BACTERIOLOGY OF URINARY TRACT INFECTION AMONG THE PATIENTS VISITING TRIBHUVAN UNIVERSITY TEACHING HOSPITAL (TUTH)

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BY

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RECOMMENDATION

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………………...

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ABSTRACT

Urinary Tract Infection (UTI) is one of the most common diseases encountered worldwide and is a major public health problem in terms of morbidity and financial costs.

A prospective cross-sectional study was carried out from Aug 2012 to Jan 2013 in department of microbiology at TUTH. A total of 1,063 urinary tract samples (Mid stream urine, catheter, and suprapubic aspirate) from patients visiting TUTH were included. Samples were processed for routine microscopy and culture and the organisms were identified by standard microbiological methods. Antibiotic susceptibility testing was done by Kirby-Bauer Disk Diffusion method according to CLSI guidelines. ESBL producing organisms were detected by Combination Disk method, MBL producing organisms were detected by EDTA-Imipenem combined disk assay and Methicillin resistance *Staphylococcus aureus* (MRSA) was detected by Cefoxitin disk.

Of the total 1,063 samples processed, 276(26.0%) samples showed significant bacteriuria. Twelve different genera of bacteria were isolated from total 276 isolates among which *E.coli* (57.6%) was the most common isolate followed by *Staphylococcus aureus* (14.1%), *Klebsiella* spp (6.2%), *Enterococcus faecalis* (5.4%), *Staphylococcus epidermidis* (3.9%), *Pseudomonas aeruginosa* (3.3%), *Acinetobacter* spp (2.5%), *Enterobacter* spp (2.2%), *Burkholderia cepacia* Complex (1.8%), *Staphylococcus saprophyticus* (1.4%), *Citrobacter* spp (1.1%) and others (<1%). Among the gram negative isolates (210), majority (61.0%) of the isolates were found MDR. Nearly 58% of MDR isolates were ESBL producer and 5% were MBL producers. Most of the ESBL and MBL producing isolates were detected in *E.coli* and *Klebsiella* spp respectively. Most of the Gram negative bacterial isolates were sensitive to Polymyxin B followed by Imipenem, Amikacin and Cotrimoxazole. Among the gram positive isolates (66), 42.4% of isolates were MDR. All the MDR *S. aureus* (21/39) isolates were found MRSA. All Gram positive isolates were sensitive to Vancomycin and Teicoplanin followed by Amikacin. Among the oral antibiotics tested, Nitrofurantoin was found to be the most sensitive antibiotic for gram positive as well as gram negative bacterial isolates.

It revealed that *E. coli* is the predominant Uropathogens in TUTH, Nepal. There is an increasing resistance to many antibiotics in the both community and hospital settings. Now-a- days, ESBL and MBL producing uropathogens are emerging.

Keywords: Antimicrobial Susceptibility Profile, ESBL, MBL, MDR, MRSA, UTI

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LIST OF ABBREVIATIONS

A/A	Acid/Acid
ALK/A	Alkaline/Acid
ATCC	American Type Culture Collection
BA	Blood Agar
CA	Clavulanic Acid
CAZ	Ceftazidime
CFU	Colony Forming Units
CLSI	Clinical and laboratory Standards Institute
CONS	Coagulase negative Staphylococci
CTX	Cefotaxime
DDST	Double Disk Synergy Test
DNA	Deoxyribonucleic Acid
ESBL	Extended spectrum β-Lactamase
H_2S	Hydrogen Sulphide
HPF	High Power Field
IMP	Imipenem
LF	Lactose Fermenter
MA	MacConkey Agar
MBL	Metallo-β-Lactamase
MDR	Multi-drug resistance
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
MR/VP	Methyl Red/Voges Proskauer
MSU	Mid stream Urine
N/No	Number
NA	Nutrient Agar

NLF	Non-lactose Fermenter
O/F	Oxidative/Fermentative
OPD	Out patient Department
RBC	Red Blood Cells
Rpm	Revolution per Minute
SIM	Sulphide Indole Motility
TSI	Triple Sugar Iron
TUTH	Tribhuvan University Teaching Hospital
UTI	Urinary Tract Infection
VP	Voges Proskauer
WBC	White Blood Cells
WHO	World Health Organization
ZOI	Zone of Inhibition
GNB	Gram Negative Bacteria

CHAPTER I

1.1 INTRODUCTION

Urinary tract infection is one of the commonest domiciliary and nosocomial bacterial infections, comprising of a variety of clinical conditions caused by microbial invasion of tissue lining the urinary tract which extends from renal cortex to urethral meatus. Infection of adjacent structures such as prostate and epididymis is also included in this entity. It also refers to the presence of bacteria undergoing multiplication in urine within the urinary drainage system and presence of more than 10^5organisms/ml in the mid-stream sample of urine (Jha and Bapat, 2005; Leigh, 1990).

Urinary tract infection (UTI) is considered to be the most common bacterial infection. More than 80 million people in the United State develop UTI each year (Coker *et al*., 2000; Nicolle, 2001). Worldwide, about 150 million people are diagnosed each year with UTIs, costing in excess of 6 billion dollars (Gupta, 2001). In the developing countries, the disease has more prevalence due to poor personal hygiene, life style, malnutrition and environmental condition.

Urinary tract infection (UTI) is one of the most important causes of morbidity in the general population, and is the second most common cause of hospital visits**.** Urinary tract infection (UTI) is one of the most common bacterial infections seen in children. It is estimated that at least 1% of boys and 3% of girls develop urinary tract infection during first ten years of life (Watson *et al*., 2003).

UTI is one of the commonest diseases among female due to short urethra compared with the male and lies close proximity to the anal region. Thus, the bacteria can reach the urethra and bladder more easily in the female. In the community, women are more prone to develop UTI. About 20% of women

experience a single episode of UTI during their lifetime, and 3% of women have more than one episode of UTI per year (Gebre-Selassie, 1998).

Asymptomatic bacteriuria among pregnant women has been reported worldwide and becoming a clinical threat due to absence of signs and symptoms of the infection. Asymptomatic bacteriuria has been reported in 2 to 7% of pregnancies (Dutta, 2007; Mohammad *et al*., 2002). Specific subpopulations at increased risk of UTI include infants, pregnant women, the elderly, patients with spinal cord injuries and/or catheters, patients with diabetes or multiple sclerosis, patients with acquired immunodeficiency disease syndrome/human immunodeficiency virus, and patients with underlying urologic abnormalities (Siroky, 2002; Stapleton, 2002).

Catheter-associated UTI is the most common nosocomial infection, accounting for >1 million cases in hospitals and nursing homes. Nosocomial UTI among newly catheterized patients is frequently asymptomatic (90%), and the risk of UTI increases with increasing duration of catheterization. In catheterized patients, the risk of bacteriuria is estimated to be 5 to 10% per day and most patients with an indwelling urinary catheter for 30 days or longer develop bacteriuria (Sedor and Mulholland, 1999; Stamm, 1991; Warren *et al.,* 1982).

Resistance bacteria are emerging world wide as a threat to the favorable outcome of common infection in the community and hospital settings. Among the wide array of antibiotics, β-lactams are the most widely used agents accounting for over 50% of all systemic antibiotics in use (Pitout *et al.,* 2005). Acquired resistance to these antibiotics in gram-negative bacteria is mainly mediated by bacterial β-Lactamases and the emergence of extended-spectrum β-Lactamases (ESBLs) is of great clinical importance (Bradford, 2005).

ESBLs have the ability to inactivate most β-lactam antibiotics, including oxyimino-β-lactams such as ceftazidime, ceftiofur, and aztreonam. They do not hydrolyze Cephamycins and carbapenems and they are inhibited by clavulanic acid. Because of the increasing importance of multiresistant ESBL-producing E. coli, clinicians should be aware of the possibility of treatment

failures of serious infections caused by these bacteria. The acquired metallo–β-Lactamases (MBLs) represent an emerging threat in the field of infectious diseases because of their rapid spread, increasing diversity, and broad hydrolytic spectrum, which includes the carbapenems enzymes. Increasing rates of resistance among bacterial uropathogens has caused growing concern in both developed and developing countries (Bradford, 2005; Gupta, 2002; Rodriguez-Villalobos *et al.,* 2011).

To the date, UTI is the second most common diseases next to respiratory tract infection. In modern medical practice, newer antibiotics have been used extensively resulting in emergence and rapid dissemination of resistant bacterial strains. The pathogens traditionally associated with UTI are changing many of their features, particularly because of antimicrobial resistance. The etiology of UTI is also affected by underlying host factors that complicate UTI, such as age, diabetes, spinal cord injury, or catheterization (Nicolle, 2001; Ronald *et al.,* 2001).

Up to now every few studies are done in Nepal regarding the multidrug resistant ESBL and MBL producing bacteria causing UTI. Also, in many parts of Nepal, the facilities for urine culture and antimicrobial susceptibility testing are still not available, leading to improper diagnosis and irrational antibiotic treatment (e.g. self-medication) of UTI. The updated knowledge and situation of the prevailing bacterial uropathogens that are multidrug resistant (MDR) is of prime importance for the proper use of antimicrobial drugs and the policy making to combat multidrug resistance in UTIs.

So, this study is intended to address the issues regarding the prevalence of ESBL and MBL producing strains in community and hospital acquired bacterial isolates causing UTI. The result of this study will also help clinicians in order to facilitate the empirical treatment of patients and management of patients with symptoms of UTIs. Moreover, the data would also help relevant authorities to formulate antibiotic prescription policies.

1.2 OBJECTIVES OF THE STUDY

1.2.1 General objective

To determine the bacteriological prevalence of Urinary Tract Infection among the inpatients and outpatients visiting TUTH and their antibiotic susceptibility pattern

1.2.2 Specific objective

i. To isolate and identify the pathogenic bacterial isolates from urinary tract specimens (mid stream urine, catheter and suprapubic aspirate).

ii. To describe the antibiotic susceptibility pattern of bacterial isolates isolated from different urinary tract samples.

iii. To determine Multi Drug Resistance pattern (MDR) of bacterial isolates, isolated from different urinary tract samples.

iv. To screen for Extended Spectrum β- Lactamase (ESBL), Metallo β-Lactamase (MBL) producing bacterial isolates on MDR isolates.

v. To determine the prevalence of Methicillin Resistance S*taphylococcus aureus* causing UTI

CHAPTER II

2. LITERATURE REVIEW

2.1 Urinary Tract Infection (UTI)

A urinary tract infection (UTI) is a condition where one or more parts of the urinary system (the kidneys, ureters, bladder, and urethra) become infected. UTIs are the most common of all bacterial infections and can occur at any time in the life of an individual. Urinary tract infection simply means the presence of bacteria undergoing multiplication in urine within the urinary drainage system (Collier *et al.,* 1998). UTI is defined as the detection of both bacteriuria ($\geq 10^5$ CFU/ml and pyuria (≥ 10 leucocytes per HPF) (Goya *et al*., 1997).

Almost 95% of cases of UTIs are caused by bacteria that typically multiply at the opening of the urethra and travel up to the bladder. Much less often, bacteria spread to the kidney from the bloodstream (Azzarone, 2007).

The clinical manifestations of UTI depend on the portion of the urinary tract involved, the etiologic organism(s), the severity of the infection and the patient's ability to mount an immune response to it (Foxman and Brown, 2003). UTI encompasses a wide variety of clinical entities whose common denominator is the microbial invasion of any tissue of the tract from the renal cortex to the urethral meatus. Infection of prostate and epididymis is also included in the definition. Urethritis caused by Chlamydia and Gonococci is not included in the definition because of their unique characters and strict localization to the urethra and genital (Pokhrel, 2004).

In properly obtained midstream urine, the presence of $\geq 10^5$ CFU of bacteria per ml indicates significant urinary tract infection (Kass, 2002) and made possible a clear distinction between infection and contamination.

In order to confirm UTI with reasonable confidence, the following criteria must be met.

- Clinical features
- Significant bacteriuria
- Pyuria

Significant bacteriuria is the term that has been used to describe the numbers of bacteria in voided urine that exceed the numbers usually due to contamination from the anterior urethra.($\geq 10^5$ bacteria/ml) (Sobel and Kaye, 2000).

Kass, Marple and Sandford criteria to interpret significant bacteriuria

Less than 10,000 CFU/ml= Contaminants

Equal to / or more than 100000 CFU/ml= significant bacteriuria

10,000-100,000= low count significant bacteriuria, subject to the following conditions:

Urine was collected before the organisms reached to the phase of growth after the entry of bacteria into the urinary tract; Patient under treatment; sometimes in younger females; the count is low as honey moon cystitis; Patient with certain endocrine disorders e.g. diabetes; Chronic infection where concentrating power of kidney is low; Obstruction of the ureters; Infection with relatively slow growing organisms e.g. *Staphylococcus saprophyticus*, *Streptococci* other than *Enterococci*, *Haemophilus influenzae* (Pokhrel, 2004). Stamm *et al.*, (1982) suggested that a low count of coliform bacteria (≥100 organisms/ml) should always be considered significant in women with symptoms, particularly when there was pyuria.

Significant bacteriuria may sometimes occur in the absence of symptoms and pyuria in patients who subsequently develop symptoms of UTI e.g. in pregnancy. The detection of such “Asymptomatic Significant Bacteriuria” is of value for there is good evidence of its association with the development of pyelonephritis in some patients. Bacteriuria occurs in 2 to 7 % of pregnancies,

particularly in multiparous women, a similar prevalence as in non-pregnant women (Stenqvist *et al*., 1987).

The term asymptomatic bacteriuria refers to the presence of high quantities of an uropathogens in the urine of an asymptomatic person. Initial studies showed that colony counts $\geq 10^5$ CFU/ml more often predicted persistently high levels of bacteriuria compared with lower colony counts (Kass, 1956).

2.2 Types of Urinary Tract Infection

UTI encompasses a broad range of clinical entities that differ in terms of clinical presentation, degree of tissue invasion, epidemiological settings and requirement for antibiotic therapy. UTIs encompass a spectrum of clinical entities ranging in severity from asymptomatic infection to acute cystitis, prostatitis, pyelonephritis and Urethritis (Fowler, 1986).

2.2.1 On the basis of symptoms, severity and inflammatory changes, UTI can be classified as:

a. Uncomplicated UTI

Uncomplicated urinary tract infection refers to infection in structurally and neurologically normal urinary tract (Sobel and Kaye, 2000). In uncomplicated UTI, microbial invasion of the urinary tract occurs but no any inflammatory changes are seen i.e. shows no structural and neurological lesions in the tract. The first episode in this type of UTI usually caused by *E.coli* and has been found particular in female. Most common organisms encountered in uncomplicated UTI are *E.coli*, *Klebsiella* spp, *Proteus* spp, *Citrobacter* spp, *Enterobacter* spp etc.

Escherichia coli is the major causative pathogen in both uncomplicated upper and lower urinary tract infection, being present in approximately 70 to 95 % of cases. *Staphylococcus saprophyticus* is found in 5 to 20 % of cases of cystitis or even higher in some studies (Hooton and Stamm, 1997). Occasionally, other Enterobacteria, such as *Proteus mirabilis* and *Klebsiella* spp, are isolated according to Guidelines on urological infections (Grabe, 2011).

b. Complicated UTI

A complicated UTI is an infection associated with a condition, such as a structural or functional abnormality of the genitourinary tract (Nicolle, 2005), or the presence of an underlying disease that interferes with host defence mechanisms, which increase the risks of acquiring infection or of failing therapy. Enterobacteria are the predominant pathogens, with *E. coli* being the most common pathogen. However, non-fermenters (e.g. *Pseudomonas aeruginosa*) and Gram-positive cocci (e.g. *staphylococci* and *Enterococ*ci) may also play an important role, depending on the underlying conditions.

Factors that suggest a potential complicated UTI

i. The presence of an indwelling catheter, stent or splint (urethral, ureteral, renal) or the use of intermittent bladder catheterization
ii. Post-void residual urine of > 100 mL
iii. An obstructive uropathy of any aetiology, e.g. bladder outlet obstruction (including neurogenic urinary bladder), stones and tumour
iv. Vesicoureteric reflux or other functional abnormalities
v. Urinary tract modifications, such as an ileal loop or pouch
vi. Chemical or radiation injuries of the uroepithelium
vii. Peri- and postoperative UTI
viii. Renal insufficiency and transplantation, diabetes mellitus and immunodeficiency

2.2.2 UTI is classified as primary or recurrent depending on whether the infection is occurring for the first time or is a repeat event

Recurrences may be either “relapse” after treatment cessation with the pretherapy isolate or as a “reinfection” with a different organism after initial treatment cessation (Foxman, 2002). In cases of recurrent UTI the dominant organisms are the uropathogens, generally *Escherichia coli* (Stamey, 1981).

Recurrent UTI occurs in 27-48 % of healthy women, even though they generally have anatomically normal urinary tract (Hooton, 2000).

2.2.3 On the basis of source of infection, UTI can be classified as:

a. Community Acquired UTI (non-catheter associated UTI)

This occurs in patients who are not admitted to the hospital at the time they become infected. *E.coli* is by the most frequent cause of uncomplicated community acquired UTI. Other bacteria isolated from patients with UTI include *Klebsiella* spp, other Enterobacteriaceae and *Staphylococcus saprophyticus* and *Enterococci*. In more complicated UTI or recurrent infections, *Proteus, pseudomonas, Klebsiella* and *Enterobacter* spp increases (Forbes, 2002). *E.coli* is the commonest urinary pathogen accounting for over 80% of community-acquired infection.

b. Hospital acquired UTI (Catheter associated or nosocomial UTI)

The hospital environment plays an important role in determining the organisms involved in the UTI. Hospital acquired UTI are those developing in patients after admission to the hospital which were neither present nor in incubation at the time of hospitalization. As many as 20% of all hospitalized patients are most likely to be infected by *E.coli, Klebsiella* spp, *Proteus mirabilis, Staphylococcus* spp, other Enterobacteriaceae, *Pseudomonas aeruginosa and Enterococcus* spp. The distribution of urinary pathogens in hospitalized patients is different, with *E.coli* accounting for about 50% of infections. *Enterococcus, Klebsiella, Enterobacter, Citrobacter, Serratia, Pseudomonas aeruginosa, Providencia* and *Staphylococcus epidermidis* account for most of the rest (Bryan and Reynolds, 1984).

Urinary tract infections (UTIs) are among the most common infectious diseases acquired in nosocomial settings (Rebecca, 2010). Hospital acquired urinary tract infections (HAUTI) account for 35-45 % of the nosocomial infections (Thoburn *et al*., 1968). About 80 % of these are associated with the use of urinary catheters (Krieger *et al.,* 1983).

Risk of infection is about 3-10% for each day of catheterization. Patients' own bowel flora may colonize the periurethral area and reach bladder via the external surface of the catheter, especially in females due to anatomical proximity (Stamm, 2005).

2.2.4 On the basis of anatomic site of infection and clinical forms UTI can be classified as:

a. Urethritis

Urethritis is an infection or inflammation of the urethra. This can be due to other things besides the organisms usually involved in UTI's. In particular, many sexually transmitted diseases (STD's) appear initially as Urethritis. Urethritis, the infection of urethra, is a common infection because *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Trichomonas vaginalis* are common cause of urethritis and are considered to be sexually transmitted. Colonization of the urethra with gram-negative aerobic bacilli commonly occurs in catheterized patients; in men they may also be acquired by sexual intercourse (Stamey, 1980).

b. Cystitis

Infection of urinary bladder is called cystitis. The term cystitis has been used to describe lower UTI, which is characterized by a syndrome involving dysuria, frequency, urgency and occasionally suprapubic tenderness (Sobel and Kaye, 2000).

Escherichia coli cause 75 to 90 % of episodes of acute uncomplicated cystitis and *Staphylococcus saprophyticus* accounts for 5 to 15 % mainly in younger women. *Enterococcus* spp and aerobic gram-negative rods other than *E. coli*, such as *Klebsiella* spp and *Proteus mirabilis*, are isolated in the remainder of the cases (Ronald, 2002).

The most important risk factors for acute cystitis in young women are a history of previous episodes of cystitis and frequent or recent sexual activity (Scholes, 2000). Cystitis can often occur at the same time as Urethritis.

c. Acute urethral syndrome

Patients with this syndrome are primarily young, sexually active women who experience dysuria, frequency and urgency but yield fewer organisms than 10^5 CFU per ml urine in culture. Almost 50% of all women who seek medical attention for complaints of symptoms of acute cystitis fall into this group. *Chlamydia trachomatis* and *N. gonorrhoeae* Urethritis, anaerobic infection, genital herpes and vaginitis account for some of the causes of acute urethral syndrome. Most of these women are infected with organisms identical to cystitis but in number less than 10^5 CFU per ml urine. Approximately 90% of these women have pyuria, an important discriminating feature of infection (Forbes, 2002).

d. Pyelonephritis

Pyelonephritis refers to inflammation of the kidney parenchyma, calices and pelvis, usually caused by bacterial infection. The typical clinical presentation of an upper UTI includes fever, flank pain and frequency. Patients can also exhibit systemic signs of infection such as vomiting, diarrhoea, chills, increased heart rate and lower abdominal pain. Of significance, 40% of patients with acute pyelonephritis will be bacteremic (Forbes, 2002). In one study carried out by Czaja *et al.* (2005), *E. coli* accounted for about 82 % of pyelonephritis cases in women and 73 % in men.

e. Renal infection other than pyelonephritis

Renal carbuncle due to *Staphylococcus aureus* usually follows skin infection such as boil or carbuncle or an infection of intravenous line; the organism reaches the kidney through the blood stream. Other infection are perinephric abscess and pyonephrosis, it results from bacterial infection arising in an obstructed ureter and is usually unilateral (Collier *et al.*, 1998).

f. Bacterial prostatitis

The term prostatitis has been used for various inflammatory conditions affecting the prostate including acute and chronic infections with specific bacteria and more commonly instances in which signs & symptoms of

prostatic inflammation are present but no specific organisms can be detected. Bacterial prostatitis is bacterial infection of the prostate gland (Nickel, 1996).

2.3 Resident Microorganisms of the Urinary Tract

In a healthy person, the kidney, urinary bladder and ureters are free of microorganisms. However, bacteria are commonly found in the lower portion of the urethra of both males and females and colonize its epithelium in the distal portion. Some of these organisms are:

i. CONS (excluding *S. saprophyticus*)
ii. Viridans and non-haemolytic Streptococci
iii. Lactobacilli
iv. Diphtheroids
v. Nonpathogenic *Neisseria* spp
vi. Anaerobic cocci
vii. *Propionibacterium* spp
viii. Anaerobic gram-negative bacilli
ix. Commensal *Mycobacterium* spp
x. Commesal *Mycoplasma* spp (Forbes, 2002)

Potential pathogens including gram-negative aerobic bacilli (primarily Enterobacteriaceae) and occasional yeasts are also present as transient colonizers. Potential source of contamination of the bladder urine specimen are the urethra, vaginal vestibule, vagina, labia and pubic hair in the female (Pfau and Sacks, 1970).

