Characterization of common bacterial pathogens from patients of Urinary Tract Infection from Sikkim and Darjeeling

A Thesis Submitted

To

Sikkim University

In Partial Fulfilment of the Requirement for the

Degree of Doctor of Philosophy

By

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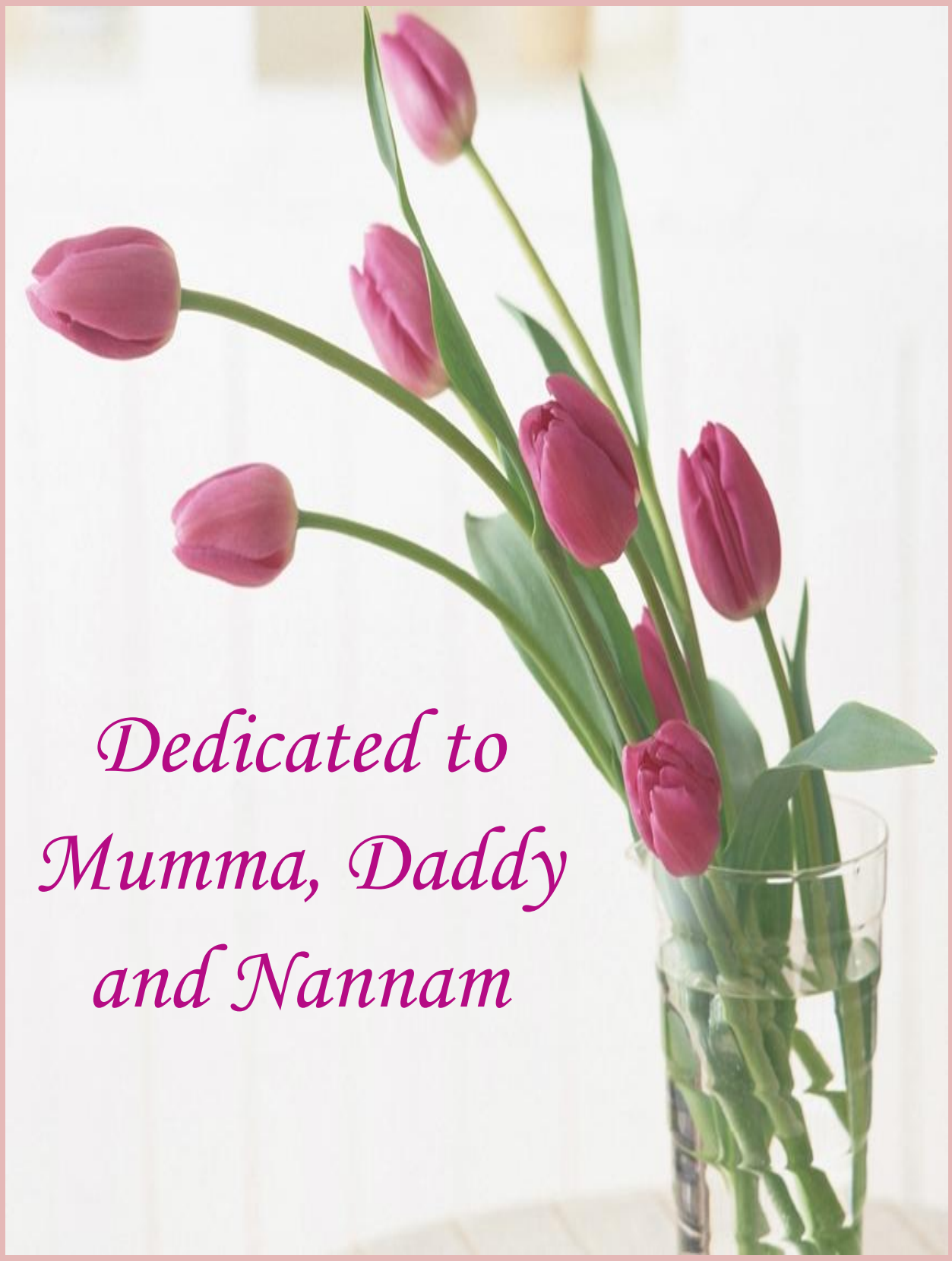
Dedicated to
Mumma, Daddy
and Nannam

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"Characterization of common bacterial pathogens from patients of Urinary Tract Infection from Sikkim and Darjeeling"

Submitted by Ms Varsha Rani Gajamer under the supervision of Dr. Hare Krishna Tiwari, Associate Professor, Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, 737102, Sikkim, India.

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Dr. Hare Krishna Tiwari

Countersigned by the supervisor

DECLARATION

I declare that the present Ph.D. thesis entitled "**Characterization of common bacterial pathogens from patients of Urinary Tract Infection from Sikkim and Darjeeling"** submitted by me for the award of the degree of **Doctor of Philosophy in Microbiology** of Sikkim University under the supervision of **Dr. Hare Krishna Tiwari,** Associate Professor, Department of Microbiology, School of Life Sciences, Sikkim University, is my original research work solely carried out by me in the Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok. No part thereof has been submitted for any degree of diploma in any University/institution

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Varsha Rani Gajamer

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Abbreviations

- ABC- ATP-binding cassette
- ACC- Ambler class C
- AMP- Ampicillin
- AmpC- ampC beta-lactamases
- ASB- Asymptomatic bacteriuria
- *bla*- β-lactamase
- BLAST- Basic local alignment tool
- CAC- Ceftazidime + clavulanic acid
- CAZ- Ceftazidime
- CEC- Cefotaxime + clavulanic acid
- CEP- Cephalothin
- CFU- Coloning Forming Unit
- CIAT- Ceftazidime-Imipenem Antagonism test
- CIP- Ciprofloxacin
- CLED- Cystine lactose Electrolyte deficient agar
- CLSI- Clinical Laboratory Standard Institute
- CMY- Cephamycins
- CRE- Carbapenem –resistance Enterobacteriaceae
- CTX- Cefotaxime
- CTX-M- Cefotaxime hydrolyzing capabilities
- CXM- Cefuroxime
- DHA- Dhahran Hospital
- *dhfr*- dihydrofolate reductase
- DHPPP- Dihydro-6-hydroxymethyipterin-pyrophosphate
- DNA- Deoxyribonucleic acid
- EARS-net- European antimicrobial Resistance Surveillance Network
- EAU- European Association of Urology
- ECDC -European Centre for Disease Prevention and Control

- ESBL-Extended –Spectrum β-lactamases
- ExPEC- Extra-interstinal pathogenic *E.coli*
- FDA- Food and Drug Administration
- FOX- Cefoxitin
- GCC- Gulf Cooperation Council
- GEN- Gentamicin
- GES- Guiana extended-spectrum
- GP- Gram positive
- GPUI- Global Prevalence of Infection in Urology
- HAI- Hospital acquired infection
- HAIs- Healthcare-associated infection
- HLA- Human leukocyte antigens
- IMI- Imipenem
- IMI- imipenem hydrolyzing β-lactamases
- Inc- Incompatibility group
- KPC- *Klebsiella pneumoniae* carbapenemase
- MATE- Multidrug/toxic compound extrusion
- MBLs- Metallo-beta-lactanases
- MDR- Multi-drug resistant
- MGE- Mobile-genetic element
- MOX- Moxalactam
- MR- Methyl Red
- MTCC- Microbial Type Culture Collection
- NaCL- Sodium chloride
- NDM- New Delhi Metallo- β-lactamases
- NET- Netillin
- NIT- Nitrofurantoin
- NOR- Norfloxacin
- OXA- Oxacillin bydrolyzing
- PABA- Para-aminobenzoic acid
- PBP- Penicillin-binding protein
- PBS- Phosphate buffer saline

- PCR- Polymerase Chain Reaction
- PER- *Pseudomonas e*xtended resistant
- PPA- Produces phenylpyruvic acid
- QS- Qurom sensing
- RND- Resistance nodulation division
- SCC- Staphylococcal-cassette chrosome
- SHV- Sulf-hydryl variable
- SIM- Seoul imipenamse
- SME- *Serratia marcescens* enzyme
- SMR- Samll multidrug resistance
- SOC- Super optimal broth with catabolite repression
- SXT- Trimethoprim /Sulfamethoxazole
- TEM- Temoneirae
- TSI- Triple Sugar Iron Agar
- TZP- Piperacillin /Tazobactum
- UP1a- Uroplakin 1a
- UP1b- Uroplakin 1b
- UPEC- Uropathogenic E coli
- UTI- Urinary Tract Infection
- VEB- Vietnam Extended-Spectrum-β-lactamase
- VIM- Verona integron –encoded metallo- β-lactamase
- VP-Voges proskaver
- WHO- World Health Organization

Contents

Chapter 1

Introduction

1. INTRODUCTION

Urinary tract infections (UTIs), are infections anywhere in the urinary tract which includes urethra, bladder, ureters, or kidneys (B. Foxman, 2014). UTIs is a very common infection and it represents one of the most common diseases encountered by clinicians (Tandogdu and Wagenlehner, 2016). It affects people from all ages from neonate to geriatric group (N. Ramesh *et al*., 2008).

An estimated population of 150–250 million cases of UTIs occurs globally annually, resulting in more than 6 billion dollars in direct health care expenditure (Costelloe C *et al*., 2010). It occurs most frequently in developing countries among the low socioeconomic populations (W.F. Nabbugodi, 2013). UTIs make use of a considerable impact on economic and public health resources and substantially affect the quality of life of those tormented with recurrent infections (B. Foxman, 2014). Consequently, updated knowledge of uropathogens and their susceptibility patterns are important for an appropriate selection and use of antibiotics, as well as for proper prescribing policy. Based on the recent European Centre for Disease Prevention and Control (ECDC) reports, 6995 (8%) of ICU patients presented with at least one ICU-acquired Hospital acquired infection (HAI) under surveillance which 3% presented with UTI and 98% of UTIs were associated with the use of a urinary catheter. Moreover, according to World Health Organization (WHO) report, in the USA and Europe, UTI was the most frequent type of infection hospital-wide (36% and 27%, respectively) 97% of which occurred in catheterized patients (W.H.O 2014). The recent Global Prevalence Infection in Urology studies have revealed that 10–12% of patients hospitalised in urology wards hava a health care–associated infection. The strains retrieved from these patients are even more resistant (Kandil, Cramp and Vaghela, 2016).

UTIs are mostly caused by Gram-negative uropathogenic bacteria including *Escherichia coli*, *Klebsiella spp*., *Pseudomonas spp*., *Enterobacter spp*., and *Proteus spp*. and Gram- positives such as *Staphylococcus saprophyticus* and *Enterococcus spp* (M. Heidary, 2016).

For the laboratory diagnosis of UTI, urine culture is done in routine microbiology laboratory. The samples yielding more than or equal to10^5organisms/ml of urine is known as significant bacteriuria, and that is regarded as UTI.

Urinary tract infection occurs in all populations and ages, however, some important risk factors include genetic factors, age, gender, sexual activity, nocturnal enuresis, and history of UTI, diabetes, obesity diaphragm/spermicide use, trauma and circumcision in boys. (Heffner V, 2008). In addition, infrequent micturition and incomplete emptying of urine from the bladder in children, faecal elimination and poor toilet habits are considered possible causes for UTI (Lindstorm TC, Baerheim, 2000).

Additionally, pregnancy in women and prostate enlargement in men can predispose the infection. It is reported that UTI is much more common in women than in men, due to their anatomical and physiological reasons. Women are particularly susceptible, because of the shorter distance between the urethral and anal opening and vaginal cavity, where potential uropathogens live. The distance from the urethral opening to the bladder is also shorter in females which make it easier for bacteria to ascend into the bladder. Moreover, females have more moist areas in the periurethrum where bacteria can grow. By virtue of its position urinogenital tract is more susceptible to bacterial infections caused by both internal and external flora (Maripandi *et al*., 2010). It is reported that about 3% of all women in the United

States visit a physician at least once each year for UTIs, and at least 50% of women once in a lifetime (Nicolle *et al*., 2006).

It is observed that the overall prevalence of asymptomatic bacteriuria (ASB) in women is 3.5%, and becomes much higher after sexual intercourse (B. Foxman, 2014). Among both men and women, the prevalence of asymptomatic bacteriuria increases with age. Laboratory findings for ASB are similar as for other UTI syndromes that is a positive urine culture and urinalysis, but ASB is without signs or symptoms (LE., 2009). However, symptoms only occur when the host response is occupied.

The symptoms of UTI include burning or pain, dark urine, strong smell and urination urgency (Danielle *et al*., 2009). Fever, malaise and chills are common along with other systemic symptoms and broad spectrum therapy is required (Mohsin *et al*., 2010). In cystitis, urinary symptoms are confined to the bladder; frequency, urgency, and dysuria are the most common symptoms. Urinary symptoms may or may not be present in pyelonephritis though the patient may present with fever and chills, back pain, nausea, and vomiting (B. Foxman, 2014).

Though UTIs are commonly curable with antibiotics, in recent decades, extensive use of antibiotics given empirically without proper antibiotic susceptibility testing has inevitably led to a massive increase in the prevalence of UTI globally (Kandil *et al*., 2016). Therefore, UTIs have become a major contributor to worldwide antibiotic use and resistance (Zalmanovici *et al*., 2010 and H.M. Zowawi *et al*., 2015). Since many years, resistance profiles for common Gram- negative uropathogens such as *Escherichia coli* or *Klebsiella pneumoniae* were predictable and steady over time. However, in the past few decades, this scenario has changed dramatically (Zowawi *et al*., 2015). The widespread usage of antibiotics have made the uropathogens resistant

to current antibiotics (Zandi H., Tabatabaei S.M., Ehsani F., 2017). During the 1980s, emergence of Extended-Spectrum β-lactamases (ESBLs) in Enterobacteriaceae was witnessed. But, currently, ESBL-producing *E. coli* or *K. pneumoniae* are now commonplace, not only in health-care facilities but in the community as well (Y Doi *et al*., 2013). It has been reported that approximately 15% of *E. coli* strains were found to be ESBL-positive and were isolated from all countries. Turkey had the highest percentage of ESBL-positive bacteria with a prevalence rate of 25% (N.Allocati *et al*., 2013). CTX-M-type ESBL is the most common globally disseminated ESBL associated with uropathogenic Enterobacteriaceae. The most prevalent type of CTX-M-type enzyme in *E. coli* CTX-M-15 is associated with the epidemic clone known as sequence type 131 (ST131) which has emerged as a dominant global strain causing extraintestinal infections (Bhullar, 2012 and (Zowawi *et al.*, 2015).

Other than routine diagnosis of UTI, to study the antibiotic resistance in gram negative uropathogens, the detection of ESBL is performed both by phenotypic and genotypic method. Phenotypic method is performed using appropriate CLSI guidelines. The phenotypic methods are comprised of screening of ESBL producers using five antibiotics namely cefotaxime, ceftazidime, ceftriaxone, aztreonam 1µg/ml and cefpodoxime 4 µg/ml (1µg/ml for *Proteus mirabilis*) in Mueller Hinton Agar by agar dilution method. The isolates that grow in any of the antibiotic containing medium is suspected to be ESBL producer and subjected to confirmatory test consisting of cefotaxime (30µg) and ceftazidime (30µg) antibiotic discs with and without clavulanic acid (10µg). A greater than or equal to 5mm increase in zone diameter for either antimicrobial tested in combination with clavulanic acid versus its zone when tested alone confirms ESBL production (CLSI, 2014). However, molecular characterization of ESBL producing bacteria is done by the detection

of ESBL genes namely bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA-2}, bla_{OXA-10}, bla_{GES}, bla_{VEB} and bla_{PER} (CLSI, 2014).

Apart from conventional PCR and multiplex PCR, real time PCR (Nijhuis *et al*., 2012) and quantitative PCR are also used for the detection of ESBL genes (Engel *et al.*, 2017). Pulsed-field gel electrophoresis of chromosomal DNA (Paterson DL, 2005), PCR single-strand conformational polymorphism (PCR-SSCP) analysis and early detection of beta-lactamase genes was performed using DNA probes (Bradford PA., 2001) are some of the molecular methods used for the identification of ESBL genes. Recent advances in traditional and real-time PCR have led to the use of loop-mediated amplification (LAMP), transcription-mediated amplification (TMA), and helicase-dependent amplification (HDA) are also used for the detection of drug resistant genes (T. Engel a *et al.*, 2018).

ESBL resistance genes are primarily carried by plasmids. It has been reported that $bla_{CTX-M-15}$ gene has been located on specific plasmids belonging to the IncF group (B, Woodford *et al*., 2009). The IncF plasmids have also been associated with the spread of other resistance determinants, including serine carbapenemase KPC, plasmid-mediated AmpC , quinolone and aminogycoside resistances genes (Alessandra Carattoli, 2011). Plasmids may also carry genes encoding resistance to other antibiotic classes, such as ampA, ampC, aminoglycosides, chloramphenicol, macrolides, or quinolone. Therefore, treatment options are limited for bacteria that produce ESBLs due to the multiple resistance genes encoded in the plasmids (Antunes LC, Visca P, 2014).

Over the past two decades, carbapenems have been the most frequently recommended antibiotics for treatment of infections caused by these resistant strains, worldwide particularly in developing countries, because of their resistance to hydrolysis by

ESBLs (Paterson *et al*., 2005). Due to this carbapenem resistance tends to emerge in areas where prevalence of ESBL is high, driven by selection pressure from use of carbapenem, and can disseminate subsequently. Carbapenem resistance in key Gram-negative pathogens is now a rapidly developing phenomenon, facilitated by international travel and globalization (Rogers, 2014). The increasing prevalence of carbapenem- resistant Enterobacteriaceae (CRE) causes the vast majority of clinical infections which arises as a result of the production of carbapenemases, and other mechanisms, like high-level expression of ESBL or AmpC β-lactamases in conjunction with outer- membrane porin changes and increased activity of efflux pumps (Zowawi *et al.,* 2015).

WHO also reported that *E. coli,* specially cephalosporin and fluoroquinolone resistant isolates, is the common causative pathogen among community- and hospital-acquired healthcare associated infections (WHO, 2014). The mortality rate associated with UTIs is significantly higher with strains which are multidrug resistant (MDR) strains (L.E. Nicolle *et al.,* 2005). The emergence and spread of MDR Gram-negative uropathogens, in the community and hospitals, have increased the therapeutic challenges (R. Ikram, R. Psutka, A. Carter, 2015). These problems have arisen due to an inappropriate traditional antimicrobial therapy.

The increasing prevalence of drug- resistant is a matter of concern because the treatment is often costly and failure of treatment and risk of death threatens the patients. Moreover, in developing countries the facilities for common tests such as urine culture and antimicrobial susceptibility tests are still unavailable which leads to improper diagnosis and irrational antibiotic treatment of UTI, this expedites the emergence of MDR uropathogens (S. Eshetie, *et al.,* 2015). Multidrug resistance of uropathogens is a global problem due to very fast arisen and spread of mutant strains

which are insusceptible to treatment, because of this the treatment of UTIs, are reduced and options for treating patients with these infections are extremely limited. The emergence of drug-resistant strains in the management of UTIs is a public health issue, particularly in the developing countries because of high level of poverty, ignorance and poor hygienic practices (N. Qadeer *et al*., 2013). Therefore, various works are being performed in the drug resistance of uropathogens in different parts of the world. However, very preliminary works have been done in this field in this part of North East India. Till date, no extensive phenotypic and molecular study has been conducted on the uropathogens isolated from the patients of study area. Therefore, the present study was undertaken to characterize the common uropathogens associated with the females of Sikkim and Darjeeling.

With this background, the present study was designed with the following objectives:

General objective:

To study and characterize the common bacterial pathogens from suspected female urinary tract infected patients of Sikkim and Darjeeling.

Specific objectives:

- To isolate and identify *Escherichia coli, Klebsiella pneumoniae, Citrobacter, Proteus mirabilis* from urine sample by culture and different biochemical tests.
- To perform antimicrobial susceptibility testing of isolated strains by disc diffusion method and minimum Inhibitory concentration.
- To perform Extended Spectrum ß lactamase test for above isolates by phenotypic methods and genotypic methods.

Chapter 2

Review of literature

2. REVIEW OF LITERATURE

2.1 Definitions of UTI

Urinary Tract Infection (UTI) is defined as the occurrence of microorganisms in the urinary tract which has the ability to invade the tissues of the urinary tract and adjacent structures (Sharon *et al*., 2018). UTIs include infections of the urethra which is known as urethritis, and infection of bladder, ureters and kidney is known as cystitis, urethritis, and pyelonephritis respectively (Barber *et al.*, 2013). UTI is the second most common infectious presentation in the community as well as in hospital settings. It has been estimated that about 150 million people are diagnosed with UTI each year worldwide (Flores-Meireles *et al.*, 2015). UTI is a major reason for antibiotic usage globally as it is the second most common reason for empirical antibiotic treatment (Magill *et al*., 2014).

2.2 Classifications of UTI clinically

UTIs are classified into 7 categories, such as:

2.2.1 Uncomplicated UTIs: Uncomplicated infection occurs to the one who is healthy individuals with either no structural or functional abnormalities of the urinary tract (Flores-Mireles *et al*., 2015 and Bartani *et al*., 2018). In uncomplicated UTI the urinary tract is normal, both structurally and physiologically, and there is no associated disorder that impairs the host defense mechanisms (Al-Badr and Al-Shaikh, 2013).

2.2.2 Complicated UTIs: Complicated UTIs are associated with predisposing lesion of the urinary tract, such as a distortion of the urinary tract, urinary stone, indwelling catheter, prostatic hypertrophy, obstruction or congenital abnormality or neurologic

deficit and it interferes with the normal flow of urine and urinary tract defenses. Complicated infections occur in both the genders and it frequently involves the upper and lower urinary tract (Sunil Sharon, Nair A Anjali A, Sharifi Saman, 2018). It is associated with 20% of high mortality (Davenport *et al.*, 2017).

2.2.3 Isolated infection: Isolated infection is an infection which is seen in the first episode of UTIs. It mostly affects the young females (25–40%).

2.2.4 Unresolved infection: The fourth category is an unresolved infection; it occurs when therapy fails due to bacterial resistance or due to infection by two different bacteria with equally limited susceptibilities.

2.2.5 Reinfection: The fifth category is reinfection and it occurs when there has been no growth after the infection is treated, but then the same organism re grows after two weeks therapy, or when a different microorganism grows during any period of time (ACOG, 2008). This accounts for 95% of Recurrent UTIs in women.

2.2.6 Relapse: The sixth category is relapse. It occurs when the same microorganism causes a UTI within two weeks of therapy. However, it is usually difficult to distinguish a reinfection from a relapse (Al-Badr and Al-Shaikh, 2013).

2.2.7 Asymptomatic bacteriuria: Asymptomatic bacteriuria (ASB) is defined as the occurrence of at least 10^5 colony-forming units of a urinary tract pathogen per milliliter in a culture of a midstream urine specimen, which is obtained from an asymptomatic woman on a routinely programmed visit (Hooton TM. *et al*., 1996). The presence of ASB increases the risk of infection for patients those who are undergoing urinary tract interventions in which mucosal trauma can be expected. For this reason, ASB should be actively required in such cases, and if found it should be treated (Kranz *et al.*, 2017).

ASB is not a treatable condition; with the exception of during pregnancy Treatment of ASB in the otherwise healthy individual often increases selection for antibiotic-resistant bacteria and results in a symptomatic UTI. The Infectious Disease Society of America recommends against screening for and treating ASB among catheterized patients (Hooton TM, Bradley SF, Cardenas DD, 2010). However, physiologic changes during pregnancy make the pregnant woman with ASB more susceptible to pyelonephritis (Ramos NL, Sekikubo M, Dzung DT, 2012). The prevalence of ASB in pregnancy ranges from 2% to 20%; and one-fifth to two-fifths of these may develop pyelonephritis if untreated. Pyelonephritis can be life threatening to both the mother and infant.11 Screening and treating ASB during pregnancy may reduce this risk by 77% (Smaill F, 2007).

2.3. Etiological agents

It is not surprising that many bacteria can grow in the urinary tract, and do so frequently as urine is a good medium for bacterial growth. The bacteria that colonize the urinary tract do not cause disease in most cases because the host has many rapid effective methods for removing bacteria from the system. These methods include urination, innate and adaptive host immune response. Bacteria that causes UTI either have special features which enables them to survive in the urinary tract or inhabit a host that is compromised in a way that limits their ability to remove bacteria.

It is reported that UTIs are caused by both Gram-negative and Gram-positive bacteria, and by certain fungi. Bacteria are responsible for more than 95% of UTI cases (Davenport *et al.*, 2017)(Farajnia *et al.*, 2009). Among bacteria, Gram-negative pathogens, mainly from the Enterobacteriaceae family accounts for UTI. It includes *Escherichia coli* (74.20%), *Klebsiella pneumoniae* (6.20%), *Enterococcus spp.* (5.30%), *Pseudomonas spp.* (3.20%) *Proteus mirabilis* (2.00%), and *Staphylococcus*

saprophyticus (1.40%) and other bacteria also account for (8.70%) of UTIs (Vasudevan, 2014 and Flores-Mireles *et al.,* 2015).

In the majority of UTIs both *E. coli* and *P. mirabilis* can be found in the intestinal tract, which is the source of microorganisms infecting the urinary tract (Nielubowicz and Mobley, 2010). The subgroups of *E.coli* namely O1, O2, O4, O6, O7, O8, O18, O25, O68, and O75 are frequently isolated from UTIs patients. It reported 85% of community-acquired and 50% of hospital-acquired UTIs is caused by *E.coli*. Gram-negative bacteria such as *Klebsiella* and *Proteus*; and Gram-positive *Enterococcus faecalis* and *Staphylococcus saprophytic* are causative agents for the remainder of community-acquired infections. The remainder of hospital-acquired infections usually occurs after colonization with *Klebsiella, Enterobacter, Citrobacter, Pseudomonas aeruginosa, E. faecalis, or S. Epidermidis* (Saeed Khoshnood *et al.*, 2017). The studies have demonstrated associations between Group B streptococcal bacteremia, *Candida,* and *Enterococci* with complicated UTIs in the elderly population (Khan and Ahmed, 2001). Children with co morbidities are more likely to develop complicated UTIs and *Staphylococcus aureus* is the most commonly isolated microorganism in pediatric patients with indwelling catheters (Schalger, 2001). *Candida* and coagulase-negative staphylococci are causative agents of complicated UTIs. *Enterobacteriaceae* are the most commonly isolated uropathogen in children with uncomplicated UTIs (Schalger, 2001). UTIs are included among the top 10 complicating illnesses in patients with diabetes mellitus caused by *E. coli*, *Klebsiella*, Group B Streptococci and *Enterococcus* as the most common uropathogens (Ronald, 2003). Common uropathogens causing complicated UTIs among patients with spinal cord injuries and indwelling catheters include *E.coli, Pseudomonas aeruginosa* and *Proteus mirabilis*.

Proteus mirabilis is particularly associated with complicated UTIs as it possesses unique virulence factors that enhance its invasive potential (Coker *et al.,* 2000).

2.3.1 Gram-negative uropathogens

2.3.1.1 Escherichia coli

Escherichia member of this group is Gram-negative, rod in shape, facultative anaerobic and motile in nature (Croxen *et al.*, 2013). *E.coli* is the normal intestinal flora in both a human being and warm-blooded animals. They are the most common uropathogen in both the community and hospitals settings (Sheerin, 2011), where bacterial infections of the UTIs present clinically with a variety of signs and symptoms and may be caused by an array of organisms such as uropathogenic *E.coli* are main etiologic agent of UTI, which is responsible for >80% of all community-acquired infections (McLellan and Hunstad, 2016). The common mechanisms of neuropathogenesis utilized by uropathogenic *E.coli* are adherence, toxin production, motility, metal acquisition, and invasion of host immune defenses (Nielubowicz and Mobley, 2010). These organisms produce hemolysin, which is cytotoxic and it facilitates tissue invasion and strains that cause pyelonephritis.

2.3.1.2 *Klebsiella pneumoniae*

Klebsiella is the second leading causative agent of UTIs after *Escherichia coli*. It is known as an opportunistic bacterial pathogen associated with UTIs (Gorrie *et al.*, 2018). Approximately 12%-15% of UTI is caused by *K. pneumoniae,* its incidence is increasing at an alarming rate all over the world, especially in Asia (Mahmudunnabi *et al.,* 2018). *K.pneumoniae* accounts for 6% to 17% of all nosocomial UTIs (Manjula N. *et al*.,2018).

K. pneumoniae carried asymptomatically in the gastrointestinal (GI) tract is a risk as it causes healthcare-associated infections in at an individual (Gorrie *et al.*, 2018)**.**

2.3.1.3 Pseudomonas aeruginosa

They are widely distributed in nature and commonly present in the moist environments. It causes the disease in human with abnormal host defenses. *P. aeruginosa* is a primary nosocomial pathogen (Brooks *et al.*, 2013) which infects people with low resistance such as cystic fibrosis patients and invades burn areas or causes urinary tract infections (Prescott and Klein, 2002).

2.3.1.4 Proteus mirabilis

It is a gram-negative bacterium belonging to a member of the Enterobacteriaceae family. It commonly causes UTI with long-term urinary catheters in or individuals with complicated UTIs and despite its antibiotic sensitivity can be difficult to clear by antibiotic treatment (Manjula N. G *et al*., 2013). Approximately 10-15%, *P. mirabilis* infections cause the formation of urinary stones (urolithiasis) (Thomas and Tolley, 2008)(Schaffer and Melanie, 2015) Moreover, some serious problems such as fever, pain, kidney damages, bacteremia and death can also occur (Bameri *et al.*, 2017).

2.3.1.5 Citrobacter

The genus *Citrobacter* was discovered by Werkman and Gillen in 1932. Members of the genus *Citrobacter* are gram-negative, non-spore forming, rods belonging to family Enterobacteriaceae. For a carbon source they solely usually utilize citrate. Although *Citrobacter* spp. is less commonly isolated from urine, they are emerging as a common nosocomial multidrug-resistant pathogen, especially in developing countries. UTI caused by *Citrobacter* spp. have been seen in 12% of patients in 1961,

and since then, its prevalence has been increasing (K. P. Ranjan and Neelima Ranjan, 2013).

Invasive procedures like catheterization or genitourinary instrumentation encourage the organism in colonization and infection of the urinary tract. The emergence of multidrug-resistant *Citrobacter* spp. results in reduced therapeutic options which further complicate the situation. (Rizvi M, Fatima N, Shukla I, 2010).

2.3.2 Gram positive uropathogens

Hoonton 2012, has reported that Gram-negative bacteria are responsible for 75% to 95% of Uncomplicated UTI (Hooton TM, 2012).The remaining proportions of uncomplicated UTI are associated with a variety of organisms, which includes the Gram-positive bacteria like *Staphylococcus saprophyticus*, *Enterococcus faecalis*, *Streptococcus agalactiae* (Group B Streptococcus, GBS), and other less frequently isolated organisms. Complicated UTIs often occur in nosocomial settings, particularly in individuals with structural or functional alterations of the urinary tract, underlying renal, metabolic, or immunological disorders (Wagenlehner FM, 2006); these populations are at greater risk of Gram-positive and polymicrobial UTI (Matthews SJ, 2011).

2.3.2.1 Staphylococcus saprophyticus

Staphylococcus saprophyticus is a Gram-positive, coagulase-negative, non-hemolytic cocci shaped bacteria. Colonies of *S. saprophyticus* are often yellow-pigmented. *S. saprophyticus* causes 5% to 20% of community-acquired UTIs (Hooton TM, 1997) and up to 42% of UTI among 16 to 25-year-old women (Hovelius B, 1984). *S. saprophyticus* is the most common cause of uncomplicated UTI in this population

second to UPEC (Zong Z, Peng C, 2011). Similar to UPEC infection recent sexual intercourse is also a risk factor for *S. saprophyticus* UTI.

S. saprophyticus infection rarely causes UTI in males and is associated with urethritis and up to 17% of prostatitis (Kline and Lewis, 2016).

UTI symptoms caused by *S. saprophyticus* are similar in spectrum to those caused by *E. coli*. Approximately 40% of patients with *S. saprophyticus* UTI present with acute pyelonephritis (Jellheden B, Norrby RS, 1996).

Novobiocin resistance is a laboratory feature for identification of *S. saprophyticus.* However, antibiotic resistance in *S. saprophyticus* is not common (Kahlmeter G, 2003).

Methicillin-resistance can occur in *S. saprophyticus* and is found in ∼1% to 8% of urine isolates by acquisition of a penicillin-binding protein (PBP) with low β-lactam affinity encoded by the mecA gene (Higashide M, Kuroda M, Ohkawa S, 2006). mecA has harboured on the staphylococcal-cassette chromosome (SCC) mobile-genetic element (MGE). UTI symptoms caused by *S. saprophyticus* are similar to those caused by *E. coli*, but can be more severe than in patients with *E. coli* UTI. Approximately 40% of patients with *S. saprophyticus* UTI suffers from acute pyelonephritis (Kline and Lewis, 2016).

2.3.2.2 Non-saprophyticus Staphylococcus

S. aureus UTI more frequently occurs in urinary-catheterized and pregnant individuals (Gilbert NM, *et al.,* 2013). The majority of *S. aureus* UTI isolates are methicillin-resistant. *S. aureus* bacteriuria is associated with subsequent development of invasive infection (Muder RR, et al., 2006). Like *S. saprophyticus, S. aureus* also encodes an active urease enzyme (Remy L *et al*., 2013).

S. *epidermidis* are a leading cause of hospital-acquired infections where they are often methicillin resistant and are associated with 2.5% of Community-Acquired Urinary Tract Infection. S. *epidermidis* is able to colonize the bladder at a similar frequency, but with significantly delayed kinetics, compared to *E. coli* or *S. saprophyticus* (Kline and Lewis, 2016).

2.3.2.3 Enterococci

Enterococci are a genus of Gram-positive lactic-acid bacteria. It typically occurs as diplococci or in short chains. *E. faecalis* and *E. faecium* are responsible for a minority of community-acquired UTI, but together cause 15% to 30% of catheter-associated UTI. They are the third leading cause of hospital-acquired UTIs (Richards MJ *et al*.,1999).

The incidence of UTIs due to *E. faecalis* has risen steadily over the years and *E. faecalis* UTI now out numbers *E. faecium* UTI at a ratio of 5:1. Infection due to multiple-drug-resistant enterococcal strains presents a significant medical problem as vancomycin resistance has increased and are prevalent among *E. faecium* isolates. *E. faecalis* readily adheres to and develops biofilms on abiotic surfaces such as urinary catheters (Huycke MM, Sahm DF, 1998). Many enterococcal virulence factors involved in UTI described till now are also biofilm determinants. However, it is not clear whether these virulence factors function in a similar manner during biofilm formation and in infection in the absence of abiotic devices (Kline and Lewis, 2016).

2.3.2.4 Group B Streptococcus (GBS)

Streptococcus agalactiae, also known as group B *Streptococcus* (GBS), is a Gram-positive β-hemolytic chain-forming coccus that is a common asymptomatic inhabitant

of the lower gastrointestinal and female reproductive tracts. GBS is estimated to cause approximately 1% to 2% of all monomicrobial UTIs (B., 2003)

Previous studies of elderly populations with UTI show an involvement of GBS in as many as 39% of nursing- home residents over 70 years of age. UTI caused by GBS are common not only among the elderly, but also in pregnant, diabetic, and immunocompromised individuals, as well as those with pre-existing urologic abnormalities. These groups are at a higher risk of ascending pyelonephritis that can progress to bacteremia and urosepsis (Edwards MS, 2005).

Although GBS may represent only a small fraction of total UTIs, the burden of GBS UTI is a major public health concern, with approximately 160,000 cases annually in the United States. Among (Farley MM *et al*., 1993). Common underlying conditions of individuals with GBS urosepsis include diabetes mellitus, malignancy, chronic kidney disease, recurrent urinary- tract infections, obstructive neuropathy, and neurogenic bladder (Chaiwarith R *et al*., 2011).

2.3.2.5 Rare, emerging, and under-reported gram-positive and polymicrobial etiologies

Several specific examples of Gram-positive bacteria that are rare, emerging, or under-reported include species of *Aerococcus*, *Corynebacterium*, *Actinobaculum*, and the potential uropathogen *Gardnerella vaginalis*. These organisms may be missed out as causes of UTI due to misclassification owing to lack of distinguishing phenotypic criteria, dismissal of significant growth as ‘microbiota contamination’, or lack of detection by standard approaches (Kline and Lewis, 2016).

2.4 Why is UTI more common in females?

The Urinary tract infection varies from sex, age, and associated genitourinary abnormalities. UTIs are the most prevalent infection affecting almost 50% of the population at least once in their lifetime which accounts for nearly 25% of all infections(Nicolle, 2002)(Davenport *et al.*, 2017). Telkar et al.,2015 reported (Telkar and Baragundi, 2015) that, female gets UTI frequently as compared to males. Kakaria et al., 2018 also found the higher incidence of Community-Acquired Urinary Tract Infection (56.46%) was found in female sex as compared to males (43.54%) (Mangukiya, Patel and Vegad, 2018).

It has also been documented that urinary tract infection is more common in females; at a ratio of 8:1 UTI is the most common infectious disease among the women due to their physiology (Jackson *et al.*, 2004). Unlike men, women have a shorter urethra, which shortens the distance for the bacteria to where it gets colonized (Gupta, Hooton and Stamm, 2001). The shorter distance to the bladder in women makes possible for bacteria to reach the bladder and colonize more easily before they are removed by micturition (Foxman, 2010). In addition, the urethral opening in women is proximate to the vaginal cavity and rectum, which harbor large bacterial communities.

Moreover, apart from anatomical factors, women are more prone to developing UTI due to poor hygiene; sexual intercourse, pregnancy and the use of contraceptive (Vasudevan, 2014). Post-menopausal women have higher rates of UTIs because of pelvic prolapse, lack of estrogen, loss of *lactobacilli* in the vaginal flora, increased periurethral colonization by *Escherichia coli* and a higher incidence of medical illnesses such as diabetes mellitus (Raynor *et al.*, 2016).

Recurrent urinary tract infections (UTIs) present a significant problem for women (Mohsin Raheela, 2010). One in four women with UTI will suffer from recurrences, in healthy, young or pre-menopausal women (Foxman B. *et al.*, 2014). The population at risk of UTI includes new born child including the premature one, mature girls, sexually active females and elderly females.

UTI in men are rare but when they occur it usually comes with severity and are most times refers to as complicated. Elderly men are at increased risk of developing UTI due to kidney stones or prostate problems. Any abnormality of the urinary tract that interferes with the flow of urine is at the increased risk of complicated UTI.

2.5 Risk factors for UTI

Risk factors specific to women for UTIs include such as:

- **Pregnancy**

Stenqvist *et al*., found that the frequency of bacteriuria increases by about 1% during pregnancy. He revealed that the risk of acquiring bacteriuria increases with the duration of pregnancy. That range from 0.8% of bacteriuric women in the 12^{th} gestational week to 2% till the end of pregnancy (Stenqvist *et al*., 1989) (B. *et al.*, 2014).

- **Complication in Pregnant Women**

The lack or improper treatment of UTIs can result to obstetric and neonatal complications. Among them, the early rupture of membranes, premature delivery and labor, restriction of intrauterine growing, low birth weight, abortion and fetal death are highlighted (Jacociunas LV, 2007). Other complications have been associated with UTIs are hypertension, preeclampsia, anemia, chorioamnionitis, endometritis, septicaemias and deterioration of kidney function (Nicolle LE, 2005).

- **Sexual activity**

UTI has long been associated with sexual activity; hence, uncomplicated uti has been dubbed 'honeymoon cystitis'. UTIs occur most frequently among women aged 18–29 years a time of life when women are most likely to be initiating sexual activity. Recent, frequent vaginal intercourse is a major risk factor in this age group (Foxman, 2000). If a woman has a UTI caused by UPEC, the same UPEC is twice as likely as an E. coli strain isolated from her rectal specimen to be found in a urethral or rectal specimen of her most recent sexual partner (IDSA.,1999). The women who are sexually active have more UTIs, due to sexual intercourse as it felicitates the entry of bacteria to the bladder as compared that to the women who are not sexually active. Therefore, having multiple sexual partners would increase the risk of recurrent UTI (Nicolle *et al.*, 1982). Buckley *et al*, 1978 reported that 30% of women after sexual intercourse have at least one log increase in bacteria in the bladder. Women who have been sexually active within the past month are six-times more likely to have UTI and woman with a new sexual partner also has an increased risk of infection (Scholes D *et al*., 2005).

- **Certain types of birth control**

Women who use spermicides for birth control have an increased vaginal pH and increased colonization with potential uropathogens, especially *E. coli* (Gupta K, Hillier SL, Hooton TM, Roberts PL, 2000). These women have five-time greater rate of infection compared with women who do not use spermicide (Hooton, 2000). Foxman *et al*., 2000 found that diaphragm use may also contribute to infection, irrespective of concomitant spermicide us. The birth control pill or condom without spermicide is not associated with increased urinary infection (Foxman *et al.*, 2000).

- **Menopause**

After menopause, estrogenic hormones level is reduced which causes or changes the circulation in the urinary tract that makes an individual more vulnerable to common infection such as acute cystitis (Jackson *et al.*, 2004) (Hooton, 2001).

- **Formation of stone**

Due to the disturbance or changes in the kidney, where the kidney stones can also trap urine in the bladder and leads to increased risk of UTIs (Bichler *et al.*, 2002)

- **Catheter use**

Catheter associated UTI is a common nosocomial infection. People who cannot urinate on their own, and use a catheter to urinate have an increased risk of UTIs (Muramatsu *et al.*, 2018). This may include hospitalized patients with neurological problems that make them difficult to control their ability to urinate (Hu *et al.*, 2018). Placement of a catheter moves bacteria into the bladder, and creates an additional portal for bacterial invasion; catheter placement increases the risk of UTI by as much as 4-fold (Uckay I *et al*., 2013).

- **Anameia**

Bacteriuria in pregnancy is associated with maternal anaemia (Kremery *et al*., 2001; Lavanya *et al*., 2002) But Fatima et al 2006 reported that there is no any association between bacteriuria and anemia (Fatima *et al*.,2006).

- **Diabetes**

The prevalence of UTI and risk factors for asymptomatic bacteriuria (ASB) in women with and without diabetes was checked by Geerlings *et al.*, 2000 and they concluded

that the prevalence of ASB is increased in women with diabetes as compared to women without non- diabetic women (Geerlings *et al.,* 2000).

- **Unhygienic Practices**

Studies report that widespread use of unsanitary absorbents and inadequate washing and drying of reused absorbents across Africa, South East Asia and the Middle East are one of the risk factors of UTI. (Baisley *et al.,* 2009).

2.6 Symptoms of UTI

Cystitis and Pyelonephritis are the most common in UTI which encounter infections among the community and hospital setting (Zhanel *et al.*, 2006), UTI is also said as one of the most common community-onset infections (Rossignol *et al.*, 2017).

- **Lower UTI such as cystitis:** It is characterized by symptoms like dysuria, frequency, and urgency, However, these symptoms may be related to lower urinary tract inflammation caused by urethritis (eg: gonorrhea or Chlamydial urethritis) (Nicolle, 2008).
- **Upper UTI known as pyelonephritis or kidney infections**: It is associated with a clinical syndrome characterized such as fever, nausea, vomiting, flank pain and refers to pathologic changes in the kidney caused by infection only (Nielubowicz and Mobley, 2010) and it reaches to more complicated risk to the bacteremia (Vasudevan, 2014).

In chronic pyelonephritis, one or both kidneys contain gross scars, through involvement is bilateral, the kidneys are not equally damaged. This uneven scarring is helpful in differentiating chronic pyelonephritis from diseases that cause symmetrical contracted kidneys- for e.g.: chronic glomerulonephritis. Even HIV positive patients

are also more prone to the UTIs which is obviously related to its immune functions and infection (Skrzat-Klapaczyńska *et al.*, 2018).

2.7 Pathogenesis in UTI

2.7.1 *Virulence factors*

Bacterial virulence factors play an important role in determining whether an organism will invade the urinary tract and the infection level acquired. Uropathogenic *E.coli* (UPEC) is the most common microorganism which infects the urinary tract by expressing specific virulence factor which allows them for the colonization and attachment (Bien, Sokolova and Bozko, 2012). The three important environmental characteristics for adherence of the microorganism are the bacteria's own adhesive characteristics, the receptive features of the urothelium and the fluid that is present between both surfaces (Davis and Flood, 2011).

The adherences of most uropathogens originate in the rectal flora and enter through the urethra, particularly in patients with indwelling catheters and also by the sexual intercourse. The pathogen is subsequently migrated to the bladder followed by colonization. As a host-pathogen complex interactions determines whether they are successful in colonization or elimination. Adhesions found on the surface of the bacterial membrane are responsible for initial attachment onto urinary tract tissues where UPEC start to bind to uroepithelium cell in a type 1 pilus dependent manner (Hannan *et al.*, 2012). Then the UPEC start producing toxins and proteases from the host cells to release nutrients and synthesizing siderophores to obtain iron. By multiplying and overcoming, the uropathogens slowly infect the kidneys, attaching by pili to colonize the renal epithelium, then produces tissue-damaging toxins where it crosses the tubular epithelial barrier to access the bloodstream (bacteremia). UPEC, *K. pneumoniae,and S. saprophyticus* have the ability to bind directly to the bladder

epithelium, which are superficial facet cells, intermediate cells and basal cells. They form a crystalline array protecting the mammalian bladder tissue from damaging agents in urine (Flores-Mireles *et al.*, 2015).

2.7.1.1 Type 1pili

Mannose-sensitive pili are also referred to as Type 1 pili which are commonly expressed in pathogenic and non-pathogenic strains of *E. coli* .Type 1 pili are composed of a helical rod with repeating Fim A subunits that are bound to a distal tip structure containing the Fim H adhesin (Jones *et al.*, 1995)(Davis and Flood, 2011). During the colonization process in the host's uroepithelium Fim H adhesins binds to mannosylated receptors and processthe inflammation which occurs shortly after the binding(Wu, Sun Ii and Medina, 1996) (Davis and Flood, 2011). This specific 'adhesin-epithelial cell' binding process occurs when type 1 pili bind to uroplakin 1a (UP1a) and uroplakin 1b (UP1b) (Malaviya and Abraham, 1998). Uroplakins are membrane proteins, found on umbrella cells that line the luminal surface of the urinary bladder. The Fim H containing pili bound to the central cavity of uroplakin hexameric rings and this binding process is responsible for the initial steps leading to active UTI.

After binding to the epithelial surface the Fim H adhesins is activated and migrate towards urothelial layers and penetrate the cell membrane. Once the uropathogen is intracellular the invasive process continues as bacteria proliferate within the cytosol to form clusters (Anderson, Martin and Hultgren, 2004).

2.7.1.2 P Fimbriated pili

P fimbriated pili of *E. coli* are associated with uncomplicated pyelonephritis because the receptor for P fimbriae is the major glycolipid component present on renal cell membranes (Mulvey, 2002)(Davis and Flood, 2011).They are mannose resistant as

they are not affected by mannose during the haemagglutination process for human erythrocytes. It has been reported that P pili are present in 91% of strains that caused pyelonephritis

2.7.2 *Pathogenesis of some common uropathogens*

2.7.2. 1 Uropathogenic Escherichia coli (UPEC)

E. coli are the most common causes of UTI but all strains are not successful to infect the urinary tract. Its strains that have several virulence factors, which increases their ability to invade the urinary tract to the natural reservoir of the human gastrointestinal tract, as it presents the most common facultative anaerobe bacterium. The strains promote their ability to cause infections outside the gut that is extra-intestinal pathogenic *E coli* (ExPEC). Entry of UPEC is followed by fimbrial adhesin known as pili are surface monofilaments which serve a variety of functions which allows bacteria to adhere unto the host cells, aggregation of bacteria and exchange of genetic material (Anderson, Martin and Hultgren, 2004). The key role of 'type I' fimbriae is it enables the bacterial binding to bladder uroepithelial cells, due to mannose presences containing receptor such as uroplakin(Flores-Mireles *et al.*, 2015)(Walsh and Collyns, 2017).

2.7.2.2 Klebsiella

K.pneumoniae which causes bloodstream infections ((Martin and Bachman, 2018), is a second most causal organism than to *E.coli*, which associated with a UTI**.** It produces various types of fimbriae, such as type 1(Walsh and Collyns, 2017). It also produces a polysaccharide capsule surrounding its outer cell membrane, of which there are over 70 antigenic types. The capsule inhibits phagocytosis, and its presence has been shown to have a role in UTIs in animal models (Flores-Mireles *et al.*, 2015).

2.7.2 3 *Proteus mirabilis*

There are various species of *Proteus*, but *P. mirabilis* and *P. Vulgaris* are most common agents which causes human infections. Uncomplicated UTI is caused by *P. mirabilis*, but more commonly it causes infection in the setting of an abnormal urinary tract, including if an indwelling catheter is present. (Walsh and Collyns, 2017) *P. mirabilis* produces the potent urease enzyme, which hydrolyzes urea into carbon dioxide and ammonia and in turn, the pH value of urine rises. Other bacteria such as *Klebsiella, Pseudomonas, and Staphylococcus* also produce ureases (Thomas and Tolley, 2008). Viable bacteria may be embedded within the crystal, which can then act as a nidus for recurrent infections.

