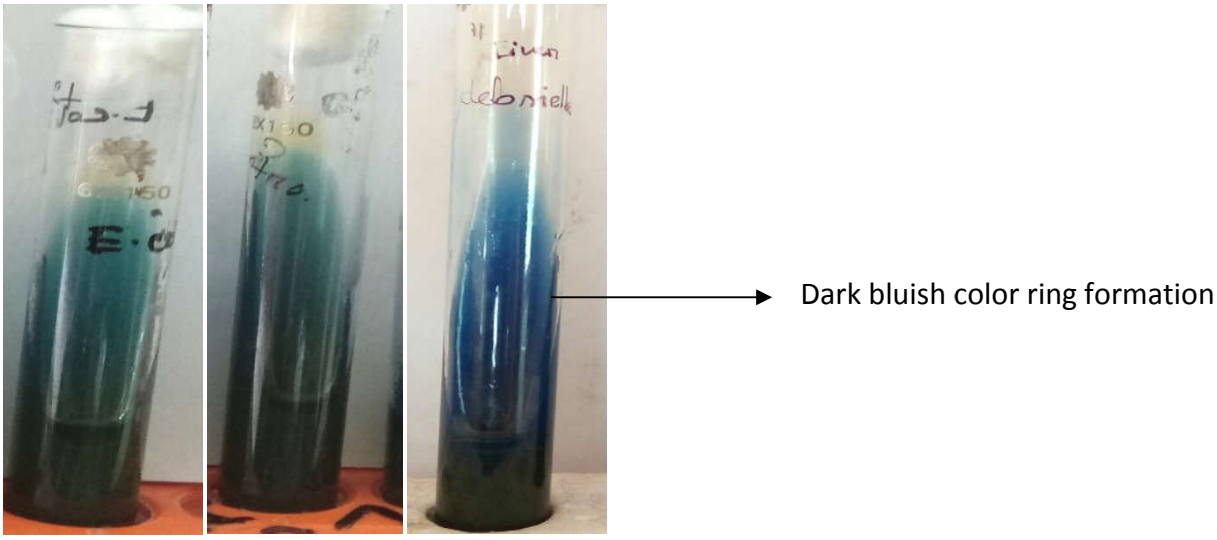


Plate no 73: Simmons citrate test showing positive result (right), negative result (left) and control middle.

4.2.3.7 TSI (Triple Sugar Iron) Test

E.coli & Klebsiella spp were showed yellow coloration both butt and slant. *Proteus spp; Staphylococcus spp & Streptococcus spp* were showed yellow coloration for butt and red coloration for slant. Only *Pseudomonas* spp was showed red coloration for both butt and slant.

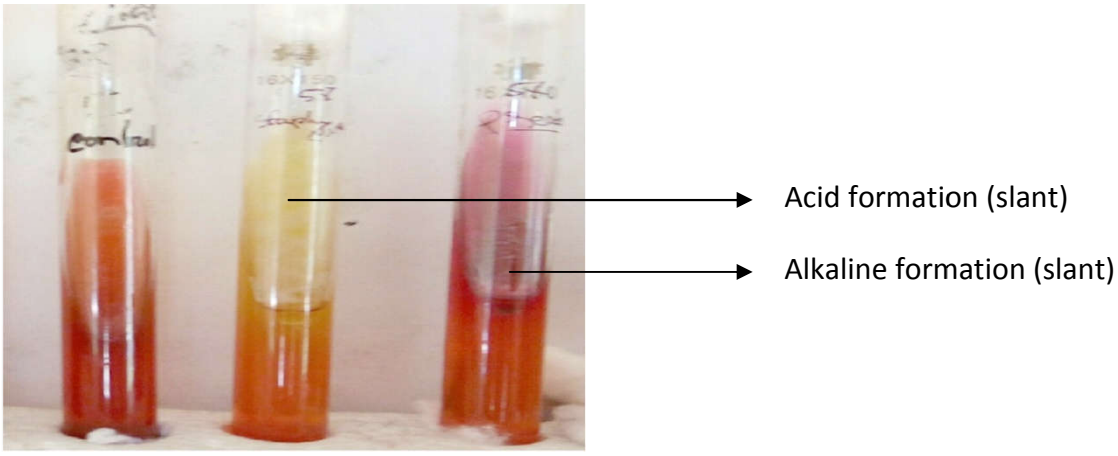
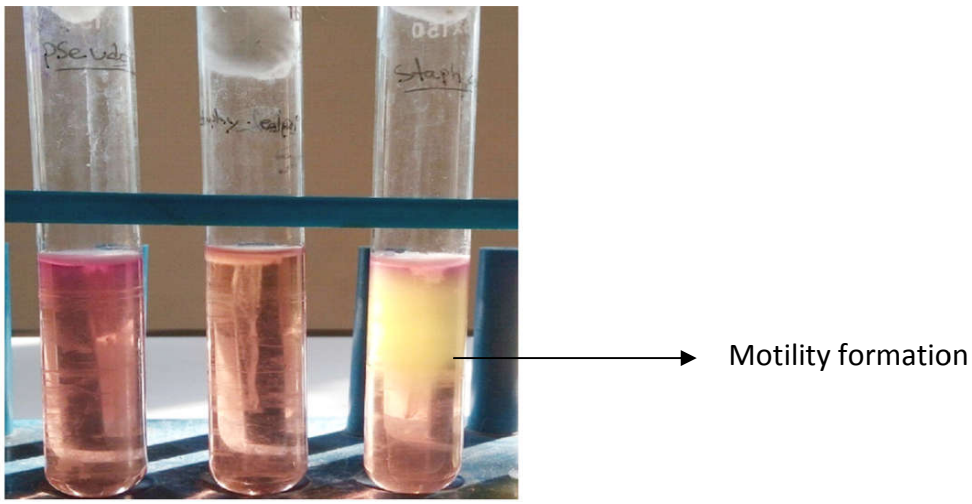


Plate no. 74: Triple Sugar Iron test show control (left), positive middle and negative (right)

4.2.3.8 MIU (Motility Indole Urease) Test

E.coli; Proteus spp; Pseudomonas spp; *Staphylococcus spp* & *Streptococcus spp* were positive results by medium changed to colorless & yellow coloration and *Klebsiella spp* was negative results by medium no changed to colorless & yellow coloration for MIU test.



Negative Control Positive

Plate no 75: Motility Indole Urease test showing negative (left), control (middle) and positive (right).

Plate no 76: Result of indole test

A= *Pseudomonas spp*, B= *Klebsiella spp*, C= Control, D= *E. coli*, E= *Proteus spp*, F= *Streptococcus spp* and G= *Staphylococcus spp*

D, E, F, G are Indole positive with cherry red color ring formation

Plate no 77: Results of Methyl –Red test

A= *Klebsiella spp*, B= *Proteus spp*, C= *Streptococcus spp*, D= *Pseudomonas spp*, E =Control, F= *Staphylococcus spp* and G= *E. coli*

B, F, G are Methyl red positive with red color ring formation

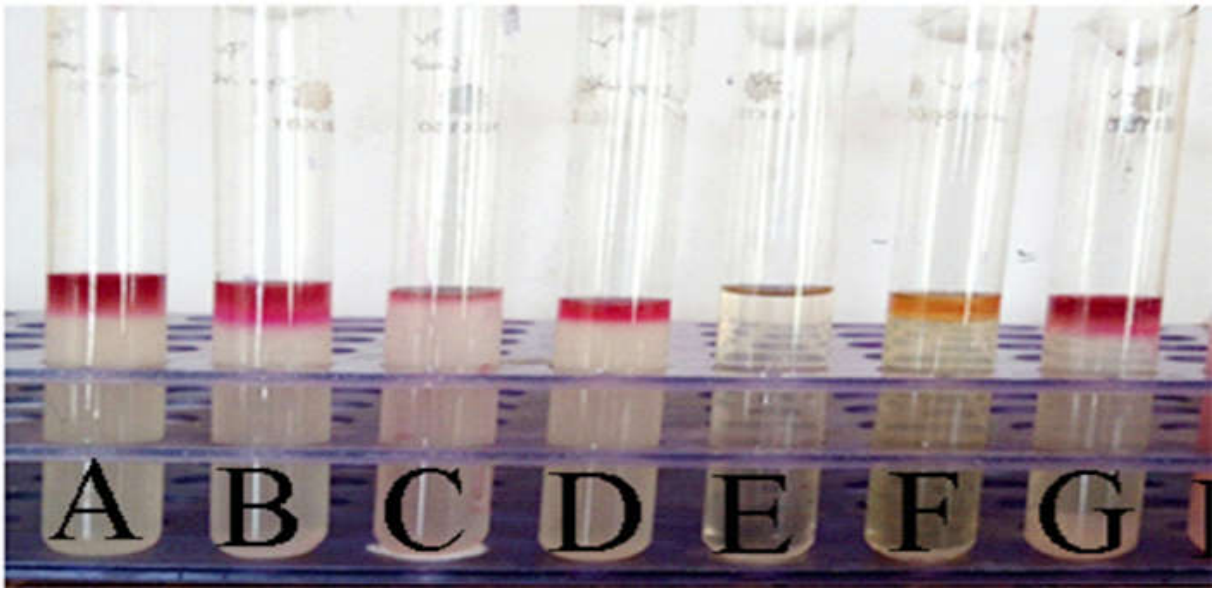


Plate no 78: Result of Voges-proskaeur test

A= *Klebsiella spp*, B= *Staphylococcus spp,* , C= *E. coli*, D= *Streptococcus spp,* E =Control, F= *Pseudomonas spp* and G= *Proteus spp*

A, B, D, G are Voges proskaeur red positive with red color ring formation

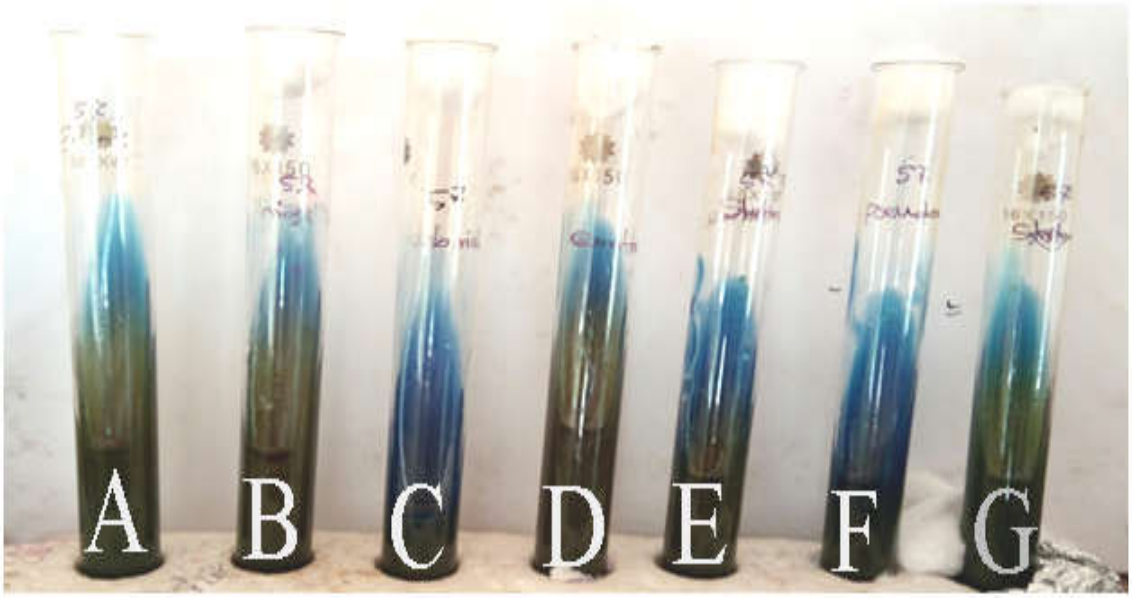


Plate no 79: Result of Simmons citrate test

A= *E. coli*, B= *Proteus spp*, , C= *Klebsiella spp*, D= Control, E =*Streptococcus spp*, F= *Pseudomonas spp* and G= *Staphylococcus spp*

C, E, F, G are Simmons Citrate positive with dark blue color formation

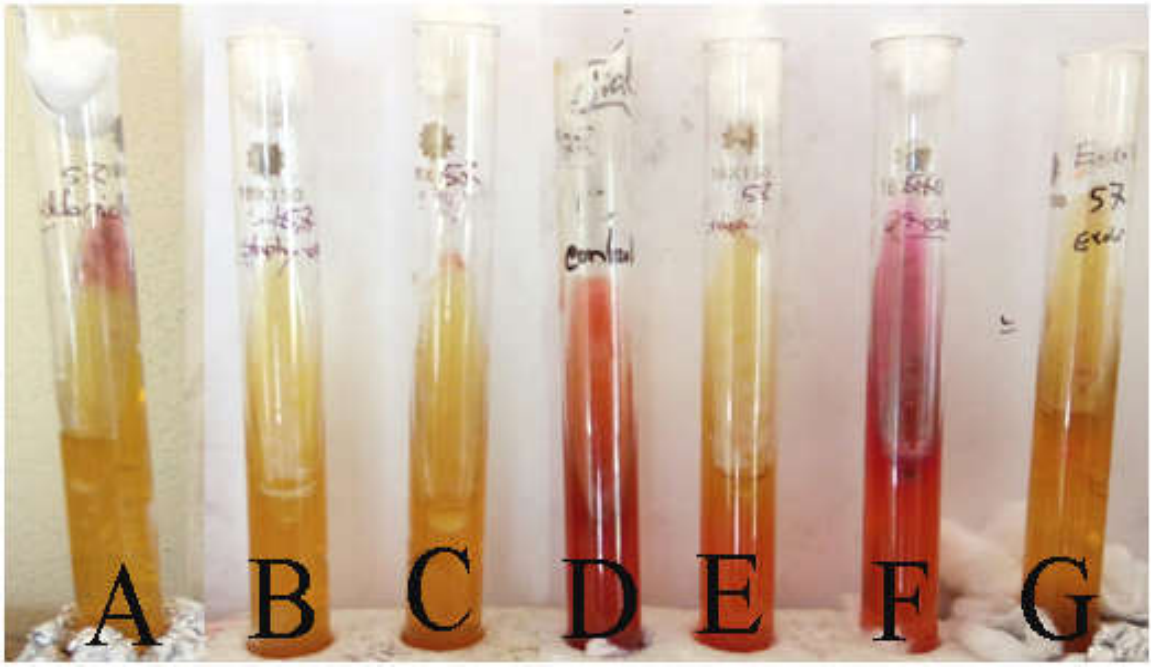


Plate no 80: Result of Triple Sugar Iron agar

A= *Klebsiella spp*, B= *Staphylococcus spp*, , C= *Proteus spp*, D= Control, E =*Streptococcus spp*, F= *Pseudomonas spp* and G= *E. coli*

B, C, G are Triple Sugar Iron positive with yellow color (acid) formation on slant and butt, F is TSI negative with red color (alkaline) on slant and butt.

