MULTIDRUG RESISTANCE AND EXTENDED SPECTRUM β-LACTAMASE PRODUCING *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* ISOLATES FROM URINE SAMPLES OF PATIENTS ATTENDING A HOSPITAL OF SUNSARI, NEPAL

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By

Romi Karki

TU Registration No: 5-2-459-91-2010

Exam Roll No: MB 164/071

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RECOMMENDATION

This is to certify that **Mrs. Romi Karki** has completed this dissertation work entitled "**MULTIDRUG RESISTANCE AND EXTENDED SPECTRUM β-LACTAMASE PRODUCING *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* ISOLATES FROM URINE SAMPLES OF PATIENTS ATTENDING A HOSPITAL, OF SUNSARI NEPAL"** as a partial fulfilment of the requirements of M.Sc. degree in Microbiology (Medical) under our supervision. To our knowledge this work has not been submitted for any other degree.

……..……..	……..……..
Mr. Hemanta Khanal, Supervisor	Dr. Madan Krishan Mandal
Department of Microbiology	Apex Hospital Ltd
Central Campus of Technology	Itahari, Sunsari, Nepal
Dharan, Sunsari, Nepal	

Date: ……

CERTIFICATE OF APPROVAL

On the recommendation of the supervisor **Mr. Hemanta Khanal** this dissertation work of **Mrs. Romi Karki** entitled "**MULTIDRUG RESISTANCE AND EXTENDED SPECTRUM β-LACTAMASE PRODUCING *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* ISOLATES FROM URINE SAMPLES OF PATIENTS ATTENDING A HOSPITAL, OF SUNSARI NEPAL**" has been approved for the examination and is submitted to the Tribhuvan University in the partial fulfilment of the requirements of M.Sc. Degree in Microbiology (Medical).

..……..

Dr. Kamana Sahani

M.Sc. Program Co-ordinator,

Department of Microbiology

Central Campus of Technology, Hattisar (CCT)

Dharan, Nepal

Date : ……..

BOARD OF EXAMINERS

Recommended by : …….

Mr. Hemanta Khanal

Supervisor

Approved by :

…….

Mr. Hemanta Khanal

Head of Department

Examined by :

…….

Internal Examiner

Date : …….

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Date : ……. Romi Karki

ABSTRACT

Multi drug resistant *Escherichia coli* and *Klebsiella pneumoniae* expressing extended spectrum β-lactamase enzymes (ESBLs) has become a serious challenges to clinicians for the therapeutic management of clinical cases in urinary tract infection. The main objective of the study was focused to determine the dominance of MDR *E.coli* and *Klebsiella pneumoniae* and the evaluation of status of β-lactamase enzyme produced by them. The study was carried out in Apex Hospital, Itahari between June and November, 2019. A total of 350 midstream urine samples were processed among suspected cases of urinary tract infection. The bacteria were isolated by semi quantative culture technique and identified by conventional biochemical tests. The antimicrobial susceptibility testing was performed by modified Kirby Bauer disc diffusion method following Clinical and Laboratory Standards Institute guidelines and were tested for ESBL by combination disc method. The p-value <0.05 was considered as statistically significant. A total of 85 samples showed significant bacteriuria with 62 *E. coli* and 23 *Klebsiella pneumoniae.* Among the isolates, 62.35% were found MDR strains. By combined disk test, 86.67% *E. coli* and 13.33% *Klebsiella* spp. were found ESBL producers. There is significant association between MDR and ESBL production as well as between age group of patients and ESBL producing organisms (P=0.01).

Higher prevalence of ESBL producing *E. coli* and *Klebsiella* spp. was observed warranting prompt need of surveillance for effective management of such MDR strains. Imipenem, Meropenem and Nitrofurantoin seemed to be drug of choice for UTI. Amoxycillin should no longer considered as drugs for empirical treatment of clinically evident UTI, because of high resistance rates. There is an increasing need for periodic monitoring of drug susceptibility pattern to prevent the spread and development of antimicrobial resistant strains and ESBL producers.

Keywords: *Escherichia coli*, Extended Spectrum β-lactamase (ESBL), *Klebsiella pneumoniae*, Multidrug resistance (MDR), Urinary Tract Infection (UTI)

TABLE OF CONTENTS

Title i
RECOMMENDATION ii
CERTIFICATE OF APPROVAL iii
BOARD OF EXAMINERS iv
ACKNOWLEDGEMENTS v
ABSTRACT vi
TABLE OF CONTENTS vii
LIST OF TABLES x
LIST OF PHOTOGRAPHS xii
LIST OF APPENDICES xiii
LIST OF ABBREVATIONS xiv
CHAPTER I INTRODUCTION AND OBJECTIVES 1
1.1 Background 1
1.2 Objectives 5
1.2.1 General objective 5
1.2.2 Specific objectives 5
CHAPTER II LITERATURE REVIEW 6
2.1 Urinary tract infection 6
2.1.1 Site of infection 6
2.1.2 Symptoms and sign of UTI 7
2.1.3 Factors predisposing to infection 7
2.1.4 Etiological agents of UTI 8
2.2 Enterobacteriaceae 8
2.2.1 Escherichia coli 9
2.2.2 Klebsiella 9
2.3 β-lactam antibiotics 10
2.4 Antibiotic resistance 11
2.4.1 Mechanism of antibiotic resistance 11
2.5 Multidrug resistance 11
2.6 β-lactamases production 12
2.6.1 Classification of β-lactamases 12

2.6.2 Extended spectrum beta-lactamases (ESBLs) 15
2.6.3 TEM β-lactamases 15
2.6.4 SHV β-lactamases.....16
2.6.5 CTX-M β-lactamases.....16
2.7 Laboratory procedure for ESBL detection 16
2.7.1 Screening for ESBL producers.....17
2.7.1.1 Disc Diffusion Method 17
2.7.1.2 Screening by dilution antimicrobial susceptibility test.....17
2.7.2 Phenotypic Confirmatory test for ESBL production.....18
2.8 Risk factors for colonization and infections with ESBL-producing Enterobacteriaceae 18
2.9 Prevention.....19
2.10 Treatment of patients infected with Enterobacteriaceae producing ESBLs 19
2.10 Global epidemiology of ESBLs 20
2.11 Nepalese scenario.....23
CHAPTER III 25
MATERIALS AND METHODS.....25
3.1 Materials.....25
3.2 Study design and sites 25
3.3 Study population 25
3.3.1 Inclusion criteria 25
3.3.2 Exclusion criteria.....25
3.4 Sample size.....26
3.5 Specimen collection and transportation 26
3.6 Processing of specimen 26
3.6.1 Macroscopic examination of specimens.....26
3.6.2 Culture of specimens 27
3.6.3 Identification of the isolates 27
3.6.4 Purity plate.....27
3.6.5 Antimicrobial susceptibility testing.....28
3.6.7 Tests for ESBL-production in strains of *E. coli* and *Klebsiella pneumoniae* 28
3.6.8 Analysis of MDR isolates.....29

3.6.9 Antibiotic susceptibility testing of ESBL producer.....29
3.6.10 Quality control.....29
3.6.11 Data analysis and entry.....30
CHAPTER IV RESULTS.....31
4.1 Growth profile of bacteria isolated in urine samples.....31
4.2. a. Growth pattern of *E.coli* and *K.pneumoniae* among inpatients and outpatients.....32
4.2. b. Gender wise distribution of patient and growth pattern.....33
4.3 Age wise distribution of the growth positivity.....34
4.4 Microbiological profiling of urine isolates.....35
4.5 Antibiotic susceptibility pattern of uropathogens.....36
4.5.1 Antimicrobial susceptibility pattern of *E.coli*.....36
4.5.2 Antimicrobial susceptibility pattern of *Klebsiella pneumoniae*.....37
4.6 Profile of MDR among uropathogens.....38
4.7 Prevalence of ESBL producing uropathogens.....39
4.8 Association between MDR and ESBL production.....40
4.9 Antimicrobial susceptibility of ESBL producing organism.....41
CHAPTER V Discussion.....45
CHAPTER VI CONCLUSION AND RECOMMENDATIONS.....53
6.1 Conclusion.....53
6.2 Recommendations.....54
REFERENCES.....55
APPENDIX-A.....73
APPENDIX B.....74

LIST OF TABLES

Table 1: Growth positivity in relation to age of patient 34

Table 2: Pattern of Enterobacteriaceae isolates causing UTI 35

Table 3: Antimicrobial susceptibility pattern of *E.coli* 36

Table 4: Antimicrobial susceptibility pattern of *Klebsiella pneumoniae* 37

Table 5: Pattern of MDR among uropathogens 38

Table 6: Prevalence of ESBL producing uropathogens 39

Table 7: Association between MDR and ESBL production 40

Table 8: Antimicrobial susceptibility of ESBL producing organism 41

LIST OF FIGURES

Fig 1: Microbial growth profile in urine samples 31

Fig 2: Growth pattern of *E.coli* and *Klebsiella pneumoniae* among inpatients and outpatients. 32

Fig 3: Gender wise distribution of patient and growth pattern. 33

LIST OF PHOTOGRAPHS

Photograph 1: *Klebsiella pneumoniae* on Mac Conkey Agar

Photograph 2: *Klebsiella pneumoniae* on EMB Agar

Photograph 3: Citrate Utilization Test (+ve for *Klebsiella pneumoniae*, -ve for *Escherichia coli*)

Photograph 4: Biochemical test for *Klebsiella pneumoniae*

Photograph 5: Confirmatory test for ESBL by combination disc method

Photograph 6: Confirmatory test for ESBL by combination disc method

LIST OF APPENDICES

Appendix A: List of equipments and materials

Appendix B: Method of collection of mid-stream urine

Appendix C: Clinical and microbiological profile of urine sample

LIST OF ABBREVATIONS

ASB:	Asymptomatic Bacteriuria
AST:	Antibiotic susceptibility test
ATCC:	American Type Culture Collection
CD:	Combination Disk
CLSI:	Combination Disk
CTX-M:	Cefotaxime Resistance Gene
ESBL:	Extended Spectrum Beta Lactamase
MA:	MacConkey Agar
MDR:	Multi Drug Resistance
MHA:	Muller Hinton Agar
MIC:	Minimum Inhibitory Concentration
OPD:	Out Patient Department
PBP:	Penicillin Binding Protein
SPSS:	Statistical Package for Social Science
UPEC:	Uropathogenic *Escherichia coli*
UTI:	Urinary Tract Infection

CHAPTER I
INTRODUCTION AND OBJECTIVES

1.1 Background

Urine is a sterile ultrafiltrate of blood. Urinary tract infection (UTI) is a variety of clinical conditions ranging in severity from asymptomatic which is carrier status in the urine to symptomatic acute infection of the kidney with resultant sepsis. UTI is defined as a spectrum of diseases that involves microbial invasion of any of the urinary tissues that is extending from the renal cortex to the urethral meatus (Singh 1991). It is the commonest bacterial infection that is highly prevalent to both genders; female and male. It is expected that about 35% of healthy women experiences warning signs of UTIs (Rezwana et al 2015). The incidence is more frequent in women than men because of the squatness of female urethra, dearth of prostatic secretions, easy contamination chance with fecal flora and pregnancy. It is estimated that about 150 million urinary tract infections occur annually worldwide (Stamm and Norrby 2001).

UTI infection may be expressed predominantly as pyelonephritis, pyelitis, ureteritis, cystitis, prostitis and urethritis but urinary tract is always at risk of invasion by bacteria (Cheesbrough M 2000).The most common uropathogenic Gram negative bacteria that causes UTI are *Escherichia coli* and *Klebsiella pneumoniae*. The common bacteria isolated causing UTI in Nepal were *E. coli* (77.5%), *Klebsiella* spp (7.1%), *Pseudomonas aeruginosa* (1.3%) among Gram - negative isolates and *Staphylococcus aureus* (5.7%), *S. saprophyticus* (2.3%) and *Enterococcus faecalis* (1.2%) among Gram positive isolates (Basnet et al 2009).

In healthy patients most uropathogens originate from rectal flora and enter the urinary tract via the urethra into the bladder (Handley et al 2002). This is known as the ascending route and uropathogens initially attach to and colonize urothelium of the distal urethra. Enhancement of this route is worsen in patients with swelling around the perineum, in patients with urinary catheters and in females that uses spermicidal agents (Foxman 2002). In patients with

established cystitis up to 50 % of infections ascend into the upper urinary tracts and pyelonephritis are caused by ascension of bacteria from the bladder through the ureter and into the renal pelvis (Busch and Huland 1984). Ureteral peristalsis can occur in conditions such as pregnancy and ureteral obstruction. Bacteria that reaches the renal pelvis penetrate the renal parenchyma through the collecting ducts and disrupt the renal tubules.