2.4 Host Defense Mechanism

Normal urinary tract is resistance to colonization by bacteria and efficiently and rapidly eliminates both pathogenic and non-pathogenic microorganisms that gain access to the bladder. Certain factors (antibacterial defense mechanism) normally play an important role in limiting the multiplication of bacteria in urinary tract though urine acts as good culture medium where bacteria may multiply. Some of the host defense factors are:

a) **Flushing mechanism** of the bladder exerts a major protective effect. When bacteria introduced into bladder there is tendency of spontaneous clearance.

b) **Low vaginal PH** results in lack of colonization of pathogenic bacteria. In normal fertile women, the vagina is colonized by Lactobacilli that maintains highly acidic environment. Acid is hostile to other bacteria. Lactobacilli also produce H_2O_2 which helps to eliminate bacteria and reduces the ability of *E.coli* to adhere to the vaginal cells.

c) Prostatic secretions: Prostatic secretions have been reported to have antibacterial activity in men (Stamey *et al.,* 1965)

d) **Urinary inhibitors of bacterial adherence**: Mucosal defence mechanisms consists of secreted substances-Tamm-horsfall protein, sIgA, Lactoferrin, bladder mucopolysaccharides etc are increased in urinary tract infection that inhibit the adhesion of bacteria or kill them.

e) Chemotactic Cytokine: Chemotactic Cytokine IL-8 is released at the mucosal site recruiting PMNs, resulting in pyuria, and contributes to the eradication of bacteriuria.

f) **Valvular structures**: Urinary tract is full of valvular structures .It prevents the ascent of bacteria by reflux of urine. Ureterovesicular valve permit a constant flow of urine from ureter to bladder but prevent when bladder is full or during micturation (Stephens and Lengaham, 1962).

2.5 Epidemiology of Urinary tract infection

a. Infecting organisms:

More than 95 % UTIs are caused by single bacterial species. *E.coli* is most frequent cause of uncomplicated UTI. In recurrent and complicated UTI in the presence of structural abnormalities as well as in hospital acquired UTI, the relative frequency of infection caused by *Proteus, Klebsiella, Enterobacter,* and *Pseudomonas, Staphylocooci* and *Enterococci* greatly increases (Sobel and Kaye, 2000).

b. Bacteriuria in children

The frequency of UTI in infants is about 1-2 % (Sobel and Kaye, 2000). It is estimated that at least 1% of boys and 3% of girls develop urinary tract infection during first ten years of life (Watson et al., 2003). The incidence of UTI varies depending on age and sex. In the first year of life, mostly the first 3 months, UTI is more common in boys (3.7%) than in girls (2%), after which the incidence changes, being 3% in girls and 1.1% in boys (Grabe, 2011).

c. Bacteriuria in adults

Females are more frequently affected by UTI (particularly cystitis) due to colonization of urethra with colonic gram-negative bacilli because of its proximity to anus, short length of urethra and sexual intercourse. The prevalence of UTI in young non-pregnant women is 1-3 % Sobel and Kaye, 2000). About 20% of women experience a single episode of UTI during their lifetime, and 3% of women have more than one episode of UTI per year (Gebre-Selassie, 1998).

It is estimated that 20-30% of women who have UTI will have a recurrent UTI (Hooten, 2000). Predisposition for recurrent UTI can partially be attributed to genetics, and partially depends on behavioral risk factors. The occurrence of UTI in age younger than 15 years, history of UTI in mother, spermicidal use and frequency of sexual intercourse are risk factors associated with recurrent UTI (Naber *et al.*, 2006)

d. Bacteriuria in Elderly population

With increasing age the prevalence of urinary tract infection (UTI) increases in both women and men. More than half of all women have at least one UTI in their lifetime and the risk of contracting a UTI increases in postmenopausal women. UTI is more common among old women because of a variety of anatomic and functional changes which arise with aging, such as hormonal changes, reduced uromucoid secretions, decreased renal ability and increased bacterial adherence to uroepithelial cell. According to Sobel and Kaye (2000), 10 % of men and 20 % of women older than 65 years suffer from bacteriuria.

e. Bacteriuria in patient with diabetes

Diabetics are predisposed to develop acute pyelonephritis and renal papillary necrosis. UTI is 3 times more common in diabetic than in non-diabetic women. In the study of Sibi *et al.*,(2011), the most frequent causative agents of UTI were *Escherichia coli* accounting for 39.4% of the isolates followed by *Staphylococcus* (18.4%), *Klebsiella* (15.7%), *Enterococcus* (13.1%), *Proteus* (7.8%), *Pseudomonas* and *Candida* (2.6% each).

f. Bacteriuria in pregnancy:

Pregnancy predisposes to upper tract infection due to dilation of ureters and renal pelvis, stasis in right ureter, atony-reduced tone in uretic musculature and temporary incompetence of the Vesicoureteric valves. UTIs are common during pregnancy. Most women acquire bacteriuria before pregnancy, and 20-40% of women with asymptomatic bacteriuria develop pyelonephritis during pregnancy (Grabe, 2011).

g. Other conditions:

Use of catheters: There is higher prevalence of developing UTI in hospitalized patient especially catheterized patients. Approximately 10-30 % of catheterized patients will develop bacteriuria (Forbes, 2002). Duration of catheterization is the important risk factor the development of catheter associated UTI. In the study of Ojha (2008) showed Bacteriuria develops in up to 25% of patients who require a urinary catheter for > 7 days, with a daily risk of 5%.

Neurogenic bladder dysfunction (interference with the nerve supply to the bladder as in spinal cord injury, tabes dorsalis, multiple sclerosis, diabetes and other disease may be associated with UTI),

Vesicoureteral reflux (reflux of urine from bladder up into ureters and sometimes in the renal pelvis) and Obstruction to flow of urine (by tumor, stricture, stone or prostatic hypertrophy) are the important risk factor for urinary tract infection.

2.6 Laboratory Diagnosis of Urinary tract infection

Among patients with symptoms suggestive of a UTI, the diagnosis can be confirmed by sending a clean-catch mid stream urine specimen for culture and for urinalysis.

2.6.1 Specimen collection

For the isolation and identification of bacteria in urine sample, the sample collection is very important. Contamination by normal vaginal, perineal and anterior urethral flora must be prevented for collection of a clinically relevant urine specimen. Generally there are four types of sample collection

- Clean-Catch, Midstream Urine
- Catheter specimen
- Suprapubic Aspiration
- Sterile urinary bag attached on the genital organs for infants

Urine collected in a normal individual by suprapubic aspiration of the bladder is sterile and does not contain leukocytes. This method represents the gold standard in the diagnosis of UTI. It is, however, not performed routinely in clinical practice in which urine samples are generally obtained after natural micturation; in this setting, some degree of artifactual contamination with normal urethral organisms must be accepted (Stamm, 1980).

Since urine itself is a good culture medium, all specimens should be processed by the laboratory within 2 hours of collection or be kept refrigerated at 4℃ until delivery to the laboratory and processed no longer than 18 hours after collection. Transport medium that can be used for urine specimens are 1.8% boric acid, sodium chloride or polyvinylpyrolidine (Pokhrel, 2004).

i) Macroscopic examination of Urine

Color and turbidity of urine is noted in the very initial step through which preliminary test results for infection are identified.

ii) Microscopic examination of urine

Urinalysis for detection of pyuria by dipstick or microscope has a sensitivity of 80-90% and a specificity of 50% for predicting UTI. Urine culture has a sensitivity of 50% (if threshold for positive is >10^5 organisms); sensitivity can be increased to >90% if threshold is >10^2 organisms (*National Guidelines Clearing House,* 2005).

The specimen is ideally examined within 30 to 60 minutes of voiding. The urine should be centrifuged at 3000 rpm for five minutes, most of the supernatant poured out, and the pellet resuspended with gentle shaking of the tube. A small amount of the resuspended sediment is poured on the slide.

Urine is examined microscopically as a wet preparation to detect:

Significant pyuria i.e., WBC is in excess of 10 cells/ µl of urine, Red cell, Epithelial cells, Casts, Yeast cells and Bacteria (providing the urine is freshly collected)

In a normal patient, for example, one HPF may contain 0 to 4 white blood cells and 0 to 2 red blood cells, and one cast may be observed in 10 to 20 low powered fields (Wright, 1959).

Stamm has defined pyuria as the presence of at least eight thousand leukocytes per ml of uncentrifuged urine, which corresponds to two to five leukocytes per high-power field in centrifuged sediment (Stamm, 1980).

The accuracy of the findings on a culture of a midstream, “clean-catch” specimen of urine depends on how a positive culture is defined. When the traditional criterion, 100,000 bacteria per milliliter, is applied to a voided urine sample, the specificity is high, but the sensitivity is only about 50 percent. Lowering the threshold to 1000 bacteria per milliliter in the cases of young women with symptoms of cystitis raises the sensitivity considerably, with minimal reduction in specificity (Stamm, 1982). Although true infection without pyuria is unusual, pyuria can occur in the absence of apparent bacterial infection, particularly in patients who have already taken antimicrobials. Other causes of sterile pyuria include: (Eagan, 1989)

- Contamination of the urine sample by sterilizing solution
- Contamination of the urine sample with vaginal leukocytes
- Chronic interstitial nephritis (such as analgesic abuse nephropathy)
- Nephrolithiasis
- Uroepithelial tumor.

When the more common causes of sterile pyuria have been eliminated, patients with dysuria and frequency should be tested for atypical organisms such as *Chlamydia spp*, *Ureaplasma urealyticum*, or *M. tuberculosis* (McDonald *et al.,* 1982)

iii) Biochemical test of urine

Biochemical tests which are helpful in investigating UTI include

- Protein
- Nitrate reductase (Greiss) test
- Leucocyte esterase test
- Triphenyl tetrazolium chloride reduction
- Catalase test

iv) Culture of urine

Known volume of urine specimen obtained is inoculated on MacConkey agar and Blood agar without delay with the help of calibrated loop. The urine should be mixed thoroughly before plating. The calibrated loop method using plates of culture media is recommended because it is inexpensive, simple to perform and provides individual colonies that are easier to identify and remove for antimicrobial susceptibility testing.

v) Interpretation of urine culture results

Count the approximate number of colonies. Estimate the number of bacteria, i.e. colony forming units (CFU) per ml of urine. The standard definition of a positive urine culture is $\geq 10^5$ CFU/ml (Kass, 1956).

However, this definition does not apply to all patients. If fecal contamination has been ruled out, a lower colony count ($>10^2$/ml) may be indicative of UTI.

This was best demonstrated in studies in women who had dysuria and frequency but a midstream culture contained less than 10^5CFU/ml. This condition had been called the acute urethral syndrome (Komaroff, 1984).

In a Gynecology clinic at a student health center, 33 percent of women with urinary tract symptoms had $\geq 10^5$CFU/ml while another 46 percent had CFU counts between 10^2 and 10^4/ml. (Kunin *et al*., 1993).

vi) Identification and antibiotic susceptibility testing of causative organism

The causative organisms isolated are identified using various techniques. Gram staining and biochemical tests are performed as required. If similar colonies are found in numbers suggesting significant bacteriuria, a separate colony or a portion of apparently pure growth should be sub-cultured for identification and testing of its susceptibility to antibiotics. The appearance of the primary growth on MacConkey medium will suggest the kind of organism that is present. Probably coliform bacilli should be differentiated into *E. coli*, *Klebsiella* spp. *Proteus* spp etc and *Staphylococcus aureus* should be distinguished from other *Staphylococci* and *Enterococci* should be distinguished from other *Streptococci.*

2.6.2 Antibiotic Susceptibility Testing

An antibiotic now refers to a substance produced by a microorganism or to a similar substance (produced wholly or partly by chemical synthesis) which in low concentration inhibit the growth of other microorganisms (Hugo and Russel, 1983).

The primary goal of antimicrobial susceptibility testing is to determine whether the bacterial etiology of concern is capable of expressing resistance to the antimicrobial agents that are potential choices as therapeutic agents for managing the infection. These tests are assays designed to determine the extent of an organism's acquired resistance.

Modified Kirby-Bauer disk diffusion technique is followed for antibiotic susceptibility testing of causative organism.

For laboratory tests to accurately determine organism-based resistance the potential influence of environmental factors on antimicrobial activity must be minimized. To control the impact of environmental factors, the conditions for susceptibility testing are extensively standardized. The components of antimicrobial susceptibility testing that are standardized and controlled include the following:

- Bacterial inoculum size
- Growth medium: pH, cation concentration, blood and serum supplements and thymidine content
- Incubation temperature, atmosphere and duration
- Antimicrobial concentration tested

However there are some limitations for the use of standard conditions. Most notably the laboratory test conditions in no way mimic the in-vivo environment at the infection site where the antimicrobial agent and bacteria will actually interact. Because of the lack of correlation between in-vitro test conditions and the in-vivo setting, antimicrobial susceptibility testing cannot and should not be used as predictors of therapeutic outcome use for the use of particular antimicrobial agents. Additionally, several other important factors that play key roles in patient outcome are not taken into account by susceptibility testing. Some of these factors include:

- Antibiotic diffusion in tissues and host cells.
- Serum protein binding of antimicrobial agents.
- Drug interactions and interference.
- Status of patient defense and immune system.
- Multiple simultaneous illnesses.
- Virulence and pathogenesis of infecting bacterium.
- Site and severity of infection.

2.6.3 Measurement of antimicrobial activity

Determination of the susceptibility of a bacterial pathogen to antimicrobial drugs can be done by one of two principle methods: dilution or diffusion. Using an appropriate standard test organism and a known sample of drug for

comparison, these methods can be employed to estimate either the potency of antibiotic in the sample or the susceptibility of the microorganism.

a) Dilution method

b) Diffusion method

There are various types of disc diffusion sensitivity tests which vary in their methods of standardization, reading and control:

Modified Kirby-Bauer disc diffusion method

The Kirby-Bauer method (Bauer *et al*., 1966) and its modifications recognize three categories of susceptibility: susceptible, intermediate and resistant.

Susceptible: An organism is called "susceptible" to drug when the infection caused by it is likely to respond to treatment with this drug, at the recommended dosage.

Intermediate: It covers two situations. It is applicable to strains that are moderately susceptible to an antibiotic that can be used for treatment at a higher dosage because of its low toxicity or because the antibiotic is concentrated in the focus of infection (e.g. urine). The classification also applies to strains that show intermediate susceptibility to a more toxic antibiotic that cannot be used at a higher dosage.

Resistant: This term implies that the organism is expected not to respond to a given drug, irrespective of the dosage and on the location of the infection.

For clinical and surveillance purpose and to promote reproducibility and comparability or results between laboratories, WHO recommends the CLSI recommended modified Kirby-Bauer disc diffusion technique (Cheesbrough, 2000).

2.6.4 ESBL Detection

i) By Combination disc method (CLSI phenotypic method)

ii) By Double Disc Synergy Test

The CLSI-ESBL phenotypic confirmatory test with Ceftazidime, Cephotaxime, Ceftriaxone and Cefixime are performed for all the isolates by disk diffusion method on Mueller-Hinton agar plates with and without 10 µg of Ampicillin salbactam. A ≥ 5-mm increase in the zone of diameter of third generation cephalosporins, tested in combination with Ampicillin salbactam versus its zone when tested alone are considered indicative of ESBL production. *E. coli* ATCC 25922 is used as ESBL negative and *K. pneumoniae* ATCC 700603 is used as ESBL positive reference strains (CLSI, 2011).

2.6.5 MRSA Detection

MRSA Detection by Cefoxitin disc (CLSI, 2011). Cefoxitin is a cephamycin antibiotic and has been described as an inducer of Methicillin resistance (Okonogi *et al*., 1989).

2.6.6 MBL detection

Several phenotypic methods are practiced though there is no recommended phenotypic method available from CLSI for their detection. All these methods are based on the ability of metal chelators, such as ethylene diamine tetra acetic acid (EDTA) and thiol-based compounds, to inhibit the activity of MBLs (Picao et al., 2007; Lee *et al*., 2003; Pitout *et al*., 2007).

i) Screening test using CAZ (Pitout *et al*., 2007)

ii) Combined disk (CD) method (Franklin *et al*., 2006)

2.7 Multiplication of Bacteria in Urine

Infection of urinary tract occurs when bacteria capable of proliferating in urine get access into the tract because human urine contains no hormonal or cellular defenses against bacterial growth (Fowler and Mariano, 1990).

The capacity of the urine to support bacterial growth depends on urinary pH, osmolality and chemical constituents such as glucose, amino acids and organic acids. Glucose is the main energy source for the growth of urinary pathogens. The number of bacteria in the urine of diabetic patients was significantly higher than in that of nondiabetic controls due to high level of glucose (' Sullivan and Fitzerald, 1961).

2.8 Pathogenesis

2.8 .1 Source of infection

The great majority of bacterial infection, whether or not with symptoms, occurs after the ascending migration of the bacteria from the urethra or perineum. Cystitis is most common. Infection of the kidney may follow the haematogenous spread of bacteria, but more often the organism ascend from the bladder via the ureter and the renal pelvis and calyces (Forbes *et al.,* 2002).

2.8.2 Routes of infection

Bacteria can invade and spread within the urinary tract by three possible routes:

Ascending route: Urethra is usually colonized with bacteria, UTI much more common in female than male due to ascending route of infection. It has been shown that organisms that cause UTI in women colonize the vaginal introitus and periurethral region before UTI results. Instrumentation such as Urinary catherization, cystoscopy is the most common cause of hospital acquired UTIs in both sexes (Forbes *et al.,* 2002).

Haematogenous route: Infection of renal parenchyma by blood-borne organisms occurs in human usually kidney is frequently the site of abscesses in patient with staphylococcal bacteremia or endocarditis but rarely with gram-negative bacilli. Haematogenous spread accounts for less than 5 percent of UTIs (Forbes *et al.,* 2002).

Lymphatic route: Importance of lymphatic spread of uropathogens to the urinary tract in the pathogenesis of UTI is not known.

2.8.3 Bacterial virulence factors

Bacterial adhesion onto mucosal or urothelial cells is an important phenomenon determining bacterial virulence. Infection in the urinary tract is related in part to the ability of bacteria to adhere and colonize the gut,

perineum, urethra, bladder, renal pelvocalyceal system, and renal interstitium (Oelschlaeger, 2002).

Uropathogenic bacteria have evolved a range of virulence factors that promote colonization and infection of the urinary tract. The virulence factors most commonly associated with these organisms include possession of fimbriae with adhesin tips, protectins, bacterial capsule including lipopolysaccharide (LPS), and production of toxins such as haemolysin and colony necrotizing factor, fimbriae (Pili). Fimbriae are thin, hair-like, surface adhesive organelles made of protein subunits. They bind glycoprotein or glycolipid moieties on urothelial cells allowing the bacteria to attach to the epithelium and persist within the urinary tract (Abraham *et al.*, 2001).

Majority of strain of *E.coli* and most other Enterobacteriaceae possess type 1 fimbriae which attach to unidentified receptors on polymorphonuclear leucocytes (Collier,1998).These fimbriae recognize kidney glycosphingolipids carrying the Gal (1–4) Gal determinant via its papG adhesin. They are important in the pathogenesis of ascending UTI and pyelonephritis in humans and have been identified in 80% of pyelonephritis *E coli* isolates (Plos *et al.*, 1995).

The pathophysiology of bacterial adhesion in the urinary tract is complex. Uropathogenic Enterobacteriaceae is electronegative and too small to overcome repulsion by the net negative charge of epithelial cells. As a result, bacterial adhesion cannot occur in the absence of fimbriae or other surface adhesion systems. These systems have favorable electrical charge and also promote adhesion via hydrophobicity. Fimbriae allow irreversible attachment to the uroepithelial cell membrane via adhesins (Oelschlaeger *et al.*, 2002).

Proteus mirabilis is armed with various virulence factors, including the production of hemolysin and IgA protease, iron acquisition, flagella, fimbriae and, most importantly, the secretion of urease (Mobley *et al.*, 1994).

S. saprophyticus adheres strongly to the urothelium, a process that appears to be linked to a lactosamine residue (Hovelius and Mardh, 1984). *S. epidermidis* represents the most common source of infections on indwelling

medical devices. This likely stems from the fact that *S. epidermidis* is a permanent and ubiquitous colonizer of human skin and the resulting high probability of device contamination during insertion (Uckay, 2009).

2.9 Beta-Lactamases in general

Resistance bacteria are emerging world wide as a threat to the favourable outcome of common infection in the community and hospital settings (Johann *et al*., 2005).Among the wide array of antibiotics, beta lactams are the most widely used agents accounting for over 50% of all systemic antibiotics in use (Philip *et al.,*2005*).* B-lactam antibiotics now include penicillins, cephalosporins, carbapenems, and monobactam (Babic *et al*., 2006).

Beta-lactamases are enzymes that are produced by some bacteria and confer resistance to beta-lactam antibacterials (such as penicillins and cephalosporins). These antibacterials are so called because of the four-atom beta-lactam ring in their molecular structure (with the ring mimicking two amino acids in the pentapeptide cross-links of the peptidoglycan bacterial cell wall). Beta-lactamase enzymes break this ring open, deactivating the molecule's antibacterial properties. Many Gram-negative bacteria possess a naturally occurring, chromosomally mediated beta-lactamase that is thought to help the bacteria compete with other beta-lactamase producing bacteria, or to remove beta-lactam-like molecules that bacteria may use as natural regulators of cell wall synthesis (Turner, 2005).

Enterobacteriaceae (including *Escherichia coli*) commonly express plasmid-encoded beta-lactamases, such as TEM-1 and SHV-1, which confer resistance to penicillins but not to expanded-spectrum cephalosporins (Turner, 2005). As these enzymes are plasmid-mediated, they can spread to other members of the *Enterobacteriaceae* family (e.g. *Klebsiella pneumoniae*, *Proteus mirabilis*) and non-enteric organisms such as *Pseudomonas* and *Acinetobacter* species.

2.9.1 Extended spectrum β-lactamase (ESBL)

Urinary tract infection (UTI) is a common disease in the community, and a matter of concern due to the increasing resistance of microorganisms to first

line antibiotics and the emergence of multiresistant strains producing extended spectrum beta lactamases (ESBL) in the community.