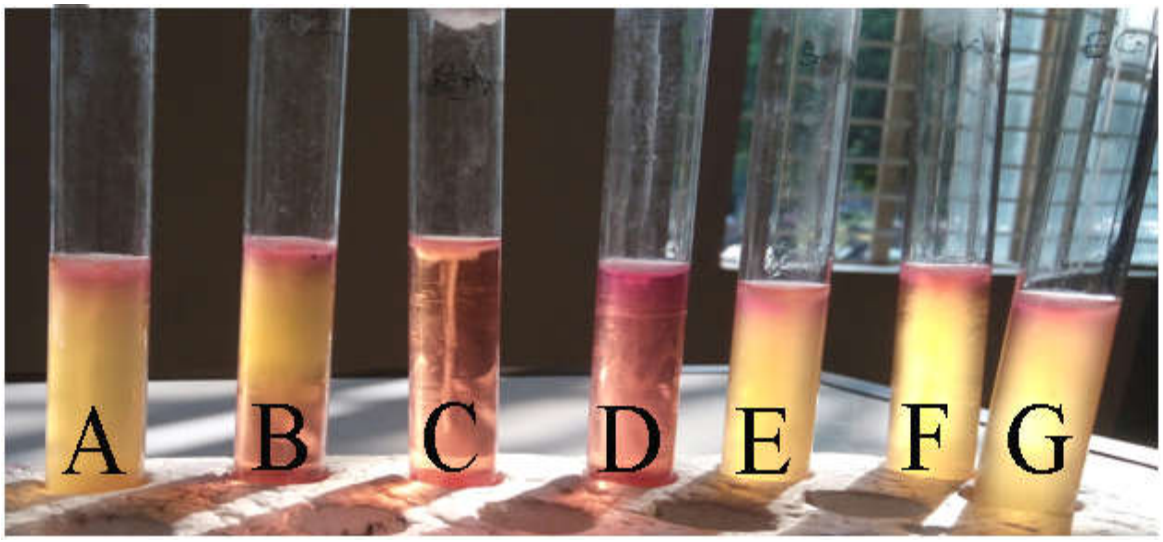


Plate no 81: Result of Motility Indole Urease Test

A= *Proteus spp*, B= *Streptococcus spp,* , C= *Staphylococcus spp*, D= Control, E =*Pseudomonas spp*, F= *Klebsiella spp* and G= *E. coli*

A, B, E, F, G are MIU positive with turbidity, yellow color and red color ring formation.

4.3 Result of PCR Amplification, Sequencing of 16S rRNA Genes with Universal Primers and Phylogenetic Analysis of *Escherichia coli*

Out of 33 positive cases *E.coli* was present in 30 cases and the percentage was very high (40%) other than all identified isolates. 16S rRNA gene region was amplified with the universal primers, Forward primer- 27F (5'AGAGTTTGATCCTGGCTCAG 3') Reverse primer- 1492R (5' TACCTTGTTACGACTT 3'). PCR Amplification band was found at 1470bp.

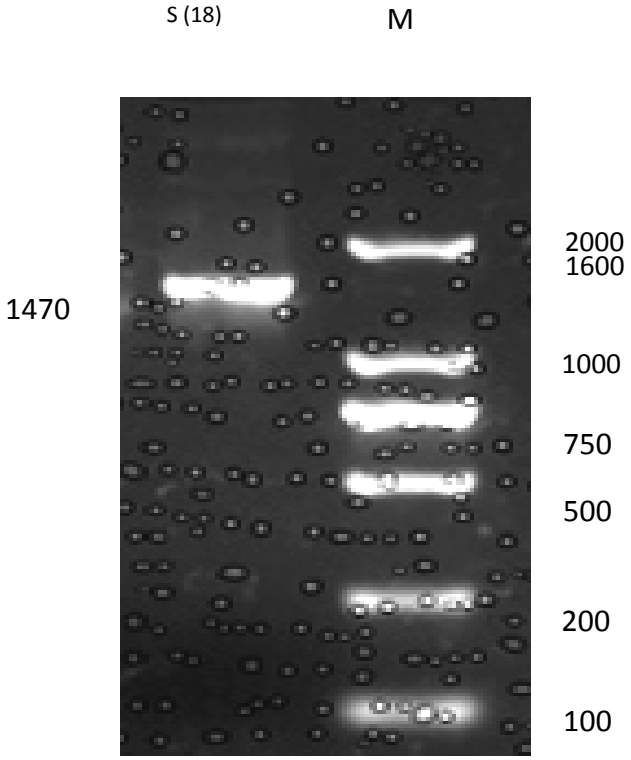


Fig. 3: Result of amplification of 16S rRNA gene region of *E.coli* by PCR.

M: Marker, 2kb DNA ladder

Note: PCR= Polymerase Chain Reaction, kb= kilo base.

4.3.1 Electropherogram

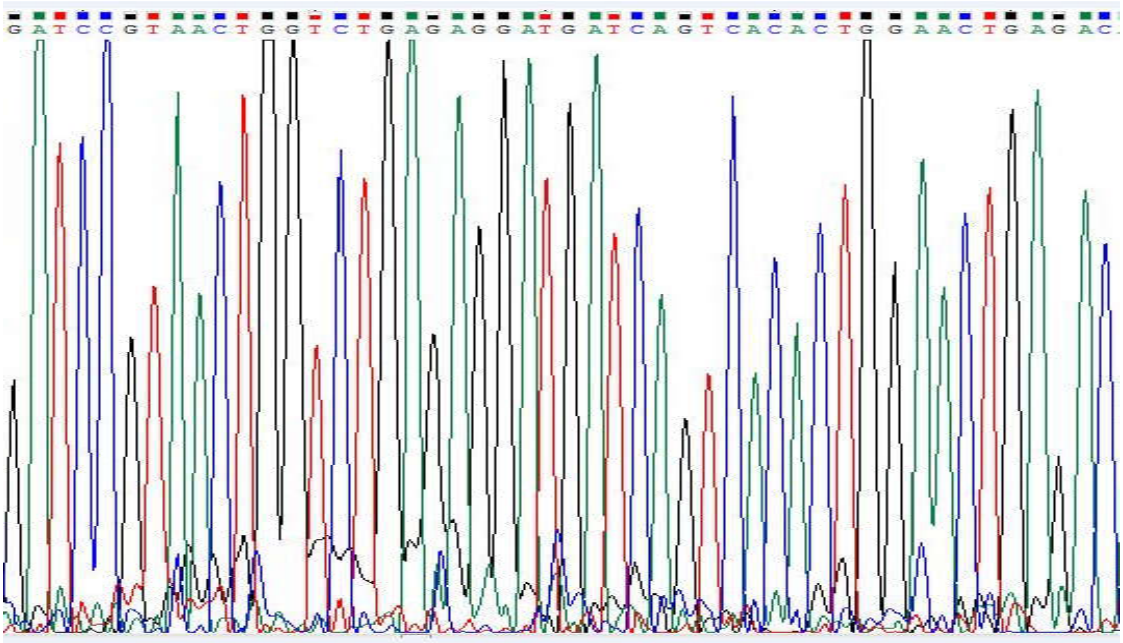


Fig. 4: Electropherogram of 16s rRNA Gene Sequence of *E.coli*

4.3.2 Contig Sequence of *E.coli*

Contig 18 (Sample no)

AAATTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACAC
ATGCAAGTCGAACGGTAACAGGAAGAAGCTTGCTCTTTGCTGACGAGTGGCGGA
CGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAAC
GGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTC
TTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACC
TAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAG
ACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCG
CAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGT
ACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACC
CGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGT
GCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAG
TCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCT
TGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGA
TCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAG
GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCG
TTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGA
ACCTTACCTGGTCTTGACATCCACGGAAGTTTTCAGAGATGAGAATGTGCCTTCG
GGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTG
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CAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
TGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACC
ACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAACCGTAGGGGAACC
TGCGGTTGGATCACCTCCTTA

4.3.3 Phylogenic tree analysis of *E.coli*

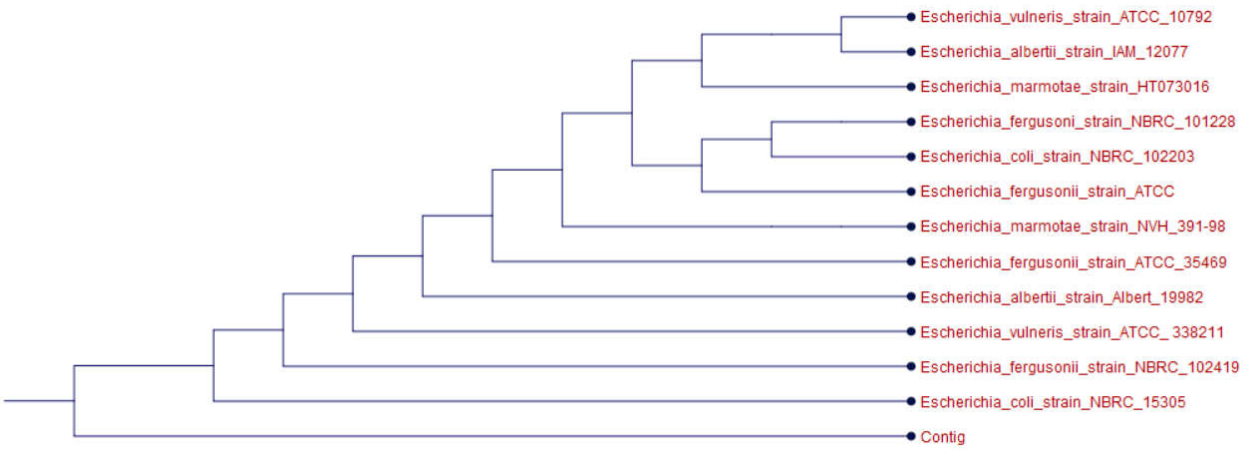

Fig. 5: Phylogenic tree analysis of *E.coli*

4.4 Frequency of UTI among pregnant women based on different catagories

Out of 100 pregnant women, positive cases of UTI were found to be 33% and total 75 bacteria were isolated. Frequencies of UTI among pregnant women based on different catagories were shown in table 11, 12, 13, 14, 15, 16, 17 and 18.

Total 33% positive cases of UTI in pregnant women were shown in table 11.

Table 11. Frequency of UTI among pregnant women

Total number of cases	No. of positive Cases	Percentage
100	33	33%

Highest frequency of UTI was seen in pregnant women of age <25 yrs and lowest frequency was noticed in age >30 yrs of age group which were shown in Table 12.

Table 12. Frequency of UTI in pregnant women in relation to age

Age	No. examined	No. positive	Percentage
<25yrs	60	25	**75.76**
25-30yrs	35	6	18.18
>30yrs	5	2	6.06
Total	100	33	100

The highest numbers of pus cells were found in 20 positive cases whereas 10-20 pus cells were found in 13 positive cases. The results were shown in table 13.

Table 13. Frequency of UTI in pregnant women in relation to pus cells

Pus cells	No. examined	No. positive cases	Percentage
0-10	67	0	100
10-20	13	13	100
>20	20	20	100
Total	100	33	

The highest number of UTI found in primary level of education were 44% and lowest number in higher educated were 16%. This percentage were shown in table 14.

Table 14. Frequency of UTI in pregnant women in relation to education

Level of education	No. examined	No. positive	Percentage
Primary	50	22	**44**
Secondary	25	7	28
Higher	25	4	16
Total	100	33	

The highest number of positive case (54.5%) found in M Abdur Rahim Medical College and Hospital and followed by 36.36% in Saint Vincent Hospital and 9.09% in Sheba Diagnostic and Consultancy Center. The results were shown in table 15.

Table 15. Distribution of UTI in pregnant women according to sample collection and types of bacteria

Name of hospital	No. of sample	No. of positive case(%)	No. of gram positive bacteria(%)	No. of gram negative bacteria (%)
M Abdur Rahim medical college	50	18(**54.5**)	9(**60**)	30(**50**)
Saint Vincent Hospital	40	12(36.36)	5(33.33)	21(35)
Sheba Diagnostic and Consultancy Center	10	3(9.09)	1(6.67)	9(15)
Total	100	33(100)	15(100)	60(100)

In the study 3rd trimester of the pregnancy 45.46% pregnant women were UTI positive and it was the highest percentage and also showed in table 16.

Table 16. Percentage of UTI by stage of pregnancy

Stage of pregnancy	No: tested	No: positive (%)
First trimester	45	8(24.24)
Second trimester	25	10(30.30)
Third trimester or above	30	**15(45.46)**
Total	100	33(100)

The highest number of *E. coli* (73.33%) and lowest number of *E. coli* (6.67%) were found in < 25 years of age which were shown in table 17.