UTIs can be classified as either complicated or uncomplicated depending on underlying host factors and on underlying uropathogens. Age, catheterization, diabetes mellitus and spinal cord injury are some of the underlying host factors that predispose to complicated UTIs. In complicated UTIs less virulent uropathogens that rarely cause disease in a normal urinary tract can cause significant damage to an abnormal urinary tract.

UTIs are usually treated with broad-spectrum cephalosporin, fluoroquinolones and aminoglycosides. Cephalosporins are cell wall inhibitors and commonly for treating infection caused by Gram-negative bacteria. These includes cefataxime, ceftazidime, cephradine, cefaclor etc. Fluoroquinolones are antibiotics which act by inhibiting the activity of DNA gyrase and topoisomerase enzymes essential for bacterial DNA replication and includes ciprofloxacin, ofloxacin, enoxacin, sparfloxacin etc. Aminoglycosides include gentamycin, kanamycin, amikacin etc. These act by inhibiting bacterial protein synthesis (Trevor et al 2001).

Nowadays, the haphazard uses of antibiotics have resulted in worldwide spread of antibiotic resistance among the bacteria causing a major problem (Goldsteine et al 2000). Clinical experience has indicated the presence of numerous case of antibiotics by uropathogens in both developed and developing countries (Gupta et al 2002). Usually antibiotics are given empirically before the laboratories results of urine are available to ensure appropriate therapy. The spread of antimicrobial resistance may be due to failure to adhere to proper infection control technique, increase use of antibiotics in animals and plants, unhygienic practices, availability of antibiotics without prescription, which is also responsible for multidrug resistant bacterial pathogens (Poudyal et al 2011).

Multi drug resistance (MDR) is the resistance acquired by bacteria to a vast range of antibiotics, at least two or more. MDR is resistance to at least two antibiotics of different classes including aminoglycosides, chloramphenicol, tetracyclines and erythromycin (Yadav et al 2016). MDR in many bacteria is due to the action of multidrug efflux pump and by the accumulation on resistance plasmid's or transposons of genes with each coding for resistance to a specific agent. Nowadays, in UTI's Extended spectrum β- lactamase expressing Gram negative bacilli (ESBL-GNB) generally cause community acquired infections (Dotis et al 2013). The resistance of gram negative bacteria is typically owed to plasmid mediated enzymes called Extended spectrum β-lactamases (ESBL's).

β- lactamases are the most widely used class of antibiotics all characterized by having a β- lactam ring as the chemical base and includes penicillins, cephalosporins, carbapenems, monobactams and cephamycins (Yao et al 2003). Hydrolysis by enzymes is the most common mechanism for resistance towards this class of antibiotics (Pitout 2012; Canton et al 2012; Bush and Jacoby 2010). β-lactamases belong to a very large, diverse family of enzymes capable of hydrolyzing β- lactam antibiotics by cutting open the β- lactam ring and the hydrolyzing ability of β- lactamases can be of narrow or broad spectres (Paterson and Bonomo 2005). Beta lactamases are commonly classified according to two general schemes: the Ambler classification molecular and the Bush-Jacoby-Medeiros functional classification (Bush et al 1995). Several families of β- lactamases exist, with most common plasmid mediated β- lactamases being Temoniera (TEM) and sulfhydryl variable (SHV), accounting for greater than 60% of *E. coli* resistance to ampicillin (ECDC 2012; Paterson and Bonomo 2005).

ESBL's confer resistance to penicillin, first, second and third generation cephalosporin and aztreonam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics and are inhibited by β- lactamases inhibitors such as clavulanic acid (Paterson and Bonomo 2005). The presence of ESBL producing organism in a clinical infection can cause significant treatment problems because ESBL mediated resistance may result in treatment failure if

any of the third generation cephalosporins (e.g ceftazidime, cefotaxime and ceftriaxone) or a monobactam (aztreonam) are used (Drieux et al 2008).

Antimicrobial resistance among bacterial strain is an emerging problem worldwide. *E. coli* and *Klebsiella pneumoniae* are the two predominant pathogens commonly isolated in urine. These uropathogens have also developed resistance to commonly prescribed antimicrobial agent. These severely limits the treatment options of an effective therapy. Primarily, these uropathogens exerts their anti-microbial resistance against β- lactams by producing ESBL'S (Coque et al 2008; Bradford 2001).

The true incidence is very difficult to determine because of difficulty in detecting ESBL production and due to inconsistencies in testing and reporting (Yusau et al 2010). The prevalence of bacterial isolates expressing the ESBL phenotype varies across different geographical regions.

There is ample evidence to suggest the spread of ESBL infections is higher in resource poor countries. The prevalence of multidrug resistance bacterial uropathogens is high in Nepal which is about 41.1% (Baral et al 2012) and 16% of bacterial isolates from urine where ESBL producers (Pokharel et al 2006). *E. coli* and *Klebsiella pneumoniae* being the most commonly isolated pathogens with high level of MDR and production of β- lactamases. Resistance to β- lactam antibiotics has increased significantly in the last two decades and has been documented in both community and hospitals settings (Kaye et al 2005; Lausch et al 2013; Hansen et al 2012).

The increasing level of antimicrobial resistance of uropathogenic organism is of great concern since it can limit the therapeutic choices used for treating common bacterial infection of UTI's and highlights the growing threat of the emergence. Current updated knowledge of the susceptibility pattern of bacteria is vital for the appropriate assortment and utilization of antimicrobial drugs and also for the succession of suitable prescribing guidelines. Thus, the detection of ESBL production is important, it is necessary to investigate the prevalence of ESBL positive strain in hospital so as to formulate a policy of

empirical therapy in high risk units where infection due to resistant organism are much higher (Mathur et al 2002).

This study was conducted with an objective to determine the presence of multidrug strains and the presence of ESBL producing *E.coli* and *Klebsiella pneumoniae* in order to formulate effective antibiotic strategy to control infection and to prevent the spread of these strains including the sex and age group of people who are more susceptible to infection.

1.2 Objectives

1.2.1 General objective

To determine multidrug resistant and extended spectrum β-lactamase (ESBL) producing *E. coli* and *Klebsiella pneumoniae* among bacterial uropathogens.

1.2.2 Specific objectives

i) To identify *Escherichia coli* and *Klebsiella pneumoniae* associated with UTI.
ii) To assess the antibiotic susceptibility pattern of isolates.
iii) To determine the MDR strains of the urinary isolates.
iv) To determine resistance to any of third generation cephalosporin among *E. coli* and *Klebsiella pneumoniae* due to ESBL production.

CHAPTER II
LITERATURE REVIEW

2.1 Urinary tract infection

Urinary tract infection (UTI) is called an infection that is caused by the presence and growth of microbes anywhere in the urinary tract (Tazebew et al 2012). UTIs are the most common bacterial infection that accounts for 25 % of all infection (Gupta et al 2013). UTI is diagnosed using a combination of urinary symptoms and urine culture demonstrating uropathogens above a given threshold (>1,000 cfu/ml of urine) (Rubin et al 1992) but thresholds as low as 100 cfu/ml and as high as 100,000 cfu/ml are also used (Warren et al 1999). UTI is due to invasion of the urinary tract by a nonresident infectious organism. Asymptomatic bacteriuria (ABU) is present if a patient does not exhibit the clinical signs of UTI and the upper limit of $\geq 10^5$cfu/ml is exceeded in two consecutive properly collected samples of midstream urine (from women). A single detection is adequate for men (Lin et al 2008).

2.1.1 Site of infection

Urethra is a portal for the exit of urine but allows the entry of different microbes that includes uropathogens into the urinary tract. Bacteria survives around the urethral opening in both men and women and they routinely colonize urine in the urethra, but are washed out during micturition. The shorter distance to the bladder in women makes it possible for bacterial colonizers to reach the bladder more easily before they are removed by micturition. Urogenital manipulations associated with activities of daily living and medical interventions facilitate the movement of bacteria from the vaginal cavity, rectal opening and periurethral area into urethra; however, classification of UTI depends on infection site like kidneys, prostate, bladder, ureter (Jha et al 2005). Given human anatomy, UTIs is not surprising, are among the most common bacterial infections; indeed, we should perhaps be surprised that, given the constant assault on our urinary system by microbes, UTIs are not more common.

2.1.2 Symptoms and sign of UTI

Most people suffering from UTIs have some symptoms like frequent urge to urinate and a bit painful, burning feeling in the area of the bladder or urethra during urination (Das et al 2006). Dysuria, frequent urination, urgency, suprapubic pain and possible hematuria are the common symptoms of UTI (Shahi 2015). The urine may appear cloudy, even reddish if blood is present with an unpleasant odour (Gupta et al 2013). Unseen fever is possible if UTI occur in the bladder or urethra but a fever may means that the infection has reached the kidney (Warren et al 2004).

2.1.3 Factors predisposing to infection

UTI is present as one the commonest bacterial infection despite the presence of different host defense mechanism against microbial infection (Awasthi et al 2015). There are different factors that facilitates the colonization and entry of uropathogens into the urinary tract. It is usually due to the bacteria from digestive tract which climb the opening of the urethra and begin to multiply to cause infection (Rahimkhani et al 2008). As compared to men, women are more susceptible to UTI due to short urethra, absence of prostatic secretion pregnancy and easy contamination of the urinary tract with faecal flora (Haider et al 2010). The main risk factors for the occurrence of UTI is the use of diaphragms, condoms and/or spermicides for contraception, and frequency of sexual intercourse among premenopausal women (Foxman 2002). Patients with diabetes mellitus have a high risk of asymptomatic bacteriuria, recurrent UTIs and polynephritis (Al-Badr and Al-shaikh 2013). As a clinical entity, UTIs may be divided into complicated or uncomplicated patients groups; uncomplicated UTIs may occur without a clear causative factor, while complicated UTIs occur in patients with immunosupression, an anatomically or functionally abnormal urinary tract, or patients at the extremes of age (Saemann and Horl 2008; Nitzan et al 2015; Arshad and Seed 2015; Matthews and Lancaster 2011). Further increased rate of UTI is also seen in patient with catheters or tube placed in urinary tract and patient with problems with body's natural defense mechanism (Awasthi et al 2015).

2.1.4 Etiological agents of UTI

There are different etiological agents of community acquired and hospital acquired UTIs. There are many different organisms that can infect the urinary tract, the most common agents are the Gram-negative bacilli mainly the family Enterobacteriaceae (Braunwald et al 2001). *E. coli* is the primary cause of uncomplicated infections of the urinary tract including cystitis (Gunther et al 2001). *E. coli* is the most frequent case of uncomplicated community acquired UTIs followed by *Klebsiella* spp, other *Enterobacteriaceae* and *S. saprophyticus*, *Pseudomonas* spp (Akram et al 2007; Forbes et al 2007). Other Gram-negative rods especially *Proteus* spp, *Klebsiella* spp and occasionally *Enterobacter* spp accounts for a smaller proportion of uncomplicated infections. *Serratia* spp and *Pseudomonas* spp assumes increasing importance in recurrent infections, associated with urologic manipulation, calculi, or obstruction (Braunwald et al 2001). *Salmonella typhi* and *Salmonella paratyphi* can be found in the urine of about 25 % of patients with enteric fever from the third week of infection (Cheesbrough 2000). *Enterococcus* spp, the most common Gram-positive cocci were isolated more frequently from a hospital setting (Haryniewicz et al 2001). More commonly, Enterococci and *S. aureus* cause infections in patients with real stones or previous instrumentation or surgery. The rarest infecting organisms include *Streptococcus agalactiae, Streptococcus milleri,* other Streptococci and *Gardnerella vaginalis* (Tabibian et al 2008).

2.2 Enterobacteriaceae

In human medicine, the most important family of bacteria is Enterobacteriaceae. The members of this family are Gram-negative, rod-shaped, non-spore-forming facultative anaerobes that ferment glucose and other sugars, reduce nitrate to nitrite, and produce catalase but seldom oxidase. These bacteria can cause many different infections, such as septicaemia, urinary tract infections, pneumonia, peritonitis, wound infections, meningitis, and gastroenteritis, and they can give rise to sporadic infections or outbreaks. Clinically important isolates are: *Enterobacter aerogenes, E.cloacae, E. coli, Klebsiella pneumoniae, K. oxytoca, Proteus mirabilis, P. stuartii, Salmonella*

enteritica, Serratia marcescens, Shigella sonnei, and *Yersinia pestis* (Aboot et al 2007; Donnenberg 2005; Faemer et al 2007).