Therefore, recurrent isolation of *P. mirabilis* should raise the suspicion of renal stones (Reyes *et al.*, 2006, Walsh and Collyns, 2017).

2.7.2.4 Pseudomonas aeruginosa

P. aeruginosa does not mark the portion of the normal resident microbial flora, in healthy hosts such as in the gastrointestinal or vaginal tracts. It usually causes urinary infections in a healthcare setting, due to association with a urinary catheter, but it rarely causes community 'acquired' infections, until there are some urological issues such as obstruction, recent instrumentation or neurogenic bladder. The antibiotics such as trimethoprim, nitrofurantoin, co-amoxiclav, and most cephalosporins are used for the treatment of UTI caused by these bacteria, but *P. aeruginosa*is intrinsically resistant to the antibiotics. The various virulence factors, including a well-developed quorum sensing (QS) pathway, which enables communication between bacterial cells (Walsh and Collyns, 2017).

2.7.2.5 Staphylococcus aureus

S.aureus ia also regarded as the 'prince of pathogens', in humans. This bacterium is successful in colonizing, and causing infections, due to its virulence factors. The most of the *S. aureus* detected in urine is due to contamination by perineal flora, or be due to hematogenous spread (Walsh and Collyns,2017).

2.7.4.6 Staphylococcus saprophyticus

S.saprophyticus has a specific niche as a cause of UTIs than to *E.coli* in which causing uncomplicated infections in sexually active women, but it rarely causes infections in men or older women. It has a unique protein which helps in adhesion (UafA), facilitates its adhesion to uroepithelial cells, and produces urease and various transport proteins, for its survival and multiplies, to face the osmotic and pH changes.

2.7.2.7 Candida

Candida species are normal human commensal flora and can be found harmlessly in the gastrointestinal and female genital tracts. *Candida spp*. is commonly found in the urine of patients with indwelling catheters without causing symptomatic infection. Due to disturbance of antibiotics, enables *Candida species* to multiply, which yield mucous membrane infections, such as vaginitis. In men, it acquires urethral candidiasis after sexual contact with *Candida vaginitis*, than to women (Walsh and Collyns, 2017).

2.7.3 Cell receptivity

Recurrent UTI occurs in a female patient who is susceptible to the pathogen due to epithelial cell receptivity. The receptivity concept was recognized after vaginal epithelial cells were collected from patients susceptible to recurrent UTI with *E. coli.* Further analysis of this genetic concept by assessing human leukocyte antigens (HLA) in females with recurrent UTIs has demonstrated that HLA-A3 may be a contributing

factor. It has also been shown that a greater number of uropathogens attach to the epithelial cell surface in females that are greater than 65 years of age compared to premenopausal females (i.e. age 18-40) (Schaeffer *et al*, 1983) (Davis and Flood, 2011).

2.7.4 Predisposing factors for pathogenic adherence

2.7.4.1 Alterations to the host's natural defense mechanisms

Normal flora including lactobacilli, coagulase-negative staphylococci and streptococci are found around the vaginal opening, periurethral region and urethra. Normal flora forms the barrier against the pathogenic colonization where it changes the vaginal activity and decreases the pH value. The disruption of mucosal barrier of Host factors which alter normal flora and induce receptivity increased for uropathogens by the spermicidal and antimicrobial agents (Winter *et al.*, 1996) (Davis and Flood, 2011). Diabetes mellitus, sickle cell disease, hyperphosphataemia, gout, and analgesics are associated with the host's natural defense mechanism (Davis and Flood, 2011).

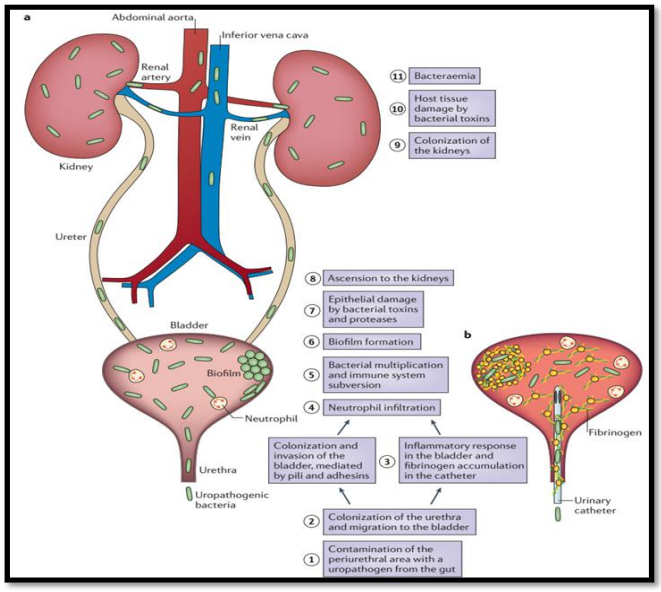


Figure 1: Pathogenesis of urinary tract infection

Adopted after permission from A review entitled "Drug-resistant gram-negative uropathogens: A review" (Khoshnood et al., 2017-Elsevier review)

2.7.4.1.1 For Uncomplicated UTIs

In uncomplicated UTIs, the uropathogens begin to reside in the gut contaminate the periurethral area and colonized the urethra. Subsequently,uropathogens migrate to the bladder and then colonize by expressing pili and then results in invasion of the umbrella cells. The inflammatory response in the bladder takes place, including the filtration of neutrophil by the uropathogen. Then the uropathogens undergoes multiplication followed by the suppressesion of immune system. After Multiplication of pathogenesis, it forms the biofilm formation. Uropathogens produce the toxins and proteases that induce host cell damage. It releases nutrients that promote bacterial survival and ascension to the kidneys.

Then the kidney gets colonized by bacteria which results in bacterial toxin production, host tissue damage and if left untreated; the pathogen crosses the tubular epithelial barrier in the kidneys and its progress to bacteremia.

2.7.4.1.2 For Complicated UTIs

The uropathogens that cause complicated UTIs follow the same initial steps as those described for uncomplicated infections, including periurethral colonization, progression to the urethra and migration to the bladder. The most common cause of complicated UTIs, the compromised bladder is catheterization. Due to catheter and indwelling, it develops the inflammatory response, where infiltration of the neutrophil, multiplication of the uropathogenic and immune suppression and formation of biofilm takes place.

Epithelium cells get damaged and reach to the kidney and get colonized. The toxins and proteases induce the host cell damage. If left untreated; the pathogen crosses the

tubular epithelial barrier in the kidneys and its progress to Bacteraemia (Flores-Mireles *et al.*, 2015) (Saeed Khoshnood *et al.*, 2017).

2.8 Treatment of urinary tract infection

Assessment of a patient's earlier urinary pathogen and susceptibility profile is essential in selecting appropriate empiric antibiotic therapy. Prior microbiologic data improved the rate of accuracy of the empiric treatment against the pathogen for UTIs from 32% to 76% (Linsenmeyer K, 2015).

Treatment options for complicated UTI

Empiric quinolone therapy is suggested in clinical guidelines for complicated UTIs, the prevalence of quinolone-resistant Gram-negatives has increased during the last decade. Ceftolozane-tazobactam, a novel antibacterial with Gram-negative activity, is a promising treatment option for patients with complicated lower-UTIs and pyelonephritis (F.M. Wagenlehner *et al.*, 2015). The drug of choice in complicated UTI with Multidrug-Resistant infection includes carbapenems and piperacillin-tazobactam as first-choice antibiotics and combination therapy including colistin plus an aminoglycoside, colistin plus carbapenem, and carbapenem plus aminoglycoside as second- choice antibiotic therapy *(F.M. Wagenlehner et al.*, 2015).

- ***Treatment options for uncomplicated UTI***

Use of antibiotics for uncomplicated UTI shows in vitro resistance and significantly reduced response rates, which can lead to serious consequences, particularly for patients with pyelonephritis (E.B. Hirsch *et al*., 2016). There is a lack of published data on the current in vitro susceptibility profiles for drug-resistant Gram-negative uropathogens. However, the European Association of Urology (EAU) guidelines

recommended fosfomycin, nitrofurantoin TMP-SMX, trometamol and pivmecillinam as first-line therapy for uncomplicated UTI (I. Linhares *et al*., 2015).

❖ ***Treatment of ESBL-producing uropathogens***

The increasing prevalence of infections due to ESBL-producing gram-negative uropathogens limited the treatment options and caused a serious universal threat to public health. Doripenem, like other carbapenems, is resistant to hydrolysis by ESBLs and has great activity against ESBL-producing *E. coli* and *P. aeruginosa* isolates. Therefore it is a valuable option for the parenteral treatment of UTI, including pyelonephritis (Saeed Khoshnood *et al.*, 2017). Understanding the local susceptibility patterns for common Gram-negative pathogens is important to guide empiric antibiotic therapy for UTIs (Koningstein M *et al*.,2014). Ceftazidime–avibactam has recently been approved for the treatment of complicated UTIs and is effective against ESBL- producing Gram-negative bacteria (A.L. Flores-Mireles, J.N. Walker, M. Caparon, 2015). Eravacycline is a new fluorocycline compound that has a broad spectrum of activity against ESBLs- producing tetracycline-resistant Enterobacteriaceae. Furthermore, Plazomicin, a next-generation aminoglycoside derived from sisomicin, has been found to have a potent in vitro activity against ESBL-producing *E. coli* and *K. pneumoniae* (E. Cerceo *et al*., 2016).

2.8.1 Common antibiotics prescribed for the treatment of UTI

❖ **Nitrofurantoin**

Nitrofurantoin remains a reliable first-line agent for the empirical treatment of acute uncomplicated cystitis (Edwards MS, 2005b). *E. coli* resistance to nitrofurantoin among outpatient adult females with UTIs is low (0.9%) and have not changed in the period of 2003–2012 in the US (Sanchez GV *et al*., 2016).

The increasing resistance of ESBL-producing organisms against nitrofurantoin, particularly in hospital-acquired infections is a matter of concern. Risk factors of resistance to nitrofurantoin include male gender, hospital-acquired infection, resistant to other oral antibiotics including ciprofloxacin, and ESBL-producing organisms (Liu H-Y *et al*., 2011).

- **Trimethoprim–sulfamethoxazole**

Unfortunately, due to the high rate of resistance to TMP/SMX, in many communities, the drug is not anymore recommended as first-line empiric therapy for outpatients with uncomplicated cystitis, complicated UTIs, and acute pyelonephritis (Liu H-Y, Lin H-C, Lin Y-C, 2011). The resistance of *E. coli* to TMP/SMX has shown a considerable increase from 17.2% to 22.2% among outpatient adult females during the period of 2003–2012 in the United States (Sanchez GV *et al*., 2016).

In the regions where the resistance of TMP–SMX is found lower than 20%, it is not considered a reliable empiric treatment option for UTIs in patients with history of recent exposure to either TMP–SMX or ciprofloxacin, in patients with previous UTIs with history of ESBL-producing *E. coli* or to those with recent exposure to the area with high resistance rate of TMP-SMX while travelling (Karlowsky JA, Denisuik AJ, Lagacé-Wiens PR, 2014). It is also not recommended as an empiric treatment option for UTIs caused by ESBL producing *E. coli* or *K. pneumonia* and AmpC β-lactamase-producing *E. coli* as high levels of resistance, >66% and 35–40%, respectively have been observed (Oteo J, Bautista V, Lara N, 2010).

- **Fluoroquinolones**

Fluoroquinolones are the second-line drug of choice in case of uncomplicated cystitis and are also one of the treatment options for community UTIs and pyelonephritis

when in general resistance rate observed is less than 10% (Gupta K *et al.*, 2011). In both the community and hospital settings, the rise of fluoroquinolone resistance among urinary pathogens has found to be rising. During the period 2003-2012, the resistance of *E. coli* to ciprofloxacin among outpatient adult females increased from 3.6% to 11.8% in the United States (Sanchez GV *et al*.,2016).

Due to ESBL production among Enterobacteriaceae, these drugs are not suggested for empiric treatment of UTIs. In a report published in North America among hospitalized UTI patients, it was found that only 7% of ESBL-*E. coli* and 18% of ESBL- *K. pneumonia*, are susceptible to ciprofloxacin (Bouchillon SK *et al*., 2013). Infection with ESBL-producing organism particularly CTX-M-15 strain, urinary catheterization, recent hospitalization, hospital-acquired infection, patients, male gender, previous UTI, previous exposure to TMP–SMX, metronidazole, cephalosporins, fluoroquinolones are risk factors associated with increased fluoroquinolones resistance (Bader, Loeb and Brooks, 2017).

- **Cephalosporin**

Ceftriaxone is considered as sound empiric treatment option for community UTIs and acute pyelonephritis as the drug has been found to be susceptible for most of the uro-pathogens (Hooton TM, Bradley SF, Cardenas DD, 2010b). Ceftazidime exhibits parallel antibacterial action as ceftriaxone and is also recommended as an effective empiric treatment option for community UTIs and acute pyelonephritis (Karlowsky JA *et al*., 2014). Cefepime and ceftazidime also exhibits have alike antibacterial action. Cefepime is reasonably stable to AmpC hydrolysis, however, 96.6% AmpC β-lactamase-producing *E. coli* are susceptible to cefepime; Hence, it is recommended for treating cUTIs and pyelonephritis caused by the above-said microorganisms

(Blanchette LM, Kuti JL, Nicolau DP, 2014).

The use of ceftazidime–avibactam combination for treatment of cUTIs, as well as pyelonephritis among patients with limited alternatives has gained approval from the Food and Drug Administration (FDA). Avibactam, a semi-synthetic β- lactamase inhibitor, a non β -lactam is a diazabicyclooctane, highly active against Ambler class A, and some class D serine β-lactamases. Hence, avibactam by improving the antimicrobial activity of ceftazidime makes it highly effective against AmpC β-lactamases, ESBL-producing Enterobacteriaceae, ceftazidime-resistant E. cloacae, KPC-producing *K. pneumoniae*, and MDR *P. aeruginosa* (Blanchette LM *et al*., 2014).

- **β-Lactam and the β-lactamase inhibitor**

Amoxicillin and ampicillin should be avoided as empiric treatment for UTIs; however, these agents are effective as definitive therapy if the pathogen proves susceptible (Gupta K, Hooton TM, Naber KG, 2011)

The resistance rate of *E. coli* against ampicillin was 41% in outpatient adult females with uncomplicated cystitis. The percentage of resistance of *E. coli*, *K. pneumoniae*, and P. *mirabilis* to amoxicillin–clavulanate was 3.9%, 3.1%, and 0.8%, respectively (Sanchez GV, Babiker A, Master RN, 2016).

Although amoxicillin–clavulanate is considered a second-line agent for UTIs, the increasing prevalence of *E. coli* resistance to TMP–SMX and fluoroquinolones may be driving increased reliance on this agent for the management of cystitis. Piperacillin–tazobactam is a broad-spectrum antibiotic that has activity against Enterobacteriaceae and *Pseudomonas spp* (Hooton TM, Bradley SF, Cardenas DD, 2010b). The role of β-lactam/β-lactamase inhibitors in the treatment of infections

caused by ESBL producers is controversial. It should be noted that these organisms could confer resistance to piperacillin–tazobactam and amoxicillin-clavulanic acid by AmpC β-lactamases, efflux pumps, loss of porin channels, and alterations in penicillin-binding proteins (Tamma PD, Han JH, Rock C, 2015b) (Blanchette LM, *et al*., 2014).

- **Carbapenems**

In a study conducted in North America and Europe, it was found that Carbapenems broad-spectrum antibiotics retained the greatest level of activity against ESBL-negative Enterobacteriaceae an ESBL-positive *E. coli* (Vazquez JA, González Patzán LD, Stricklin D, 2012). Carbapenems are considered as a drug highly effective against most ESBL-producing strains and therefore has gained recommendation as first-line empiric therapy. These agents are used as voluntary targeted therapy in critically ill patients with severe infections when there is a threat or record of ESBL-producing Gram-negative bacteria(Tamma PD *et al.*, 2015a).

However, reduced susceptibility of *Pseudomonas spp*., *K. pneumoniae*, particularly ESBL-producing strain and *P.mirabilis* to carbapenems is becoming a matter of concern (Sader HS, Castanheira M, Flamm RK, 2016). Whenever possible the use of carbapenems must be terminated as any previous use of carbapenems is associated with colonization and infection due to carbapenem-resistant bacteria. However, non-carbapenem treatment options can be chosen in mild UTIs and to avoid treatment by carbapenems (Armbruster CE, Smith SN, Yep A, 2014).

- **Aminoglycosides**

Aminoglycosides, approved by the FDA for treatment of UTIs are almost solely renally excreted, achieve high urinary levels (Vidal L *et al*., 2007). Amikacin is

considered useful for empiric treatment of cUTIs and acute pyelonephritis. Role of amikacin for treatment of Amikacin is due to CRE as ceftazidime–avibactam has activity against some class D (e.g. OXA-48) serine β-lactamases and no activity against class B metallo-β-lactamases (Paño-Pardo JR *et al*., 2013).In order to reduce the risk of nephrotoxicity, a single dose of amikacin or in combination with other antibiotics can be used as an optional empiric treatment of cUTI or acute pyelonephritis in patients with threat of infection with antibiotic resistant organisms like ESBL-producing organisms or MDR *pseudomonas spp*. (Bader, Loeb and Brooks, 2017).

- **Gentamicin or tobramycin**

Gentamicin or tobramycin is not a feasible option for Gram-negative organisms such as ESBL-producing organisms and MDR-Pseudomonas spp due to high antibiotic-resistant. Similar to other antimicrobials, resistance to aminoglycosides is emerging in Gram-negative organisms and is commonly encountered among hospital-acquired ESBL produving *E. coli*, ESBL producing *K. pneumoniae,* and *Acinetobacter* spp. (Sorlozano A, Jimenez-Pacheco A, de Dios Luna Del Castillo J, 2014).

- **Polymixins**

Polymixins include polymixin B and polymixin E (colistin) also known as colistimethate, is given in the form of the sodium salt of colistin methanesulfonate. The polymixins are used as a final option treatment of MDR causing bacteria. Mode of administration of colistin is intravenous for to treatment cUTIs and acute pyelonephritis (Bader, Loeb and Brooks, 2017). Although the rate of resistance to colistin has remained low; with increased use, the emergence of resistance is observed in *A. baumannii, K. pneumonia, MDR-Pseudomonas,* and *E. coli*. The resistance to

colistin occurs as a result of alterations in lipid A as a consequence of chromosomal mutations and by MCR-1 gene that is carried on a plasmid and can transfer colistin resistance (Sorlozano A, Jimenez-Pacheco A, de Dios Luna Del Castillo J, 2014)

Approach to probable decreased failure rate and resistance comprises- optimizing dose, interval of dosage, and duration of therapy along with combination therapy (for carba- penemase-producing *K. pneumoniae* strains, pathogen exhibiting MIC greater than 1 mg/l, or patients with creatinine approval greater than approximately 80 ml/min) (Nation RL, Li J, Cars O, 2015).

- **Tigecycline**

Tigecycline is a glycylcycline antibiotic, a derivative of minocycline .however, the antibiotic Tigecycline is generally not recommended for treatment of UTIs because of low peak serum concentrations, limited urinal excretion, and simultaneous resistance development(van Duin D, Cober ED, Richter SS, 2014). As a safe and alternative option for UTIs, high dose of tigecycline (usually 200–400 mg followed by 100–200 mg daily) can be used to treat infections caused by organisms resistant to antibiotics. Although, tigecycline is not recommended as a monotherapy for the treatment of moderate to acute UTIs, mainly in case of bacteremia and must be used in combination with other antimicrobial agents such as carbapenems, colistin, or aminoglycosides (van Duin D, Cober ED, Richter SS, 2014).

Antimicrobial agents ceftazidime–avibactam has lately gained approval to be used in treatment of complex UTIs and is also efficient against ESBL- producing Gram-negative bacteria (A.L. Flores-Mireles, J.N. Walker, M. Caparon, 2015).

Eravacycline a novel fluorocycline compound has a broad spectrum activity against ESBLs- producing tetacycline-resistant Enterobacteriaceae. In addition, Plazomicin, a

next generation aminoglycoside derivative of sisomicin, has shown effective in vitro activity against ESBL-producing *E. coli* and *K. pneumoniae* (E. Cerceo *et al*., 2016).

2.9 Drug resistance in gram negative uropathogens

At present, UTIs is the most common infection as an encounter in the clinical practice, which is affecting people of all ages (Santo, Salvador, and Marin, 2007). As UTIs is estimated 150 million cases which occurs annually in the developing countries frequently, so many urological procedures need a treated without any effective antibacterial agent to against uropathogens (Prakash and Saxena, 2013). Even it states that uropathogens and their antibiotic susceptibility patterns are very important for the selection and use of antibiotics. In recent studies, the widespread use of an antibiotic has been increased for antibiotic resistance against uropathogens. World Health Organisation (WHO) reported that *E.coli* resistant the antibiotic such as cephalosporin and fluoroquinolone especially(WHO, 2014). The mortality rate of UTIs is higher due to bacteremia (strains), which are resistant to antibiotic classes, known as as multidrug-resistant (MDR) strain (Laupland *et al.*, 2007). Due to the emergence and spread of MDR Gram-negative pathogens, it increases in therapeutic challenges, as these are affected by the drug-resistant uropathogenic which is infected from community to the hospital setting and vice-versa, so it extended beyond the problems (Arana, Rubio and Alós, 2017), it concern that the increasing growth of drug-resistant uropathogenic may result in the problem in its treatment due to costs for treatment, or it may cause to death. Therefore the effective number of drugs is used for treatment

2.9.1 Mechanism of drug resistance Gram-negative uropathogens

Though resistance of uropathogens to antibiotics is reported globally, in the developing countries, UTI is always treated globally. Due to the traditional therapy, it has increased the spread of antibacterial in the uropathogens (Henry Oladeinde *et al.*, 2011). There are various mechanism of resistance to β-lactam agents in drugs-resistant Gram-negative uropathogens such as (Malhotra *et al.*, 2016). Different types of mechanism of drug resistance in gram-negative uropathogens are as follows:

2.9.1.1 Production of β-lactamase

Beta-lactam antibiotics are broadly divided as antibacterial agents, due to its favorable characteristics and conditions, so that drug is classified into broad range. It is classified by its structural and functional characteristics of the enzyme. It is classified by using amino acid sequence and divides the β-lactamase into the classes such as A, B, C, and D respectively. Class A enzyme represents the largest group of Beta-lactamases (serine β-lactamases) for the identification of extended-spectrum beta-lactamases (ESBLs), it expresses the Gram-negative bacilli frequently causing community-acquired the infection, such as UTIs.Class B enzyme can easily inactive the antibiotics such as carbapenems, cephalosporins, and penicillins so called as Metallo-β-lactamases (MBLs) (Palzkill, 2013).

Class C β-lactamases can easily spread among the Gram-negative organism due to catalytic cephalosporin efficiency (Ghafourian *et al.*, 2015)**.** The main enzymes of class C are AmpC β-lactamases. Class D enzyme has a higher hydrolysis rate for oxacillin than benzylpenicillin (Saeed Khoshnood *et al.*, 2017).

2.9.1.2 Target sites

The production of 16S rRNA methylases by bacilli has emerged with the high level of resistance mechanism to aminoglycosides. Methylating a nucleotide in the AG-binding is contributed to resistance by 16S rRNA methylases using S-adenosyl-methionine as co-substrate. In the worldwide, Enterobacteriaceae family member have been found with plasmid-mediated 16S rRNA (Sylvie Garneau-Tsodikovaa, 2016). Even the *Pseudomonas aeruginosa* and *Acinetobacter spp*. are found to be the production of methylation of guanine and methylation of adenosine, the range rate of 16S rRNA methylases from 0.03% to 95% shows in different reports due to the resistance level to aminoglycosides. In 2003 the first 16S rRNA methylases gene, armA was identified. armA and rmtB genes are frequent 16S rRNA methylase genes are seen in Enterobacteriaceae family, where these genes can transfer to the other bacteria (Ayad *et al.*, 2016) (Saeed Khoshnood *et al.*, 2017).

2.9.1.3 Efflux proteins

Bacteria have a system to pump out or efflux all the waste products and toxic substances, it occurs to both single and multi-component system. Pumping out the solute from cell called as efflux, it has a five types of efflux proteins which include major facilitator superfamily (MSF), resistance nodulation division (RND), ATP-binding cassette (ABC), multidrug/toxic compound extrusion (MATE) and small multidrug resistance (SMR**)** (Santajit and Nitaya, 2016)**.** Over-expression efflux pump can develop the mutations in local repressor genes in *E.coli* by the global transcriptional regulator(Sylvie Garneau-Tsodikovaa, 2016). Mutation influences the function and has a direct effect on uropathogens to an antibiotic, such effect is porin loss, size modification, reduced porin expression to resistance in *Pseudomonas aeruginosa* (Webber and Piddock, 2003).

2.9.1.4 Overproduction of enzymes

An enzyme substrate is an overproduction by *E.coli* due to the presence of sulphonamide resistance mechanisms. So sulphonamides mimic the para-aminobenzoic acid (PABA), where over production of antibiotics may have difficult to reach in their target. Therefore DHPS is inhibited by sulphonamide drugs, as an enzyme which involved and catalysis the reaction of PABA and dihydro-6-hydroxymethylpterin-pyrophosphate (DHPPP) to form dihyropteroic. DHPS or DHFR may overproduces resulting that sulphonamide resistance in *E.coli* isolate, where it may affect the expression of the extracellular enzymes (Saeed Khoshnood *et al.*, 2017).

2.9.1.5 Drug modification

Gram negative uropathogens can become resistance by modifing the expression of drugs or enzymes activity to the group of transferases, where it inactivates the enzyme such as chloramphenicol, aminoglycosides, streptogramin and rifampicin by substitution of the chemical as adenylyl, phosphoryl or acetyl group. For co-substrate activity, covalent has to modified to restricted in the cytoplasm (Saeed Khoshnood *et al.*, 2017) (Etebu and Arikekpar, 2016).

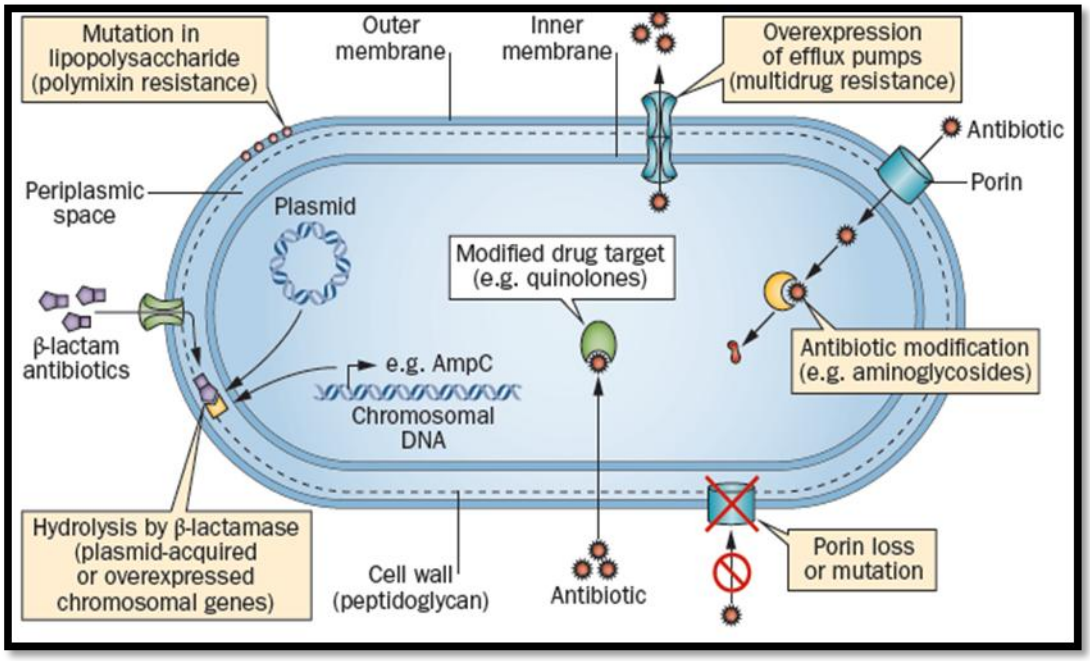


Figure 2: Antibiotic resistance mechanism in gram negative bacteria

Adopted after permission from a review entitled "The emerging threat of multidrug-resistant Gram-negative bacteria in urology" (Hosam M. Zowawi et al., 2015-Nature reviews)

2.10 Multi-drug-resistant Gram-negative bacteria

UTIs represent an important public health problem with a substantial economic burden as it is the most frequent infectious diseases affecting humans. Due to the massive use of antibiotics for the treatment of UTI, antibacterial resistance for Enterobacteriaceae, specifically *Escherichia coli* and *Klebsiella pneumoniae*, has significantly increased globally (Mazzariol, Bazaj and Cornaglia, 2017). Infections due to Gram-negative bacteria are causing morbidity and mortality worldwide, and the production of β-lactamase remains the most important contributing factor to β-lactam resistance (Koshesh *et al.*, 2017)

Currently, the rapid spread of resistance to β-lactam antibiotics among Gram-negative bacteria poses a clinical and public health challenge and increasing rates of drug

resistance among them is a major concern globally (Koshesh *et al.*, 2017). Monobactams, cephalosporins, penicillins, and carbapenems can be hydrolyzed by the enzymes of the β- lactamase group, which renders them microbiologically ineffective (Bush, K., and Mobashery, 1998). In the early 1980s, a new family of beta-lactamases namely Extended-Spectrum β-lactamases was identified and introduced that hydrolyze penicillins and expanded-spectrum cephalosporins have emerged (Kliebe *et al*.,1985). They are effective against β-lactam antibiotics like ceftazidime, ceftriaxone, cefotaxime, and oxyiminomonobactam, but not cephamycins or carbapenems ESBL production has been associated with Enterobacteriaceae, mostly *Klebsiella pneumoniae* isolated from Intensive care unit (ICU) patients; however, it is found also in a community setting and, more recently, other Gram-negative bacteria such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa*(Paterson, D. L., and Bonomo, 2005).

Currently, more than 600 ESBL variants have been described, the majority of which belong to the Cefotaxime hydrolyzing capabilities (CTX-M), Sulfhydryl variable (SHV) and Temoneira (TEM). Most of the detected ESBLs are either of SHV or TEM types and associated with nosocomial infections caused by Gram negative bacteria (Paterson, D. L., and Bonomo, 2005). In the current years, the spread of CTX-M-producing *E. coli* has been dramatic and they are considered the primary ESBL producers that are always associated with community-acquired infections (Leylabadlo *et al*., 2016).

In general, ESBL has been found all over the world. Earlier studies have reported that the prevalence of ESBL in Europe is higher than the United States, but lower than South America and Asia. Asia is almost certainly a part of the world, in which the

prevalence of ESBLs is very high among Gram Negative bacteria; however, different countries have different prevalence rates (Leylabadlo *et al.*, 2017).

2.10.1 Definition of ESBL producing bacteria

ESBLs are plasmid-encoded or chromosomally encoded β-lactamases with broad activity against penicillins and cephalosporins group of antibiotic. They are a diverse group of bacterial enzymes that break down and inactivate most β-lactam antibiotics. They are inhibited by the available β-lactamase inhibitors like clavulanic acid, sulbactam, avibactam, and tazobactam and do not affect cephamycins (e.g., cefoxitin and cefotetan) or carbapenems. β-Lactamases are divided into A, B, C, and D classes according to their amino acid sequence homology (Ambler classification) (Van Duin D, 2017). Enterobacteriaceae, primarily *Escherichia coli* and *Klebsiella pneumoniae*, are among the most frequently ESBL producing bacteria (Magiorakos AP, Srinivasan A, 2012).

These bacteria are usually multi-resistant, as they are frequently capable of resisting other antibiotics, such as the aminoglycosides, tetracyclines, and trimethoprim/sulfamethoxazole though other mechanisms, leaving few treatment options (de Kraker ME, Wolkewitz M, 2011). As these resistant genes are plasmid-mediated, they can be easily disseminated to other bacterial species (Padmini N, Ajilda AA, 2017). UTIs caused by these organisms are seen at alarming rates in both hospital infection and in the community settings (Padmini N, Ajilda AA, 2017).

Although 95–100% ESBL organisms are still considered sensitive to carbapenems, rapid emergence of carbapenem resistance has been documented globally, and was linked to the over usage of these agents (Briongos-Figuero LS, Gomez-Traveso T, 2012).

Arguably the most important development of the last two decades in the field of antibiotic resistance is the emergence and spread of extended-spectrum b-lactamases (ESBLs) of the CTX-M group(Hawkey PM, 2000).

Careful work by a group in Paris has shown that CTX-M resistance genes are present on the chromosomes of at least three species of Kluyvera, environmental bacteria closely associated with the rhizosphere (Humeniuk C, Arlet G, Gautier V, Grimont P, Labia R, 2002).

CTX-M was first recognized in 1989 in an isolate from a cancer patient of E. coli which was resistant to third generation cephalosporins. It was located on an 85 kilobase conjugative plasmid and the enzyme was originally designated MEN-1.6 Four major groups of genetically distinct but related genotypes of CTX-M have emerged: 1, 2, 25, and 9.7 Of the genotypes within these groups, two have been immensely successful, i.e. CTX-M 15 and to a lesser extent CTX-M 14 (Hawkey PM, 2009).

CTX-M 15 is the only genotype present throughout India. Bearing in mind the early recognition there of the ESBL phenotype and the genotyping of strains from the early 2000s, it seems most plausible that this gene emerged in the Indian subcontinent and has then spread throughout the world (Ensor VM, Shahid M, Evans JT, 2006).

CTX-M 14 was first described in Enterobacteriaceae from Guangzhou in Southern China in 1998 (Chanawong A, M'Zali FH, Heritage J, Xiong JH, 2002).

The study indentifying CTX-M 14 observed a very high rate (35%) of ESBL production among E. coli in Guangzhou and, by reasoning similar to that applied to CTX-M 15 in India, it is most likely that CTX-M 14 emerged in China or an adjacent

country during the mid-1980s, when locally produced cefotaxime was very widely used in hospital practice (Hawkey, 2015).

2.10.2 Diversity in ESBL Types

2.10.2.1 *SHV type*: It refers to sulfhydryl variable and was first reported in 1983 in *Klebsiella ozaenae*. They are the most frequently found ESBL type in clinical isolates than any other type. SHV refers to sulfhydryl variable (Christine K, 1985).SHV is a beta lactamase that originated *in K. pneumoniae*. It can be plasmid or chromosomal mediated (Blomberg B, 2005). The SHV-type of ESBLs is frequently found in clinical isolates than any other type of ESBLs. It is responsible for up to 20% of the plasmid-mediated ampicillin resistance in *K. pneumoniae*. The outbreaks of SHV-producing *Pseudomonas aeruginosa* and *Acinetobacter spp.* have also been reported worldwide and SHV-type ESBLs have been detected in a wide range of Enterobacteriaceae (Blomberg B, 2005). The majority of SHV variants possessing an ESBL phenotype are characterized by the substitution of a serine for glycine at position 238 and lysine for glutamate at position 240. The serine residue at position 238 is critical for the efficient hydrolysis of ceftazidime, and the lysine residue is critical for the efficient hydrolysis of cefotaxime.

2.10.2.2 *TEM:* TEM is a plasmid mediated beta lactamase that originated in *E.coli* TEM-1 is the most commonly encountered beta-lactamase in Gram-negative bacteria. Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1. TEM-1 is able to hydrolyze penicillins and early cephalosporins such as cephalothin and cephaloridine (Blomberg B, 2005). TEM-2, the first derivative of TEM-1 had a single amino acid substitution from the original beta-lactamase. But it did not change the substrate profile. TEM-3, originally reported in 1989, was the first TEM-type beta-lactamase that displayed the ESBL phenotype (Bradford PA., 2001).

More than 100 TEM type beta-lactamases have been described, the majority of which are ESBLs. When substitutions occur at that position a number of amino acid residues are important for producing the ESBL phenotype. They consist of glutamate to lysine at a position of 104, arginine to either serine or histidine at a position of 164, glycine to serine at position 238, and glutamate to lysine at position 240. The combinations of these amino acid changes result in various restrained alterations in the ESBL phenotypes. TEM-type ESBLs are the result of random selective pressure from several beta-lactams. Though TEM-type beta-lactamases are most often found in *E. coli* and *K. pneumoniae*, it is reported that they are also found in other species of Enterobacteriaceae such as *Enterobacter aerogenes, Morganella morganii*, *Proteus mirabilis*, *Proteus rettgeri*, and *Salmonella sp*p. and in *non-Enterobacteriaceae* like *P. aeruginosa* and *Capnocytophaga ochracea* (Asma M, 2006).

2.10.2.3 CTX - M: The *CTX* - M beta-lactamases reflects the potent hydrolytic activity of β-lactamases toward cefotaxime than ceftazidime (Kmtrc, 2016). CTX-M is a recently described family of the extended- spectrum beta-lactamases. The name CTX reflects the potent hydrolytic activity of these beta-lactamases against cefotaxime. These enzymes hydrolyze cephalothin better than benzylpenicillin and they preferentially hydrolyze cefotaxime over ceftazidime (Rice LB, 1990). Some organisms may harbor both CTX-M-type and SHV-type ESBLs or CTX-M-type ESBLs and AmpC-type beta-lactamases, which may alter the antibiotic resistance phenotype (Paterson DL, 2005). They have evolved separately, at least some of them via the escape and mutation of chromosomal β-lactamases of Kluyvera species (Livermore DM, 2004b). Another unique feature of these enzymes is that they are inhibited better by the beta-lactamase inhibitor tazobactam than by sulbactam and clavulanate. Serine residue at position 237, which is present in all of the CTX-M

enzymes, plays an important role in the extended-spectrum activity of the CTX-M-type beta lactamases. CTX-M type beta-lactamases may be the most frequent type of ESBLs worldwide. The number of CTX-M-type beta-lactamases is rapidly expanding. More than 40 CTX-M variants are currently known. Although, they are mainly found in strains of *Salmonella enterica* serovar *Typhimurium* and *E. coli*, but have also been seen in other species of *Enterobacteriaceae* like *P. mirabilis, E. cloacae, E. aerogenes* (Paterson DL, 2005).

2.10.2.4 OXA types: These β-lactamases are characterised by hydrolysis of cloxacillin and oxacillin greater than 50% that for benzylpenicillin and found predominantly in Pseudomonas aeruginosa (Kmtrc, 2016). The OXA-type enzymes are another family of ESBLs which arengrowing rapidly. The OXA-type beta lactamases are so named because they have the ability of hydrolyzing oxacillin. OXA types differ from the TEM and SHV enzymes as they belong to molecular class D and functional group 2d. They are characterized by their high hydrolytic activity against oxacillin and cloxacillin and the fact that they are poorly inhibited by clavulanic acid (Bradford PA., 2001). These confer frank resistance to cefotaxime and sometimes ceftazidime and aztreonam. The simultaneous production of a carbapenem-hydrolyzing metalloenzyme and an aztreonam hydrolyzing OXA enzyme can readily lead to resistance to all beta lactam antibiotics. They predominantly occur in Pseudomonas aeruginosa and Escherichia coli isolates (Paterson DL, 2005).

2.10.2.5 PER types: These types of ESBLs efficiently hydrolyze penicillins and cephalosporins. However, shares only 25- 27% homology with SHV and TEM types (Mavroidi A, Tzelepi E, Tsakris A, Miriagou V, Sofianou D, 2001).

2.10.2.6 VEB-1, GES-1, and other ESBLs: These are either plasmid-mediated or integron-associated class A enzymes (Bonnet R *et al*.,2000), (Mavroidi A, Tzelepi E,

Tsakris A, Miriagou V, Sofianou D, 2001)(Kmtrc, 2016). VEB stands for Vietnamese ESBL named so because it was found in Vietnam hospital (Poirel L, 1999). They confer high-level resistance to ceftazidime, cefotaxime, and aztreonam, and inhibited by clavulanic acid. VEB was first reported in *E. coli.* The gene encoding VEB was found to be plasmid mediated; such plasmids also confer resistance to non-beta lactam antibiotics. It is found in *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Enterobacter sakazakii, and Pseudomonas aeruginosa* (Kmtrc, 2016).

2.11 Prevalence of ESBL producing uropathogens

According to the recent reports of United States National Health Care Safety Network, Gram-negatives are responsible for approximately 30% of healthcare–associated infections, and these bacteria predominate (45%) in cases of UTIs. Furthermore, recently Global Prevalence of Infections in Urology studies (GPU) have reported that 10–12% of patients hospitalised in urology wards have a HAI (A.Y. Peleg, 2010a). UTIs are the second most common infectious presentation in community medical practice. Approximately 150 million people are diagnosed with UTI each year worldwide and lactose fermenters *E. coli* and *Klebsiella* are the most common pathogens associated with UTIs (R.F. Polse, S.Y. Yousif, 2016).

2.11.1 Global Scenario

- ***Europe***
  - The first report of ESBL producer strains was from Europe, specifically Germany (Knothe, H., et al., 1983). During the period of 1990s, nearly about 25 to 35% of nosocomial infections by *K. pneumoniae* isolates in France were found to be ESBL positive (Marty and Jarlier, 1998).

- In 2008, the frequency of ESBL among Enterobacteriaceae in France was on rise which increased the prevalence of CTX-M which was under 1%, in 1998 (Galas et al., 2008).

- In 2009, in a study conducted in Spain, Jesus Oteo et al., investigated the population structure of ESBL producing *Escherichia coli* and found that among Ninety-two ESBL-producing *E. coli* isolates from 11 Spanish hospitals. The predominant ESBLs in this collection were CTX-M-14 (45.7%), SHV-12 (21.7%) and CTX-M-9 (20.6%) (Jesus Oteo et al., 2009).

- In 2010, M. Carroll et al., in Italy found *E. coli* ST131 was prevalent and this clone invariably and carried a specific pattern of substitutions in the topoisomerase genes and one isolate produced CTX-M-15. One ST131 isolate produced SHV-12. The new ST131 variant described here is of particular concern because it combines fluoroquinolone resistance and chromosomally encoded CTX-M-15 (Carattoli, 2010).

- In 2011, a study was conducted in Sweden and it was found that 200 ESBL producing *E. coli* and *K. pneumoniae* isolates were collected over a 10-year period. Interestingly it was found that 87% were producing CTX-M, belonging to subgroup CTX-M-1 (64%), CTX-M-9 (34%), or CTX-M-2 (2%). The remaining isolates were producing variants of SHV and TEM. Sequencing of the $bla_{\text{CTX-M}}$ genes revealed 10 different CTX-M types, with a dominance of CTX-M-15 (*E. coli* 54%, *K. pneumoniae* 50%) followed by CTX-M-14 (*E. coli* 28%, *K. pneumoniae* 27%) (Anna Onnberg Paula, 2011).

- Between 2008 and 2011, the percentages of *E. coli* isolates resistant to third-generation cephalosporins significantly increased in 18 countries out of 28 reporting countries. No country showed a decreasing trend during this period. The corresponding rates for *K. pneumoniae* ranged from the 2% noted in Sweden to the incredibly high levels of 81% in Bulgaria. The most common ESBL type disseminated globally and associated with uropathogenic Enterobacteriaceae is the CTX-M-type ESBL.

- In 2012, approximately 97% of ESBL- producing *E. coli* reported from Europe and North America have been found to produce a CTX-M-type ESBL (Hoban, 2012).

- In 2013, in a study performed in 42 centres of Europe, antibiotic resistance among *E. coli* isolates was found to be on the rise and 15% of *E. coli* strains were ESBL- producing and were isolated from all countries. Turkey had the highest percentage with 25% of all ESBL-producing organisms. Drug-resistant *E. coli* were also isolated in Russia, Switzerland, Croatia and Bosnia and Herzegovina (N. Allocati, M. Masulli, M.F. Alexeyev, 2013).

- In 2016, a study on uropathogens was conducted among urinary tract infected patients in 41 centers in Poland. The most prevalent pathogen was *Escherichia coli* (71.4 %), followed by *Klebsiella* spp. (10.8 %) and the *Proteus* group (7.6 %). *Escherichia coli* were responsible for 80.6 % of cases of uncomplicated and 65.8 % of complicated infections. Only 65.8 % of *E. coli* isolates were susceptible to ciprofloxacin (uncomplicated 75.9 %, complicated 58.3 %), 64.0 % to nitrofurantoin (67.2 %, 62.8 %), 65.1 % to trimethoprim/ sulfamethoxazole (68.1 %, 62.8 %), and 66.4 % to

fosfomycin (77.6 %, 62.2 %). Thirty-five strains (10.4 %) were capable of producing extended-spectrum β-lactamases (ESBLs (E. Stefaniuk *et al*., 2016).

- In 2017, in a study performed in the United Kingdom, 380 uropathogenic *E. coli* ESBL-producing isolates were collected from the Leicester Royal Infirmary in Leicestershire, which harboured $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8d}}$, $bla_{\text{CTX-M-9}}$ and $bla_{\text{CTX-M-25}}$, bla_{SHV}, bla_{TEM} and bla_{OXA} and was detected by first multiplex PCR. Multiple resistance genes were detected in 10.2% of isolates. In the second multiplex assay, the order of prevalence of CTX-M genes were as follows: $bla_{\text{CTX-M-1}}$ (56%), $bla_{\text{CTX-M-9}}$ (11%), $bla_{\text{CTX-M-25}}$ (6%), $bla_{\text{CTX-M-8}}$ (0.5%) and $bla_{\text{CTX-M-2}}$ (0.2%) (Reid, R et al., 2017).
- The prevalence of ST131 *Escherichia coli* clone which is associated with global dissemination of ESBLs was found to be 20.5% (141/688), C1/*H*30R1 isolates were significantly more prevalent in Geneva (49%) and C2/ *H3*0Rxn in Madrid (67%). C1/H30R1 isolates showed resistance to amikacin than C2/H30Rx (4% versus 35%) and all were susceptible to penicillin/inhibitor combinations. CTX-M was the most common enzyme (49%) followed by CTX-M -27 (27%). C1/*H3*0R1 isolates were significantly associated with CTX-M-27 (72%) and all of these isolates belonged to C1- M27 clade (Irene *et al*., 2018).

❖ ***Asia***

- In 2009–2010, 28% of Enterobacteriaceae isolates from UTIs in eleven countries in Asia had extended-spectrum β-lactamases, and the proportion

of extended-spectrum cephalosporin-resistant isolates were 26–50% (S. Khoshnood *et al.*, 2017).

- In 2010, In Hong Kong, ESBLs were found in 7.3% *of E. coli* from uncomplicated community cystitis, including in 11/219 isolates from women aged 18-35 years, a much younger age group than typically affected by ESBL *E. coli* elsewhere (Ho PL, Yip KS, Chow KH, Lo JY, Que TL, 2010).
- In 2011, multiple surveys have shown that the highest ESBL rates for *E. coli* and *Klebsiella spp.* occurred in India (≥ 80%) and China (≥ 60%) (Hsueh PR, Badal RE, Hawser SP, 2011) with rates ≥ 30% elsewhere in East and Southeast Asia, Latin America, vs. 5-10% in North America (Hsueh PR, Badal RE, Hawser SP, 2011). Some studies have suggested that prevalence of ESBL in India is equally high in community and hospital *E. coli* isolates, probably reflecting free antibiotic use coupled with recycling of gut bacteria by an overloaded sewage infrastructure (Hsueh PR, Badal RE, Hawser SP, 2011).
- In 2012, in Nepal Baral et al., reported that among the significant bacterial growth 41.1% isolates were MDR out of 219 isolates. The most prevalent uropathogen was *E. coli* (81.3%, 178 isolates) and 38.2% was MDR. The second most common organism, *Citrobacter* spp. (5%, 11 isolates) was found 72.7% MDR. ESBL production was detected in 55.2% of MDR *E. coli* isolates. The most common plasmid of size 32 kb was detected in all of the plasmids harboring *E. coli* strains (Baral *et al.*, 2012).

- In 2013, a study was conducted in Arabian Peninsula and it was found that Gulf Cooperation Council (GCC), namely, Saudi Arabia, United Arab Emirates, Kuwait, Qatar, Oman, and Bahrain share a high prevalence of extended-spectrum-β-lactamase (ESBL) and most of which are associated with nosocomial infections. Well-known and widespread β-lactamases genes like CTX-M-15, OXA-48, and have found their way into isolates from the GCC states. However, less common and unique enzymes have also been identified which includes PER-7, GES-11, and PME-1 (Zowawi *et al.*, 2013).

- In 2014, alarming reports on the prevalence of drug-resistant Gram-negative bacteria in low- and middle-income countries in Asia, particularly the Indian subcontinent, have been published (S.J. Leopold *et al.*, 2014). The main resistance mechanisms in Asian countries include fluoroquinolone resistance and class A β-lactamase enzymes (NDM-1 and ESBLs). In Asia, there is an alarming number of antibiotic-resistant species, including, MDR-*A. baumannii*, ESBL- producing *K. pneumoniae* (CTX-M-9, CTX-M-14 and CTX-M-15), and NDM-1-producing Enterobacteriaceae. Particularly, in the Asian-Pacific region and in India, the prevalence of ESBL producing fluoroquinolone-resistant uropathogenic strains is extensively higher (S.J. Leopold, F. van Leth, H. Tarekegn, 2014).