ESBLs are enzymes capable of conferring resistance to the penicillins, first-, second-, and third-generation cephalosporin, and aztreonam. Cephamycins (Cefoxitin and cefotetan) and carbapenems still remain active as well as commonly used inhibitors like clavulanic acid, sulbactam, and tazobactam (Livermore, 2008); Paterson *et al*., 2005). With the exception of OXA-type enzymes, which are class D enzymes, the ESBLs are of molecular class A and can be divided into three groups: TEM, SHV, and CTX-M types (Pitout *et al.*, 2008).

First described in Germany (1983) and France (1985) among *Klebsiella* spp, ESBLs exist in every region of the world and in most genera of enterobacteria (Paterson *et al*., 2005).

The ESBL family is heterogeneous. SHV and TEM-type ESBLs arose by amino acid substitutions that allowed narrower spectrum enzymes to attack the new oxyimino beta-lactams. Others, notably members of the CTX-M family, represent plasmid acquisition of broad spectrum beta-lactamases originally determined by chromosomal genes. Traditionally, the SHV and TEM type ESBLs have predominated, which is still the case in the US; however, the CTX-M type are now becoming more common worldwide and the plasmids carrying these ESBLs often carry other enzymes conferring resistance to flouroquinolones as well (Peleg *et al*., 2010).

Enterobacteriaceae producing expanded-spectrum β-lactamases (ESBLs), those of the CTX-M type in particular, are a major problem worldwide, causing outbreaks as well as sporadic infections (Paterson *et al*., 2003). ESBLs have been found exclusively in Gram-negative organisms, primarily in *Klebsiella pneumoniae, Klebsiella oxytoca,* and *Escherichia coli* but also in *Acinetobacter, Burkholderia, Citrobacter, Enterobacter, Morganella, Proteus, Pseudomonas, Salmonella, Serratia,* and *Shigella spp* (Kliebe *et al*., 1985).

Although ESBLs have been described in a range of Enterobacteriaceae and Pseudomonadaceae from different parts of the world, they are most often

identified in *Klebsiella pneumoniae* and *Escherichia coli.* These enzymes belong to the Ambler class A and D beta-lactamases (Ambler *et al*., 1991).

ESBL varieties: TEM beta-lactamases, SHV beta-lactamases, CTX-M beta-lactamases, OXA beta-lactamases, others problems in identification arise because ESBLs are heterogeneous. OXA-type ESBLs, for example, are poorly inhibited by clavulanate. Some ESBLs are best detected with ceftazidime and others with cefotaxime (such as most CTX-M enzymes). Consequently, susceptibility to several oxyimino-beta-lactams must be tested; criteria for ESBL detection have changed over time; and clinical laboratories vary in their success in diagnosis.

Clinical laboratories vary greatly in their success in identifying ESBLs. In a study of 77 rural hospitals in the United States, only eight percent reported specifically screening for ESBLs in Gram-negative bacilli (Stevenson *et al*., 2003).

Molecular class B metallo-β-lactamases (MBLs) are a group of enzymes that require a metal ion (Zn2+) for β-lactam hydrolysis. Therefore unlike serine β-lactamases, they are not inhibited by common inhibitors but can be inhibited by chelating agents like EDTA. Most MBLs can hydrolyze all β-lactams but not aztreonam. The first MBLs discovered were chromosomal enzymes from *Bacillus cereus, Aeromonas* spp., and *Stenotrophomonas maltophilia*. Of greater importance are the acquired or transferable families of MBLs which include VIM, IMP, GIM, SIM, SPM, and NDM which are located within gene cassettes as part of integron structures (Walsh *et al.,* 2005).

Resistance to the carbapenems among Gram-negatives can arise through hyperproduction of class C β-lactamases or ESBLs and porin loss, augmented drug efflux, alterations in PBPs, and carbapenemases production (serine carbapenemases or metallo-β-lactamase) (Patel and Bonomo, 2011 ; Zhanel *et al*., 2007).

2.10 Various study on urinary tract infection

The majority of urinary tract infections are caused by bacteria and occasionally by fungi and viruses. The spectrum of causative organisms differs between community-acquired and hospital- acquired infections and the occurrence of antibiotic resistance. *E. coli* accounts for up to 70 % of community acquired infections, in hospital - acquired infections approximately 50 % by *E. coli*, 15 % by *Enterococcus faecalis* (Stuart *et al.*, 2003).

According to Ullah (2005), Common micro organisms in decreasing order of yield are *E. coli, Klebsiella* spp*, Staph aureus, Proteus* spp and *Pseudomonas* spp isolated from 80, 7.1, 5.0 & 1.5% samples respectively. *E.coli* has been found to be the most common infective agent in this series, being grown in 168 (80%) of cases. Imipenum is found to be the most effective for *E. coli.*

In Complicated nosocomial UTI caused by nonfermenters*, Pseudomonas spp.* were the commonest (45.4%) followed by *Acinetobacter* spp (39.0%), *Alcaligenes* spp (8.1%) and *Flavobacter* spp (3.63%) (Meharwal *et al.*, 2002).

The study by Tinelli *et al.,* (2012) involved 297 long-term care facilities patients (99 with ESBL+ GN UTI, 99 with ESBL- GN UTI and 99 without GN infection). ESBL+ GN UTIs were due to *Escherichia coli* (64%), *Proteus mirabilis* (25%) and *Klebsiella pneumoniae* (11%). The CTX-M-type enzymes were the most prevalent (73% of isolates), whereas TEM- and SHV-type ESBLs and AmpC-type enzymes were less prevalent (10%, 2% and 15% of isolates, respectively).

The prevalence of UTIs in the referral hospital in Nigeria was 67.2%. The incidence was higher in females with a prevalence rate of 54.3, while in males was 45.7%. Among the total isolates, Gram-negative isolates had a prevalence of 74.7%, while gram-positive isolates had 25.30%. The bacteria isolated in order of ranking were *E. coli* (24.5%)*, K. pneumoniae* (17.3%), *P. mirabilis* (14.6%), *S. faecalis* (13.4%), *S. aureus* (5.3 %)*, P. vulgaris* (4.7%), *P. stuartii* (4.1%), *S. epidermidis* (3.8%), *A. faecalis* (3.4%), *S. saprophyticus* (2.8%), *P. aeruginosa* (2.5%), *S. marsescens* (2.0%) and *C. fruendii* (1.7%) (EI-Mahmood, 2009).

A total of 520 urine samples were analyzed out of which 129(24.8%) showed significant growth and 115(89.1%) were gram-negative bacteria. The most frequently detected gram-negative bacteria were *E.coli* with 85% cases (73.9%). Female (79.1%) was more prone to develop UTI than males (20.9%) in the study. The most resistant antibiotics for *E.coli* was Ampicillin (98.4%) and Amoxicillin (83.7%) and the most sensitive antibiotic were Amikacin (93.3%), ciprofloxacin (91.5%), nitrofurantoin (89.8%) respectively (Mohammadi *et al*., 2010).

Richards *et al.*, (1999) concluded from survey in ICU in United States that Gram-positive cocci play a lesser role in UTI. *Staphylococcus saprophyticus* is now a common cause of UTI, particularly cystitis in young, sexually active women.

Nitrofurantoin is the drug of choice in community acquired UTI. For patients with lower UTI, empirical first-line treatment with Nitrofurantoin rather than Trimethoprim, Quinolones or Cephalexin might be more appropriate, as most uropathogens, including ESBL producing *E.coli,* are sensitive to Nitrofurantoin (Paterson *et al*., 2005).

The susceptibility of the isolates to the quinolone antibiotics were assessed using the disc diffusion method. Among the gram-negative bacteria, *P. aeruginosa* was less susceptible with a profile of (71.4%) to Ciprofloxacin, Ofloxacin (42.9%), Pefloxacin (57.1%), and Sparfloxacin (42.9 %), but no activity for nalidixic acid. The susceptibility pattern followed similar trend for the other gram-negative bacteria. Among the gram-positive bacteria, *S. aureus* was least sensitive with a profile of 64.0% for Ciprofloxacin, Ofloxacin (56.0%), pefloxacin (52.0%), Sparfloxacin (52.0%) and Nalidixic acid (8.0%). The quinolone antibiotics were still effective against the uropathogens, but should be reserved for only complicated UTIs to avoid the development of resistance (El-Mahmood *et al*., 2009).

Particularly, resistance patterns were alarmingly higher for Amoxycillin, Co-trimoxazole, Flouroquinolones and third-generation Cephalosporins, which

necessitate the re-evaluation of first and second line therapies for UTI (Baral, Shrestha *et al.,* 2012). In the study of UTI in diabetics, Trimethoprim was found to be effective for empirical treatment of UTI and has covered the majority of urinary pathogens followed by Nalidixic acid, Chloramphenicol and kanamycin (Sibi *et al.*, 2011).

In a recent nationwide study in Spain, 93% of ESBL producing *K. pneumoniae* strains were isolated from inpatients, while 51% of ESBL-producing *E. coli* strains were isolated from outpatients (Herna´ndez et al., 2003). A laboratory study published in 2001 from the northern part of Israel analyzing susceptibility patterns of 8338 bacteria isolated from community urines found that 1.25% of the Gram-negative uropathogens were ESBL producers (Colodner *et al*., 2001).

The study of Yasmin, revealed a higher occurrence of multidrug resistant ESBL producing *Klebsiella spp* (80%), *Proteus* spp (72%), *Enterobacter* spp. (71.4%), *E.coli* (67.3%) and *pseudomonas* spp (88.8%) from various clinical isolates (Yasmin, 2012).

The only current proven therapeutic option for severe infections caused by ESBL producing organisms is the carbapenem family (Imipenem, Meropenem, and Ertapenem). ESBL-producing isolates typically show greater than average resistance to other agents including aminoglycosides and fluoroquinolones. These relationships were illustrated in a review of 85 episodes of bacterimia due to ESBL producing *K. pneumoniae* from 12 hospitals in seven countries. All isolates were susceptible to Imipenem or Meropenem, while 71 % were resistant to Gentamicin, 47 % to Piperacillin plus tazobactum, and 20 % to Ciprofloxacin (Paterson e*t al*., 2004). Treatment with Imipenem or Meropenem has produced the best outcomes in terms of survival and bacteriologic clearance. Ertapenem has good in vitro activity (Jacoby *et al.,* 1997).

When an oxyimino beta-lactam (e.g., Cefotaxime, Ceftazidime, Ceftriaxone, or Cefepime) is used to treat severe infections caused by ESBL producing *K.*

pneumoniae, treatment failure is likely even if the organism tests susceptible to the antibiotic in-vitro. In a review of 28 patients with ESBL-producing *Klebsiella pneumoniae* with reported susceptibility to cephalosporins, 15 failed to respond to cephalosporin therapy (Paterson DL *et al*., 2001). Clinical and Laboratory Standards Institute (CLSI) recommends that such ESBL-producing organisms should be reported as resistant (CLSI, 2007). Plasmids responsible for ESBL production may also carry genes encoding resistance to other drug classes, for example, Aminoglycosides, Trimethoprim, and Fluoroquinolones (Turner, 2005).

However, overuse of carbapenems should be avoided and there should be prompt discontinuation of such therapy if an infection is found to be due to a non ESBL producing organism (Yang *et al*., 2007).

2.11 Pattern of etiological agents of UTI in Nepal

In the study of Jha and Bapat (2005) conducted in Kathmandu Valley, the most common organism to cause UTI was found to be *E. coli* (49%), followed by *S. aureus* (23%) and *Klebsiella* (9.71%). All the organisms causing UTI were sensitive to nitrofurantoin and Amoxycillin and Ciprofloxacin was found to be least effective.

The study done by Ojha (1999) at TUTH, establishes that Foley catheter was associated with increased risk of bacteriuria in postoperative women following obstetrical and gynecological surgery**.**

A study carried out by Shrestha *et al.,* (2005) found *E. coli* as the most predominant pathogen (60.2%) followed by *Staphylococcus epidermidis* (16.7%), *Staphylococcus aureus* (4.9%), *Klebsiella* spp (3.7%), *Proteus* spp (3.6%), *Pseudomonas aeruginosa* (3.1%), *Citrobacter freundii* (2.4%), *Morganella morganii* (2.4%) and others.

In the study conducted by Das *et al.,*(2006) the *Enterobacteriaceae* group, namely, *E. coli* (59.4%), *Klebsiella* spp (15.7%), *Enterococcus faecalis* (8.1%), and *Proteus mirabilis* (7.4%), were the most common pathogens isolated, followed by gram-positive cocci, namely, *Staphylococcus aureus*

(3.4%) and *Staphylococcus saprophyticus* (1.4%). Similarly, Chhetri *et al.,* (2001) also showed that *E. coli* was the most common isolate accounting for 77.5% of all bacterial isolates and was followed by *Proteus* spp, *Klebsiella* spp and *Staphylococcus* spp.

In a study in Nepal, Karki *et al.,* (2004) also found five different bacterial species as *E. coli* (33.3%), *Proteus* spp (27.7%), *Klebsiella* spp (16.6%), *Staphylococcus aureus* (8.8%) and *Pseudomonas aeruginosa* (1.1%).

Similarly the study carried in Pokhara, Nepal by Jha *et al*.,(2007) showed the prevalence of asymptomatic bacteriuria in school going children was 1.39% and in Bharatpur, Chitwan in 2009 ,the prevalence of asymptomatic bacteriuria among diabetic patients found to be 9.43% (12.07% in females and 5.08% in males.

A study carried out in 2008 in Tribhuvan University Teaching Hospital (TUTH), Nepal by Kattel *et al.,* (2009) showed 27% significant bacteriuria with *E.coli* most predominant among gram negative bacteria followed by *Klebsiella* spp and others. About 35% were ESBL producers which were higher in indoor patients compared to outdoor patients.

In the retrospective study done by Basnet *et al.,* (2009) in tertiary care hospital of Nepal, prevalence rate of UTI was 23.3%, most common isolates being *E.coli*(77.5%) followed by *Klebsiella* spp and others. UTI was found significantly higher in females compared to males.

According to Baral *et al.,* (2012) high prevalence of multidrug resistance in bacterial uropathogens was observed. Particularly, resistance patterns were alarmingly higher for amoxycillin, Co-trimoxazole, Flouroquinolones and third-generation Cephalosporins, which necessitate the re-evaluation of first and second line therapies for UTI.

In a prospective study conducted in Nepalese patients in TUTH by Dhakal *et al*.,(2002) found that *E. coli* was the most predominant microorganism causing

UTI and Nitrofurantoin was the most effective antibiotic against the isolated urinary pathogens.

The study carried out by Tuladhar *et al*., (1990) in TUTH showed that UTI was the most prevalent nosocomial infection which accounted (62.7%) of total nosocomial infection. Similarly, *E. coli* was the dominant etiological agent (48%) followed by *Pseudomonas* spp (25.8%), *Klebsiella* spp (22.5%) and *Staphylococcus aureus* (3.2%).

CHAPTER III

3. MATERIALS AND METHODS

3.1 Materials

The materials, equipments, media, chemicals and reagents used in this study are listed in the appendices III and IV.

3.2 Methodology

This prospective cross-sectional study was carried out in Department of Microbiology at Tribhuvan University Teaching Hospital (TUTH), Kathmandu from August 2012 to January 2013. A total of 1063 urinary tract samples received from outdoor patients as well as indoor patients were studied. This study included all urinary tract samples suspected of UTI (mid stream urine, catheter tip, supra pubic aspirate) received for culture and antibiotic sensitivity testing in the laboratory on the collection day. Patients of all age groups and both sexes visiting TUTH were included in the study. The demographic parameters, clinical history, prior antibiotic use were recorded in the data collection form (Appendix I).

3.3 Sample size and sample types

A total 1063 urinary tract samples including mid stream urine (950), Catheter (111) and suprapubic aspirate (2) were studied during the study period. Specimen not properly representing from urinary tract, (like stool mixed urine), specimen not properly labeled, sample vial without cover, sample in unsterilized vial was not processed for culture. Duplicate isolates from the same patient was also excluded in the study.

3.4 Sample Design

3.4.1 Specimen Collection

Patient suspected of UTI attending TUTH from August 2012 to Jan 2013 was be included in this study. Questionnaires were filled up regarding all the clinical information by asking to the participants as well as by looking into their physicians check up file. They were instructed how to collect clean catch midstream urine and provided with sterile universal containers to obtain 10 to 15 ml urine sample. The collected urine samples then were transported to the laboratory for processing within one hour. Other urinary tract samples- catheter tips, supra pubic aspiration were also received for culture**.** The methods for the collection, isolation and identification were followed as described by American Society for Microbiology (Isenberg, 2004).

Detailed guidelines for collection of clean midstream urine are mentioned in appendix V.

3.4.2 Sample processing

All the samples collected were labeled with lab no. and urine samples were processed for routine microscopic examination and culture whereas Catheter samples were directly processed for culture. Control strains of American Type Culture Collection (ATCC) were used parallel as a part of quality control of test system. Both positive and negative controls were included during the tests. Urine samples showing mixed growths were not repeated for culture during the study period.

3.4.2.1 Microscopic Examination

Five ml of urine sample was taken in a clean sterile centrifuge tube and centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded. The sediment was then examined microscopically at high power magnification for the presence of pus, red blood cells, epithelial cells, casts, crystals, and yeast cells. Pus cells $\geq$5/HPF for female and $\geq$3/HPF for Male was considered significant for infection.

3.4.2.2 Culture of specimens

Culture of each uncentrifuged urine sample was done by semi quantitative technique using a standard calibrated loop on Blood agar (BA) and MacConkey agar (MA) plates before microscopic examination.

A calibrated loop was immersed just below the surface of well-mixed uncentrifuged urine specimen. A loopful of urine was then streaked on to the plate to make straight line inoculum down the centre of the plate and the urine was streaked by making series of passes at 90^0 angles throughout the inoculum. Similarly, catheter samples were directly inoculated on Blood Agar and MacConkey Agar plates. Plates were then incubated aerobically at 37^0C for 18-24 hours.

3.4.2.3 Examination of culture plates

The culture plates were observed after 18-24 hours. Colonies were counted. Samples showing $\geq 10^5$colony forming unit (CFU) per milliliter (ml) of urine were taken significant. Low count significant bacteriuria (10^4-10^5CFU/ml) was taken into consideration if there was any indication which can lower the concentration of bacteria in the urine. Growth of more than two contaminating organisms was reported as mixed growth and request for repeat sample for culture. In case of Catheter and suprapubic aspirate samples, growth of pure colonies of bacteria was also processed rather than colony count. Blood agar was observed for haemolysis and MacConkey agar for lactose fermentation and lactose non-fermentation.

3.4.2.4 Identification of isolates

The identification of various gram negative bacterial isolates from positive culture plates was done with the use of standard Microbiological techniques as described in Bergey's Manual of Systemic Bacteriology which comprises of studying the colony morphology, Gram staining reactions, various biochemical properties.

3.4.2.5 Pure Culture for identification

Before performing biochemical and other tests each of the organisms was isolated in pure form. Gram staining of an isolated colony was done from primary culture. For gram-negative organism, a speck of single isolated colony from MacConkey agar and for gram-positive the same from blood agar was transferred into the nutrient broth and incubated at 37ºC for 4 hours. It was then sub cultured on dried nutrient agar plate and incubated at 37^oC for 18-24 hours. Thus obtained overnight incubated culture of organism on nutrient agar was used to perform Catalase, Oxidase, other biochemical and antibiotic susceptibility test.

3.4.2.6 Biochemical Tests

Different biochemical tests were performed for the identification of the bacterial isolates.

Gram-positive isolates were identified primarily on the basis of their response to gram's staining, Catalase, Oxidase, Coagulase tests, Novobiocin tests etc.

Gram-negative bacterial isolates were identified on the basis of different biochemical reaction by employing various biochemical tests such as Catalase test, Oxidase test, Indole test, Methyl red test, Voges Proskauer test, Citrate utilization test, Oxidation/Fermentation test, Triple Sugar Iron (TSI) test, Motility test, Gas production tests, Hydrogen sulphide production test, Urease test, Decarboxylase tests etc. Pure colonies of bacteria on the media plates were inoculated on different biochemical media and test result were noted.

The composition and preparation of biochemical media and reagents used in the biochemical tests are mentioned in the appendix IV. The gram-staining procedure is mentioned in the appendix V.

3.4.2.7 Purity plate

Purity plate culture of biochemical test was performed to observe whether the tests were preceded in an aseptic condition or not. The 4 hours incubated broth culture prepared for biochemical test was inoculated on one half of the nutrient agar plate just before preceding the biochemical tests. The other half of the

same nutrient agar plate was inoculated immediately after completing the biochemical test. The plate was incubated at 37ºC for overnight. The growth of same organism in both the pre and post inoculated portion of the plate is the indication of maintenance of aseptic condition throughout the experiment.

3.5 Antibiotic susceptibility testing

The antibiotic sensitivity tests of the pathogens isolated from the clinical specimen against different antibiotics was determined by Kirby-Bauer method of disk diffusion technique as recommended by CLSI (2011) using Mueller Hinton agar (MHA).

At least three to five well- isolated colonies of the same morphological types was selected from Nutrient Agar plate. The base of each of colony was touched with a inoculating wire and the growth was transferred into a tube containing 5 ml of nutrient broth and was incubated at 37°C (usually 2 to 6 hours) until it achieve the turbidity equivalent to the McFarland tube number 0.5.In case of overgrowth, the broth was diluted with sterile physiological saline to match with McFarland tube number 0.5.

A sterile cotton swab was dipped into the broth and the swab was rotated several times and pressed firmly on the inner side wall of the tube above the fluid level to remove excess inoculum from the swab. Then the dried surface of a MHA plate was inoculated by streaking the swab over the entire Agar surface three times, turning the plate 60°C between streaking. Finally, the inoculum was left to dry for few minutes at room temperature with the lid closed.

The predetermined battery of antimicrobial disks were placed on the surface of the prior inoculated agar plate such that there will be 25 mm distance from disk to disk and 15 mm from the side. The disks were pressed down to ensure complete contact with the agar surface. For about 15 minutes of applying the disks, the pates were left at room temperature to allow the antimicrobials to diffuse from the disk. Then they were incubated aerobically at 37°C overnight.

After overnight incubation, the diameter of zone of inhibition (ZOI) of each disk was measured (including diameter of the disk) and recorded in millimeter.

It will then compared with standard chart developed by Kirby-Bauer to determine bacterial susceptibility towards different antimicrobial agents in terms of “sensitive”, ‘resistant’ and moderately sensitive (Intermediate). The measurement was made with a ruler on the under surface of the plate without opening the lid.

Staphylococcus aureus ATCC 25923, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 was also be tested in every set of experiment, in parallel, as a part of quality control.