2.2.1 Escherichia coli

Escherichia coli is the most prevalent facultative anaerobic species in the human gastrointestinal tract (10^4 CFU/g faeces)but it also colonizes the intestine of animals and is thus used as indicator of faecal contamination of drinking water and food. *E.coli* is usually a harmless microbe, although it is also the most common cause of community-acquired bacteraemia and the fifth most common cause of nosocomial bacteraemia (Friedman et al 2000). The more virulent pathotypes often have a larger genome compared to the non-pathogenic *E.coli* and there also many different virulence factors which are usually encoded on plasmids, chromosomes or bacteriophages (Welch et al 2002). Pathogenic *E.coli* strains are divided into two major groups based on the disease they cause and on the site of infection: Extra-intestinal pathogenic *E.coli* (ExPEC) cause infections in organs other than the gut and Intestinal pathogenic *E.coli* (InPEC) cause gastroenteritis or colitis when ingested (Johnson and Nolan 2009; Russo and Johnson 2000). The most common infections are urinary tract infections (UTIs), abdominal sepsis, septicemia and neonatal meningitis. Extended spectrum beta-lactamase (ESBL) producing strains are usually community acquired and only a few hospital outbreaks of such bacteria have been reported (Nicolas et al 2008).

2.2.2 Klebsiella

Klebsiella pneumoniae (including subspecies *K. ozaenae), K. oxytoca,* and *K. granulomatis* are the three major species of this genus. Like *E. coli, Klebsiella* spp. are usually found in the human gastrointestinal tract (10^4 CFU/g faeces).The major virulence factor of *Klebsiella* is the polysaccharide capsule, which is also responsible for the mucoid colony phenotype. *K .pneumoniae* is the species which is isolated most often from human infections, and it may cause a wide variety of (nosocomial) infections, including urinary tract infections (UTIs), septicemia, wound infections, cholecystitis, and pneumonia (Friedlander's disease) (Podschum and Ullman 1998).

2.3 β-lactam antibiotics

β-lactam antibiotics contain a β-lactam ring which is a hetero-atomic ring structure consisting of three carbon atoms and one nitrogen atom. The β-lactam ring of natural or semi-synthetic penicillin is fused with a thiazolidine ring. In cephalosporin, the β-lactam ring is merged with a dihydrothiazine ring. In the carabapenem, the β-lactam ring is combined with hydroxyethyl side chain, lacking an oxygen or Sulphur atom in the bicyclic nucleus. In contrast to the antibiotics, clavulanic acid, a β-lactamase inhibitor, is composed of a β-lactam ring fused with an oxazolidine ring and does not possessed an amide function (Amyes 2010). The β-lactam antibiotics can be divided into six different groups: penicillin, cephalosporin, carbapenem, cephamycin, monobactam, and β-lactamase inhibitors (Smet et al 2008).

The bactericidal effect of β-lactam antibiotics involves inhibition of cell wall synthesis, and this effect occurs through covalent attachment to penicillin-binding protein (PBP), which is a peptidoglycan transpeptidase enzyme that catalyzes the final steps in cell wall formation (Kohanski et al 2007; Ghuysen 1994). A rigid cell wall comprised of alternating N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) unit preserve osmotic stability. These glycosidic units are linked by transglycosidases. NAG is attached to each NAM unit, and the cross-linking of two d-alanine-d-alanine of NAM pentapeptides is catalyzed by PBPs, which acts as transpeptidases (Sauvage et al 2008). The β-lactam ring is similar to the d-alanine-d-alanine of the NAM pentapeptide, and PBPs mistakenly use the β-lactam as a building block during cell wall synthesis (Zapun et al 2008). This results in acylation of the PBP, which renders the enzyme unable to catalyze further transpeptidation reactions (Fisher and Mobashery 2009). As cell wall synthesis shows to a halt, constitutive peptidoglycan autolysis continues. Thus, the β-lactam-mediated inhibition of transpeptidation causes cell lysis, although the specific details of penicillin's bactericidal effects are still unclear (Bayles 2000).

2.4 Antibiotic resistance

Antibiotic resistance is the reduction in effectiveness of a drug such as an antimicrobial or an antineoplastic in curing a diseases or condition (Shahi et al 2015). It is the temporary or permanent ability of an organism and its progeny to remain viable or multiply under environmental conditions that would otherwise destroy or inhibit other cells (Denyer et al 2005).

2.4.1 Mechanism of antibiotic resistance

Antibiotic resistance can be intrinsic or acquired. Intrinsic resistance is the bacterial capability to resist a specific antibiotic naturally (Blair et al 2015; Poole 2004).

Acquired resistance is when a bacterium develop or acquire resistance through gene mutation or horizontal gene transfer. Acquired resistance can be achieved by main three mechanisms: i) decreased uptake and increased efflux of antibiotics from the bacterial cells, ii) target modification by mutation and iii) modification or hydrolysis of the antibiotic which makes it inactive. Inactivation by hydrolysis is clinically the most important resistance mechanism and consists of thousands of different enzymes. It includes a large group of different β-lactamases which hydrolyse a range of important antibiotics like penicillins, cephalosporins and carbapenems. The mechanism of action of β-lactamases towards penicillin is the hydrolysis of β-lactam ring which is responsible for the drug activity (Paterson and Bonomo 2005).

2.5 Multidrug resistance

Multidrug resistance has been defined by various organizations and researchers in different ways in different clinical settings. Multidrug resistance is defined as resistance to two or more classes of antimicrobial agent (CDC 2006). Concurrent resistance to antimicrobials of different classes has arisen in a multitude of bacterial species complicating the therapeutic management of infections and is considered multidrug resistant if they show resistance to three or more routinely used antibiotics (Daniel et al 2001).

2.6 β-lactamases production

The production of β-lactamases is the most important mechanism of β-lactam resistance, especially amongst Gram negative bacteria. These enzymes can hydrolyze β-lactam ring that results in the antimicrobial ineffective (Helfand and Bonomo 2003). These antibiotics are so called because of four atom β-lactam ring in their molecular structure (with the ring mimicking two amino acids in the pentapeptide crosslinks of the peptidoglycan bacterial cell wall) (Shaikh et al 2015). They were first described in *Escherichia coli* isolates before the release of the first β-lactam drug, penicillin (Abraham and Chain 1940). After the, these enzymes have been identified in Gram negative and Gram positive bacteria where they are found either plasmid or chromosomally encoded, and are associated with mobile genetic structures such as transposons and integrons (Rowe Magnus and Mazel 2002). By 2009, the number of individual protein sequences for β-lactamases was found to be more than 890. β-lactamase enzyme production is most commonly suspected in Gram negative bacteria that exhibit resistance to a β-lactam antibiotic (Bush and Jacoby 2010).

2.6.1 Classification of β-lactamases

Classification based on molecular structure was first suggested by Ambler in 1980 with two classes. Class A included PC1 β-lactamases encoded by the chromosome of *Staphylococcus aureus*, with a serine residue at the active site, and class B metallo *coli* K12, with a serine residue at the active site. Class D β –lactamases were separated from other serine β -lactamases after sequencing of PSE-2 and OXA-1 hydrolysing carbencillin and oxacillin (Houvinen et al 1988). Many kinetic, mutagenesis and structural reports have been done on these enzymes, contributing significant information about their substrate specificities and catalytic mechanisms (Helfand and Bonomo 2003). Some of these enzymes can target the expanded spectrum β-lactams such as the cephalosporins, including the extended spectrum β-lactamases (ESBL) (classes A and D) (reviewed by Bradford 2001), the AmpC (class C cephalosporinases) enzymes (Philippon Arlet and Jacoby 2002) and the

carbapenemases that can hydrolyze most β-lactams agents, including the carbapenems (classes A, B and D) (Poirel and Nordmann 2002).

The Ambler molecular scheme was modified to one based on a combination of both functional and molecular schemes. According to both systems classification, there are groups 1, 2, and 3, and subgroups 2a, 2c, 3a, etc. (Bush and Jacoby 2010). Group1 cephalosporinases which are not inhibited by clavulanate, group 2 broad spectrum enzymes which comprise the largest category and are generally inhibited by clavulanate with the exception of the 2d and 2f sub-groups, and the group 3 metallo- β -lactamases. However, most ESBLs are allocated to group 2 be which can hydrolyse penicillins, monobactams and cephalosporins and inhibited by clavulanate according to the Ambler classification. The CTX-M enzymes fulfill the criteria for group 2 be enzymes (Dhillon and Clark 2012).

Table 1.1: β-lactamases classification schemes modified from (Bush and Jacoby

2010).

Bush – Jcoby Group	Ambler class	Main Substrate	Inhibited by		Representative enzymes
			CA/TZB	EDTA	
1	C	Cephalosporins	-	-	Amp C, P 99, ACT-1, CMY-2, FOX-1, MIR-1
1e	C	Cephalosporins		-	GC-1, CMY-37
2a	A	Penicillins	+	-	PC 1
2b	A	Penicillins, early cephalosporins	+	-	TEM-1, TEM-2, SHV-1
2be	A	Extended Spectrum cephalosporins, Monobactams	-	-	TEM-3, SHV- 2, CTXMs, PER, VEB
2br	A	Penicillins Extended -spectrum cephalosporins, Monobactams	-	-	TEM-30, SHV-10
2ber	A	Carbenicillin Carbenicillin, Cefepime	-	-	TEM-50
2c	A	Cloxacillin	+	-	PSE-1, CARB-3
2ce	A	Extended -spectrum cephalosporins	+	-	RTG-4
2d	D	Carbapenems	V	-	OXA-1, OXA-10
2de	D	Extended -spectrum cephalosporins	V	-	OXA-11, OXA-15
2df	D	Carbapenems	V	-	OXA-23, OXA-48
2e	A		+	-	CEPA
2f	A		V	-	KPC-2, IMI-1, SME-1
3a (B1)	B	Carbapenems	-	+	IMP-1, VIM-1, IND-1, CcrA L1, CAU-1, GOB-1, FEZ-1
3b(B3)	B	Carbapenems	-	+	CphA, Sfh-1
4	Unknown	-			

(V), Variable, (+), Yes, (-), No, CA, Clavulanic acid, TZB, Tazobactam

2.6.2 Extended spectrum beta-lactamases (ESBLs)

ESBLs are primarily found in the Enterobacteriaceae family of Gram negative bacteria, particularly *Escherichia coli* and *Klebsiella pneumoniae* (Paterson and Bonomo 2005). ESBLs are defined as β-lactamases that have the following characteristics: they are transferable; they can hydrolyze penicillins, first, second and third generation cephalosporins, and aztreonam (but not the cephamycins); they can be blocked in vitro by β-lactamases inhibitors such as clavulanic acid. There are many ESBLs genotypes. The most common of these are the SHV, TEM, and CTX-M (Rupp and Fey 2003). But there are also other clinically important genotypes that include VEB, PER, BES-1, BEEL-1, SFO-1, TLA and IBC (Jacoby and Munoz Price 2005).

According to the Bush and Jacoby scheme (Table 1) (Bush and Jacoby 2010) ESBLs enzymes can be divided into three main groups: i) cephalosporinases which are not inhibited by clavulanate, ii) broad spectrum enzymes which comprise the largest group and are generally inhibited by clavulanate except the 2d and 2f groups and iii) metallo-β-lactamases. However, most ESBLs are allocated to group 2 be which can hydrolyse penicillins, monobactams, and cephalosporins and are inhibited by clavulanate, they are class A according to the Ambler molecular scheme. CTX-M genotype still suits the former criteria for group 1 be enzymes (Dhillon and Clark 2012).

2.6.3 TEM β-lactamases

The TEM family of ESBLs represents the largest and widely distributed group among these enzymes. TEM-1 and TEM-2 penicillinases are their evolutionary precursors (Livermore 1995; Medeiros 1997 and Bradford 2001). They hydrolyse the β-lactam ring of penicillins, cephalosporins and related antibiotics and are detected at high rates in hospitals and clinics worldwide (Matagne et al 1998). Over 200 TEM variants have been reported and new genes continue to appear. TEM-3 was the first TEM-type β-lactamase that revealed the ESBL phenotype (Sougakoff et al 2012).

2.6.4 SHV β-lactamases

SHV is universally found in *K.pneumoniae* and is the most frequently found ESBL type in clinical isolates than any other type. It confer resistance to broad – spectrum penicillins. SHV β-lactamases is responsible up to 20% of plasmid mediated ampicillin resistance in this species (Shakil et al 2015).

2.6.5 CTX-M β-lactamases

A new family of β-lactamase that preferentially hydrolyzes cefotaxime has arisen (Shakil et al 2015). These enzymes were named for their greater activity against cefotaxime than other oxyamino β-lactam substrate (ceftriaxone, ceftazidime) (Khoteja 2014).

2.7 Laboratory procedure for ESBL detection

Observation of organism harboring ESBLs provides clinicians with helpful information (Shakil et al 2015). The method for detection methods that use non-molecular techniques, which is based on the principle that most ESBLs hydrolyze third generation cephalosporin although they are inhibited by clavulanate (Shakil et al 2015) and genotypic methods, which use molecular technique to detect the gene responsible for the production of the ESBL. These all depend on detecting synergy between clavulanic acid and the indicator cephalosporin is used in the primary screening. Failure to detect ESBL production by routine disk diffusion tests has been well documented (Paterson and Bonomo 2005; Tenover et al 2009).