Table 17: Distribution of organisms based on age difference

Age	Organisms						Total
	E.coli	***Klebsiella spp***	***Proteus spp***	***Pseudomonas spp***	***Staphylococcus spp***	***Streptococcus spp***	
< 25 years	22 (73.33%)	8(50%)	5 (55.55%)	3(60%)	7(58.33%)	2(66.67%)	
25-30 years	6(20%)	5 (31.25%)	3 (33.33%)	2(40%)	3(25%)	1(33.33%)	
> 30 years	2(6.67%)	3(18.75%)	1 (11.11%)	0	2(16.67%)	0	
Total	30	16	9	5	12	3	75

Gram negative (80%) and gram positive (20%) bacteria were found from urine sample of pregnant women which are shown in table 18.

Table 18. Percentage of gram positive and gram negative bacteria isolated from UTI

Bacterial isolates	Total organisms number	Total (%) of UTI isolates	Individual % of gram(-) and gram (+) isolates
Gram negative	**60(80%)**		
E. coli	30	**40**	**50**
Klebsiella spp	16	21.33	26.67
Proteus spp	9	12	15
Pseudomonas spp	5	6.67	8.33
Gram positive	**15(20%)**		
Staphylooccus spp	12	16	**80**
Streptococcus spp	3	4	20
Total	**75**	**100**	

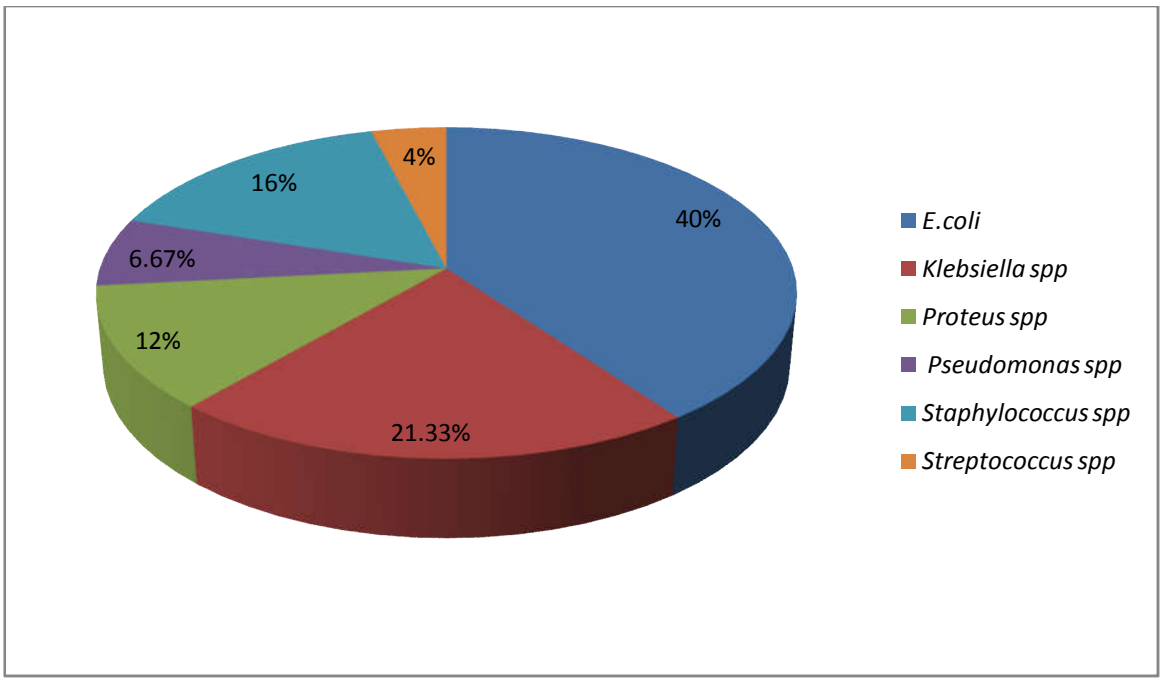

Fig. 6: Percentage of gram positive and gram negative bacteria isolated from UTI cells.

4.4.1 Prevalence of UTI among pregnant women

The table 19 represents prevalence of UTI based on study area, age, education and stage of pregnancy. The present results revealed that the study area had no significant (P>0.05) effect but age, level of education and stage of pregnancy had significant (P<0.05) effect on the prevalence of urinary tract infection in pregnant women. In the study area, the highest prevalence was found in M Abdur Rahim Medical College (36%), followed by Saint Vincent Hospital (30%) and Sheba Diagnostic Center (30%) respectively. Among the age group, the prevalence of UTI was 41.67% and 40% in < 25 years and > 30 years which were significant by (P<0.05) higher than 25-30 years (17.14%). The primary educated women had significant by higher prevalence of UTI than secondary and higher. According to pregnancy stage, the higher prevalence of UTI was found in 3rd trimester (50%) which was followed by 2nd trimester (40%) and 1st trimester (17.78%) respectively.

Table 19: Prevalence of UTI based on study area, age, education and stage of pregnancy

Parameter		No. examined	No. Positive	Prevalence (%)	P value
Study area	M Abdur Rahim Medical College	50	18	36	0.13
	Saint Vincent Hospital	40	12	30	
	Sheba Diagnostic Center	10	3	30	
Age	< 25 years	60	25	41.67	0.047
	25-30 years	35	6	17.14	
	> 30 years	5	2	40	
Education	Primary	50	22	44	0.043
	Secondary	25	7	28	
	Higher	25	4	16	
Stage of pregnancy	1st trimester	45	8	17.78	0.01
	2nd trimester	25	10	40	
	3rd trimester	30	15	50	

P<0.05 means significant at 5% level of significance

P>0.05 means Not Significant

4.5 Results of Antibiotic Sensitivity Tests

Several commercially available antibiotics were used for *E. coli, Klebsiella spp, Proteus spp, Pseudomonas spp, Staphylococcus spp* and *Streptococcus spp* are given in table 20.

Table 20: Result of antibiotic sensitivity test for *E. coli*

Name of antibiotic with disc concentration	Zone of diameter(mm)	Interpretation
Ciprofloxacin(5 µg)	33	S
Chloramphenicol(30 µg)	25	S
Amoxicillin(30 µg)	-	R
Ampicillin(25 µg)	-	R
Amikacin(30 µg)	-	R
Cephalexin(30 µg)	-	R
Colistin(10 µg)	16	S
Penicillin(10 µg)	-	R
Neomycin(30 µg)	20	S
Kanamycin(30 µg)	15	I
Gentamycin(10 µg)	24	S
Vancomycin(30 µg)	-	R

Legends: S= Sensitive, R= Resistant and I= Intermidiate

Table 21: Result of antibiotic sensitivity test for *Klebsiella spp*

Name of antibiotic with disc concentration	Zone of diameter(mm)	Interpretation
Ciprofloxacin(5 µg)	22	S
Chloramphenicol(30 µg)	20	S
Amoxicillin(30 µg)	-	R
Ampicillin(25 µg)	-	R
Amikacin(30 µg)	-	R
Cephalexin(30 µg)	-	R
Erythromycn(15 µg)	-	R
Penicillin(10 µg)	-	R
Levofloxacin(5 µg)	20	S
Kanamycin(30 µg)	24	S
Gentamycin(10 µg)	26	S
Vancomycin(30 µg)	-	R
Cloxacillin(1 µg)	-	R

Legends: S= Sensitive, R= Resistant

Table 22: Results of antibiotic sensitivity test for *Pseudomonas spp*

Name of antibiotic with disc concentration	Zone of diameter(mm)	Interpretation
Ciprofloxacin(5 µg)	16	R
Chloramphenicol(30 µg)	-	R
Amoxicillin(30 µg)	-	R
Ampicillin(25 µg)	-	R
Amikacin(30 µg)	-	R
Cephalexin(30 µg)	-	R
Penicillin(10 µg)	-	R
Kanamycin(30 µg)	-	R
Gentamycin(10 µg)	-	R
Vancomycin(30 µg)	-	R
Neomycin(30 µg)	-	R
Colistin(10 µg)	14	S

Legends: S= Sensitive, R= Resistant

Table 23: Result of antibiotic sensitivity test for *Proteus spp*

Name of antibiotic with disc concentration	Zone of diameter(mm)	Interpretation
Ciprofloxacin(5 µg)	16	S
Chloramphenicol(30 µg)	-	R
Amoxicillin(30 µg)	-	R
Ampicillin(25 µg)	-	R
Amikacin(30 µg)	-	R
Cloxacillin(1 µg)	-	R
Penicillin(10 µg)	-	R
Kanamycin(30 µg)	18	S
Gentamycin(10 µg)	20	S
Vancomycin(30 µg)	-	R
Neomycin(30 µg)	18	S
Tetracycline(15 µg)	-	R

Legends: S= Sensitive, R= Resistant

Table 24: Result of antibiotic sensitivity test for *Staphylococcus spp*

Name of antibiotic with disc concentration	Zone of diameter(mm)	Interpretation
Ciprofloxacin(5 µg)	26	S
Chloramphenicol(30 µg)	24	S
Amoxicillin(30 µg)	-	R
Ampicillin(25 µg)	-	R
Amikacin(30 µg)	-	R
Cloxacillin(1 µg)	-	R
Levofloxacin(5 µg)	20	S
Kanamycin(30 µg)	18	S
Gentamycin(10 µg)	20	S
Vancomycin(30 µg)	-	R
Neomycin(30 µg)	18	S
Novobiocin(30 µg)	22	S
Norfloxacin(10 µg)	13	R
Cephalexin(30 µg)	-	R
Tobramycin(10 µg)	12	S

Legents: S= Sensitive, R= Resistant

Table 25: Result of antibiotic sensitivity test for *Streptococcus spp*

Name of antibiotic with disc concentration	Zone of diameter(mm)	Interpretation
Cephalexin(30 µg)	-	R
Chloramphenicol(30 µg)	-	R
Amoxicillin(30 µg)	-	R
Ampicillin(25 µg)	-	R
Amikacin(30 µg)	-	R
Cloxacillin(1 µg)	-	R
Penicillin(10 µg)	-	R
Kanamycin(30 µg)	18	S
Gentamycin(10 µg)	20	S
Vancomycin(30 µg)	-	R
Neomycin(30 µg)	18	S
Tetracycline(30 µg)	-	R
Ofloxacin(2 µg)	16	S

Legends: S= Sensitive, R= Resistant

4.5.1Percentage of Antibiotic Sensitivity pattern for gram negative bacteria

Antibiotic sensitivity test was carried out using disc diffusion technique on Mueller Hinton agar for all the bacterial isolates to the most common antibiotic agents that used in this study are given below the table. Antibiotic sensitivity pattern *of E. coli* (10), *Klebsiella spp* (5), *Proteus spp* (3) and *Pseudomonas spp* (3) were shown below the table. The antibiotic study revealed that all of the isolates *E. coli* (10) were sensitive to Gentamicin, Chloramphenicol and Ciprofloxacin (80%), followed by Neomycin and Colistin (70%). The isolates were resistant to Penicillin, Amoxicillin and Amphicillin (100%), followed by Vancomycin (90%) and Cephalexin (80%). *Klebsiella spp* were sensitive to Ciprofloxacin, Chloramphenicol and Levofloxacin (100%), followed by Norfloxacin (60%). The isolates were resistant to Amikacin, Amoxicillin, Cloxacillin (100%). Proteous spp were sensitive neomycin and Gentamycin (66.67%) and resistant to Ciprofloxacin, Chloramphenicol, Penicillin, Amoxicillin, Amphicillin (100%), followed by Cloxacillin and Amikacin (66.67%). *Pseudomonas spp* were sensitive to Colistin (100%) and resistant to all other antibiotic (100%) except Ciprofloxacin and Amikacin.