In this study, if the isolates were resistant to at least three classes of antimicrobial agents will be regarded as MDR (Magiorakos *et al*., 2012).

3.6 Test for ESBL Producing Gram negative bacterial isolates

Screening test:

The initial screen test for the production of ESBL was performed by using both Ceftazidime (CAZ) (30µg) and Cefotaxime (CTX) (30µg) disks. The organism was swabbed on to a Mueller-Hinton agar plate as done for screening test in antibiotic sensitivity test. Plates are incubated 18-24 hrs at 37°c. Next day the zone size is measured and if the zone of inhibition was ≤ 22mm for CAZ and /or ≤ 27 mm for CTX, the isolate was considered a potential ESBL producer as recommended by CLSI (2011).

The organism was swabbed on to a Mueller-Hinton agar plate as done for screening test in antibiotic sensitivity test and confirmation is done by:

Combination disk method:

This method was used for the confirmation of ESBL producing strains in which CTX and CAZ (30µg) alone and in combination with clavulanic acid (CA) (10µg) was used. An increase ZOI of ≥ 5mm for either antimicrobial agent tested in combination with CA versus its zone when tested alone was

confirmed ESBL producer. *E. coli* ATCC 25922 and K. pneumoniae ATCC 700603 were used as negative and positive controls respectively.

3.7 Test for MBL producing gram negative isolates

Screening:

The MBL producing isolates were screened by using Ceftazidime (30μg) disk (MAST, UK). Isolates resistant to CAZ will be considered potential MBL producers. The organisms were swabbed on to a MHA plate. Then the combination disk method was applied.

Combination disk method:

In Combination disk test, two IMP_S (10μg), one containing 10μl of 0.1M (292μg) anhydrous EDTA (Sigma chemicals, ST. Louis, MO), were placed 25mm apart centre to centre. An increase in Zone diameter of >4mm around the IMP-EDTA disk compared to that of IMP disk alone was considered positive for MBL. Ten μl of 0.1M (292μg) EDTA was chosen as higher concentration was suggestive of possessing inhibitory effects on bacteria.

For MBL test standardization, *P. aeruginosa* ATCC 27853 and *P. aeruginosa* PA 27853 were used as negative and positive controls respectively (Franklin et al., 2006).

3.8 Tests for MRSA

30μg Cefoxitin disc method by the methodology recommended by CLSI (2011) was put up and agar plates were incubated at 37ºC. The diameter of the zone of inhibition of growth was recorded and interpreted as susceptible or resistant by the criteria of CLSI. Organisms with “intermediate” levels resistance were included in the percentage of resistant organisms for final analysis.

The *S. aureus* strains ATCC 25923 and WHO-2 were used as negative and positive controls respectively. Organisms were regarded Methicillin resistant when zone of inhibition was ≤21mm for *S. aureus* with the Cefoxitin disc method.

3.9 Quality Control

During this study, quality control was applied in various areas.

During sample collection, aseptic technique was followed for collecting midstream urine in sterile bottles in order to avoid contamination. All the tests were carried out appropriately in aseptic conditions during processing.

While using readymade dehydrated media, the manufacturer's instructions for preparation, sterilization and storage were followed to prevent the alteration of the nutritional, selective, and inhibitory and biochemical properties of media. The performances of newly prepared media were tested using control species of bacteria (i.e. known organisms giving positive and negative reactions).For stains and reagents, whenever new batches of them were prepared, control smear was stained to ensure correct staining reaction.

Control strains of *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 27853) were used for the standardization of the Kirby-Bauer test and also for correct interpretation of zone of diameter. For ESBL test standardization, *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as negative and positive controls respectively. For MBL test standardization *P. aeruginosa* ATCC 27853 and *P. aeruginosa* PA 105663 were used as negative and positive controls respectively. For MRSA test standardization, *S. aureus* strains ATCC 25923 and WHO-2 were used as negative and positive controls respectively (Isenberg, 2004).

3.10 Statistical analysis

All the results were entered in the worksheet of Statistical Package for social science (SPSS 17.0) and Statistical analysis was performed. Main focus was on frequency and percentages. Chi-square (X^2) test was done wherever applicable with a P value <0.05 regarded as significant.

METHODOLOGY FLOWCHART

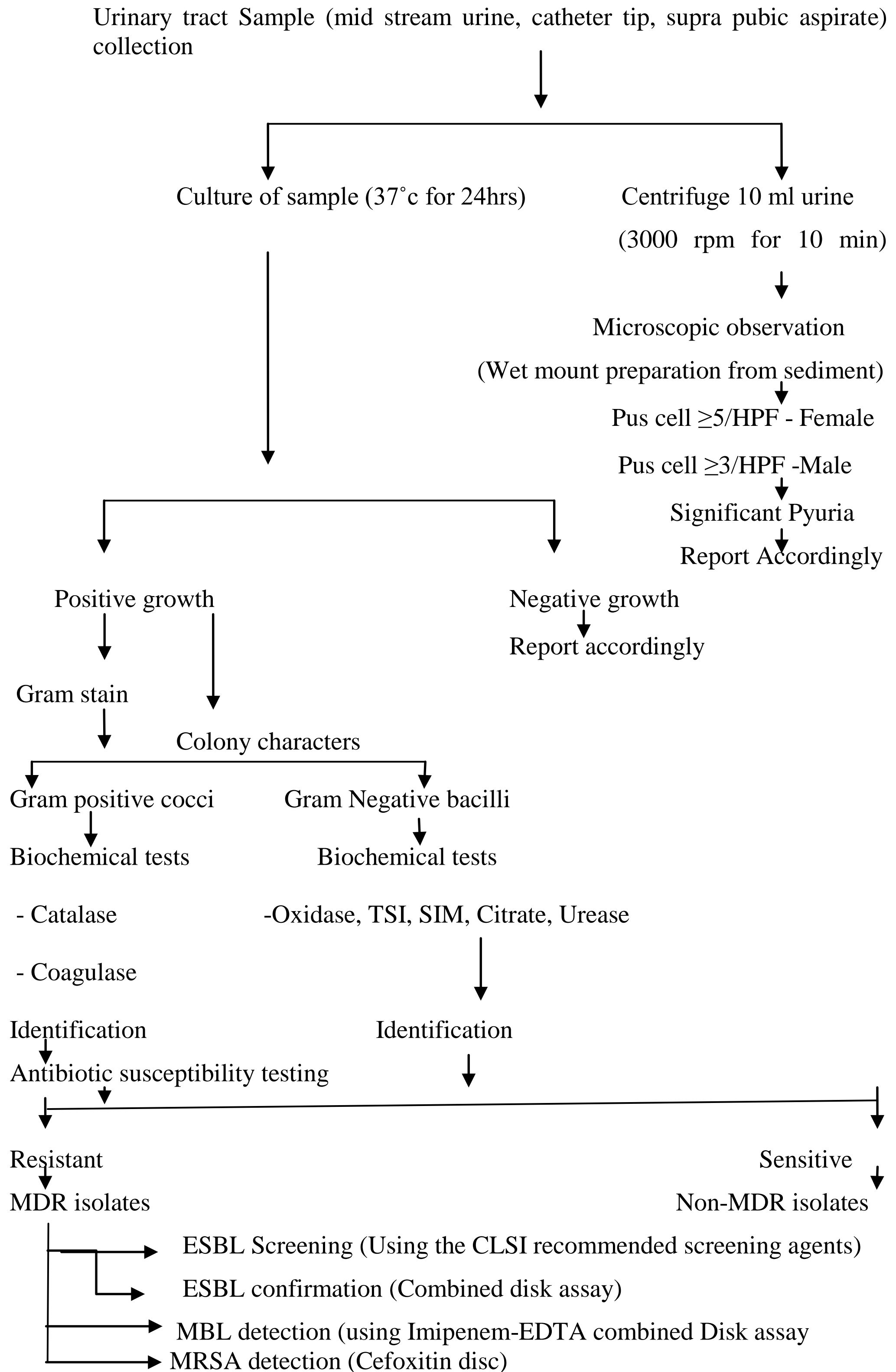


Fig 1: Flowchart showing various stages in the detection and confirmation of ESBL, MBL and MRSA.

CHAPTER IV

4. RESULTS

A total of 1063 urinary tract samples (urine, catheters, Suprapubic aspirate) submitted to the bacteriology laboratory for culture and sensitivity from patients suspected to have UTI within the study period from August 2012 to January 2013 were analyzed which are shown in the following tables and figures.

4.1 CLINICAL PATTERN OF RESULT

4.1.1 Distribution pattern of different urinary tract samples received for culture from patient visiting TUTH

Out of total samples received in laboratory for culture, 950(89.4%) samples were mid stream urine, 111(10.4%) samples were catheter and remaining 2(0.2%) samples were suprapubic aspirate. The results are shown in figure 2.

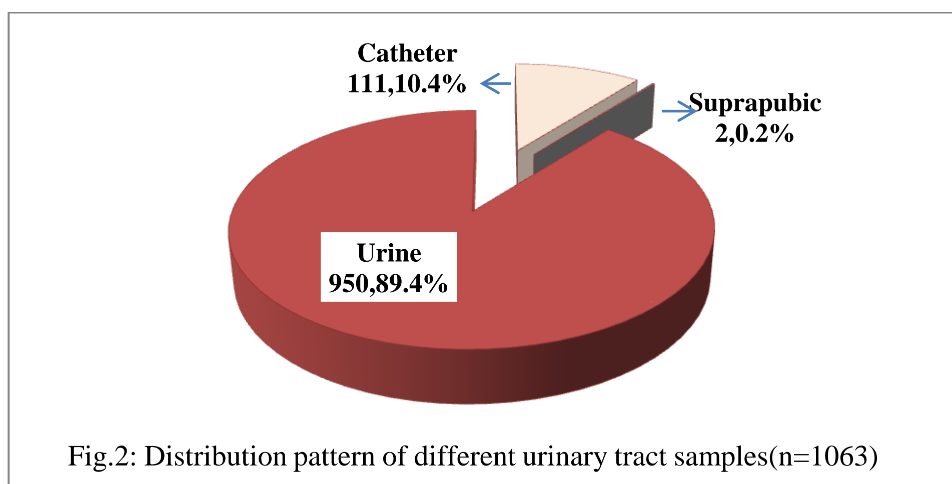


Fig.2: Distribution pattern of different urinary tract samples(n=1063)

4.1.2 Indoor and Outdoor distribution of patients visiting TUTH

Out of the total 1063 patients, majority cases (75.8%, 806) were from outpatient department and 257(24.2%) were from patients admitted to the hospital. Out of the total 714 female patients, the distribution between outdoor and indoor patients was 537 (75.2%) and 177 (24.7%) respectively. Similarly, out of 349 male patients, 80 (29.7%) were from hospitalized patients and 269

(77.0%) were from outpatient department of the hospital. In comparison to males, higher number of urine samples was collected from females which accounts for 714 (67.1%). The results are shown in Table 1.

Table 1: Indoor & Outdoor distribution of patients visiting TUTH

Gender	Indoor Patients	Outdoor Patients	Total Number	%
Male	80	269	349	32.8
Female	177	537	714	67.1
Total	257	806	1063	
%	24.2	75.8		

4.1.3 Age and Gender wise distribution of patients visiting TUTH

In our study, the age of the patients ranged from 10 days to 99 years. The highest number of patients belonged to the age group 21-30 (396, 37.2%), followed by age group 31-40 (157, 14.7%). Maximum number of female patients were found in the age group 21-30 (317, 44.4%), followed by age group 31-40(112 (15.7%). Number of female patients in outpatient department is higher than in admitted patients for all age group. Similarly higher number of male patients in outpatient and inpatient were found in age group 21-30 (66, 24.5%) and 51-60 (16, 20.0%) respectively. The results are shown in table 2.

Table 2: Age and Gender wise distribution

Age Group	Indoor patients				Outdoor patients				Total
	Male		Female		Male		Female		
	No	%	No	%	No	%	No	%	
0-10	5	6.3	6	3.9	8	2.9	10	1.8	29
11-20	9	11.2	26	14.6	25	9.3	60	11.2	120
21-30	13	16.2	80	45.1	66	24.5	237	44.1	396
31-40	10	12.5	24	13.5	35	13.0	88	16.9	157
41-50	9	11.2	14	7.9	41	15.2	43	8.0	107
51-60	16	20.0	9	5.0	36	13.4	48	9.1	109
61-70	9	11.2	8	4.5	27	10.0	24	4.5	68
71-80	6	7.5	5	2.8	22	8.2	17	3.2	50
81-90	2	2.5	4	2.3	8	2.9	8	1.5	22
91-100	1	1.3	1	0.6	1	0.4	2	0.4	5
Total	80	100	177	100	26	100	537	100	1063

4.2 MICROBIOLOGICAL PATTERN OF RESULTS

4.2.1 Pattern of culture result

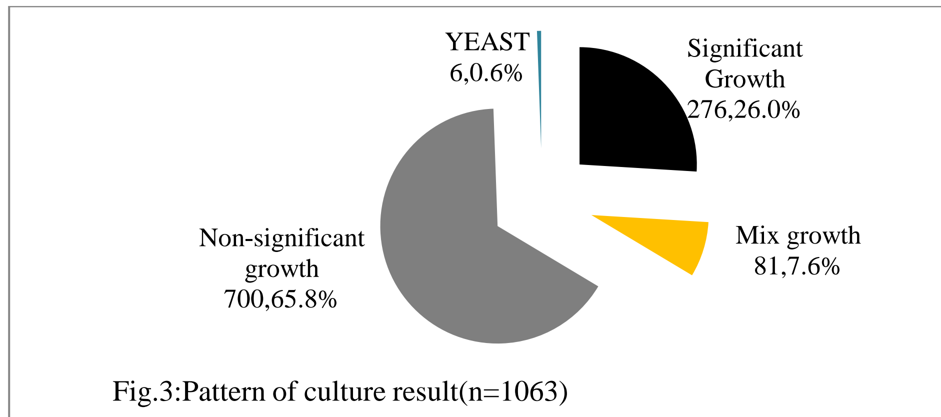


Fig.3:Pattern of culture result(n=1063)

Out of the total samples, 276(26.0%) showed significant bacteriuria, 6(0.6%)samples showed significant growth of yeast cells and 81 (7.6%) samples showed mixed type of growth. The results are shown in Figure 3.

4.2.2 Significant growth pattern in mid stream urine, catheter and suprapubic aspiration samples

Out of the total samples, 25.7 %(245/950) mid stream urine samples (msu), 30.6 %(34/111) catheter samples and 50% (1/2) suprapubic samples showed significant bacterial growth. The results are shown in figure 4.

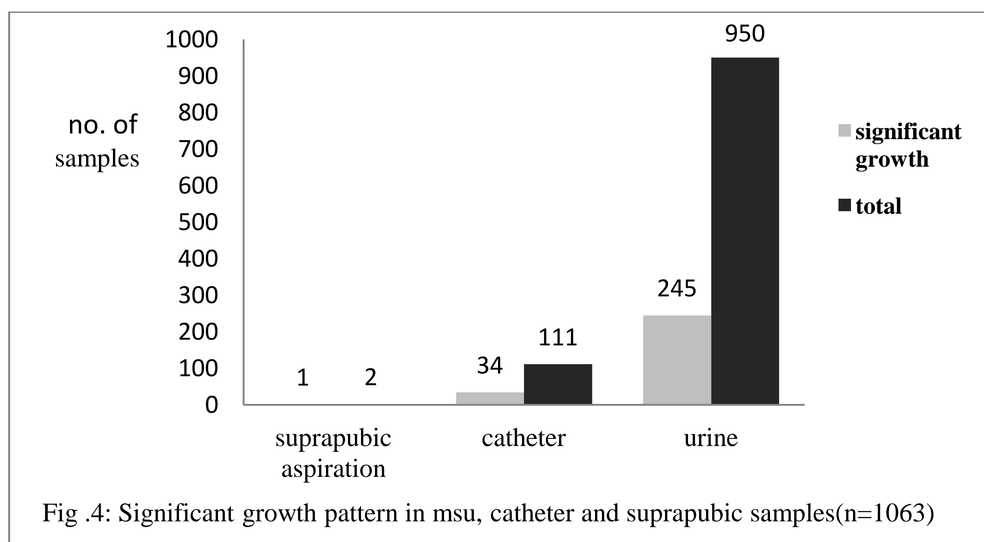


Fig .4: Significant growth pattern in msu, catheter and suprapubic samples(n=1063)

4.2.3 Significant growth pattern in Indoor and Outdoor patients

Out of the total 806 samples from outdoor patients, 200 (24.8%) samples and out of 257 samples from indoor patients, 76(29.5%) samples showed significant growth of bacteria respectively. There was higher number of significant growth from indoor patients ($P<0.05$). The results are shown in table 3.

Table 3: Significant Growth Pattern of Indoor and Outdoor Patients

Source	No	Significant Growth	%
Outdoor patients	806	200	24.8
Indoor patients	257	76	29.5

4.2.4 Significant growth pattern in male and female patients

Out of the total 349 samples from male patients, 84 (24.0%) samples were found to show significant growth. Similarly, 192 (26.8%) out of 714 samples from female patients showed significant growth. However, there was no significant difference in growth ($P>0.05$) between male and female patients. The results are shown in table 4.

Table 4: Significant Growth Pattern in Male and Female patients

Gender	No	Significant Growth	%
Male	349	84	24.0
Female	714	192	26.8
Total	1063	276	26.0

4.2.5 Significant Growth Pattern in various age groups

Among the 276 significant growth cultures, highest percent (34.4%) of significant growth culture was obtained from age group 21-30, which was higher than age group 31-40 (13.4%) with ($P<0.05$). Similar results were obtained from male and female age group. Results are shown in Table 5.

Table 5: Significant Growth Pattern in various age groups

Age Group	Male		Female		Total	
	No	%	No	%	No	%
0-10	4	1.4	2	0.7	6	2.1
11-20	10	3.6	26	9.4	36	13.0
21-30	18	6.5	77	27.8	95	34.4
31-40	7	2.5	30	10.8	37	13.4
41-50	15	5.4	13	4.7	28	10.1
51-60	15	5.4	18	6.5	33	11.9
61-70	6	2.1	13	4.7	19	6.8
71-80	5	1.8	6	2.1	11	3.9
81-90	4	1.4	6	2.1	10	3.6
91-100	0	0	1	0.3	1	0.3
Total	84	30.1	192	68.3	276	100

4.2.6 Pattern of growth results

Among the 276 significant bacterial growths, 11.2 % (31) positive growths were < 10^5 colony but > 10^3 colonies per ml of urine and 88.7 % (245) positive growths were > 10^5. The results are shown in Figure 6.

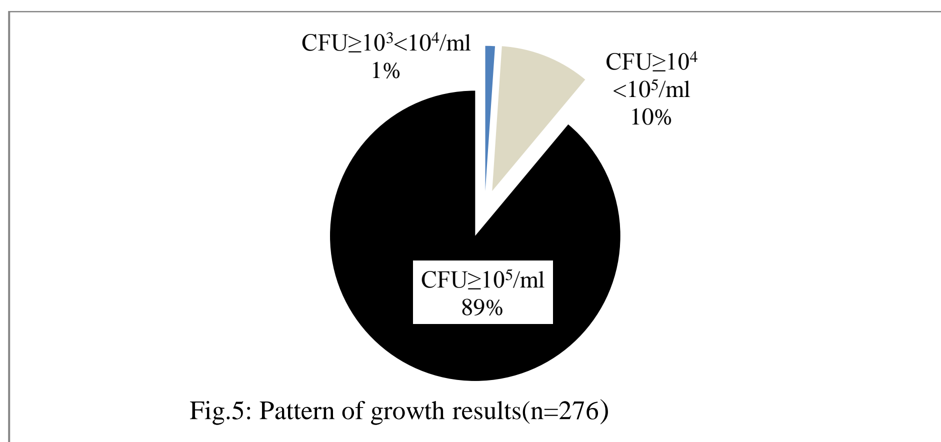


Fig.5: Pattern of growth results(n=276)

4.2.7 Pyuria versus bacteriuria in Male and Female Patients

The culture positive rate increases with increase in pus cells/HPF but in 16.1% of male and 18.8% of female there is significant bacterial growth in absence of

pyuria(pus cells<5/HPF) which is shown in Table 6. Microscopic examination is not done for 111 catheter samples.

Table 6: Pyuria versus bacteriuria in Male and Female Patients

Pus cells	No of	Male			Female		
No/HPF	sample	Culture negative	Mix growth	Culture positive	Culture negative	Mix growth	Culture positive
0 -1	582	169	7	34(16.1%)	280	22	70(18.8%)
2-5	158	33	3	17(31.4%)	61	11	33(31.5%)
6-10	56	8	3	3(21.4%)	20	5	17(40.4%)
11-15	32	4	1	3(37.5%)	9	0	14(60.8%)
*NT	111	6	3	3(30.0%)	46	21	31(31.6%)
Plenty	124	27	0	24(47.0%)	39	5	27(38.0%)
Total	1063	246	17	84(24.0%)	454	64	192(26.8%)

*NT: Not tested for pus cells

4.2.8 Presence of pus cells versus significant bacterial growth

Table 7: describes the pattern of pyuria of urine sediment against the number of samples showing significant bacteriuria. However, there was no significant difference (P>0.05) between male and female.

Table 7: Presence of pus cells versus significant growth

Pyuria	No. of Samples			Total
	Significant growth	Non-significant growth	Mix growth	
Male	30(43.8%)	39	4	73
Female	58(35.5%)	68	10	78
Total	88(37.6%)	107	14	151

4.2.9 Pattern of bacterial isolates causing UTI

Out of 276 bacterial isolates from mid stream urine, catheter and suprapubic aspirate samples in our study, 12 different bacterial genera were isolated which are tabulated in table 8. Among the bacterial isolates, *E. coli* (57.6%, 159) was found to be the most predominant organism (P<0.05) followed by *Staphylococcus aureus* (14.1%, 39), *Klebsiella* spp (6.2%, 17) and others.

Table 8: Pattern of bacterial isolates causing UTI

S.N.	Organisms isolated	Number	%
1	*Escherichia coli*	159	57.6
2	*Staphylococcus aureus*	39	14.1
3	*Klebsiella* spp	17	6.2
4	*Enterococcus faecalis*	12	5.4
5	*Staphylococcus epidermidis*	11	3.9
6	*Pseudomonas aeruginosa*	9	3.3
7	*Acinetobacter* spp	7	2.5
8	*Enterobacter* spp	6	2.2
9	*Burkholderia cepacia* complex	5	1.8
10	*Staphylococcus saprophyticus*	4	1.4
11	*Citrobacter* spp	3	1.1
12	*Proteus* spp	2	0.7
13	*Providencia* spp	1	0.4
14	*Morganella morganii*	1	0.4
	Total	276	100

4.2.10 Distribution of Gram positive and Gram negative bacterial isolates

Fig.6: showed out of total bacterial isolates, 210(76%) samples showed growth of Gram negative bacteria whereas 66(24%) showed growth of Gram positive bacteria.