The Clinical and Laboratory Standard Institute (CLSI) recommendations (2007) for detection of ESBL's in *Klebsiella* spp and *E.coli* includes an initial screening test with any two of the following β-lactam antibiotics: cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone. Isolates exhibiting a MIC >1µg/ml should be confirmed phenotypically using ceftazidime plus ceftazidime/clavulanic acid and cefotaxime plus cefotaxime/clavulanic acid.

2.7.1 Screening for ESBL producers

Clinical and laboratory Standards Institute (CLSI) has developed disc diffusion and broth micro dilution screening tests for the possible ESBL production.

2.7.1.1 Disc Diffusion Method

The CLSI has proposed disc diffusion methods for screening of ESBL production by *klebsiella* spp and *E. coli*. The possible ESBL production can be detected by noting specific zone diameters that indicate a high level of suspicion for ESBL production. Cefpodoxime, ceftriaxone, cefotaxime, ceftazidime or aztreonam can be used for screening improves the sensitivity of detection (CLSI 2013).

2.7.1.2 Screening by dilution antimicrobial susceptibility test

The CLSI has proposed dilution methods for screening ESBL production by *Klebsiella* spp and *E.coli*. Ceftazidime, cefotaxime, ceftriaxone or aztreonam can be used for screening at concentration of 1µg/ml (CLSI 2013).

The isolates should be considered as potential ESBL producer if the test result is as follows:

Disc diffusion

Cefpodoxime (10mcg) ≤17mm

Ceftazidime (30mcg) ≤22mm

Aztreonam (30mcg) ≤27mm

Cefotaxime (30mcg) ≤27mm

Ceftriaxone (30mcg) ≤25mm

(CLSI 2013)

The ideal indicator cephalosporin is one to which all ESBLs confer resistance, even when their production is scanty. Choice is predicted by the following general traits; TEM and SHV ESBLs obvious resistance to ceftazidime, variable to cefotaxime. CTX-M ESBLs obvious resistance to cefpodixime. All ESBLs obvious resistance to cefpodoxime. Thus, the logical indicator is either cefpodoxime or both of cefotaxime and ceftazidime resistance (HPA 2005).

2.7.2 Phenotypic Confirmatory test for ESBL production

The CLSI has recommended phenotypic confirmatory test for the suspected ESBL producers. Several phenotypic confirmatory tests are:

Cephalosporins/ clavulanate combination Disc: CLSI advocates the use of cefotaxime (30 µg), ceftazidime (30µg) disks with or without clavulanate (10 µg) or cefpodoxime (10 µg) plus clavulanate (10 µg) for phenotypic confirmation of the presence of ESBL. A difference of ≥5mm between the zone diameters of either of the cephalosporin discs and their respective cephalosporin/clavulanate disc is taken to be phenotypic confirmation of ESBL production.

Double Disc Synergy Test: This test incorporate the use of cefotaxime (30 µg) and ceftazidime (30 µg) disks which are placed on either side of Coamoxiclav (20+10 µg) at a distance of 20-30 mm onto a Mueller Hinton Agar plate already inoculated with test organism. ESBL production is inferred when the zone of either cephalosporin is expanded by the clavulanate. Disc spacing should be highly considered (Livermore and Woodford 2004).

2.8 Risk factors for colonization and infections with ESBL-producing Enterobacteriaceae

Studies have identified risk factors for acquiring and being infected with ESBL-producing Enterobacteriaceae and most of them have focused on risk factors in health care but there are some important risk factors in the community as well (Valverde et al 2008). Transmission of such bacteria is mainly due to the faecal oral route, either directly or indirectly through hand contact with healthcare workers which further spreads by overcrowding. The

prior use of antibiotics, particularly quinolones and third-generation cephalosporins, but also co-trimoxazole, aminoglycoside, and metronidazole is also another risk factors mentioned in the literature including the prolonged hospital stays, living in nursing homes or long-term care facilities, underlying medical conditions, recent surgery and haemodialysis (Paterson and Bonomo 2005) (Lytsy et al 2008) (Colodner et al 2004). In the case of disseminating *Klebsiella* spp is more as compared to *E.coli*. A study done by Harris et al (2007) seemed that there was more extensive transmission of *K. pneumoniae* despite a larger reservoir of *E. coli* at ICU admission. In another study done by research group (Tham et al 2010) and other investigators (Tangden et al 2010) have found evidence that international travel to highly endemic areas (i.e. Asia, the Middle East, and Africa) represents one of the most important risk factors for ESBL carriage especially in the community.

2.9 Prevention

All of the hospitals should adopt infection control procedures that aims to prevent the diseases caused by ESBL-producing bacteria and their plasmids. When ESBL-producing bacteria are isolated from inpatients those patients should be placed in single patient rooms or isolation depending on whether they are colonized or infected by organisms. Some precautions should be followed like:

- A limited number of staff treating the patient
- Protective clothing
- Well organized bio-wastes
- Protective clothing should be removed before leaving the ward
- Washing, drying and sanitizing hands before leaving the ward

2.10 Treatment of patients infected with Enterobacteriaceae producing ESBLs

Due to the progressive increase of extended spectrum β-lactamasae (ESBL) producing enteric bacteria, it has called for a re-evaluation of current antibiotic therapy for these infections (Garau 2008). Since, ESBLs are clinically

significant, the patients infected with ESBL producing Enterobacteriaceae experiences a greater likelihood of poor outcome when treated with inappropriate antimicrobial agents. Problem that arises when treating patients with these infections is that the plasmids carrying the ESBL gene often have additional mechanisms that gives rise to co-resistance to many other antibiotics (Rupp and Fey 2003). The clinical efficacy of the treatment does not always reflect the in vitro susceptibility to antibiotics. The first choice for treatment of patients infected with ESBL producing Enterobacteriaceae especially in cases involving severe septicemia or septic shock are Carbapenem (such as imipenem, meropenem etc.) (Paterson 2000). An alternative to carbapenem can be cefepime plus β-lactamase inhibitor as the activity of cefepime plus tazobactam was significantly better compared with that of cefoperazone+sulfbactam and piperacillin+tazobactam (Sharma et al 2012). However, the emergence of CTX-M-15 producing bacteria also regularly leads to production of OXA-1-β-lactamase, which is alarming and renders the β –lactamase inhibitor ineffective (Rodriguez et al 2011). Cefepime, which is often referred to as fourth-generation cephalosporin, an oxyimino β-lactam with an amino thiazolyl side chain is active against most ESBL producing organisms, particularly those with SHV derived enzymes. But treatment failure have been observed in some cases, therefore, until more clinical data is available, clinicians should not regard cefepime as a first line therapy for ESBL producing organisms and if used, it should be given a high dose (≥2 g every 12 hours) usually in combination with other active agents (aminoglycosides, fluoroquinolones) (Rupp and Fey 2003). An alternative drug of choice for patients infected with ESBL producing bacteria can be Tigecycline, a new semisynthetic glycylcycline (Ku et al 2008; Nicasio et al 2009).

2.10 Global epidemiology of ESBLs

The epidemiology of ESBLs is quite complex. There are several different levels to consider: the wider geographical area, the country, the hospital, the community and the host. In Europe, ESBLs were first described in 1983 from Germany and England (Shaikh 2015). ESBLs have been reported from all

parts of the world. However, very limited data have been collected regarding the prevalence of these microbes over time in the developing countries and some other parts of the world as very few investigations have examined on this aspect. Nonetheless, a number of studies have been published in recent years that have given a somewhat clearer picture of the situation.

The European Antimicrobial Resistance Surveillance System (EARSS) showed 2.6% of *E. coli* and 1.7% of *K. pneumoniae* strains in Sweden were resistant to third-generation cephalosporins in 2010 (Denisuik et al 2013). Most ESBLs were found in *K. pneumoniae* in nosocomial outbreak situations, mainly in intensive care units (ICUs) and they were primarily SHV and TEM enzymes until the late 1990s. New TEM and the SHV enzymes are still evolving in Europe and specific epidemic clones have been found, for example *Salmonella* isolates with TEM-52 in Spain (Fernandez et al 2006) and *E. coli* and *K. pneumoniae* isolates with SHV-12 in Italy (Perilli et al 2011). A study performed in Turkey showed prevalence of 21% ESBL producer among *E.coli* causing community acquired UTI during 2004 and 2005. This percentage is seen higher than the 5.2% observed in Spanish multicenter study 2006 (Coque et al 2008).

A few investigations have been conducted in sub-Saharan Africa. In Tanzania, in 2001-2002 first study of ESBLs was performed and analyzed blood isolates from neonates and it was found that 25% of the *E.coli* and 17% of the *K. pneumoniae* produced ESBLs, mainly the CTX-M-15 and TEM-63 types (Blomberg et al 2005). In Mwanza, Tanzania, the prevalence of ESBLs in all Gram-negative bacteria (377 clinical isolates) in a more recent investigation was 29%. The ESBL prevalence was 64% in *K. pneumoniae* and 24% in *E.coli* (Mshana et al 2009). A study from Saudi Arabia in 2008, it was found that 26% of *K. pneumoniae* isolates produced ESBLs, the majority of which were SHV-12 and TEM-1 enzymes and 36% were CTX-M-15 (Tawfik et al 2011).

In Asian countries like China, 27% of *E. coli* and *K. pneumoniae* were identified to be ESBL producers (Du et al 2002). It is estimated that 5% to 8% of *E.coli* isolates from Korea, Japan, Malaysia and Singapore were positive for ESBL while it was 12% to 24% in Thailand, Taiwan, The Philippines and

Indonesia (Ensor et al 2006). It was noted that the 25% of the Enterobacteriaceae in Thailand were producing ESBL (Hawkwy 2008). Investigation from India and Pakistan shows alarming and rapid increase in the prevalence of Enterobacteriaceae rate from 6.9% in a hospital Varanasi, India, to 18.5% in Raawalpindi, Pakistan (Nordmann et al 2011).

K. pneumoniae isolates with ESBL genotype were more prevalent in Latin America 45.4% followed by western pacific region 24.6%, Europe 22.6%, the United State 7.6% and Canada 4.9% (Winokur et al 2001). Amongst the world, the highest prevalence of ESBL producing *K. pneumoniae* is seen primarily in Latin America, where approximately 50% of the bacterial isolates harbor ESBLs and 8% to 18% for *E. coli* (Winokur et al 2001; Sader et al 1999). At a cancer centre in Texas, the studies revealed that 9% of *E. coli* isolated in 2009 produced ESBLs (Bhusal et al 2011). The prevalence of ESBLs in Europe is higher than in USA but lower than in Asia and South America (Girlich et al 2004).

The study conducted in Khyber Teaching Hospital, Peshawar, Pakistan found that *E. coli* was the major isolates 33.9% from the urine specimens and females were more susceptible to UTI as compared to males. The most effective antibiotic was imipenem with 98.3% of the isolates susceptible and followed by meropenem with 97.4% of the isolates being susceptible. Among cephalosporins, 62% resistance was recorded to cefotaxime, 65% to cefaclor and ceftazidime both and 72% to cephradine while the highest resistance was recorded to penicillin group (ampicillin) being 89%. In total 83% isolates were multi drug resistant (MDR), that is, resistant to at least 3 or more drug classes and all of these were ESBL positive. The most prevalent MDR pattern was resistance to β-lactams, doxycycline, flouroquinolones and co-trimoxazole. In this study, 56.9% *E. coli* were found to be ESBL producers, 71.2% isolated from females and 28.8% from male patients. Prevalence of ESBL was almost similar in medical and gynaecology wards being 58 and 58.3% respectively while it was 52% in children ward (Ullah et al 2009).

A report from North India on uropathogens such as *K. pneumoniae, E.coli*, *Enterobacter, Proteus* and *Citrobacter* spp, showed that 26.6% of the isolates

were ESBL producers. Similarly, a study from Nagpur showed 48.3% were ESBL producers and other report from India showed 41% in *E.coli* and 40% in *K. pneumoniae* showed ESBL positive (Umadevi et al 2011).

2.11 Nepalese scenario

There were a limited number of studies on prevalence of ESBL showing a high rate in Nepal, where Enterobacteriaceae were 28% to 67% (Hammer et al 2007) and at tertiary care hospital of Eastern Nepal it was found to be 14.8% in *Klebsiella* spp followed by *Proteus* spp 12.9%, *E. coli* 53.7% (Shrestha et al 2011). According to the study conducted by Chaudhary et al 2014, out of a total 1986 specimens investigated, *E. coli* was isolated in 309 (83.9%) and *K. pneumoniae* in 38 (10.3%) cases among which 7 (18.4%) *K. pneumoniae* isolates were ESBL producers.