- In 2015, several studies focused on the prevalence of ESBL producing UPEC, which is known to be associated with 90% of the community-acquired and approximately 50% of the nosocomial UTIs worldwide. Increasing prevalence of ESBL-producing Enterobacteriaceae in Asian

countries is an emerging threat to clinical therapeutics. In China, 50% of *K. pneumoniae* isolates produce ESBLs and ESBL-producing *E. coli* accounted for 70% of *E. coli* strains. Furthermore, ESBL-producing *E.coli* isolates increased to 60% in New Delhi (S. Dahiya, P. Singla, U. Chaudhary, 2015).

- In 2016 in Pakistan, over the last decade, although an increase in resistance against quinolones has been witnessed, however, not much is known about the fluoroquinolone resistance in ESBL producing UPEC strains (I. Ali, Z. Rafaque, S. Ahmed, S. Malik, 2016).
- Surveillance in Asia, Latin America, and Europe revealed dramatically increasing resistance to cephalosporins *among E. coli* and *Klebsiella* spp. (Zhang J, Zhou K, 2016). In outpatient populations, regardless of sex, surveillance data suggest that clinicians are more frequently facing antimicrobial resistance among uropathogens. The prevalence of drug-resistant *E. coli* among outpatient isolates in the U.S. increased from 9% to 17% from 2001 to 2010 (E.B. Hirsch. E. Walker, A. Lyman, K. Gupta, M.V. Mahoney, G.M. Snyder, 2016).
- In 2016, in a large study in Turkey (SMART), the rate of ESBL in *E. coli* isolated from urine samples was high (50% hospital isolates and 38% community acquired isolates (Koksal I, Yilmaz G, 2017). In one study, 21,414 positive urine cultures were collected from a University hospital in the UK. There were 1420 ESBL-positive specimens. There was 44% increase, from 4.6 to 6.6%, of the ESBL-positive organisms over 2 years. Multidrug resistance were detected in 75% of ESBL + *Klebsiella spp*. against >6 antibiotic classes (Toner L, Papa N, 2016). In the CHINET

surveillance system data from 2005 to 2014, ESBL production among *E. coli* isolates was between 51.7 and 55.8% (Hu FP, Guo Y, 2016).

- According to the data reported in 2017 , it is clear that Iran has a lower prevalence of MDR-UPEC compared to other Asian countries (such as India, Pakistan, Nepal, Saudi Arabia and Bangladesh) (S. Hadifar *et al.*, 2017). In Nepal, in 2017 incidence of urinary tract infection among pediatric patients was found to be 19.68% and Ecoli (68.4%) was leading pathogen involved. Out of 739 *E.coli* isolates, 64.9% were multidrug resistant (MDR) and 5% were extensively drug resistant (XDR). Extended spectrum beta lactamase (ESBL) was detected in 288 (38.9%) of the *E coli* isolates (Narayan Prasad Parajuli *et al*., 2017). In Nepal, of total 1568 different clinical samples processed, 268 (17.1%) samples were culture positive which included *E. coli* and *K. pneumoniae* as 138 (51.5%) and 39 (14.6%) numbers respectively. Of the total isolates 61 (34.5%) were ESBL producers indicating high rate of ESBL production was found in the *E. coli* and *K. pneumoniae* isolated from outpatients suggesting the dissemination of ESBL producing isolates in community (Fernando *et al*., 2017).

- Based on recent reports of 2018 from the USA, Japan, China, India, Saudi Arabia, Brazil, and Nepal, the prevalence of drug-resistant *E. coli* causing UTIs is on rise (Rajput and Sarsaiya, 2018). According to the European Centre for Disease Prevention and Control, antibiotic resistance caused about 25,000 deaths annually which were equal to about half the number of deaths in a road accident in Europe (Rajput and Sarsaiya, 2018). The mortality rate in patients with ESBL-producing UTIs has ranged from 42%

to 100% (W.H. Organization, 2014). In India, empirical use and easy availability of antibiotics over the country has led to widespread resistance among the isolates (Bharara,2018). The spreading of such isolates in the community is well documented, so control of this type of bacterial infection will be real challenging There were many outbreaks caused by these organisms all over the world with high morbidity and mortalities (Hamza and Khalil, 2018).

❖ ***America***

- In 2008, the prevalence of ESBL-producing Enterobacteriaceae in community-acquired urinary infections in southern Brazil was found to be 1.5%. A significant presence of CTX-M-producing species among strains isolated from community patients has been reported in other studies conducted in Brazil (Afonso Gomes Abreu *et al*., 2008).
- In 2009 in Canada, 18 multidrug-resistant CTX-M–producing *Escherichia coli* isolates were isolated from patients in Canadian intensive care units. The spread of the $bla_{\text{CTX-M}}$ genes among *E. coli* in Canada occurs through diversified different mechanisms and does not correspond to a single CTX-M determinant (Patricia J. Baudrya *et al.*, 2009).
- In 2010, in North America, uropathogenic *Escherichia coli* were found to be resistant towards ampicillin and sulfamethoxazole trimethoprim and one tenth of isolates of these Gram negative pathogens seem to produce extended spectrum beta lactamases (ESBL). About one third of hospital acquired *E. coli* and *Klebsiella* isolates are ESBL producers. Approximately 40% of *Pseudomonas aeruginosa isolates* are resistant

to ceftazidime, imipenem or levofloxacin (Amábile Cuevas, 2010). Healthcare-associated urogenital tract infections (HAUTI) are some of the most-frequently occurring HAIs (F. Wagenlehner *et al*., 2010). Gram-negative organisms account for about 70% of these types of infections in intensive care units in the U. S (A.Y. Peleg, 2010b).

- In 2012 in Brazil, ESBL production was detected in 12 (11.7%) *E. coli* and 4 (19%) *K. Pneumonia*. They were isolated out of 102 *E.coli* and 21 *Klebsiella* isolates respectively. TEM ESBLs were detected in seven *E. coli* and three *K. pneumoniae* isolates. SHV ESBLs were found in four *K. pneumoniae* isolates followed by CTX-M-1 phylogenetic subgroup was positive in seven *E. coli* and three *K. pneumoniae* isolates (Pedro Martinez, Denisse Garzón, 2012).

- Moreover, in 2013, the TEM- and SHV-type ESBLs have predominated among hospital-acquired bacteria, and this is still the case in the U. S (Villar HE, Aubert V, 2013).Of total 1568 different clinical samples processed, 268 (17.1%) samples were culture positive still the case in the U. S (K.M. Papp-Wallace *et al*.,2013). UTIs caused by gram-negatives have features that are of main threat in American countries. They are highly efficient at acquisition and up-expression of genes that code for mechanisms of resistance to antibiotics (E.N. Berezin, 2014).

- In 2015 Abubaker A. et al, reported that uropathogens were found in 371 (20.7%) urine specimens examined. *E. coli* and *Klebsiella* spp. were the predominant uropathogens at 55.8% and 18.5% respectively. Other uropathogens were detected in 25.7% of total urine samples. Of the *E. coli* and *Klebsiella* spp. tested, 69.2 and 100% were resistant to ampicillin, 6.7

and 33.3% to ceftriaxone, and ciprofloxacin, respectively. MDR was found in 69 (33.2%) of *E. coli* and in 29 (42%) of *Klebsiella pneumonia* isolates. ESBLs were detected in 14 (6.7%) of *E. coli* and in 15 (21.7%) of *Klebsiella* spp. Isolates by phenotypic detection methods. bla_{OXA} gene was found to be present in 10 isolates followed by bla_{TEM} gene in seven isolates, and $bla_{\text{CTX-M}}$ gene in six isolates. bla_{SHV}gene was not detected in the present study (Abubaker A. *et al*., 2015).

- In 2016, it has been documented that in outpatient populations, regardless of sex, surveillance data suggest that clinicians are more frequently facing antimicrobial resistance among uropathogens. For instance, the prevalence of drug-resistant *E. coli* among outpatient isolates in the U.S. increased from 9% to 17% from 2001 to 2010 (E.B. Hirsch E *et al*.,2016).
- A molecular survey was conducted in Cochabamba, Bolivia in 2017 to characterize the mechanism involved in the resistance to clinically relevant antibiotics. Extended Spectrum β-lactamase encoding genes were investigated in a total of 101 oxyimino-cephalosporin-resistant enterobacteria recovered from different health centres during four months. CTX-M enzymes were detected in all isolates, being the CTX-M-1 group the most prevalent (88.1%) (D.M. Arana, M. Rubio, J.I. Alos, 2017).
- In 2018, out of 12 community-associated *E coli* isolates tested in America, all were confirmed to harbor ESBL genes; the *CTX-M1* β-lactamase gene was found in 8 (67%) isolates which belong to genotype ST131 (Bradley W. *et al*., 2018).

❖ ***Africa***

- In 2011 in Morocco, out of 803 numbers of *Escherichia coli* (*n*=767) and *Klebsiella pneumoniae* (*n*=36) isolates collected from patients with urinary tract infections in three Moroccan cities, 10 *E. coli* (1.3%) and 2 *K. pneumoniae* (5.6%) isolates were shown to produce ESBLs. PFGE revealed that the *E. coli* isolates comprised seven distinct genotypes. Nucleotide sequencing indicated that the plasmids encoded the *bla* $_{CTXM}$, *bla* $_{OXA}$, *bla* $_{TEM}$ and *bla* $_{SHV}$ genes, including genes for CTX-M-15 (*n*=11), OXA-1 (*n*=11), TEM-1b (*n*=4), SHV-5 (*n*=1) and SHV-1 (*n*=2) (Abouddihaj Barguigua, *et al*., 2011)**.**

- In 2016 in Libya, out of the 1153 urine samples tested, 160 (13.9%) samples were positive, from which 17 different, solely Gram negative, uropathogens were identified. *Escherichia coli* were the most prevalent (55.6%) bacteria, followed by *Klebsiella pneumoniae* subspecies *pneumoniae* (16.3%), *Proteus mirabilis* (6.3%), *Pseudomonas aeruginosa* (5.6%), *Enterobacter cloacae* and *Klebsiella oxytoca* (2.5%, each), *Citrobacter koseri* and *Providencia rettgeri* (1.9%, each), *Acinetobacter baumannii*, *Enterobacter aerogenes* and *Proteus vulgaris* (1.3%, each), and *Aeromonas caviae*, *Citrobacter freundii*, *Cronobacter sakazakii*, *Enterobacter amnigenus biogroup* , *Pseudomonas putida* and Serratia marcescens (0.6%, each).

- The isolated uropathogens showed increased levels of resistance ranged from 10.5% to 64.5%, with an overall resistance of 28.9%. Amikacin was the most effective antimicrobial followed by Imipenem and Meropenem

with resistance percent of 0%, 0.6% and 2.5% resistance, respectively (Mohammed *et al.*, 2016).

- In 2018, in Nigeria, five hundred urine samples were analyzed. Out of these, a total of 175 Gram-negative isolates were obtained with a prevalence rates of *E. coli* as 56% followed by *K. pneumoniae* (20%,) *Proteus mirabilis* (16%) and *Pseudomonas aeruginosa* (4%). ESBL production was observed in 34.3% of all the isolates. Fifty percent (50%) of *E. coli* and 40% of *K. pneumoniae* were identified as ESBL producers and were found to be resistant to multiple antimicrobial agents. Imipenem and nitrofurantoin had sensitivity of 100% and 70%, respectively, while susceptibility to ciprofloxacin and gentamicin was low at 35% and 30%, respectively and 96% sensitivity was observed with amikacin (Fatima Jummai Giwa *et al*., 2018).

- In 2018 in Algeria, *Enterobacteriaceae* were identified in 1,561 analysed samples (87%). *Escherichia coli* were the dominant uropathogen (66.15%) in both hospitalized and non-hospitalized patients. The other main detected *Enterobacteriaceae* memberswere *Klebsiella pneumoniae* (11.96%) and *Proteus mirabilis* (5.42%). Analysis of results showed also that women were more prone to UTI than men. The susceptibilities of isolated *Enterobacteriaceae* to antibiotics revealed that they had acquired resistance to several classes, particularly toward β-lactams. Resistance frequencies were relatively high to ampicillin and sulfamethoxazole, while being very low to aminoglycosides and furans. Results obtained revealed that 7% of isolates were resistant to third

generation cephalosporins by the production of extended spectrum β-lactamases (ESBL) (Fatima Jummai Giwa *et al*., 2018).

2.11.2 Indian Scenario

- In 2008, in South India, a total of 23 clinical isolates (*Escherichia coli* n=15 and Klebsiella *pneumoniae* n=8) resistant to cefotaxime and ceftazidime were investigated for the production of CTX-M extended spectrum β-lactamase (ESBL) by phenotypic and molecular methods. Polymerase chain reaction revealed the presence of CTX-M type ESBLs in 19 isolates. Further sequencing identified the CTX-M-15 type of ESBLs (Padmini S B, Raju B A, 2008).
- In 2012 in Mumbai, a total of 195 gram negative urine isolates were identified as *Pseudomonas aeruginosa* (n=13), *Proteus mirabilis* (n=21), *Klebsiella pneumoniae* (n=29), *Escherichia coli* (n=96), *Enterobacter aerogenes* (n=1), *Enterobacter cloacae* (n=1), *Enterococcus fecalis* (n=1), *Morganella morganii* (n=1), *Citrobacter diversus* (n=16), *Citrobacter amalonaticus* (n=5) and *Proteus vulgaris* (n=11). Antimicrobial Susceptibility Testing showed that 43.07 % (84/195) of the isolates were resistant to more than 70 % of the antibiotics used. Overall prevalence of ESBL producing bacteria was found to be 34.71 % (68/195). The study showed 72.05 % of the ESBL producers to be resistant to fluoroquinolones, showing its extensive use in the region of south Mumbai (Aruna and Mobashshera, 2012).
- In 2013 in Kolkata, Forty UPEC was isolated from 200 urine samples of clinically UTI suspected hospitalized patients. All the 40 isolates except

three were multidrug resistant. They showed the highest sensitivities for nitrofurantoin (72.5%) and amikacin (70%). A high level of resistance was observed against ampicillin (97.5%), nalidixic acid and cefelexin (95%), amoxicillin (92.5%), cotrimoxazole (82.5%) and ciprofloxacin (80%) respectively. Thirty different antibiotic resistance patterns were observed against the different antibiotics. Twenty-eight out of the 40 isolates were resistant to the third generation cephalosporins. However, the phenotypic test for the ESBL confirmation indicated that eighteen out of the twenty-eight isolates were ESBL producers (Mukherjee M, Basu S, Mukherjee SK, 2013).

- In 2014, in Madhya Pradesh a significantly higher number of IPD and OPD patients (55.1% and 55.5%) were found to be urine culture positive. *Escherichia coli* (55.3%) was the most frequently isolated uropathogen followed by *Klebsiella pneumoniae* (23%). However, strains of *Escherichia coli* (41.6%) were the highest ESBL producing isolates followed by *Pseudomonas aeruginosa* (36.1%). ESBL producing isolates were found to be multidrug-resistant when compared to non-ESBL producers (Bajpai T, Pandey M, Varma M, 2014) Out of 15 ESBL-producing *E. coli* isolates obtained from urine samples , 15 *E.coli* isolates harboured the $bla_{\text{CTX-M-15}}$ gene.(Dureja Chetna, Mahajan Sakshi, 2014).
- In 2016, in a tertiary hospital of North East India a study was conducted where 85 multidrug-resistant isolates of *E. coli* was obtained from clinical samples and were screened for resistance to third generation cephalosporins. Imipenem was found to be most effective against *E. coli* (S=96.47%) while ciprofloxacin was the least effective antibiotic (R=

60%). The most prevalent gene was *bla*$_{SHV}$ (63.04%) followed by *bla*$_{TEM}$ and *bla*$_{CTX-M}$ (60.86 and 54.34%, respectively) in ESBL of the extensively used antibiotics, appear to be ineffective against the ever-mutating bacteria.(Kumar *et al.*, 2016).

- In 2017 Bajpai *et al*. observed that *bla*$_{TEM}$ as the dominant ESBL type followed by *bla*$_{CTX-M}$ and *bla*$_{SHV}$ in tertiary hospital in central India among male and female UTI patients of Central India (and G. S. Bajpai, T., M. Pandey, M. Varma, 2017).
- In 2018, in a study conducted in Madhya Pradesh, it was found that the prevalence *of E. coli* in all uropathogenic microbial strains among the 500 samples studied was 68.8%. Out of these, more than half (60%) were reported resistance for all major antibiotics. *E. coli* was reported as a major MDR and ESBL (2% of MDR) producing uropathogenic microbial strains associated with onset and progression of infection in Bhopal city (Shrivastava, Gupta and Tripathi, 2018).
- In 2018 in Tripura, out of 150 clinical isolates from patients of the tertiary care hospital, 85 strains were identified as *E. coli* strains. Out of 85 isolates, 52 were detected as ESBL producers. Molecular analysis of the ESBL gene indicated predominance of *bla*$_{TEM}$ 42% followed by *bla*$_{CTX-M}$ 35%, *bla*$_{OXA}$ + *bla*$_{TEM}$ 15% and *bla*$_{CTX-M}$ +bla$_{TEM}$ 8% respectively (Dasgupta Arunabha *et al.*, 2018).

2.12 Mechanism of ESBL resistance

The most common mechanisms of ESBL resistance include enzymatic inactivation, target modification, reduced permeability, and active efflux. Antibiotic resistance can

be intrinsic to specific microorganisms, which can be explained by their inherent characteristics. Point mutations on β-lactamase genes are responsible for emergence of ESBLs. These new genes could be transmitted through small mobile genetic element DNA to other bacteria from same or other species (Majeed A, Alarfaj S, 2017). A more distinct type of ESBL including CTX-M-type, AmpC, and carbapenemase, can confer phenotypic resistance that widens the resistance abilities against more antibiotics than the classical isolates (Zowawi HM, Balkhy HH, 2013)

2.13 Necessity of ESBLs detection:

Detection of ESBLs is important because:

- They destroy cephalosporins, antibiotics which are given as first-line agents to many severely ill patients including those with intraabdominal infections, community-acquired pneumonia and bacteremia.
- They delay recognition and inappropriate treatment of severe infections caused by ESBL producers with cephalosporins has been associated with increased mortality.
- Many ESBL producers are multi-resistant to non-β-lactam antibiotics such as quinolones, aminoglycosides and trimethoprim, and leads to narrowing treatment options.
- Some producers achieve outbreak status, spreading among patients and locales, perhaps owing to particular pathogenicity traits (Livermore DM, 2004a).

2.14 Methods for ESBL detection

The basic strategy to detect ESBL producers is to use an indicator cephalosporin to screen for ESBL producers, then to seek cephalosporin/clavulanate synergy, which

distinguishes ESBL producers from hyper producer AmpC or K1 enzymes (Livermore DM, 2004a).

2.14.1 Screening test for ESBL detection

Screening for ESBL detection includes the following steps:

2.14.1.1 Screening by disk diffusion methods: The CLSI has proposed disk diffusion methods for screening for ESBL production by bacteria. Mostly all laboratories use disk diffusion methods for antibiotic susceptibility testing. They can be screened for ESBL production by noting specific zone diameters that indicate a high level of suspicion for ESBL production. Antibiotics like Cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone are used for screening. The use of more than one of these agents for screening improves the sensitivity of detection. If any of the zone diameters indicate doubt for ESBL production, phenotypic confirmatory tests should be performed to ascertain the diagnosis (Morris D, 2006).

2*.14.1.2 Screening by dilution antimicrobial susceptibility tests:* The CLSI has proposed dilution methods for screening for ESBL production by Enterobacteriaceae. Ceftazidime, aztreonam, cefotaxime, ceftriaxone and cefpodoxime can be used at a screening concentration of 1 mg/ml. If growth at this screening antibiotic concentration is found to be ≥2 mg/ml (MIC of the cephalosporin of ≥2 mg/ml) they are suspicious of ESBL production and is an indication for the sted by a phenotypic confirmatory test (Consensus guidelines for the management of infections by ESBL producing bacteria, 2001).

2.14.2 Phenotypic confirmation test for ESBL detection

Phenotypic confirmation test for ESBL detection includes the following steps:

2.14.2.1 Disc Diffusion Test (PCDDT): The CLSI advocates use of cefotaxime (30 μg) or ceftazidime disks (30 μg) with or without clavulanic acid (10 μg) for phenotypic confirmation of production. Disks for use in phenotypic confirmatory tests are available commercially. The CLSI recommends that the disk tests be performed with confluent growth on Mueller-Hinton agar. A difference of ≥5 mm between the zone diameters of either of the cephalosporin disks and their respective cephalosporin/clavulanate disk is taken to be phenotypic confirmation of ESBL production (CLSI, 2001).

2.14.2.2 Broth microdilution: Phenotypic confirmatory testing can also be performed by broth microdilution assays using ceftazidime (0.25 to 128 μg/ml), ceftazidime in combination with clavulanic acid (0.25/4 to 128/4 μg/ml), cefotaxime (0.25 to 64 μg/ml), and cefotaxime in combination with clavulanic acid (0.25/4 to 64/4 μg/ml) (Paterson DL, 2005).

2.14.2.3 Double Disk Synergy Test (DDST): The Jarlier double disk approximation or double disk synergy was the first detected and described in the 1980's. DDST is a disk diffusion test in which 30 μg antibiotic disks of ceftazidime, ceftriaxone, cefotaxime and aztreonam are placed on the Petri plate, 30 mm (center to center) from the amoxicillin/clavulanate (20μg/10μg) disk. A clear extension of the edge of the antibiotic's inhibition zone toward the disk containing clavulanate is interpreted as synergy which indicates the presence of an ESBL (Paterson DL, 2005).

2.14.3 Commercially available methods for ESBL detection

Commercially available methods for ESBL detection include the following steps:

2.14.3.1 *E-test for ESBLs*

AB Biodisk (Solna, Sweden) produces plastic drug- impregnated strips, one end of which contains a gradient of ceftazidime (MIC test range 0.5 to 32 μg/ml) and the other with a gradient of ceftazidime in combination with a constant concentration of clavulanate (4 μg/ml). These strips are useful for both screening and phenotypic confirmation of ESBL production. A ≥8-fold reduction in cephalosporin MICs in the presence of clavulanate is indicative of the presence of ESBL (Vercauteren E, 1997). Strips containing Cefotaxime and cefotaxime/clavulanate are now available.

2.14.3.2 *The Vitek ESBL test*

It utilizes cefotaxime and ceftazidime, alone (at 0.5 μg/ml), and in combination with clavulanic acid (4 μg/ml). Inoculation of the cards is similar to that performed for regular Vitek cards (bioMerieux Vitek, Hazelton, Missouri). Analysis of all wells is performed automatically once the growth control well has reached a set threshold (4 to 15 hours of incubation). A predetermined reduction in the growth of the cefotaxime or ceftazidime wells containing clavulanic acid, compared with the level of growth in the well with the cephalosporin alone, indicates a positive result (Sanders CC, 1996).

2.14.3.3 BD Phoenix Automated Microbiology System

Becton Dickinson Biosciences (Sparks, Md) have introduced a short-incubation system for bacterial identification and susceptibility testing, known as BD Phoenix. The Phoenix ESBL test uses a growth response to cefpodoxime, ceftazidime, Ceftriaxone and cefotaxime, with or without clavulanic acid, to detect the production of ESBL (Turng B, 2002).

2.14.4 Molecular methods for ESBL detection

The different tests that are described above only presumptively identify the presence of an ESBL; however, with the advent of molecular technology determination of the specific ESBL at the nucleic acid level is made possible (Bradford PA., 2001).

- Early detection of beta-lactamase genes was performed using DNA probes that were specific for TEM and SHV enzymes. However, using DNA probes can rather labour demanding. The first molecular method for the identification of beta-lactamase was the oligotyping method which was developed by Ouellette et al., and was used to discriminate between TEM-1 and TEM-2, this method used oligonucleotide probes that are designed to detect point mutations under stringent hybridization conditions (Bradford PA., 2001).

- Pulsed-field gel electrophoresis of chromosomal DNA is probably the most widely used technique of genotyping ESBL-producing organisms (Paterson DL, 2005). Ribotyping, a Southern blot analysis in which strains are characterized by the restriction fragment length polymorphisms, which is potentially very useful in typing ESBL producing organisms. The easiest and most common molecular method used to detect the presence of a gene coding for beta-lactamase enzymes is by Polymerase Chain Reaction (PCR) using specific oligonucleotide primers.

- PCR will not discriminate among different variants of TEM or SHV, however, Multiple variations of PCR have been applied to the typing of ESBL-producing organisms (Bradford PA., 2001).

- Restriction site insertion PCR is a recently developed technique used for detection of mutations of the SHV genes which uses amplification primers designed with one to three base mismatches near the 3b end to engineer a desired

restriction site. Combination of PCR- restriction fragment length polymorphism and restriction site insertion PCR techniques can be readily applied to the epidemiological study of SHV beta-lactamases.

- Another method used to characterize SHV-type ESBLs is PCR single-strand conformational polymorphism (PCR-SSCP) analysis. This method has been used to detect a single base mutation at specific locations within the gene (Bradford PA., 2001).

- Ligase chain reaction is a recently developed technique also used to discriminate SHV variants. This technique uses a thermostable ligase and biotinylated primers. It can detect single base pair changes (Paterson DL, 2005)

- Pyrosequencing has been suggested as a method for rapid identification of few ESBL types (Poirel L, 1999). Nucleotide sequencing remains the standard for determination of the specific beta-lactamase gene present in a strain (Bradford PA., 2001).

2.15 Incompatibility typing of plasmids harboring ESBL genes

A plasmid is defined as a double-stranded, circular DNA molecule capable of independent replication. By definition, plasmids do not carry genes essential for the growth of host cells under non-stressed conditions but they have systems which assure their autonomous replication also controlling the copy number and ensuring stable inheritance during cell division. Generally, all the plasmids confer positively selectable phenotypes by the presence of antimicrobial resistance genes. Plasmids evolve as an important part of the bacterial genome, providing resistance genes that can be easily exchanged among bacteria of different origin and source by conjugation. A multidisciplinary approach is currently applied to study the acquisition and spread

of antimicrobial resistance in clinically important bacterial pathogens and the established surveillance can be implemented by replicon typing of plasmids. Plasmid families are more frequently detected among Enterobacteriaceae and plays major role in the diffusion of specific resistance genes. For instance, IncFII, IncA/C, IncL/M, IncN and IncI1 plasmids carrying extended-spectrum beta-lactamase genes and acquired AmpC genes are currently considered to be "epidemic resistance plasmids", as they are detected worldwide in Enterobacteriaceae of different origin and sources. The occurrence of IncX type plasmids among enterobacterial populations has reported which comprises least four subtypes IncX1-IncX4 (Johnson *et al*., 2012).

Plasmids bekonging to the IncL/M family are mostly responsible for the spread of $bla_{\text{CTXM-3}}$ gene in the European eastern countries, France, Belgium and Korea and very often the aminoglycoside resistance genes armA has been co-localized on the same IncL/M plasmid with the $bla_{\text{CTXM-3}}$ gene (Golebiewski *et al*., 2007). The European spread of the $bla_{\text{CTXM-9}}$ gene in the clinical *E.coli* and *S.enterica* strains was largely due to the dissemination of plasmids of the InCHI2 grou p (Novais *et al*., 2006). Plasmids of th the IncHI2 group were also associatrd with the dissemination of the $bla_{\text{CTXM-2}}$ gene in the animal reservoirs (García Fernández *et al*., 2007).

The recognition of successful plasmids is an essential first step to design intervention strategies preventing their spread (Alessandra Carattoli, 2011).

The identification and spread of plasmid-encoded ESBL genes has recently changed the degree of the problem in Gram-negative bacteria. Especially the rapid and extensive worldwide dissemination of carbapenem-resistance is associated with bacterial infection in human. This transfer increase bacterial genetic diversity,

acquiring and losing genes and may be useful to their bacterial host such as antimicrobial resistance or virulence genes (Amabile-Cueves and Chicurel, 1992).

The *bla*$_{\text{CTX-M}}$ genes were found primarily located on diverse IncF plasmids of multiple replicon types downstream of the ISEcp1 element. The spread of the *bla*$_{\text{CTX-M}}$ genes among *E. coli* in Canada occurs through a diverse different mechanisms and does not correspond to a single CTX-M determinant, or a single clone, or a single plasmid but rather through the combination of clonal spread of virulent strains and acquisition of diverse CTX-M–bearing plasmids (Baudry *et al.*, 2009).

Ingti et al 2017 detected DHA-1 genes from clinical isolates obtained from Silchar Medical College. DHA-1 genes were detected in plasmid and incompatibility typing from the transformants indicated that the plasmid encoding *bla* $_{\text{DHA-1}}$ was carried mostly by the FIA and L/M Inc group (Ingti *et al.*, 2017). He also detected *bla*$_{\text{CMY-42}}$ on IncI1-type plasmids, which are considered to be the main vehicles for the spread of *bla*$_{\text{CMY-42}}$ in this hospital setting (Ingti *et al.*, 2018).

In addition to vertical gene transfer, the ESBL genes located on plasmids could disseminate through horizontal gene transfer among different species of bacteria contributing to the global spread of ESBL-resistant bacteria. Horizontal gene transfer is a driving force behind the evolution of bacteria. Horizontal transfer of plasmid that encoded ESBL plays an important role in the spread nosocomial and community antibiotic-resistant infections worldwide by multi drug resistance infections. Horizontal transmission of ESBL genes is often accompanied by transmission of flanking genes which confers resistance to other antimicrobials.

2.16 Stability of ESBL genes

The widespread dissemination of ESBL producing organisms in healthcare settings is a rising global public health threat. The survival of resistance plasmids in the absence of selective pressure for the antibiotic resistance genes they harbor is important for assessing the value of interventions to combat resistant bacteria. The fitness of an antibiotic-resistant bacteria is determined by the relative rates at which resistant and sensitive bacteria i) grow and die in hosts and environment ii) are transmitted between hosts and iii) are cleared from infected hosts (Anderson *et al*., 1999). The biological relevance of fitness cost depends not only on the growth rate but also on the ability of its transmission and colonization of the resistant strain (Petersen *et al*., 2009). Bacterial resistance can sometimes be accompanied by a decrease in biological fitness in the absence of antibiotic selection. The less fit resistant bacteria may be outcompeted and removed by fitter, susceptible bacteria in the absence of antibiotic use, leading to the suggestion that it may be possible to lessen the occurrence of antibiotic resistance for the interim by restricting prescribing. It is likely that the fitness cost of a particular antibiotic resistant plasmid confers on a given bacterial species is variable depending on the plasmid as well as on the genotype of the host strain (Humphrey *et al*., 2012). The three ways by which a bacterial host can neutralize the potential fitness cost exerted by antibiotic resistance genes carried on mobile genetic elements; the first is to acquire compensatory mutations, and the second one is an outright loss of the mobile genetic element. The bacteria could also switch off the expression of resistance genes when they are not required whilst retaining the genes themselves in order to lower costs, that could be a third possibility. Plasmids can carry multiple resistance genes that can disseminate multidrug-resistant gene through horizontal gene transfer in and between bacterial populations. After,

successful transfer events, the stability and persistence of newly acquired plasmids largely depend on the fitness cost imposed on new hosts in the absence of selective pressure on traits harbored by the plasmid. The fitness cost of plasmid carriage depends on the individual plasmid-host combinations. When the time of horizontal transfer outweighs the costs involved in plasmid carriage to a cell plasmids are favored. It is due to the reason that carrying a plasmid and expressing resistance has a cost which has to be offset by the benefit of infecting susceptible cells. In the absence of antibiotics, hosts which carry multiple copies of resistance genes would be at a disadvantage due to the extra cost of producing more antibiotic resistance proteins.

2.17 Co-resistance genes among ESBL producing isolates

The sudden rise in the prevalence of antibiotic resistance and the lack of new antibiotic drug development has gradually reduced the treatment options for bacterial infections (Lee C, Cho I, Jeong B, 2013). Extensive use of antibiotics plays a crucial role in the emergence of antibiotic resistance among gram-negative bacteria worldwide (Singh *et al.*, 2018). Due to the massive selective pressure of antibiotics, and the presence of various ESBLs in different countries ß-lactamases are remarkably diversified. Since ESBL genes are mostly plasmid mediated, it may also harbor genes encoding resistance to other class of antibiotics, such as aminoglycosides, macrolides, chloramphenicol, quinolone or carbapenems. Due to the presence of the multiple resistance genes encoded in the plasmids, treatment options become restricted for ESBL producing bacteria (Alyamani *et al.*, 2017).

The emergence and rapid distribution of ESBL producing bacteria which are capable of hydrolyzing penicillins, broad-spectrum cephalosporins, and monobactams, also harbor resistance genes for other antibiotics, thus making carbapenem limiting treatment options for infections over the last couple of decades (Shibl *et al.*, 2013).

Carbapenemase-producing *E. coli* are of major clinical concern (Gajmer *et al.*, 2018). It has been reported that carbapenem-resistant Enterobacteriaceae causes mortality by up to 50% of patients who acquire bloodstream infections (Okoche *et al.*, 2015). As a consequence of this, increased utilization of carbapenems has led to the emergence of isolates with resistance genes that encode for carbapenem-resistant genes. AmpC harboring strains are challenging as it confers resistance to broad-spectrum cephalosporins and this further limits treatment option when expressed to higher levels (Ingti *et al.*, 2017). Moreover, aminoglycosides are frequently used in combination with the ß-lactam group of antibiotics for the treatment of severe infections in hospital patients. However, the bacterial population has developed various mechanisms of resistance and shortly, the therapeutic use of this drug will be inadequate (J. *et al.*, 2017). Infections due to ESBL producers with the capacity of antibiotic resistance with other precious antibiotics like carbapenem, Cephalosporins aminoglycosides makes the treatment challenging.

2.17.1 Carbapenem-resistant organisms: The carbapenems are the most potent antibacterial agents with wide spectra of coverage. However, resistance to these agents has increasingly been reported worldwide, making them increasingly ineffective. These organisms are also capable of resisting other classes of antibiotics such as aminoglycosides, fluoroquinolone, and co-trimoxazole, due to the frequent coexistence of other resistance genes on the same mobile genetic elements, rendering them superbugs. The most recent example is the emergence of colistin resistant genes in isolates which are already resistant to the carbapenems.

K. pneumoniae carbapenemase (KPC-class A) was the first CRE enzyme to be reported in 2001. New Delhi Metallo-β-lactamase (NDM-class B) is one of the most recently reported metalloenzymes which has spread widely in the Indian sub-

continent and now worldwide. The oxacillinase-48 type (OXA-48-class C) has been observed mostly in Mediterranean and southern European countries. Other mechanisms of resistance include efflux pump overactivity, hyperproduction of AmpC β-lactamase in the already highly resistant ESBL organisms, and changes in porin permeability (Ruppe E, Woerther PL, 2015). Infections due to such resistant isolates resulted in high morbidity, prolonged hospital stay, and mortality (Ben-David D, Kordevani R, 2012).

In a pooled analysis of the 9 studies (985 patients), the mortality rate was found to be higher among CRE-infected than carbapenem susceptible Enterobacteriaceae-infected patients (RR 2.05, 95% CI 1.56–2.69 (Villegas MV, Pallares CJ, 2016).

It was reported that a multicenter observational study in 11 hospitals from 7 Latin American countries included 255 patients with bacteremia. Twenty-three percent of the isolates were CRE/CRP (Villegas MV, Pallares CJ, 2016).

According to the latest data collected by the European Antimicrobial Resistance Surveillance Network (EARS-Net), the rate of CRE raised from 6.2% in 2012 to 8.1% in 2015 (Weist K, 2016) CRE are more prevalent in Italy and Greece. In an active surveillance study, rectal swabs were collected from 15,104 hospitalized patients (over 2 years) and *K. pneumoniae* CRE was detected in 496 consecutive non-replicated samples (Hamza and Khalil, 2018).

The detection of Carbapenemase includes antimicrobial susceptibility testing, modified Hodge testing and inhibitor- based testing. Several phenotypic assays are available commercially for detection of carbapenemase production from the bacterial culture within hours. The Carba NP test has high sensitivity and specificity that it can differentiate between class A, B, and C CRE. In one study, its specificity and

sensitivity were almost 96% (PoirL, 2015). There are many commercially available PCR-based testing for early recognition and confirmation for Carbapenemase producing bacteria.

2.17.2 AmpC β lactamase producing uropathogens

*Amp*C β-lactamases can be chromosomally or plasmid- mediated that hydrolyzes all β-lactam antibiotics except cefepime and carbapenems. The plasmid-mediated Amp*C* genes are derived from inducible chromosomal genes that have been mobilized among a variety of organisms. The commonly reported genotypes include ACC, FOX, MOX, DHA, CMY, CIT and EBC. These mobilized plasmid-mediated enzymes confer a resistance pattern similar to the overproduction of chromosomal *Amp*C β-lactamases, which also involve all β-lactam antibiotics except for carbapenems and cefepime (Gunjan Gupta, Vibhor Tak, 2016).

AmpC ß-lactamases can be detected both phenotypically or genotypically by AmpC Disk Test, Ceftazidime-Imipenem Antagonism Test (CIAT), Disc Antagonism Test and Modified Three-dimensional Test and PCR (Mwinga, Rashmi and Sumana, 2018).

For AmpC detection, a conventional disk diffusion susceptibility assay is carried out with a susceptible strain, such as *E. coli* ATCC 25922 are spread as the lawn and a suspension of the test organism is added to a circular slit in the agar 3 mm from a disk containing cefoxitin or some other agent. Distortion of the zone of inhibition indicates a positive test since cefoxitin is hydrolyzed by the presence of an AmpC enzyme (Jacoby, 2009).

Etest strips with a gradient of cefotetan or cefoxitin on one half and the same combined with a constant concentration of cloxacillin on the other half have been

evaluated for AmpC detection (Bolmstro ¨m, A., A. Engelhardt, L. Bylund, P. Ho, 2006).

Boronic acids have long been known as AmpC inhibitors. As the current "gold standard" for plasmid-mediated AmpC β-lactamase detection, multiplex PCR has been developed by utilizing six (Perez-Perez, F. J., 2002) to which the seventh pair for CFE-1 β-lactamase (Nakano, R., R. Okamoto, Y. Nakano, K. Kaneko, N. Okitsu, Y. Hosaka, 2004) could be added. Infections caused by AmpC-positive bacteria are of particular clinical and epidemiological importance and as they cause higher patient morbidity and mortality (Helmy MM, 2014). Indeed, mortality rates of 14.3 – 46 % have been reported (Ogefere, Osikobia and Omoregie, 2016).

2.17.3 Aminoglycoside modifying enzyme producing uropathogens

Aminoglycosides are very potent antibiotics, however; their use is associated with significant renal and auditory toxicities. They have been successful in treating ESBL-UTIs as a mono- therapy or in combinations with other agents. Combinations with other agents were effective in the treatment of CRE infections if the strain is sensitive to aminoglycosides (Kaye KS, Gales AC, 2017).

Many of the plasmids that carry ESBL-producing genes also carry genes that encode resistant to aminoglycosides, mostly against tobramycin and gentamicin. In contrast, amikacin has retained high susceptibility rates, particularly against *E. coli* (Hamza and Khalil, 2018).

In a small study of UTI caused by highly resistant ESBL were also resistant to nitrofurantoin, fosfomycin, and quinolones and trimethoprim/ sulfamethoxazole, amikacin intramuscular injections for 10 days achieved clinical success in 97.2%. In

general bacteriological success rate was 94.1% on the 7–10 days after treatment (Kaye KS, Gales AC, 2017)

In a review of 20 studies evaluating CRE infections therapy, a combination of aminoglycosides and carbapenems displayed the lowest mortality rate (11.1%) (Tzouvelekis LS, Markogiannakis A, 2014).

2.18 MIC Creep phenomenon in gram negative bacteria

In recent years, a continuous increase in the prevalence of drug-resistant bacteria has been observed. MICs have increased to the high end of the Clinical and Laboratory Standards Institute (CLSI) susceptibility range. This MIC shift has been defined as "MIC creep" (Soriano A, Marco F, Martinez J, Pisos E, Almela M, Dimova V, 2008).

Vancomycin MIC creep in methicillin-resistant *Staphylococcus aureus* (MRSA) isolates have been observed from 2006 to 2010 in a hospital in China by Chang et al., 2015 (Chang W, Ma X, Gao P, Lv X, Lu H, 2015). However, Diaz *et al*, 2017 assess the vancomycin MIC distribution for *Staphylococcus aureus* isolates over a period of 4 years in Centro Hospitalar Baixo Vouga (Aveiro, Portugal) to check the presence of MIC creep by different susceptibility testing methods. Unfortunately, the there result did not suggest the presence of vancomycin MIC creep during the study period (Diaz R *et al*., 2017). Few studies were conducted on the evidence of MIC creep phenomenon in Latin America and Europe which analyzed ertapenem susceptibility trends of *E. coli* over 5 years but no significant "MIC creep" was observed in the in vitro activity of ertapenem vs. *E. coli* from 2008-2012 (Lob S *et al.*, unpublished data). Jean *et al*, 2015 reported in China the escalations in MIC levels for *E. coli* and *E. cloacae* isolates and ESBL producing *Klebsiella pneumoniae* against imipenem during the period of 2005 to 2009.

Chapter 3

Methodology

3. METHODOLOGY

The objectives of the present study were to characterize the common bacterial uropathogens isolated from the UTI suspected female patients of Sikkim and Darjeeling. In order to meet these objectives, the following methods were used in the study.

3.1. Sample collection

3.1.1 Sample collection site

From June 2014 to September 2016 a total of 1516 urine samples were collected from UTI suspected female patients of age group 18 to 48. The urine samples were collected from in-patient and out-patient departments of tertiary hospitals viz. (Sikkim Manipal Institute of Medical Sciences (SMIMS) Sikkim. Sikkim is a landlocked state and it is situated in the North-Eastern region of India with an area of about 7,096 square kilometers. The climatic condition ranges from subtropical to high alpine. As per 2011 census, Sikkim has a population size is 6,07,688, density is 86 persons per km2 and more than 20% of the population classified as tribal (Publication Division, 2010). The literacy rate is about 56.9% as stated in Sikkim human development report,2001 (Publication Division, 2010).

Moreover, samples were also collected from Neotia Get well Healthcare Siliguri. Siliguri is a city which spans areas of the Darjeeling and Jalpaiguri districts in the Indian state of West Bengal. The city is located on the banks of the Mahananda River and the foothills of the Himalayas. It the largest metropolitian city of the region (West Bengal Govt.2011). The samples were also collected from various diagnostic centers like Planters hospital Darjeeling, Omega Diagnostic Centre Siliguri, Primary Health Centre, Pakhim and Ruchi Diagnostic Centre, Gangtok. Moreover, urine

samples were also collected randomly from females of the study areas to check the prevalence of asymptomatic UTI.

3.1.2 Sample collection procedure

Midstream urine specimens were collected from females of age group 18-48 years. The samples were collected in a sterile wide-mouthed plastic container. The UTI suspected female patients were asked to pass urine with labia separated and middle stream urine sample was collected for examination (Collee, J.G., R.S. Miles, 2011).

3.1.3 Sample and data Collection from the Community: The study was carried out on a randomly selected female population of same age group by direct interview using comprehensive pre-structured English questionnaire (explained in local languages) based feedback model system for data collection (Annexure I). Consent was obtained from the participants before the collection of the sample (Annexue II). Random locations including villages, markets, panchayats, and health centers were selected for sampling. Participants were well informed about the purpose and outcome of the study. To check the presence of asymptomatic bacteriuria, after proper instruction, a total of 207 urine samples were collected from the participants who did not show the symptoms of UTI. Questionnaire-based anonymous feedback system was used for the collection of data. The field survey used the observations method and the rapport building method to gain the information.

- Inclusion criteria: Females between age group 18-48 years, who did not show the symptoms of UTI.
- Exclusion criteria: Men, participants less than 18 and more than 48 years, participants consuming antibiotics.

3.1.4 Statistical Analysis

The questionnaire was checked for errors and data was entered into MS-excel and it was converted into Microsoft Excel format (.xls) and statistically analyzed using Graph pad prism V5.01.exe software (San Diego, USA). Relative Risk Calculation Relative Risk analyses were carried out using online software ‘‘MEDCALC’’ (Version 12.2.1- 1993–2012, MedCalc Software, Broekstraat 52, 9030 Mariakerke, Belgium). Relative risk = [a/ (a ? b)]/[c/(c ? d)] (Table 1).

Table 1: *Relative Risk Calculator*

Exposed group	
Number with positive outcome:	a=
Number with negative outcome:	b=
Control group	
Number with positive outcome:	c=
Number with negative outcome:	d=

Criteria for Significance of Relative Risks

RR = 1 means the association between exposure and disease unlikely to exist;

RR >1 means the increased risk of disease among those that have been exposed.

RR <1 means the decreased risk of disease among those that have been exposed (Table 1)

3.1.5 Ethical Considerations: Ethical clearance for this work was obtained from the Institutional Ethical Committee, Sikkim University and written informed consent from community participants samples were collected as the questionnaire was in English, symptoms were described in Nepali/Hindi for those subjects who did not understand scientific or English terms. Symptoms were explained to respondents to

identify the exact disease/ailment and then after confirmation were marked on the questionnaire.

3.2 Sample transportation

Once a specimen of urine was collected it was transported to the laboratory without delay, as urine is an excellent culture medium and contaminating bacteria can readily multiply to reach apparently significant numbers. If a delay of more than 1-2 hour was unavoidable, the multiplication of bacteria in the urine was prevented by storing in a refrigerator at 4C, or by transport in some form of refrigerated container, or by collection and transport in a container with boric acid at a final, bacteriostatic concentration of 1.8% (Collee, J.G., R.S. Miles, 2011)

3.3 Isolation of pathogen

The samples were observed carefully for adult parasites, consistency, blood, mucous, color and pH. In microscopic observation, the samples were observed carefully for pus cells and red blood cells. For isolation of bacteria from the urine sample semiquantitative method was used in which a calibrated loop that delivers 0.01 ml of sample is used to inoculate the plate. The number of colonies that appear from this 1/100th-ml sample is multiplied by 100 to give the number per milliliter.

The samples were inoculated onto High Chrome UTI, Cystine Lactose Electrolyte Deficient Agar (CLED) and Mac Conkey agar plate Agar (Hi-Media, India) by the semi-quantitative method and incubated aerobically at 37ºC for complete 24 h incubation. The specimen yielding more than or equal to 10^5organisms/ml of urine was interpreted as significant (Collee, J.G., R.S. Miles, 2011).

3.4 Identification of the isolates

3.4.1 Gram staining (Gram, 1884)

It demonstrates the morphology of bacteria along with two categories: Gram-positive and Gram-negative. The methodology followed for staining is given below:

The clean and grease free slide was taken for making the smears

⇩

A loopful of culture suspension was taken and placed on the clean slide

⇩

The cell suspension was spread into a thin area with a circular movement of the loop

⇩

The smear was air dried and heat fixed over the flame

⇩

The smear was then covered with crystal violet and allowed to stand for 1 minute

⇩

The smear was rinsed gently under tap water

⇩

The smear was covered with Grams iodine and allowed to stand for 1 minute

⇩

The smear was rinsed gently under tap water

⇩

The smear was decolorized by 95% ethanol

⇩

The smear was then rinsed gently under tap water

⇩

The smear was covered with safranin for counterstain for 30-60 second

⇩

The smear was again rinsed gently under tap water and air dried

⇩

The smear was observed first under low power (40X) objective and then under oil immersion (100X) objective to characterize the Gram stains reaction.

Interpretation:

- **Gram positive bacteria:** Stained as **purple** color
- **Gram negative bacteria:** Stained as **pink** color

Quality control:

- **Gram positive:** *Staphylococcus aureus*
- **Negative reaction:** *Escherichia coli*

3.4.2 Cultural characteristics of the isolates

After complete incubation at 37° C for 24 hours, the cultural characteristics of the isolates were observed on CLED agar, MacConkey agar, and Hi Chrome UTI agar. The shape, size, color and opacity of the colonies were taken into account.

Table 2: Typical colony morphology of Enterobacteriaceae in a different culture media (Collee, J.G., R.S. Miles, 2011)

Microorganism	Observation on CLED Agar	Observation on Macconkey Agar	Observation on Hi chrome UTI agar
Escherichia coli	Opaque yellow colonies with a slightly Deeper yellow center	Lactose fermenter; flat, dry, pink colonies with a surrounding darker pink area of precipitated bile salts.	Pink to red colony
Klebsiella pneumoniae	Yellow to whitish-blue colonies, extremely mucoid	Mucoid, lactose fermenter, pink in color	Blue-green, mucoid
Proteus mirabilis	Yellow to whitish-blue colonies, extremely mucoid	Non- lactose fermenter, swarming depending on the amount of agar in the medium; characteristic foul smell	Light brown
Pseudomonas aeruginosa	Large green colonies with a brownish center, surrounded by a blue halo	Non- lactose fermenter, colorless	Colorless (greenish pigment may be observed)
Citrobacter Sp.	Large golden-yellow colonies surrounded by a yellow halo	Late lactose fermenter; therefore Non-LactoseFermenter (NLF) after 24 hours; Lactose fermenter after 48 hours; colonies are light pink after 48 hours.	Violet to the purple colony with a pink border which made these strains easier to identify

3.4.3 Phenotypic method for bacterial identification

Biochemical tests are among the most important methods for microbial identification. Microbial biochemistry tests shorten the time required to identify microbes, reduce costs, and ensure or enhance the accuracy of identification of an unknown sample. It is the fastest developing trend in microbial identification (Atlas of Oral Microbiology, 2015).