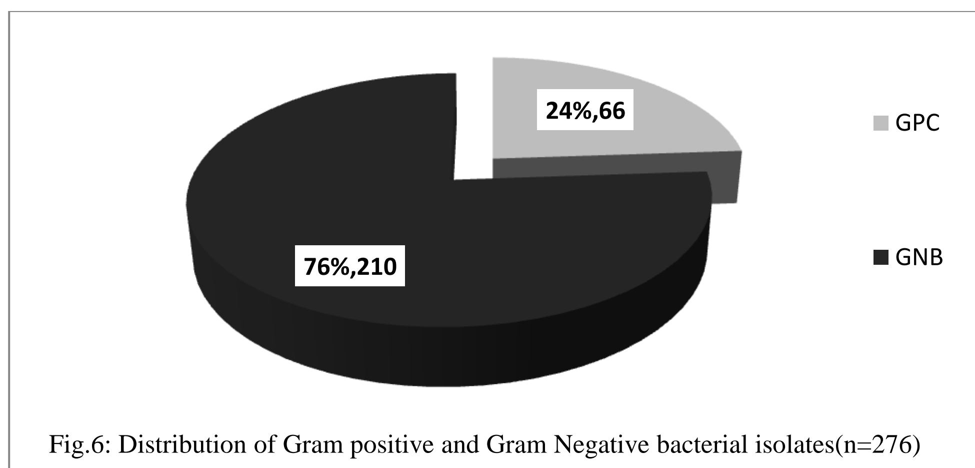


Fig.6: Distribution of Gram positive and Gram Negative bacterial isolates(n=276)

4.3 ANTIBIOTIC SUSCEPTIBILTY PATTERN OF ISOLATES

4.3.1 Antibiotic susceptibility profile of *Escherichia coli*

In case of *E. coli*, sensitivity pattern to different antibiotics was as follows: polymyxin B (100%) followed by Imipenem (97.4%), Amikacin (89.3%), Piperacillin plus tazobactam (71.2%), Gentamycin (70.0%), Cotrimoxazole (62.0%), and Nitrofurantoin (61.2%). Ceftazidime, ceftriaxone, cefepime, Ampicillin plus salbactum were found to be moderately sensitive. High rate of resistance was found to Ampicillin (88.0%) followed by Cephalexin (73.9%), Cefixime (66.0) and fluoroquinolones. The results are shown in table 9.

Table 9: Antibiotic susceptibility profile of *Escherichia coli* (n=159)

Antibiotics	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Ampicillin	19	12	0	0	140	88.0
Cephalexin	42	26.2	1	0.6	116	72.9
Cotrimoxazole	99	62			60	37.7
Ofloxacin	61	38.1	1	0.6	98	61.6
Norfloxacin	61	38.2			98	61.6
Levofloxacin	64	40.2			98	61.6
Nitrofurantoin	98	61.2	1	0.6	60	37.7
Gentamycin	112	70.0	1	0.6	46	28.9
Amikacin	142	89.3	1	0.6	16	10.0
Cefixime	54	33.7			105	66.0
Ceftazidime	70	44.0			90	56.6
Ceftriaxone	88	55.0			72	45.2
Cefepime	89	56.0			69	43.3
Imipenem	155	97.5			4	2.5
Piperacillin + Tazobactum	114	71.2			45	28.3
Ampicillin + salbactum	72	45.0			87	4.7
Polymyxin B	159	100				

4.3.2 MDR, ESBL and MBL in *Escherichia coli*

Fig.7: showed that majority of strains of *E.coli* were ESBL producers (65, 63.1%) and only 1(0.97%) strain was MBL producer among 103 MDR isolates which is 67.7% of total isolates.

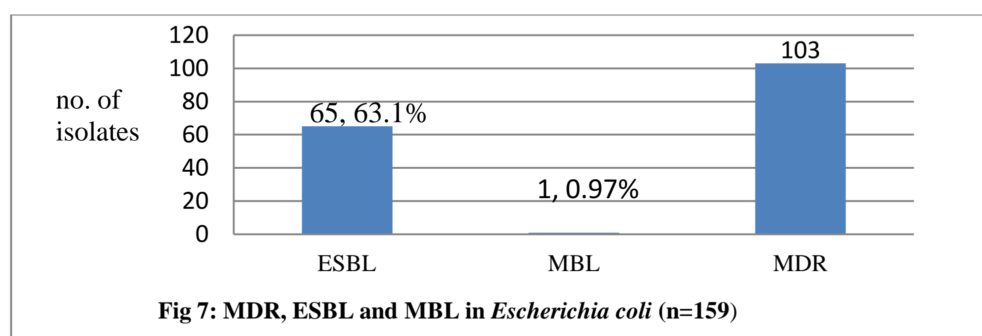


Fig 7: MDR, ESBL and MBL in ***Escherichia coli*** **(n=159)**

4.3.3 Antibiotic susceptibility profile of *Staphylococcus aureus*

The most sensitive antibiotic against *Staphylococcus aureus* was found to be Vancomycin (100%) and Teicoplanin (100%) followed by Amikacin (97.4%), Doxycycline (95.0%), Nitrofurantoin (87.0%), Cefotaxime (59.0%), Gentamycin (54.0%). Flouroquinolones were moderately sensitive whereas Amoxycillin/Ampicillin and Cotrimoxazole were found to be the least sensitive drug. The results are shown in table 10.

Table 10: Antibiotic susceptibility profile of *Staphylococcus aureus (n*= 39)

Antibiotics	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Ampicillin	9	23.0			30	76.9
Cephalexin	17	43.5			22	41.0
Cefotaxime	17	43.5			22	29.0
Cotrimoxazole	9	23.0			30	76.9
Nitrofurantoin	34	87.0	2	5.1	3	7.6
Amikacin	38	97.4			1	2.5
Gentamycin	21	54.0			18	46.1
Norfloxacin	14	35.8			25	64.1
Ciprofloxacin	16	41.0			23	58.9
Ofloxacin	13	33.3			26	66.6
Levofloxacin	19	48.7			20	51.2
Cefoxitin	18	46.1			21	53.8
Cloxacillin	17	43.5			22	56.4
Piperacillin + tazobactam	18	46.1			21	53.8
Doxycycline	37	95			2	5.1
Vancomycin	39	100				
Teicoplanin	39	100				

4.3.4 MRSA Vs MDR in *Staphylococcus aureus*

Fig.8: showed that all isolates were MRSA among the 21 MDR isolates.

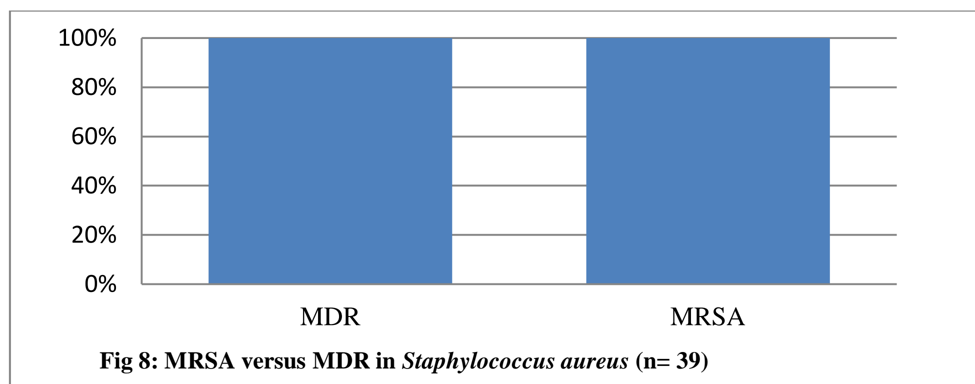


Fig 8: MRSA versus MDR in *Staphylococcus aureus* (n= 39)

4.3.5 Antibiotic susceptibility profile of *Enterococcus* spp

In case of *Enterococcus* spp, sensitivity pattern to different drugs was as follows: Vancomycin and Doxycycline (100%) followed by Nitrofurantoin (83.3%), Piperacillin plus Tazobactam (75.0%), Ampicillin (66.7%). Similarly, Levofloxacin (58.3%) was found to be the more sensitive drug than other flouroquinolones (33.3%).Gentamycin was 100%resistant. The results are shown in table 11.

Table 11: Antibiotic susceptibility profile of *Enterococcus* spp (n=12)

Antibiotics	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Ampicillin	8	66.7			4	33.3
Norfloxacin	4	33.3			8	67.7
Ciprofloxacin	4	33.3			8	67.7
Ofloxacin	4	33.3			8	67.7
Levofloxacin	7	58.3			5	41.7
Nitrofurantoin	10	83.3			2	16.6
Gentamycin	0	0			12	100
Amikacin	1	8.3			11	91.6
Piperacillin + tazobactam	9	75.0			3	25
Doxycycline	12	100				
Vancomycin	12	100				

4.3.6 Antibiotic susceptibility profile of *Klebsiella* spp

Klebsiella spp was found to be highly sensitive towards Polymyxin B (100%) followed by Imipenem (65%), Piperacillin plus Tazobactum(53%), Ofloxacin as well as Cefepime(47%), Amikacin and Gentamycin (41%), and all were found to be resistant towards Ampicillin and Cephalexin. The results are shown in table 12.

Table 12: Antibiotic susceptibility profile of *Klebsiella* spp (n=17)

Antibiotics	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Amoxycillin/Ampicillin	0	0			17	100
Cephalexin	0	0			17	100
Cotrimoxazole	5	29.4			12	70.5
Norfloxacin	5	29.4	1	5.8	11	64.7
Ciprofloxacin	8	47.0			9	52.9
Ofloxacin	8	47.0			9	52.9
Levofloxacin	6	35.2	1	5.8	10	58.5
Nitrofurantoin	3	17.6	1	5.8	13	76.4
Gentamycin	7	41.0			10	58.5
Amikacin	7	41.0	2	11.7	8	47.0
Cefixime	5	29.4			12	70.5
Ceftazidime	6	35.2			11	64.7
Ceftriaxone	6	35.2			11	64.7
Cefepime	8	47.0			9	52.9
Ampicillin + Salbactum	0	0			17	100
Piperacillin +Tazobactum	9	52.9	2	11.7	6	35.2
Imipenem	12	70.5			5	29.4
Polymyxin B	17	100				

4.3.7 MDR, MBL and ESBL in *Klebsiella* spp

Figure 9: shows that out of 11 MDR isolates of *Klebsiella* spp, 4(36.3%) were MBL producer and 5(45.5%) were ESBL producer.

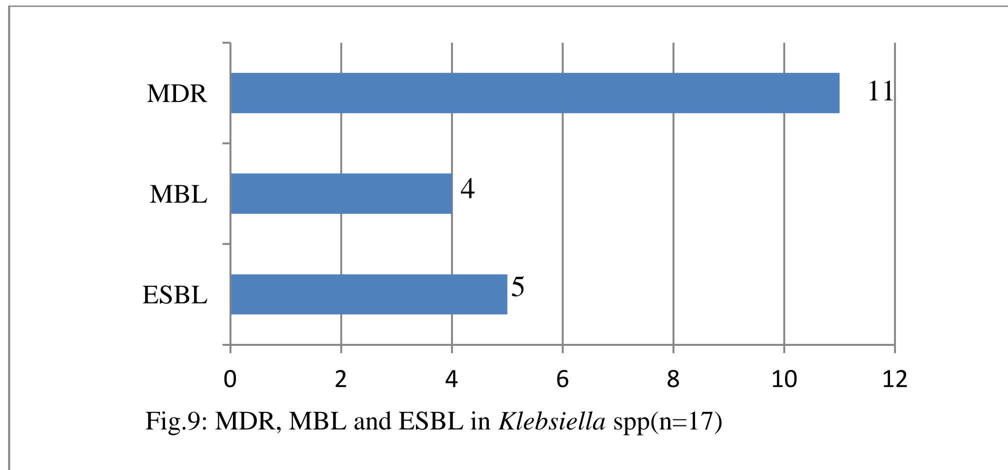


Fig.9: MDR, MBL and ESBL in *Klebsiella* spp(n=17)

4.3.8 Antibiotic susceptibility profile of *Staphylococcus epidermidis*

The most sensitive antibiotic against *Staphylococcus epidermidis* was found to be Vancomycin and Teicoplanin followed by Doxycycline (90.9), Piperacillin plus Tazobactum and Cefotaxime (86.6%) each, Cephalexin (72.7%). Gentamycin, Cloxacillin and Nitrofurantoin (63.6%) each were moderately sensitive whereas Cotrimoxazole, Ofloxacin, Ciprofloxacin, Levofloxacin and Amoxycillin/Ampicillin were least sensitive drug. The results are shown in table 13.

4.3.8 Table 13: Antibiotic susceptibility profile of *Staphylococcus epidermidis* (n=11)

Antibiotics	Sensitive		Resistant	
	No	%	No	%
Amoxycillin/Ampicillin	2	18.1	9	81.8
Cephalexin	8	72.7	3	27.2
Cefotaxime	9	81.8	2	18.1
Cotrimoxazole	3	27.2	8	72.7
Nitrofurantoin	7	63.6	4	36.3
Gentamycin	7	63.6	4	36.3
Norfloxacin	4	36.3	7	63.6
Ciprofloxacin	3	27.2	8	72.7
Ofloxacin	4	36.6	7	63.6
Levofloxacin	4	36.3	7	63.6
Cloxacillin	7	63.3	4	36.3
Piperacillin +Tazobactum	9	81.8	2	18.1
Doxycycline	10	90.9	1	9.1
Vancomycin	11	100		
Teicoplanin	11	100		

MDR	Number	%
	4	36.3

4.3.9 Antibiotic susceptibility profile of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa showed 100% sensitive towards Polymyxin B and Piperacillin plus Tazobactam and good susceptibility to Imipenem, Amikacin, Piperacillin, Cefepime, and Levofloxacin. Ciprofloxacin, Norfloxacin and Ofloxacin were the least sensitive drug. 5/9(55.5%) isolates were found to be MDR. The results are shown in table 14.

Table 14: Antibiotic susceptibility profile of *Pseudomonas aeruginosa* (n=9)

Antibiotics	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Levofloxacin	6	67			3	33
Ciprofloxacin	3	33	1	11	5	55
Ofloxacin	3	33	1	11	5	55
Norfloxacin	3	33	1	11	5	55
Gentamycin	4	44			5	55
Amikacin	6	67			3	33
Ceftazidime	7	78			2	22
Piperacillin	6	67			3	33
Piperacillin +Tazobactum	9	100			0	0
Ampicillin + Salbactam	0	0			9	100
Imipenem	8	89			1	11
Cefepime	8	78			1	11
Tobramycin	5	55			4	11
Polymyxin B	9	100			0	0

MDR	Number	%
	5	55.5

4.3.10 Antibiotic susceptibility profile of *Acinetobacter* spp

All *Acinetobacter spp* were found to be 100% sensitive towards Polymyxin B. Imipenem, Amikacin, Gentamycin, Levofloxacin, Cotrimoxazole, Piperacillin plus Tazobactam, Cefepime were moderately sensitive. 42.8% isolates were found MDR. The results are shown in table 15.

Table 15: Antibiotic susceptibility profile *Acinetobacter* spp (n=7)

Antibiotics	Sensitive		Resistant	
	No	%	No	%
Ampicillin	0	0	7	100
Cephalexin	0	0	7	100
Cotrimoxazole	4	50.0	3	42.8
Ciprofloxacin	3	42.8	4	50.0
Norfloxacin	3	42.8	4	50.0
Ofloxacin	3	42.8	4	50.0
Levofloxacin	4	50.0	3	42.8
Nitrofurantoin	1	12.0	6	66.6
Gentamycin	5	62.0	2	37.0
Amikacin	5	62.0	2	37.0
Cefixime	0	100	7	100
Ceftazidime	2	25.0	5	62.0
Ceftriaxone	1	12.0	6	66.6
Cefepime	2	37.0	5	62.0
Piperacillin + Tazobactum	4	50.0	3	42.8
Ampicillin + Salbactum	5	75.0	2	25.0
Imipenem	5	75.0	2	25.0
Polymyxin B	7	100	0	0

MDR	Number	%
	3	42.8

4.3.11 Antibiotic susceptibility profile of *Enterobacter spp*

All *Enterobacter* spp isolates were found to be sensitive to Imipenem and Polymyxin B followed by Amikacin (83.3%). Nitrofurantoin, Flouroquinolones, Gentamycin, Cefepime, Ceftriaxone and ceftazidime were moderately sensitive. All isolates were resistant to Ampicillin, Cephalexin, Ampicillin plus salbactum and Cefixime. The results are shown in table 16.

Table 16: Antibiotic susceptibility profile of *Enterobacter* spp (n=6)

Antibiotics	Sensitive		Resistant	
	No	%	No	%
Ampicillin	0	100	6	100
Cephalexin	0	100	6	100
Cotrimoxazole	4	66.6	2	33.3
Norfloxacin	3	50.0	3	50.0
Ciprofloxacin	3	50.0	3	50.0
Ofloxacin	3	50.0	3	50.0
Levofloxacin	4	66.6	2	33.3
Nitrofurantoin	4	66.6	2	33.3
Gentamycin	4	66.6	2	33.3
Amikacin	5	83.3	1	16.6
Cefixime	1	16.6	5	83.3
Ceftazidime	4	66.6	2	33.3
Ceftriaxone	4	66.6	2	33.3
Cefepime	4	66.6	2	33.3
Piperacillin + Tazobactum	4	66.6	2	33.3
Ampicillin + Salbactam	0	0	6	100
Imipenem	6	100	0	
Polymyxin B	6	100	0	

4.3.12 MDR, MBL and ESBL in *Enterobacter* spp

Figure 10: showed that Two *Enterobacter* spp isolates were found MDR (33%) and one was ESBL producer (17%).

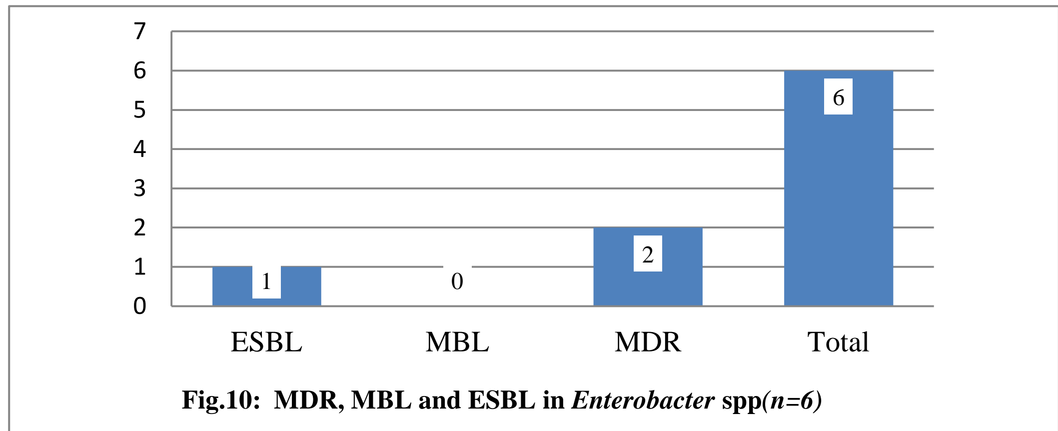


Fig.10: MDR, MBL and ESBL in *Enterobacter* spp(*n=6*)

4.3.13 Antibiotic susceptibility profile of *Burkholderia cepacia* complex

All isolates were sensitive towards Cotrimoxazole, Imipenem, Ceftazidime, Piperacillin plus Tazobactam and Gentamycin followed by Amikacin (80%), Cefepime (60%), and Cefixime (60%). Fluoroquinolones and Ceftriaxone were least sensitive. Similarly, all isolates were resistant to Ampicillin plus Salbactum, Polymyxin B, Nitrofurantoin, Cephalexin and Ampicillin. The results are shown in table 17.

4.3.13 Table 17: Antibiotic susceptibility profile of *Burkholderia cepacia* complex (n=5)

Antibiotics	Sensitive		Resistant	
	No	%	No	%
Ampicillin	0	0	5	100
Cephalexin	0	0	5	100
Cotrimoxazole	5	100	0	0
Norfloxacin	1	20	4	80
Ciprofloxacin	1	20	4	80
Ofloxacin	1	20	4	80
Levofloxacin	1	20	4	80
Nitrofurantoin	0	0	5	100
Gentamycin	5	100	0	0
Amikacin	4	80	1	20
Cefixime	2	40	3	60
Ceftazidime	3	60	2	40
Ceftriaxone	2	40	3	60
Cefepime	3	60	2	40
Piperacillin + Tazobactam	5	100	0	0
Ampicillin + Salbactum	0	0	5	100
Imipenem	5	100	0	0
Polymyxin B	0	0	5	100

MDR	Number	%
	3	100

Antibiotic susceptibility profile of *Staphylococcus saprophyticus*

All isolates of *Staphylococcus saprophyticus* (n=4) were susceptible to most of tested antibiotics like Vancomycin, Teicoplanin, Doxycycline, Piperacillin plus Tazobactum, Cefotaxime, Cephalexin, Gentamycin, Cloxacillin and Nitrofurantoin, Ofloxacin, Ciprofloxacin, Levofloxacin whereas Cotrimoxazole and Ampicillin were least sensitive drug.

4.3.15 Antibiotic susceptibility profile of *Citrobacter* spp

In case of *Citrobacter* spp (n=3), sensitivity pattern to different drugs was as follows: Polymyxin B (100%) followed by Imipenem (67%). Only 33% of isolates were Sensitive towards Cefepime, Ceftazidime, Ceftriaxone, Cefixime, Piperacillin plus Tazobactum. Similarly, all isolates were resistant to Cephalexin, Gentamycin, Amikacin, Cotrimoxazole, Norfloxacin, Ciprofloxacin, Levofloxacin, Nitrofurantoin, and Ampicillin. All isolates were MDR among which one was ESBL Producer.

4.3.16 Antibiotic susceptibility profile of *Proteus* spp *(P.mirabilis, P.vulgaris)*

All *Proteus* spp isolates (n=2) were found to be sensitive towards Polymyxin B, Imipenem, Amikacin, Cefixime, Cefepime, Ceftriaxone, Ceftazidime, Piperacillin plus Tazobactam, Norfloxacin, Ciprofloxacin, Levofloxacin, Ofloxacin, and Gentamycin. All were resistant to Ampicillin, Cotrimoxazole and Nitrofurantoin.