Similarly, a study reported, out of 977 clinical specimens, 254 (35.99%) were found to be gram negative bacterial isolates, among them *K. pneumoniae* 83(32.67%) was the most predominant organism followed by *E.coli* 51 (20.07%), *Pseudomonas aeruginosa* 36 (14.17%), *K. oxytoca* 32 (12.59%), *Proteus mirabilis* 13 (5.11%) and *P. vulgaris* 13 (5.11%), *Acinetobacter* spp 11 (4.33%), *Citrobacter* spp 10 (3.93%) and *Enterobacter* spp 5 (1.96%) respectively. 83 (32.67%) isolates were found to be MDR, 38(14.96%) were positive for ESBL (Pramila et al). In a study conducted at Tribhuvan University Teaching Hospital (TUTH), 68.3% of the urinary isolates were MDR with 12 urinary isolates demonstrating ESBL activity (Bomjan 2005) and 27.5% ESBL producing *E. coli* and *Klebsiella* spp was found in 2006 (Manandhar et al 2006). The similar study conducted at TUTH, 60.4% of urinary isolates were MDR strains among which 16% of the isolates were ESBL producers (Pokharel et al 2006).

The study conducted at National Public Health Laboratory (NPHL) by Thakur et al (2013), in the urinary isolates 31.6% were ESBL producing *E. coli* among the Enterobacteriaceae family. The similar study at National Institute of Neurological and Allied Science, Kathmandu by Khanal et al (2013) concluded that out of 146 tracheal aspirates, ESBL producer was confirmed in

25% of the isolates, among which the major microbes were *Pseudomonas* spp (42.8%) and *K. pneumoniae* (34.3%). The necessity of investigation of the prevalence of ESBL positive strains in a hospital helps to formulate a policy of empirical therapy in high-risk units where infections due to resistant microbes are much higher (Mathur et al 2002).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

The materials, equipment and various reagents used in different stages of this study are listed in Appendix A.

3.2 Study design and sites

A cross sectional study was conducted from June to November, 2019 in Apex Hospital, Itahari and Microbiology Laboratory of Central Campus of Technology, Dharan for further analysis of the sample. The study site was the pathological department in Apex Hospital, Itahari. It is a 25 bed hospital in Itahari town and is a secondary care hospital with outpatients and inpatients facilities.

3.3 Study population

The study populations were the patients coming for routine culture and antibiotic susceptibility testing to Apex Hospital and Research Centre in Itahari. All the patients including all age group and both sexes suspected of UTIs were screened.

3.3.1 Inclusion criteria

All the patients attending for routine culture in the hospital in that region between June to November 2019.

3.3.2 Exclusion criteria

Patients suspected with any symptoms other than UTI and patients with any intake of antibiotics for UTI within 7 days and were excluded.

3.4 Sample size

The sample size is estimated based on 23.68% prevalence rate according to the study done by Rimal et al 2016 in Maharajgunj, Kathmandu, Nepal and the sample size was calculated by using Fisher's formulae:

$$N = \frac{Z^2 P(1-P)}{D^2}$$

$= (1.96)^2 * 0.2368 * 0.77/(0.05)^2$

$= 3.8416 * 0.182336/0.0025$

$=280.18 \sim 280$ (Required number of sample size)

Where, N= Sample size

Z= level of significance

P= probability of outcome

D= deviation error

350 urine samples was taken in this study.

3.5 Specimen collection and transportation

Three hundred and fifty mid-stream urine samples were collected from all the patients who came for routine culture to Apex Hospital, Itahari. Mid-stream urine (Appendix B) was collected by the patients with full aseptic precautions in a sterile, dry, wide mouthed, leak proof container. The sample labeled properly with demographic information of patients such as name, age, sex, hospital number, date and time of collection of specimen was accepted, otherwise a second sample was requested. Some of the specimens were transported to Microbiology Laboratory of Central Campus of Technology, Dharan for further processing within one hour.

3.6 Processing of specimen

3.6.1 Macroscopic examination of specimens

Macroscopic examination of urine samples collected was conducted by observing for its color, turbidity, appearance and pH and reported accordingly (Collee et al 2001).

3.6.2 Culture of specimens

The urine samples were cultured on Mac-Conkey Agar and 5% Blood Agar plates by semi-quantitative culture technique using the standard loop. The protocol was followed as recommended by WHO (Vandipitte et al 2003). An inoculating loop of standard dimension was used to take up approximately fixed and a known volume (0.001ml) of mixed urine for inoculation. The plates were incubated at 37°C overnight. Colony count was performed to calculate the number of colony forming unit (CFU) per millimeter (ml) of urine and the bacterial count was reported. If the cultured indicates presence of two uropathogens both showing significant growth, definitive identification and antimicrobial susceptibility testing of both were performed whereas in the case of ≥ 3 pathogens, it was reported as multiple bacterial morphotypes and asked for appropriate recollection with timely delivery to laboratory (Isenberg 2004).

3.6.3 Identification of the isolates

Identification of bacterial isolates was done using standard microbiological techniques as described in Bergey's Manual of Systematic Bacteriology which comprises of studying the colonial morphology, staining reactions and various biochemical properties. Isolated colonies from the pure culture were identified by standard conventional biochemical tests and identified accordingly. The biochemical media employed were Sulphide Indole Motility (SIM) media, MR-VP broth, Simmon's citrate media, Triple Sugar Iron Agar (TSI), Christensen's urease media and Fermentative media.

3.6.4 Purity plate

The purity plate was used to ensure that the inoculation used for the biochemical test was pure culture and to see whether the biochemical tests were performed in an aseptic condition or not. Thus, while performing biochemical tests, the same inoculum was sub cultured and incubated. The media were then checked for the appearance of pure growth of organisms.

3.6.5 Antimicrobial susceptibility testing

The antibiotic sensitivity tests of the pathogens isolated from the urine specimen against different antibiotics were determined by Kirby-Bauer method of disc diffusion technique as recommended by CLSI (2013) using Muller Hinton Agar (MHA). At least three to five well isolated colonies of the same morphological types were selected from the MA and BA plate. The tip of each colony was touched with an inoculating wire and the growth was transferred into a tube containing 5 ml of nutrient broth and was incubated at 37℃ (usually 2 to 6 hours) until it achieved turbidity that matched to the McFarland tube number 0.5. A sterile cotton swab was dipped into the broth and the swab was rotated on MHA plate. Then the antimicrobials discs were placed on the surface such that there was 25 mm distance from disc to disc. The commercial antibiotics used includes Amoxycilin (30µg), Ciprofloxacin (30µg), Gentamycin (10µg), Nitrofurantoin (300µg), Ceftazidime (30µg), Aztreonam (30 µg) and Ceftriaxone (30 µg), Imipenem (10µg) and Meropenem (10µg). For about 15 minutes of applying the discs, the plates were left at room temperature to allow antimicrobials to diffuse from the disc. Then they were incubated aerobically at 37℃ overnight. After overnight incubation, the diameter of zone of inhibition (ZOI) of each disc was measured (including the diameter of the disc) and recorded in millimeter. It was then compared with Standard Chart developed by Kirby-Bauer to determine bacterial susceptibility towards different antimicrobial agents in terms of ‘susceptibility’, ’resistant’ and ‘intermediately susceptible’. The measurements were made with the ruler on the under surface of the plate without opening the lid.

3.6.7 Tests for ESBL-production in strains of *E. coli* and *Klebsiella pneumoniae*

3.6.7.1 Screening of ESBL producer

Screening of the suspected ESBL isolates was performed according to the guidelines for screening issued by CLSI in 2014. The screening test for the production of ESBL was performed using both Ceftazidime (CAZ) (30µg) and

Ceftriaxone (CTR) (30µg) disks. If the zone of inhibition was less than or equal to 22mm for CAZ and/or less than or equal to 25mm for CTR, the isolate was considered a potential ESBL-producer as recommended by CLSI.

3.6.7.2 Confirmation of ESBL producer by phenotypic method

Isolates those were suspected as ESBL producer by screen test were tested further by Combination Disc Method (CD). Combination disc method was used for the confirmation of ESBL producing strains in which ceftazidime (30µg) and cefotaxime (30µg), each alone and in combination with clavulanic acid (CA) (10µg) were used. After incubating overnight at 37°C, ≥ 5mm increase in the zone diameter for either antimicrobial agent which was tested in combination with clavulanic acid versus its zone when tested alone, was interpreted as positive for ESBL production (CLSI 2013).

3.6.8 Analysis of MDR isolates

As antibiotic used for AST were from different class, resistance to more than two antibiotics were considered as MDR isolates (Huys et al 2005).

3.6.9 Antibiotic susceptibility testing of ESBL producer

The susceptibility of the ESBL producing strains of *E. coli* and *Klebsiella pneumoniae* to alternative drugs such as Imipenem, Meropenem was determined by the Kirby-Bauer disc diffusion method according to the Clinical Laboratory Standards Institute guidelines (CLSI 2013).

3.6.10 Quality control

3.6.10.1 Monitoring and regular evaluation of laboratory equipment, reagents and media

Laboratory equipments like incubator, refrigerator, autoclave and hot air oven were regularly monitored for their efficiency. The temperature of the incubator and refrigerator was monitored every day. Reagents and media were regularly monitored for their manufacture and expiry date and proper storage. After preparation, they were properly labeled with preparation date, expiry date. The

quality of media prepared was checked by incubating one plate of each lot for sterility (Cheesbrough 2000).

3.6.10.2 Quality control during antimicrobial susceptibility testing

Muller Hinton Agar and the antibiotics disc were checked for their lot number, manufacture and expiry date and proper storage. For the standardization of Kirby-Bauer test and for performance testing of antibiotics and MHA, control strains of *E.coli* ATCC 25922 *K. pneumoniae* ATCC 700603 were tested primarily. Quality of sensitivity test was maintained by maintaining the thickness of Muller-Hinton Agar at 4mm and the pH at 7.2-7.4.

3.6.11 Data analysis and entry

All the data collected were analyzed using Statistical Software SPSS version 22.0. Descriptive analysis was done. Chi-square test was used to determine association of variables. A P-value of less than or equal to 0.05 was considered to be statistically significant ($p \leq 0.05$).

CHAPTER IV

RESULTS

The study was carried out at the Microbiology Lab of Central Campus of Technology, Hattisar to determine the status of MDR and ESBL producing *E. coli* and *Klebsiella pneumoniae* isolated from the urine sample, from patients suspected of urinary tract infections (UTI). 350 mid-stream urine samples were collected from the patients complaining of urinary tract infection.

A total of 350 urine samples who fulfil the inclusion criteria were screened for the study population. 113 (32.28%) were from male population and 237(67.72%) were from female population. Among the population, the most studied cases were obtained from age group (21-30) years with the male 34% and female 32% followed by age group (31-40) years with the female 23% and male 22%.

4.1 Growth profile of bacteria isolated in urine samples

Out of 350 urine samples processed, 85 (24%) showed significant growth while rest of the samples 265(76%) showed no growth (Fig: 1).

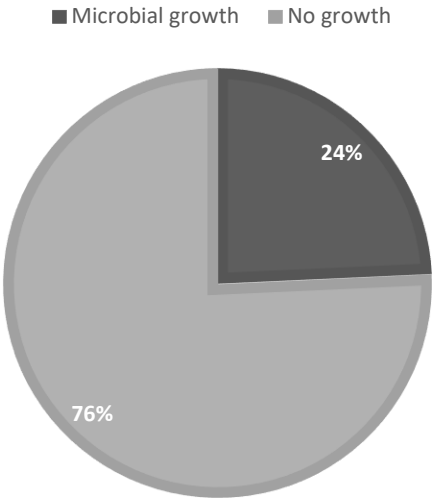


Fig 1: Microbial growth profile in urine samples

4.2. a. Growth pattern of *E.coli* and *K.pneumoniae* among inpatients and outpatients

Among the total 350 mid-stream urine samples, 277 (79.14%) were from outpatients and 73 (20.86%) were from inpatients. Among 277 outpatients 64 (23.10%) showed significant growth, while among 73 inpatients 21 (28.76%) showed significant growth (Fig: 2). The difference in growth is statistically insignificant among inpatient and outpatients (P>0.05).