Different biochemical tests that were performed to identify and characterize the isolates are as follows (Collee, J.G., R.S. Miles, 2011)

3.4.3.1 Indole test

This test demonstrates the ability of certain bacteria to degrade the amino acid tryptophan to indole which is mediated by the enzyme tryptophanase produced by some indole positive bacteria.

Bacterial culture was inoculated in peptone water containing peptone and NaCl

⇩

The culture was incubated overnight at 37^0C

⇩

0.5ml of Kovac's indole reagent (Hi-media, Mumbai, India) was added to the culture

Interpretation:

- **Positive reaction:** Pink/red color ring at the interface of reagent and broth
- **Negative reaction:** No color development

Quality control:

- **Indole positive:** *Escherichia coli*
- **Indole negative:** *Klebsiella pneumoniae*

3.4.3.2 Methyl red test

The methyl red test is a quantitative test which detects acid production during the fermentation of glucose to strong acid like lactic acid, acetic acid and formic acid. Methyl red is a pH indicator and it shows red color in pH under 4.4, yellow in pH over 6.2 and orange in between.

Bacterial culture was inoculated in MR-VP medium (Hi-media, Mumbai, India)

⇩

Incubated at 37^0C for overnight

⇩

Few drops of methyl red indicator (Hi-media, Mumbai, India) was added to the medium

Interpretation:

- **Positive reaction:** Formation of bright red color
- **Negative reaction:** Formation of yellow color

Quality Control:

- **Positive reaction:** *Escherichia coli*
- **Negative reaction:** *Klebsiella pneumoniae*

3.4.3.3 Voges-Proskauer test

This test determines the capability of some bacteria to produce nonacidic or neutral end products such as acetyl methyl carbinol from the organic acids produced as a result of glucose metabolism.

The MR-VP medium was prepared in-house and bacterial culture was inoculated

⇩

The culture was incubated at 37^0C for overnight

⇩

Barritt's reagent (Hi-media) which consists of a mixture of 5% α-naphthol and 40% potassium hydroxide solution was added in the medium and mixed

Interpretation:

- **Positive reaction:** Formation of pink-red color at the surface of the medium
- **Negative reaction:** Yellow color at the surface of the medium

Quality control:

- **Positive reaction:** *Klebsiella pneumoniae*
- **Negative reaction:** *Escherichia coli*

3.4.3.4 Citrate utilization test

This test was performed to detect the ability of bacteria to utilize citrate as a sole source of carbon in the absence of glucose or lactose and this depends on the presence of the enzyme citrate permease.

The slants of Simmons citrate agar (Hi-media, Mumbai, India) were prepared in a test tube

⇩

The bacterial culture was stabbed in the butt and streaked on the slant with the help of a straight wire

⇩

The test tube was incubated at 37^0C for overnight

Interpretation:

- **Positive reaction:** Growth with intense blue color in the slant
- **Negative reaction:** Absence of growth and no color change in the medium (remains green)

Quality control:

- **Citrate positive:** *Klebsiella pneumoniae*
- **Citrate negative:** *Escherichia coli*

3.4.3.5 Triple sugar iron test

Triple sugar iron medium is a differential medium that can distinguish between numbers of Gram-negative enteric bacteria based on their physiological ability. TSI medium indicates whether the bacteria ferment glucose only or lactose and sucrose also with or without production of gas. The medium can detect the production of hydrogen sulphide (H_2S) as well as other bacteria which utilizes only glucose but not lactose or sucrose.

The slant of TSI agar (Hi-media, Mumbai, India) was prepared

⇩

The bacterial culture was stabbed in the butt with the help of a straight wire and streaked on the slope

⇩

The tube was incubated at 37^0C for overnight.

Interpretation:

The result of the TSI test was interpreted according to the reaction obtained in the test and the details are given in table 2

Table 3: Summary of the TSI interpretation

Reaction	**Carbohydrate fermented**	**Typical organisms**
Alkaline slant/alkaline butt (K/K reaction)	No carbohydrate formation. Bacteria are non-fermented	*Pseudomonas aeruginosa*
Alkaline slant/acidic butt (K/A reaction)	Glucose is fermented, lactose and sucrose Is not fermented. Organism is non-lactose fermenter	*Shigella* spp., *Vibrio* spp.
Alkaline slant/acidic butt/black precipitate of H2S (K/A, H_2S positive reaction)	Glucose is fermented; lactose and sucrose are not fermented. Characteristics of non-lactose fermenting, H_2S producing bacteria	*Salmonella* spp., *Proteus* spp.
Acidic slant/acidic butt (A/A reaction)	Glucose, lactose and sucrose are fermented. Characteristics of lactose fermenting	*Escherichia coli, Klebsiella* spp., *Enterobacter* spp.

3.4.3.6 Urease test

The urease enzyme produced by many bacteria is able to hydrolyze urea and releases ammonia and carbon dioxide. Ammonia reacts in the solution to form ammonium carbonate, which is alkaline leading to an increase in the pH of the medium. Phenol red that is generated in the medium changes the color from yellow to red in alkaline pH, thus indicating the presence of urease activity.

The slant of Christensen's urea agar was prepared and a pure colony was streaked onto it

⇩

The tube was then incubated at 37^0C for overnight

Interpretation:

- **Positive reaction:** The slant became pink in color
- **Negative reaction:** No color change in the medium (remains yellow)

Quality control:

- **Positive reaction:** *Proteus mirabilis*
- **Negative reaction:** *Escherichia coli*

3.4.3.7 Oxidase test

The Oxidase test is performed to demonstrate the presence of an intracellular enzyme cytochrome oxidase which is produced by oxidase positive bacteria. This enzyme oxidizes the reagent N-N-N tetramethyl para-phenylene diamine hydrochloride (a colorless reagent).

Oxidase disc (Hi-media, Mumbai, India) was placed in a clean Petri plate

⇩

A sterile capillary tube was used to pick an isolated colony and was rubbed in the disc

Interpretation:

- **Positive reaction:** Formation of indophenols blue, an intense purplish color product
- **Negative reaction:** Absence of coloration later than 60 seconds

Quality control:

- **Oxidase positive organism:** *Pseudomonas aeruginosa*
- **Oxidase negative organism:** *Escherichia coli*

3.4.3.8 Phenylalanine deaminase test

Phenylalanine deaminase test is used to test the ability of an organism to produce enzyme deaminase. This enzyme removes the amine group from the amino acid phenylalanine and produces phenylpyruvic acid (PPA) and ammonia.

Phenylalanine was dissolved in 1 ml of sterile distilled water

⇩

Pure cultures of the test organism were inoculated and incubated at 35^0C for 24 hours

⇩

Four to five drops of 10% ferric chloride ($FeCl_3$) was added to the inoculated media and observed

Interpretation:

- **Positive reaction:** Intense green color was observed
- **Negative reaction:** No color change

Quality control:

- **Deaminase** positive organism: *Proteus mirabilis*
- **Deaminase** negative organism: *Escherichia coli*

3.4.3.9 Sugar fermentation

This test was used to detect an organism's ability to ferment the sugars namely glucose, lactose, sucrose and mannitol as well as its ability to convert the end product of glycolysis, pyruvic acid into gaseous byproduct.

A carbohydrate fermentation broth was prepared at pH 7.4 and four different sugars were added in respective test tubes

⇩

The broth contains 0.5% of carbohydrate to be tested (sucrose/ mannitol/ lactose / glucose), nutrient broth and the pH indicator phenol red

⇩

Inverted Durham tubes filled with carbohydrate fermentation broth was placed in the test tube to detect the production of gas

⇩

Bacterial culture was inoculated in the tubes and incubated at 37°C for overnight

Observation		**Interpretation**
The color of the media changes to pink with gas in Durham's tube	⇨	Fermentation with the production of gas
The color of the media changes to pink with gas in Durham's tube	⇨	Fermentation with the production of gas
The color of media remains yellow	⇨	Not fermented

Quality control:

- **Sugar fermentative** positive organism: *Escherichia coli*
- **fermentative** negative organism: *Pseudomonas aeruginosa*

3.4.4 Automated method for bacterial identification (Standard Operating Procedures, 2018)

Some of the bacterial isolates were identified by Vitek 2 instruments (VITEK 2 compact; Biomerieux) using the ID-GNB and ID-GPC cards for gram negative and gram positive bacteria respectively, in accordance with the manufacturer's instructions.

The Vitek 2 Compact (30 card capacity) system uses a fluorogenic methodology for organism identification and a turbidimetric method for susceptibility testing using a 64 well card that is barcoded with information on card type, expiration date, lot number and unique card identification number. Test kits available include ID-GN (gram-negative bacillus identification), ID-GP (gram-positive cocci identification), AST-GN (gram- negative susceptibility) and AST-GP (gram-positive susceptibility). The Vitek 2 ID-GN card identifies 154 species of Enterobacteriaceae and a select group of glucose nonfermenting gram-negative organisms within 10 hours. The Vitek 2 ID-GP card identifies 124 species of *Staphylococci, Streptococci, Enterococci* and a select group of gram positive organisms within 8 hours or less.

3.4.5 Molecular method for bacterial identification

3.4.5.1 16s rDNA sequencing

Sometimes phenotypic methods cannot identify the isolates correctly therefore in the present study 16s rDNA sequencing was used for molecular identification of bacterial isolates. Since molecular methods are a reliable method, the molecular method was used for bacterial identification.

3.4.5.1. Selection of organisms

The representative isolates were selected and identified by 16s rDNA sequencing.

3.4.5.2 Preparation of DNA template

DNA was extracted by boiling centrifugation method (Freschi C R *et al*., 2005) and used as a template for PCR reaction.

Isolates were inoculated in Luria Bertani broth (Hi-Media, Mumbai, India) and incubated at 37^0C for overnight

⇩

The aliquots of the overnight culture were transferred to a sterile 1.5 ml of the microcentrifuge tube

⇩

Tubes were centrifuged at 10,000 rpm for 10 minutes

⇩

The pellets were re-suspended in 200-300 µl of nuclease-free water

⇩

The suspended pellets were then heated at 86°C in the dry block for 15 minutes

⇩

Tubes were then cooled in ice and centrifuged at 10,000 rpm for 10 minutes

⇩

The supernatant was used as a template for PCR reaction

3.4.5.3. Preparation of reaction mixture

A total of 25 µl reaction mixture was prepared which contained 2 µl of template DNA (100ng/µl), 1 µl of each primer (10 picomole) (Table 3), 12.5 µl of Go Taq Green Master Mix, 2X DNA polymerase (Promega, Madison, USA) and the rest of the volume to make up with nuclease-free water

Reaction conditions:

Initial denaturation: 95^0C for 3 mins

Final denaturation: 95^0C for 30 sec
Annealing: 54^0C for 1 min,
Extension: 72^0C for 1.30 mins
} 32 cycles

Final extension: 72^0C for 10 mins.

Table 4: Oligonucleotides used as primers for 16s rDNA amplification

Primer Pairs	Target	Sequence(5′-3′)	Amplified Product Size	Reference
27-F	16s rDNA	5'-AGAGTTTGATCMTGGCTCAG-3'	1476	(Woo PC, Leung PK, Leung KW, 2000)
1492R		5'-TACGGYTACCTTGTTACGACTT-3'		

3.4.5.5 Gel electrophoresis and analysis of PCR products

The amplified product of 16s rDNA was run in agarose gel electrophoresis. A volume of 10 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 60 volts for two hours with 1X Tris Borate EDTA (TBE) buffer. A molecular marker of 100 bp DNA ladder (Genei, Banglore, India) was run concurrently and the gel was visualized in Gel Doc EZ imager (Bio-Rad, California, USA).

Interpretation

Presence of a single band of molecular weight of 1476 bp confirmed the amplification of 16s rDNA gene.

3.4.6 PCR product purification

The amplified PCR products were purified using the MinElute® PCR Purification Kit (Qiagen, Hilden, Germany). The steps used are as follows:

5 volumes of binding buffer were mixed to 1 volume of PCR reaction mixture

⇩

The color of the mixture turned yellow (if not, then 10µl 3 M sodium acetate of pH 5.0 was added and mixed)

⇩

The mixed solution was transferred to the MinElute column and centrifuged for 1 min

⇩

The flow-through was discarded and the column was placed in the same collection tube

⇩

750 µl of wash buffer was added to the MinElute column and centrifuged for 1 min

⇩

The flow-through was discarded

⇩

The column was placed in a 2 ml collection tube and centrifuged for an additional 1 min to completely remove the residual wash buffer

⇩

The MinElute column was placed in a clean 1.5 ml centrifugation tube

⇩

10 µl of Elution buffer was added to the center of the MinElute membrane and the column kept for 1 min at room temperature

⇩

The column was centrifuged for 1 min

⇩

The MinElute purification column was discarded and the centrifuge tube containing purified DNA was collected into a fresh microcentrifuge tube (1.5 ml)

⇩

Purified DNA was stored at 4^0C

3.4.7 Sequencing of the 16s rDNA amplified product

30 µl of purified PCR products send for sequencing along with 16s rDNA primers of 20 µl each (10 picomoles). Sequence results were analyzed using the BLAST suite program of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.5 Antimicrobial susceptibility testing of isolated uropathogens (CLSI, 2014)

3.5.1 Disc diffusion method

The antimicrobial susceptibility of the isolates was determined by the Kirby-Bauer disc diffusion method (Bauer AW, Kirby WM, Sherris JC, 1996) which is described as follows:

The test organisms were inoculated into Nutrient agar plate to obtain pure and isolated colonies

⇩

Three to four pure culture colonies were picked up and transferred into peptone water for preparation of inoculums

⇩

The inoculums were picked up and incubated at 37°C for 1-2 hours till light to moderate turbidity was achieved.

⇩

The turbidity of the suspension was adjusted to McFarland's Standard 0.5 (1.5×10^8 CFU/ml).

⇩

A sterile nontoxic cotton swab was dipped into the suspension.

⇩

The soaked swab was rotated firmly against the upper inside wall of the tube to remove the excess fluid.

⇩

It was streaked evenly onto the entire agar surface of the plate three times, turning the plate 60^o angle between each streaking to make sure that inoculums were evenly spread onto the Muller Hinton Agar (MHA) plate

⇩

The MHA plate was allowed to dry for 5-10 minutes at 37°C.

⇩

Commercially prepared antibiotic discs (Hi-Media, Mumbai, India) were placed onto the lawn culture of test organism with the help of sterile forcep

⇩

The forcep tips were sterilized by the red heat using flame of spirit lamp

⇩

In one plate 5 antibiotics were placed. All together 10 antibiotics were tested for each organism.

- The discs were placed with centers at least 24 mm apart and incubated 37^{o}C for 14-19 hrs.
- The zone of inhibitions was measured in millimeter with standard chart provided by manufacturer which is as per CLSI guidelines 2014.
- MTCC *E. coli* 1089 was used as a gram-negative control in every batch of the AST experiments.

Table 5: Antibiotics were used in disc diffusion method

	Antibiotics	**Group**
1	Ampicillin (10µg)	Penicillin
2	Gentamicin (120µg)	Aminoglycoside
3	Piperacillin/tazobactam (100/10µg)	β -lactam& β- lactamase inhibitor
4	Netillin (30µg)	Aminoglycosides
5	Norfloxacin (10µg)	Fluoroquinolones
6	Ciprofloxacin (30µg)	
7	Cephalothin (30µg)	First-generation cephalosporin
8	Cefoxitin (30µg)	Second-generation cephalosporin
9	Cefuroxime (30µg)	Second-generation cephalosporin
10	Ceftazidime (30µg)	Third-generation cephalosporin
11	Co-trimoxazole	Anti- metabolite inhibitor Trimethoprim/Sulfamethoxazole (1.25 + 23.75 µg)
12	Nitrofurantoin (300µg)	-
13	Imipenem (10µg)	Carbapenem

3.5.2 Minimum inhibitory concentrations (MICs)

The MIC is the least amount of antimicrobial that will inhibit the visible growth of an organism after overnight incubation. MICs were determined using the agar dilution

method (Collee, J.G., R.S. Miles, 2011). The CLSI 2014 recommended MIC breakpoints were used to categorize the susceptibility pattern of the test organism. The pure form of powdered antibiotics (cefotaxime, ceftriaxone, cefepime, ceftazidime, cefpodoxime), were obtained from pharmaceuticals. The remaining other two antibiotics Aztreonam and Imipenem were procured from a pharmacy shop.

3.5.2.1 Preparation of stock solution

Fresh stock antibiotics were prepared based on serial dilution Dilutions of antibiotics was prepared based on the 1mg/ liter. Ten different concentrations of antibiotics were prepared i.e. 0.25, 0.5, 1, 2, 4,8,16, 32, 64, 128 and 256 mg/litre etc. The stock solution was prepared by using the following formula:

1000/P x V x C = W

Where P= potency of preparation in relation to base, V= volume (ml) required; C=final concentration of the solution in multiples of 1000 and W= weight (mg) of antibiotic to be dissolved in V. (Collee JG *et al*., 1996).

A method for preparing working dilutions of antibiotic is shown in Figure 1

For each antibiotic, a serial dilution ranging from 0.25 mg/l-256mg/l was prepared in sterile distilled water aseptically. The detailed procedures as follows:

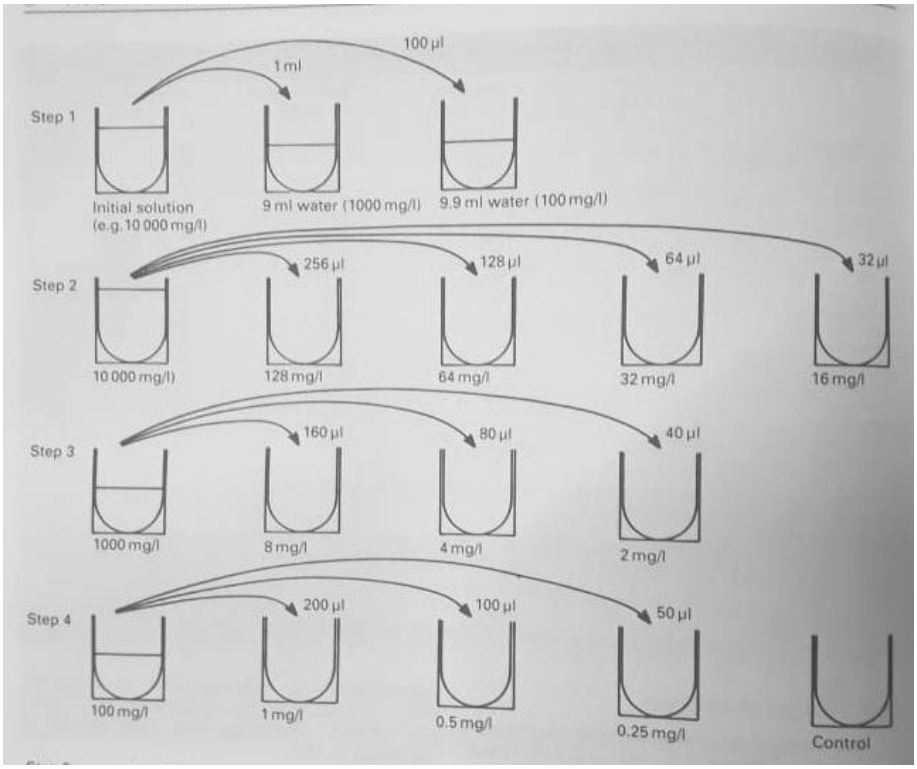


Figure 3: Preparation of dilution of antibiotics

(Adopted from Mackie & Mc Cartney Practical Medical Microbiology Book) (CLSI, 2014)

Step 5

3.5.2.2 Preparation of agar dilution plates

Sterile empty plates were marked with a particular dilution of antibiotics.

⇩

A series of dilution of five beta-lactam antibiotics (cefotaxime, ceftriaxone, cefepime, ceftazidime, cefpodoxime), monobactam (aztreonam), a carbapenem (imipenem) ranging from 2-256 µg/ml were placed onto the marked sterile empty plates accordingly.

⇩

Then after 20ml of sterile molten MHA agar approx 42-45℃ were poured into all Petri dishes

⇩

The plates were gently rotated in a clockwise and anticlockwise direction in order to mix the antibiotic solution and media

3.5.2.3. Preparation of test organism

The test organisms were inoculated into Nutrient agar plate to obtain pure and isolated colonies

⇩

Three to four pure culture colonies were picked up and transferred into peptone water for preparation of inoculums

⇩

The inoculums were picked up and incubated at 37°C for 1-2 hours till light to moderate turbidity was achieved.

⇩

The turbidity of the suspension was adjusted to McFarland's Standard 0.5 (1.5×10^8 CFU/ml).

⇩

With the help of ruler and marker backside of these plates were divided into 20-25 squares too place the test inoculums

⇩

Test inoculums were added into each square and incubated

⇩

After 12 to 14 hours the MIC of the bacterial isolates was noted

Table 6: Antibiotics used for performing Minimum Inhibitory Concentration

Sl no.	Antibiotics	Group
1.	Cefotaxime	Cephalosporin
2.	Ceftriaxone	
3.	Cefepime	
4.	Ceftazidime	
5.	Cefpodoxime	
6.	Aztreonam	Monobactam
7.	Imipenem	Carbapenem

3.6 Extended Spectrum ß-lactamase detection methods

3.6.1 Phenotypic method for Extended Spectrum ß-lactamase detection (CLSI, 2014)

ESBL production in the isolates was characterized by a phenotypic method using a screening test followed by combined disc diffusion method.

3.6.1.1 Screening for the ESBL producing organisms

The screening test of the isolates was done using five antibiotics namely;

- Cefotaxime,
- Ceftazidime,
- Ceftriaxone,
- Aztreonam at 1µg/ml and
- Cefpodoxime 4 µg/ml (1 µg/ml for *Proteus mirabilis*) in Muller Hilton Agar by agar dilution method.

3.6.1.1.1 Preparation of stock solution

10 mg of the above-mentioned antibiotics was dissolved in 10 ml of sterile triple distilled water in separate vials to make a final concentration of 1 mg/ml.

3.6.1.1.2 Preparation of screen agar plate

1 µg/ml of each antibiotic from stock solution was added into molten cool Mueller Hinton agar (Hi-media, Mumbai, India).

⇩

The agar and antibiotics were mixed thoroughly and poured into Petri dish. The agar was allowed to solidify at room temperature and stored at 4-8^{o}C.

3.6.1.1.3 Inoculation of test isolates in screen agar plates

Preparation of test organism was done

(As mentioned in Antibiotic Susceptibility Testing of bacteria)

⇩

Dilution of inoculum was adjusted to 10^6 CFU/ml and 2 µl of the inoculums was applied to the surface of the agar with the help of a micropipette.

⇩

A control plate (without any antibiotics) was inoculated first followed by the screen agar plates and were incubated at 37°C for 12-16 hours. MTCC *Escherichia coli* 1089 was used as a negative control.

Interpretation

The isolates which grew in that antibiotic-containing medium were suspected to be ESBL producer and were subjected to the confirmatory test.

3.6.1.2 Confirmatory tests for ESBL production (Combined disc diffusion test)

- It was performed by using an ESBL kit consisting of:
- Ceftazidime (30 µg) (CAZ),
- Ceftazidime + clavulanic acid (30/10 µg) (CAC),
- Cefotaxime (30 µg) (CTX),
- Cefotaxime + clavulanic acid (30/10µg) (CEC) (CLSI 2011).

3.6.1.2.1 Inoculation of test isolates in agar plates

The test organisms were inoculated into Nutrient agar plate to obtain pure and isolated colonies

⇩

Three to four pure culture colonies were picked up and transferred into peptone water for preparation of inoculums

⇩

The inoculums were picked up and incubated at 37°C for 1-2 hours till light to moderate turbidity was achieved.

⇩

The turbidity of the suspension was adjusted to McFarland's Standard 0.5 (1.5×10^8 CFU/ml).

⇩

A sterile nontoxic cotton swab was dipped in to the suspension.

⇩

The soaked swab was rotated firmly against the upper inside wall of the tube to remove the excess fluid.

⇩

It was streaked evenly onto the entire agar surface of the plate three times, turning the plate 60^o angle between each streaking to make sure that inoculums was evenly spread onto the Muller Hington Agar (MHA) plate

⇩

The MHA plate was allowed to dry for 5-10 minutes at 37°C.

⇩

Commercially prepared antibiotic discs (Hi-Media, Mumbai, India) were placed onto the lawn culture of test organism with the help of sterile forcep

⇩

The forcep tips were sterilized by the red heat using flame of spirit lamp

⇩

The antibiotics discs from the ESBL kit (Hi-media, India) were applied using aseptic technique

⇩

The discs were placed with centers at least 24 mm apart and incubated at 37°C for 14-19 hours.

Interpretation

A greater than or equal to 5mm increase in zone diameter for either antimicrobial tested in combination with clavulanic acid versus its zone when tested alone confirms ESBL production.

Indicator organism

This test uses *Escherichia coli* MTCC 1089 as an indicator organism, at a turbidity of 0.5 McFarland standards.

3.6.2 Genotypic method for Extended Spectrum ß-lactamase detection

The genotypic characterization of ESBL genes was performed by targeting eight different genes in multiplex I and multiplex II PCR: bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA-2}, bla_{OXA-10}, bla_{VEB}, bla_{GES}.

3.6.2.1 Multiplex I PCR

PCR assay was performed targeting different *bla* genes namely bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA-2}, bla_{OXA-10} for all the isolates which were confirmed to be ESBL producer by phenotypic methods. The specific oligonucleotides used for amplification of the partial gene are mentioned in Table 7.

Table 7: Detailed list of primers used for the detection of ESBL genes

List of primer pairs	Target	Sequence (5'-3')	Product size (bp)	References
TEM-F TEM-R	TEM	ATGAGTATTCAACATTCCG CTGACAGTTACCAATGCTTA	867	(Bert, 2002)
SHV-F SHV-R	SHV	AGGATTGACTGCCTTTTTG ATTTGCTGATTTCGCTCG	392	(Colom *et al*., 2003)
CTX-M-F CTX-M-R	CTX-M -1, -2, -9 Group	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT	550	(Bert, 2002)
OXA-10-F OXA-10-R	OXA-2 group	TCAACAAATCGCCAGAGAAG TCCCACA CCAGAAAAACCAG	478	(Bert, 2002)
OXA-2-F OXA-2-F	OXA-2	AAGAAACGCTACTCGCCTGC CCACTCAACCCATCCTACCC	276	(Bert, 2002)
VEB-F VEB-R	PER	CATTTCCCGATGCATGCAAAGCGT CGAAGTTTCTTTGGACTCTG	650	(Lee *et al*.,2005)
GES-F GES-R	GES	AGTCGGCTAGACCGGAAAG TTTGTCCGTGCTCAGGAT	863	(Lee et al.,2005)
PER-F PER-R	VEB	AATTTGGGCTTAGGGCAGAA ATGAATGTCATTATAAAAGC	923	(Lee *et al*.,2005)

3.6.2.1.1 Preparation of DNA template

DNA was extracted by boiling centrifugation method (Freschi C R *et al*., 2005) and used as a template for PCR reaction.

⇩

Isolates were inoculated in Luria Bertani broth (Hi-Media, Mumbai, India) and incubated at 37^0C for overnight

⇩

The aliquots of the overnight culture were transferred to a sterile 1.5 ml of micro centrifuge tube

⇩

Tubes were centrifuged at 10,000 rpm for 10 minutes

⇩

The pellets were re-suspended in 23.5.2.300-300 µl of nuclease-free water

⇩

The suspended pellets were then heated at 86°C in dry block for 15 minutes

⇩

Tubes were then cooled in ice and centrifuged at 10,000 rpm for 10 minutes

⇩

The supernatant was used as a template for PCR reaction

3.6.2.1.2 Preparation of reaction mixture and reaction conditions

A total volume of 25 µl reaction mixture was prepared and each reaction mixture contained 2 µl of template DNA (100 ng/µl), 1 µl of each primer (10 picomoles), 12.5 µl of Go Taq Green Master Mix, 2X DNA polymerase (Promega, Madison, USA) and the rest of the volume was made up with nuclease-free water.

Reaction conditions were as follows:

Initial denaturation: 95°C for 5 mins
Final denaturation: 95°C for 1 min
Annealing: 54°C
Extension: 72°C for 1 min
Final Extension: 72°C for 7 mins

32 cycles

3.6.2.2 Multiplex II PCR

PCR assay was performed targeting different *bla* genes namely bla_{VEB}, bla_{GES}, bla_{PER} for all the isolates which were phenotypically confirmed to be ESBL production. The specific oligonucleotides used for amplification of the partial gene are mentioned in table 9.

3.6.2.2.1 Preparation of reaction mixture and reaction conditions

A total volume of 25 µl reaction mixture was prepared and each reaction mixture contained 2 µl of template DNA (100 ng/µl), 1 µl of each primer (10 picomole), 12.5 µl of Go Taq Green

Master Mix, 2X DNA polymerase (Promega, Madison, USA) and the rest of the volume was made up with nuclease free water.

Reaction conditions were as follows:

Initial denaturation = 95C for 2 mins
Final denaturation =95C for 20s
Annealing=50°C for 1min
Extension=72°C for min
Final Extension=72°C for 5 mins

32 cycles

3.7 Determination of horizontal transferability of plasmid encoding ESBL genes

3.7.1 Isolation of plasmid

The plasmid of the isolates carrying single or multiple carbapenemase genes was isolated using a QIAGEN® Plasmid Mini kit (Qiagen, Hilden, Germany) for each

isolate. QIAGEN plasmid purification protocol was based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to QIAGEN Anion-Exchange Resin under appropriate low salt and pH conditions. RNA, proteins, dyes, and low molecular weight impurities were removed by a medium-salt wash. Plasmid DNA was eluted in the high salt buffer and then concentrated and desalted by isopropanol precipitation.

Procedure

A single colony from a fresh selective plate was inoculated into a10 ml LB broth containing cefoxitin (0.25 µg/ml)

⇩

The broth was incubated overnight at 37^0C with vigorous shaking at 200 rpm

⇩

Bacterial cells were pelleted by centrifugation at 10,000 rpm for 10 min at room temperature

⇩

Bacterial pellets were mixed with 300 µl of re-suspension buffer

⇩

Then 300 µl of lysis buffer was added into the solution and mixed thoroughly by inverting 4-6 times until the solution became clear

⇩

400 µl of neutralization buffer was immediately added and mixed thoroughly by inverting 4-6 times

⇩

The mixture was centrifuged at 13,000 rpm for 10 minutes

⇩

The supernatant was then transferred to the QIAprep spin column by decanting

⇩

The column was centrifuged for 1 minute and the flow through was discarded

⇩

500 µl of binding buffer was then added in to the QIAprep spin column

⇩

The column was centrifuged for 1 minute and the flow through was discarded

⇩

The column was then washed with 750 µl of wash buffer

⇩

Column was centrifuged for 1 minute and the flow through was discarded

⇩

The residual wash buffer was removed by an additional centrifugation of the column for 1 minute

⇩

The QIAprep spin column was then placed in a clean 1.5 ml micro-centrifuge tube

⇩

50 µl of elution buffer was then added into the center of the column and the column was allowed to stand for 1 minute

⇩

The column was centrifuged at 13,000 rpm for 1 minute

⇩

The plasmid DNA was eluted and kept in a fresh centrifuge tube at -20^{0}C

3.7.1.1 Visualization

The plasmids were separated in 1% agarose gel at a very low voltage (40V) and size of the plasmid was determined by comparing with a molecular marker.

Horizontal transferability of ESBL resistant determinants was determined by transformation assay.

3.7.2 Transformation assay

The transformation was carried out by the heat shock method using *Escherichia coli* JM107 competent cell as a recipient. **.**

3.7.2.1 Preparation of SOC medium

The composition of SOC medium (per liter)

Tryptone……..2 g

Yeast extracts……..0.5 g

Glucose…….. 0.4 g

NaCl……...0.584 g

KCl……....0.186 g

$MgCl_2$……0.952 g

$MgSO_4$……2.408 g

3.7.2.2 Preparation of media for selection of transformants

The transformants carrying ESBL genes were selected in a Luria Bertani medium containing 0.25 µg/ ml. The specific amount of antibiotic was added into molten cool LB agar medium (Hi-Media, Mumbai, India), mixed thoroughly and poured into Petri dish. The agar was allowed to solidify at room temperature and stored at 4-8^{0}C.

3.7.2.3 Preparation of antibiotic stock solution

The stock solution for the cefoxitin antibiotic was made at 1 mg/ml concentration in nuclease-free water and was stored at -20^{0}C.

3.7.2.4 Component of Luria Bertani agar (per litre)

Peptone…….10 g

Yeast extracts…….5 g

NaCl……. 10 g

Agar…….15

3.7.2.5 Preparation of competent cells

A single fresh colony of *E. coli* JM107 was inoculated in LB broth and incubated overnight

⇩

An overnight culture of *E. coli* was transferred at 1:100 ratio into fresh LB broth

⇩

Cells were grown at 37^{0}C until OD600 was obtained

⇩

The culture was chilled in ice for 15 min to slow growth

⇩

Cells were pelleted by centrifugation at 5000 rpm for 15 min at 4^0C

⇩

Supernatant was decanted

⇩

Cells were re-suspended in 1/5 volume of original culture of ice-cold 0.1 M $CaCl_2$

⇩

Cells were incubated on ice for 20 min

⇩

Cells were pelleted by centrifugation at 5000 rpm for 15 min at 4^0C

⇩

Supernatant was decanted

⇩

Cells were re-suspended in 1/25 volume of original culture of ice-cold 0.1 M $CaCl_2$, 10% glycerol

⇩

100 µl aliquots were made into pre-chilled micro centrifuge tubes (1.5 ml)

⇩

Stored at -20^0C

3.7.2.5 Procedure of Transformation: (Sambrook, J., Fritsch , E.F., Maniatis, 1989)

The transformation was done by heat shock method involving the following steps-

⇩

The competent cells were removed from -20^0C and thawed on ice

⇩

The cells were mixed gently by flicking tubes and 50 µl of cells were taken in sterile micro-centrifuge tubes (1.5 ml)

⇩

5 µl of plasmids were added into the cell suspension and the tube was gently swirled for a few seconds to mix

⇩

The tubes were incubated in ice for 30 minutes

⇩

The tubes were placed in a water bath at 42^0C for 40 seconds without shaking

⇩

Tubes were removed from the water bath and immediately placed on ice for 10 minutes

⇩

The tubes were diluted by adding 500 µl of SOC medium

⇩

The tubes were kept in a shaker incubator at 37^0C and 200 rpm for 2 hours

⇩

The cells were centrifuged for 2 minutes at 5000 rpm

⇩

The pellets were spread on LB agar medium containing cefoxitin abiotic

⇩

The plate was incubated for 12-16 hours in an incubator at 37^0C

3.7.2.6 Spreading of cells on LB agar plates

Cells were centrifuged for 2 minutes and pellets were spread on LB agar medium containing specific antibiotic containing medium and incubated the plate at 37^0C.

Interpretation

After overnight incubation, the presence of any growth on the antibiotic containing LB agar plate confirms the successful transformation.

3.7.3 PCR detection of ESBL genes within transformants

The transformants carrying ESBL genes were further confirmed by PCR assay targeting single or multiple ESBL genes (Table 7)

3.8 Incompatibility typing of plasmids encoding ESBL resistant determinants

Incompatibility type of the transformant plasmid carrying different ESBL resistant determinants was determined by PCR based replicon typing targeting 18 different

replicons viz. FIA, FIB, FIC, HI1, HI2, I1-Ig, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA in 5 multiplex and 3 simplex PCR (Carattoli, 2011) (Table 8).

3.8.1 Preparation of DNA template

Transformant plasmids carrying single or multiplex ESBL genes were isolated using QIAGEN® Plasmid Mini kit (Qiagen, Hilden, Germany). Purified plasmid DNA was used as template for the reaction.

Table 8: Oligonucleotides used for amplifying different incompatibility types

Inc types	Primer pairs	DNA sequence	Product size (bp)
HI1	HI1-F HI1-R	5'-GGAGCGATGGATTACTTCAGTAC-3' 5'-TGCCGTTTCACCTCGTGAGTA-3'	471
HI2	HI2-F HI2-R	5'-TTTCTCCTGAGTCACCTGTTAACAC-3' 5'-GGCTCACTACCGTTGTCATCCT-3'	644
I1	I1-F I1-R	5'-CGAAAGCCGGACGGCAGAA-3' 5'-TCGTCGTTCCGCCAAGTTCGT-3'	139
X	X-F X-R	5'-AACCTTAGAGGCTATTTAAGTTGCTGAT-3 5'-TGAGAGTCAATTTTTATCTCATGTTTTAGC-	376
L/M	L/M-F L/M-R	5'-GGATGAAAACTATCAGCATCTGAAG-3' 5'-CTGCAGGGGCGATTCTTTAGG-3'	785
N	N-F N-R	5'-GTCTAACGAGCTTACCGAAG-3' 5'-GTTTCAACTCTGCCAAGTTC-3'	559
FIA	FIA-F FIA-R	5'-CCATGCTGGTTCTAGAGAAGGTG-3' 5'-GTATATCCTTACTGGCTTCCGCAG-3'	462
FIB	FIB-F FIB-R	5'-GGAGTTCTGACACACGATTTTCTG-3' 5'-CTCCCGTCGCTTCAGGGCATT-3'	702
W	W-F W-R	5'-CCTAAGAACAACAAAGCCCCCG-3' 5'-GGTGCGCGGCATAGAACCGT-3'	242
Y	Y-F Y-R	5'-AATTCAAACAACACTGTGCAGCCTG-3' 5'-GCGAGAATGGACGATTACAAAACTTT-3'	765
P	P-F	5'-CTATGGCCCTGCAAACGCGCCAGAAA-3'	534

Inc types	Primer pairs	DNA sequence	Product size (bp)
	P-R	5'-TCACGCGCCAGGGCGCAGCC-3'	
FIC	FIC-F FIC-R	5'-GTGAACTGGCAGATGAGGAAGG-3' 5'-TTCTCCTCGTCGCCAAACTAGAT-3'	262
A/C	A/C-F A/C-R	5'-GAGAACCAAAGACAAAGACCTGGA-3' 5'-ACGACAAACCTGAATTGCCTCCTT-3'	465
T	T-F T-R	5'-TTGGCCTGTTTGTGCCTAAACCAT-3' 5'-CGTTGATTACACTTAGCTTTGGAC-3'	750
FII_S	FII_S-F FII_S-R	5'-CTGTCGTAAGCTGATGGC-3' 5'-CTCTGCCACAAACTTCAGC-3'	270
F_{repB}	F_{repB}-F F_{repB}-R	5'-TGATCGTTTAAGGAATTTTG-3' 5'-GAAGATCAGTCACACCATCC-3'	270
K	K/B-F K/B-R	5'-GCGGTCCGGAAAGCCAGAAAAC-3' 5'-TCTTTCACGAGCCCGCCAAA-3'	160
B	B-F B-R	5'-GCGGTCCGGAAAGCCAGAAAAC-3' 5'-TCTGCGTTCCGCCAAGTTCGA-3'	159

3.8.1.1 Replicon types targeted in multiplex PCR

Five multiplex PCR was performed to target 15 incompatibility types of plasmids, which are as follows:

First Multiplex: HI1, HI2, I1-Ig

Second multiplex: X, L/M, N

Third multiplex: FIA, FIB, W

Fourth Multiplex: Y, P, FIC

Fifth multiplex: A/C, T, FIIA

3.8.1.2 Replicon type targeted in simplex PCR

(i) First simplex: $F_{rep}B$, (ii) Second simplex: K and (iii) Third simplex: B/O

Primers

The different oligonucleotides used for determining the incompatibility type of the plasmids are described in Table 8.

3.8.1.2 Preparation of reaction mixture

A total volume of 25 µl of reaction mixture was prepared containing 2 µl of template DNA (100 ng/µl), 1 µl of each primer (10 picomole), 12.5 µl of Go Taq Green Master Mix, 2X DNA polymerase (Promega, Madison, USA) and nuclease free water.

Reaction condition for multiplex PCR:

For PCR based replicon typing, PCR reaction was run under the following conditions:

Initial denaturation: 95^0C for 2 mins

Final denaturation: 95^0C for 20 secs

Annealing : 60^0C for 30 secs

Extension: 72^0C for 1 min

} 30 cycles

Final extension: 72^0C for 5 mins

Reaction condition for simplex PCR:

For PCR based replicon typing, PCR reaction was run under the following conditions:

Initial denaturation; 95^0C for 2 mins

Final denaturation: 95^0C for 20 secs

Annealing: 52^0C for 30 sec

Extension: 72^0C for 1 min

} 30 cycles

Final extension: 72^0C for 5 mins

3.8.1.3 Gel electrophoresis and Visualization of PCR products:

The amplified PCR products were identified by agarose gel electrophoresis. 10 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 60 volts

for two hours with TBE buffer. A molecular marker of 10 kb Hyper ladder (Genei, Bangalore, India) was run concurrently. The gel was visualized in the Gel Doc EZ imager (Bio-Rad, California, USA).

Interpretation

Presence of different molecular weights of bands confirms the presence of different Inc groups in plasmids.

3.8.2 Purification of amplified PCR products

The amplified PCR products of different incompatibility type were purified by PCR purification kit (Qiagen, Hilden, Germany).

3.8.3 DNA Sequencing

A total of 30µl of purified PCR products were used for sequencing along with the specific incompatibility type primers of 20 µl each (10 picomoles each primers). Sequence results were analyzed using BLAST suite program of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

3.9 Plasmid stability test of ESBL isolates

3.9.1 Selection of ESBL isolates

Twenty numbers of representative ESBL resistant isolates were selected for ESBL testing.

3.9.1.1 Serial Passage of the ESBL isolates

Plasmid stability analysis of all ESBL producers as well as their transformants were analyzed by serial passages method for 28 consecutive days at 1:1000 dilutions in Luria Bertani (LB) broth without antibiotic pressure (Locke JB *et al*., 2012).

Step 1(First day):

- The ESBL producing organism was cultured in LB broth in a falcon tube

Step 2 (Second day):

- 10 ml freshly prepared LB were transferred in Falcon tube
- 100ul of the bacterial culture from step 1 was inoculated in 10ml LB broth
- The broth was kept overnight in an incubator at 37°C

Step 3 (Third day):

- ESBL gene were detected from this incubated broth
- 100µl of this broth were further inoculated into fresh LB broth. These tubes were further incubated overnight at 37^0C
- From the step 1 to 3 were repeated until all the bacteria lost its ESBL genes

3.9.2 Detection of ESBL genes by PCR assay

PCR assay was carried out for the presence of *bla* genes in the isolates after each passage as per the above mentioned methods.

3.10 Detection of co-resistance gene in ESBL pathogens

Since, ESBL resistance genes are mostly plasmid mediated it may also carry genes encoding resistance to other class of antibiotic, such as ampA, ampC, aminoglycosides, macrolides, chloramphenicol, quinolone or carbapenems (Alyamani EJ *et al*., 2017). Therefore, the occurrence of co-resistance gene of following antibiotics was detected among ESBL uropathogens using the genotypic method. The Carbapenem-Resistant genes, Amp C resistant genes and Aminoglycoside resistant genes were detected in the study.

3.10.1 Molecular characterization of carbapenemases

The genotypic characterization of carbapenemase genes was performed by targeting three different classes such as class A, class B and class D carbapenemase genes.

3.10.1.1 Class A carbapenemase

PCR assay was performed targeting different class A carbapenemase genes namely *bla*$_{KPC}$, *bla*$_{IMI/NMC}$, *bla*$_{SME}$ (Table 9) for all the isolates which were ESBL positive. The specific oligonucleotides used for amplification of the partial gene are mentioned in table 7.

3.10.1.1.1 Preparation of DNA template

DNA was extracted by boiling centrifugation method (Freschi *et al*., 2005) as described earlier and used as a template for PCR reaction.

3.10.1.1.2 Preparation of reaction mixture

A total volume of 25 µl reaction mixture was prepared and each reaction mixture contained 2 µl of template DNA (100 ng/µl), 1 µl of each primer (10 picomole), 12.5 µl of Go Taq Green Master Mix, 2X DNA polymerase (Promega, Madison, USA) and the rest of the volume was made up with nuclease free water.

Reaction conditions:

The reaction condition followed for amplification of class A carbapenemase gene was as follows:

Initial denaturation: 95^{0}C for 2 min
Final denaturation: 95^{0}C for 20 secs
Annealing: 52^{0}C for 30 secs
Extension: 72^{0}C for 1 min
} 32 cycles
Final extension: 72^{0}C for 2 mins

Table 9: Oligonucleotides used as primers for amplification of class A carbapenemase genes

Primer Pairs	**Target**	**Sequence(5′-3′)**	**Amplified product size**	**Reference**
KPC F KPC R	KPC	5′-CATTCAAGGGCTTTCTTGCTGC-3′ 5′-ACGACGGCATAGTCATTTGC-3′	538	(Nass T *et al*.,1998)
SME F SME R	SME	5′-AACGGCTTCATTTTTGTTTAG-3′ 5′-GCTTCCGCAATAGTTTTATCA-3′	831	(Nass T *et al*.,1998)
IMI F IMI R	IMI/NMC	5′-CCATTCACCCATCACAAC-3′ 5′-CTACCGCATAATCATTTGC-3′	440	(Rasmussen *et al*.,1996)

3.10.1.1.4 Gel electrophoresis and analysis of PCR products

The DNA fragments of class A carbapenemase genes amplified by PCR were identified by agarose gel electrophoresis. 10 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 60 volts for two hours with 1X Tris Borate EDTA (TBE) buffer. Molecular marker of 100 bp DNA ladder (Genei, Banglore, India) was run concurrently and the gel was visualized in Gel Doc EZ imager (Bio-Rad, California, USA).

3.10.1.1.5 Interpretation

Presence of bands of molecular weight of 538 bp, 831 bp and 440 bp confirm the presence of KPC, SME and IMI or NMC genes respectively.

3.10.1.2 **Class B carbapenemase**

PCR assay was performed for characterizing different Metallo-beta-lactamase gene viz. bla_{NDM}, bla_{IMP}, $bla_{VIM,}$ bla_{GIM}, bla_{SIM}, bla_{SMB} and bla_{SPM}. The different oligonucleotides used are mentioned in a Table 10.

3.10.1.2.1 Preparation of DNA template

DNA was extracted by boiling centrifugation method as described earlier (Freschi et al., 2005).

3.10.1.2.2 Preparation of reaction mixture

25 µl reaction mixture was prepared which contained 2 µl of template DNA (100 ng/µl), 1 µl of each primer (10picomole), 12.5 µl of Go Taq Green Master Mix, 2X DNA polymerase (Promega, Madison, USA) and the remaining volume was made up with the addition of nuclease-free water.

Reaction conditions:

Two multiplex PCR was performed for amplification of Metallo-beta-lactamase genes

- ***Multiplex PCR-1***

First multiplex PCR was performed targeting the genes *bla*$_{NDM}$, *bla*$_{VIM}$ and *bla*$_{IMP}$. The reaction condition followed for amplification of these metallo-beta-lactamase genes were as follows:

Initial denaturation: 94^0C for 5 min,

Final denaturation: 94^0C for 30 secs
Annealing: 53^0C for 1 min
Extension: 72^0C for 1 min
} 32 cycles

Final extension: 72^0C for 5 mins

- ***Multiplex PCR-2***

Second multiplex PCR was performed targeting the other metallo-beta-lactamase genes *bla*$_{GIM}$, *bla*$_{SIM}$, *bla*$_{SMB}$ and *bla*$_{SPM.}$ The reaction condition followed for amplification of class A carbapenemase gene were as follows:

Initial denaturation: 95^0C for 5 mins
Final denaturation: 95^0C for 30 secs
Annealing: 52^0C for 1 min
Extension: 72^0C for 1 min
} 32 cycles

Final extension: 72^0C for 5 mins

3.10.1.2.4 Gel electrophoresis and analysis of PCR products

The DNA fragments amplified by PCR were identified by agarose gel electrophoresis. 5 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 60 volts for two hours with 1X Tris Borate EDTA (TBE) buffer. A molecular marker of 100 bp DNA ladder (Genei, Banglore, India) was run concurrently and the gel was visualized in Gel Doc EZ imager (Bio-Rad, California, USA).

Interpretation:

- ***Multiplex PCR-1*:**

  Presence of bands of molecular weight of 476 bp, 390 bp and 139 bp confirm the presence of NDM, VIM and IMP genes respectively.

- ***Multiplex PCR-2*:**

  Presence of bands of molecular weight of 614 bp, 571 bp, 492 bp and 784 bp confirm the presence of GIM, SIM, SMB and SPM genes respectively.