4.3.17 Antibiotic susceptibility profile of *Providencia* spp

Only one MDR *Providencia* spp isolate was found to be sensitive towards Imipenem, Piperacillin plus Tazobactam and Cefepime and resistant to Polymyxin B, Nitrofurantoin, Amikacin, Gentamycin, Cefixime, Ceftriaxone, Ceftazidime, Ampicillin plus Salbactum, Fluoroquinolones, Cotrimoxazole and Ampicillin.

4.3.18 Antibiotic susceptibility profile of *Morganella morganii*

Only one *Morganella morganii* isolate was found to be sensitive towards Imipenem, Piperacillin plus Tazobactum, Nitrofurantoin, Amikacin, Gentamycin, Ceftazidime, Levofloxacin and Cefepime and resistant to Polymyxin B, Nitrofurantoin, Cefixime, Ceftriaxone, Ampicillin plus Salbactum, Norfloxacin, Ciprofloxacin, Ofloxacin, Cotrimoxazole and Ampicillin.

4.3.19 Antibiotic susceptibility profile of Gram negative bacteria

The most sensitive antibiotic against gram-negative bacteria was found to be Polymyxin B (96.6%) followed by Imipenem (93.8%), Amikacin (82.3%),

Piperacillin plus Tazobactum (70%) Gentamycin (66.6%), Cefepime (56.6%), Cotrimoxazole (56.1%), Nitrofurantoin (51.4%). Ampicillin (11.9%) and Cephalexin (16.6%) were found to be highly resistant antibiotics among the tested antibiotics for gram-negative bacteria. The results are shown in table 19.

Table 18: Antimicrobial susceptibility profile of Gram negative bacteria (n=210)

Antibiotics	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Ampicillin	19	9.0	2	1.0	189	88.0
Cephalexin	43	20.4	3	4.3	165	85.2
Cotrimoxazole	118	56.1	1	0.5	92	58.6
Norfloxacin	80	38.0	2	1.0	129	60.0
Ciprofloxacin	79	37.6	1	0.5	130	69.5
Ofloxacin	79	37.6	0	0	131	62.3
Levofloxacin	89	42.3	1	0.5	96	57.6
Nitrofurantoin	108	51.4	1	5.0	92	47.1
Gentamycin	140	66.6	1	0.5	69	36.0
Amikacin	173	82.3	1	0.5	36	16.7
Cefixime	66	31.4	4	1.9	132	64.3
Ceftazidime	96	45.7	0	0	114	45.3
Ceftriaxone	106	50.4	1	0.5	103	57.6
Cefepime	119	56.6	2	1.0	89	42.3
Piperacillin + Tazobactam	148	70.4	2	1.0	60	20.0
Ampicillin + Salbactam	77	36.6	0	0	133	75.2
Imipenem	197	93.8	3	1.0	13	5.0
Polymyxin B	203	96.6	0	0	8	2.4

4.3.20 MDR, ESBL AND MBL in Gram negative isolates

Fig.11: showed that out of total 210 Gram negative bacterial isolates, 61.0% (128/210) were MDR isolates. Among the 128 MDR isolates, 74/128(57.8%) were ESBL producers and 6/128(4.7%) were MBL producers.

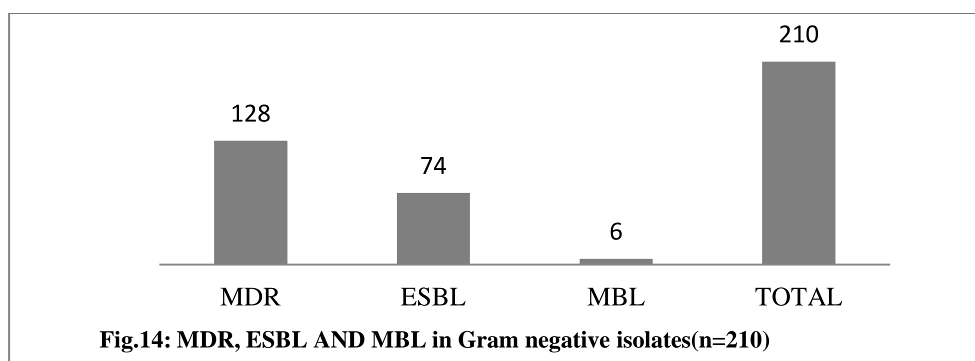


Fig.14: MDR, ESBL AND MBL in Gram negative isolates(n=210)

4.3.21 Antibiotic susceptibility profile of Gram positive bacteria

The most sensitive antibiotic against gram-positive bacteria was found to be Vancomycin and Teicoplanin (100%) followed by Doxycycline (93.9%), Nitrofurantoin (83.3%), Amikacin (78.7%), Piperacillin plus tazobactam (59.0%). Only 21.2% isolates were sensitive to Cotrimoxazole followed by Ampicillin (32%). The results are shown in table 19.

Table 19: Antimicrobial susceptibility profile of Gram positive bacteria (n=66)

Antibiotics	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Ampicillin	21	31.8			34	51.5
Cephalexin	36	54.5	1	1.5	15	22.7
Cefotaxime	30	45.4			19	28.7
Cotrimoxazole	14	21.2			30	45.4
Nitrofurantoin	55	83.3			11	16.6
Gentamycin	32	48.4			34	51.5
Amikacin	52	78.7			14	21.2
Norfloxacin	25	37.8			41	62.6
Ciprofloxacin	25	37.8			41	62.6
Ofloxacin	25	37.8			41	62.6
Levofloxacin	34	51.5			32	48.4
Cloxacillin	25	37.8			29	43.9
Piperacillin + Tazobactum	39	59.0			27	40.9
Doxycycline	62	93.9			4	6.0
Vancomycin	66	100				
Teicoplanin	66	100				

4.3.22 MDR in Gram positive bacterial isolates

Fig.12: showed that out of 66 gram positive bacterial isolates, 21/39(53.8%) *Staphylococcus aureus* isolates, 3/12 *Enterococcus* isolates and 4/11 *Staphylococcus epidermidis* isolates were MDR.

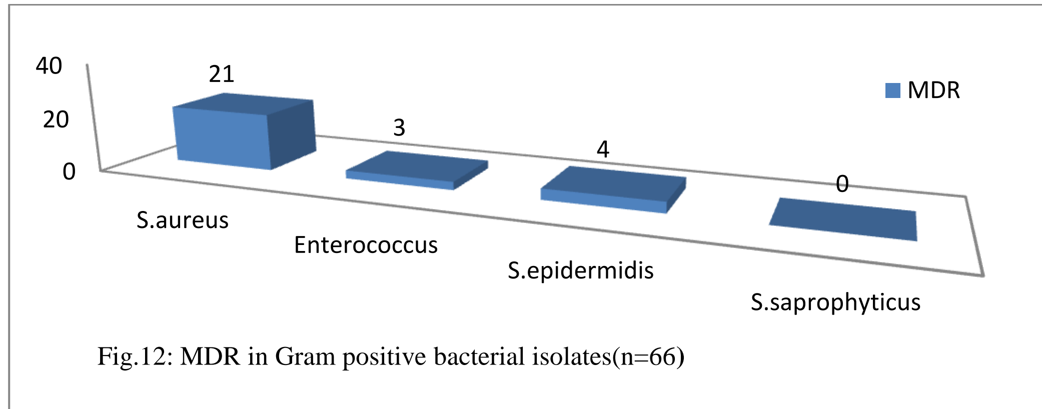


Fig.12: MDR in Gram positive bacterial isolates(n=66)

4.3.23 Antibiotic susceptibility profile of ESBL producing bacteria

All the ESBL producing bacteria were found sensitive towards Polymyxin B. Majority showed susceptible to Imipenem (97.2%) followed by Amikacin (89.1%), Nitrofurantoin (58.1%), Gentamycin (55.4%), Piperacillin plus Tazobactum (54.0%). High rate of resistance was found towards Fluoroquinolones, Cotrimoxazole and Ampicillin plus Salbactam .The results are shown in table 20.

Table 20: Antimicrobial susceptibility profile of ESBL producing bacteria (n=74)

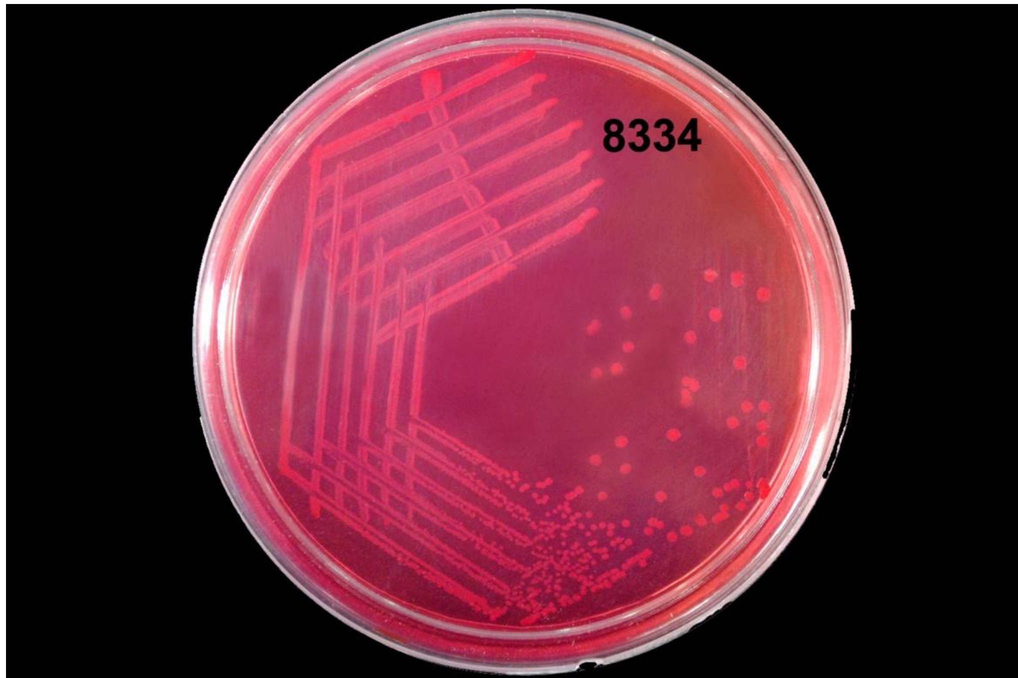
Antibiotics	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Imipenem	72	97.2			2	2.7
Amikacin	66	89.1	2	2.7	6	8.1
Gentamycin	41	55.4			32	43.2
Nitrofurantoin	43	58.1	4	5.4	26	35.1
Piperacillin + Tazobactum	40	54.0			35	47.2
Cotrimoxazole	19	25.6			54	72.9
Ampicillin +Salbactum	21	25.3			53	71.6
Norfloxacin	9	12.1			66	89.1
Ciprofloxacin	8	10.8			66	89.1
Ofloxacin	10	13.5			64	86.4
Levofloxacin	10	13.5			64	86.4
Polymyxin B	74	100			0	0

4.3.24 MDR versus ESBL and MBL producing bacteria

Maximum percentage of MDR was seen in *E.coli*, followed by *Klebsiella* spp. *E.coli* was major ESBL producer whereas *Klebsiella* spp was major MBL producers in comparison to other gram negative bacteria. The results are shown in table 21.

Table 21: MDR versus ESBL and MBL producing bacteria (n=276)

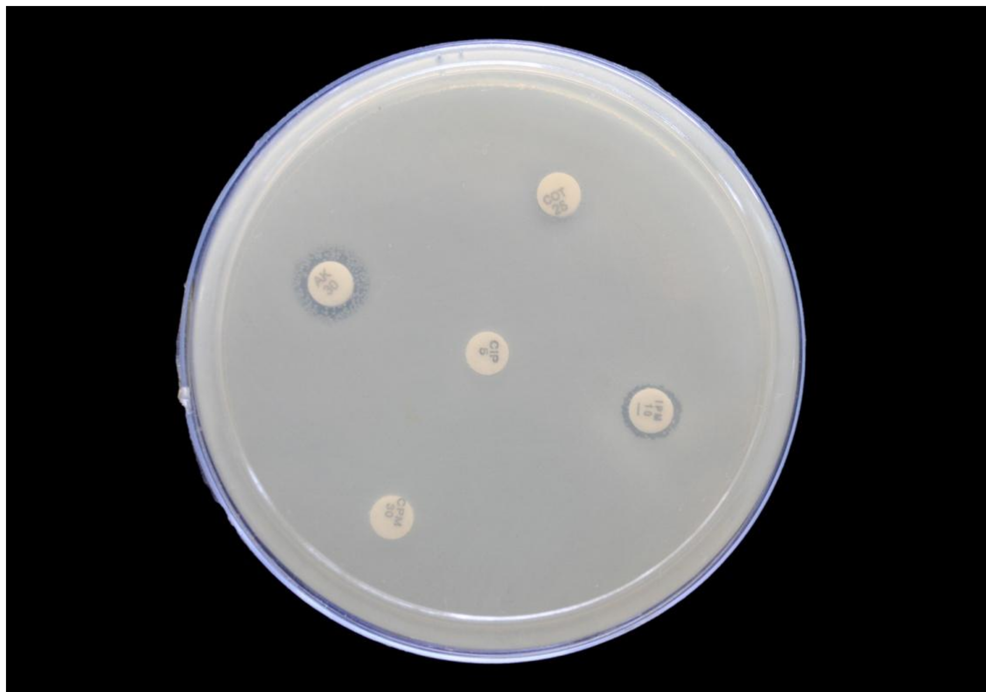
Isolates	MDR		ESBL		MBL	
	No	% of n	No	% of n	No	% of n
Escherichia coli	103	37.3	66	23.9	1	0.36
Klebsiella spp	11	3.9	5	1.8	4	1.44
Citrobacter spp	3	1.0	1	0.3	0	0
Enterobacter spp	2	0.7	1	0.3	0	0
Acinetobacter spp	3	1.0	1	0.3	0	0
P.aeruginosa	5	1.8	0	0	0	0
Total	128	46.3	74	20.9	5	1.8



Photograph 1: Significant growth of *E.coli* isolates from urine sample on MacConkey Agar

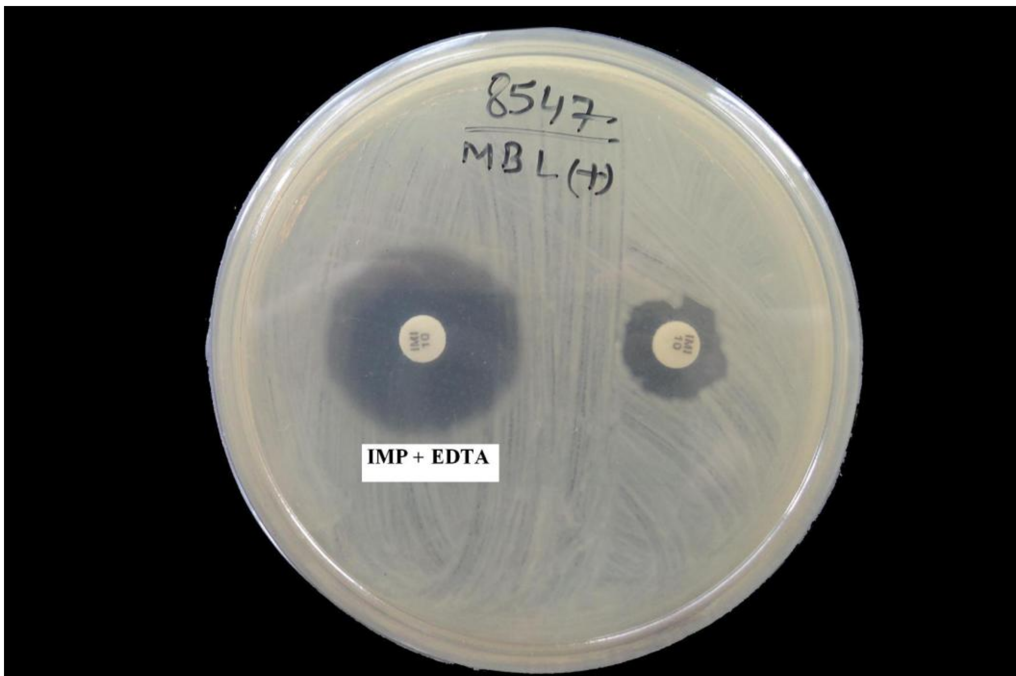
Photograph 2: *E. coli* (Test) showing different biochemical reactions along with Controls (From left to right: TSI, SIM, Citrate, Urease, O/F)

Photograph 3: Significant growth of *Klebsiella pneumoniae* isolates on MacConkey Agar



Photograph 4: Multidrug resistant *Klebsiella pneumoniae* isolates on Mueller Hinton Agar

Photograph 5: Confirmed ESBL production in *E.coli* isolates by Combined Disk Method



Photograph 6: *Klebsiella* spp demonstrating MBL activity in Imipenem-EDTA combined disk assay

CHAPTER V

5. DISCUSSION

Urinary Tract Infection (UTI) is the most commonly encountered community acquired as well as hospital acquired infection. This study was conducted among the patient suspected of UTI visiting Tribhuvan University Teaching Hospital (TUTH), Kathmandu, Nepal. A total of one thousand and sixty three urinary tract samples (urine, Catheter, and Suprapubic aspirate) submitted to the bacteriology laboratory for culture and sensitivity was studied within the period from Aug 2012 to Jan 2013 in Department of microbiology of the Hospital.

Out of the total (1063) sample received, majority were mid stream urine samples (89.4%) followed by Catheter samples (10.4%) and remaining (0.2%) suprapubic aspirate samples.

In this study, the samples from outpatient department were more (75.8%) in compared to hospital admitted patient samples (24.2%) for both male and female. Among the total patients, 714 were female and 349 were Male with female to male ratio being 2:1.The study population ranged from 16 days to 93 years of patient. The highest number of female patient belongs to age group 21-30 both in case of indoor and outdoor patient whereas number of male is higher in age group 51-60 in case of hospital admitted patient compared to outdoor patient where higher number is of age range 21-30.

In our study, Out of the total urinary tract samples received, 26.0 %(276/1063) showed significant bacteriuria and small percentage (0.6%) showed growth of yeast cells. Significant bacteriuria in case of indoor (24.8%) and outdoor patient (29.5%) was of not much difference. This results correlates to the research done by Mohammadi *et al.*,(2010), Basnet *et al.,* (2009) and the study carried out in TUTH, Nepal by kattel *et a*l., (2009) where significant growth rate was 24.8% ,23.3% and 27% respectively. However, our findings was slightly lower than significant bacterial growth (28.3%, 500/1766) shown by the study of Mishra *et al.,* (2005) and Shrestha *et al*., (2005). The possible cause of low rate of growth positivity is that the samples might be from

patients taking antibiotics, infection due to slow growing organisms or due to those organisms that were not able to grow on the routine media or patients might not have given proper history of UTI to physician due to lack of proper education.

Out of 1063 samples, 245/950(25.7%) mid stream urine, 34/111(30.6%) catheter and (1/2)50% suprapubic aspirate showed significant growth. Urinary tract infections (UTIs) are the most common type of nosocomial (hospital-acquired) infections, accounting for 40% of all infections in hospitals per year (Burke and Zavasky, 1999). Several studies have reported that about 80% of nosocomial UTIs occur following instrumentation, primarily catheterization (Asher *et al.,* 1986). Because nearly 10% of all hospitalized patients are catheterized, Preventing UTIs is a major factor in decreasing nosocomial infections. Our study showed 30.6% growth in catheter samples which were received from ward admitted patient mostly from female surgical wards. In several prospective studies, rates of catheter-associated UTIs ranging from 9% to 23% have been reported (Johnson *et al*., 1990). Notably, 88% of nosocomial UTIs were catheter related in the study done by Bronsema *et al.,* (1993).

In our study, 24.8% (200/806) of samples from outdoor patients and 29.5% (76/257) of samples from indoor patients showed significant bacterial growth. Though there is no significant difference($P>0.05$) between significant growth in indoor and outdoor cases but growth rate is slightly higher in indoor cases which may due to iatrogenic infection such as use of catheters, probes and swabs in the hospital, also other complication such as obstruction to flow of urine (by tumor, stricture, stone or prostatic hypertrophy).

Females are more frequently affected by UTI (particularly cystitis) due to colonization of urethra with colonic gram-negative bacteria because of its proximity to anus, short length of urethra and sexual intercourse (Forbes *et al*., 2002) but our study showed 26.8% of female and 24.0% of males were affected by UTI .There was no significant difference ($P>0.05$) in significant growth number between male and female patients.

Our study showed higher number of growth was obtained from age group 21-30 in female patients compared to other age group($P<0.05$). Out of the 192 female patients showing significant growth of urine culture, 77 (27.8%) female patients were from this age group. Female anatomy, sexual intercourse and pregnancy may be the causes as majority of female marry in this age group. In young sexually active women, sexual activity is the cause of 75–90% of bladder infections, with the risk of infection related to the frequency of sex (Nicolle, 2008). Urinary tract infections are more concerning in pregnancy due to the increased risk of kidney infections. During pregnancy, high progesterone levels elevate the risk of decreased muscle tone of the ureters and bladder, which leads to a greater likelihood of reflux, where urine flows back up the ureters and towards the kidneys (Dielubanza et *al.,* 2011). However, there was no significant difference ($P>0.05$) of growth in age of male patients.

Among the 276 significant bacterial growth, majority (88.9%) were $> 10^5$ cfu/ml and few were $< 10^5 > 10^3$cfu/ml. Although Kass's (1956) definition of true or significant bacteriuria based on the presence of $\geq 10^5$ CFU per ml in a carefully collected sample of clean-voided or midstream urine, our study found 11.0% (31/276) of bacterial growth showing $<10^5 \geq 10^3$ CFU/ml with pyuria. In certain condition, the Kass, Marple and Sandford have suggested CFU between10^4-10^5 might be regarded low count significant bacteriuria. (Pokhrel, 2004). According to National clearing house (NCG), 2005, urine culture has a sensitivity of 50% (if threshold for positive is $>10^5$ organisms); sensitivity can be increased to >90% if threshold is $>10^2$ organisms. A lower colony count ($>10^2$/mL) may be indicative of UTI if fecal contamination has been ruled out, which was best demonstrated in studies carried out by Komaroff (1984). Stamm *et al.,*(1980) also found $\geq 10^2$ CFU/ml on culture of a midstream specimen who also had pyuria underwent suprapubic bladder aspiration and all of these women responded to appropriate antimicrobial therapy. Similar result was also found in a study in Gynecology clinic at a student health center (Kunin *et al.,* 1993).