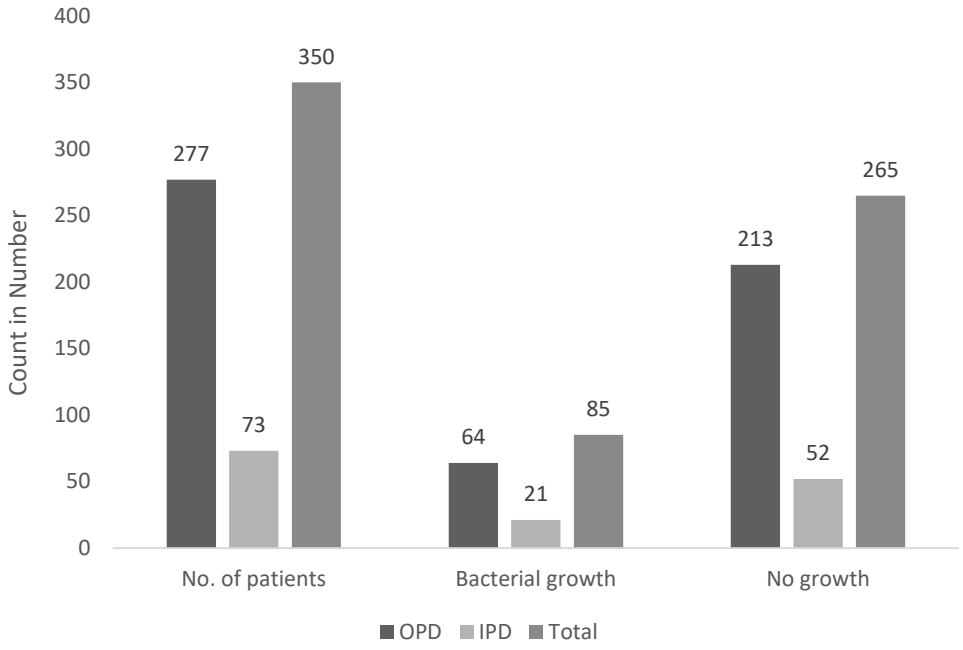


Fig 2: Growth pattern of *E.coli* and *K. pneumoniae* among inpatients and outpatients

4.2. b. Gender wise distribution of patient and growth pattern

Among the total 350 mid-stream urine samples 113(32.28%) were from male patients and 237 (67.71%) were from female patients. Out of 113 male patients, 16 (14.15%) and out of 237 female patients, 69 (29.11%) showed significant growth (Fig 4). The association of growth between male and female patient is found statistically significant (P<0.05).

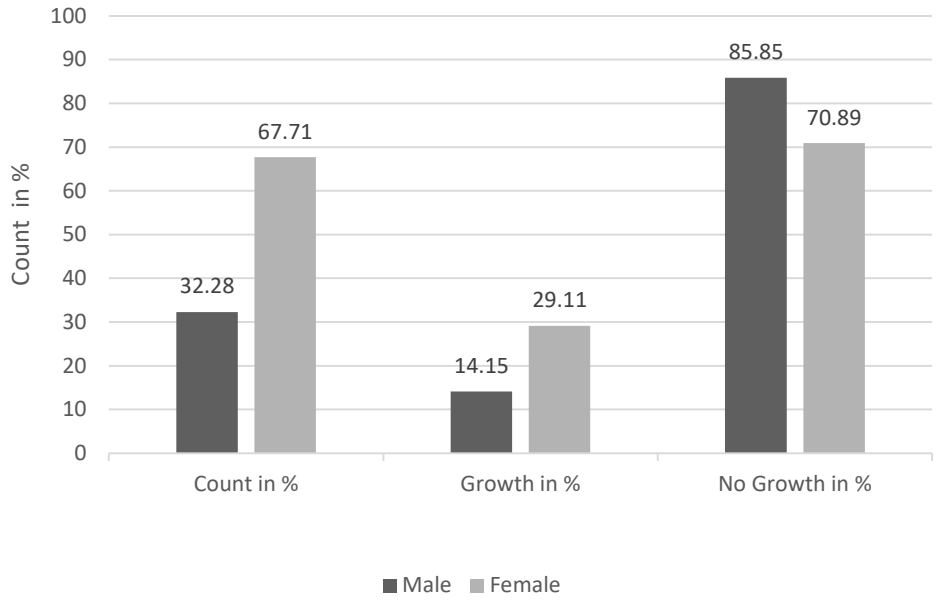


Fig 3: Gender wise distribution of patient and growth pattern

4.3 Age wise distribution of the growth positivity

Among the 85 significant bacteriuria cases, the highest percentage of bacteriuria were obtained from age group (21-30) years (31.76%) followed by age group (31-40) years (22.35%) and (41-50) years (14.11%) (Table 1). There is significant association ($P<0.05$) between age group of patients and growth of bacteriuria.

Table 1: Growth positivity in relation to age of patients

Age of patients in years	Growth		No growth		Total
	Number	%	Number	%	Number
0-10	2	2.35	6	2.26	8
11-20	10	11.76	26	9.81	36
21-30	27	31.76	86	32.45	113
31-40	19	22.35	61	23.01	80
41-50	12	14.11	39	14.71	51
51-60	6	7.05	20	7.54	26
61-70	4	4.7	12	4.52	16
71-80	3	3.5	8	3.01	11
81-90	2	2.35	7	2.64	9
Total	85	24.28	265	75.72	350

4.4 Microbiological profiling of urine isolates

Out of 85 bacterial isolates from mid-stream urine samples in this study, 2 different genera of Enterobacteriaceae were isolated (Table 2). Among the bacterial isolates, *E.coli* (72.94%) was found to be most predominant organism and then *Klebsiella pneumoniae* (27.06%) was least in number.

Table 2: Pattern of Enterobacteriaceae isolates causing UTI

S.N.	Bacterial isolates	Number	Percentage (%)
1	*Escherichia coli*	62	72.94
2	*K. pneumoniae*	23	27.06
	Total	85	100

4.5 Antibiotic susceptibility pattern of uropathogens

4.5.1 Antimicrobial susceptibility pattern of *E.coli*

The antibiotic susceptibility pattern of *E. coli* (n=62) revealed that sensitivity was seen for aztreonam (75.8%) followed by nitrofurantoin (56.45%). The resistance was seen against amoxicillin (69.6%), followed by ceftriaxone (51.61%). (Table 3).

Table 3: Antimicrobial susceptibility pattern of *E.coli*

Antibiotics	Susceptibility pattern (n=62)					
	Sensitivity		Intermediate		Resistance	
	No	%	No	%	No	%
Amoxycillin	5	8.06	14	22.58	43	69.35
Aztreonam	40	75.8	9	14.51	13	9.67
Ciprofloxacin	19	30.64	14	22.58	29	46.77
Ceftazidime	20	32.25	18	29.03	24	38.7
Gentamycin	29	46.77	13	20.96	20	32.25
Nitrofurantoin	35	56.45	11	17.74	16	25.8
Ceftriaxone	16	25.8	14	22.58	32	51.61

4.5.2 Antimicrobial susceptibility pattern of *Klebsiella pneumoniae*

The antibiotic susceptibility pattern of *K. pneumoniae* (n=23) revealed that sensitivity was seen for gentamycin (95.65%) followed by ciprofloxacin (82.6%). The resistance was seen against amoxicillin (91.3%), followed by ceftazidime (56.52%) (Table 4).

Table 4 : Antimicrobial susceptibility pattern of *Klebsiella pneumoniae*

Antibiotics	Susceptibility pattern (n=23)					
	Sensitivity		Intermediate		Resistance	
	No	%	No	%	No	%
Amoxycillin	1	4.34	1	4.34	21	91.3
Aztreonam	9	39.13	3	13.04	11	47.82
Ciprofloxacin	19	82.6	1	4.34	3	13.04
Ceftazidime	6	26.08	4	17.39	13	56.52
Gentamycin	22	95.65	0	0	1	4.34
Nitrofurantoin	10	43.47	2	8.69	11	47.82
Ceftriaxone	8	34.78	3	13.04	12	52.17

4.6 Profile of MDR among uropathogens

Among 85 isolates, 53(62.35%) were found to be MDR in which maximum MDR was found in *K. pneumoniae* (69.5 %), followed by *E.coli* (59.6%) (Table 5).

Table 5: Pattern of MDR among uropathogens

S.N	Isolated organisms	Multi drug resistant				Total
		Yes		No		
		No.	%	No.	%	
1	*E.coli*	37	59.6	25	40.3	62
2	*K. pneumoniae*	16	69.5	7	30.4	23
	Total	53	62.35	32	37.6	85

4.7 Prevalence of ESBL producing uropathogens

Among 85 isolates, 15 bacteria were found to be ESBL producer. The prevalence of ESBL production in *E.coli* (20.9%), *K. pneumoniae* (8.69%) (Table 6).

Table 6: Prevalence of ESBL producing uropathogens

S.N.	Isolated organisms	ESBL production				Total
		Yes		No		
		No.	%	No.	%	
1	*E.coli*	13	20.9	49	79	62
2	*K. pneumoniae*	2	8.6	20	86.9	23
	Total	15	17.64	69	81.17	85

4.8 Association between MDR and ESBL production

Among 85 isolates of bacteria, 15 were ESBL producer and 53 were MDR positive. There is significant association between MDR and ESBL production at ($P<0.05$) (Table 7).

Table 7: Association between MDR and ESBL production

	MDR			
ESBL	**Yes (%)**	**No (%)**	**Total**	**p-value**
Positive	15(28.3)	-	15	
Negative	38(71.7)	32(60.67)	70	0.001
Total	53(100)	32(60.67)	85	

4.9 Antimicrobial susceptibility of ESBL producing organism

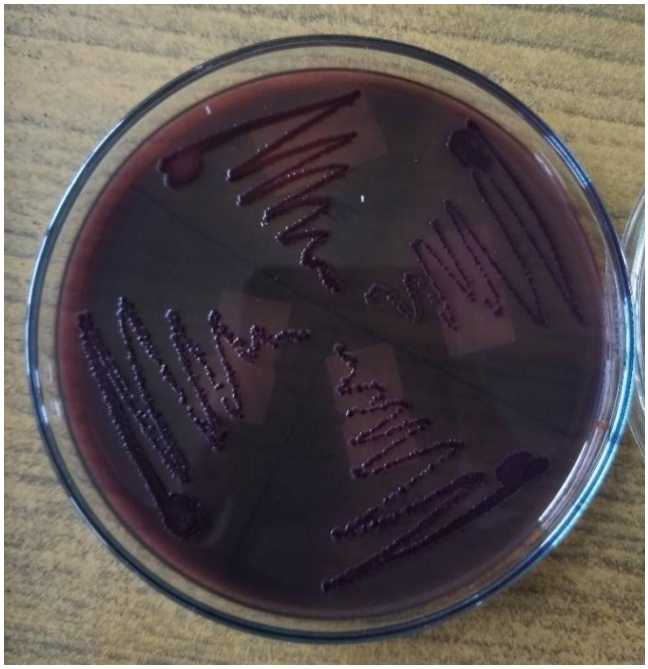
Fiveteen bacterial isolates were ESBL producers. All 13 ESBL producing isolates of *E. coli* were sensitive to Imipenem. 92.3% and 84.6% were resistant to Ceftriaxone and Ceftazidime, respectively. Similarly, 69.2% of *E. coli* were resistant to both Ciprofloxacin and Cotrimoxazole. Furthermore, 30.7% of *E. coli* were resistant to Meropenem. All of the 2 ESBL producing *K. pneumoniae* isolates were sensitive to Imipenem, Meropenem. All of the isolates were resistant to Amoxycillin (Table 8).

Table 8: Antimicrobial susceptibility of ESBL producing organism

Antibiotics	***E.coli*(n=13)**			
	Sensitive No.	**Sensitive %**	**Resistance No.**	**Resistance %**
Amoxycilin	-	-	13	100
Ciprofloxacin	4	30.7	9	69.2
Nitrofurantoin	12	92.3	1	7.6
Gentamycin	8	61.5	5	38.4
Ceftazidime	2	15.3	11	84.6
Ceftriaxone	1	7.6	12	92.3
Cotrimoxazole	4	30.7	9	69.2
Imipenam	13	100	-	-
Meropenam	9	69.2	4	30.7

PHOTOGRAPHS

Photograph 1: *Klebsiella pneumoniae* on Mac Conkey Agar (pink mucoid colony, lactose fermentor)