Table 26: Resistant and susceptibility percentage for isolated gram negative pathogens

Antibiotics with disc concentration	*E. coli*(10)		*Klebsiella spp* (5)		*Proteus spp* (3)		*Pseudomonas spp* (3)	
	%R	%S	%R	%S	%R	%S	%R	%S
Ciprofloxacin(5)	2(20)	8 (80)	0(0)	**5(100)**	**3(100)**	0(0)	2(66.67)	1(33.33)
Chloramphenicol(30)	2(20)	8 (80)	0(0)	**5(100)**	**3(100)**	**0(0)**	**3(100)**	0(0)
Penicillin(10)	**10(100)**	0 (0)	NT	NT	3(100)	**0(0)**	**3(100)**	0(0)
Cloxacillin(1)	NT	NT	**5(100)**	0(0)	2(66.67)	1(33.33)	NT	NT
Kanamycin(30)	5(50)	2 (20)	0(0)	5 (100)	0(0)	**3(100)**	**3(100)**	0(0)
Gentamycin(10)	2(20)	8 (80)	0(0)	5(100)	1(33.33)	**2(66.67)**	**3(100)**	0(0)
Vancomycin(30)	9(90)	1 (10)	**5(100)**	0(0)	3(100)	**0(0)**	**3(100)**	0(0)
Neomycin(30)	1(10)	7 (70)	2(20)	**4(80)**	1(33.33)	**2(66.67)**	**3(100)**	0(0)
Amoxicillin(30)	**10(100)**	**0(0)**	5(100)	**0(0)**	**3(100)**	**0(0)**	**3(100)**	0(0)
Ampicillin(25)	**10(100)**	**0(0)**	4(80)	**2(20)**	**3(100)**	**0(0)**	**3(100)**	0(0)
Amikacin(30)	6(60)	4 (40)	5(100)	0(0)	2(66.67)	0(0)	2(66.67)	1(33.33)
Norfloxacin(10)	NT	NT	2(40)	3(60)	**NT**	**NT**	**NT**	NT
Levofloxacin(5)	NT	NT	0(0)	5(100)	**NT**	**NT**	**NT**	NT
Colistin(10)	2(20)	7 (70)	NT	NT	**NT**	**NT**	0(0)	3(100)
Erythromycin(15)	NT	NT	0(0)	5(100)	2(66.67)	1(33.33)	NT	NT
Cephalexin(30)	8(80)	2 (20)	4(80)	0(0)	2(66.67)	1(33.33)	3(100)	0(0)
Cefotaxime(30)	NT	NT	**3(100)**	0(0)	3(100)	0(0)	NT	NT

Legends: S= Sensitive, R= Resistant, %=Percentage and NT= Not tested

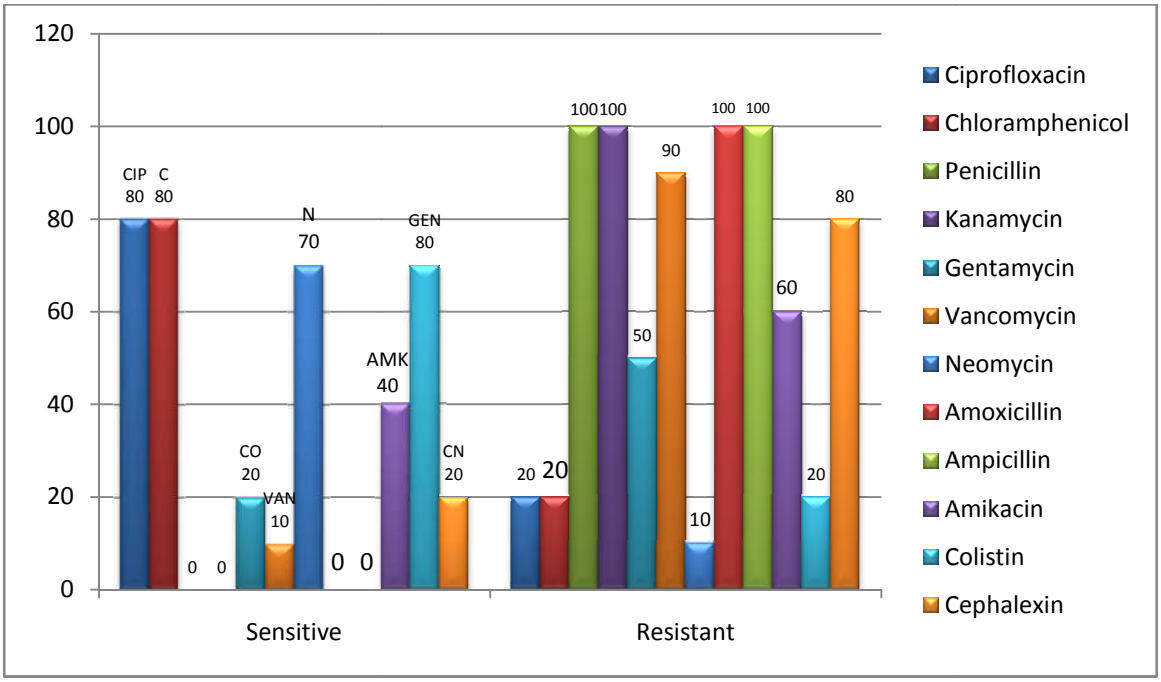

Fig. 7: Antibiotic susceptibility pattern of *E.coli* from urine sample of pregnant women

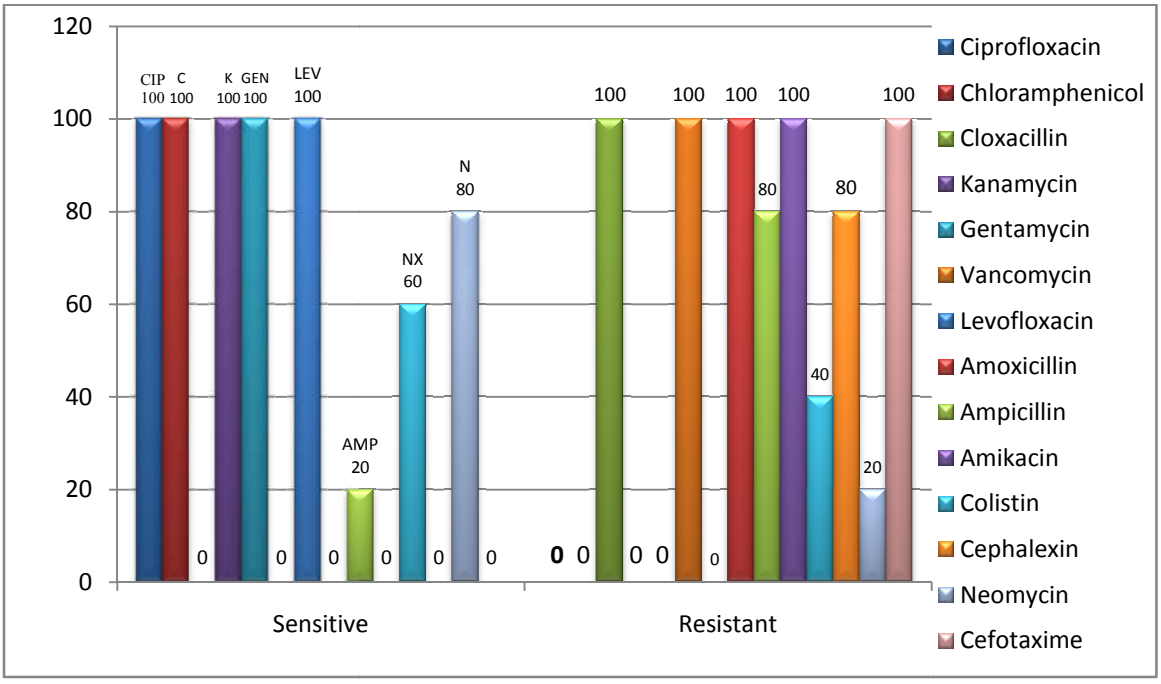

Fig. 8 Antibiotic susceptibility pattern of *Klebsiella spp* from urine sample of pregnant women

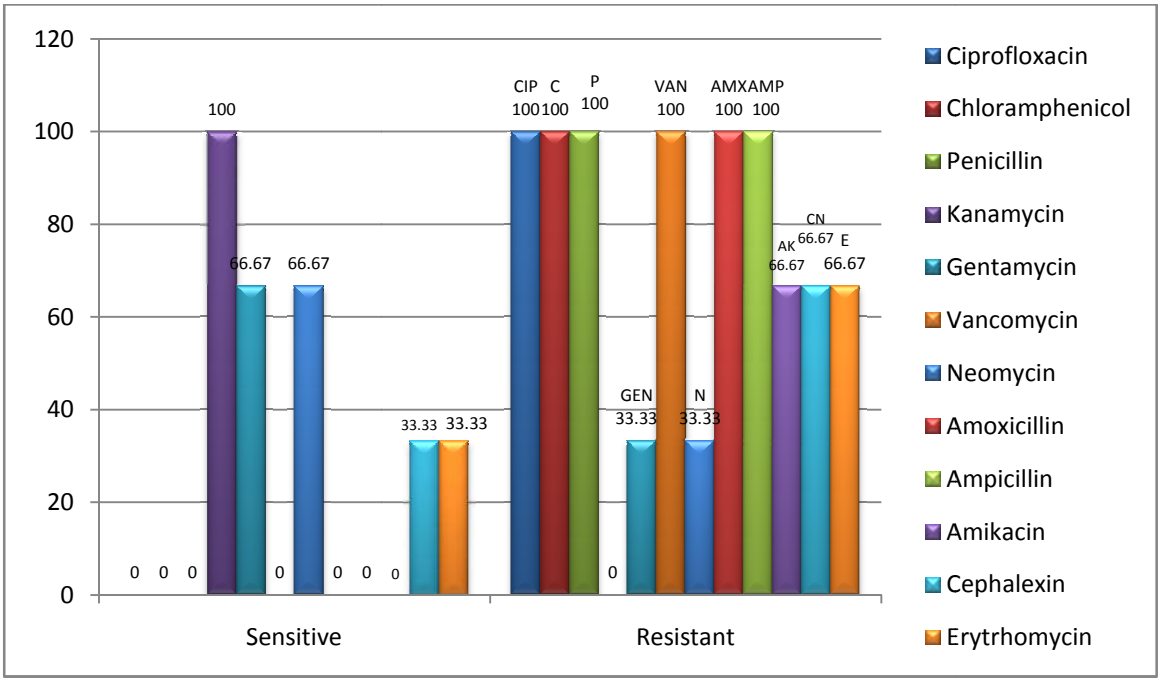

Fig. 9: Antibiotic susceptibility pattern of *Proteus spp* from urine sample of pregnant women

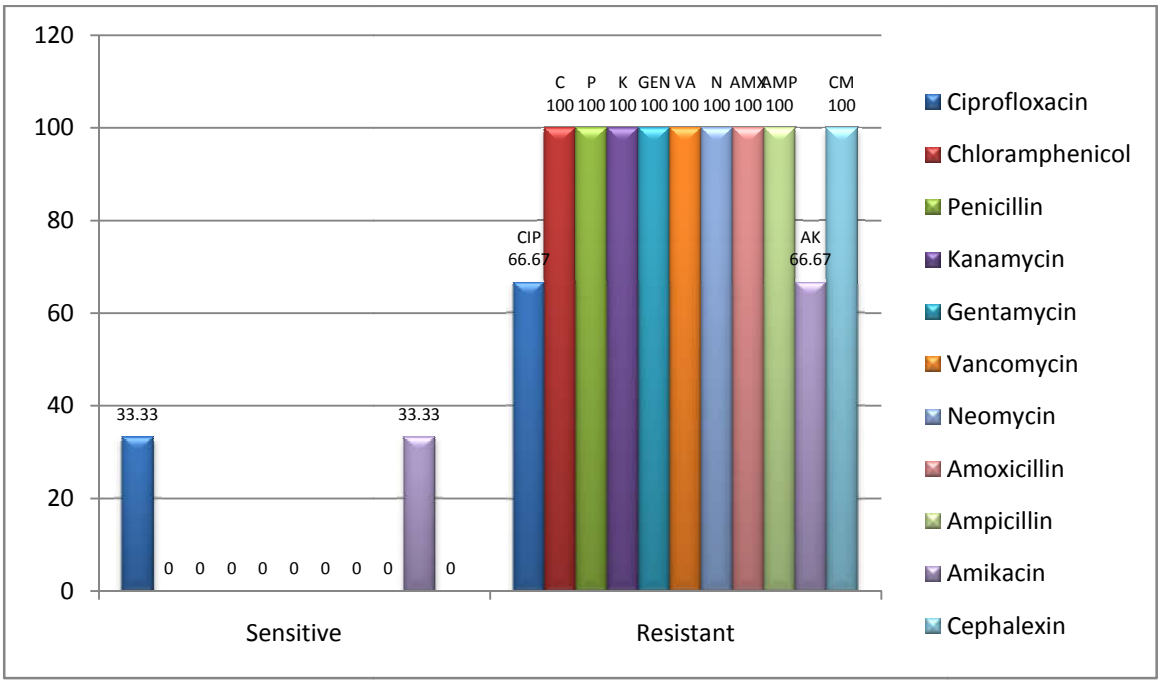

Fig. 10: Antibiotic susceptibility pattern of *Pseudomonas spp* from urine sample of pregnant women

4.5.2 Percentage of Antibiotic Sensitivity pattern for gram positive bacteria

Urinary tract infection mainly caused by gram negative bacteria. Gram positive bacteria was found some urine sample. Antibiotic sensitivity pattern of *Staphylococcus spp* (5) and *Streptococcus spp* (1) were shown in table 27. The antibiotic study of all isolates *Staphylococcus spp* (5) were sensitive to Levofloxacin, Ofloxacin, Chloramphenicol and Gentamycin (100%), followed by Novobiocin, Kanamycin and Tobramycin (80%). The isolates were resistant to Chephalexin, Amoxicillin and Amphicillin(100%), followed by Norofloxacin and Colistin (60%). *Streptococcus spp* were sensitive to Kanamycin, Gentamycin and Ofloxacin (80%) and highly resistant to Cephalexin, Penicillin-G and Cloxacillin (100%).