Here, our study showed, as the number of pus cells increased per HPF the chance of getting culture positive results was also high in case of male patients. However, in female, culture positiveness was higher in 11-20 pus cells/HPF than in plenty pus cells/HPF. There was significant difference ($P<0.05$) of pyuria versus significant growth between male and female patients. In this study, Criteria for pyuria (≥5 pus cells/HPF) were made according to Stamm *et al*., 1980 and Wright (1959).

Out of the total 740 urine samples without pyuria, 154 (20.8%) urine samples showed significant bacterial growth. Likewise, out of the total 212 urine samples with pyuria, only 88 (41.5%) urine samples showed significant bacterial growth. Our findings of pyuria and without pyuria versus significant growth were obtained in accordance with National clearing house (NCG, 2005) that have mentioned microscopic examination of pyuria has a sensitivity of 80-90% and a specificity of 50% for predicting UTI and Urine culture has a sensitivity of 50% (if threshold for positive is $>10^5$ organisms); sensitivity can be increased to >90% if threshold is $>10^2$ organisms.

According to Stenqvist *et al., (1987)*, significant bacteriuria may sometimes occur in the absence of symptoms and pyuria in patients who subsequently develop symptoms of UTI e.g. in pregnancy. The detection of such asymptomatic bacteriuria is of value for there is good evidence of its association with the development of pyelonephritis in some patients. Bacteriuria occurs in two to seven percent of pregnancies, particularly in multiparous women, a similar prevalence as in nonpregnant women. According to Smail (2001), 30 to 40 % of pregnant women with untreated asymptomatic bacteriuria will develop symptomatic UTI including pyelonephritis, during pregnancy. This risk is reduced by 70 to 80 % if bacteriuria is eradicated.

Bacteriuria without significant pyuria often occur in cases of asymptomatic patients, patients with diabetes, enteric fever or bacterial endocarditis whereas significant pyuria with sterile bacterial culture occur in patients with prior antibiotic use, renal tuberculosis, corticosteroid administration, analgesic nephropathy, renal calculi or in the presence of bacteria that are not able to

grow in the media used (Forbes, 2005). Our study also showed pyuria without bacteriuria and bacteriuria without pyuria in certain number of patients which could be due to inclusion of all kinds of patients mentioned above. Kattel *et al.,* 2009 also showed similar results to our study where 11.2% of the samples showed significant Bacteriuria without pyuria.

Mostly, UTI is originated from colonic bacteria which comprise mainly gram negative bacteria. Also in our study, majority 76.0 %(210/276) were Gram negative bacterial isolates which were found significantly higher (P<0.05) than Gram positive bacterial isolates. These findings were consistent with the findings of previous studies done by Chhetri *et al.,*(2001), Mishra *et al.,* (2005),), Karki *et al*.,(2004), Khurana *et al.,*(2002*)*, Shrestha *et al.,*(2005).

Regarding the pattern of bacterial isolates, *E.coli* (57.6%) was the predominant bacteria among the total gram positive and gram negative bacteria isolated in our study followed by staphylococcus aureus (14.1%), *K. pneumonia*e (6.2%) and others which is in favour of the study done by Sibi *et al.*, (2011) where the most frequent causative agents of UTI were *Escherichia coli* accounting for 39.4% of the isolates followed by *Staphylococcus* (18.4%), *Klebsiella* (15.7%), *Enterococcus* (13.1%), *Proteus* (7.8%), *Pseudomonas* and *Candida* (2.6%) each. Our study also showed similar result with the study of Ullah (2005), where common micro organisms in decreasing order of yield are *E.coli*, *Klebsiella, Staph aureus*, *Proteus* species and *Pseudomonas* isolated from 80, 7.1, 5.0 & 1.5% samples respectively. *E. coli* has been found to be the most common infective agent in this series, being grown in 168 (80%) of cases.

Majority of *E.coli* were isolated from Outpatient department in our study. *E.coli* accounts for 50% - 90% of all the uncomplicated urinary tract infections (Vgaarali *et al.,* 2008). These *E.coli* are primarily derived from the faecal flora, which can colonize the periurethral area, overcome the local host defenses and enter and multiply within the urinary tract. These *E. coli* strains are designed as Uropathogenic *E.coli* (UPEC) which possess distinctive traits that confer an enhanced extra intestinal virulence potential (Forbes *et al.*, 2007; Johnson *et al.,* 2002; Oelschlaeger *et al*., 2002). According to Ronald

(2002), *Escherichia coli* cause 75 to 90 % of episodes of acute uncomplicated cystitis, and *Staphylococcus saprophyticus* accounts for 5 to 15 %, mainly in younger women. *Enterococci* and aerobic gram-negative rods other than *E. coli,* such as *Klebsiella* spp and *Proteus mirabilis,* are isolated in the remainder of the cases. Many virulence factors of the *E. coli* might be the reason to be the most frequent organism to cause UTI in both sexes all over the World. As the most common bacteria causing UTI is *E. coli*, our study also exhibited *E. coli* as significantly ($P<0.05$) the most predominant organism in most of age group of both sexes.

In our study, *Staphylococcus aureus* was found the second most common isolates. Generally *Staphylococcus aureus* reaches the kidney through the blood stream. Other infection are perinephric abscess and pyonephrosis, it results from bacterial infection arising in an obstructed ureter. UTI due to *E. faecalis* are usually associated with the use of instrument or catheterization (Collier *et al*., 1998).

Among the Gram positive bacteria, coagulase negative *Staphylococcus are* now a common cause of UTI, particularly cystitis in young, sexually active women (Hovelius *et al.,* 1984). Of total 5.4% (15/276) coagulase negative *Staphylococcus* isolated, four isolates were *Staphylococcus saprophyticus* and 11 isolates were *Staphylococcus epidermidis* in our study. Similar to our results, Shrestha *et al.,* (2005) also found *E. coli* as the most predominant pathogen (60.2%) followed by *Staphylococcus epidermidis* (16.7%), *Staphylococcus aureus* (4.9%), *Klebsiella spp* (3.7%), *Proteus* spp (3.6%), *Pseudomonas aeruginosa* (3.1%), *Citrobacter freundii* (2.4%), *and Morganella morganii* (2.4%). In contrast to our study, *Staphylococcus epidermidis* (16.7%) was the second most predominant isolate. Most of the *Staphylococcus epidermidis* isolates were obtained from catheter samples, it may be due colonization of catheters by the bacteria. Among the Gram positive bacteria, *Staphylococcus saprophyticus* is now a common cause of UTI, particularly cystitis in young, sexually active women (Hovelius and Mardh, 1984). However, only four isolates of *Staphylococcus saprophyticus* were isolated in our study.

P. aeruginosa is one of the most important nosocomial pathogens, being a major cause of pneumonia, bacteremia, and urinary tract infections (Pier and Ramphal, 2005) and similarly in our study 3.3% isolates were *P.aeruginosa* and most of them were isolated from ward admitted patients.

Urinary tract infections with *Burkholderia cepacia* complex have been associated after bladder irrigation or use of contaminated hospital objects. In renal transplant recipients, UTI is the most common infectious complication which contributes significantly to mortality and morbidity (Sousa *et al.,* 2010). Structural abnormalities, urological procedures, placement of stents, colonization of indwelling urinary catheters or bladder irrigation with contaminated fluids are the established risk factors. These risk factors may provide nidus for bacterial growth leading to infections and also contribute to recurrence (Mitra *et al*., 2011). Rare opportunist organisms which are usually considered as usually considered as contaminants could cause infections. Burkholderia cepacia is not a common genito-urinary tract infection causing pathogen and is usually introduced after some urological procedures or catheterization (Bennett *et al.,* 2005). Exposure to contaminated hospital instruments and chemicals contributes to B.cepacia associated nosocomial infections (Harumasa *et al.,* 2010). Mohammad et al reported case of recurrent urinary tract infection with B. cepacia in renal transplant recipients. In our study also, 1.8% *Burkholderia cepacia* complex (5/276) was isolated and majority were from catheter samples which favours the above various research.

In our study, major causative agents of UTI were gram negative bacteria (76%) in compared to gram positive bacteria (24%) which in support of the various study done by Sibi *et al.*, 2011; Ullah (2005). EI-Mahmood *et al.,* (2010) also showed high prevalence rate (74.7%) of gram negative bacteria compared to gram positive bacteria (25.3%).

Through genetic exchange mechanisms, many bacteria have become resistant to multiple classes of antibacterial agents, and these bacteria with multidrug resistance (defined as resistance to ≥3 antibacterial drug classes) (Magiorakos

et al., 2012) have become a cause for serious concern, particularly in hospitals and other healthcare institutions where they tend to occur most commonly.

Resistance to antibiotics is increasing now-a-days in community patient in our country Nepal. It may be due to preliminary use antibiotics without urine culture or before urine culture is done. Patients do not have knowledge about resistivity and sensitivity of the drug to the organism causing UTI. Since many people in Nepal are illiterate or they can't afford high pay for doctor visit or doing urine culture. They are not aware of effect of irrational use of drugs, wrong dose and dose taken insufficient length of time. UTI is treated with antibacterial drugs. The Kirby-Bauer method and its modifications recognize three categories of susceptibility: susceptible, intermediate and resistant. The choice of drug and length of treatment depend on the patient's history and the urine tests that identify the offending bacteria and their sensitivity profile (Biswas et *al.*, 2006).

Our study showed that all isolates of *E.coli* were sensitive to Polymyxin B and Imipenem was found 97.5% (145/159) sensitive in vitro for *E. coli* isolates. Likewise, more than 80% of the isolates were susceptible to Amikacin. Gentamycin and Piperacillin plus Tazobactum were found sensitive for more than 70% of isolates. Cotrimoxazole and Nitrofurantoin were moderately sensitive about 60%. Remaining other drugs was not found satisfactory. Similar to our study, Imipenem was 100% sensitive for *E.coli* in a study of Mohammed *et al.,* (2007). Our study also showed 67.7% (103/159) of *E.coli* were found MDR and 63.1% (65/103) were ESBL producer only one isolates was MBL producer which is in favor of the study done by Poudyal *et al*., (2011) where *Escherichia coli* (80%) was the major ESBL producer followed by *Klebsiella pneumoniae* (5.8%).

Our study found 17 isolates of *Klebsiella* spp and among this majority (11/17) were MDR strains and 45.5 % (4/11) were ESBL producers and 36.3% were MBL producers. Polymyxin B was 100% sensitive, Imipenum 65% sensitive and less than 50% isolates were sensitive to other antibiotics tested.

Our study showed that only Vancomycin and Teicoplanin were found 100% (39/39) sensitive for *Staphylococcus aureus* even for MRSA. However, most of the isolates were found susceptible towards Doxycycline (95%) and Nitrofurantoin (87%). Majority (53.8%, 21/39) isolates were MDR as well MRSA isolates. Most sensitive antibiotics among the Coagulase negative staphylococci (*S. epidermidis* and *S.saprohpyticus*) were Doxycycline, Teicoplanin and Vancomycin. Majority of the isolates were susceptible towards Nitrofurantoin, Cloxacillin, Gentamycin and Piperacillin plus Tazobactum. Other drugs among tested drugs were not found satisfactory. Only few isolates of *S. epidermidis* (4/11) were MDR.

In recent years enterococcal infections have become much more significant in hospital admitted patients (Richards *et al*., 1999). Similarly, our study found 4.3% isolates (12/276) were *Enterococcus* spp and Vancomycin and Doxycycline were found 100% sensitive for *Enterococcus* spp. Majority of the isolates showed susceptibility towards Nitrofurantoin and Piperacillin plus Tazobactum. Other drugs among tested were not found satisfactory and few (3/12) isolates were found MDR.

Nine isolates of *Pseudomonas aeruginosa* were isolated in our study that showed 100% sensitive toward Polymyxin and Piperacillin plus Tazobactum. Good susceptibility towards Imipenem (89%), Cefepime and ceftazidime (78%). In addition, MDR isolates were not found. In our study, all seven isolates of *Acinetobacter* spp were 100% susceptible only towards Polymyxin B. However, 75% of isolates were sensitive towards Imipenem Ampicillin plus Salbactum. Amikacin and Gentamycin were sensitive for more than 60% of isolates. Other remaining drugs among tested were not found satisfactory and majority (42.8%, 3/7)) were found MDR isolates and one was ESBL producer. Similar to our study, Meharwal *et al.*, (2002) found *Pseudomonas* spp were commonest (45.4%) followed by *Acinetobacter* spp (39.0%) in complicated nosocomial UTI caused by non-fermenters.

Only Imipenem and Polymyxin B was found 100% (4/4) sensitive for *Enterobacter* spp isolates then followed by Amikacin (71%). Other tested antibiotics were below 60% sensitive. Two isolates were found MDR (33.3%)

and 16.6% (1/6) isolates were ESBL producer. Five isolates of *Burkholderia cepacia* complex were sensitive to Imipenem, Gentamycin, Cotrimoxazole and Piperacillin Tazobactum. Ceftazidime and Cefepime were sensitive to only 60% of strains. Other drugs among tested were not found satisfactory.

Three isolates of *Citrobacter* spp isolated were all MDR and mostly sensitive towards Polymyxin B and Imipenem. All two *Proteus* spp isolates were sensitive (100%) towards Imipenem, Amikacin, Norfloxacin, Piperacillin plus Tazobactum, Cefepime, Cefixime, all Fluoroquinolones tested and Gentamycin. All were resistant to remaining tested drugs. Only one *Providencia* spp isolated was MDR and showed sensitive towards Imipenem, Piperacillin plus tazobactam and Cefepime only. Only one *Morganella morganii* was isolated that was found both MDR and ESBL producer which was sensitive only to Imipenem, Nitrofurantoin, Gentamycin, Ceftazidime, Piperacillin plus Tazobactum, Amikacin, Cefepime.

The most sensitive antibiotic against Gram negative bacteria was found to be Polymyxin B (196.6%) followed by Imipenem (93.3%), Amikacin (82.3%), Piperacillin plus Tazobactum (70.4%). Similarly, the most sensitive antibiotic against Gram positive bacteria was found to be Teicoplanin and Vancomycin (100%, 66/66) followed by Doxycycline (88.0%), Nitrofurantoin (81.8%). 36.3% (24/66) gram positive isolates were found MDR.

Of the total 1063 samples, 276(26.0%) showed significant growth among which 128/210 (61.0%) gram negative isolates and 28/66(42.4%) gram positive isolates were multi-drug resistant being 56.5% MDR in total isolates. Poudyal *et al*., (2011) found slightly higher (64.5%) MDR strains among 237 significant growths as compared to our results. Of the total isolates, more than half were MDR, *E. coli* being the major contributor followed by *S. aureus*, *K. pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter* spp. In gram-negative pathogens, the most important resistance problems are encountered in *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter*, with increasing trends observed for all major anti-gram-negative agents (β-lactams, Fluoroquinolones and Aminoglycosides). Our study was in contrast to the

study of Yasmin, which revealed a higher occurrence of multidrug resistant ESBL producing *Klebsiella* spp (80%), *Proteus* spp (72%), *and Enterobacter* spp (71.4%), *E.coli* (67.3%) and *pseudomonas* spp (88.8%) from various clinical isolates (Yasmin, 2012).

A matter of major concern is the emergence of new β-lactamases capable of degrading the expanded-spectrum cephalosporins and/or carbapenems, such as the extended-spectrum β-lactamases (ESBLs) and the carbapenemases. These β-lactamase genes are often associated with resistance determinants to non-β-lactam agent (e. g. aminoglycosides and fluoroquinolones), and strains producing ESBLs or carbapenemases often exhibit complex multidrug resistant phenotypes and sometimes are panresistant. Of various ESBLs types, enzymes of the CTX-M-type have proved to be extremely successful at spreading and, in several settings, they are now the most common ESBLs in *E. coli* and *K. pneumoniae* (Livermore *et al*., 2007 and Peleg *et al*., 2010).

Staphylococcus aureus has developed resistance to newer antibiotics over the years. Methicillin resistance is quite frequent approaching and at time exceeding 50% in tertiary care centres. Vancomycin resistance has been very low (Arakere *et al.,* 2005). Likewise Coagulase negative staphylococci have acquired multiple resistance and become important nosocomial pathogens (Singhal *et al.,* 2006).In our study, 53.8% (21/39) of *Staphylococcus aureus* isolates were MDR as well as MRSA whereas 4/11(26.3%) of *S.epidermidis* was MDR.

Extended spectrum beta-lactamase (ESBL) producing organisms create a major problem for clinical therapeutics. These resistant bacteria are emerging world wide as a threat to human health in both the community and hospital settings. Among the total bacterial gram negative bacterial isolates in our study, 57.8% (74/128 MDR isolates) were ESBL producers. *E.coli* (63.1%) followed by *Klebsiella* (45.5 %) were the major ESBL producers. A similar study from North India on uropathogens such as *Klebsiella pneumoniae, Escherichia coli, Enterobacter, Proteus* and *Citrobacter* spp., showed that 26.6% of the isolates were ESBL producers (Tankhiwale *et al.,* 2004). A

report from Coimbatore (India) also showed that ESBL production was 41% in *E. coli* and 40% in *K. pneumoniae* (Babypadmini *et al.,* (2004). In our study, ESBL was not detected in *P.aeruginosa*, *Burkholderia cepacia* complex and *proteus* spp which is in favour of study of Poudyal *et al*., (2011). Urine (70.4%) was the main source of ESBL-producing isolates from all patients, followed by blood (16.5%) in a study of Alipourfard *et al.,* (2010) and our study also showed high percentage of ESBL and MBL producers among the total (128/210) MDR isolates in Gram negative bacteria.

In our study, almost all ESBL producing bacterial isolates were susceptible to Polymyxin B (100%) and Imipenem (97.2%). Of all isolates, 89.0% were susceptible to Amikacin and 55.1% to Gentamycin. Nitrofurantoin (58.1%) and Piperacillin plus Tazobactum (54.0%) were moderately sensitive. High rate resistance was observed to all Quinolones tested- Ciprofloxacin, Ofloxacin and Norfloxacin (89.1%) followed by Levofloxacin (86.4%). Cotrimoxazole (72.9%) and Ampicillin plus Salbactum (71.6%) were highly resistant. Cephalosporin and Ampicillin were 100% resistant. This is in favour of the study done by Alipourfard *et al.,* (2010) and Umadevi *et al.,* (2011). Paterson *et al*., (2005) also found Nitrofurantoin as the more appropriate antibiotics rather than Quinolones which supports our research study that nitrofurantoin is most effective oral antibiotics for uropathogens, including ESBL producers.

The recent increase of MDR, ESBL, MBL and MRSA strains in hospital has started to pose great difficult in selecting antimicrobial agents for the management of the infection they caused and the cost will be definitely high because of need of new costly drugs as well as prolong hospital stay. However, results of this type of study must be used to implement preventive measure to prevent the emergence and spread of antimicrobial resistance.

CHAPTER VI

6. CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

Majority of uropathogens causing UTI in both sexes of all age groups from both Indoor patients and Outdoor patients were Gram negative bacterial isolates. Among them *E.coli* was significantly the most predominant one followed by others.

Majority (56.5%) of the isolates included in our study was found resistant to three or more antibiotics (MDR), 57.8% MDR isolates were ESBL producers and 4.7% isolates were MBL producers. Among the *Staphylococcus aureus,* 53.8% isolates were MRSA. Majority of MDR isolates were found *in E. coli* followed by *S. aureus*. Most of the ESBL producing isolates were detected in *E.coli* and *Klebsiella* species. MBL production was found in 36.3% MDR *Klebsiella* spp isolates. All the ESBL and MBL producing bacterial isolates were found MDR and these isolates may give false susceptibility reaction towards expanded spectrum Cephalosporins.

Moreover, our study concludes that all *E. coli* isolates and most of the other Gram negative bacterial isolates were sensitive to Polymyxin B and among the other antibiotics tested, Imipenem, Amikacin, and Gentamycin were found sensitive for majority of cases; therefore, these may be the drugs of choice for the treatment of UTI in our region. Similarly, Vancomycin, Teicoplanin and Doxycycline were found the most sensitive drug for Gram positive bacteria. Among the oral antibiotics tested, Nitrofurantoin was found to be sensitive for majority of gram positive as well as gram negative isolates. So, Nitrofurantoin may be the best choice of antibiotics for community acquired UTI.

Imipenem and Polymyxin B was most effective drug for ESBL and MBL producers followed by Amikacin and Nitrofurantoin. This study shows that the frequency of ESBL producing strains of *E. coli* and *K. pneumoniae* is high in both hospital and community levels and it has a significant implication for patients' management.

6.2 RECOMMENDATION

1. As majority of uropathogens were found MDR, it is strongly recommended to request for culture and sensitivity test of Urinary tract samples before prescribing antibiotics for the treatment of UTI.
2. Since majority of strains of Gram negative bacteria especially *Escherichia coli* showed high incidence of ESBL producer and few *Klebsiella* spp showed MBL production. It is essential to screen and report ESBL and MBL production along with the routine sensitivity reporting, which will help the clinician in prescribing the proper antibiotics.
3. Phenotypic MRSA screening by Cefoxitin disk should be done for *Staphylococcus aureus* along with Antibiotic sensitivity testing.
4. Study of this type should be carried out in large scale in the country and everyone related to health care system should be made aware of improper use of antibiotics in UTI and increasing drug resistance among bacterial isolates.

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APPENDIX I

QUESTIONNAIRE

Tribhuvan University Teaching Hospital, Department of Microbiology

Clinical and Microbiological profile of patient

Serial no: **Lab no:** **Date:**

Clinical profile

Name: [ ] Age: [ ]

Previous Participation in the study....

Symptoms of UTI: fever..........; burning micturation... ; Dysuria..... ; Hematuria.....

Previous history of UTI.................... recurrent UTI.............

On antibiotics: …………………..

Pregnancy: ……………….... age factor…… …... Diabetics: …………..

Other infection................

Microbiological profile

Specimen: urine collection time: Time of lab receipt:

Day 1

Inoculate on BA, MA

Incubate aerobically at 37°c

Macroscopic examination

Colour: pH: Alb/ Sug :

Microscopic examination

Pus cells: ……………………..

Day 2

C/S result

Significant growth

Colony count: ....

Colony characteristics:

Blood Agar:

MacConkey Agar:

Gram's stain:

Catalase: Oxidase: Coagulase:

Others:

Provisional Identification:

Biochemical test performed.