Table 10 : Oligonucleotides used as primers for amplification of metallo-beta-lactamase genes

Primer Pairs	Target	Gene	Sequence(5′-3′)	Product size	Reference
NDM F NDM R	NDM	bla_{NDM}	5′-GGGCAGTCGCTTCCAACGGT-3′ 5′-GTAGTGCTCAGTGTCGGCAT-3′	476	(Yong D *et al*., 2009)
VIM F VIM R	VIM	bla_{OXA}	5'-GATGGTGTTTGGTCGCATA-3' 5'-CGAATGCGCAGCACCAG-3'	390	(Tsakris A *et al*., 2009)
IMP F IMP R	IMP	bla_{IMP}	5'-TTGACACTCCATTTACDG-3' 5'-GATYGAGAATTAAGCCACYCT-3'	139	(Ito H *et al.,* 1995)
GIM-F GIM-R	GIM	bla_{GIM}	5'-GCTCAGGGTCATAAACCGCT-3' 5'- CTTCCAACTTTGCCATGCCC-3'	614	(Castanheira M *et al.*,200
SIM-F SIM-R	SIM	bla_{SIM}	5'- TACAAGGGATTCGGCATCG -3' 5'- TAATGGCCTGTTCCCATGTG -3'	571	(Lee K *et al.,* 2005)
SMB-F SMB-R	SMB	bla_{SMB}	5'- CAGCAGCCATTCACCATCTA-3' 5'- GAAGACCACGTCCTTGCACT-3'	492	(Wachino J *et al.*,2011)
SPM-F SPM-R	SPM	bla_{OXA}	5'- CTGCTTGGATTCATGGGCGC-3' 5'-GATCAAGGTCGCGGAAAAGG-3'	784	(Toleman MA *et al.,* 200

3.10.1.3 Class D carbapenemase

Multiplex PCR assay was performed for characterizing different class D carbapenemase genes viz. bla_{OXA-23}, bla_{OXA-48}, bla_{OXA-58} and $bla_{OXA-198}$. The different oligonucleotides used are mentioned in a Table 11.

3.10.1.3.1 Preparation of DNA template

DNA was extracted by boiling centrifugation method as mentioned earlier (Freschi *et al*., 2005).

3.10.1.3.2 Preparation of reaction mixture

A total volume of 25 µl reaction mixture was prepared for each sample which contained 2 µl of template DNA (100 ng/µl), 1 µl of each primer (10 picomole), 12.5 µl of Go Taq Green Master Mix 2X DNA polymerase (Promega, Madison, USA) and the remaining volume made up with nuclease-free water.

Reaction conditions:

The reaction condition followed for amplification of class D carbapenemase genes was as follows:

Initial denaturation: 95^0C for 2 mins
Final denaturation: 95^0C for 45 secs
Annealing: 54^0C for 1 min
Extension: 72^0C for 1.20 min
Final extension: 72^0C for 5 min

32 cycles

Table 11: Oligonucleotides used for amplification of class D carbapenemase genes

Primer Pairs	Target	Sequence(5′-3′)	Amplified product size	Reference
OXA-23 F OXA-23 R	OXA-23	5′-CATTCAAGGGCTTTCTTGCTGC-3′ 5′-ACGACGGCATAGTCATTTGC-3′	501	(Woodford N *et al.*,2004)
OXA-48 F OXA-48 R	OXA-48	5′-AACGGCTTCATTTTTGTTTAG-3′ 5′-GCTTCCGCAATAGTTTTATCA-3′	845	(Cuzon G, Bonnin RA, 2013)
OXA-58 F OXA-58 R	OXA-58	5′-CCATTCACCCATCACAAC-3′ 5′-CTACCGCATAATCATTTGC-3′	529	(Poirel L *et al.*, 2005)
OXA-198 F OXA-198 R	OXA-198	5′-TTCGTCGCTACACAACGGAA-3′ 5′-GGCTCGCGTAATTCTCCGTA-3′	312	(Garch FE *et al.*,2011)

Multiplex PCR was performed targeting all the AmpC genes by using a pair of primers as listed in Table 3. Isolates were investigated for the presence of other AmpC gene families namely; DHA, CIT, ACC, FOX and EBC (Dallenne *et al.*, 2010). PCR amplification was performed using 30 µl of total reaction volume.

Reactions conditions:

Initial denaturation: 95 °C for 2 mins
Final denaturation: 95 °C for 15s
Annealing: 51 °C for 1 min
Extension: 72 °C for 1 min
Final extension: 72 °C for 7 mins

34 cycles

3.10.2 Molecular characterization of AmpC resistant genes

The genotypic characterization of AmpC resistant genes was performed by targeting

PCR assay was performed targeting different types of AmpC resistant genes namely *bla*$_{DHA}$, *bla*$_{ACC}$, *bla*$_{FOX,}$ *bla*$_{EBC}$ and *bla*$_{MOX}$ (Table 12) for all the isolates which were ESBL positive isolates. The specific oligonucleotides used for amplification of the partial gene are mentioned in Table 12.

3.10.2.1 Preparation of DNA template

DNA was extracted by boiling centrifugation method (Freschi C R *et al*., 2005) as, mentioned earlier and used as template for PCR reaction.

3.10.2.2 Preparation of reaction mixture

A total volume of 25 µl reaction mixture was prepared and each reaction mixture contained 2 µl of template DNA (100 ng/µl), 1 µl of each primer (10 picomole), 12.5 µl of Go Taq Green Master Mix, 2X DNA polymerase (Promega, Madison, USA) and the rest of the volume was made up with nuclease-free water.

Reaction conditions

The reaction condition followed for amplification of class AmpC gene was;

Initial denaturation: 95 °C for 2 mins

Final denaturation: 95 °C for 15secs
Annealing: 51 °C for 1 min
Extensuin: 72 °C for 1 min
} 34 cycles

Final extension: 72 °C for 7 mins

3.10.2.4 Gel electrophoresis and analysis of PCR products

The DNA fragments of AmpC genes were amplified by PCR were identified by agarose gel electrophoresis. 10 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 60 volts for two hours with 1X Tris Borate EDTA (TBE) buffer. Molecular marker of 100 bp DNA ladder (Genei, Banglore, India) was run concurrently and the gel was visualized in Gel Doc EZ imager (Bio-Rad, California, USA).

3.10.2.5 Interpretation

Presence of bands of molecular weight of 997 bp, 538 bp, 346, 162,683 and 895 bp confirm the presence of DHA, CIT, ACC, FOX, EBC and MOX genes respectively.

Table 12: Detailed information of primers used in multiplex PCR for detection of AmpC resistant genes (Caroline D *et al.*, 2010)

List of primer pairs	**Target**	**Sequence (5'-3')**	**Product size (bp)**
DHA F DHA R	DHA-1 and DHA-2	5'-TGATGGCACAGCAGGATATTC-3' 5'-GCTTTGACTCTTTCGGTATTCG-3'	997
CIT F CIT R	LAT-1 to LAT-3, BIL-1, CMY-2 to CMY-7, CMY-12 to CMY-18 and CMY-21 to CMY-23	5'-CGAAGAGGCAATGACCAGAC-3' 5'-ACGGACAGGGTTAQGATAQ-3'	538
ACC-F ACC-R	ACC-1 and ACC-2	5'-CACCTCCAGCGACTTGTTAC-3' 5'-GTTAQCCAGCATCACGATCC-3'	346
FOX-F FOX-R	FOX-1 to FOX-5	5'-CTACAGTGCGGGTGGTTT-3' 5'-CTATTTGCGGCCAGGTGA-3'	162
EBC-F EBC-R	ACT-1 and MIR-1	5'-CGGTAAAGCCGATGTTGCG-3' 5'-AGCCTAACCCCTGATACA-3'	683
MOX –F MOX R	MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11 and CMY-19	5'-GCAACAACGACAATCCATCCT-3' 5'-GGGATAQGCGTAACTCTCCCAA-3'	895

3.10.3 Molecular characterization of aminoglycoside-resistant genes

The genotypic characterization of aminoglycoside-resistant genes was performed by PCR assay targeting different types of 16s methyl transferase resistant genes namely *ArmA,RmtB, NpmA* (multiplex 1) *and RmtA, RmtC, Rmt D* (multiplex II) (Table) for all the isolates which were ESBL positive isolates. The specific oligonucleotides used for amplification of the partial gene are mentioned in a Table 13.

3.10.3.1 Preparation of DNA template

DNA was extracted by boiling centrifugation method (Freschi *et al*., 2005) and used as a template for PCR reaction.

3.10.3.2 Preparation of reaction mixture

A total volume of 25 µl reaction mixture was prepared and each reaction mixture contained 2 µl of template DNA (100 ng/µl), 1 µl of each primer (10 picomoles), 12.5 µl of Go Taq Green Master Mix, 2X DNA polymerase (Promega, Madison, USA) and the rest of the volume was made up with nuclease-free water.

Reaction conditions:

The reaction condition followed for amplification of class Aminoglycoside gene was as follows:

Initial denaturation: 94°C for 5 mins
Final denaturation: 94°C for 30 secs
Annealing: 52°C for 40 secs
Extension: 72°C for 1 min 20 secs
Final extension: 72°C for 7 mins

34 cycles

3.10.3.4 Gel electrophoresis and analysis of PCR products

The DNA fragments of 16s methyl transferase genes amplified by PCR were identified by agarose gel electrophoresis. 10 µl of each PCR product was run in 1% agarose gel (Hi-Media, Mumbai, India) at constant 60 volts for two hours with 1X Tris Borate EDTA (TBE) buffer. A molecular marker of 100 bp DNA ladder (Genei, Banglore, India) was run concurrently and the gel was visualized in Gel Doc EZ imager (Bio-Rad, California, USA).

3.10.3.5 Interpretation

Presence of bands of molecular weight of 1153 bp, 635 bp, 756 bp, 1000, 744 bp and 981 confirm the presence of *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD* and *npmA* genes respectively.

Table 13: Detailed information of primers used in multiplex PCR for detection of Aminoglycoside resistant genes (Wu Q *et al.*, 2009)

List of primer pairs	**Target**	**Sequence (5'-3')**	**Product size (bp)**
armA-F armA-R	armA	5'- GGTGCGAAAACAGTCGTAGT-3' 5'-TCCTCAAATATCCTCTATGT-3'	1153
rmtA-F rmtA-R	rmtA	5'- CTAGCGTCCATCCTTTCCTC-3' 5'- TTTGCTTCCATGCCCTTGCC-3'	635
rmtB-F rmtB-R	rmtB	5'-GGAATTCCATATGAACATCAACGATGCC-3' 5'-RCCGCTCGAGTCCATTCTTTTTTATCAAGT-3'	756
rmtC-F rmtC-R	rmtC	5'- CGAAGAAGTAACAGCCAAAG-3' 5'-GCTAGAGTCAAGCCAGAAAA-3'	1000
rmtD-F rmtD-F	rmtD	5'- TCATTTTCGTTTCAGCAC -3' 5'- AAACATGAGCGAACTGAAGG-3'	744
npmA-F npmA-R	npmA	5'-CGGGATCCAAGCACTTTCATACTGACG-3' 5'-CGGAATTCCAATTTTGTTCTTATTAG-3'	981

3.11 Minimum Inhibitory Concentration (MIC) tests for Carbapenem positive isolates:

MIC test was performed for the isolates harboring carbapenem-resistant gene which included *bla*$_{NDM}$, *bla*$_{OXA-48}$, *bla*$_{VIM}$ gene against commonly used carbapenem antibiotics like imipenem, meropenem and ertapenem (CLSI, 2014). The MIC value of isolates harboring NDM gene was detected by agar dilution method against imipenem (Lupin, India), ertapenem (MSD, India) and meropenem (Ameropem, India). The antibiotics were procured from the pharmaceuticals. The MIC interpretive standards for the susceptibility categories were categorized as per the breakpoints recommended by the CLSI, 2014.

3.11.1 Preparation of stock solution

A fresh stock of antibiotic solutions was prepared based on serial dilution Dilutions of antibiotics was prepared based on the 1mg/litre. Ten different concentrations of antibiotics were prepared i.e. 0.25, 0.5, 1, 2, 4,8,16, 32, 64, 128 and 256 mg/litre etc. The stock solution was prepared by using the following formula:

1000/P x V x C = W

Where P= potency of preparation in relation to base, V= volume (ml) required; C=final concentration of solution in multiples of 1000 and W= weight (mg) of antibiotic to be dissolved in V. (Collee JG *et al*., 1996).

A method for preparing working dilutions of antibiotic is shown in Figure 3.

For each antibiotic, a serial dilution ranging from 0.25 mg/l-256mg/l was prepared in sterile distilled water aseptically. The detailed procedures as follows:

3.11.1.2 Preparation of agar dilution plates

Sterile empty plates were marked with a particular dilution of antibiotics.

⇩

A series of dilution of carbapenem antibiotics like imipenem, meropenem and ertapenem ranging from 2-256 µg/ml were placed onto the marked sterile empty plates accordingly.

⇩

Then after 20ml of sterile molten MHA agar approx 42-45°C were poured into all Petri dishes

⇩

The plates were gently rotated in clockwise and anticlockwise direction in order to mix the antibiotic solution and media

3.11.1.3 Preparation of test organism

The test organisms were inoculated into Nutrient agar plate to obtain pure and isolated colonies

⇩

Three to four pure culture colonies were picked up and transferred into peptone water for preparation of inoculums

⇩

The inoculums were picked up and incubated at 37°C for 1-2 hours till light to moderate turbidity was achieved.

⇩

The turbidity of the suspension was adjusted to McFarland's Standard 0.5 (1.5×10^8 CFU/ml).

⇩

With the help of ruler and marker backside of these plates were divided into 20-25 squares too place the test inoculums

⇩

Test inoculums were added into each square and incubated

⇩

After 12 to 14 hours the MIC of the isolates was noted.

3.12 Detection of MIC creep phenomenon

NDM positive isolates having (MIC≤2, MIC≤4) for imipenem were subjected to serial passage in LB broth with a gradually increasing concentration exposure of imipenem from 0.25 µg/ml to 8µg/ml for consecutive six days (Locke JB *et al*., 2012).

The detailed procedure for the detection of MIC creep phenomenon is as follows:

Step 1(First day):

- NDM positive isolates having (MIC≤2, MIC≤4) for imipenem were cultured into a 10 ml LB broth in a falcon tube

Step 2 (Second day):

- 10 ml of freshly prepared LB broth were transferred in a Falcon tube
- 100µl of the bacterial culture from step 1 was inoculated into 10ml of freshly prepared LB broth containing0.25 µl solution of antibiotic (imipenem)
- The broth was kept overnight in an incubator at 37°C.

Step 3 (Third day):

- A loopful of broth from the broth of step 2 was inoculated on LB agar to check the growth of bacteria.
- 100µl of the bacterial culture from step 2 was inoculated into 10ml of freshly prepared LB broth containing 0.5 µl solution of antibiotic (imipenem)
- The broth was kept overnight in an incubator at 37°C.

Step 4 (Fourth day):

- A loopful of broth from the broth of step 3 was inoculated on LB agar to check the growth of bacteria.

- 100µl of the bacterial culture from step 3 was inoculated into 10ml of freshly prepared LB broth containing 1 µl solution of antibiotic (imipenem)
- The broth was kept overnight in an incubator at 37°C.

Step 5 (Fifth day):

- A loopful of broth from the broth of step 4 was inoculated on LB agar to check the growth of bacteria.
- 100µl of the bacterial culture from step 4 was inoculated into 10ml of freshly prepared LB broth containing 2 µl solution of antibiotic (imipenem)
- The broth was kept overnight in an incubator at 37°C.

Step 6 (Sixth day):

- A loopful of broth from the broth of step 5 was inoculated on LB agar to check the growth of bacteria.
- 100µl of the bacterial culture from step 5 was inoculated into 10ml of freshly prepared LB broth containing 4 µl solution of antibiotic (imipenem)
- The broth was kept overnight in an incubator at 37°C.

Step 7 (Seventh-day):

- A loopful of broth from the broth of step 6 was inoculated on LB agar to check the growth of bacteria.
- 100µl of the bacterial culture from step 6 was inoculated into 10ml of freshly prepared LB broth containing 8 µl solution of antibiotic (imipenem)
- The broth was kept overnight in an incubator at 37°C.

3.12.1 Detection of Revertants

The isolates tested for MIC creep with elevated MIC against imipenem were subjected to serial passage in LB broth at 1:1000 dilutions in the absence of imipenem for 30 consecutive days (Locke JB *et al*., 2012). MIC of each strain was subsequently determined at the 10th, 20th and 30th day of passage respectively.

Step 1

- 10 ml of freshly prepared LB broth were transferred in a Falcon tube
- 100µl of the bacterial culture from step 7 was inoculated into 10ml of freshly prepared LB broth without any antibiotic pressure
- The broth was kept overnight in an incubator at 37°C.

Step 2

- 10 ml of freshly prepared LB broth were transferred in a Falcon tube
- 100µl of the bacterial culture from step 1 was inoculated into 10ml of freshly prepared LB broth without any antibiotic pressure
- The broth was kept overnight in an incubator at 37°C.

Step 3

- Step 2 was repeated for 9 days and MIC of the strain was checked.

Step 5

- Step 2 was repeated for 10 days and on 20^{th} day MIC of the strain was checked.

Step 6

- Step 2 was repeated for 10 days and on 30^{th} day MIC of the strain was checked.

Chapter 4

Results

4. RESULTS

4.1 Isolation and Identification of bacteria

From June 2014 to September 2016 a total of 1516 urine samples were collected from UTI suspected female patients of age group 18 to 48 years. Among the 1,516 urine samples collected from female patients suspected to have a UTI, 454 showed significant growth (significant bacteriuria) of a single type of microorganism with a prevalence rate of 29.94%. *E. coli* were found to be the most predominant uropathogen among the 454 samples with a percentage of 74.88% followed by *K. pneumoniae* (20.2%), *Pseudomonas aeruginosa* (2.4%), and *P. mirabilis* (1.98%).

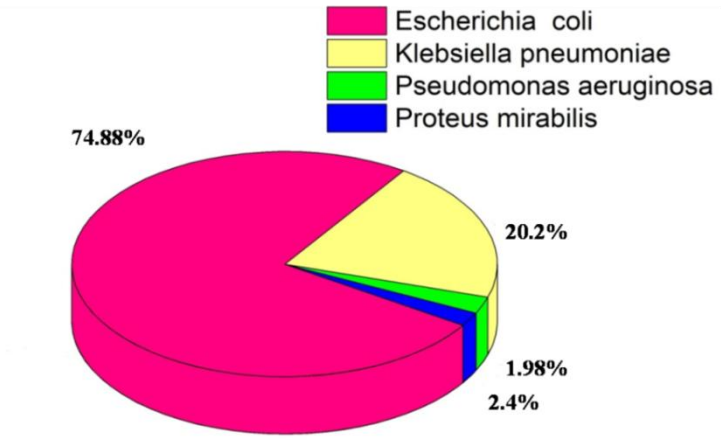


Figure 4: Distribution of uropathogens

The bacterial isolates were identified based on Gram staining, cultural characteristics and standard biochemical tests (Table 14).

Table 14: Tests performed for the identification of bacteria

	Gram staining	**Cultural Characteristics**		**Biochemical Tests**											
		MacConkey agar	CLED Agar	Lacose	Motility	Gas production	Indole	VP	Citrate	PDA	Urease	H_2S	Oxidase	TSI	*Bacteria*
Group A (n=340)	Gram negative	Dry pink, Small colonies	Yellow opaque, Small colonies	+	+	+	+	-	-	-	-	-	_	A/A	*Escherichia coli*
Group B (n=92)	Gram negative	Sticky pink, Mucoid colonies	Mucoid yellow to blue Colonies	+	-	++	_	+	+	_	+	_	-	A/A	*Klebsiella pneumoniae*
Group C (n=11)	Gram negative	Pale yellow colonies	Pale yellow colonies	-	+	+	_	+/-	+/-	+	++	+	-	K/A	*Proteus mirabilis*
Group D (n=9)	Gram negative	Pale yellow colonies	Pale yellow colonies	-	+	_	_	_	+	-	_	_	+	K/K	*Peeudomonas aeruginosa*

Lactose- Fermentation of lactose; Motility-Motility of bacteria; Gas production- Gas production from glucose; Indole- Indole production; VP-Voges Proskauer; Citrate- Citrate utilization; PDA- Phenylalanine deaminase; Urease-Urease production; H_2S- H_2S production; Inositol-Inositol production, Oxidase- Oxidase production; TSI-Triple sugar Iron Agar

Genotypic identification by 16srRNA sequencing: Sanger sequencing of 16srRNA gene of representative uropathogen was performed for the identification of bacteria. Accordining to 16srRNA sequencing results, the uropathogens were identified as *Escherichia coli* (n-340), *Klebsiella pneumoniae* (n=92), *Pseudomonas aeruginosa* (n=11) and *Proteus mirabilis* (n=9). *E.coli* was the prevalent uropathogen whereas the prevalence of uropathogen was least. The sequences were submitted to gene bank and the accession numbers were provided (Table 15).

Table 15: Identification of uropathogens and their accession numbers

Sl no.	Microorganism	Accession number
1.	*Escherichia coli*	MH393633
2.	*Klebsiella pneumoniae*	MH393634
3.	*Pseudomonas aeruginosa*	MH393636
3.	*Proteus mirabilis*	MH393635

4.1.1 Study of Asymptomatic bacteriuria (ASB)

Additionally, the prevalence of asymptomatic bacteriuria among 207 females was checked.. The prevalence of asymptomatic bacteriuria was found to be 4.34% only. Asymptomatic bacteriuria is defined by the occurrence of at least 10^5cfu/ml of a urinary tract pathogen per milliliter in a culture of a midstream urine specimen obtained from an asymptomatic woman (Rowe et al., 2014). Risk factor of asymptomatic bacteriuria was calculated using online software, Relative risk calculator "MEDCALC".

Table 16: Risk Factors Associated with Asymptomatic UTI

Risk factor of asymptomatic UTI	Status	Total	Total no. of respondents	Statistics			
				RR	95% Cl	Z-test	P-value
Sexual activity	Sexual activity (Yes)	144	207	1.3913	1.2194 to 1.5875	4.907	P < 0.0001
	Sexual activity (No)	63	207				
Frequency of water consumption	Less than 1litre	109	207	1.0531	0.8964 to 1.2373	0.630	0.5288
	More than 1 litre	98	207				

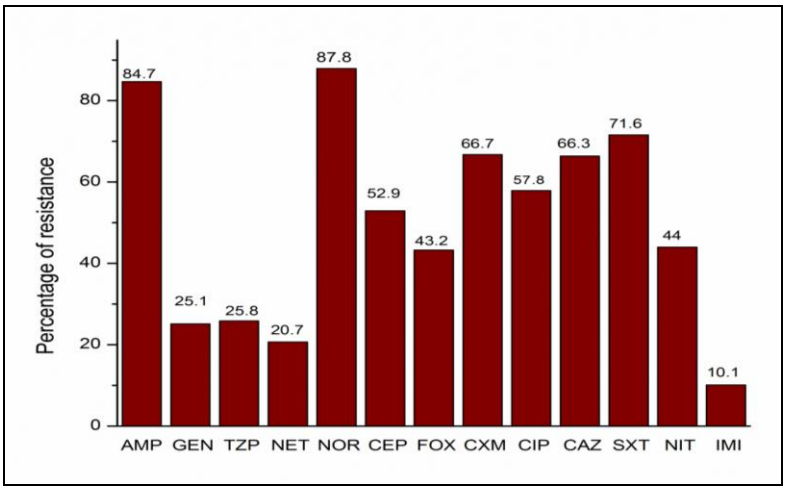
Among all the risk factors of UTI mentioned in the questionnaire namely; sexual activity, frequency of water consumption in a day, sanitary condition, process of cleaning their private part after excretion of faeces, distance of toilet from their work place or home. From the current study it was revealed that sexual activity and frequency of water consumption in a day are the two main risk factors for UTI found in the study area as its Relative risk value was found to be more than 1 i.e. 1.3913 and 1.03.1 respectively (Table 16).

4.2 Antibiotic Susceptibility testing of bacteria

4.2.1 Antibiotic Susceptibility testing of bacteria by Kirby Bauer disc diffusion method

A total of 454 uropathogens were subjected to 13 numbers of commonly used antibiotics against urinary tract infection. The uropathogens showed different antibiotic resistance profile against various group of antibiotics. *Escherichia coli* showed maximum resistance to Norfloxacin (87.88), followed by ampicillin (84.7%), Trimethoprim /Sulfamethoxazole (71.6%), cefuroxime (66.7%), ceftazidime (66.3%),

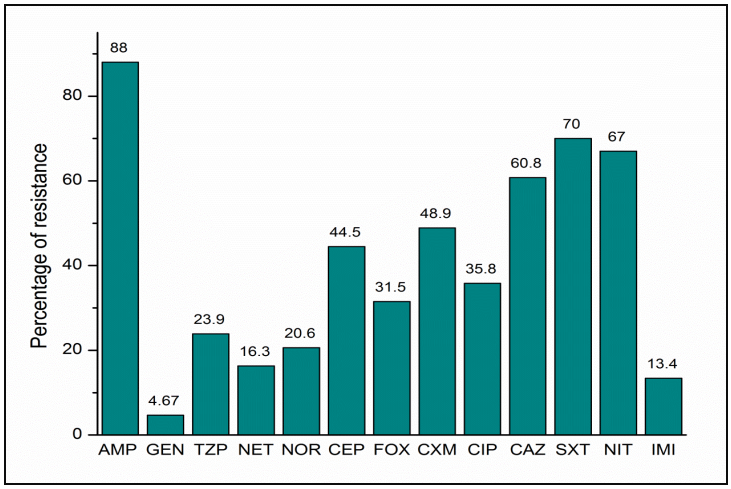
Ciproflocaxin (57.8%), Cephalothin (52.9%), Cefoxitin (43.27%) Nitrofurantoin (44%), Piperacillin /Tazobactum (25.88%), Gentamicin (25.14%), Netillin (20.7%) and Imipenem (10.1%) **(Figure 5)**



AMP-Ampicillin; **GEN**-Gentamicin; **TZP**-Piperacillin /Tazobactum; **NET**- Netillin; **NOR**-Norfloxacin; **CEP**-Cephalothin ; **FOX**-Cefoxitin; **CXM**-Cefuroxime; **CIP**-Ciprofloxacin; **CAZ**-Ceftazidime; **SXT**-Trimethoprim /Sulfamethoxazole; **NIT**-Nitrofurantoin ; **IMI**- Imipenem

Fig 5: Antibiotic resistance pattern of *Escherechia coli*

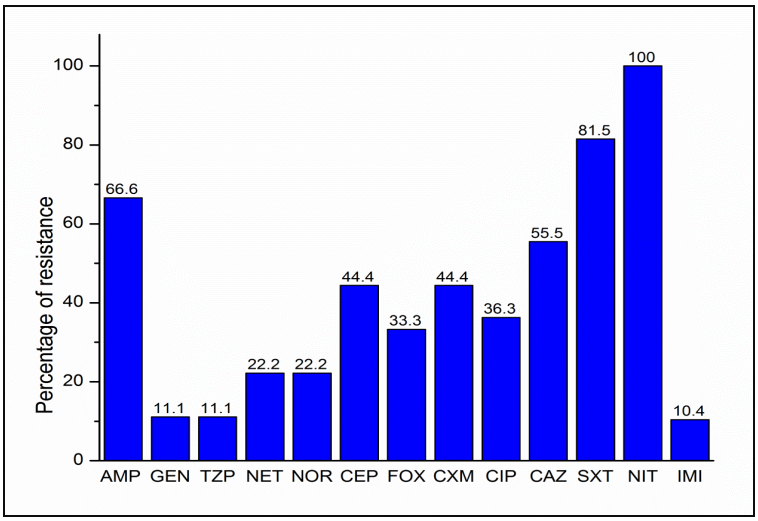
Klebsiella pneumoniae showed highest resistance to ampicillin (88.7%) followed by Trimethoprim /Sulfamethoxazole (70%), nitrofurantoin (67%), ceftazidime (60.86%), cefuroxime (48.91%), Cephalothin (44.56%), Ciprofloxacin (35.86%), Cefoxitin (31.52%), Piperacillin /Tazobactum (23.91%), Norfloxacin (20.65%), Netillin (16.30%), ceftazidime (13.4%) and gentamicin (4.67%) imipenem (13.4%) **(Figure 6).**



AMP-Ampicillin; **GEN**-Gentamicin; **TZP**-Piperacillin /Tazobactum; **NET**- Netillin; **NOR**-Norfloxacin; **CEP**-Cephalothin ; **FOX**-Cefoxitin; **CXM**-Cefuroxime; **CIP**-Ciprofloxacin; **CAZ**-Ceftazidime; **SXT**-Trimethoprim /Sulfamethoxazole; **NIT**-Nitrofurantoin ; **IMI**- Imipenem

Fig 6: Antibiotic resistance pattern of *Klebsiella pneumoniae*

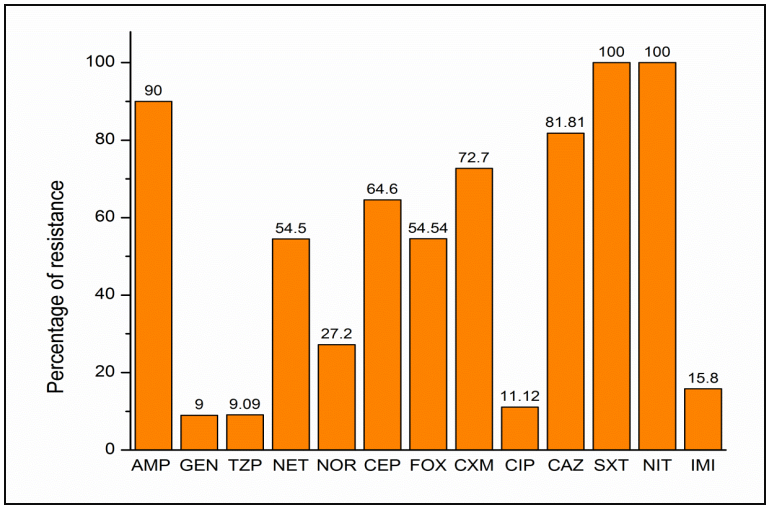
Proteus mirabilis showed highest resistance to Nitrofurantoin (100%) Trimethoprim/ Sulfamethoxazole (81.5%), ceftazidime (55.56%), Cephalothin (44.45%), cefuroxime (44.45%), Ciprofloxacin (36.37%), Cefoxitin (33.34%), Netillin (22.23%), Norfloxacin (20.65%), Piperacillin /Tazobactum (11.12%), gentamicin (11.12%), imipenem (10.4%) **(Figure 7)**



AMP-Ampicillin; **GEN**-Gentamicin; **TZP**-Piperacillin /Tazobactum; **NET**- Netillin; **NOR**-Norfloxacin; **CEP**-Cephalothin ; **FOX**-Cefoxitin; **CXM**-Cefuroxime; **CIP**-Ciprofloxacin; **CAZ**-Ceftazidime; **SXT**-Trimethoprim /Sulfamethoxazole; **NIT**-Nitrofurantoin ; **IMI**- Imipenem

Fig 7: Antibiotic resistance pattern of *Proteus mirabilis*

Pseudomonas aeruginosa showed highest resistance to Nitrofurantoin (100) Trimethoprim /Sulfamethoxazole (100%), Ampicillin (90%), Ceftazidime (81.81%), Cefuroxime (72.7%), Cephalothin (64.6%), Cefoxitin (54.45%), Netillin (54.5%), Norfloxacin (27.2%), Piperacillin /Tazobactum (9.09%), Gentamicin (9%), Ciprofloxacin (11.2%), and (15.8%) **(Figure 8)**



AMP-Ampicillin; **GEN**-Gentamicin; **TZP**-Piperacillin /Tazobactum; **NET**- Netillin; **NOR**-Norfloxacin; **CEP**-Cephalothin ; **FOX**-Cefoxitin; **CXM**-Cefuroxime; **CIP**-Ciprofloxacin; **CAZ**-Ceftazidime; **SXT**-Trimethoprim /Sulfamethoxazole; **NIT**-Nitrofurantoin ; **IMI**- Imipenem

Fig 8: Antibiotic resistance pattern of *Pseudomonas aeruginosa*

4.2.2 Antibiotic susceptibility testing of bacteria by Minimum Inhibitory Concentration method

Minimum Inhibitory Concentration for all the isolated uropathogens was performed by agar dilution method (Figure 9). Out of 340 *E.coli isolates* 214 isolates were found to be resistant to cefotaxime and remaining (n=126) sensitive. In case of *Klebsiella pneumoniae* (n=54) numbers of isolate showed resistance to cefotaxime and only 28 isolate was found to be sensitive. Highest number of *K.pneumoniae* (n=18) showed M/C value of 64 µg/ml (n=18) followed by 256 µg/ml (n=17). Out of 11 isolates of *Pseudomonas aeruginosa* 10 isolates were found to be on the category of resistance with the maximum number of isolates having MIC value of 256 µg/ml (n=3) and 64

µg/ml (n=3). In case of *Proteus mirabilis* five isolates were found to be under the category of resistance with the MIC distribution of 32 µg/ml (n=1), 128 µg/ml (n=2) and 256 µg/ml (n=2).

Highest number of *E.coli* (n=85) isolates had MIC value of 85 µg/ml followed by MIC value of 128 µg/ml. Out of total isolates highest number of *E.coli* were observed to be on the category of resistance (n=242) and remaining sensitive similarly for *Klebsiella pneumonia* maximum number of isolates were found to be resistant (n=65) with highest number of isolates showing the MIC value of 256 µg/ml (n=85).

Out of 81 isolates of *Pseudomonas aeruginosa* 8 isolates were found to be resistant with the MIC value of 256 µg/ml & 128 µg/ml (n=4). Whereas, in case of *Proteus mirabilis* 5 isolates were found to be sensitive and 4 isolates resistant with MIC value of 8 µg/ml (n=2) and 256 µg/ml (n=2).

In case of cefepime and ceftazidime highest number of isolates (n=77) and (n=33) respectively, were found to be under the category of intermediate, which on increase of one fold dilution will fall into the category of resistance. Highest number of *E.coli* isolates (n=151) had MIC value of 256 µg/ml for cefpodoxime which indicates the maximum use of cefpodoxime for the treatment of UTI.

Cefepime was found to be a little effective against *E.coli* as compared to other antibiotics of cephalosporin as 133 isolates were found to be sensitive, and 80 numbers of isolates being resistant. Out of 11 isolates of *Pseudomonas aeruginosa* 7 isolates were found to be resistant with the MIC value of 64 µg/ml (n=3) and 256 µg/ml (n=4). In case of ceftazidime highest number of *E.coli* (n=83) and *Klebsiella pneumonia* (n=22) were found to be intermediate. Highest number of Proteus

mirabilis n= (7) were found to be resistant with maximum of them having MIC value of 256 µg/ml (n=5)

MIC result showed that 55.28% (n=251), 25.11% (n=114), 19.82% (n=90), 17.62% (n=80) and 16.74% (n=76) isolates had MIC value of 256 µg/ml against cefpodoxime, ceftriaxone, cefotaxime, ceftazidime and cefepime respectively. On the contrary, 22.69% (n=103) and 24.2% (n=110) of isolates were intermediate against cefepime and ceftazidime with their MIC values of 16µg/ml and 8µg/ml respectively. In case of aztreonam higher number of isolates (22%, n=75) showed MIC value of 128µg/ml. Imipenem showed good sensitivity against a maximum number of uropathogens with the MIC value as 2µg/ml for 26.43 % of isolates. Interestingly, 14.4% of *E. coli* and 15.2% of *Klebsiella pneumoniae* has a MIC value of 4µg/ml which if increases its MIC by two-fold will fall into the category of resistance (Table 17).

Table 17: MIC pattern of different uropathogens against common antibiotics

Bacteria	Antibiotic concentration									
	0.5 µg/ml	1 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	256 µg/ml
Cefotaxime										
***E.coli* (n=340)**	45	16	17	48	15	23	20	48	37	71
***K.pneumoniae*(n=92)**	16	2	4	6	10	4	3	18	12	17
***P.aeruginosa* (n=11)**	1	0	0	0	0	2	1	3	1	3
***P.mirabilis* (n=9)**	2	0	0	2	0	0	1	0	2	2
Ceftriaxone										
***E.coli* (n=340)**	21	10	31	36	44	12	21	33	47	85
***K.pneumoniae*(n=92)**	6	6	7	8	12	2	10	3	15	23
***P.aeruginosa* (n=11)**	0	0	3	0	0	0	0	0	4	4
***P.mirabilis* (n=9)**	2	1	1	1	2	0	0	0	0	2
Cefepime										
***E.coli* (n=340)**	60	6	7	45	15	77	10	36	32	52
***K.pneumoniae*(n=92)**	15	2	8	4	4	22	3	10	6	18
***P.aeruginosa* (n=11)**	0	0	0	1	0	3	0	3	0	4
***P.mirabilis* (n=9)**	3	0	0	0	1	1	0	2	0	2
Ceftazidime										
***E.coli* (n=340)**	0	14	14	35	83	31	46	18	28	55
***K.pneumoniae*(n=92)**	2	2	4	5	22	14	15	3	7	18
***P.aeruginosa* (n=11)**	1	0	2	0	2	2	0	0	3	1
***P.mirabilis* (n=9)**	1	0	0	0	3	2	0	0	0	5

Bacteria	Antibiotic concentration									
	0.5 µg/ml	1 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	256 µg/ml
Cefpodoxime										
***E.coli* (n=340)**	8	15	0	0	0	13	8	75	61	151
***K.pneumoniae*(n=92)**	9	3	0	0	0	2	0	20	18	40
***P.aeruginosa* (n=11)**	3	0	0	0	0	0	0	3	0	5
***P.mirabilis* (n=9)**	0	1	0	0	0	0	0	0	5	5
Aztreonam										
***E.coli* (n=340)**	18	25	22	55	53	23	16	31	65	32
***K.pneumoniae*(n=92)**	3	5	2	12	12	12	7	5	7	17
***P.aeruginosa* (n=11)**	0	2	0	0	1	0	0	3	0	5
***P.mirabilis* (n=9)**	0	1	0	0	0	0	0	2	3	3
Imipenem										
***E.coli* (n=340)**	41	51	76	48	16	27	12	20	22	27
***K.pneumoniae*(n=92)**	30	7	20	14	5	7	3	3	7	6
***P.aeruginosa* (n=11)**	3	0	5	1	0	1	0	1	0	0
***P.mirabilis* (n=9)**	2	2	1	2	0	0	0	2	0	0

Sensitive **Intermediate** **Resistant**

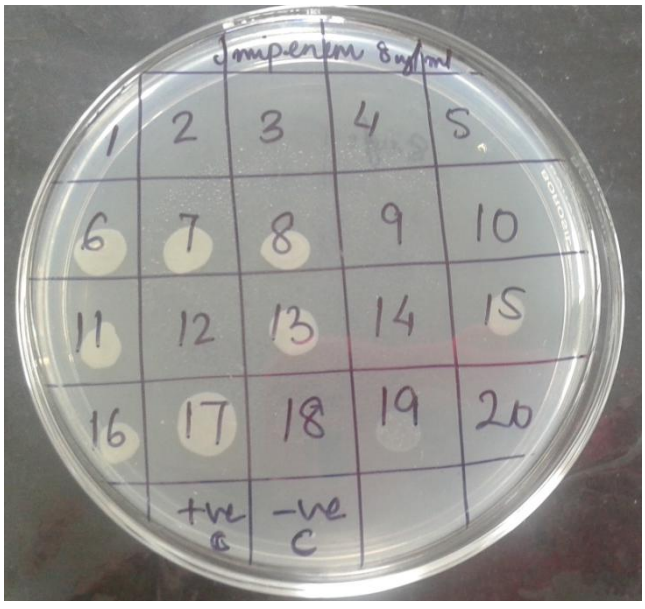


Figure 9: Image showing Minimum Inhibitory Concentration of uropathogens against imipenem at 8µg/ml in a Muller Hinton agar

4.3. Detection of ESBL producing uropathogen

4.31. Phenotypic method of ESBL detection

Phenotypic method for ESBL detection consisted of screening test followed by disc diffusion test for ESBL production.

4.3.1.1 Screening for ESBL production: A total 454 isolates were subjected to a screening test for ESBL production, 112 isolates were found to be positive.

4.3.1.2 Disc diffusion confirmatory test for ESBL production: A total of 454 isolates were subjected to a disc diffusion confirmatory test for ESBL production and 86 isolates were found to be positive with a prevalence rate of 19.60 % (Figure 10 and Figure 11) (Table 18).

Table 18: Distribution of ESBL producing uropathogen by disc diffusion test

Sl no.	Sample code	Bacteria	Sl no	Sample code	Bacteria
1	D:011	*Escherichia coli*	18	D:061	*Escherichia coli*
2	D:013	*Escherichia coli*	19	D:064	*Escherichia coli*
3	D:016	*Escherichia coli*	20	D:065	*Escherichia coli*
4	D:017	*Escherichia coli*	21	D:066	*Escherichia coli*
5	D:024	*Escherichia coli*	22	D:068	*Escherichia coli*
6	D:035	*Escherichia coli*	23	D:071	*Escherichia coli*
7	D:036	*Escherichia coli*	24	D:072	*Escherichia coli*
8	D:037	*P. aeruginosa*	25	D:074	*Escherichia coli*
9	D:046	*Escherichia coli*	26	D:088	*Escherichia coli*
10	D:047	*Escherichia coli*	27	D:090	*Escherichia coli*
11	D:049	*Escherichia coli*	28	D:092	*Escherichia coli*
12	D:050	*Klebsiella pneumoniae*	29	D:093	*Escherichia coli*
13	D:052	*Escherichia coli*	30	D:100	*Escherichia coli*
14	D:053	*Klebsiella pneumoniae*	31	D:102	*Escherichia coli*
15	D:054	*Escherichia coli*	32	D:103	*Escherichia coli*
16	D:057	*Escherichia coli*	33	D:112	*Escherichia coli*
17	D:060	*Escherichia coli*	34	D:115	*Escherichia coli*

Sl no.	**Sample code**	**Bacteria**	**Sl no**	**Sample code**	**Bacteria**
35	D:123	*Klebsiella pneumoniae*	65	SK:084	*Escherichia coli*
36	D:126	*Escherichia coli*	66	SK:091	*Escherichia coli*
37	D:131	*Escherichia coli*	67	SK:099	*Escherichia coli*
38	D:145	*Escherichia coli*	68	SK:104	*Escherichia coli*
39	D:157	*Escherichia coli*	69	SK:105	*Escherichia coli*
40	D:177	*Proteus mirabilis*	70	SK:121	*Klebsiella pneumoniae*
41	D:178	*Escherichia coli*	71	SK:124	*Escherichia coli*
42	SK:02	*Escherichia coli*	72	SK:129	*Escherichia coli*
43	SK:03	*Escherichia coli*	73	SK:130	*Escherichia coli*
44	SK:08	*Escherichia coli*	74	SK:131	*Escherichia coli*
45	SK:10	*Escherichia coli*	75	SK:133	*Klebsiella pneumoniae*
46	SK:011	*Escherichia coli*	76	SK:135	*Escherichia coli*
47	SK:012	*Klebsiella pneumoniae*	77	SK:136	*Klebsiella pneumoniae*
48	SK:014	*Escherichia coli*	78	SK:170	*Klebsiella pneumoniae*
49	SK:018	*Escherichia coli*	79	SK:173	*Escherichia coli*
50	SK:021	*Escherichia coli*	80	SK:221	*Escherichia coli*
51	SK:022	*Escherichia coli*	81	SK:334	*Escherichia coli*
52	SK:024	*Escherichia coli*	82	SK:335	*Escherichia coli*
53	SK:031	*Klebsiella pneumoniae*	83	SK:338	*Escherichia coli*
54	SK:32	*Klebsiella pneumoniae*	84	SK:339	*Escherichia coli*
55	SK:034	*Klebsiella pneumoniae*	85	SK:343	*Escherichia coli*
56	SK:035	*Klebsiella pneumoniae*	86	SK:344	*Escherichia coli*
57	SK:036	*Klebsiella pneumoniae*			
58	SK:043	*Escherichia coli*			
59	SK:047	*Escherichia coli*			
60	SK:049	*Escherichia coli*			
61	SK:051	*Escherichia coli*			
62	SK:059	*Escherichia coli*			
63	SK:060	*Escherichia coli*			
64	SK:068	*Escherichia coli*			

Figure 10: Image showing confirmatory test of ESBLproducing *Klebsiella pneumoniae* (A)

Figure 11: Image showing confirmatory test of ESBLproducing *Escherichia coli* (B)

4.31. Genotypic method of ESBL detection

Phenotypically confirmed ESBL isolates were subjected to genotypic method for ESBL detection. DNA was isolated from all 86 isolates by boiling method. All the isolates were subjected to multiplex PCR for the detection of various kinds of ESBL genes. Out of a total number of phenotipically positive ESBL isolates (n=86), 63 isolates were found to harbour different types of ESBL resistant genes. Chromosomal DNA from representative strains was prepared and purified by

procedures described previously. Sequencing was performed to identify specific ESBL genes. Sanger sequencing confirmed the occurrence of ESBL genes and also revealed its different variants.

Four different ESBL gene variants were detected by sanger sequencing which included CTX-M-15 (*n* = 32), OXA-2 (*n* = 5), TEM (*n* = 1), CTX-M-15+OXA-2 (*n* = 15), TEM+CTX-M-15 (*n*=2), CTX-M- 15+OXA-2+TEM (*n* = 6), and TEM+OXA-2+SHV-76 (*n* = 2) (Fig.20). CTX-M-15 gene was found to be more prevalent (87.03%). The ESBL genes were distributed among different uropathogens (Table 19). It was observed that in 25 isolates, $bla_{\text{CTX-M-15}}$ was present with other coexisting ESBL genes (Figure 12, 13 and 14) (Table 19, 20).