Table 27: Resistant and susceptibility percentage for isolated gram positive pathogens

Antibiotics with disc concentration	***Staphylococcus spp(5)***		***Streptococcus spp(1)***	
	%R	**%S**	**%R**	**%S**
Levofloxacin(5)	0(0)	**5(100)**	NT	NT
Ciprofloxacin(5)	2(40)	3(60)	NT	**NT**
Norfloxacin(10)	3(60)	2(40)	NT	NT
Novobiocin(30)	**1(20)**	**4(80)**	NT	NT
Cephalexin(30)	**5(100)**	0(0)	**100**	0
Chloramphenicol(30)	0(0)	**5(100)**	80	0
Kanamycin(30)	1(20)	4(80)	20	80
Colistin(10)	3(60)	2(40)	20	80
Penicillin –G(10)	NT	NT	**100**	0
Neomycin(30)	2(40)	0(0)	40	60
Gentamycin(10)	0(0)	**5(100)**	20	80
Amoxicillin(30)	5(100)	0(0)	80	0
Ampicillin(25)	**5(100)**	0(0)	80	0
Amikacin(30)	2(40)	3(60)	66.67	33.33
Ofloxacin(2)	0(0)	**5(100)**	20	80
Tobramycin(10)	1(20)	4(80)	NT	NT
Cloxacillin(1)	NT	NT	100	0
Vancomycin(30)	NT	NT	20	80
Cephalexin(30)	**5(100)**	0(0)		

Legends: S= Sensitive, R= Resistant, %= Percentage and NT= Not tested

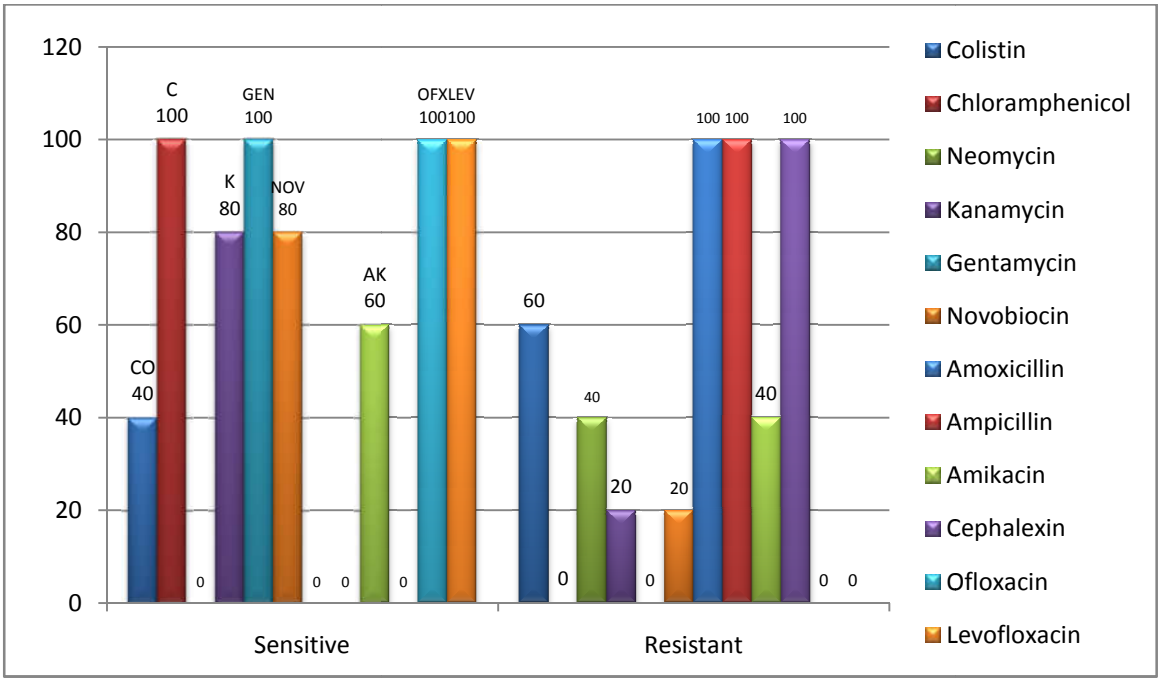

Fig. 11: Antibiotic susceptibility pattern of *Staphylococcus spp* from urine sample of pregnant women

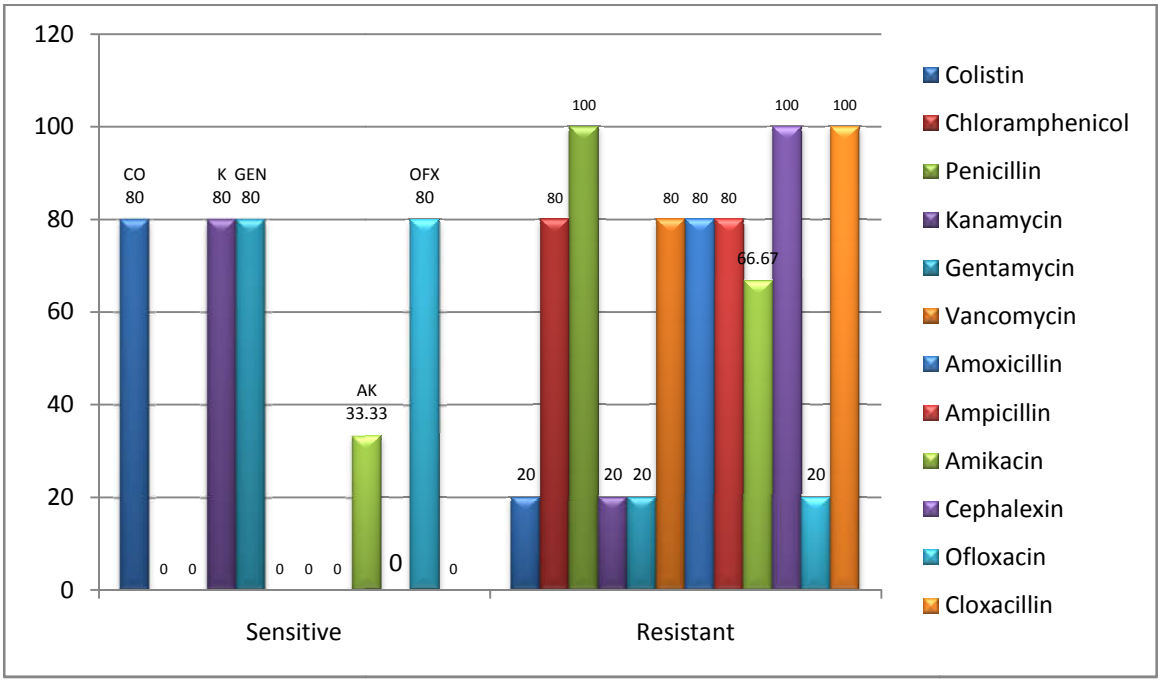

Fig. 12: Antibiotic susceptibility pattern of *Streptococcus spp* from urine sample of pregnant women