<u>Day 3</u>

Interpretation of biochemical tests:

a) TSI: b) SIM: c) Citrate:

d) Urease: e) Serotyping if needed: f) Others:

Organism identified as:

Antibiotic sensitivity profile of the isolate :(disk diffusion Method)

Antibiotics	ZOI of control (mm)	ZOI of test (mm)	Interpretation

Comments on Drug Resistance Pattern: MDR ☐ Non MDR ☐

Comments on MRSA (Cefoxitin test):

Comments on ESBL production:

Comments on MBL production:

Performed by: --------------- **Checked by: ------------------**

APPENDIX-II

LIST OF EQUIPMENTS AND MATERIALS USED DURING THE STUDY

A. Equipments:

1.	Hot air oven:	Sakura (Japan)
2.	Incubator:	Sanyo (Japan)
3.	Autoclave:	Sakura (Japan)
4.	Refrigerator:	Toshiba (Japan)
5.	Microscope:	Olympus (Japan)
6.	Centrifuge:	Hitachi (Japan)
7.	Weighing Machine:	Chyo MP 300 (Japan)
8.	Water distillation Plant:	Yamato (Japan)
9.	Laminar Flow:	Dalton (USA)
10.	Water bath:	NSW (India)

B. Antibiotic discs

All the antibiotics discs used for the susceptibility tests were from MAST, UK. The antibiotics used were as follows:

1 Ampicillin (10µg)
2. Cefotaxime (30µg)
3. Cephalexin (30µg)
4. Ciprofloxacin (5µg)
6. Cotrimoxazole (25µg)
7. Nitrofurantoin (300µg)
8. Norfloxacin (10µg)
9. Novobiocin (30µg)
10. Amikacin (30µg)
11. Ceftazidime (30µg)
13. Ampicillin plus Salbactum (10µg)
14. Ceftazidime plus Clavulanic acid (40µg)
15. Piperacillin (100µg)
17. Piperacillin plus Tazobactum (110µg)
18. Imipenem (10µg)
19. Cefepime (30µg)
20. Cefotaxime plus Clavulanic acid (40µg)
21. Vancomycin (30µg)
22. Cefoxitin (30µg)
23. Doxycycline (10µg)
24. Teicoplanin (1µg)
25. Levofloxacin (10µg)
26. Ceftriaxone (30µg)
27. Cloxacillin (30µg)

APPENDIX-III

I. Composition and preparation of different culture media

The culture media used were from three companies:

a. Oxoid Unipath Ltd. Basingstoke, Hampsire, England
b. MAST Group Ltd, Mast House, Derby Road ,UK
c. Hi-Media Laboratories Pvt. Limited, Bombay, India

(All compositions are given in grams per liter and at 25ºC temperature)

1**. Blood agar base** (MAST, UK)

Blood agar base (Infusion agar) + 5-10% sheep blood

Ingredients	gm/liter
Protease peptone	15.0
Liver Digest	2.5
Yeast extract	5.0
Sodium Chloride	5.0
Agar	12.0

Final pH (at 25ºC) 7.3±0.2

Preparation: 40 grams of the blood agar base medium was suspended in 1000 ml distilled water, dissolved by boiling and sterilized by autoclaving at 121ºC (15 lbs pressure) for 15 minutes. After cooling to 45-50ºC, 5-7% sterile defibrinated sheep blood was added aseptically, then mixed with gentle rotation and immediately poured in sterile petriplates.

2. **MacConkey Agar** (MAST, UK)

Ingredients	gm/liter
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral Red	0.075
Agar	12.0
Final pH (at 25ºC)	7.4±0.2

Preparation: 52 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at

121ºC (15 lbs pressure) for 15 minutes. After cooling to 50ºC immediately poured in sterile petriplates.

3. **Mueller Hinton Agar** (MAST, UK)

Ingredients	gm/liter
Beef, infusion form	300.0
Casein Acid Hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH (at 25ºC)	7.4±0.2

Preparation: 38 grams of the medium was suspended in 1000 ml distilled water and the medium was warmed to dissolve completely. Then the medium was sterilized by autoclaving at 121ºC (15 lbs pressure) for 15 minutes. After cooling to 50ºC immediately poured in sterile petriplates.

4. **Nutrient Agar** (MAST, UK)

Ingredients	gm/liter
Peptone	10.0
Sodium Chloride	5.0
Beef Extract	10.0
Yeast Extract	1.5
Agar	12.0
Final pH (at 25ºC)	7.4±0.2

Preparation: 37 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121ºC (15 lbs pressure) for 15 minutes

4**. Nutrient Broth** (MAST, UK)

Ingredients	gm/liter
Peptone	5.0
Sodium Chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5
Final pH (at 25ºC)	7.4±0.2

Preparation: 13 grams of the medium was dissolved in 1000 ml distilled water and autoclaved at 121ºC for 15 minutes. After cooling, poured in sterile tubes and bottles.

5. **Peptone Water** (Oxoid, England)

Ingredients	gm/liter
Peptone	10
Sodium Chloride	5

Preparation: 15 grams of the medium was dissolved in 1000 ml distilled water and autoclaved at 121ºC for 15 minutes. After cooling, poured in sterile tubes and bottles.

II. Composition and preparation of different biochemical tests media

1. **MR-VP Medium** (Hi-Media laboratories)

Ingredients	gm/liter
Buffered Peptone	7.0
Dextrose	5.0
Dipotassium Phosphate	5.0
Final pH (at 25ºC)	6.9±0.2

Preparation: 17 grams was dissolved in 1000 ml distilled water. 3 ml of medium was distributed in each tube and autoclaved at 115ºC for 15 minutes.

2. **Hugh and leifson's Medium** (Hi- media laboratories)

Ingredients	gm/liter
Tryptone	2.0
Sodium chloride	5.0
Dipotassium Phosphate	0.3
Bromothymol Blue	0.08
Agar	2.0
Final pH (at 25ºC)	6.8±0.2

Preparation: 9.4 grams of the medium was rehydrated in 1000 ml cold distilled water and then heated to boiling to dissolve completely. The medium was distributed in 100 ml amounts and sterilized in the autoclave for 15 minutes at 15 lbs pressure (at 115ºC). To 100 ml sterile medium aseptically added 10 ml of sterile Dextrose and mixed thoroughly and dispensed in 5 ml quantities into sterile culture tubes.

3. **Sulphide Indole Motility (SIM) medium** (Oxoid, England)

Ingredients	gm/liter
Tryptone	20.0

Peptone	6.1
Ferrous ammonium sulphate	0.2
Sodium Thiosulphate	0.2
Agar	3.5
Final pH (at 25ºC)	7.3±0.2

Preparation: 30 grams of the medium was suspended in 1000 ml distilled water and dissolved completely. Then it was distributed in tubes to a depth of about 3 inches and sterilized by autoclaving at 115ºC for 15 minutes.

4. **Simmon's Citrate Agar** (Oxoid, England)

Ingredients	gm/liter
Magnesium Sulphate	0.2
Ammonium dihydrogen Phosphate	0.2
Sodium ammonium phosphate	1.0
Sodium Citrate, tribasic	2.0
Sodium Chloride	5.0
Agar	15.0
Bromothymol Blue	0.08
Final pH (at 25ºC)	6.8±0.2

Preparation: 23 grams of the medium was dissolved in 1000 ml distilled water. 3ml medium was distributed in test tubes and sterilized by autoclaving at 115ºC for 15 minutes. After autoclaving tubes containing medium were tilted to form slant.

5. **Triple Sugar Iron Agar (TSI)** (Oxoid, England)

Ingredients	gm/liter
Lab lemco powder	3.0
Yeast Extract	3.0
Peptone	20.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0
Ferric Citrate	0.3
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Phenol Red	0.024
Agar	12.0
Final pH (at 25ºC)	7.4±0.2

Preparation: 65 grams of the medium was dissolved in 1000 ml of distilled water and sterilized by autoclaving at 115ºC pressure for 15 minutes. The medium was allowed to set in slope form with a butt about 2.5 inches of length.

6. **Christensen Urea Agar** (Oxoid, England)

Ingredients	gm/liter
Peptone	1.0
Dextrose	1.0
Sodium Chloride	5.0
Dipotassium Phosphate	1.2
Mono-potassium phosphate	0.8
Phenol Red	0.012
Agar	15.0
Final pH (at 25ºC)	7.4±0.2

Preparation: 24 grams of the medium was suspended in 950 ml distilled water and sterilized by autoclaving at 115ºC for 15 minutes. After cooling at about 45ºC, 50 ml of 40% urea was added and mixed well. Then 5 ml was dispensed in test tube and set at slant position.

7. **Phenylalanine deaminase medium** (Oxoid, England)

Ingredients	gm/liter
Yeast extract	3
L-phenylalanine	1
Disodium hydrogen phosphate	1
Sodium chloride	5
Agar	12
Final pH (at 25ºC)	7.4±0.2

Preparation: 22 grams of the medium was dissolved in 1000 ml distilled water. 3ml medium was distributed in test tubes and sterilized by autoclaving at 115ºC for 15 minutes. After autoclaving tubes containing medium were tilted to form slant.

8. **Amino acid Decarboxylase test medium** (Oxoid, England)

Ingredients	gm/liter
Peptone	5
Meat extracts	5

Glucose	0.5
Pyridoxal	5 mg
Bromocresol purple	5 ml
(1 in 500 solution)	
Cresol red (1 in 500 solution)	2.5 ml

Preparation: Dissolved the solids in water and adjusted the pH to 6.0±0.2 before the addition of the indicators. This was the basal medium and to it was added the amino acid whose decarboxylation was to be tested. Divided the basal medium into four portions and treated separately as follows

1. added 1% L- lysine hydrochloride
2. added 1% L- ornithine hydrochloride
3. added 1% L- arginine hydrochloride
4. No additions (Control)

Re adjusted the pH to 6.0±0.2 .Distributed 1 ml quantities in small tubes containing sterile liquid paraffin to provide a layer about 5 mm thick above the medium. Autoclaved at 115ºC for 15 minutes

9. **Pyruvate fermentation medium** (Oxoid, England)

Ingredients	gm/liter
Tryptone	10 g
Yeast extracts	5 g
K2HPO4	5 g
NaCl	5 g
Sodium pyruvate	10 g
Bromothymol blue	0.1 g

Preparation: After adjusting the pH 7.2, dispensed into screw-capped tubes, and sterilized at 121ºC for 15 min.

III. Composition and preparation of different staining and tests reagent

1. Preparation of Gram stain Reagent

(a) Crystal violet solution (Hucker's crystal violet)

I. Crystal violet stock solution

Crystal violet (90% to 95% dye content	40 g
Ethanol, 95%	400 ml

Preparation: Dissolved and mixed in a glass bottle, labeled with a 1-year expiration date, and stored at room temperature.

II. Ammonium oxalate solution (1%)

Ammonium oxalate (reagent grade)	16 g
Distilled water	1600 ml

Preparation: Dissolved and mixed in a brown glass bottle, labeled with a 1-year expiration date, and stored at room temperature.

III. Crystal violet working solution

Crystal violet stock solution	40 ml
Ammonium oxalate solution (1%)	160 ml

Preparation: Filtered crystal violet stock solution into a glass. Allowed to filter completely, and then filtered ammonium oxalate solution. Labeled with earliest expiration date of stock solutions.

(b) Gram's iodine

I. Stock lugol's iodine solution

Iodine crystals (reagent grade)	25 g
Potassium iodide (reagent grade)	50 g
Distilled water	500 ml

Preparation: Mixed and let stand until dissolved in a brown glass bottle, labeled with a 6- month expiration date, and stored at room temperature.

II. Sodium bicarbonate, 5% (w/v)

Sodium bicarbonate (reagent grade)	50 g
Distilled water	1000 ml

Preparation: Dissolved in a glass bottle, labeled with a 1-year expiration date, and stored at room temperature.

III. Gram's iodine

Stock lugol's iodine solution	60 ml
Ditilled water	220 ml
Sodium bicarbonate (5%)	60 ml

Preparation: Mixed in a brown glass bottle, labeled with a 6-month expiration date, and stored at room temperature

(c) Acetone-Alcohol Decolorizer

Acetone	500 ml
Ethanol (absolute)	475
Distilled Water	25 ml

Preparation: To 25 ml D/W, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then immediately, 500 ml acetone was added to the bottle and mixed well.

(d) Safranin (Counter Stain)

Safranin	10.0 gm
Distilled Water	1000 ml

Preparation: In a clean piece of paper, 10 gm of safranin was weighed and transferred to a clean bottle. Then 1 liter D/W was added to the bottle and mixed well until safranin dissolved completely.

2. **Normal Saline**

Sodium Chloride	0.85 gm
Distilled Water	100 ml

Preparation: The sodium chloride was weighed and transferred to a leak- proof bottle premarked to hold 100 ml. Distilled water was added to the 100 ml mark, and mixed until the salt was fully dissolved. The bottle was labeled and stored at room temperature.

Biochemical Tests Reagents

a. **Catalase Test**

Catalase Reagent (3% H_2O_2)

Stock Hydrogen Peroxide (30%)	10ml
Distilled Water	90ml

Preparation: To 90 ml of D/W, 10 ml of hydrogen peroxide was added and mixed well

b. **Oxidase Test**

Oxidase Reagent (impregnated in Whatman's No. 1 filter paper)

Tetramethyl p-phenylene

diamine dihydrochloride (TPD) 1 gm

Distilled Water 100 ml

Preparation: This reagent solution was made by dissolving 1 gm of TPD in 100 ml D/W. To that solution strips of Whatman's No.1 filter paper were soaked and drained for about 30 seconds. Then these strips were dried in incubator and stored in a dark bottle tightly sealed with a screw cap.

c**. Indole Test**

Kovac's Indole Reagent

Isoamyl alcohol 30 ml

P-dimethyl aminobenzaldehyde 2.0 gm

Hydrochloric acid 10 ml

Preparation: In 30 ml of Isoamyl alcohol, 2 gm of p-dimethyl aminobezaldehyde was dissolved and transferred to a clean brown bottle. Then to that, 10 ml of conc.HCL was added and mixed well.

d. **Methyl Red Tests**

Methyl Red Solution

Methyl red 0.05 gm

Ethyl alcohol (absolute) 28 ml

Distilled water 22 ml

Preparation: To 28 ml ethanol, 0.05 gm of methyl red was dissolved and transferred to a clean brown bottle. Then 22 ml of D/W was added to that bottle and mixed well.

e. **Voges- Proskauer Test (Barritt's Reagent**)

Solution A

Alpha-Naphthol 5.0 gm

Ethyl alcohol (absolute) 100 ml

Preparation: To 25 ml D/W, 5 gm of alpha-Naphthol was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

Solution B

Potassium hydroxide (KOH) 40.0 gm

Distilled water 1000 ml

Preparation: To 25 ml D/W, 40 gm of KOH was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

4**. McFarland tube (No. 0.5)**

0.5 ml of 0.048 M $Bacl_2$ (1.17% w/v $Bacl_2H_2O$) was added to 99.5 ml of 0.18 M H_2SO4 (1% w/v) with constant stirring. The McFarland standard was thoroughly mixed to ensure that it is evenly suspended. Using matched cuvettes with a 1 cm light path and water as a blank standard, the absorbance was measured in a spectrophotometer at a wavelength of 625 nm. The acceptable range for the turbidity standard is 0.08-0.13. The standard was distributed into screw-cap tubes of the same size and volume as those used to prepare the test inoculum. The tubes were sealed tightly to prevent loss by evaporation and stored protected from light at room temperature. The turbidity standard was then vigorously agitated on a vortex mixer before use. Standards may be stored for up to 6 months, after which they should be discarded.

APPENDIX-IV

Gram staining procedure

The test was originally developed by Christian Gram in 1884. The modification currently used for general bacteriology was developed by Hucker in 1921. Gram-stain can be used effectively to divide all bacterial species into two large groups: those that take up the basic dye, crystal violet (Gram-positive) and those that allow the crystal dye to wash out easily with the decolourizer alcohol or acetone (Gram-negative). The following steps are involved in Gram stain:

1. A thin film of the material to be examined was prepared and dried.
2. The material on the slide was heat- fixed and allowed to cool before staining.
3. The slide was flooded with crystal violet stain and allowed to remain without drying for 30 to 60 seconds.
4. The slide was rinsed with tap water, shaking off excess.
5. The slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet is in contact with the slide surface.
6. The slide was rinsed with tap water, shaking off excess.
7. The slide was flooded with acetone alcohol decolorizer for 10 seconds and rinsed immediately with tap water until no further colours flow from the slide with the decolourizer. Thicker smear requires more aggressive decolorizing.
8. The slide was flooded with counter stain (Saffranin) for 30 seconds and washed off with tap water.
9. The slide was blotted between two clean sheets of blotting paper and examined microscopically under oil immersion at 100X.

APPENDIX-V

Method of collection of midstream urine

Midstream urine was collected from patients with full aseptic precautions and the samples were processed within half an hour.

Whenever possible, the first urine passed by the patient at the beginning of the day was requested for examination. This specimen is the most suitable for culture, microscopic examination and biochemical analysis.

Midstream urine (MSU) for microbiological examination was as follows:

WOMEN

Women who were ambulatory, they were requested as

- Wash her hands thoroughly with soap and water and dry them with a clean towel.
- Undress in a suitable room, spread the labia and cleanse the vulva and labia thoroughly using sterile cotton gauze pads and warm soapy water wiping from front to rear.
- Rinse thoroughly with warm water and dry with a sterile cotton gauze pad. During the entire process, the patient should keep the labia separated and do not touch the cleansed area with fingers.
- Pass urine, discarding the first part of the stream. Collect the remaining urine in the sterile container, closing the lid as soon as the urine has been collected.
- The specimen should be transported promptly to the laboratory.

MEN

Men who were ambulatory, they were requested as:

- Wash his hands.
- Pull back the foreskin (If not circumcised), wash and dry the glans with soapy water and gauze pads and pass urine, discarding the first part of the stream.
- Still holding back the foreskin, pass most of remaining urine into a sterile container. This is a midstream urine specimen.
- Place the cover on the container and the specimen should be transported promptly to the laboratory.

For Bedridden patients, the same procedure was followed, except that a nurse assisted the patient.

APPENDIX-VI

Table: Some of the distinguishing reactions of the isolated Gram negative bacteria

Key: +, ≥ 85% of strains positive; -, ≥ 85% of strains negative; ±, 16-84% of strains positive after 24-48 h at 36ºC. Key reactions are in bold type.

Species	Test/Substrate									
	lac	mot	gas	ind	vp	cit	PDA	ure	lys	H_2S
E.coli	+	+	+	+	-	-	-	-	+	-
S.typhi	-	+	-	-	-	-	-	-	+	+
C. freundii	±	+	+	-	-	+	-	±	-	±
C. koseri	±	+	+	+	-	+	-	±	-	-
K. pneumoniae	+	-	++	-	+	+	-	+	+	-
K. oxytoca	+	-	++	+	+	+	-	+	+	-
E. aerogenes	+	+	++	-	+	+	-	-	+	-
P. mirabilis	-	+	+	-	±	±	+	++	-	+
M. morganii	-	+	+	+	-	-	+	++	-	±
P. aeruginosa	-	+	-	-	-	+	-	-	-	-
A. calcoaceticus	-	-	-	-	-	-	-	±	-	-

Lac: fermentation of lactose; mot: motility; gas: gas from glucose, ind: indole production; vp: Voges-Proskauer; cit: citrate utilization (Simmons'); PDA: phenylalanine deaminase; ure: urease; lys: lysine decarboxylase; H2S: H2S production in TSI agar.

Source: Mackie and McCartney practical medical microbiology, fourteenth edition, edited by J.G. Collee/A.G.Fraser, B.P. Marmion/ A. Simmons

APPENDIX-VII

Zone size interpretative chart

Antimicrobial Agent	**symbol**	**Disc content**	**Resistant (mm or less)**	**Intermediate (mm)**	**Sensitive (mm)**
Amikacin	AK	30µg	14	15-16	17
Amoxycillin plus Salbactum when testing Gram negative enteric organism	SAM	30µg	13	14-17	18
Amoxycillin/Ampicillin When testing Gram negative enteric organism When testing Staphylococci When testing Enterococci	AML/AMP	10µg	13 28 16	14-16	17 29 17
Cefepime	CPM	30µg	14	15-17	18
Cefotaxime	CTX	30µg	14	15-22	23
Ceftazidime	CAZ	30µg	14	15-17	18
Cephalexin	CL	30µg	14	15-17	18
Ciprofloxacin	CIP	5µg	15	16-20	21
Cotrimoxazole	SXT	25µg	10	11-15	16
Doxycycline Hydrochloride	DO	10µg	12	13-15	16
Gentamicin	CN	10µg	12	13-14	15
Imipenem	IPM	10µg	13	14-15	16
Nitrofurantoin	F	300µg	14	15-16	17
Norfloxacin	NOR	10µg	12	13-16	17
Ofloxacin	OFX	5µg	12	13-15	16

Piperacillin When testing for Pseudomonas spp When testing for Gram negative bacteria	PRL	100µg	 17 17	 18-20	 18 21
Piperacillin plus Tazobactum When testing for Pseudomonas spp When testing for Gram negative bacteria	TZP	110µg	17 17 17	18-20 18-20	21 18 21
Teicoplanin	TEC	30µg	17		18
Vancomycin When testing for Staphylococci When testing for Enterococci	VA	30µg	 14 16		 15 17

SOURCE: OXOID UNIPATH LTD. BASINGSTOKE, HAMPSIRE,

APPENDIX-VIII

The following antibiotics were used for different bacteria:

i. Enterococcus

1st line: Ampicillin, Nitrofurantoin, Norfloxacin, Ofloxacin, Gentamycin

2nd line: Teicoplanin, Vancomycin, Ciprofloxacin, Levofloxacin, Chloramphenicol

ii. Staphylococcus

1st line: Ampicillin, Nitrofurantoin, Norfloxacin, Ofloxacin, Cloxacillin, Cephalexin Cotrimoxazole, Gentamycin

2nd line: Cefoxitin, Teicoplanin, Vancomycin, ciprofloxacin, Levofloxacin, Doxycycline, cefotaxime

iii. Gram negative bacilli

1st line: Ampicillin, Nitrofurantoin, Norfloxacin, Amikacin, Gentamycin, Ofloxacin, Cephalexin, Cotrimoxazole, Gentamycin, cefixime

2nd line: Ampicillin plus salbactum, ceftriaxone, ceftazidime, Piperacillin plus Tazobactum, ciprofloxacin, Levofloxacin, cefepime, Imipenem, Meropenem, polymyxin B

iv. Pseudomonas

1st line: Norfloxacin, Amikacin, Ofloxacin, Gentamycin, Piperacillin, ceftazidime,

2ndline: Ampicillin plus salbactum, Ceftriaxone, Cefepime, Piperacillin plus Tazobactum, Ciprofloxacin, Levofloxacin, Tobramycin, Imipenem, Polymyxin B.