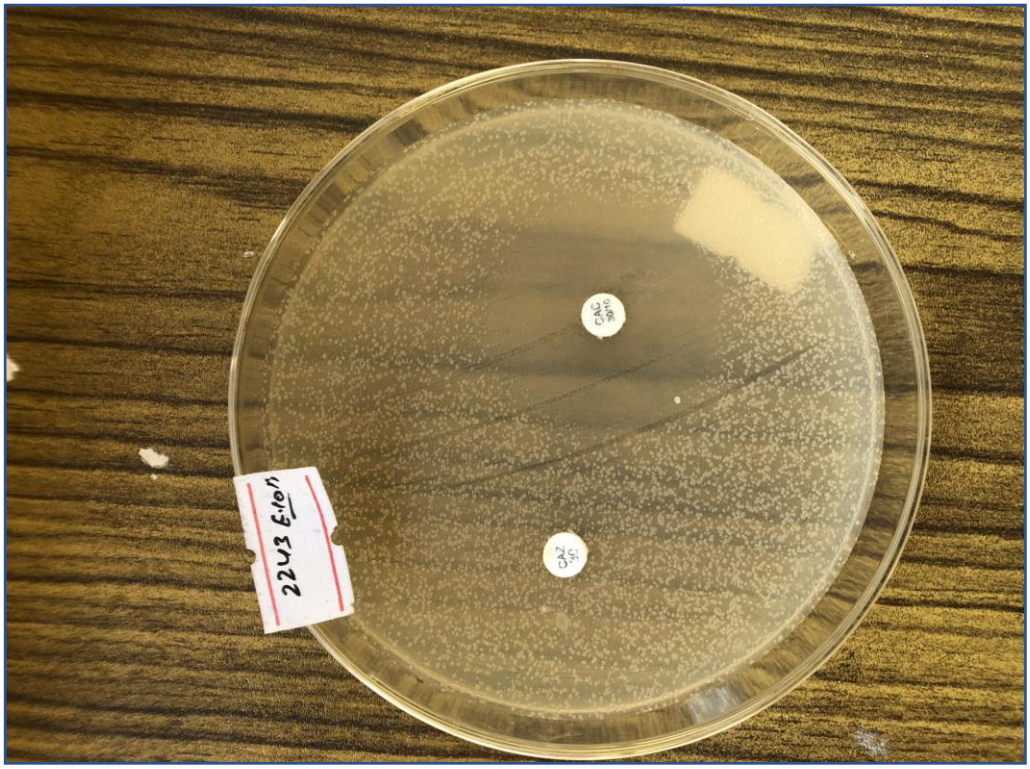


Photograph 2: *Klebsiella pneumoniae* on EMB Agar (pink to purple colonies without metallic green sheen)

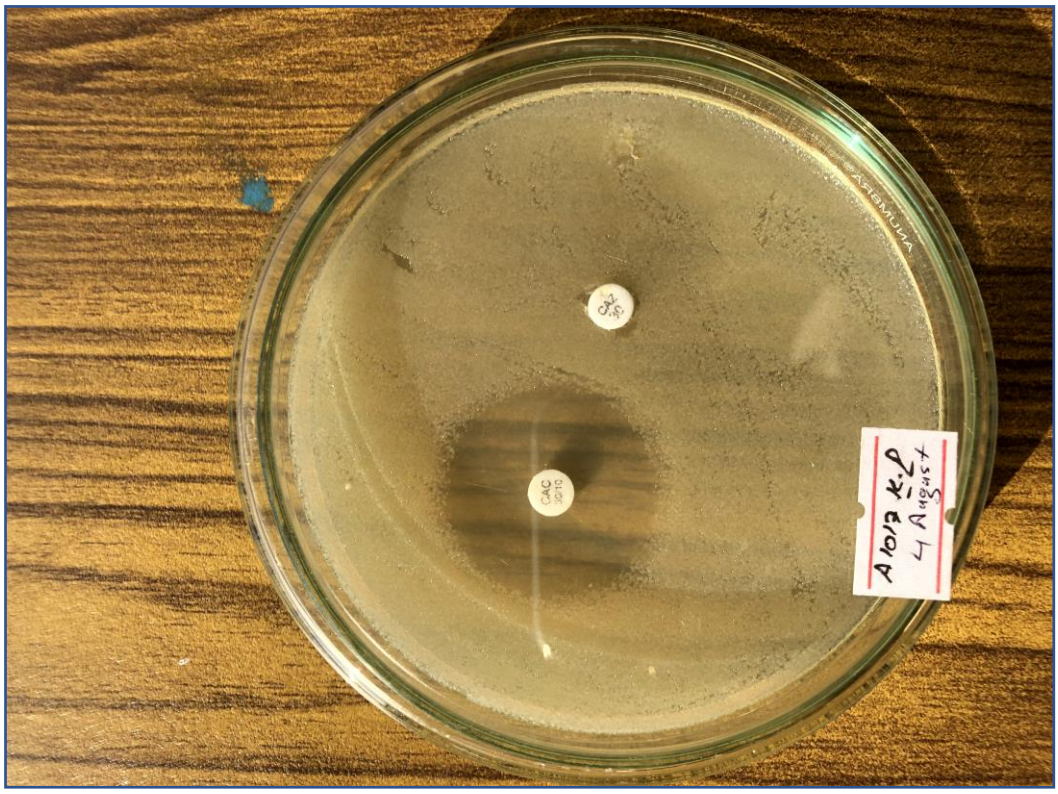
Photograph 3: Citrate Utilization Test (+ve for *Klebsiella pneumoniae*, -ve for *Escherichia coli*)

Photograph 4: Biochemical test for *K.pneumoniae*

1: Indole^{-ve}, 2 :MR^{-ve}, 3: Citrate^{+ve}, 4: Urease^{+ve} 5: Urease^{-ve}(*E.coli*), 6:SIM(S^{-ve}, I^{-ve}, Non-motile)



Photograph 5: ESBL positive *E. coli*, zone diameter 22mm for CAC and no zone of inhibition for CAZ alone



Photograph 6: ESBL positive *K. pneumonae*, zone diameter 22mm for CAC and no zone of inhibition for CAZ alone.

CHAPTER V
DISCUSSION

Urinary tract infection is a serious health problem that is affecting millions of people each year. Recently, antibiotics have been used extensively and newer antibiotics are continuously being added for the treatment of UTI. A major problem has created leading to increased morbidity, mortality and health care costs due to the extensive use of β-lactam antibiotics in hospital and community. The use of proper antibiotics is very important for various reasons. The emergence of multidrug resistant isolates and rapid spread are of great concern worldwide; among them, ESBL producing Enterobacteriaceae has been major concern. During the past decades, ESBLs producing Gram-negative bacilli especially *E.coli and K.pneumoniae* have emerged as serious pathogens both in hospital and community acquired infections worldwide. This study was conducted to isolate *E.coli* and *K.pneumoniae* causing UTI and determining the status of MDR and ESBL producing uropathogens from the patients suspected of urinary tract infection visiting Apex Hospital, Itahari.

This study enrolled a total of 350 mid-stream urine samples who fulfil the inclusion criteria were screened for the study population. In analyzing my study, 85 (24.28%) showed significant growth out of the screened population. Similar results were obtained by Awasthi et al (2015), Sharma et al (2013) and Paudel et al (2013) showed with the percentage of growth positivity of 23.87%, 25.5%, 27.3% and 29.9% respectively. Similarly, the study carried out in India by Niranjan et al (2014) obtained 18.5% significant growth. However, our result is low as compared to that reported from South Africa (51%) by Habte et al (2009).

The majority of urine specimen showed no growth (75.72%). The possible cause of low rate might be due to relatively small sample size and differences in the study population. It might also be due to urine sample obtained from patients were on antibiotic therapy.

The samples from outpatient department were 277 which was more as compared to hospital admitted patient samples 73. In the study, 64 of samples from outdoor patients and 21 of samples from indoor patients showed significant bacterial growth. This signifies more prevalence of UTI in community.

In this study, higher rate of infections was found in female patients 69/237 (29.11%) and the rate of infections was found to be 16/113 (14.15%) in male which was statistical significant difference between them ($P<0.05$). This result confirms and expands the previous finding of Shakya et al (2017), Chander and Shrestha (2013), Chhetri et al (2001), Jha and Bapat (2005) and Rajbhandari and Shrestha (2002), Yadav et al (2015) in Nepal. Significant microbial growth was higher in case of female. The patient's sex is risk factor of UTI. Even though, everyone is susceptible to UTI, there are specific subpopulations that are at increased risk of UTI, including infants, pregnant women, and elderly patients with catheters, patients with diabetes, multiple sclerosis or acquired immunodeficiency syndrome (AIDS)/ human immunodeficiency virus (HIV) and patients with underlying urologic abnormalities. Females are more frequently affected by (particularly cystitis) due to colonization of urethra with colonic Gram-negative bacteria because of its proximity to anus and short length of urethra Forbes et al (2007).

In this study, the highest percentages of growth were obtained from age group (21-30) years (31.76%) followed by age group (31-40) years (22.35%) and age group (41-50) years (14.11%). The highest percentage in some group was neglected because of relatively small sample size. There was significant association ($P<0.05$) between age group of patients and bacterial uropathogens. This study revealed a higher occurrence of uropathogens in the adult age group of 21-30 years, which is similar to that reported in a study done by Kattel et al (2012). The female may be the reason behind the maximum growth in these age groups because this age group consist sexually active women, frequent or recent sexual activity. The most important risk

factor for UTIs in young women is frequent or recent sexual activity (Yadav et al 2015). Nearly 80% of all UTIs in premenopausal women occur within 24 hour of intercourse. In celibate women, UTIs are rare. The risk of UTI can also be increased by the use of certain types of contraceptives (Yadav et al 2015). Furthermore, the use of spermicidal coated condoms dramatically alters the normal bacterial flora and has been associated with increase in vaginal colonization with *E.coli* and in the risk of UTI (Braunwald et al 2001).

The predominant pathogens of the UTI were Enterobacteriaceae. All together 85 bacterial isolates of two different genera were isolated. *E.coli* (72.95%) was the most common uropathogens isolated followed by *Klebsiella pneumoniae* (27.06%). Higher prevalence of *E.coli* seen in this study also resembled the study done by different other researchers viz. Singh et al (2015), Bawankar et al (2015), Baral et al (2012), Manges et al (2006) and Khanfar et al (2009). From all the above study, *E.coli* was the major pathogen concerned with UTI. As *E.coli* is a common pathogen which is usually a commensal bacterium of humans. Intestinal and extra-intestinal infections, including gastroenteritis, urinary tract infection, meningitis, peritonitis, and septicemia are caused by pathogenic variants (Von and Marre 2005). *E.coli* is a predominant isolate, because *E.coli* can bind to the glycol-conjugate receptor of the uroepithelial cells of human urinary tract so it can initiate infection itself. *E.coli* is isolated in 90% of infection and strains are characterized by presence of unique virulence determinant the pilus (Gal-Gal) receptor Yadav et al (2015). *E. coli*, including other enterobacteria, are likely to have caused infection after colonization of the periurethral area by gastrointestinal tract flora LK and DA (2016). Also, the fact that strain of *E.coli* affecting the Urinary tract possess a variety of virulence characteristics that facilitate their intestinal carriage, persistence in vagina and then ascension and invasion of the anatomically normal urinary tract (Annabelle et al 1999).

Antimicrobial resistance is now accepted as a major problem in public health and patient care. It is more troublesome to developing countries. In this study, *E.coli* was highly resistant to amoxicillin (69.6%) and least resistant to aztreonam (9.6%). The high sensitivity rate was seen towards aztreonam

(75.8%) which was followed by nitrofurantoin (56.4%). This is similar to the finding by Shakya et al (2017) were resistance towards amoxicillin was found to be 73.3%. In case of *Klebsiella pneumoniae* the maximum resistance was seen against amoxicillin (91.3%), followed by ceftazidime (56.52%) and Gentamycin was found to be effective drugs with the sensitivity of (95.65%), followed by ciprofloxacin (82.6%). A report by Chandar and Shrestha (2013) showed among the gram-negative bacteria, highest percentage of resistance towards first line antibiotics was found for Amoxycilin. Some of the isolates were also found resistant to third generation cephalosporin i.e, cefotaxime. Also, some isolates were found resistance to Cotrimoxazole, Ciprofloxacin. The study conducted by Perez et al accounted 94% *E.coli* isolates to be resistant to ceftriaxone. This high rate of resistance may be due to the lack of antibiotic policy and the irrational use of third generation cephalosporins, mainly ceftriaxone in the hospital Shobha et al (2007). The cause of increasing resistance is due to outrageous and unnecessary use of antibiotics for non-therapeutic complaints and treatment of UTIs empirically (Mukherjee et al 2013). However, overall aztreonam, nitrofurantoin, ciprofloxacin, gentamycin showed a less resistance and can be considered as the first line therapy.

A major problem in the management of uropathogens is MDR. MDRs were classified as resistant to two or more antibiotics (CDC 2006). In this study, (62.3%) isolates were MDR. The result accords with other studies showing 55.9% (Poudyal et al 2011), 64% (Shakya et al 2017), and 64.9% (Parajuli et al 2017) of MDR isolates. The maximum MDR was found in *K.pneumoniae* (69.5 %), followed by *E.coli* (59.6%). The fact that drugs are easily available without doctor's prescription from pharmacy is the main reason behind the high degree of resistance. In developing countries like Nepal self-medication is a common practice and this too is a major cause of antibiotic resistance in clinical isolates. The development of resistance in clinical isolates is also promoted by expired antibiotics, self-medication counterfeit drugs, inadequate hospital control measures (Thakur et al 2013).

The leading cause of resistance to beta lactam antibiotics in Gram-negative bacilli is the production of beta lactamases. *E.coli* and *Klebsiella pneumoniae*

were subjected to phenotypic laboratory detection of ESBL production. Among 85 bacterial isolates tested for ESBL production, 15(29.5%) bacteria were found to be ESBL producer. The majority consisted of *E.coli* 13/15 (20.9%) followed by *K.pneumoniae* 2/15 (8.69%). The result was confirmed by combined disc approximation test in which 3rd generation cephalosporin were combined with β-lactamases inhibitor clavulanic acid (i.e. CTX30+Clav10 and CAZ30+Clav10) in which the structural analog of β-lactam antibiotics (clavulanic acid i.e. inhibitor) inhibits the action of β-lactamase and antibiotic can act on the cell wall of the bacteria, and the result confirmed by at least or more than 5 mm increase in zone of inhibition than cephalosporin alone (Rawal et al 2010).