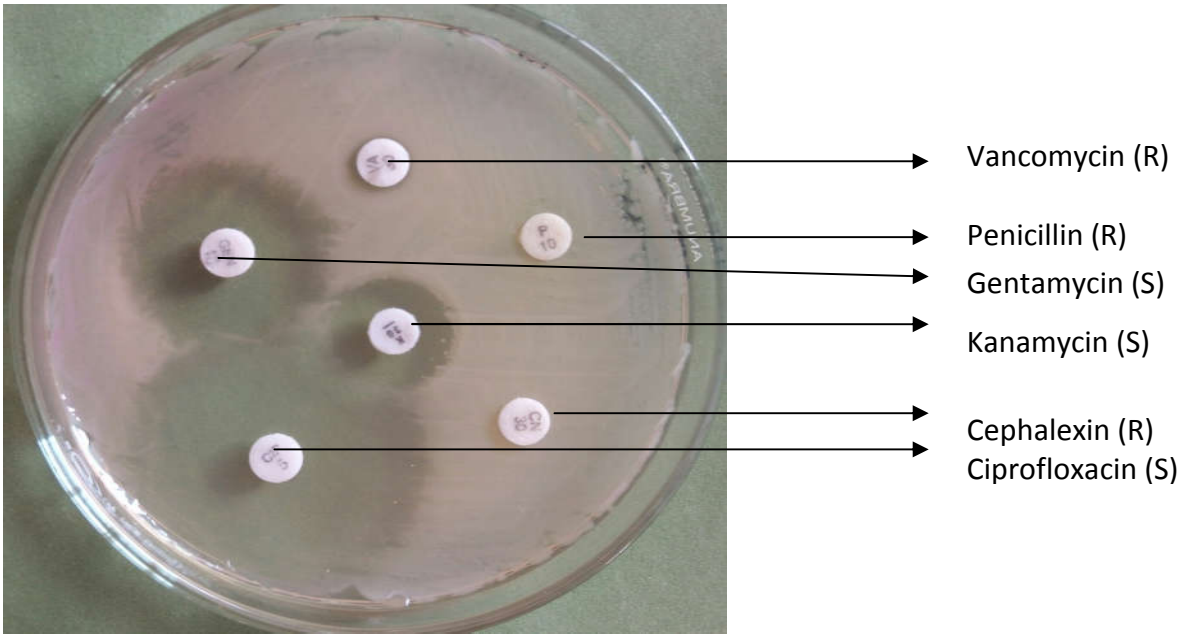

Plate 82: Antibiotic sensitivity test for *E. coli*

Legends: S = Sensitive

R= Resistant

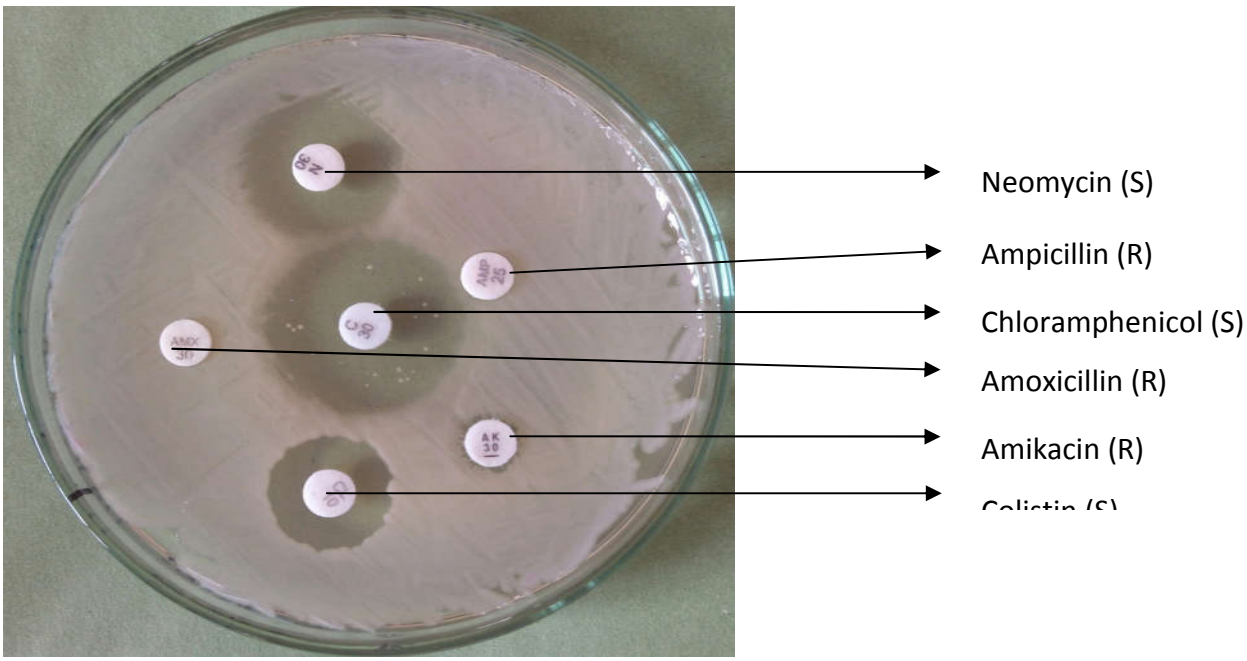

Plate 83: Antibiotic sensitivity test for *E. coli*

Legends: S = Sensitive

R= Resistant

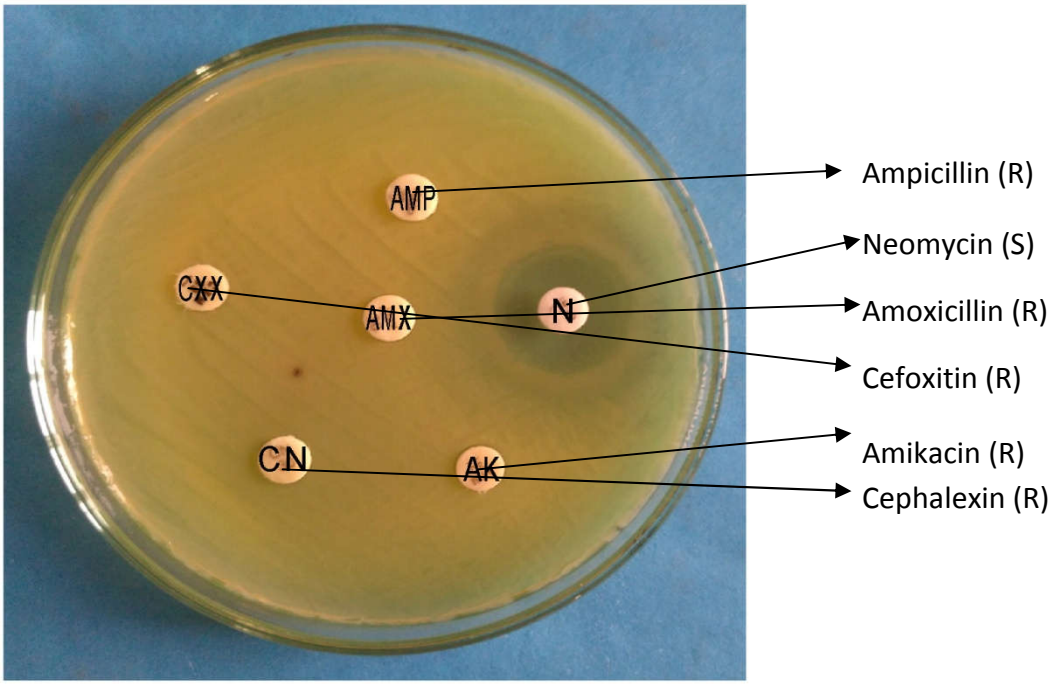

Plate 84: Antibiotic sensitivity test for *Klebsiella spp*

Legends: S= Sensitive

R= Resistant

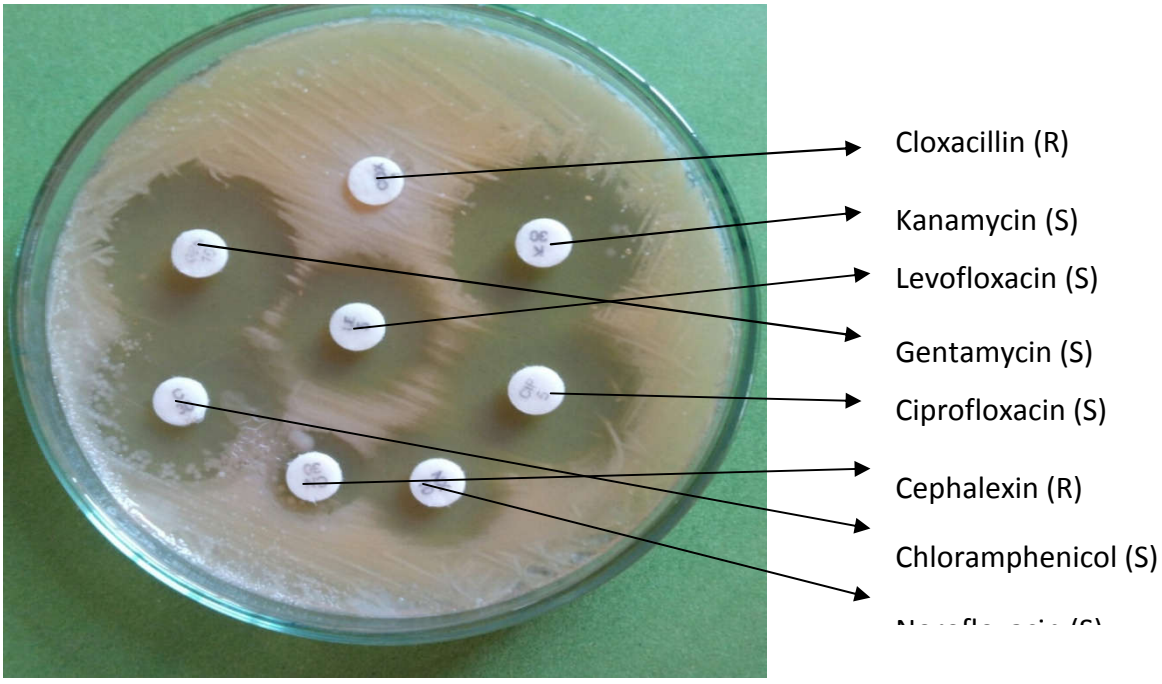

Plate 85: Antibiotic sensitivity test for *Klebsiella spp*

Legends: S= Sensitive

R= Resistant

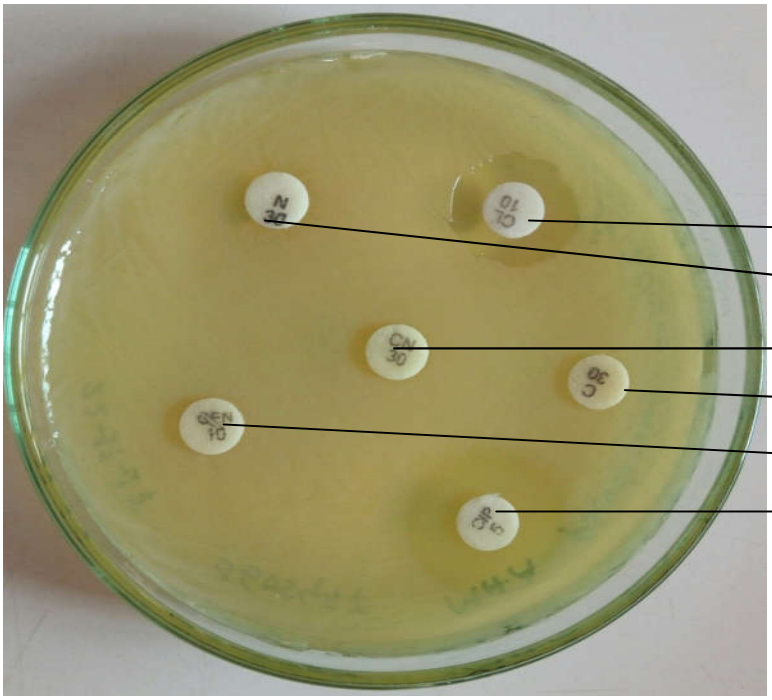

Plate 86: Antibiotic sensitivity test for *Pseudomonas spp*

Legends: S= Sensitive

R= Resistant

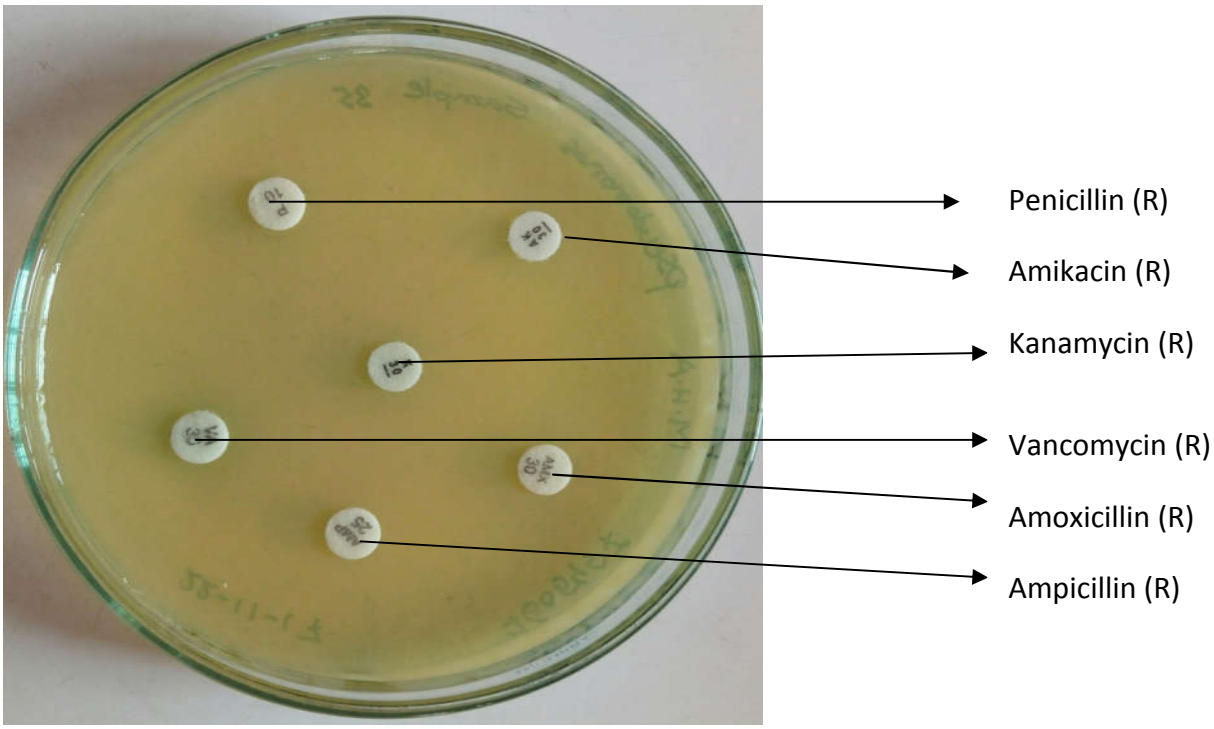

Plate 87: Antibiotic sensitivity test for *Pseudomonas spp*

Legends: S= Sensitive

R= Resistant

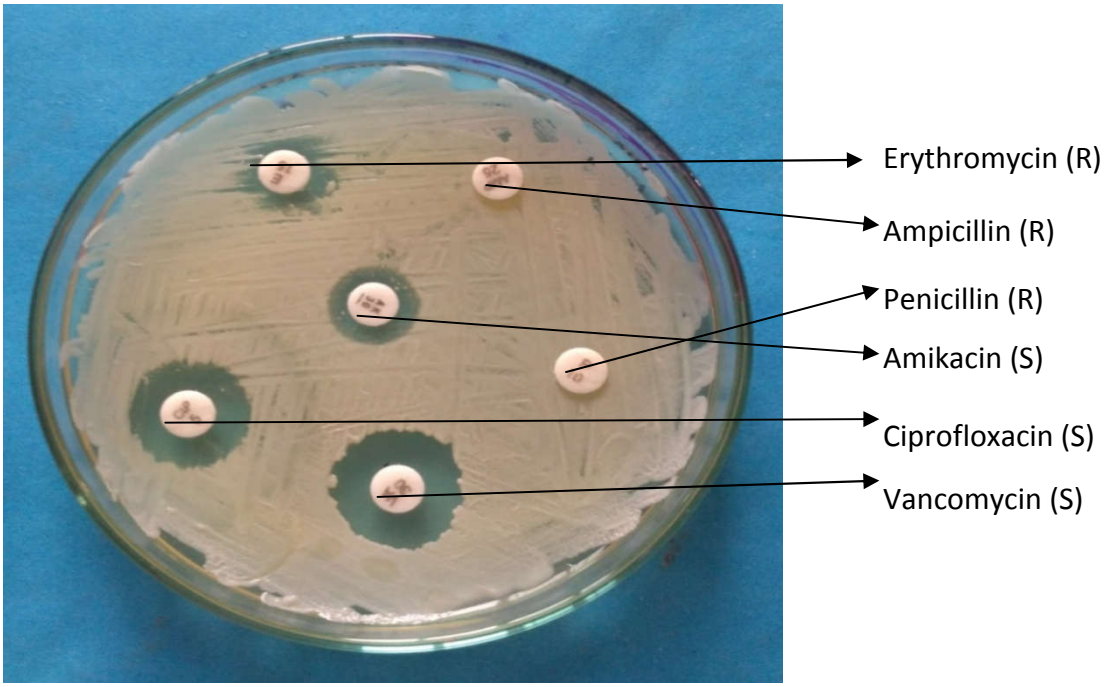

Plate 88: Antibiotic sensitivity test for *Proteus spp*

Legents: S= Sensitive

R= Resistant

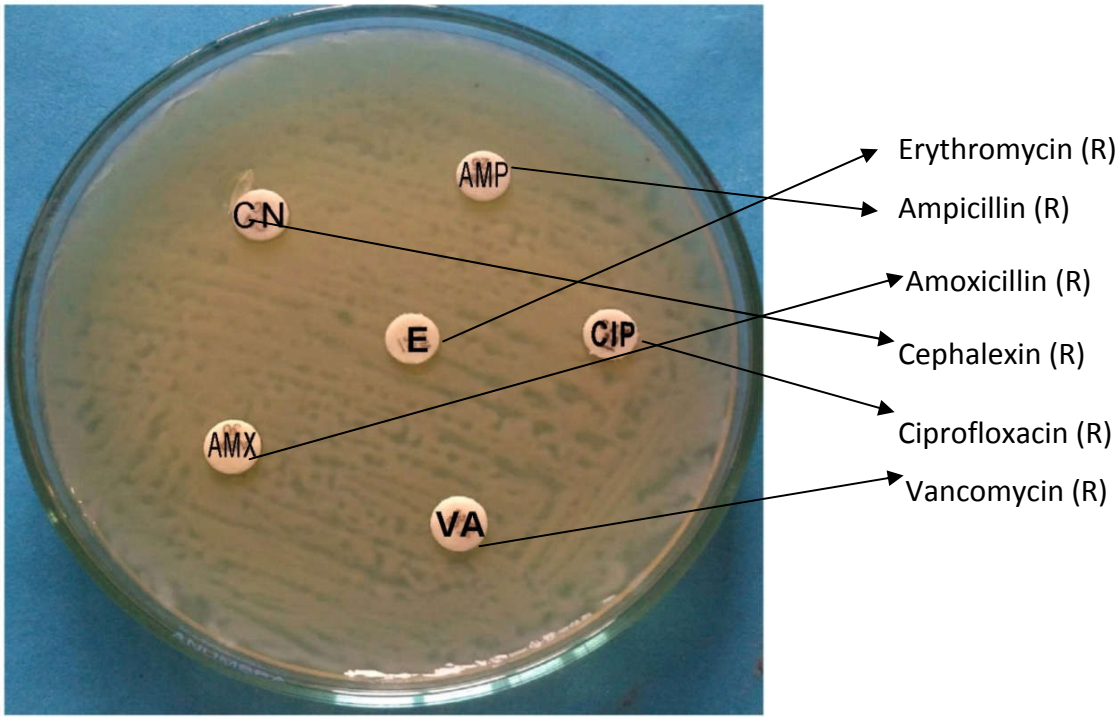

Plate 89: Antibiotic sensitivity test for *Proteus spp*

Legents: S= Sensitive

R= Resistant

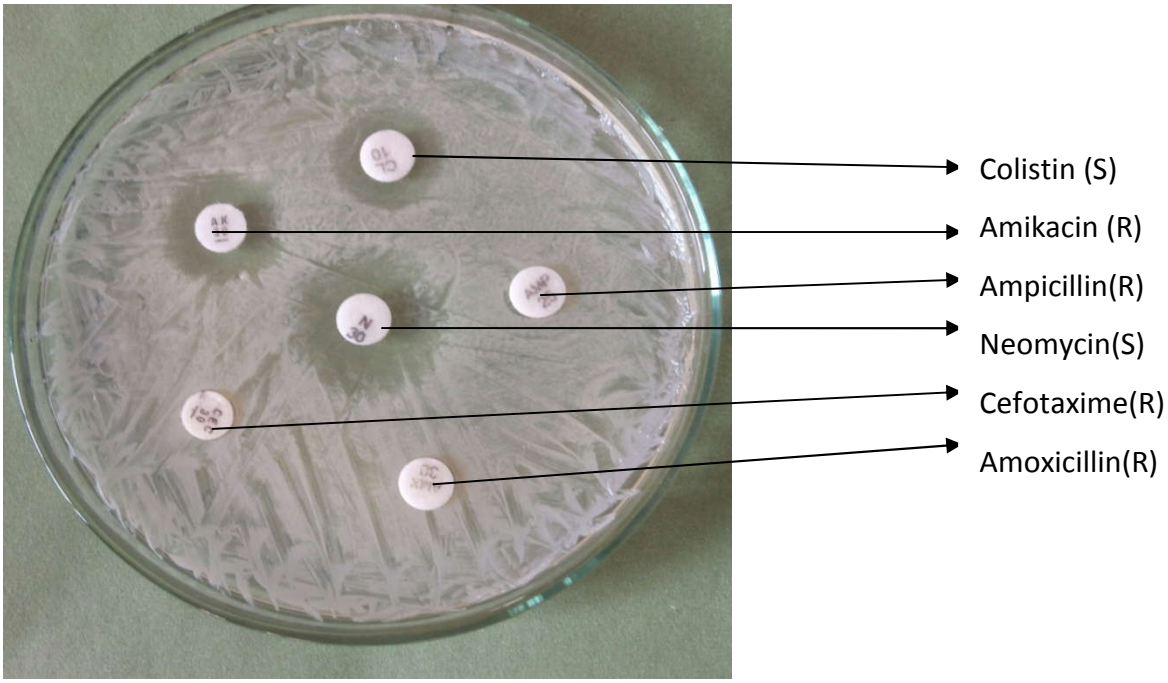

Plate 90: Antibiotic sensitivity test for *Staphylococcus spp*

Legends: S= Sensitive

R= Resistant

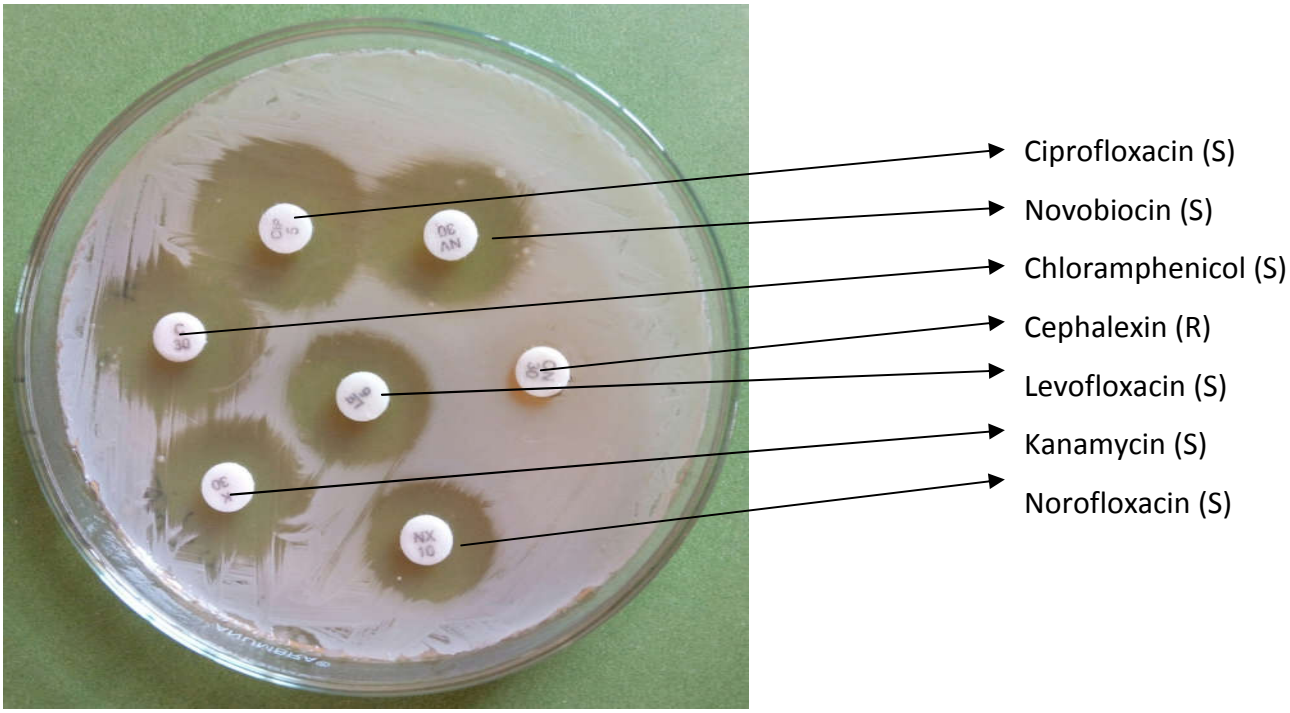

Plate 91: Antibiotic sensitivity test for *Staphylococcus spp*

Legends: S= Sensitive

R= Resistant

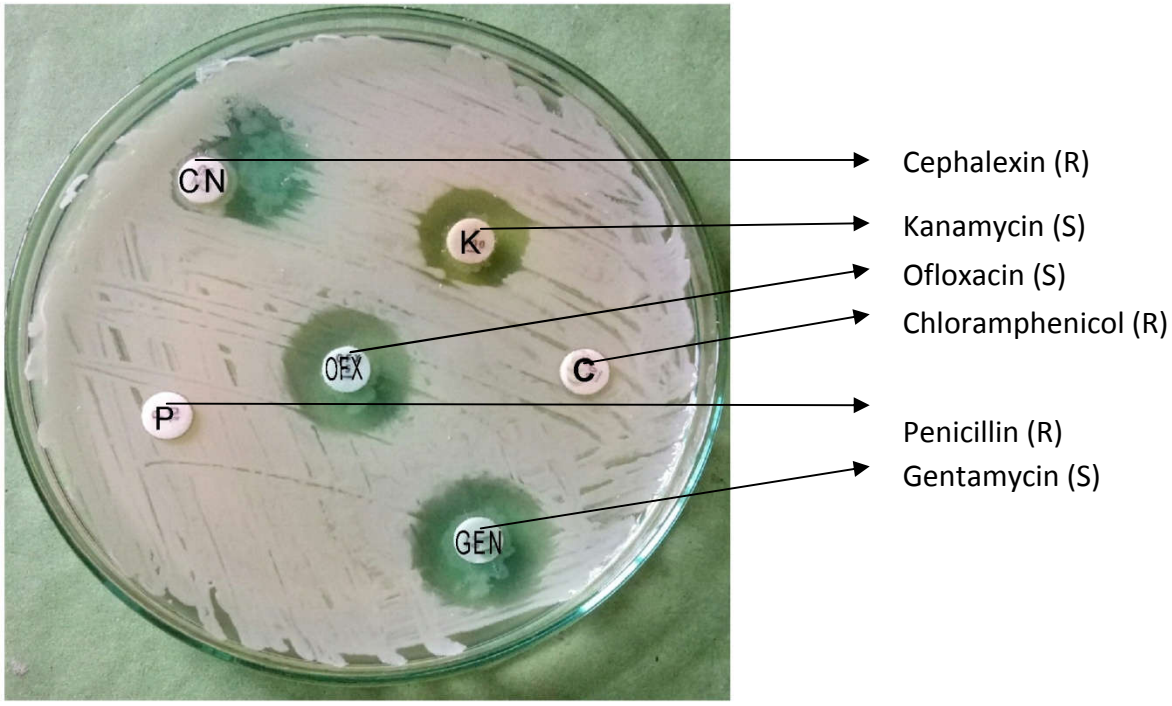

Plate 92: Antibiotic sensitivity test for *Streptococcus spp*

Legends: S= Sensitive

R= Resistant

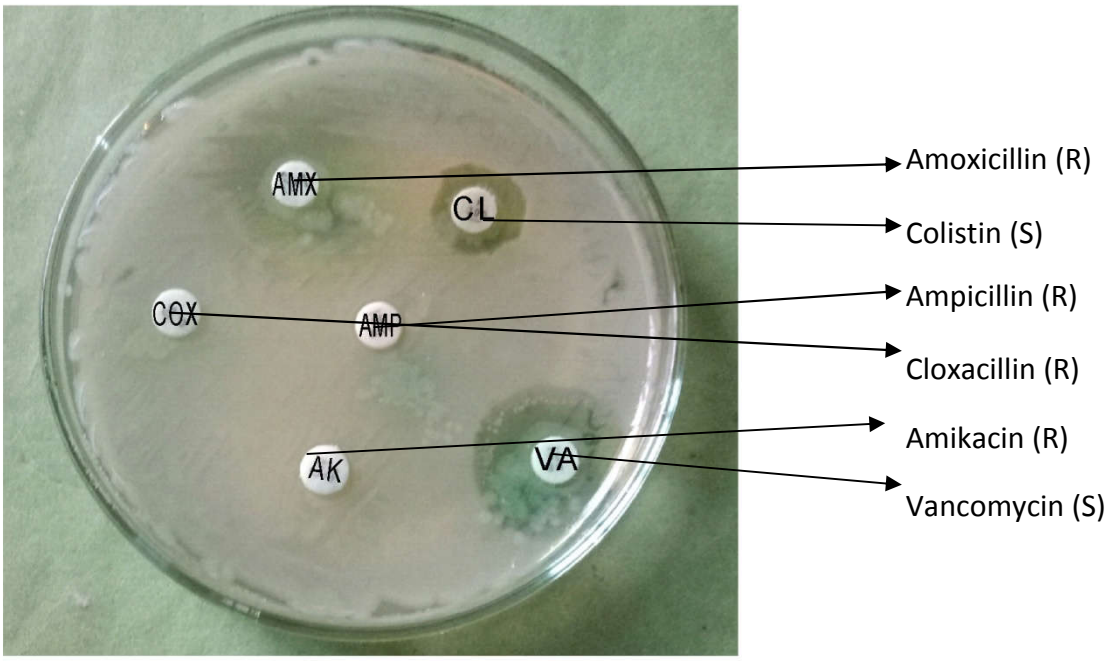

Plate 93: Antibiotic sensitivity test for *Streptococcus spp*

Legends: S= Sensitive

R= Resistant

CHAPTER- 5

DISCUSSIONS

The experiment was carried out for the isolation, identification and characterization of the different types of bacteria isolated from urine sample of pregnant women from different hospitals (M Abdur Rahim Medical College and Hospital, Saint Vincent Hospital and Sheba Diagnostic and Consultancy Center) in Dinajpur district. A total of 100 samples were collected for isolation, identification and antibiotics sensitivity tests. In this study, 6 different types of bacteria were isolated and identified. Isolated bacteria were identified on the basis of colony morphology, gram reaction, microscopic character, biochemical characteristics and molecular characteristics of selected isolate. **16S rRNA** Gene Sequencing was used to identify *E. coli species*. In case of PCA the highest total viable count 8.6 $\times 10^{9}$ cfu/ml was found in 10^{-7} dilution and lowest 1.6×10^{9} cfu/ml in 10^{-6} dilution. In EMB agar the highest total viable count 5.8 $\times 10^{7}$ cfu/ml was found in 10^{-4} dilution and lowest 1.0×10^{9} cfu/ml in 10^{-7} dilution. In MAC agar the highest total viable count 2.4×10^{9}cfu/ml was found in 10^{-7} dilution and lowest was $1.\times 10^{9}$ cfu/ml in 10^{-6} dilution. In MSA agar the highest total viable count 8.2 $\times 10^{8}$ cfu/ml was found in 10^{-6} dilution and lowest was 1.5 $\times 10^{8}$ cfu/ml in 10^{-5} dilution. The result of our study was not significant at ($p<0.05$) where P value is 0.13, in case of study area and result were significant at $p<0.05$ in case of age difference, education level and stage of pregnancy where P value was 0.047, 0.043 and 0.01. This results were more or less similar to (Amit R*, et al.,* 2017).