Table 19: Distribution of different types of ESBL genes among uropathogen (I)

Sl no.	Sample code	Bacteria	Resistant gene
1.	SK:036	*Klebsiella pneumoniae*	$bla_{\text{CTX-M}}$
2	SK:057	*Escherichia coli*	$bla_{\text{CTX-M+OXA-2}}$
3	D:017	*Escherichia coli*	$bla_{\text{CTX-M+TEM}}$
4	D:023	*Escherichia coli*	$bla_{\text{CTX-M}}$
5	SK:021	*Escherichia coli*	$bla_{\text{CTX-M}}$
6	D:028	*Klebsiella pneumoniae*	$\text{bla}_{\text{OXA-2}}$
7	SK:053	*Escherichia coli*	$bla_{\text{CTX-M}}$
8	D:024	*Escherichia coli*	$bla_{\text{CTX-M}}$
9	D:064	*Escherichia coli*	$bla_{\text{CTX-M}}$
10	SK:001	*Escherichia coli*	$bla_{\text{CTX-M}}$
11	D:100	*Escherichia coli*	$bla_{\text{CTX-M}}$
12	SK:046	*Escherichia coli*	$bla_{\text{CTX-M}}$
13	D:091	*Escherichia coli*	$bla_{\text{CTX-M}}$
14	D:011	*Escherichia coli*	$bla_{\text{CTX-M}}$
15	D:047	*Escherichia coli*	$bla_{\text{CTX-M}}$
16	SK:049	*Escherichia coli*	$bla_{\text{CTX-M}}$
17	SK:016	*Escherichia coli*	$bla_{\text{CTX-M+TEM}}$

Sl no.	Sample code	Bacteria	Resistant gene
18	SK:032	*Klebsiella pneumoniae*	$bla_{\text{CTX-M}}$
19	SK:041	*Escherichia coli*	bla_{TEM}
20	SK:035	*Klebsiella pneumoniae*	$bla_{\text{CTX-M+TEM+OXA-2}}$
21	SK:021	*Escherichia coli*	$bla_{\text{CTX-M+OXA-2}}$
22	SK:024	*Proteus mirabilis*	$bla_{\text{CTX-M+OXA-2+TEM}}$
23	SK:053	*Escherichia coli*	$bla_{\text{CTX-M+OXA-2+TEM}}$
24	SK:031	*Klebsiella pneumoniae*	$bla_{\text{CTX-M+OXA-2}}$
25	SK:017	*Klebsiella pneumoniae*	$bla_{\text{CTX-M+OXA-2+TEM}}$
26	**S** SK:037**:**	***E** Escherichia coli**coli***	$bla_{\text{CTX-M}}$
27	SK:043	*Escherichia coli*	$bla_{\text{CTX-M+OXA-2}}$
28	SK:020	*Klebsiella pneumoniae*	$bla_{\text{CTX-M+OXA-2}}$
29	SK:023	*Klebsiella pneumoniae*	$bla_{\text{CTX-M+OXA-2}}$
30	SK:042	*Klebsiella pneumoniae*	$bla_{\text{CTX-M}}$
31	D:044	*Klebsiella pneumoniae*	$bla_{\text{OXA-2}}$
32	D:012	*Escherichia coli*	$bla_{\text{CTX-M+OXA-2}}$
33	SK:056	*Escherichia coli*	$bla_{\text{CTX-M+OXA-2}}$
34	D:056	*Escherichia coli*	$bla_{\text{CTX-M+OXA-2}}$
35	D:105	*Escherichia coli*	$bla_{\text{CTX-M+OXA-2}}$
36.	D:072	*Klebsiella pneumoniae*	$bla_{\text{CTX-M+OXA-2}}$
37.	D:123	*Klebsiella pneumoniae*	$bla_{\text{OXA-2}}$
38.	D:089	*Escherichia coli*	$bla_{\text{CTX-M}}$
39	D:103	*Klebsiella pneumoniae*	$bla_{\text{CTX-M+OXA-2}}$
40.	D:045	*Escherichia coli*	$bla_{\text{CTX-M+OXA-2}}$
41	D:014	*Escherichia coli*	$bla_{\text{CTX-M+OXA-2}}$
42	D:074	*Klebsiella pneumoniae*	$bla_{\text{CTX-M}}$
43	SK:013	*Klebsiella pneumoniae*	$bla_{\text{CTX-M}}$
44	SK:003	*Escherichia coli*	$bla_{\text{CTX-M+OXA-2}}$
45	SK:121	*Escherichia coli*	$bla_{\text{OXA-2}}$
46	SK:125	*Escherichia coli*	$bla_{\text{OXA-2}}$

Sl no.	Sample code	Bacteria	Resistant gene
47	SK:129	*Escherichia coli*	$bla_{\text{CTX-M}}$
48	SK:130	*Escherichia coli*	$bla_{\text{CTX-M}}$
49	SK:132	*Escherichia coli*	$bla_{\text{CTX-M}}$
50	SK:131	*Escherichia coli*	$bla_{\text{CTX-M}}$
51	D:103	*Klebsiella pneumoniae*	$bla_{\text{CTX-M+OXA-2}}$
52	SK:135	*Escherichia coli*	$bla_{\text{CTX-M}}$
53	SK:136	*Escherichia coli*	$bla_{\text{CTX-M}}$
54	SK:173	*Escherichia coli*	$bla_{\text{CTX-M}}$
55	D:126	*Escherichia coli*	$bla_{\text{CTX-M}}$
56	D:157	*Escherichia coli*	$bla_{\text{CTX-M}}$
57	SK:173	*Escherichia coli*	$bla_{\text{CTX-M}}$
58	D:185	*Escherichia coli*	$bla_{\text{CTX-M}}$
59	SK:049	*Escherichia coli*	$bla_{\text{CTX-M+ TEM}}$
60	SK:032	*K.pneumoniae*	bla_{TEM}
61	SK:046	*Escherichia coli*	$bla_{\text{TEM+SHV}}$
62	D:024	*K.pneumoniae*	$bla_{\text{OXA-2}}$
63	D:091	*Escherichia coli*	$bla_{\text{CTX-M}}$

Table 20: Distribution of different types of ESBL genes among uropathogens

Sl no	*bla*gene	Microorganism			Total frequency
		E.coli	***K.pneumoniae***	***Proteus mirabilis***	
1.	CTX-M-15	24	8	0	32
2.	OXA-2	4	1	0	5
3	TEM	0	1	0	1
2.	CTX-M-15+OXA-2	9	6	0	15
3.	CTX-M-15+TEM	2	0	0	2
	TEM +SHV-76	1	0	0	1
4	CTX-M-15 +OXA-2 + TEM	4	2	1	6
5	TEM+OXA-2 +SHV-76	1	0	0	2

❖ **Genotypic identification of ESBL resistant gene**

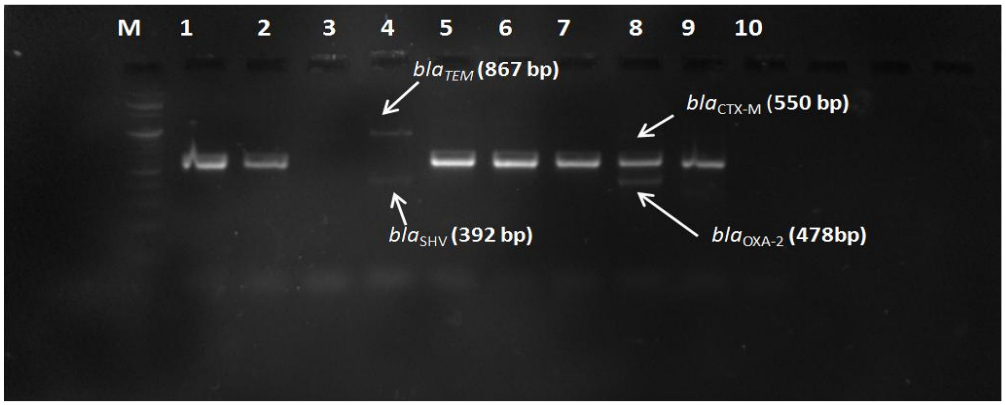


Fig 12: Agarose gel showing PCR amplified products of ESBL genes (I)

Lane M-1000 bp DNA ladder
Lane 1-*bla* CTX-M, Lane 2- *bla* CTX-M, Lane 3- No band, Lane 4- *bla* TEM+SHV, Lane 5- *bla* CTX-M, Lane 6- *bla* CTX-M, Lane 7- *bla* CTX-M, Lane 8- *bla* CTX-M+OXA-2, Lane 9- +ve control (*bla* CTX-M), Lane 10- -ve control.

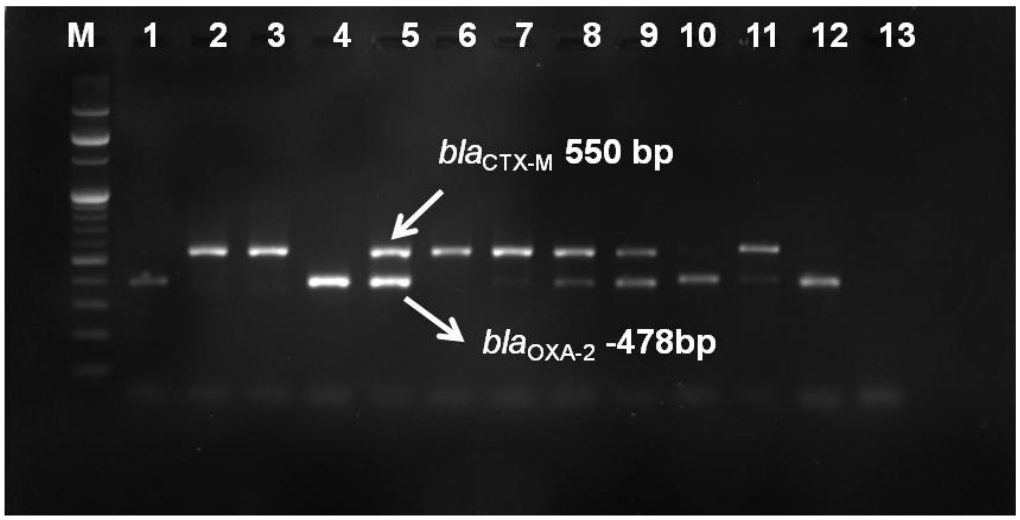


Fig 13: Agarose gel showing PCR amplified products of ESBL genes (II)

Lane M-100bp DNA ladder
Lane 1*bla*OXA-2, Lane2 -*bla*CTX-M+OXA-2, Lane3- *bla*CTX-M+OXA-2, Lane 4- *bla*OXA-2, Lane 5- *bla*CTX-M+OXA-2, Lane 6- *bla*CTX-M+OXA-2, Lane 7- *bla*CTX-M+OXA-2, Lane 8- *bla*CTX-M+OXA-2, Lane 9- *bla*CTX-M+OXA-2, Lane 10- *bla*CTX-M+OXA-2, Lane 11- *bla*CTX M+OXA-2, Lane 12-+ve control (*bla*CTX-M), Lane 13-ve control.

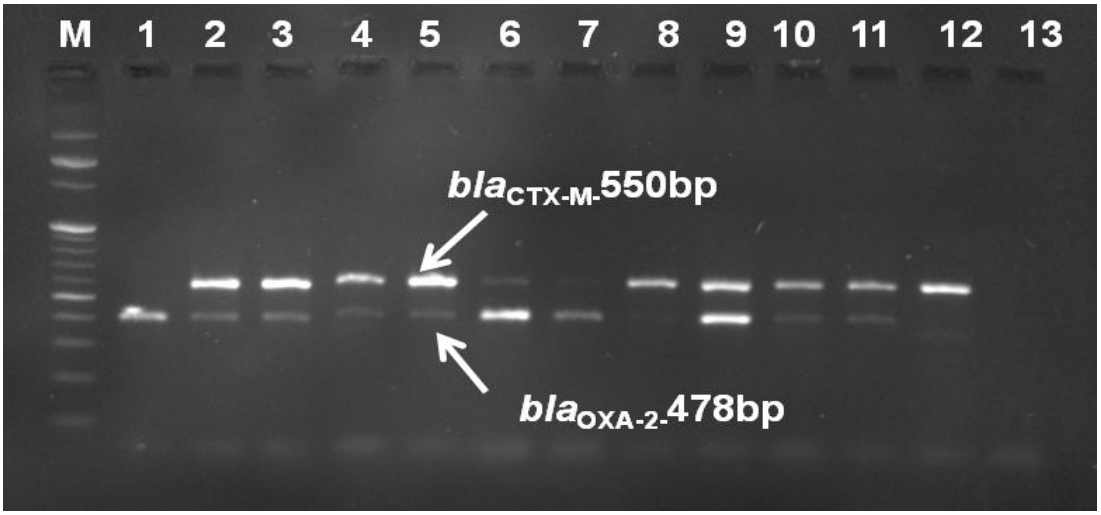


Fig 14: Agarose gel showing PCR amplified products of ESBL genes (III)

Lane M-100bp DNA ladder.
Lane 1-*bla*$_{OXA-2}$, Lane 2- *bla*$_{CTX-M+OXA-2}$, Lane 3 *bla*$_{CTX-M+OXA-2}$, Lane 4-*bla*$_{CTX-M+OXA-2}$
Lane 5-*bla*$_{CTX-M+OXA-2}$, Lane 6- *bla*$_{CTX-M+OXA-2}$, Lane 7- *bla*$_{CTX-M+OXA-2}$, Lane 8-*bla*$_{CTX-M+OXA-2}$, Lane 9 -*bla*$_{CTX-M+OXA-2}$, Lane 10-*bla*$_{CTX-M+OXA-2}$, Lane 12 -+ve control (*bla*$_{CTX-M)}$, Lane 13 -ve control.

4.4 Genetic transferability of ESBL genes

Plasmid isolated from 30 representatives ESBL positive bacterial isolates were cultured in Luria-Bertani (LB) broth (Hi-Media, Mumbai, India) containing 0.25 µg/ml of cefoxitin. Competent cells were prepared and transformation procedure was performed.

After the transformation procedure, it was found that 23 transformed bacterial cells were able to grow in the presence of .25µg of antibiotic pressure (cefotaxime). It means that the bacterial cells which were transformed are resistant to the antibiotic (Figure 15 and 16) (Table 21).

Table 21: Details of Transformants

Sl no.	Sample code	Transferability	Transferable gene
1.	D:089	Transferable	CTX-M
2	SK:001	Transferable	CTX-M
3	D:074	Transferable	CTX-M
4	SK:053	Transferable	CTX-M
5	D:100	Transferable	CTX-M
6	D:024	Non-transferable	-
7	D:064	Transferable	CTX-M
8	SK:013	Non-transferable	-
9	D:091	Transferable	CTX-M
10	D:011	Transferable	CTX-M
11	SK:024	Transferable	CTX-M+OXA2+TEM
12	SK:021	Transferable	CTX-M+OXA2+TEM
13	SK:041	Transferable	CTX-M+OXA2+TEM
14	SK:031	Transferable	CTX-M+OXA2+TEM
15	SK:017	Non-transferable	-
16	D:044	Transferable	OXA-2
17	D:123	Transferable	OXA-2
18	D:028	Transferable	OXA-2
19.	D:065	Transferable	CTX-M+OXA-2
20.	D:014	Transferable	CTX-M+OXA-2
21.	D:072	Transferable	CTX-M+OXA-2
22.	D:045	Transferable	CTX-M+OXA-2
23.	D:012	Transferable	CTX-M+OXA-2
24.	D:013	Transferable	CTX-M+OXA-2
25.	SK:003	Non-transferable	-
26.	D:105	Non-transferable	-
27.	SK:049	Transferable	CTX-M+TEM
29.	SK:046	Non-transfeable	-
30.	SK:32	Non-transferable	-

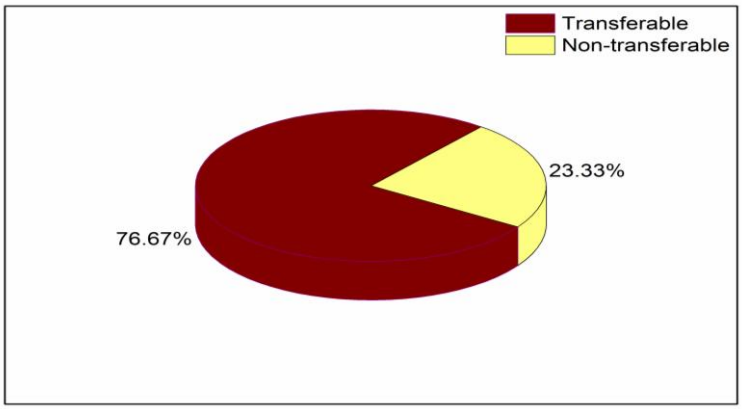
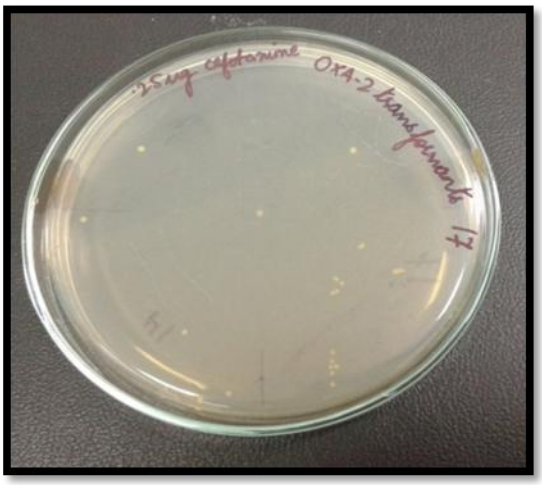
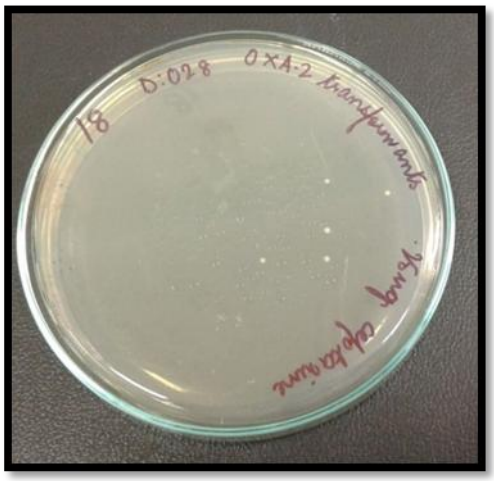


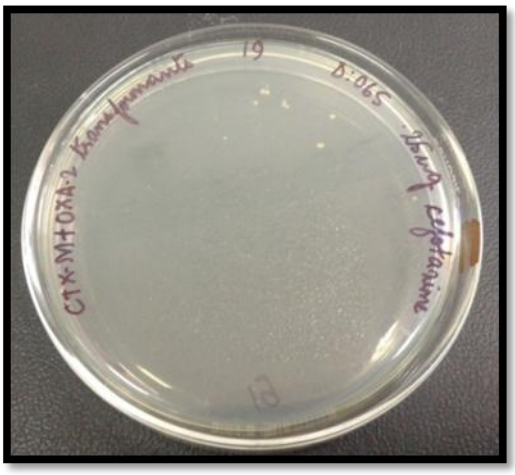
Figure 15: A pie chart showing distribution of transferable and non-transferable plasmids



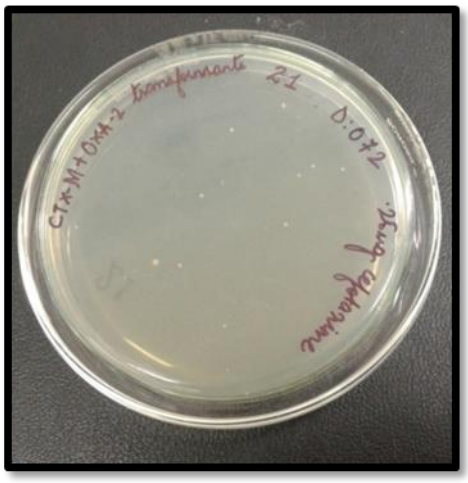
(A)



(B)



(C)



(D)

Figure 16: Transformants in *Escherichia coli*

(A) & (B) Transformants carrying $bla_{\text{OXA-2}}$ genes (white colonies)

(C) & (D)Transformants carrying $bla_{\text{CTX-M-15+OXA-2}}$ genes (white colonies)

4.5 Incompatibility typing

Replicon typing classifies plasmids by their ability to stably coexist with other plasmids in the same bacterial strain, a trait that is dependent on their replication machinery. The plasmids were characterized by PCR based replicon typing for determining the incompatibility group type of the plasmid in all *bla* genes harbouring strains A total of 18 different replicon types such as FIA, FIB, FIC, HI1, HI2, I1/Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, *F* and FIIA were targeted by 5 multiplex and 3 simplex PCR reaction (A Carattoli, 2011).

In a first multiplex PCR HI1 (n=5) replicon type was observed followed by I_1 (n=2) (Table 22).

Table 22: Replicon typing in first multiplex PCR (HI 1, H1 2, I1)

Sl no	SAMPLE CODE	ESBL TYPE	REPLICON TYPE
1.	SK:024	CTX-M+OXA-2+TEM	HI_1
2.	SK:021	CTX-M+OXA-2+TEM	I_1
3.	SK:041	CTX-M+OXA-2+TEM	I_1
4.	D:014	CTX-M+OXA-2	HI_1
5.	D:102	CTX-M+OXA-2	HI_1
6.	D:103	CTX-M+OXA-2	HI_1
7.	SK:049	CTX-M+TEM	HI_1

No band was observed in second multiplex PCR (X, LM, and N)

In a third multiplex PCR most of the plasmid had a replicon type of FIA+FIB (n=8) followed by FIA (n=4) (Table 23).

Table 23: Replicon typing in third multiplex PCR

Sl no	SAMPLE CODE	ESBL TYPE	REPLICON TYPE
1.	D:074	CTX-M	FIA+FIB
2.	SK:053	CTX-M	FIA+FIB
3.	D:100	CTX-M	FIA+FIB
4.	D:064	CTX-M	FIA+FIB
5.	D:091	CTX-M	FIA
6.	SK:024	CTX-M+OXA-2+TEM	FIA+FIB
7.	SK:021	CTX-M+OXA-2+TEM	FIA+FIB
8.	Sk:041	CTX-M+OXA-2+TEM	FIA+FIB
9.	D:014	CTX-M+OXA-2	FIA
10	D:045	CTX-M+OXA-2	FIA
11	D:102	CTX-M+OXA-2	FIA+FIB
12	D:103	CTX-M+OXA-2	FIA

In a fourth multiplex PCR only Y type of replicon type was observed (Table 24).

Table 24: Replicon typing in fourth multiplex PCR

SL NO.	SAMPLE CODE	ESBL TYPE	REPLICON TYPE
1	D:074	CTX-M	Y
2	SK:024	CTX-M+OXA-2+TEM	Y
3	SK:041	CTX-M+OXA-2+TEM	Y
4	D:014	CTX-M+OXA-2	Y
5	D:102	CTX-M+OXA-2	Y
6	D:103	CTX-M+OXA-2	Y

In a fifth multiplex PCR no band was observed PCR (**A/C, Tnand FIIS**)

In a simplex 1 only Frep B replicon type was observed in 12 numbers of plasmids (Table 25).

Table 25: Replicon typing in Simplex 1

SL NO.	SAMPLE CODE	ESBL TYPE	REPLICON TYPE
1.	D:074	CTX-M	Frep B
2.	SK:053	CTX-M	Frep B
3.	D:100	CTX-M	Frep B
4.	D:064	CTX-M	Frep B
5.	D:091	CTX-M	Frep B
6.	SK:024	CTX-M+OXA-2+TEM	Frep B
7.	SK:021	CTX-M+OXA-2+TEM	Frep B
8.	Sk:041	CTX-M+OXA-2+TEM	Frep B
9.	D:014	CTX-M+OXA-2	Frep B
10	D:045	CTX-M+OXA-2	Frep B
11	D:102	CTX-M+OXA-2	Frep B
12	D:103	CTX-M+OXA-2	Frep B

In a simplex 2 only Frep B replicon type was observed in 12 numbers of plasmids

Table 26: Replicon typing in Simplex 2

Sl no	SAMPLE CODE	ESBL TYPE	REPLICON TYPE
1.	D:014	CTX-M+OXA-2	K

In simplex 3 no band was observed.

All transformants carrying the bla_{ESBL} genes were selected on screening agar and the resistance determinant was found to be carried on plasmids of diverse incompatibility (Inc) groups, namely HI1, I1, FIA + FIB, FIA and Y types.

4.6 Stability of plasmids encoding ESBL genes

Stability of plasmids encoding ESBL genes was analyzed and it was observed that the organism harboring specific ESBL genes were stable till 28^{th} passages (Table 27).

Table 27: Plasmid stability analysis of uropathogens harboring different ESBL genes

Sl no.	Sample code	Organism	ESBL type	Stability (days)		
				1-10	11-20	20-30
1	D:089	*E.coli*	CTX-M	Stable	unstable	unstable
2	SK:001	*E.coli*	CTX-M	Stable	unstable	unstable
3	D:074	*K.pneumoniae*	CTX-M	Stable	unstable	unstable
4	SK:053	*E.coli*	CTX-M	Stable	unstable	unstable
5	D:091	*E.coli*	CTX-M	Stable	unstable	unstable
6	SK:024	*K.pneumoniae*	CTX-M+OXA-2+TEM	Unstable	unstable	unstable
7	SK:021	*E.coli*	CTX-M+OXA-2+TEM	Stable	unstable	unstable
8	D:011	*E.coli*	OXA-2	Unstable	unstable	unstable
9	SK:031	*K.pneumoniae*	OXA-2	Stable	stable	unstable
10	SK:041	*E.coli*	OXA-2	stable	stable	unstable
11	D:044	*K.pneumoniae*	CTX-M+ OXA-2	stable	unstable	unstable
12	D:123	*K.pneumoniae*	CTX-M+ OXA-2	unstable	unstable	unstable
13	D:028	*K.pneumoniae*	CTX-M+ OXA-2	stable	unstable	unstable
14	D:065	*E.coli*	CTX-M+ TEM	stable	unstable	unstable
15	D:014	*E.coli*	CTX-M+ TEM	unstable	unstable	unstable
16	D:072	*K.pneumoniae*	CTX-M+ OXA-2	unstable	unstable	unstable
17	D:045	*E.coli*	CTX-M+ OXA-2	unstable	unstable	unstable
18	D:103	*K.pneumoniae*	CTX-M+ OXA-2	unstable	unstable	unstable
20	D:017	*E.coli*	CTX-M+ TEM	unstable	unstable	unstable

4.7 Detection of co-resistance gene In ESBL pathogens

Since, ESBL resistance genes are mostly plasmid mediated it may also carry genes encoding resistance to other class of antibiotic, such as ampA, ampC, aminoglycosides, macrolides, chloramphenicol, quinolone or carbapenems (Alyamani EJ *et al*., 2017). Therefore, the occurrence of co-resitance gene of following antibiotics was detected among ESBL uropathogens using genotypic method.

Out of 59 coresistant strains, chromosomal DNA from representative strains was prepared and purified by procedures described previously. Sequencing was performed to identify specific co-resistant (carbapenem,AmpC, Aminoglycoside) genes. Sanger sequencing confirmed the occurrence of coresistant genes and also revealed its different variants.

4.7.1 Co-existence of carbapenem resistence gene in ESBL producing bacteria

Interestingly, among 63 ESBL–producing strains, 59 isolates harboured carbapenem-resistant genes which included *bla*$_{\text{NDM-5}}$ (*E.coli*-34, *K.pneumoniae*-14), *bla*$_{\text{NDM-5}}$ + *bla*$_{\text{OXA-48}}$ (*E.coli*-3, *K.pneumoniae*-2), and *bla*$_{\text{NDM-5}}$ + *bla*$_{\text{IMP}}$ (*E.coli*-5), *bla*$_{\text{NDM-5}}$ + *bla*$_{\text{IMP}}$ + *bla*$_{\text{VIM}}$ (*E.coli*-1) (Figure 17 and 18).

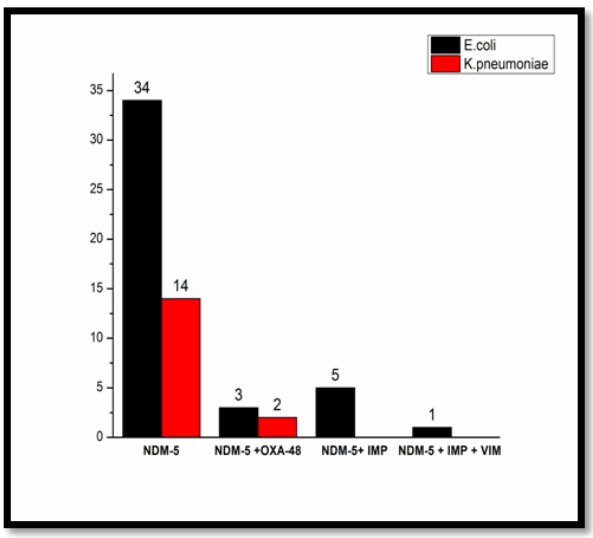


Figure 17: Distribution of Carbapenem resistant gene among ESBL isolates

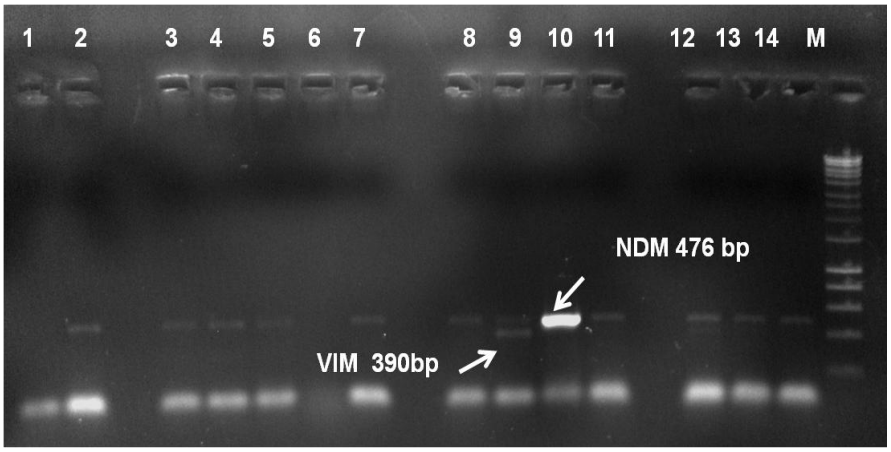


Fig 18: Agarose gel showing PCR amplified products of Carbapenem-resistant gene

Lane 1-ve control, Lane 2-+ control, Lane 3,4,5- NDM, Lane-6 No band, Lane 7,8 – NDM Lane 9 NDM+ VIM, Lane 10,11, 12, 13, 14-NDM. Lane M- 1kb Ladder.

4.7.2 Co-existennce of AmpC resistence gene in ESBL producing bacteria

Among the 63 ESBL–producing strains, only 9 isolates harboured AmpC resistant genes which included *bla*$_{CIT}$ (n=*E.coli*-6, *Klebsiella pneumoniae*-2) and *bla*$_{DHA-1}$ (n=*E.coli*-1) genes (Figure 19, 20 and 21).

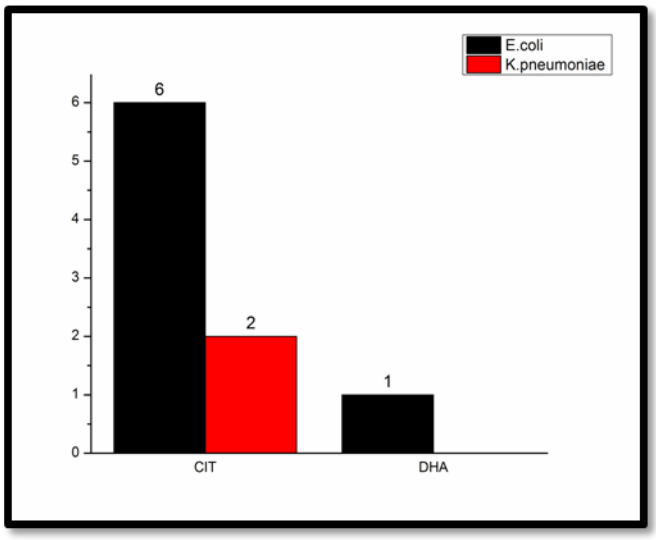


Figure 19: Distribution of AmpC resistant gene among ESBL isolates

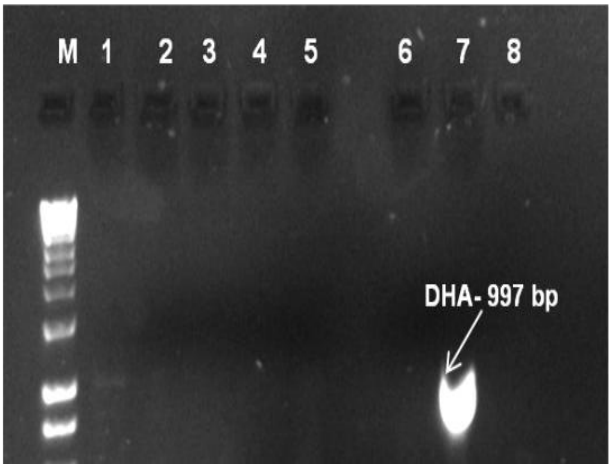


Fig 20: Agarose gel showing PCR amplified products of AmpC gene (I)

Lane M- 1kb Ladder, Lane 7- DHA gene

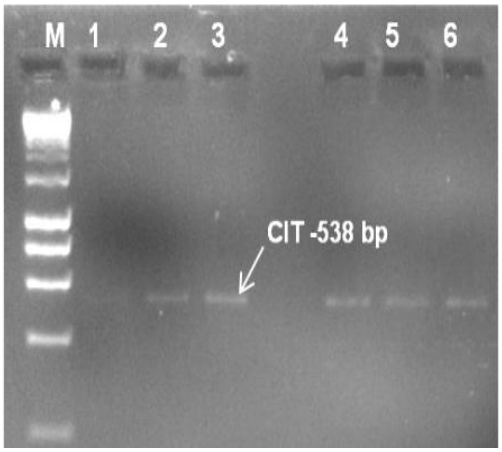


Fig 21: Agarose gel showing PCR amplified products of AmpC gene (II)

Lane M- 1kb Ladder, Lane 3- CIT gene

4.7.3 Co-existennce of Aminoglycoside resistence gene in producing bacteria

The most prevalent aminoglycoside resistant gene was found to be *rmt* B and the least prevalent was *rmt* C and *Arm* A. Among 63 ESBL–producing strains 15 isolates harbored 16S rRNA methylase genes which included *rmt*B (n=9), *rmt*A (n=4), *rmt*C (n=1) and *Arm*A (n=1) (Figure 22, 23 and 24).

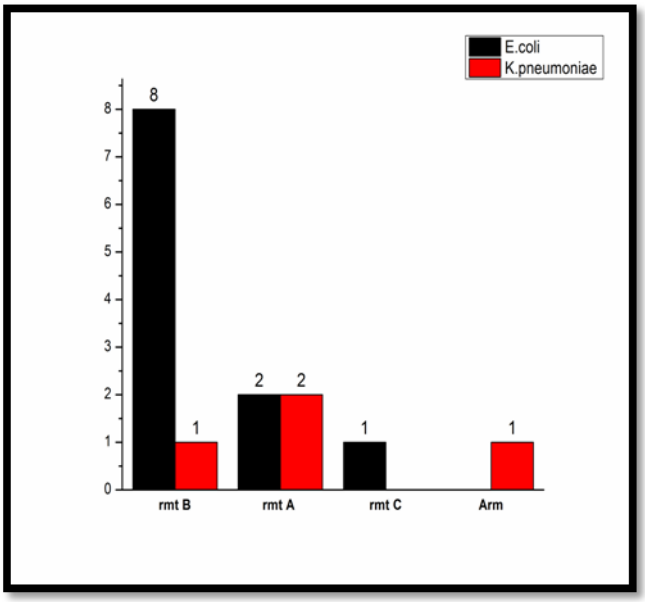


Figure 22: Distribution of Aminoglycoside resistant gene

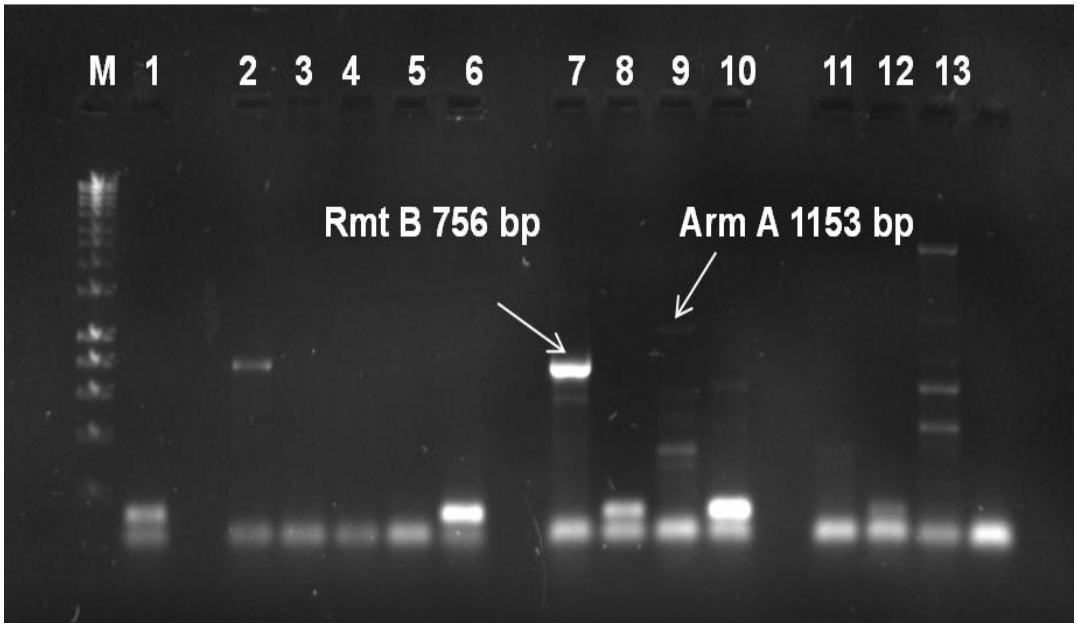


Fig 23: Agarose gel showing PCR amplified products of 16S rRNA methyltransferase genes (I)

Lane M- 1kb Ladder, Lane 1- - ve control, Lane 2- +ve control Lane 7- Rmt B, Lane 9- Rmt A

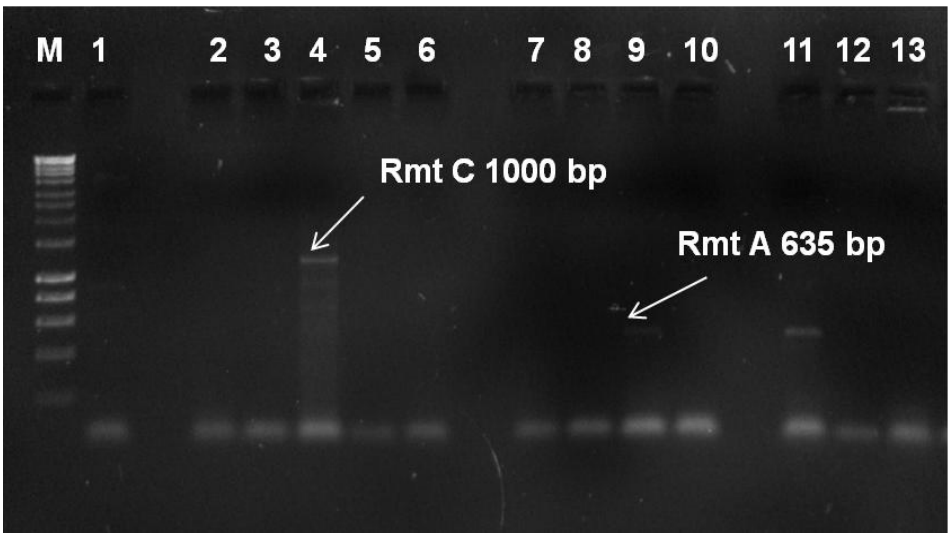


Fig 24: Agarose gel showing PCR amplified products of 16S rRNA methyltransferase gene II

Lane M- 1kb Ladder, Lane 2- Lane 4,- Rmt C, Lane 9- Rmt A, Lane 11- + control, Lane 13-ve control

4.8 MIC of Carbapenemase producing uropathogen against Carbapenem group of antibiotic

The MIC of isolates harboring carbapenem resistant gene was checked because it was observed that high number of ESBL producing isolates harbored Carbapenem resistant gene with a prevalence rate of 93.65%. MIC test was performed for the isolates harboring carbapenem resistant gene which included *bla* $_{NDM}$, *bla* $_{OXA-48}$, *bla*$_{VIM}$ gene against commonly used carbapenem antibiotics like imipenem, meropenem and ertapenem. The MIC value of isolates harboring NDM gene was detected by agar dilution method against imipenem (Lupin, India), ertapenem (MSD, India) and meropenem (Ameropem, India). The MIC interpretive standards for the susceptibility categories were categorized as per the breakpoints recommended by the CLSI, 2014. It was observed that most of the carbapenemase producing ESBL isolates had MIC value between (0.1µg/ml to 2 µg/ml) (Table 28).

Table 28: Different MIC data shown by cabapenamase producing uropathogens against commonly used carbapenem antibiotics

	Sensitive			Intermediate	Resistant				
	0.5	1	2	4	8	16	32	64	128
Ertapenem									
E.coli (n=34)	11	15	1	-	2	2	2	1	-
K.pnuemoniae(n=19)	7	3	2	-	2	3	1	1	-
Imipenem									
S	10	8	2	5	2	-	3	-	3	1
K.pnuemoniae (n=19)	12	5	-	1	-	-	2	-	-	-
Meropenem									
E.coli (n=34)	11	17	-	1	-	-	1	1	3	-
K.pnuemoniae (n=19)	9	4	3	-	-	-	2	2	-	-

Sensitive Intermediate Resistant

4.9 Observation of study of MIC Creep Phenomenon

The detection of co-resistance gene among the ESBL producing uropathogens showed that most of the isolates were NDM positive. However, when the MIC of the NDM positive isolates was performed against commonly used Carbapenem group of antibiotic it was observed that most of the isolates had a MIC value of (MIC≤2, MIC≤4). On studying the MIC creep activity, the NDM positive isolates with MIC≤2 and MIC≤4 when exposed against gradually increasing imipenem stress for consecutive 6 days in LB broth the MIC was escalated to 64µg/ml and 128µg/ml respectively (Table 29).

4.8.1 Analysis of revertants

The revertants when analyzed, after 10 consecutive serial passages without imipenem stress, it was observed that the MIC of most of the NDM positive isolates reduced to 64µg/ml and 32µg/ml while the MIC remained 128µg/ml for the rest (Table 29) (Figure 25).

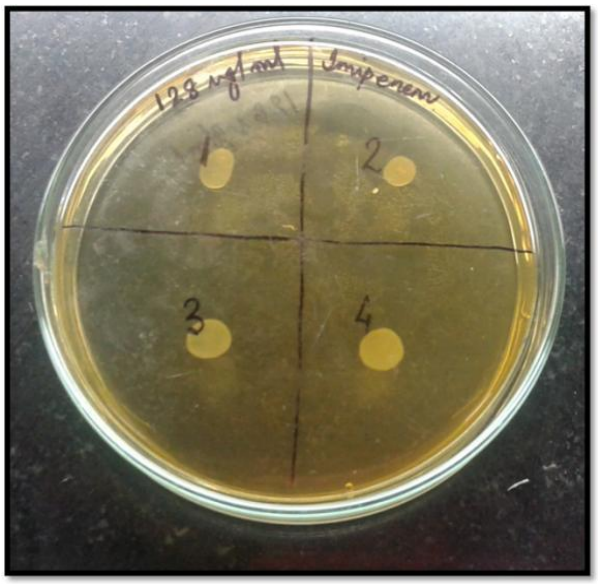


Figure 25: *E. coli* growth showing the MIC Creep phenomenon

(MIC against Imipenem increased from 1µg/ml to at 256 µg/ml) on Muller Hinton Agar (Imipenem at 128µg/ml)

Table 29: *Escherichia coli* isolate harbouring the *bla*$_{NDM-5}$ gene and showing the minimum inhibitory concentration (MIC) creep

Sl no.	Sample code	Initial MIC (μg/mL)			IPM MIC creep after exposure (μg/mL)	IPM MIC of revertants (μg/mL)		
		ETP	IPM	MEM		After 10th passage	After 20th passage	After 30th passage
1	SK:053	1	1	0.5	128	64	16	16
2	D:064	0.5	16	1	128	64	16	16
3	SK:001	0.5	1	1	128	64	16	16
4	D:067	0.5	0.5	1	64	64	16	16
5	D:065	1	4	0.5	128	64	16	16
6	SK:057	2	4	0.5	128	64	16	16
7	D:105	64	128	0.5	128	128	128	128
8	SK:125	1	4	1	128	64	16	16
9	SK:017	0.5	4	1	128	64	16	16
10	D:024	1	16	1	128	32	16	16
11	SK:016	1	1	0.5	64	32	16	16
12	SK:130	32	64	1	128	128	16	16
13	SK:014	0.5	128	1	128	128	128	128
14	SK:043	16	64	0.5	128	128	32	16
15	SK:031	8	64	1	128	64	32	16
16	SK:135	1	0.5	1	64	32	16	16
17	D:074	1	1	0.5	128	64	16	16
18	D:017	1	16	0.5	128	64	16	16
19	SK:056	0.5	1	1	128	128	64	16
20	D:089	0.5	2	1	64	64	16	16
21	D:014	1	0.5	1	64	64	32	32
22	SK:049	0.5	2	0.5	64	64	16	16
23	SK:131	0.5	4	1	128	128	64	16
24	D:057	1	0.5	0.5	64	64	16	16
25	D:024	1	0.5	1	64	64	16	16
26	D:091	2	1	32	64	64	16	16
27	SK:047	0.5	1	4	128	64	16	16
28	SK:021	1	1	64	128	64	32	16
29	D:011	1	0.5	16	128	64	16	16
30	SK:012	1	4	0.5	128	64	32	16
31	SK:003	16	0.5	64	64	32	16	16
32	SK:041	8	0.5	64	64	64	16	16
33	D:068	1	0.5	1	64	64	16	16
34	D:101	1	0.5	1	64	32	16	16

ETP, ertapenem; IPM, imipenem MEM, meropenem.

While studying the MIC creep phenomenon, NDM-positive isolates with MICs of ≥2 mg/ml and ≥4 mg/mL when exposed to gradually increasing imipenem stress for six consecutive days in LB broth, the MIC was found to be elevated to 64 mg/ml and 128 mg/ml, respectively (Table 29; Figure 25). Analysis of the revertants showed that after 10 consecutive serial passages without imipenem stress the MIC of most of the NDM-positive isolates had reduced to 64 mg/ml and 32 mg/ml, whilst the MIC remained at 128 mg/mL for the rest isolates. The MIC was further found to be reduced to 16 mg/mL for most of the isolates after the 20th and 30th serial passages, respectively (Table 29).

Chapter 5

Discussion

5. DISCUSSION

The urinary tract infection remains a major problem globally precisely in developing countries (Wiener J *et al*., 1999) (Shrivastava, Gupta and Tripathi, 2018). In the current scenario, clinical findings have demonstrated a new challenge associated with urinary tract infection. The frequent use of antibiotics makes the uropathogens resistant to current antibiotics (Zandi H., Tabatabaei S.M., Ehsani F., 2017).

The emergence of multidrug-resistant has further intensified existing problem worldwide affecting developed countries as well (Perez F., Endimiani A., 2007). Asian countries are a major sufferer of different gram-negative uropathogens. On an average, every woman in India and other developing countries suffer from moderate to severe urinary tract infections (Paterson D.L *et al*., 2005).

The management of urinary tract infections and the treatment of multidrug-resistant uropathogens have become difficult. The most significant finding in the last two decades suggested that there is the emergence of uropathogens with a capacity of producing β-lactamase. The genes encoding such enzyme selectively provide a resistance to the organism. Further, extended beta-lactamase producing uropathogens have been reported across the globe (Shrivastava, Gupta and Tripathi, 2018).

Empirical antibiotic treatment is usually the first treatment to be administered to patients with UTI. Therefore, it is essential to be aware of the epidemiological data for an appropriate initial treatment. Additionally, healthcare policy makers will require knowing of the incidence of the disease to best assign the necessary resources for management. In the current period of high-resistance rates toward antibiotics, understanding the epidemiological information is much more important (Tandogdu and Wagenlehner, 2016).

The present study was undertaken to characterize the common bacterial pathogens responsible for Urinary tract infection among the females of Sikkim and Darjeeling. The current study was also designed to perform the molecular characterization of ESBL and other co-resistant genes among the isolated uropathogens.

5.1 Prevalence of Urinary tract infection

The prevalence of UTI in the present study was found to be 29.94% which was found to be quite high as compared to the study conducted in Varanasi, India and Northern part of Bengal, India with the prevalence rate of UTI as 13.6% (Priscilla R, 2017) and of 21.8% (Gajamer VR *et al*.,2015) respectively. Barry et al., reported the prevalence of UTI in Senegal, West Africa as 26.7% (Barry *et al.*, 2017) among the UTI suspected female patients of less than 18 years age.

While comparing the prevalence of UTI with other places in India it was found that the prevalence of UTI in the current study is low as compared to the study conducted in Bihar (32%) by Pratap *et al*, 2016 (Pratap R, Kumar A, 2016) and in Gurugram (33.11%) by Rajput *et al*., 2018 (Rajput and Sarsaiya, 2018). Moreover, the prevalence of UTI was evaluated in 630 patients attending Teerthanker Mahaveer Medical College & Research Centre, Moradabad for the duration of one year from February 2016 to January 2017. There results showed significant bacteriuria in 215 patients with a prevalence rate of 34.12% (Mashkoor *et al.*, 2017) which was found to be high as compared to the present study. The prevalence of UTI in the North Western Region of Nigeria was found to be much higher as compared to that of India with the prevalence rate of 41%.

There are several gram-negative bacteria associated with the development of urinary tract infection including *Escherichia coli, Klebsiella species, Pseudomonas species,*

Proteus species, Enterobacter, Citrobacter and *Enterococcus species etc.* However, considering India and other developing countries *E. coli* remains a main uropathogenic microbial strains associated with the onset and progression of UTI (Livermore D.M., 2007). Similarly in the present study, the most prevalent uropathogen was found to be *Escherichia coli* (70.43%) followed *by Klebsiella pneumoniae* (20.1%). This finding is similar to the findings of Saha et al.,2014 where he found that in West Bengal the most prevalent uropathogen was *Escherichia coli* (67.1%) followed *by Klebsiella pneumonia* (22%) (Saha S et al., 2014). The finding is also in consistent with the study conducted in in Pakistan by (Rahman H, 2016) and (Mohammed *et al.*, 2016).

On the contrary to the present study in Mangalore India where only 44% of *E. coli* was isolated from UTI patients (Shariff *et al.*, 2013).

Similarly, in a study conducted in Bhopal Shrivastava *et al*., 2018 also reported that among the UTI patients of the study area the most prevalent uropathogen was found to be *E.coli* (68.18%) followed by *Klebsiella pneumonia* (13.64%), *Proteus* (6.06%) and *Pseudomonas* (3.03%) (Shrivastava, Gupta and Tripathi, 2018). Whereas in the current study we found that the *Escherichia coli* and *Klebsiella pneumoniae* were followed by *Pseudomonas aeruginosa* (2.4%), and *P. mirabilis* (1.98%). Our finding is very similar to the findings of the study conducted in Bihar, India where low prevalence of *Pseudomonas aeruginosa* and P*roteus mirabilis* were isolated (Pratap R, Kumar A, 2016). In a study conducted in Karnataka out of 60 microorganisms isolated 57 (95%) organisms were bacterial species among them gram negative organisms and were frequently isolated. *Escherichia coli* was the most common organism isolated (66.67%) followed by *Klebsiella* (18.33%). *Pseudomonas species* (10%) were the least isolated bacterial species (Sundaran *et al.*, 2018). In the current

study *Citrobacter* were not isolated. In a study conducted at Nigeria a total of 528 bacterial isolates were isolated from the urine samples. The most frequently isolated organism in the urine samples of patients was *Escherichia coli* (24.2%) while the prevalence of *Citrobacter intermedius* (6.1%) and *Staphylococcus saprophyticus* (6.1%) was found to be low.

5.2 Asymptomatic bacteriuria

Asymptomatic bacteriuria to acute cystitis to pyelonephritis remains one of the most frequent bacterial infections in women encountered by general practitioners (Bengtsson C, Bengtsson U, 1980). It is known that antibiotics are recommended for the treatment of symptomatic urinary infections. Since last few decades, a variety of questions have been raised against the treatment of asymptomatic bacteriuria. However, after the randomized controlled trials the use of antibiotics has been suggested in asymptomatic bacteriuria in pregnancy (Wettergren B, Jodal U, 1985). The prevalence of asymptomatic bacteriuria in healthy women of age group 18 to 40 years of age is found to be approximately 5 percent, and it rises with age to 20 percent or more in ambulatory elderly women (Zhanel GG, Harding GK, 1990).