The prevalence of ESBL producing Enterobacteriaceae varies greatly among country and among the hospitals within the country. Less than 1% to greater than 70% ESBLs producers are reported worldwide. In this study, the prevalence of ESBL production was higher in 21 - 30 years age group. The prevalence was higher in this age group as most isolates, accounting 53.84%, were isolated from this group. Moreover, self-medication practice which is high in this age group, could have further accounted for higher prevalence (Gyawali et al 2015; Shankar et al 2002). A higher prevalence of ESBL production was observed in *E.coli* (20.9%) followed by *K.pneumoniae* (8.69%). The findings are in agreement with the study by (Aminzadeh et al 2008), however, contrary to the findings of other study (Ullah et al 2009) of 365 *E.coli* isolates, 33 (9.0%) were ESBL producers. Likewise, of 23 *Klebsiella pneumoniae* isolates, 2 (8.69%) were ESBL producers. This accords with other studies (Thakur et al 2013; Chander et al 2013; Behrooozi et al 2010; Bhandari et al 2016; Han et al 2015), however, discords with following studies (Sharma et al 2013; Somily et al 2014; Mahdi et al 2016; Akpaka et al 2008).

There is significant association between MDR and ESBL production at ($P<0.05$). This results confirms and expands the previous findings of Chhetri et al (2001).

Detection methods are based on a phenotypic profile that has potential to yield false positive and false negative results. In some of the isolates, additional mechanisms of resistance, such as AmpC- β-lactamases, porin changes and inhibitor resistant TEMs (IRTs) and SHV β-lactamases with reduced affinities for β-lactamase inhibitor can mask clavulanic acid (CA) inhibition. Current Clinical and Laboratory Standards Institute (CLSI 2013) recommend the use of a screening and confirmation test, in addition to standard susceptibility testing methods, to detect extended spectrum β-lactamases (ESBLs) in the routine clinical laboratory among the strains of *E.coli* and *K.pneumoniae*. This method has proven reliable over many years in detecting the great majority of conventional ESBLs, particularly of variants of TEM and SHV enzyme class. The CLSI method, however, does not address the significance of strains that are positive on the screening test but negative on the confirmation test. By default, the result of standard susceptibility test (e.g. broth micro dilution or disc diffusion) is applied to organism with this ESBL test profile. It is important for both clinical and infection control reasons to detect strains harboring transmissible resistance mechanisms to extended spectrum cephalosporin (Bell et al 2007).

In modern medical practice, newer antimicrobial drugs have been un-extensively in emergence and rapid dissemination of resistant bacterial strain. ESBL producing strains are mostly associated with UTIs. UTIs is a common bacterial disease, often contributes to a frequent cause of morbidity in outpatients as well as hospitalized patients. Clinical experience has indicated the pressure of numerous cases of antibiotic resistance to common antibiotics by uropathogens in both developed and developing countries. 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 multi drug resistance in UTIs (Baral et al 2012).

Antibiotic sensitivity pattern of ESBL producing Enterobacteriaceae revealed that most of organism were resistant to ceftriaxone and amoxycilin and sensitive to imipenem, nitrofurantoin and meropenem. This study signifies that the antibiotic susceptibility pattern of *E.coli* showed that imipenem, nitrofurantoin and meropenem was the most effective drug. Also, similar results had seen documented in previous studies (Chaudhary et al 2016, Thakur et al 2013) which are in accordance with this study. In the study most of the ESBL producers were resistant to amoxicillin. This is due to the enzyme β-lactamase that break down the structural beta lactam ring of penicillin that inactivates the antibiotics and renders them ineffective. Similar resistance pattern was observed in the study carried out by Manandhar (2006), Pokhrel (2006). ESBL producers showed wide resistance to the non β-lactam antibiotics. This result directs the need for the inclusion of large number of samples, more sophisticated techniques in detection of these enzymes in a similar type of studies which can yield results that can be generalized.

Similarly, higher level of drug resistance seen among *K.pneumoniae* is mediated by the production of different kind of β-lactamases primarily ESBL, AmpC and Metallo β-lactamases. The fact that the carriage of resistance trait for quinolones and aminoglycoside in the plasmid along with the gene for β-lactamases have had a great impact on the drug resistance character shown by these pathogenic bacteria (Lee et al 2013; Piaco et al 2008 and Walsh et al 2005).

Transferable resistance has been identified for some antibiotic groups as β-lactams, aminoglucosides, macrolides, sulphonamide, tetracycline, chloramphenicol etc. However, the production of plasmid or chromosomal encoded β-lactamase enzymes is the most common mechanism of resistance in Gram-negative bacteria causing significant infection (Bush et al 1995).

A significant finding in this report was that for the ESBL producing Enterobacteriaceae, Nitrofurantoin was found to be effective against the isolates *E.coli* (92.3%), and impenem was found to be 100% similar to *K.pneumoniae*. Meropenem was also found to be effective against *E.coli* (69.2%). For UTIs causing isolates, Nitrofurantoin was also found to be

optimal drugs. This may be due to the restricted use of these drugs in our hospital setting and nitrofurantoin is usually reserved to be prescribed only in case of UTIs since it is excerted and concentrated in urine (Chander and Shrestha 2013). These indicated that imipenem, meropenem and nitrofurantoin are the drug of choice for treating serious infections caused by ESBL producing microorganism. Imipenem, belonging to the carbapenem group, are extremely potent and broad spectrum β-lactam antibiotics as it is resistant to most β-lactamase (Ahmed et al 2014).

Antibiotic resistance has become a major clinical and public health problem within the lifetime of most people living today. Therefore, detection of the ESBL producing strains is of significant important for all major hospital worldwide as these strains are most likely to be even more prevalent then it is currently recognized, difficulty in their detection by current clinical methods, constitute a serious threat to current Beta lactam therapy, and institutional outbreaks are increasing because of selective pressure due to the heavy use of extended-spectrum cephalosporins and also due to lapses in effective infection control measure (Rodrigues et al 2004). However, antibiotic susceptibility testing should be performed for each strain before prescribing antibiotics. Correct precaution against ESBL should be taken before the organism begins to develop resistance mechanism against antibiotics. Awareness and health problem can be effective. Although most of the outbreaks were limited to the high-risk patient care areas such as ICUs, oncology units etc.

Therefore, now a day the threat of the ESBL is not limited to only in ICUs or the tertiary care hospitals, but they are also found in OPD patients. The Clinical and Standard Laboratory Institute (CLSI) have issued recommendation for ESBL detection, for the reporting for organism other than *E.coli* and *Klebsiella pneumoniae* (Dalela et al 2012).

CHAPTER VI
CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The prevalence of multidrug resistance and Extended spectrum β-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* was 15.14 % and 4.28% respectively in patients coming for routine culture attending Apex Hospital, Itahari, Eastern Nepal. In the study, higher rate of infections was found in female patients 69/237 (29.16%) which was statistically significant difference between male patients (P>0.05). The prevalence of UTI was comparatively found higher among age group (21-30) years. The Gram-negative bacteria *E.coli* was found to be the most common uropathogens. Maximum ESBL and MDR was found. There is a significant association between MDR and ESBL production (P<0.05). For ESBL producing *E. coli*, imipenem was found to be effective with 100% sensitivity, Nitrofurantoin with 92.3% and Meropenem with 69.2%. For *Klebsiella* pneumoniae also the sensitivity of Imipenem was 100% and most of the organism were resistant to amoxicillin. In this study, *E.coli* was found to be the most predominant MDR isolate. The prevalence of ESBL producing *E.coli* and *Klebsiella pneumoniae* was higher. The majority of ESBL producing *E.coli* and *Klebsiella pneumoniae* were resistant to the in-use antibiotics used for treatment of UTI. Imipenem was the most effective antibiotic and could be the drug of choice for treatment of infections caused by ESBL strains. This clinical threat of increased ESBL prevalence is creating significant therapeutic problems prompting an immediate need to formulate strategic policy initiatives to reduce their prevalence. These findings also suggest integrating early and sensitive methods to detect ESBL producing strains should be practiced so that appropriate antibiotics can be used. To decrease the spread of ESBL producing microorganisms it has become important to formulate the appropriate antibiotic policies.

6.2 Recommendations

1. More ESBL associated UTI patients were found in female patients, they should be regularly monitored.
2. Imipenem, meropenem and nitrofurantoin can be used for the treatment of ESBL producing *E.coli* and *Klebsiella* spp.
3. If the patient is not responding to the third-generation cephalosporin antimicrobials, the clinician should think for the ESBL- producing organism and request for its test.

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APPENDIX-A

LIST OF EQUIPMENTS AND MATERIALS

A. EQUIPMENT:

Autoclave, Incubator, Hot air oven, Microscope, Refrigerator, Weighing machine, Water bath, Gas burners, Glass wares, Inoculating wire and loops.

B. MICROBIOLOGICAL MEDIA:

Blood Agar

Mac Conkey Agar

Muller Hinton Agar

Urea Agar Base

Simmon's Citrate Agar

Sulphur Indole Motility Media

Triple Sugar Iron Agar

Mueller Hinton Broth

C. CHEMICALS AND REAGENTS:

Catalase Reagent (3% H_2O_2), Oxidase Reagent (1% Tetramethyl p-phenylene diamine dihydrochloride), Kovac's Reagent, Barritt's Reagent (40% KOH, 5%α-napthol in a ratio of 1:3), Barium Chloride, Conc. H_2SO_4, Gram's reagent, etc.

D. ANTIBIOTIC DISCS

The antibiotics that will be required for the susceptibility test are as follows :

Amoxycillin (10µg), Aztreonam (30µg), Cefotaxime (30µg), Ceftazidime (30µg), Cotrimoxazole (25µg), Ciprofloxacin (5µg), Gentamycin (10µg), Imipenem (10µg), Meropenem (10µg), Nitofurantoin (300µg).

APPENDIX B

Method of collection of mid stream urine

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

Whenever possible, the first urine passed by the patient at the beginning of the day was requested for examination and biochemical analysis.

Midstream urine (MSU) for microbiological examination as follows :

WOMEN

Women who were ambulatory, they were requested as

1. Wash her hands thoroughly with soap and water and dry them with a clean towel.
2. Undress in a suitable room, spread the labia and cleanse the vulva and labia thoroughly using sterile cotton guaze pads and warm soapy water wiping from front to rear.
3. Rinse thoroughly with warm water and dry with a sterile cotton gauze pad. During the entire process, the patients should keep the labia separated and do not touch the cleansed area with fingers.
4. 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.
5. The specimen should be transported promptly to the laboratory.

MEN

Men who were ambulatory, they were requested as :

1. Wash his hands.
2. Pull back fore skin (if not circumcised), wash and dry the glans with soapy water and guaze pads and pass urine, discarding the first part of the stream.

3. Still holding back the fore skin, pass most of remaining urine into a sterile container. This is a mid-stream urine specimen.
4. Placed the cover on the container and the specimen should be transported promptly to the laboratory.

For bedridden patients, the sample procedure was followed, except that a nurse assisted the patients.

INFANTS AND CHILDREN

Collection of a clean-catch urine specimen from infants and childrens who are ill in bed or uncooperative can be a problem. Give the child water or other liquid to drink. Clean the external genitalia. The child can be seated on the lap of the mother, nurse or ward attendant, who should encourage the child to urinate and collect as much as urine as possible in sterile container. The container should be covered and delivered to laboratory for immediate processing.

APPENDIX C

CLINICAL AND MICROBIOLOGICAL PROFILE OF URINE SAMPLE

Clinical profile:

Name: …….. Lab No. ……

Age/Sex ……..... Date: ……

Address: …….

Microbiological Profile of Urine Sample

Time of collection: …….

Mode of collection: ……..

Direct Macroscopic observation

pH of urine: …….

Color of urine: ……

Turbidity: ……

Reading of Culture plates

Media used	Growth	Shape	Size	Color	Texture

Gram Staining result: …… Catalase: ……..

Coagulase: …… Oxidase: ……..

MR/VP: …….. Bacterial isolates: ……

Antimicrobial susceptibility test of the bacterial isolates from urine sample

Microorganism → / Antibiotic Discs ↓						
Gentamicin GEN						
Ciprofloxacin CIP						
Nitrofurantoin NIT						
Ceftazidime CAZ						
Aztreonam AT						
Ceftriaxone CRO						
Amoxicillin AMX						
Cotrimoxazole COT						
Imipenem IPM						
Meropenem MRP						