The cultural characterization of *E. coli* from urine sample of pregnant women revealed greenish black colony with metallic sheen in EMB agar, rose pink colour smooth transparent colony in MacConkey agar, and smooth, glistening and opalescent colony in nutrient agar (table 3) which were similar to (Carter, 1979; Buxton and Freser, 1977)

The cultural characterization of *Klebsiella spp* from urine sample of pregnant women revealed large colony on Nutrient Agar, large, red, mucoid on Mac-Conkey's agar and mucoid, no metallic sheen, with transmitted light, gray brown centers and pink color with clear edges on EMB agar (table 20) which were similar to (Carter, 1979; Buxton and Freser, 1977).

In this study the colony characteristics of *Pseudomonas spp* observed on different media were similar to the findings of (Buxton and Freser, 1977). In the present study *proteus spp* revealed Smooth. Opaque, translucent white colonies on nutrient agar, Colourless and translucent colony on Nutrient Agar, large, red, mucoid on Mac-Conkey's agar and mucoid, no metallic

sheen, with transmitted light, gray brown centers and pink color with clear edges on EMB agar (table 20) which were similar to (Carter, 1979; Buxton and Freser, 1977).

In this study the colony characteristics of *Pseudomonas spp* observed on different media were similar to the findings of (Buxton and Freser, 1977). In the present study *proteus spp* revealed Smooth. Opaque, translucent white colonies on nutrient agar, Colourless and translucent dewdrop like colonies on Mac-Conkey agar and pale colour small colonies on EMB agar which are similar to (Carter, 1979).

In this study the colony characteristics of *Staphylococcus spp* observed on different media were similar to the findings of (Buxton and Freser, 1977). The colony character of *Streptococcus spp* on Blood agar is bête hemolytic colony, dark bluish colour colony on Mitis Salivarius agar, beta hemolytic colony on TSA agar were similar to the findings of (Bergnann *et al*., 1980; Terzolo *et al*., 1979)

In Gram's staining, the morphology of the *E. coli* exhibited Gram negative short rod arranged in single or paired and in biochemical examination *E. coli* isolates fermented different sugars with the production of acid after incubation. This isolates also revealed positive reaction in MR test, negative reaction in VP test. After motility test we also found that they were motile.

In Gram's staining, the morphology of the *Staphylococcus spp* exhibited Gram positive, cocci shape, grape like cluster which was supported by several author (Freeman, 1985; Marchant and parker 1976). After motility test we also found that they were non-motile.