In the present study, the prevalence of asymptomatic bacteriuria was found to be 4.34% only. The prevalence of asymptomatic bacteriuria varies according to age, sex, sexual activity and the presence of genitourinary abnormalities. Asymptomatic bacteriuria is usually detected in women aged more than 60 years at the rate of 3%to 5%. Asymptomatic bacteriuria is more common in patients with diabetes and the elderly (Kranz *et al.*, 2017). It is reported that 25% to 50% of elderly women and 15%to 40% of elderly men in long-term care facilities are bacteriuric. Asymptomatic bacteriuria is rare in healthy young men, but its prevalence increases significantly after the age of 60 years (A *et al.*, 2012). On the contrary, Ankur *et al*., 2015 found

the prevalence of asymptomatic UTI slightly higher (8.8%) as compared to the present study (Ankur G, Namita S, Sapna G, 2015).

5.3 Antibiotic Susceptibility profiling

Antibiotic resistance shown by different isolates is a barrier that hinders a successful treatment. Extensive use of antibiotics exerts the selective pressure that acts as a driving force in the development of the resistance to various antibiotics (Rajput and Sarsaiya, 2018).

The susceptibility profile of bacteria varies from place to place depending on a particular type of antibiotic used in a particular centre. In the current study *Escherichia coli* showed maximum resistance to Norfloxacin (87.88), *Proteus mirabilis and Pseudomonas aeruginosa* showed maximum resistance to Nitrofurantoin (100%), and minimum to Imipenem with the resistance percentage of 10.1%, 10.4% and 15.8% respectively. Whereas in 2016 most of the uropathogens were found to be sensitive for Nitrofurantoin, Gentamicin and Trimethoprim-Sulfamethoxazole in a study conducted in a Medical College at Ethiopia (Mamuye, 2016). The study reveals the increasing antibiotic resistance trends of uropathogens towards Nitrofurantaoin. Shrivastava *et al*., 2018 also found that maximum resistance for *E. coli* was shown by nitrofurantoin (95%) and minimum for norfloxacin (34%) (Shrivastava *et al*., 2018). *Klebsiella pneumoniae* showed highest resistance to ampicillin (88.7%) and lowest to imipenem (13.4%). The resistance rate of *Klebsiella pneumoniae* against ampicillin in the current study is similar to the findings of (Shrivastava *et al*., 2018) with a prevalence rate of 88.9%. The rise in the resistance pattern of uropathogens to commonly used antibiotics such as ampicillin, ciprofloxacin, and cefuroxime has caused considerable alarm (Shariff V A AR, Shenoy M S, Yadav T, 2013).

For ampicillin 100% resistance is shown by *Pseudomonas aeruginosa* followed by *Klebsiella pneumoniae* (88%), *E. coli* (85.9%) and *Proteus mirabilis* (66.66%) which is consistent with the findings of Mandal *et al.* 2012 wherein 80% of the *E.coli* were resistant to ampicillin (Mandal J, Acharya NS, Buddhapriya D, 2012). The current study is similar to the findings of Saha S. *et al*., 2014 who reported that *Klebsiella spp* isolated from UTI samples of Eastern India were highly resistant to the penicillin group of antibiotics, followed by aminoglycosides and third generation cephalosporin (Saha S *et al*., 2014b). *Klebsiella pneumonia* showed second highest resistance against different classes of antibiotics followed by *E. coli* in this study. Ciprofloxacin which still remains the first drug of choice among the fluoroquinolones group in community-acquired UTI was found to be less effective against uropathogens. It was found that 58.2% of *Escherichia coli* showed resistance to ciprofloxacin followed by *Klebsiella pneumoni*ae (35.82%), *Proteus mirabilis* (11.11%) and *Pseudomonas aeruginosa* (9%). *Escherichia coli* showed lowest rate of resistance comparatively towards imipenem (10.1%) followed by piperacillin/tazobactam (25.8%) and gentamicin (25.1%) which is similar to the findings of Pant *et al* in a study conducted among the uropathogens in tertiary hospital of Nepal (Pant *et al.*, 2017). Similar to our present study imipenem appeared as the most active drug for uropathogens with less than 3% resistance of isolates in a study conducted Barry et al., 2017 at Senegal, South Africa (Barry *et al.*, 2017).

Saman Mashkoor *et al*.,2017 reported that in Uttar Pradesh out of 215 UTI positive cases, 80.94% of the isolates were gram negative organisms followed by gram positive organisms 19.06%.The most common pathogenic organisms were *Escherichia coli* accounting for 98 (45.19%) urinary isolates. Antibiotic susceptibility tests revealed that the gram negatives bacteria were sensitive to quinolones and

Carbapenems (Mashkoor *et al.*, 2017). Even in our study, Carbapenem group of antibiotics were found to be effective.

High MICs for third-generation cephalosporins make it inappropriate for empirical therapy. Therefore, MIC test of uropathogens was performed against cephalosporin group of antibiotics, aztreonam, and imipenem for 10 different concentrations which ranged from 0.5 to 256ug μg/ ml. In a current study, after performing MIC test for all the isolates (n=454) it was found that highest number of *E. coli* had a MIC value of 256ug/ml in case of cefotaxime, ceftriaxone, and cefpodoxime. In case of cefepime and ceftazidime, highest number of isolates falls into the category of an intermediate which is expected to increase its MIC in due course of time. Similar to our present study, in a study conducted in China by Jean *et al*., 2015 it was observed that maximum number of *E. coli* had a MIC value of more than 128ug/ml in case of cefotaxime. On the contrary to our study maximum isolates had a MIC value of 16ug/ml against ceftazidime and cefepime (Jean *et al*., 2015).

In the present study, the maximum number of uropathogens had a MIC value more than 256ug/ml on the contrary Jean *et* al., 2015 found the maximum number of uropathogens had a MIC value of 32ug/ml (Jean *et al*., 2015). Nineteen percent isolates of *E.coli* showed MIC value of 128ug/ml against aztreonam. Imipenem which is known as the last line of the drugs showed a good sensitivity against a maximum number of isolates. However, 14.4% of *E. coli* and 15.2% of *Klebsiella pneumoniae are* on borderline with a MIC value of 4ug/ml. Any further increase in MIC by two fold will push these isolates into resistance category that will be a matter of concern for the clinicians for the treatment of patients.

The current study indicates the high resistance pattern of these isolates towards most of the commonly used antibiotics. In the light of these findings, there is a need of

various measures to stop the development and spread of antibiotic resistance. The findings of the current study suggests that carbapenem, piperacillin tazobactam and gentamicin drugs should be a drug of choice to treat an infection caused by uropathogens.

5.4 Detection of ESBL producing bacteria

The infection caused by ESBL producing bacteria has become a significant public health challenge in the last decade and have been associated with high mortality rates and frequent treatment failure. Due to the frequent use of cephalosporins in empiric and discrete treatments caused by different uropathogens, it is important to understand the prevalence of ESBL resistance genes involved in conferring resistance (Saeed Khoshnood *et al.*, 2017).

The prevalence of ESBL producing organism varies worldwide based on the extent and rationale of use of β-lactam antibiotics. Widespread and irrational use of antibiotics exerts a selection pressure on bacteria and leads to the emergence of ESBL producing strains. The occurrence of ESBLs varies among different parts of the world. Recently an emergence of ESBL in the community has led to increased concern among the clinicians. In the present study a total of 454 uropathogens were subjected to both phenotypic and genotypic methods for ESBL detection. Out of 454 uropathogens 131 isolates showed ESBL production in screening method. Again out of 131 isolates 86 isolates were found to be positive in ESBL kit (disc diffusion method). Further out of 86 isolates, 63 isolates showed the presence of ESBL genes in a PCR assay. Diverse types of ESBL genes were detected by molecular method in the current study which included $bla_{\text{CTX-M-15}}$ (n=32), followed by $bla_{\text{CTX-M-15}} + bla_{\text{OXA-2}}$ (n=15), $bla_{\text{CTX-M-15}} + bla_{\text{OXA-2}} + bla_{\text{TEM}}$ (n=6), $bla_{\text{OXA-2}}$ (n=5), $bla_{\text{OXA-2}} + bla_{\text{SHV-76}}$ (n=1), $bla_{\text{TEM}}{}_{+\text{SHV-76}}$ (n= 1) and bla_{TEM} (n=1). It is a well known fact that CTX-M

enzymes are replacing SHV and TEM enzymes as the most predominant ESBL type (Eriksson *et al*., 2011). Moreover, it has been reported that *bla*$_{\text{CTX-M-15}}$ producing *E. coli* has spread worldwide (Pitout, J.D., 2008) and this resistance determinant is a major contributor to expanded-spectrum cephalosporin resistance in clinical settings (Maurya *et al*., 2016). Interestingly, Quijada *et al*., reported that *bla*$_{\text{CTX-M-16}}$ is more prevalent among uropathogens in Venezuela at Venezuelan University Hospital (Quijada-Martínez *et al.*,2017).

In our study, most of the Gram-negative bacilli produced *bla*$_{\text{CTX-M-15}}$. From our study, we documented that *bla*$_{\text{CTX-M-15}}$ is the dominant ESBLs in Sikkim and Darjeeling hills of India. On the contrary, Bajpai *et al.* observed *bla*$_{\text{TEM}}$ as the dominant type followed by *bla*$_{\text{CTX- M}}$ and *bla*$_{\text{SHV}}$ in central India among male and female UTI patients of the study area (Bajpai *et al*., 2017).

In a study conducted in Sikkim Manipal Institute of Medical Sciences, Tshering et al.,2017 reported that from clinical samples, 238 gram-negative bacilli were isolated they were *Escherichia coli* followed by *Pseudomonas aeruginosa, Klebsiella pneumoniae, Citrobacter freundii, Proteus mirabilis, Morganella morganii* and *Enterobacter cloacae*. By phenotypic method of ESBL detection 34% of ESBL-positive isolates were isolated (Tshering *et al.*, 2009). In a study conducted in New Delhi by Anas et al., found out that out of 414 UTI patients, 313 women were affected with UTI (75.6%). Out of 241 isolates, 165 isolates were found to be ESBL producing *E. coli* (Anas *et al.*, 2017). Rajput *et al*. reported that 293 (33.11%) urine samples showed significant bacteriuria. ESBL detection was done by phenotypic method. The most common ESBL producing uropathogens were found to be *E. coli* followed by *Klebsiella pneumonia.* All the ESBL positive isolates were found to be MDR (Rajput and Sarsaiya, 2018). In International scenario also in in the United Kingdom, 380

uropathogenic *E. coli* from a tertiary hospital was found to harbour $bla_{\text{CTX-M-1}}$(56%), $bla_{\text{CTX-M-2}}$(0.2%), $bla_{\text{CTX-M-8d}}$ (0.5%), $bla_{\text{CTX-M-9}}$(11%) and $bla_{\text{CTX-M-}}$25(6%), bla_{SHV}, bla_{TEM} and bla_{OXA} (Reid, R *et al*., 2017). Arbar et al., 2018 reported that ESBL is much higher in Pakistan. They revealed that bla_{OXA}, bla_{SHV}, bla_{TEM}, and $bla_{\text{CTX- M}}$ were the most commonly found ESBL genes in Pakistan (Abrar *et al.*, 2018).. Mashwal et al., 2017 reported that high incidence of ESBLs among the *E. coli* isolated from UTI (23.1%) in a tertiary hospital of Saudi Arabia. Their study also indicated that $bla_{\text{CTX- M}}$ genes are the most prevalent among the uropathogens followed by bla_{TEM} class (6%), they also found that a higher percentage *E. coli* (3.4%) simultaneously harbouring bla_{TEM} and $bla_{\text{CTX- M}}$ genes was isolated. None of their isolates harboured the bla_{SHV} (Mashwal *et al.*, 2017).

Alarming rate of drug resistance among pediatric uropathogens and high rate of ESBL-producing *E.coli* was observed in Nepal. Incidence of urinary tract infection among pediatric patients was found to be 19.68% and *E.coli* (68.4%) was found to be leading pathogen involved. Out of 739 *E.coli* isolates, 64.9% were found to be multidrug resistant (MDR) and 5% were extensively drug resistant (XDR) (Parajuli *et al.*, 2017).

In a study, prevalence of ESBL was observed in almost all parts of the Iran and high resistance was observed, especially in the central part of Iran. Up to 89.8% *Escherichia coli*, 72.1% *Klebsiella pneumonia*, 84.2% *Acinetobacter baumannii*, and 83.8% *Pseudomonas aeruginosa* isolates were found to be ESBL positive (Leylabadlo *et al.*, 2017).

In the current study Carbapenem was found to be effective for all the ESBL producing uropathogens similar to a hospital based cross sectional study in Sri lanka where the prevalence of meropenem resistance is 4.9% which is alarming compared to other

countries. Carbapenems seem to remain as the first line therapy for majority of ESBL-UTIs in their local setting (Fernando *et al.*, 2017).

Im our study it was found that some uropathogens harbored a combination of three ESBL genes. None of the isolates produced GES, VEB, or PER β-lactamase. The medical history of patients harboring the resistance gene was checked and it was found that most of the patients were suffering from pyelonephritis and recurrent UTI with multidrug-resistant bacteria.

In the current study, there were many uropathogens harbouring multiple ESBL genes which is alarming in context of treatment of UTI patients. Therefore, proper antimicrobial stewardship is required for optimizing therapy for patients both at individual and community level in order to preserve existing antibiotics for future use.

Through this kind of study that has been conducted in this region, we can understand the local distribution of these ESBL resistant genes and their movement, adaptability, and propagation under antibiotic exposure in different clinical environmental conditions.

5.5 Stability analysis of plasmids encoding ESBL genes

It is a known fact that plasmid carry many antibiotic resistant genes that permit bacteria to survive hostile environment found in the host and resist treatment. The dissemination of ESBL determinants is enhanced because of their presence within plasmids within different genetic backbones and association with other mobile genetic elements. It creates a potential for further spread of these resistance genes to other susceptible group of bacteria. It is very important to understand the survival of a plasmid carrying antibiotic resistance gene in absence of any selective pressure.

In the stability analysis, the different incompatibility types harboring β-lactamase genes showed progressive plasmid loss after 28 passages, unlike Maurya *et al.*, who showed a complete plasmid loss after 40 passages in P Inc type harboring $bla_{\text{CTX-M-15}}$ (Maurya *et al*., 2016). This study indicates the fitness cost and the stability of the plasmid was highly variable in different bacterial species. The highly stable plasmids may be of particular concern in the dissemination of antibiotic resistance phenotypes and as they are conjugatively transferable they will likely to move from host to host until they encounter one where fitness costs are negligible and subsequently go on to thrive with the host.

5.6 Transferability of bla_{ESBL} and incompatibility typing

Plasmid families are emerging in association with clinically relevant resistance genes and are largely common in Enterobacteriaceae. The emergence of the CTX-M-15 enzyme is one of the most relevant findings associated with the current epidemiology of ESBL worldwide (Coque, T.M., Baquero, F., Canton, 2008) and the $bla_{\text{CTX-M-15}}$ gene has been located on specific plasmids belonging to the IncF group. The IncF plasmids have been also linked with the spread of other resistance determinants. IncF plasmids possibly contribute to the fitness of the bacterial host by providing virulence and antimicrobial resistance determinants. It also encodes several addiction systems which assures their maintenance and stability in the host cell, independently by the positive selective pressure exerted by the antimicrobials (Alessandra Carattoli, 2011).

IncI1 plasmids carrying the $bla_{\text{CTX-M-1}}$ gene were identified by pMLST in the Netherlands in *E. coli* isolates from poultry meat samples (Leverstein-van Hall et al., 2011). Interestingly, in the current study, it was observed that β-lactamase genes were horizontally transferred through multiple incompatible types of plasmids. This proves their diverse source of origin and adaptation in both hospital and community settings.

ESBL genes are known to be pandemic and are often reported to be carried within Inc FII plasmid (Bajpai, T *et al*., 2017). Inc group detection has been commonly used to categorize plasmids in a homogeneous group on the basis of their phylogenetic similarities and may be helpful to investigate their transmission in nature as well as linkage with the host cell to determine the evolutionary origins of plasmids (Alessandra Carattoli, 2011).

In our study, we found the majority of plasmids belonged to an incompatibility group of FIA+FIB (*n* = 9), followed by Y (*n* = 9), HI1 (*n* = 5), FIA (*n* = 4), and I1 (*n* = 3). On the contrary, Maurya *et al*., reported their carriage through I1, F (FIA = 6; FIC = 3; FrepB = 3), W, and P Inc groups (Maurya AP *et al*., 2016).

The presence of this resistance gene in uropathogens in hospital and community might be owing to the extensive use of CTX and ceftriaxone in this setting. Supriya et al., performed transferability assay and reported that CTX-M could be horizontally transferred from diverse host range to recipient E. coli DH5α. She also revealed that that CTX-M-15 was carried within Inc I1 and Inc F II types (Upadhyay *et al*., 2015).On the contrary Maurya et al., reported Incompatibility typing of $bla_{\text{GES-5}}$ harbouring *Pseudomonas aeruginosa* isolated from human urine specimen from North-East India, the diverse Incompatibility groups was obtained which are as follows: I1/Iγ, FIA, FIB, W, FIC, FrepB and K (Maurya *et al*., 2014).

Jayalaxmi Wangkheimayum performed incompatibility typing of the plasmids of 16S rRNA methyltransferase-mediated-aminoglycoside resistant bacteria that harbored these genes showed that rmtA was carried by the FIA Inc group, whereas *rmtB* was carried by the Y Inc plasmid type. It was observed that *rmtC* was carried by the FIA and N Inc groups, whereas *rmtD* was found to be carried by the FIB and N Inc groups. Inc group A/C was found to carry armA. Isolates harboring multiple 16S

rRNA methyltransferase genes originated from diverse Inc groups, viz., FIB, FIIS, I, T, X, N, Y, L/M, and FIA(Wangkheimayum *et al*., 2017). Thus the current study establishes that ESBL genes were located in different Inc type which could predict multiple source origin of their acquisition and propagation. This finding is of epidemiological interest and helpful in formulation of hospital infection control policy. The present study establishes the possible use of PCR based replicon typing has been a significant tool in tracing the diffusion of plasmids which are conferring antimicrobial resistance and it may also track the evolution and spread of emerging plasmids. This method could be applied in routine laboratory in monitoring of transmission of plasmids within strains from diverse setting. This study also advocates that conjugative plasmids are key players in the genetic elements that fuel bacterial adaptability and diversity. Conjugative plasmids are responsible for the horizontal transfer of the ESBL genes to a new host and play a crucial role in the population dynamics of bacterial plasmids and maintenance within the host.

5.7 Detection of the co-resistant gene among ESBL producers

It is reported that ESBLs in Enterobacteriaceae coexists with resistance to other antimicrobial classes infections. In case of infections caused by ESBL producing bacteria, carbapenems are the antibiotics of choice for the treatment (Rahman M *et al*., 2015). However several studies have reported on the emerging resistance to carbapenem antibiotics due to the increased production of β-lactamases worldwide, which hydrolyze all β-lactam antibiotics including carbapenems. In the present study, 93.65% of the ESBL producing microorganisms harbored carbapenem-resistant genes (mainly NDM-5), most of the isolates (n=34) were obtained from community settings. The current prevalence is also much higher than data obtained in studies from Uganda where only 28.6% of carbapenemase producers were detected among ESBL

producing Enterobacteriaceae (Okoche *et al.*, 2015). Similarly, a very low prevalence of carbapenemase encoding gene was reported from Spain with a prevalence rate of 0.04% only (Miró *et al.*, 2013). However, $bla_{\text{NDM-5}}$ was found to be more prevalent among all carbapenemase producing genes.

The organisms harboring AmpC beta-lactamase is a major cause of therapeutic failure leaving cephalosporins inactive along with co-existing mechanism of resistance. Our study revealed a prevalence of the AmpC producing gene of 14.75% among ESBL producing Enterobacteriaceae. In the study, we found out that among all the AmpC producers, the genes producing CIT enzymes were more prevalent. But in the study conducted by Jean et al.,2017 most of the *E.coli* (11.7%) harbored CMY-2 producing enzyme (Jean *et al.*, 2017). In a study conducted in Iran 2017 by Koshesh *et al*., 2017. In a study conducted by Pal et al., 2009 it was observed that 105 *E. coli* isolates were isolated from UTI suspected both male and female patients. It was revealed that the prevalence of ESBLs, AmpC-lactamase, bla_{TEM} and bla_{OXA} in the inpatient isolates was 37.2%, 2%, 37.2% and 5.8%, respectively (Pal *et al.*, 2009).

Aminoglycosides are frequently used in combination with the ß-lactam group of antibiotics to treat severe infections in hospital patients. However, the bacterial population has developed various resistance mechanisms and very soon the therapeutic use of this drug will be limited (Wangkheimayum *et al*., 2017). Acquired 16S rRNA methyltransferases which accounts for high-level and broad-spectrum aminoglycoside resistance have been reported increasingly among enterobacterial isolates in recent years, often in association with beta-lactamases, further complicating the management of infections caused by multidrug-resistant isolates (Bartoloni *et al.*, 2016). In our study, four different types of 16S rRNA methyltransferase genes have been characterized which are responsible for

aminoglycoside resistance. Among these, *rmtB* (16.39%) was found to be sthe most predominant type in this part of India, on the contrary, Wangkheimayum J *et al*, (2017) found *Rmt C* being predominant in the Eastern part of the India (J. *et al.*, 2017). It is reported that 16S rRNA methyltransferases often coexist with bla_{NDM} and bla_{CTX-M} genes (Rahman *et al.*, 2015) (J. *et al.*, 2017) and our study was not an exception.

In a study conducted in Spain in 2017 a total of of 420 *E.coli* producing ESBL isolates and 139 *Klebsiella pneumonia* producing ESBL isolates were obtained from clinical samples of tertiary hospitals. The prevalence of aminoglycoside enzyme genes in ESBL harbouring E.coli was found to be 38.3% and in case of ESBL harbouring *Klebsiella pneumonia* was found to be (115/139, 82.7%). The most prevalent aminoglycosides modifying genes in ESBL producing *E.coli* and ESBL producing *Klebsiella pneumoniae* were aac(6¢)-Ib (16.2%and 44.6%) and aac(3)-IIa (14.7% and 43.1%), respectively (Ferna and Jesu, 2017).

The findings that ESBL producing uropathogens co-harbored carbapenem, aminoglycoside and AmpC resistant genes underscore that India as an epicenter of horizontal transfers of high-level resistance alleles among Gram-negative bacteria irrespective of community or nosocomial settings (Miro E *et al*.,2013). Since the study area shares the border of the border with countries like Nepal, Bhutan and Bangladesh a good number of patients visit these hospitals for treatment purpose. This may be one of the factors for the acquisition and spread of drug-resistant pathogens among the people of the study area.

5.8 MIC creep phenomenon

In the present study it was revealed that most of the ESBL producing isolates harbored carbapenem resistant gene with prevalence rate of 93.65%.Therefore, MIC of isolates harboring carbapenem resistant gene was checked. MIC test was performed for the isolates harboring carbapenem resistant gene which included $bla_{NDM,}$ bla_{OXA-48}, bla_{VIM} gene against commonly used carbapenem antibiotics like imipenem, meropenem and ertapenem. Surprisingly, it was found that most of the NDM positive isolates had a MIC value of MIC≤2, MIC≤4 and were falling in the category of sensitive according to CLSI MIC breakpoint 2017 (Table 29).

On studying the MIC creep phenomenon, NDM-positive isolates with MIC≤2, MIC≤4mg/ml when exposed to gradually increasing imipenem stress for six consecutive days in LB broth, the MIC was found to be elevated to 64 mg/ml and 128 mg/ml, respectively. On analysing the revertants, the revertants showed that after 10 consecutive serial passages without imipenem stress the MIC of most of the NDM-positive isolates had reduced to 64 mg/ml and 32 mg/mL, whilst the MIC remained at 128 mg/ml for the rest the isolates.The MIC was further found to be reduced to 16 mg/mL for most of the isolates after the 20th and 30th serial passages, respectively. The rising trend in MICs of antibiotics against clinical isolates is worrisome. This study identified the presence of NDM-5-mediated carbapenem resistance in community-acquired UTI cases, although with routine antibiogram profiling most of them resulted as sensitive. Carbapenems are not commonly used for the treatment of community-acquired infections in the study area, therefore we anticipate that this gene might have been acquired along with other resistance determinants through a common plasmid and are maintained within the host. Because of the absence of pressure of

carbapenem, the resistance gene was not expressed and MIC of the isolates was found to below the breakpoint.

Most of the *bla*$_{\text{NDM-5}}$ harbouring *E. coli* had MICs of 1 mg/mL and 0.5 mg/mL against carbapenems, masquerading as susceptible. Isolates with MICs of ≤2, ≤4when exposed to gradually increasing carbapenem antibiotics namely; imipenem, meropenem and ertapenem stress for six consecutive days in LB broth developed elevated MICs to 64 mg/ml and 128 mg/ ml, respectively, showing the MIC creep activity. This is the first report which shows the MIC creep phenomenon in NDM-producers. Interestingly, till date there is no report suggestive of carbapenem MIC creep in NDM-producers. This observed MIC creep phenomenon poses a global threat as these pathogens may evade phenotypic screening in routine laboratories.

On increasing the exposure to imipenem ertapenem and meropenem, the MIC of the NDM-producers increased from 0.5 mg/ml and 1 mg/ml to 64 mg/ml and 128 mg/ml, respectively. This finding suggests that continuous exposure or usage of carbapenems, which is also known as a last line of drug, may be associated with an increased probability of treatment failure, reducing therapeutic choices. Therefore, carbapenem antibiotics should be used carefully with proper molecular level analysis of carbapenemases. The *bla*$_{\text{NDM}}$ gene among the other carbapenemases has gained particular attention owing to its global dissemination and multidrug resistance phenotype.

The finding of this study is it has demonstrated a high prevalence of the *bla*$_{\text{NDM}}$ gene in *E. coli* isolated from female UTI patients in this region of India. The main finding of this study was established using the CLSI 2017 MIC breakpoints; the evidence for imipenem MIC creep in NDM-producers was found. Moreover, the evidence of a decreased rate of susceptibility to carbapenem antibiotics among *E.coli* was also

observed. Though slight but steady increase in MICs is important in long-term and thus trends in MICs must be continiously monitored. This is mainly important since ESBL-producers appear to be increasingly significant globally both in hospital and community-associated infections. Therefore, continuous monitoring the susceptibility profiles of Enterobacteriaceae against important antibiotics is still warranted.

Conclusion

CONCLUSION

The purpose of the present study was to study the prevalence of Urinary Tract infection in the UTI suspected females of Sikkim and Darjeeling. Furthermore, the drug resistance pattern of the uropathogens was studied. Moreover the study also aimed at characterizing the drug resistant uropathogens on a molecular level. High numbers of drug resistant bacteria harbouring multiple drug resistant genes was studied.

The current study has the following highlights and comments:

- Out of 1516 urine samples collected from UTI suspected female patients of the study area, 454 samples showed the significant bacteriuria. The prevalence of UTI was found to be 29.94%. The most prevalent uropathogen was found to be *E.coli* (74.3%) followed by *Klebsiella pneumoniae* (20.1%), *Pseudomonas aeruginosa* (2.43%) and *Proteus mirabilis* (1.98%). In the current study also *E.coli* was found to be the main etiological agent similar to the other studies. When the prevalence of asymptomatic bacteriuria was checked among 207 females, the prevalence of asymptomatic bacteriuria was found to be 4.34% only. Among all the risk factors of UTI mentioned in the questionnaire sexual activity and frequency of water consumption in a day are two main risk factors for UTI found in the study area.

- A total of 454 uropathogens were subjected to 13 numbers of commonly used antiobiotics. Various antibiotic resistance profiles of different uropathogens were obtained. Imipenen were found to be most effective against the uropathogens whereas ampicillin was found to be the least

effective. Apart from imipenem, piperacillin/tazobactam and gentamicin was also found to be drug of choice. The MIC of the uropathogens against Cephalosporin group of antibiotic was found to be on rise. The MIC value of most of the uropathogens against cefotaxime, ceftriaxone and cefpodoxime was found to be 256µg/ml. The MIC value of uropathogens against cefepime and ceftazidime was found to be on borderline, and if it increases its MIC by two fold it will fall into the category of resistance is which is a matter of concern.

- A total of 454 isolates were subjected to a screening test for ESBL production, 112 isolates were found to be positive. Again, total of 454 isolates were subjected to a disc diffusion confirmatory test for ESBL production and 86 isolates were found to be positive for ESBL production. A total of 86 isolates were considered for molecular characterization of ESBL genes. Out of 86 isolates, 63 isolates harboured ESBL genes. The $bla_{\text{CTX-M-I5}}$ group was the most prevalent ESBL type, followed by $bla_{\text{OXA-2}}$ and $bla_{\text{CTX-M-I5}}$ enzymes incombination. The different variants of antibiotic resistance genes in a study area reflect diverse origin, acquisition and mobilization of those antibiotic resistant determinants in the tertiary referral hospital and the community of the study area.

- In the present study different Inc group namely HI1, I1, FIA+FIB, FIA, and Y are found to be the main carrier for rapid dissemination of ESBL genes among the uropathogens. Thus, this study advocates continuous surveillance in molecular level for infection control and it demands urgent measures to slow down the spread.

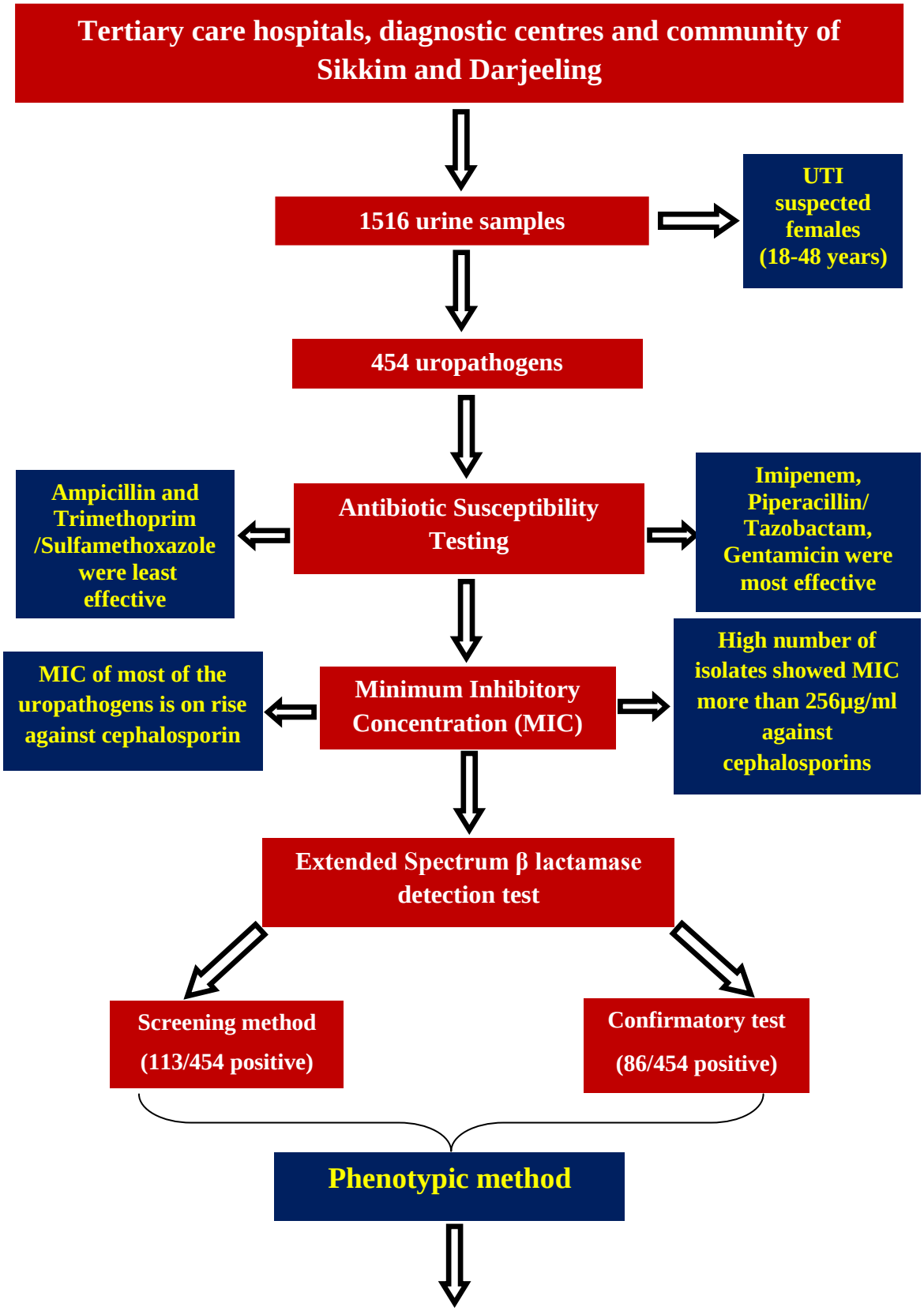
- In a stability test of ESBL isolates, complete plasmid loss was observed in 28^{th} passages. The ability to retain a resistant plasmid for several generations even after withdrawing the antibiotic pressure underscores the high stability may be the potential reason for dissemination of antibiotic resistant genes within the hospital settings as well as in the community when dispersed in an antibiotic free condition.
- The uropathogens primarily carried various types of ESBL genes coexisting with carbapenem, AmpC and aminoglycoside resistant genes. The emergence of multiple resistance mechanisms in these isolates makes these pathogens a major challenge for treating infections by such pathogens as they show huge resistance to commercially available common drugs.
- This study has demonstrated a high prevalence of bla_{NDM} genes in *E.coli* isolated from the female UTI patients of the study area. The bla_{NDM} gene among the other carbapenemases has gained particular attention due to its global dissemination and multidrug resistance phenotype. This study observed a unique phenotype of NDM producers which is not been reported earlier. The major breakthrough of this study was we found an evidence of MIC creep phenomenon in a NDM producing *E.coli* which has not been reported throughout the world. This is the first report which suggest the MIC Creep Phenomenon in NDM producing *E.coli.* This can be used as a base line data to find out epidemiological cut value, which can prevent or slow down treatment failure.
- The observed phenomenon poses a global threat as these pathogens may evade phenotypic screening by routine laboratories. Still a slight but steady increase in MICs is significant in long-term therefore trends in

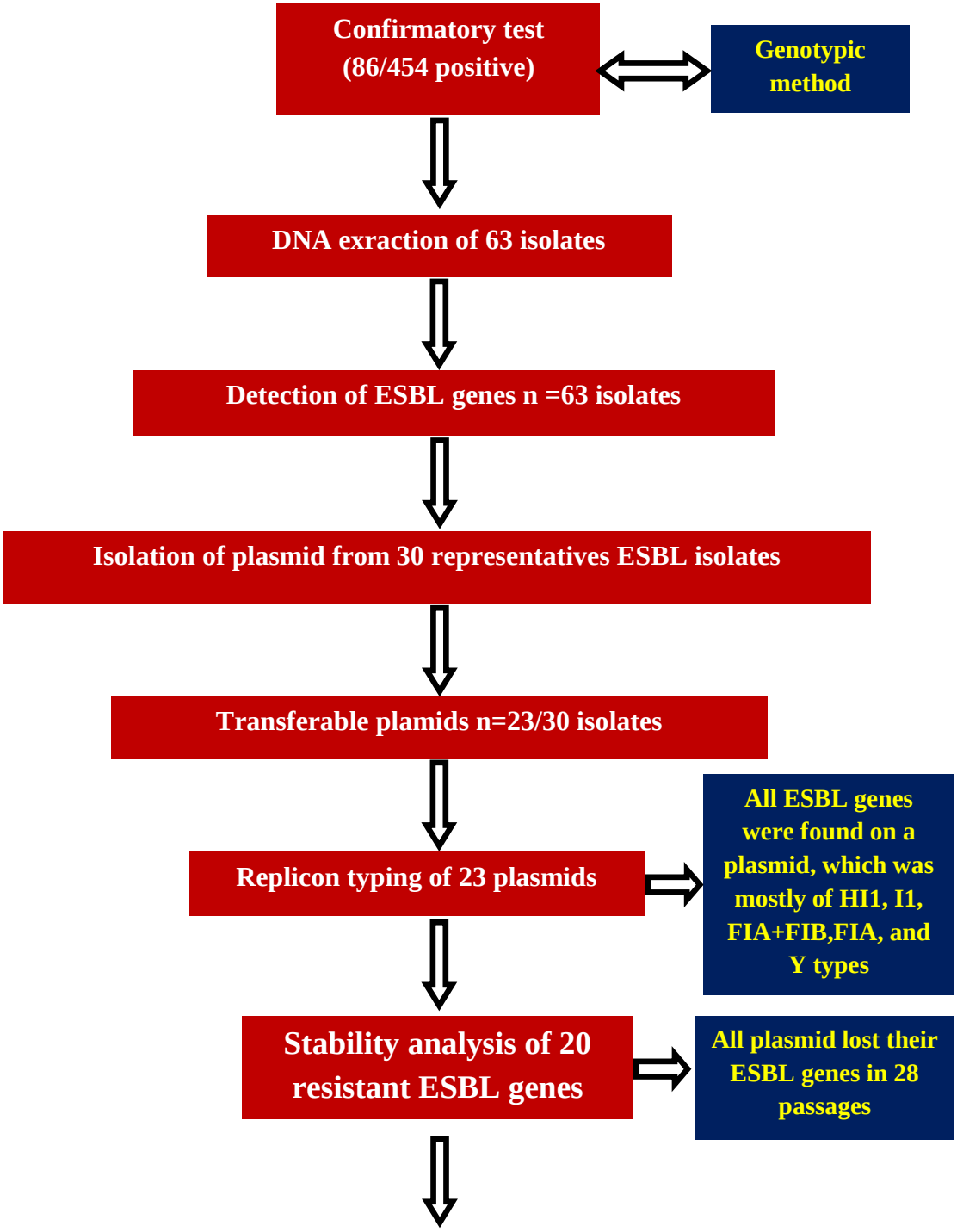
MICs must be continuously monitored. Monitoring of susceptibility profiles of Enterobacteriaceae against important antibiotics is still necessary.

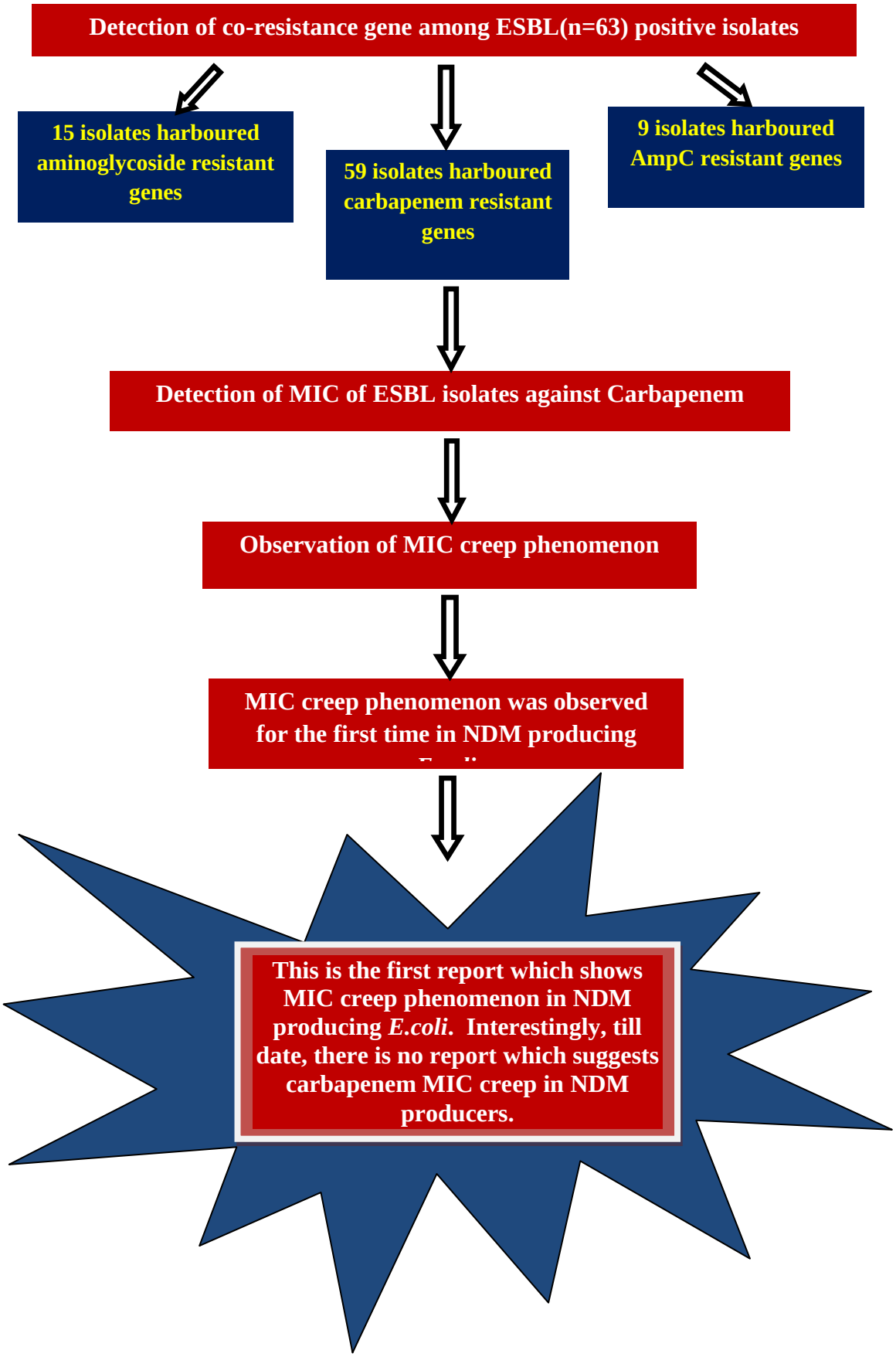
- As from the last decade no new beta-lactam antibiotics and inhibitors has come into clinical practice and nor it is in a pipeline. Moreover, carbapenamse producing bacteria may be the source of therapeutic death ends and till now no anti-Gram negative beta-lactam molecules are expected in the near future. Misuse of antibiotics, non-compliance of the prescribed dose of antibiotics, use of antibiotics without the prescription of a doctor, use of antibiotics in animal fodder are some of the factors responsible for the development of antibiotic resistance in bacteria.
- Therefore, the current study emphasizes the careful and restricted use of beta-lactam antibiotics combined with good control practices, compliance to the dose of antibiotics prescribed by the doctor and less use of antibiotics in animal fodder and formulates strict infection control procedure in order to control the spread of this resistance mechanism.
- Sikkim and Darjeeling is a hub of medicinal plants which are widely used in the treatment of various diseases as an alternative to allopathic medicines. There are less likely chances of developing drug resistance in bacteria in the study area due to its limited use. However, this is a matter of research and concern for healthcare sectors to find out the actual cause of the emergence of drug-resistant pathogens from time to time.
- Moreover there is an urgent need for exploration of newer-beta lactam antibiotics and inhibitors to prevent treatment failure and decrease morbidity and mortality rate in this part of the world. Further, more more

emphasize should be given to the use of simple and rapid diagnostic tools in order to identify the origin, acquisition and resistance mechanism to avoid the treatment failure in the near future. From the healthcare diagnostic point of view to combat the spread of antibiotic-resistant bacteria the diagnostic laboratories have to do regular surveillance programme for detection of such important pathogens, quickly identifying and isolating infected patients, disinfecting hospital equipment, and following common hospital practices which will be of immense use.

LAYOUT OF Ph.D. WORK PERFORMED BETWEEN JULY 2014 TO JUNE 2018




Confirmatory test (86/454 positive)
Genotypic method
DNA exraction of 63 isolates
Detection of ESBL genes n =63 isolates
Isolation of plasmid from 30 representatives ESBL isolates
Transferable plamids n=23/30 isolates
Replicon typing of 23 plasmids
All ESBL genes were found on a plasmid, which was mostly of HI1, I1, FIA+FIB,FIA, and Y types
Stability analysis of 20 resistant ESBL genes
All plasmid lost their ESBL genes in 28 passages


Detection of co-resistance gene among ESBL(n=63) positive isolates
15 isolates harboured aminoglycoside resistant genes
59 isolates harboured carbapenem resistant genes
9 isolates harboured AmpC resistant genes
Detection of MIC of ESBL isolates against Carbapenem
Observation of MIC creep phenomenon
MIC creep phenomenon was observed for the first time in NDM producing
This is the first report which shows MIC creep phenomenon in NDM producing *E.coli*. Interestingly, till date, there is no report which suggests carbapenem MIC creep in NDM producers.

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मन्जुरी पत्र

(सहभागी/**अभिभावक तथा सोधकर्ताले भर्नुपर्ने**)

म मेरो छोरा/छोरीलाई तपशिल शिर्षक भएको अनुषन्धान अध्ययन हेतु निजलाई सहभागी गराउन राजीखुशि छु ।

"Characterization of common bacterial pathogens from patients of Urinary Tract Infection from Sikkim and Darjeeling"

यस अध्ययनमा सहभागीको व्यक्तिगत सुचना तथा निजकोबाट मूत्र को नमुना (Sample)अध्ययनको लागीलिईने छ । मलाई वताई सकिएको छ की यस प्रकारको अध्ययन विश्वको अन्यभागमा पनि गरिएको छ र यस सम्बन्धमा स्वास्थ्य सम्बन्धि कुनै पनि नकारात्मक असर पर्ने छैन ।

यस अध्ययनको सम्भाव्य असरको बारेमा मलाई विस्तृत रुपमा वताई सकिएको छ ।

उपरोक्त सम्वन्धमा सहभागीलाई थप जानकारी आवश्यक भएमा **वर्षा रानी गजमेर (मोब**: 9647775465) संग सम्पर्क गर्ने छु ।

सहभागी / अभिभावके नाम र हस्ताक्षर

सोधकर्ताको नाम,
वर्षा रानी गजमेर
हस्ताक्षर

दिनांक

Consent Form

(To be filled by the participants/parents and Researcher)

I along with my son/daughter do hereby agree to be a participant in the research work as titled:

"Characterization of common bacterial pathogens from patients of Urinary Tract Infection from Sikkim and Darjeeling"

In this study the participant's personal information and the urine sample will be provided with. I have been explained and have understood that this type of study has been conducted in several parts of the world and has no adverse effects on the health of the respondent.

Varsha Rani Gajamer will be contacted if additional information are required in this context. (Mobile no.9647775465)

Name and signature of the participant

Researcher's name,

Varsha Rani Gajamer

Signature

Date:

Questionnaire based feedback form for the characterization of common bacterial pathogens from patients of urinary tract infection from Sikkim and Darjeeling

Demographics

- Name-
- Gender-
- Age (Yrs)-(18-49)_____
- Population type- Rural/Urban
- Name of the place _____
- Sikkim/ Darjeeling

Risk factors

- Water source - Government provided /Natural spring/river /Not known
- Water consumed- Raw /Boiled /Filtered /Purified
- Amount of water intake-
- Frequency of water consumption in a day- 1 lite/ 2 litres / more than 2 litres
- Sanitary condition-Western style/Indian style
- Process of cleaning their private part after excretion of feces- Water/ toilet paper/stone/ stick
- Do you use cleansing agent to clean their private part after excretion of feces- Yes/No
- Distance of toilet from their work place or home- nearby/ far/very far
- Do u wash your hands after toilet- Yes/No
- Sexual activity- Yes/ No
- Are you consuming antibiotics? Yes/ No

SYMPTOMS OF ACUTE URINARY TRACT INFECTION (UTI)

- Fever (high grade)
- Dysuria (burning pain on passing urine)
- Frequency in urination
- Urgency in urination
- Malaise
- Shivering
- Pain in abdomen

SYMPTOMS OF CHRONIC UTI

- Similar symptoms as of acute UTI with a low grade fever
- Tenderness in pubic region

- **History of any Infection/ diseases**
- Heart diseases
- Diabetes
- High blood pressure
- Cancer
- Spinal cord injury

- **Any specific observation:**

List of papers published in National/International journals during my Ph.D. work

1. **Gajamer VR,** Bhattacharjee A, Paul D, Deshamukhya C, Singh AK, Pradhan N and Tiwari HK. (2018) '*E. coli* encoding $bla_{\text{NDM-5}}$ associated with community-acquired UTI cases with unusual MIC creep like phenomenon against Imipenem.' *Journal of global antimicrobial resistance* 14, 228-232.

2. **Gajamer VR**, Singh AK, Pradhan N, Kapil J, Sarkar A, Tiwari HK. (2018) 'Prevalence and Antibiogram Profile of Uropathogens Isolated from Symptomatic and Asymptomatic Female Patients with Urinary Tract Infections and its Associated Risk Factors: Focus on Cephalosporin'. *Research & Reviews: Journal of Medical Science and Technology* (7):1. ISSN: 2319-3417 (Online), ISSN: 2349-1272 (Print).

3. **Gajamer VR**, Bhattacharjee A, Paul D, Kapil J, Sarkar A, Singh A K, Tiwari HK**.** (2018). The First Report of Phenotypic and Molecular Characterization of Extended-Spectrum Beta-Lactamase-Producing Uropathogens in Sikkim and Darjeeling Hills of India. *Microbial Drug Resistance* DOI: 10.1089/mdr.2017.0159.