Out of 100 samples 33 patients were found to be UTI positive and a total of 75 isolates were isolated. The isolated bacteria were *E. coli, Klebsiella spp, Proteus spp, Pseudomonas spp, Staphylococcus spp* and *Streptococcus spp.* Gram negative isolates were 60(80%) where gram positive isolates were 15 (20%). Gram negative bacteria 80% were found in highly prevalence than gram positive 20%. In this study, the most frequent UTI were gram negatives which made up 80% of all isolates and this findings were in agreement with (Angoti *et al.,* 2015). In present study 40% *E. coli* found and previous studies have also demonstrated that *E. coli* is the most frequent etiological agent causing community and hospital acquired UTIs.(Gales *et al*., 2002; Brosnema *et al*., 1993), followed by *Klebsiella spp* (21.33%), *Proteus spp* (12%), *Pseudomonas spp* (6.67%), similar to (Ranjon *et al.,* 2017) *Staphylococcus spp* (16%) and *Streptococcus spp* (4%).

Out of 100 sample 60 sample were collected from less than 25 years old pregnant women and highest number of positive cases 24(72.72%) found from them and lowest number of positive cases 2(6.06%) were found from up to 30 ages pregnant women. (Amit *et al*., 2017) found on their study 60% pregnant women were UTI infected and their age was less than 25 years old.

In present study 60.60% pregnant women had more than 20 pus cells and they were highly infected. This was similar to (Amit *et al*., 2017).

According to education 57.58% pregnant women were primary level of education and they were highly infected with UTI. Only 12.12% pregnant women were infected with UTI who were higher educated. According to present study the highest incidence of UTI is seen in third trimester (45.46%) followed by second trimester (30.30%) and least is seen in first trimester (24.24%). The increased incidence during third trimester may relate to increased mechanical obstruction due to gravid uterus. The risk of UTI is partly due to the pressure of gravid uterus on the ureters causing stasis of urine flow and is also attributed to the humoral and immunological changes during normal pregnancy. Our study is agreement with those reported by other works (Amit *et al*., 2017).

According to present study the highest incidence of UTI is seen in M Abdur Rahim Medical College and Hospital 54.5% followed by Saint Vincent Hospital 36.36% and Sheba Diagnostic and Consultancy Center 9.09%.

All the isolates were screen for drug resistance profile by disc-diffusion method with commercially available disc of Ciprofloxacin, Chloramphenicol, Penicillin, Cloxacillin, Kanamycin, Gentamycin, Neomycin, Tobramycin, Ofloxacin, Cephalexin, Cefexime, Amoxicillin, Ampicillin, Amikacin, Norofloxacin, Novobiocin, Levofloxacin, Colistin and Vankomycin. Nearly all the isolates were found to be resistant against most of the antibiotics, whereas gram negative shows more resistance as compare to gram positive organisms. Gram negative bacteria were resistant to Amoxicillin, Ampicillin, Amikacin, Penicillin, Vankomycin, Erythromycin, Cloxacillin, Neomycin, Tobramycin were more frequent in this study.

E. coli which is the predominant cause of UTI, showed high percentage of resistance to Penicillin, Amoxicillin and Ampicillin (100%), Vankomycin (90%), and Cephalexin(80%) and high percentage of sensitive to Ciprofloxacin, Chloramphenicol and Gentamycin (80%). *Klebsiella spp* which is the second most prevalent pathogen of UTI displayed high percentage of resistance to Cloxacillin, Vankomycin and Cefotaxime (100%), Ampicillin and

Cephalexin (80%) and high percentage of sensitive to Ciprofloxacin, Chloramphenicol, Kanamycin, Levofloxacin (100%). *Proteous spp* were sensitive Neomycin and Gentamycin (66.67%) and resistant to Ciprofloxacin, Chloramphenicol, Penicillin, Amoxicillin, Ampicillin (100%), followed by Cloxacillin and Amikacin (66.67%). *Pseudomonas spp* were sensitive to Colistin (100%) and resistant to all other antibiotic (100%) except Ciprofloxacin and Amikacin. *Staphylococcus spp* were sensitive to Levofloxacin, Ofloxacin, Chloramphenicol and Gentamycin (100%), followed by Novobiocin, Kanamycin and Tobramycin (80%). The isolates were resistant to Chephalexin, Amoxicillin and Ampicillin (100%), followed by Norofloxacin and Colistin (60%). *Streptococcus spp* were sensitive to Kanamycin, Gentamycin and Ofloxacin (80%) and highly resistant to Cephalexin, Penicillin-G and Cloxacillin (100%). The result of antibiotic sensitivity test was more or less similar to (Nadia *et al*., 2004; Geoffrey *et al*., 2013; Mahmood *et al*., 2016; Angoti *et al*., 2015). Indiscriminate use of antibiotic bacteria obtained more resistant power day by day and as a result UTI cannot be prevented.

It has been argued that there is a direct relation between the antibiotic used and the frequency and kinds of antibiotic- resistant strain in human beings. (Kupers, 1981)The resistance to antimicrobial agents can easily transferred among bacteria by transmissible elements or plasmids (Neu, 1994) These resistant organisms can pass their resistance genes to their offspring by replication or to related bacteria through conjugation (Tomasz, 1994). Epidemiological studies have suggested that antibiotic resistance genes emerge in microbial populations within 5 years of the therapeutic introduction of antibiotic (Chakrabarty, 1990). Further the antibiotics resistance genes (found in human and animal isolates) could have originated in the industrial microbes that are used for the production of antibiotics.(web and Davis, 1993). The results of isolation, identification, biochemical test, frequency distribution and antibiotic sensitivity of the bacteria isolated from urine sample of pregnant women in the present study, indicated that the microbial factors play an important role for the development of UTI. Detailed further epidemiological study about the extrinsic and intrinsic factors, which might have direct or indirect influence on the development of UTI in association with microbes are required.

CHAPTER -6

CONCLUSION

The prevalence rate of urinary tract infection (UTI) during pregnancy is found very high (33%). The physiological changes of pregnancy predispose women to UTI so does other factors such as age, sexual activity, previous history of UTI and socio-economic conditions. All pregnant women should be screened for UTI with a urine culture, treated with antibiotics if the culture is positive and then retested for cure. *E. coli* was the highly responsible bacteria for urinary tract infection in pregnant women found in this study. *Klebsiella spp, Proteus spp, Pseudomonas spp, Staphylococcus spp* and *Streptococcus spp* were also found in this study. In this study 20 antibiotics were used against different bacteria. Gram negative showed more resistance to these antibiotics as compared to gram positive organisms. In this present study Levofloxacin, Gentamycin, Ciprofloxacin, Kanamycin, Ofloxacin and Chloramphenicol are highly active against UTI pathogens.

Present study shows that UTI pathogens showed decreased susceptibility to most of the antibiotics usually used for the treatment of UTI. It is now very necessary to develop new antimicrobials and therapeutic agents having high effectiveness with no side effects, easy availability and less expensive.

From the above, the future study can also help in the following way

1. In this limited attempt, *E. coli., Klebsiella spp, Proteus spp, Pseudomonas spp, Staphylococcus spp and Streptococcus spp* of bacteria have been isolated from urine samples of pregnant women from few hospital at Dinajpur district. So, there is need to study other hospital at Dinajpur district to identify the bacteria associated with UTI.
2. Sero-typing and pathogenicity studies of the isolated bacteria will be required to identify their actual activities as pathogens and cellular defense mechanism of the bacteria.
3. Identify resistant plasmid of bacteria and designed antibiotic against this bacteria.
4. Molecular characterization of *Klebsiella spp, Pseudomonas spp, Staphylococcus spp and Streptococcus spp.*

CHAPTER-7

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APPENDICES

APENDIX I

A. Composition of different media

1. Nutrient broth

Ingredients	**g/L**
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final p^H (at 25°C)	7.4±0.2

2. Nutrient agar

Beef extract	3.0
Peptone	5.0
Sodium chloride	5.0
Agar	20.0
Final p^H (at 25°C)	7.1±0.1

3. Mac Conkey agar

Peptone	17.0 gm
Protease peptone	3.0 gm
Lactose	10 gm
Bile salts	1.5 gm
Sodium chloride	5.0 gm
Agar	13.5 gm
Neutral Red	0.03 gm
Crystal violet	0.001 gm
Final pH (at 250C)	7.1 ± 0.2

4. Blood Agar

Blood Agar	60 gm
Distilled Water	1000 ml
Bovine blood	5.00 ml
Or Nutrient agar	500 ml
Sterile Defibrinated blood	25 ml

5. Salmonella-Shigella agar

Peptic digest of animal tissue	5.00 gm
Beef extract	5.00gm
Lectose	10.00 gm
Bile salts mixture	8.50 gm
Sodium citrate	10.00 gm
Sodium thisulphate	8.50 gm
Ferric citrate	1.00 gm
Brilliant green	0.00033 gm
Neutral red	0.025 gm
Agar	15 gm
Disstilled water	1000 ml
Final P^{H} (at 25 ^{0}C)	7.0 ± 0.2

6. Eosine methylene blue agar

Peptone	100
Lactose	10.0
K2HP04	2.0
Eosin	0.4
Methylene blue	0.065
Agar	20.0
Final pH	6.8±0.2

7. Muller- Hinton Agar	**g/L**
Beef infusion	300.00 gm
Casein acid hydrolysate	17.50 gm
Starch	1.50 gm
Agar	17.00 gm
Final pH (at 25^{o}C)	7.3 ± 0.1 gm

8. Tryptic Soy Agar

Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g

9.Cetrimide Agar Base

Ingredients	**Gms / Litre**
Pancreatic digest of gelatin	20.000
Magnesium chloride	1.400
Potassium sulphate	10.000
Cetrimide	0.300
Agar	15.000
Final pH (at 25°C)	7.2±0.2

10.Baird Parker Agar

Ingredients	**Gms / Litre**
Casein enzymic hydrolysate	10.000
Meat extract B#	5.000
Yeast extract	1.000
Glycine	12.000
Sodium pyruvate	10.000
Lithium chloride	5.000
Agar	20.000
Final pH (at 25°C)	7.0±0.2

11. Mitis Salivarius Agar Base

Formula / Liter Supplement (# 7989)

Enzymatic Digest of Casein	15 g
Tellurite Supplement (1%) Chapman	
Enzymatic Digest of Animal Tissue	5 g
Potassium Tellurite	100 mg
Sucrose	50 g
Dextrose	1 g
Dipotassium Phosphate	4 g
Trypan Blue	0.075 g
Crystal Violet	0.0008 g
Agar	15 g
Final pH	7.0 ± 0.2 at 25°C

12. Sugar media

a. Peptone water

Bacto-peptone	10.0 gm
Sodium chloride	5.0 gm
0.5% Phenol red	0.1 ml
Distilled water	1000 ml

b. Sugar solutions

Individual sugar	5 gm
Distilled water	100 ml

c. Sugar media preparation

Peptone water	4.5 gm
Sugar solution	0.5 ml

13. Simmons citrate agar

Ingredients	**g/L**
Magnessium sulphate	0.20
Ammunium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothvmol blue	0.08
Agar	15.0

14. TSI Agar slant

Ingredients	gm/L
Lab Lamco Powder	3.00 gm
Yeast extract	3.00 gm
Peptone	20.00 gm
Sodium chloride	5.00 gm
Lactose	10.00 gm
Sucrose	10.00 gm
Glucose	1.00 gm
Ferric citrate	0.3 gm
Sodium thiosulphate	0.3 gm
Phenol red	0.3 gm
Agar	12.00 gm
Distilled water	1000 ml
Final pH	7.4 +/- 0.2 (at 25°C)

15. MR- VP medium (Himedium, India)

Composition	7.0
Buffered peptone	
Dextrose	5.0
Dipotassium phosphate	5.0
Final pH (at 25°C)	6.9±0.2

APPENDIX II

B. Preparation of reagents

1. Peptone water

peptone	1gm
Distilled water	1000 ml

2. Kovac's reagent for indole preparation

P- dimethyl aminobenzal dehyde	5 gm
Amyl alcohol	75 gm
Conc. HCL	25 ml

3. V-P reagent-1

5% alpha- naphthanol in absolute ethyl alcohol

4. V-P reagent-2

40% potassium hydroxide containing 0.3% creatine. The ingredient was dissolved by heating gently over a steam bath. When in solution, added 0.052 gm of cotton blue dye.

5. Methyl red Solution

Methyl red	0.05 gm
Ethanol(absolute)	28 ml

6. Phenol red solution

0.2% aqueous solution of phenol red

7. Gram stain solution

A. Stock Crystal violate

Crystal violate	10.0 gm
Ethyl alcohol (95%)	1000 ml

B. Stock oxalate solution

Ammonium oxalate	1.0 gm
Distilled water	1000 ml

C. Crystal violet working solution

20 ml of solution no. (**a**) Mixed with 80 ml of solution no. (**b**). Additional dilution was made when desired.

D. Ethyl alcohol	250 ml
E. Acetone	250 ml

F. Counterstain

Safranin	2.5 ml
Ethyl alcohol (95%)	100 ml

Safranine working solution:

The stock safranine is usually diluted as 1:4 with distilled water.

8. Oxidase reagent

Tetramethyl-p- phenylenediamine	0.1 ml
Distilled water	10 ml

9. 3% Hydrogen per oxide (H_2O_2) for catalase test

H_2O_2	3 ml
Distilled water	97 ml

10. Potassium Tellurite 3.5%

Components	(1 mL per vial)
Potassium Tellurite	0.35g
Distilled water	1mL

11. Shelf life of prepared cultured medium

BA	The plates may be stored at 2-8 ^{0}C
MA	4 weeks at 2-8 ^{0}C
NA/NB	Up to 2 years in cool dark place
SS	agar6 weeks at 2-8 ^{0}C