4. **Gajamer VR,** Tiwari HK, Bhutia PD, Sen SS, Ghosh R and Sarkar A. (2015). Detection of antibiotic resistance pattern with ESBL producers and MRSA among the uropathogens at Tertiary Health Care Centre, North Bengal. *Int. J. Pure App. Biosci.* 3 (2): 522-533. ISSN: 2320 – 7051.

5. Emergence of Multidrug-Resistant Uropathogens harboring ESBL, Carbapenem, Aminoglycosides and AmpC resistant genes from Northern India (Communicated).

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Research Article

OPEN ACCESS

Detection of antibiotic resistance pattern with ESBL producers and MRSA among the uropathogens at Tertiary Health Care Centre, North Bengal

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ABSTRACT

The objective of the present study was to determine distribution and antibiotic susceptibility pattern of bacterial strains isolated from patients suffering from UTI at tertiary health care centre in North Bengal, with special reference to ESBL and MRSA producers. This health care centre was chosen for the study as this centre is visited by patients from inside and outside the country. Moreover, this health care centre is also visited by patients from neighboring countries like western part of Bangladesh, Bhutan and Eastern Nepal. The present retrospective study was conducted from july 2013 to july 2014 where 457 uropathogens were isolated from 2090 consecutive urine samples. Automated identification and susceptibility (AST) system that analyzed MIC patterns was used. ESBL producers, their phenotypes and MRSA were identified. Results were analyzed using computer software, specifically designed to evaluate the results generated by the automated system. The most prevalent pathogens were Escherichia coli (48%) followed by Klebsiella spp (22%) and Pseudomonas aeruginosa (5%). Majority of the isolates (59%) were from females. Prevalence of ESBL and MRSA was found to be 33.26 % and 75% respectively. Higher than 80% resistance were observed for broad-spectrum penicillin with an increasing resistance to third generation cephalosporins and quinolone drugs. Tigegcycline was found to be effective against both gram negative and gram positive uropathogen. Daptomycin and Colistin was found to be drug of choice for both gram positive and for gram negative uropathogen respectively. The data highlights a serious need to monitor the current trend of growing antibiotic resistance. It indicates that it is imperative to rationalize the use of antimicrobials and employ them conservatively.

Keywords: *Urinary tract infection, uropathogens, Antibiotic Resistance, ESBL, MRSA etc.*

INTRODUCTION

Since the first description of plasmid-mediated extended spectrum beta lactamase (ESBL) in 1983, ESBL-producing gram-negative organisms have posed a significant threat to hospitalized patients due to their hydrolyzing activity against extended spectrum cephalosporins often employed in the treatment of hospital-acquired infections. Detection of organisms harboring ESBLs provides clinicians with helpful information. Treatment of infections caused by ESBL-producing organisms with extended-spectrum cephalosporins or aztreonam may result in treatment failure even when the causative organisms appear to be susceptible to these antimicrobial agents by routine susceptibility testing[24].

The β lactamase enzymes produced by the organisms break down the structural beta-lactam ring of β-lactam antibiotics. Many genera of gram negative bacteria possess a naturally occurring, chromosomally mediated β-lactamase and also some are plasmid mediated β-lactamases[24].

Urinary tract infections (UTI) are one of the most common infectious diseases seen in the clinical practice and community. In recent studies microbial species that cause urinary system infection are classified by their target sites, Such as urine infection (bacteriuria), bladder infection (cystitis), kidney infection (pyelonephritis), which can be asymptomatic or associated with symptoms[17,19]. It has been estimated that nearly 10% of the human population will experience a UTI during their life time[10]. UTI is the third most common cause of admission to hospitals in India. Gram negative bacteria are most often implicated in causing UTI[3]. Detection of ESBL producing organisms from urine samples will be valuable as this represents an epidemiologic marker of colonization.

It has been estimated that about 6 million patients per year are visited worldwide for UTI out of which around 30,000 are treated in the wards[5]. An estimate of patients suffering from UTI is around 150 million per annum across the Globe, which may rise to 75% in the female population by the age of 24, and 15–25% of this group will suffer from a relapse of this disease[15,16,20,25].

In eastern India, UTI is a common infection found among all ages from infants to elderly persons. However, studies on UTI and the susceptibility pattern of antibiotics in Eastern India are still underway, and there is extensive debate on the choice of antibiotics due to the lack of clear guidelines.

Knowledge of the etiology and antibiotic susceptibility pattern of the pathogen causing UTI is absolutely essential. The introduction of antimicrobial therapy has contributed significantly to the management of UTIs. However the main problem with current antibiotic therapies is the rapid emergence of antimicrobial resistance in hospitals and the community[13]. The resistance pattern of community acquired uropathogens has not been extensively studied in the Indian subcontinent[3,18]. No data concerning the antimicrobial resistance of bacteria isolated from UTIs from this part of the country that is North Bengal been documented till date.

It is important to realize that there may be marked differences between various geographic areas within a vast country like India. Since most UTIs are treated empirically the selection of antimicrobial agent should be determined not only by the most likely pathogen but also by its expected susceptibility pattern. Thus, knowledge of local antimicrobial susceptibility patterns of common uropathogens is essential for prudent empiric therapy of community acquired UTIs.

Nowadays, infectious pathogens are mostly resistant to several antibiotics, and this undermines the ability of antibiotics to control infections[14,21,29,30].

Hence, the present study was designed to study the current antibiotic susceptibility pattern among the uropathogens and to detect ESBL production and MRSA among them. The current study is of critical importance since it is useful for preparing the current antibiotic policy, in infection controlling policy and for detection and control of the outbreak of ESBL and MRSA producing organisms in a hospital. Additionally, the study also aimed at identifying possible resistance trends. As per electronic literature survey no such kind of study has been documented in the study area.

MATERIALS AND METHODS

Design & Setting

The data was taken from the WHONET software from the department of Microbiology, Tertiary health Care hospital, North Bengal during July 2013 to July 2014. Besides the people of North Bengal this health care centre is also visited by patients from nearby states like Eastern Bihar and whole Sikkim. Moreover, this health care centre is also visited by patients from neighboring countries like western part of Bangladesh, Bhutan and Eastern Nepal.

This was an analysis of data generated from the records of consecutive urine samples received in the laboratory from hospital's indoor and outdoor during the study period.

The anonymity of the patients was ensured. All data were retrospectively collected and de-identified when this was necessary to ensure patient confidentiality.

The study included all the patients who were admitted or visited the out-patient department in the hospital or health centre with symptoms of UTI during the study period and then had UTI confirmed further by positive urine culture reports. During this period a total of 2090 urine samples were collected. Majority of the samples were midstream clean catch urine followed by stream catheter, catheter site, catheter central, catheter peripheral, catheter permanent, catheter umbilical, urine bladder, urine clean voided , suprapubi caspirate, urine first voided, urine kidney, urine nephrostomy, urine non catherized and urine obtained from Foley's catheter.

Isolation of pathogen

The samples were observed carefully for adult parasite, consistency, blood, mucous, color and pH. In microscopic observation the samples were observed carefully for pus cells and red blood cells. Urine samples were cultured using a 1μmcalibrated loop onto Hichrome UTI agar plates. The samples were inoculated onto High Chrome UTI Agar (Hi Media, India) by semi quantitative method and incubated aerobically at 37ºC for complete 24 h incubation. The specimen yielding more than or equal to 10^5organisms/ml of urine was interpreted as significant**.** Isolates were identified on the basis of gram staining, colony morphology and standard biochemical tests. The isolates were further identified by vitek 2 instruments (VITEK 2 compact, Biomerieux).

Antibiotic Susceptibility Testing

All gram positive and the gram negative uropathogens were subjected for Antibiotic Susceptibility testing and the results were interpreted by modified Vitek 2 method automated system. The system included an Advanced Expert System (AES) that analyzed MIC patterns and detected the phenotype of organisms. Pure subcultures of QC and clinical organisms were suspended in aqueous 0.45% (wt/vol) NaCl to achieve a turbidity equivalent to that of a McFarland 2.0 standard (range, 1.80 to 2.20), as measured by the Densi Chek (bioMerieux) turbidity meter. Strain characterization and antimicrobial susceptibility testing were performed with the VITEK 2 automated system using the ID-GNB and AST-N280 and ID-GPC and AST- P628 cards for gram negative and gram positive bacteria respectively, in accordance with the manufacturer's instructions[11].

The VITEK 2 instrument automatically filled, sealed, and incubated the individual test cards with the prepared culture suspension. Cards were held at 35.5°C for 18 h, with optical readings taken automatically every 15 min. Based on these readings, an identification profile was established and interpreted according to a specific algorithm. The antimicrobial susceptibility testing card comprises various antibiotics which includes Ampicillin, Amoxicillin/ clavulanic acid, cefuroxime, cefuroxime/axetil, Ceftriaxone, Ciprofloxacin, Trimethoprim/Sulfamethoxazole, Pipercillin/ Tazobactum, Cefoperazone/Sulbactam, Cefepime, Imipenem, Meropenem, Amikacin, Gentamicin, Nalidixic acid, Nitrofurantoin, Colistin, Tigercycline for gram negative bacteria and for gram positive bacteria the testing card comprised the following antibiotics Benzyl penicillin, Oxacillin, Gentamicin, Ciprofloxacin, Levofloxacin, Erythromycin, Clindamycin , Linezolid, Daptomycin, Teicoplanin, Vancomycin, Tetracycline, Tigecycline, Nitrofurantoin, Rifampicin and Trimethoprim/Sulfamethoxazole. Final results were analysed using version 7.01 software, an AES specifically designed to evaluate the results generated by the VITEK 2 system**.**

ESBL and Methicillin Resistant Staphylococcus aureus (MRSA) Testing

Each isolate was tested using the VITEK 2 (software configuration version R07.01) system with the ESBL test panel with six wells containing three third generation cephalosporin, alone and in combination with clavulanic acid (CA). Growth in each well was quantitatively assessed by means of an optical scanner. The proportional reduction in growth in wells containing cephalosporin plus CA compared with those containing the cephalosporin alone was considered indicative of ESBL production. Quality control strains were included in each run. All phenotypic interpretations of ESBLs were reported as a positive ESBL screening result. Strains were reported as ESBL-negative whenever phenotypic interpretations other than ESBLs were proposed by the AES.

The Vitek (software configuration version R07.01)automated susceptibility testing system with a modified Gram-Positive Susceptibility (GPS) 106 Card (bioMerieuxVitek, Inc) were evaluated for their ability to detect oxacillin resistance in *Staphylococcus aureus*.

RESULTS

Microorganisms

Data from a total of 2090 consecutive urine samples were included in the study. Out of these, 1633 (78.2%) were sterile, 457(21.8%) showed significant growth.

Out of these positive cultures (n = 457), 391 gram negative rods and 42 gram positive cocci were isolated. Among gram negative rods major pathogens were *Escherichia coli* (56%) followed by *Klebsiella pneumoniae ss. Pneumoniae* (25%), *Pseudomonas aeruginosa* (6%), *Acinetobacter baumannii* (5%), *Citrobacter freundii* (1%), *Pseudomonas putida* (1%), *Proteus mirabilis* (1%), *Enterobacter cloacae* (1%), *Proteus rettgeri* (1%), *Klebsiella oxytoca* (1%), *Citrobacter koseri* (diversus) (1%). Among gram positive rods *Enterococcus faecalis* (32%) *Enterococcus sp* (20%), *Enterococcus faecium* (18%), *Staphylococcus aureus ss. aureus* (9%), *Staphylococcus haemolyticus* (9%), *Staphylococcus sciuri ss. lentus* (5%), *Staphylococcus epidermidis* (2%), *Staphylococcus saprophyticus ss. saprophytic* (2%) and *Staphylococcus warneri* (2%) were isolated.

Moreover, some of the fungal pathogen which was isolated was *Candida albicans, Candida tropicalis, Candida glabrata* and *Candida parapsilosis* (Table 1).

116 uropathogens were isolated from critical care unit (surgical and Medical care intensive units =73, Pediatric are intensive units=2, Neonatal care intensive units=0, High Dependency unit=41) and from non critical care (cabins=10, Surgical male and female ward=15 and 20 respectively, Medical male and Female ward= 39 and 52 respectively, emergency=3, nursery=2) (Table 2).

Table 1: All organisms isolated from urinary tract infected patient (457 isolates)

Mostly isolated uropathogens				
Organism	**No. of isolates**	**(%)**	**2013**	**2014**
Escherichia coli	220	48	75	145
Klebsiella pneumoniae ss. pneumoniae	100	22	48	52
Pseudomonas aeruginosa	24	5	9	15
Acinetobacter baumannii	20	4	9	11
Enterococcus faecalis	14	3	5	9
Occasionallv isolated uropathogens				
Candida albicans	9	2		9
Enterococcus sp.	9	2	9	
Enterococcus faecium	8	2		8
Candida tropicalis	6	1		6
Rarely isolated uropathogens				
Proteus mirabilis	4	1	2	2
Staphylococcus haemolyticus	4	1	2	2
Pseudomonas putida	4	1	3	1
Citrobacter freundii	4	1	3	1
Staphylococcus aureus ss. aureus	4	1	2	2
Enterobacter cloacae	4	1	2	2
Citrobacter koseri (diversus)	3	1		3
Proteus rettgeri	3	1	2	1
Klebsiella oxytoca	3	1		3
Staphylococcus sciuri ss. lentus	2	0	1	1
Candida glabrata	2	0		2
Stenotrophomonas maltophilia	1	0		1

Staphylococcus saprophyticus ss. saprophytic	1	0		1
Acinetobacter lwoffii	1	0	1	
Staphylococcus epidermidis	1	0		1
Sphingomonas paucimobilis	1	0	1	
Raoultella ornitholytica	1	0		1
Pseudomonas stutzeri	1	0	1	
Candida parapsilosis	1	0		1
Klebsiella sp.	1	0	1	
Staphylococcus warneri	1	0		1

Table 2: Distribution of uropathogens from different wards

	Locations	**No. of isolates**
1.	Critical care unit	116
2.	Semi critical	141

Distribution of uropathogen among gender

The distribution of uropathogen was checked in both males and females. The distribution was checked by checking the number of isolated organism from both male and female urinary tract infected patient. It was found that maximum number of uropathogens was isolated from females. A total of 184 isolates (enterobactereciae=117, gram negative =127, gram positive = 23) was isolated from males whereas a total of (enterobactereciae = 225, gram negative=229, gram positive=19) 267 isolates was isolated from females. Therefore, it was found that higher percentage of women (59%) to be suffering from UTI as compared to men (41%).

Antibiotic Susceptibility testing

Antibiotic susceptibility testing was performed for all the isolates. In general, tigegcycline was found to be effective against both gram negative and gram positive uropathogen. Daptomycin and Colistin was found to be drug of choice for both gram positive and for gram negative uropathogen respectively (Table 3 and 4).

Table 3: Resistance pattern of gram negative uropathogens

Antibiotic name	**Number**	**%R**
Cefuroxime	363	87.6
Amoxicillin/Clavulanic acid	362	83.7
Trimethoprim/Sulfamethoxazole	391	72.9
Ciprofloxacin	389	72.8
Nalidixic acid	362	67.4
Ceftriaxone	377	64.7
Piperacillin/Tazobactam	361	49
Gentamicin	388	42
Cefepime	383	40.2
Nitrofurantoin	373	37.8
Meropenem	389	29.3
Imipenem	382	27
Amikacin	390	22.8
Colistin	379	4
Tigecycline	181	0

Table 4: Resistance pattern of gram positive uropathogens

Antibiotic name	**Number**	R%
Clindamycin	12	83.3
Ciprofloxacin	41	82.9
Levofloxacin	42	65.3
Trimethoprim/Sulfamethoxazole	12	66.7
Rifampin	12	58.3
Vancomycin	42	9.5
Teicoplanin	36	8.3
Linezolid	38	2.6
Daptomycin	21	0
Tigecycline	17	0

The mostly occurring uropathogen showed the antibiotic resistance pattern in the following way where *Escherichia coli* showed 92.9% resistance to Nalidixic acid followed by Ampicillin (85.3%) Ciprofloxacin (78.9%), Trimethoprim/Sulfamethoxazole (71.6%) Cefuroxime (62.8%), Ceftriaxone (61.3%), Cefepime (32.7%), Gentamicin (32.3%) Piperacillin/Tazobactam (23.8%) Nitrofurantoin (12%) Meropenem (11.9%), Imipenem (10.1%) Amikacin (9.6%) and Colistin (0.5%) (Table 5).

It was found that *Klebsiellapneumoniae* showed 96% resitence to Ampicillin followed by Cefuroxime Cefoperazone (73.5%), Trimethoprim/Sulfamethoxazole (70%), Ceftriaxone (68.7%) Nitrofurantoin (67%) Nalidixicacid (667%) Ciprofloxacin (64%) Amoxicillin / Clavulanic acid and Piperacillin/Tazobactam(56%), Gentamicin (53.5%) Meropenem (50%) Cefepime (48.5%) Imipenem (46%) Amikacinand Colistin (1%).

Pseudomonas aeruginosa showed 100% resistance to Ampicillin, Nalidixicacid, Trimethoprim/Sulfamethoxazole, Ampicillin/Sulbactam, Ceftriaxone, Nitrofurantoin, Amoxicillin/Clavulanic acid and Cefuroxime followed by Levofloxacin (80%), Cefoperazone (69.2%), Ciprofloxacin (66.7%), Meropenem and gentamicin (54.2%), Imipenem (50%), Pipercillin/ Tazobactam, Cefepime, Amikacin (45.8%), Doripenem (44.4%), Minocycline (42.9%), Ceftazidime (36.4 %) Aztreonam (33.3 %) Colistin (4.2%). All the isolates of *Escherichia coli* and *Klebsiella pneumonia* and *Pseudomonas aeruginosa* were sensitive to Tigecycline.

Acinetobacter baumannii showed 100% resistance to Nitrofurantoin followed by imipenem (93.8%). It showed 88.2% of resistivity was towards Cefuroxime, Amoxicillin/Clavulanic acid, Ceftriaxone, Meropeneme, Nalidixic acid, Ciprofloxacin and Trimethoprim/Sulfamethoxazole. Moreover, 87.5 % of resistivity was shown towards Ampicillin and Cefepime followed by Gentamicin (82.4%) and Amikacin (64.7%).

Table 5: Antibiotic Resistence pattern of mostly isolated uropathogen

	Escherichia coli		***Klebsiella pneumoniae***		***Pseudomonas aeruginosa***		***Acinetobacter baumanii***	
Antibiotic name	**Number**	**%R**	**Number**	**%R**	**Number**	**%R**	**Number**	**%R**
Nalidixic acid	210	92.9	100	66.7	9	100	17	88.2
Ampicillin	217	85.3	100	96	9	100	16	87.5
Ciprofloxacin	218	78.9	100	64	24	66.7	17	88.2
Trimethoprim/Sulfamethoxazole	218	71.6	100	70	24	100	17	88.2
Cefuroxime	207	62.8	98	73.5	11	100	17	88.2
Ceftriaxone	217	61.3	99	68.7	13	100	17	88.2
Cefepime	214	32.7	99	48.5	24	45.8	16	87.5
Gentamicin	217	32.3	99	53.5	24	54.2	17	82.4
Piperacillin/Tazobactam	202	23.8	91	56	24	45.8		
Nitrofurantoin	216	12	100	67	9	100	17	100
Meropenem	218	11.9	100	50	24	54.2	17	88.2
Imipenem	218	10.1	100	46	24	50	16	93.8
Amikacin	218	9.6	100	39	24	45.8	17	64.7
Colistin	209	0.5	100	1	24	4.2	17	0
Tigecycline	123	0	21	0	10	0	7	0

All the isolates of *Candida albicans* showed sensitivity towards 5-Fluorocytosine, Fluconazole Amphotericin B, Caspofungin and Voriconazole (Table 6).

Table 6: Resistance pattern of *Candida albicans*

Antibiotic name	Number	S%
Amphotericin B	9	100
Caspofungin	9	100
5-Fluorocytosine	9	100
Fluconazole	9	100
Voriconazole	6	100

Distribution of ESBL pathogens and MRSA

Out of the 457 uropathogens isolated, 152 isolates were found to be ESBL producers showing the prevalence by 33.26%. Among all the ESBL isolates highest number of ESBL producer was found to be *Escherichia coli* (66%) followed by *Klebsiella pneumonia* (37.72%) and *Citrobacter freundii* (12.5%). And among the 4 isolates of *Staphylococcus aureus* isolated from urinary tract infected patient the number of MRSA was found to be 3 showing the prevalence rate of 75% (Table 7 and 8).

Table 7: Distribution of ESBL pathogens among uropathogen

S. No.	Organisms	No. of isolates	No. of ESBL producers	Percentage Of ESBL producers
1.	*Klebsiella pneumoniae*	220	83	37.72
2.	*Escherichia coli*	100	66	66
3.	*Citrobacter freundii*	24	3	12.5
	Total ESBL pathogens		**152**	

Table 8: Methicillin Resistant Staphylococcus aureus (MRSA) isolates among uropathogen

Total no. of *Staphylococcus aureus* isolates	No. of MRSA isolated	Percentage
4	3	75%

Figure 1: Resistance pattern of *Escherichia coli*

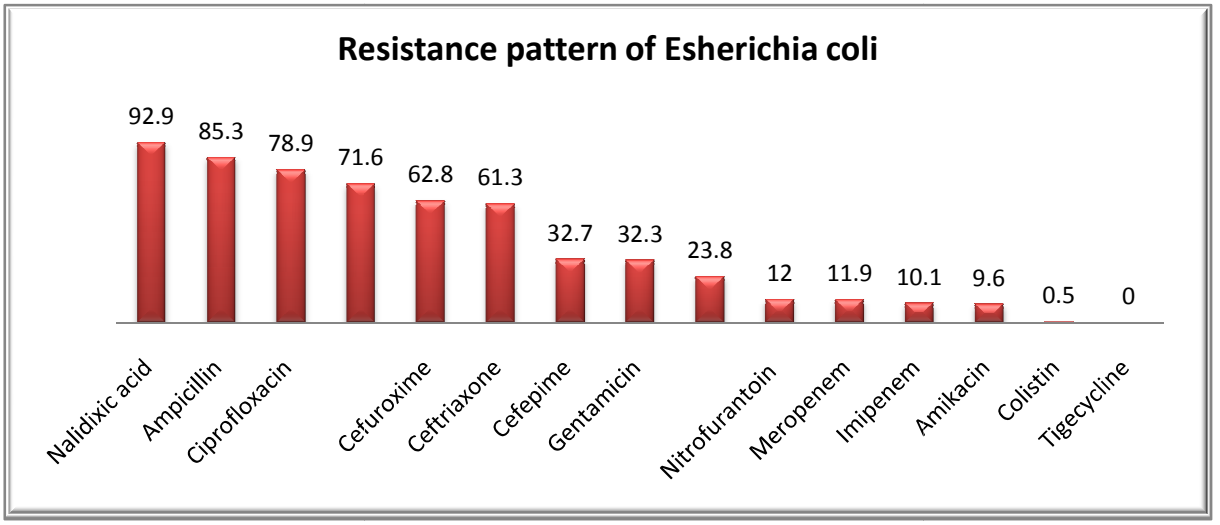


Figure 2: Resistance pattern of *Klebsiella pneumoniae*

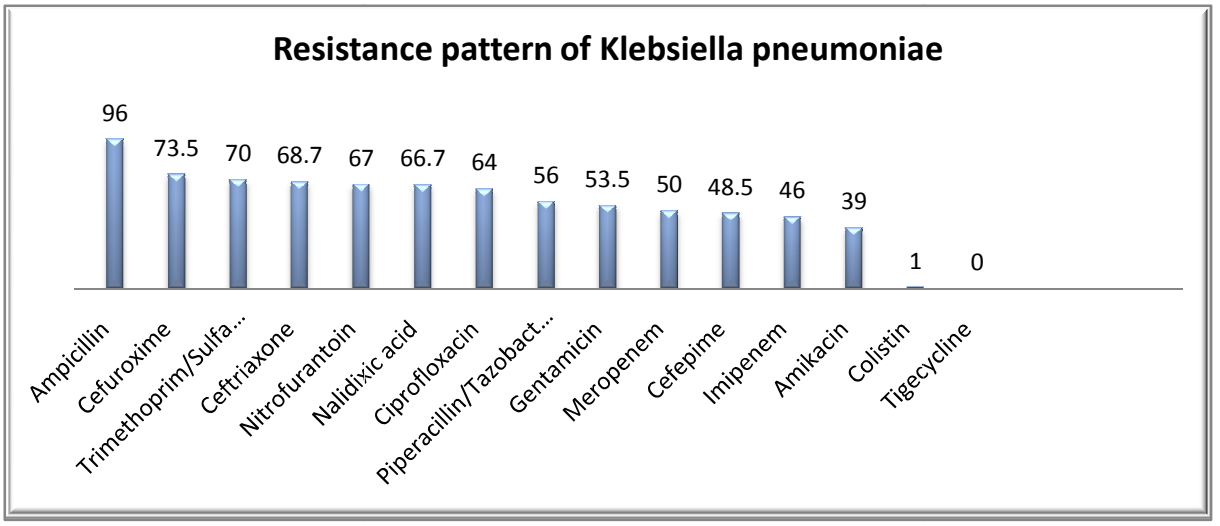


Figure 3: Resistance pattern of *Pseudomonas aeruginosa*

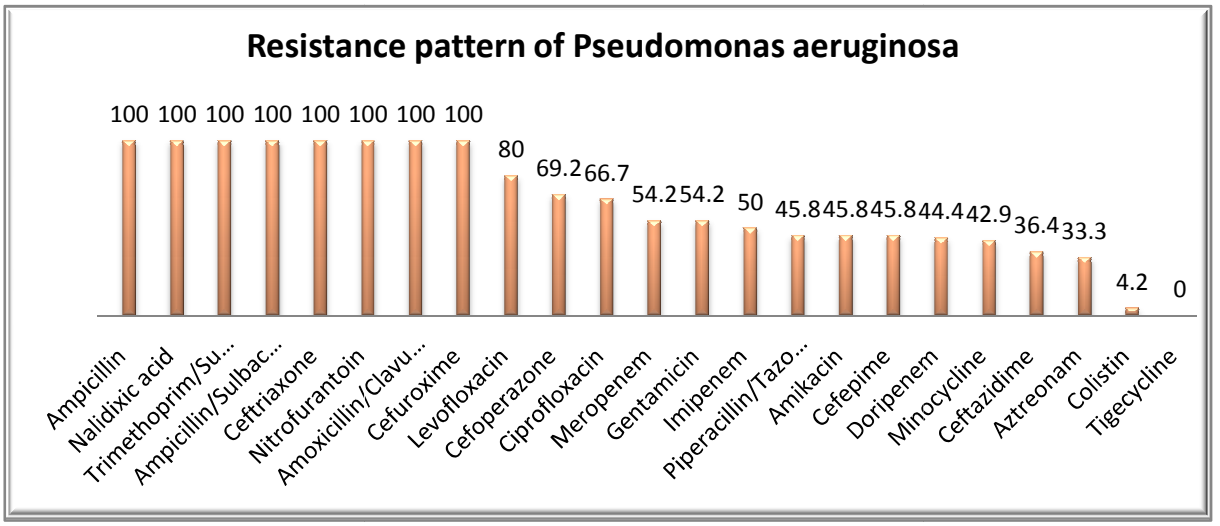
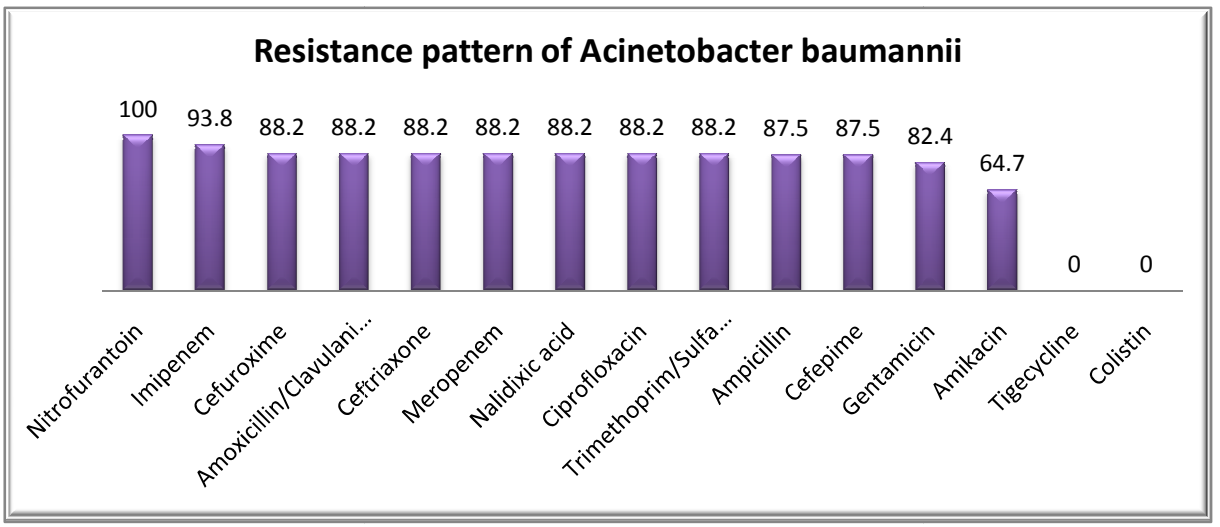


Figure 4: Resistance pattern of *Acinetobacter baumannii*



DISCUSSION

Urinary tract infection is among the most prevalent infectious disease in general population. The effectiveness of an antibiotic administered to a patient depends on the site and severity of the infection, liver and renal function, presence of implants and local resistance patterns. It is also believed that age and pregnancy in the patient determine the effectiveness of the antibiotic used[1]. Recently, with increased rates of antimicrobial resistance, treatment of complicated UTIs has become increasingly challenging for clinicians.

Amoxycillin (a β-lactam antibiotic) was traditionally used in the first line therapy for UTIs, but with the spread of drug resistance, treatment options have now changed. Complicated cases of UTI usually require a longer course or intravenous antibiotics, and in case symptoms do not improve in two or three days, further diagnostic testing is needed. Since bacterial resistance to antibiotics represents a serious problem for clinicians and pharmaceutical industry, efforts have been made recently to reverse this trend by exploring alternate methods[6,29].

For the current investigation, a total of 2090 urine specimens received and processed at Tertiary Health Care centre, located in North Bengal were studied. More than 10^5 colony forming units (cfu) of bacteria/mL of urine were considered significant bacteraemia. Gram-negative isolates were identified up to species level by VITEK 2 automated microbiology system. Of the total 457 isolates, the most commonly isolated bacteria were *Escherichia coli* (48%). Other isolates included *Klebsiella pneumonia* 100 (22%), *Pseudomonas aeruginosa* 24 (5%) and *Acinetobacter baumannii* 20 (4%). Similar kinds of studies have been reported from different regions of India and from other countries have reported that the most prevalent UTI pathogen was *E. coli*, followed by *Klebsiella* spp[12,26,20,28]. 116 uropathogens was isolated from critical unit and 141 from semi critical. Data analysis revealed that urinary tract infection was more prevalent in women (59%) to be as compared to men (41%) similar to the study of Dugal et al.,[11]. It is known that UTI occur more commonly in women, with half of them having at least one infection at some point in their lives. It is believed that bacteria are usually transmitted to the urethra from bowel, with females at greater risk due to their anatomy. During pregnancy, high progesterone levels elevate the risk of decreased muscle tone of the ureter and bladder, which leads to a greater likelihood of reflux, towards the kidneys[11].

For the study, the antibiogram pattern of the 457 isolates was checked against 16 antibiotics belonging to different groups and possessing varied modes of action.

Escherichia coli showed highest resistance against nalidixic acid (92.9%), ampicillin (85.3%) , followed by Ciprofloxacin (78.9%) similar to the result of Ahmed et al 2014 who found similar type of resistance pattern for *Escherichia coli* that is nalidixic acid (98.5%) and ciprofloxacin (86.2%).

It was reported that *Klebsiella* spp. from Eastern India UTI samples were maximally resistant to penicillin combination, followed by aminoglycosides and third generation cephalosporin. Studies conducted in West Bengal and around other parts of the country showed consistency in *Klebsiella pneumonia* which presented the second highest resistance after *E. coli*, against different classes of antibiotics[18]. Data are consistent with the findings of a northern Indian city where *Klebsiella pneumonia* showed the highest resistance to a drug from the penicillin combination similar to the present study where *Klebsiella pneumonia* showed 96% resistance to a drug from penicillin combination followed by third generation cephalosporin, cefuroxime and cefoperazone.

Data analysis showed that all *Pseudomonas aeruginosa* isolate showed 100% resistance to Ampicillin, Nalidixic acid, Trimethoprim/Sulfamethoxazole, Ampicillin/Sulbactam, Ceftriaxone, Nitrofurantoin, Amoxicillin/Clavulanic acid and Cefuroxime. *Acinetobacter baumannii* isolates showed high level of resistence to nitrofurantoin followed by imipenem.

A high level of sensitivity was noted to colistin and tigegcycline by all gram negative bacilli.

The accurate detection of extended-spectrum β-lactamases is a major clinical problem, particularly in invasive infections, frequently leading to therapeutic failure and adverse clinical outcome. In typical circumstances, ESBLs derive from genes for TEM-1, TEM-2, or SHV-1 by mutations that alter the amino acid configuration around the active site of these β-lactamases.

This extends the spectrum of β-lactam antibiotics susceptible to hydrolysis by these enzymes. Successful spread of ESBL-encoding genes within the microbial genome can be attributed to their common localization on self-transmissible or easily movable broad-range plasmid[23].

The Advanced Expert System (AES) in conjunction with the VITEK 2 automated antimicrobial susceptibility test system is widely used in clinical microbiology laboratories for the identification and evaluation of the susceptibility profiles of bacteria and helps in the detection of extended-spectrum β-lactamases (ESBLs) produced by organisms.

The phenotypic data generated in the current study, using this system, indicates a considerably significant prevalence of ESBL producers in the region of North bengal, where a total of 152 out of 457 (33.26%) uropathogens were found to be ESBL producers which was very similar to the study conducted in the Central Referral Hospital, Gangtok where the prevalence of ESBL was found to be 34.03%[30]. On the contrary 27.67% uropathogens were found to be ESBL producers by Dugal *et al.*,[11] in a similar kind of study conducted at Mumbai hospital.

The identification of the *mec*A gene is the most reliable method for detecting the MRSA isolates. However, not all laboratories can include molecular biology techniques in their routine clinical practice. So, it is important that phenotypic techniques which are able to detect the MRSA isolates in a rapid and accurate manner are made available, in order to ensure the correct antibiotic treatment and to avoid the spread of the MRSA isolates in the hospital environment[9].

The prevalence of MRSA in our study was 75% while Dalela *et al.*,[9] in Jhalawar found prevalence rate as 42.4%. Sanjana R K *et* al.,[28] in Nepal, detected the prevalence of MRSA as 39.6%, Rajaduraipandi K *et al*.,[27] in Coimbatore found 31.1% strains of MRSA and Anupurba S *et al.*,[4] in eastern Uttar Pradesh found a 54.85% prevalence of MRSA, which correlated well with the findings of our study. Onanuga A *et al*.,[22] in Nigeria have reported a high prevalence of 69%, while Coombs G W et al.,[8] in Australia found it to be very low as 16%.

CONCLUSION

Despite the advances in diagnostic methods, availability of antimicrobials and awareness among the people, urinary tract infections continue to remain a major health problem and the resistance pattern of multi drug resistant uropathogen is in rise. In the current study, among the oral drugs, broad spectrum penicillin, amoxycillin/clavulanic acid, quinolone drugs, and third generation cephalosporins like ceftriaxone and cefuroxime and should no longer be considered as the first line drugs for the empirical treatment of clinically evident UTI, because of the very high resistance rates. Moreover, a significantly higher number of ESBL and MRSA were found.

However, the present investigation was carried in a particular healthcare centre, additional studies can be carried out with a larger sample size from various hospitals in the region to obtain a more representative picture. Moreover, control measures which include the judicious use of antibiotics, antibiotic cycling, and the implementation of appropriate infection control measures and the formulation of an antibiotic policy must be done, to prevent the spread of these strains.

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The First Report of Phenotypic and Molecular Characterization of Extended-Spectrum Beta-Lactamase-Producing Uropathogens in Sikkim and Darjeeling Hills of India

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Extended-spectrum β-lactamase (ESBL)-producing bacteria are a global health threat both in hospital and in community settings. The emergence of these organisms poses major difficulty in treating infections. This study was carried out to assess major ESBL-producing uropathogens in female patients of Sikkim and Darjeeling by phenotypic and genotypic methods. Out of 1,516 urine samples, 454 uropathogens were isolated with a prevalence rate of 29.94%. Among them, *Escherichia coli* (74.3%) was the predominant type followed by *Klebsiella pneumoniae* (20.1%), *Pseudomonas aeruginosa* (2.4%), and *Proteus mirabilis* (1.98%). Four different ESBL genes were detected in 63 isolates, which included CTX-M ($n=32$), CTX-M+OXA-2 ($n=15$), CTX-M-15+OXA-2+TEM ($n=6$), OXA-2 ($n=5$), TEM+CTX-M-15 ($n=2$), TEM+OXA-2+SHV-76 ($n=2$), and TEM ($n=1$). All ESBL genes (*bla* genes) were found on a plasmid, which was mostly of HI1, I1, FIA+FIB, FIA, and Y types and was horizontally transferable. Among all ESBL genes, *bla*CTX-M-I5 group was the most prevalent. The study of urinary tract infection (UTI) caused by ESBL-producing bacteria needs to be studied in other high-altitude parts of India to understand the actual burden of UTI in the female.

Keywords: ESBL, *bla* genes, replicon typing, urinary tract infection

Introduction

PENICILLINS, BROAD-SPECTRUM cephalosporins, and monobactams hydrolyzing rapidly evolving plasmid-mediated and diverse complex enzymes are known as extended-spectrum β-lactamase (ESBL). ESBL-producing organisms that cause urinary tract infections (UTIs) are increasing in incidence and pose a major burden to healthcare.[1] ESBLs are generally derived from TEM and SHV-type enzymes. CTX-M type enzyme isolated from ESBL producers plays an important role in multidrug resistance as it has been recognized as an important subtype.[1,2] The occurrence of point mutations in the sequence of the primary β-lactamase gene results in variants with both broad- and extended-spectrum activity.[3] β-lactamases are classified into four main groups, including A, B, C, and D on the basis of their inhibitory mechanism, type of substrate, and physical characterization such as molecular weight and isoelectric point. According to this classification, broad-spectrum β-lactamase enzyme is categorized among group A.[3,4] Treatment option becomes complicated when an infection is associated with ESBL-producing organisms. *Escherichia coli* and *Klebsiella pneumoniae* are common producers of ESBL, and are a major cause of UTIs. β-lactamase-producing bacteria can play an important role in polymicrobial infections.[5] They can have a direct pathogenic impact in causing the infection as well as an indirect effect on their ability to produce the enzyme β-lactamase. These enzyme-producing Enterobacteriaceae families are associated with a higher morbidity, mortality, and economic burden.[5] More than 200 types of ESBLs have been found worldwide, most of them belong to the Enterobacteriaceae family.[1] This study was carried out to assess major ESBL-producing uropathogens in female patients of Sikkim and Darjeeling by phenotypic and genotypic characterization.

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Materials and Methods

Study design, setting, and sample collection

This study was carried out at the Department of Microbiology, Sikkim University, Sikkim, India. From May 2014 to July 2016, a total number of 1,516 urine samples were collected from female patients suspected to have UTIs. The urine samples were collected from in-patient and out-patient departments of two tertiary hospitals in India: Sikkim Manipal Institute of Medical Sciences in Sikkim and Neotia Get Well Healthcare Centre in Siliguri, Darjeeling.

Standard microbiological techniques were used for the collection, transportation, and processing of clean-catch midstream samples. The uropathogens were isolated on cystine lactose electrolyte deficient agar, Hi chrome UTI agar and Mac Conkey agar plate by the semiquantitative method.[6] Specimens yielding more than or equal to 10^5 cfu/ml of urine were interpreted as significant bacteriuria. All the isolates were identified on the basis of gram staining, colony morphology, and standard biochemical tests and a few isolates were further confirmed by Vitek 2 instruments (VITEK 2 compact; Biomerieux).

Phenotypic tests for the detection of ESBL

Screening for ESBL—the screening test of the isolates was done using five antibiotics namely cefotaxime (CTX), ceftazidime (CAZ), ceftriaxone, aztreonam at 1 μg/ml, and cefpodoxime 4 μg/ml (1 μg/ml for *Proteus mirabilis*) in Mueller Hinton agar (MHA) by the agar dilution method. The isolates that showed growth in any of these antibiotic containing media were suspected to be an ESBL producer and were subjected to a confirmatory test.[7] Confirmatory tests for ESBL production (combined disk diffusion test): isolates considered to be positive for ESBL production by the screening test were subjected to the phenotypic confirmatory test by using an ESBL kit consisting of CAZ (30 μg) (), ceftazidime+clavulanic acid (30/10 μg) (CAC), CTX (30 μg), and cefotaxime+clavulanic acid (30/10 μg) (CEC).[7]

Molecular characterization of bla*ESBL genes by multiplex PCR*

For the extraction of DNA from bacterial samples, the boiling centrifugation method was used. The organism was cultured in 5 ml Luria–Bertani (LB) broth. One milliliter of culture was transferred into an Eppendorf tube and heated at 85°C for 20 min in a thermomixer. The lysed cell obtained after heating was centrifuged at 15,680 *g* for 10 min. The supernatant containing DNA was collected in a sterile Eppendorf tube for the detection of ESBL genes.[8] ESBL genes were detected by multiplex PCR (BioRad) in a total volume of 25 μl containing 23.5 master mix and 1.5 μl of template DNA. For amplification and characterization of *bla*ESBL, a set of eight primers were used, namely *bla*TEM, *bla*CTX-M, *bla*SHV, *bla*OXA-2, *bla*OXA-1O, *bla*PER, *bla*GES, and *bla*VEB (Table 1). Reactions were run under the following conditions: initial denaturation at 94°C for 5 min, 33 cycles of 94°C for 35 sec, 51°C for 1 min, 72°C for 1 min, and the final extension at 72°C for 7 min.[8] PCR products were separated by gel electrophoresis on 1% agarose gel.

Plasmid stability test of isolates harboring ESBLs

Plasmid stability analysis of all ESBL producers as well as their transformants was analyzed by the serial passages method for 110 consecutive days at 1:1,000 dilutions in LB broth without antibiotic pressure.[9] PCR assay was carried out for the presence of *bla* genes in the isolates after each passage.

Plasmid preparation, genetic transferability, and incompatibility typing

ESBL positive bacterial isolates were cultured in LB broth (Hi-Media, Mumbai, India) containing 0.25 μg/ml of cefoxitin. After overnight incubation, plasmids were extracted by QIAprep Spin Miniprep kit (Qiagen, Germany). Plasmids of *bla* genes were subjected to transformation by heat shock method using *E. coli* JM107 as a recipient. Transformants were selected on LB agar with 0.25 μg/ml of cefoxitin, which were then confirmed both by phenotypic and by PCR analysis. The plasmids were characterized by PCR-based replicon typing for determining the incompatibility group type of the plasmid in all *bla* genes harboring strains.[10] A total of 18 different replicon types such as FIA, FIB, FIC, HI1, HI2, I1/Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA were targeted by 5 multiplex and 3 simplex PCR.[11]

DNA sequence analysis

Chromosomal DNA from representative strains was prepared and purified by procedures described previously.[8] Sequencing was performed to identify specific CTX-M and SHV type ESBL genes. The DNA was sequenced using the dideoxynucleotide chain termination method at Sci genome, Kakkanad, Cochin, India. The ABI sequence files were assembled, and contigs were prepared using Codon Code aligner software (CodonCode Aligner 7.0.1.).

Nucleotide sequence similarity searches were performed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) server on GenBank database, release 138.0.[12]

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed by the Kirby–Bauer disk diffusion method on the MHA as per Clinical Laboratory Standards Institute guidelines to determine the resistance pattern of different bacterial isolates.[7]

Results

Among the 1,516 urine samples collected from female patients suspected to have a UTI, 454 showed significant growth (significant bacteriuria) of a single type of microorganism with a prevalence rate of 29.94%.

E. coli were found to be the most predominant uropathogen among the 454 samples with a percentage of 74.3% followed by *K. pneumoniae* (20.1%), *Pseudomonas aeruginosa* (2.4%), and *P. mirabilis* (1.98%). Among the total uropathogens, ESBL producers by the phenotypic test were 18.9% ($n=86$). Among 86 samples that were phenotypically confirmed as ESBL producers, 63 isolates showed the presence of β-lactamase genes by multiplex PCR

Table 1. Detailed Information of Primers Used in Multiplex PCR for Detection of *bla* Genes in Extended-Spectrum β-Lactamase Producers

List of primer pairs	*Target*	*Sequence (5′-3′)*	*Product size (bp)*	*References*
TEM-F	TEM	ATGAGTATTCAACATTCCG	867	Bert *et al.* (2002)[16]
TEM-R		CTGACAGTTACCAATGCTTA		
SHV-F	SHV	AGGATTGACTGCCTTTTTG	392	Colom *et al.* (2003)[17]
SHV-R		ATTTGCTGATTTCGCTCG		
CTX-M-F	CTX-M	CGCTTTGCGATGTGCAG	550	Lee *et al.* (2005)[18]
CTX-M-R	-1, -2, -9 group	ACCGCGATATCGTTGGT		
OXA-10-F	OXA-2 group	TCAACAAATCGCCAGAGAAG	478	Bert *et al.* (2002)[16]
OXA-10-R		TCCCACA CCAGAAAAACCAG		
OXA-2-F	OXA-2	AAGAAACGCTACTCGCCTGC	276	Bert *et al.* (2002)[16]
OXA-2-F		CCACTCAACCCATCCTACCC		
VEB-F	PER	CATTTCCCGATGCATGCAAAGCGT	650	Lee *et al.* (2005)[18]
VEB-R		CGAAGTTTCTTTGGACTCTG		
GES-F	GES	AGTCGGCTAGACCGGAAAG	863	Lee *et al.* (2005)[18]
GES-R		TTTGTCCGTGCTCAGGAT		
PER-F	VEB	AATTTGGGCTTAGGGCAGAA	923	Lee *et al.* (2005)[18]
PER-R		ATGAATGTCATTATAAAAGC		

(13.8%). Sanger sequencing confirmed the occurrence of ESBL genes and also revealed its different variants. Four different ESBL gene variants were detected, which included CTX-M-15 ($n=32$), OXA-2 ($n=5$), TEM ($n=1$), CTX-M-15+OXA-2 ($n=15$), TEM+CTX-M-15 ($n=2$), CTX-M-15+OXA-2+TEM ($n=6$), and TEM+OXA-2+SHV-76 ($n=2$) (Fig. 1). CTX-M-15 gene was found to be more prevalent (87.03%). The ESBL genes were distributed among different uropathogens (Table 2). It was observed that in 25 isolates, *bla*CTX-M-15 was present with other coexisting ESBL genes.

Plasmid analysis showed that the ESBL gene was encoded within the plasmid of ~18 kb in size. In the transformation assay, the ESBL gene was found to be horizontally transferable and the resistance determinant was carried within diverse incompatibility (inc) group, namely HI1, I1, FIA+FIB, FIA, and Y types. Nine plasmids had an incompatibility group of FIA+FIB and Y, respectively, followed by HI1 ($n=5$), FIA ($n=4$), and I1 ($n=3$). In the stability analysis, the mentioned Inc types harboring ESBL genes showed progressive plasmid loss after 28 passages. This implicates the specialized adaptation of this plasmid for the survival of host under cephalosporin stress in both hospitals and in the community.

Imipenem, gentamicin, and piperacillin/tazobactam showed good response against ESBL uropathogens, whereas high (>60%) resistance was shown against ampicillin (Table 3). *P. aeruginosa* and *P. mirabilis* showed 100% resistance to nitrofurantoin.

Discussion

It is well known that CTX-M enzymes are replacing SHV and TEM enzymes as the most predominant ESBL type.[13] Moreover, it has been reported that *bla*CTX-M-15 producing *E. coli* has spread worldwide[14] and this resistance determinant is a major contributor to expanded-spectrum

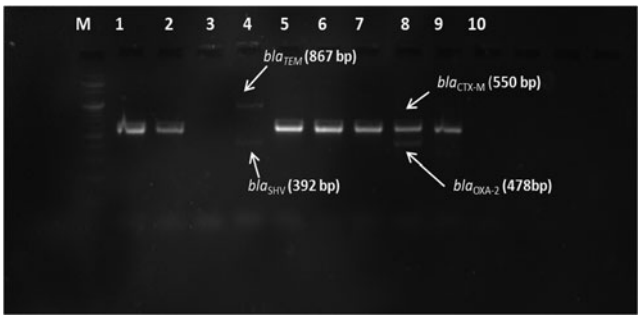

FIG. 1. Agarose gel showing PCR amplified products. Lane M: 1,000 bp DNA ladder. Lane 1: *bla*CTX-M, Lane 2: *bla*CTX-M, Lane 3: No band, Lane 4: *bla*TEM+SHV, Lane 5: *bla*CTX-M, Lane 6: *bla*CTX-M, Lane 7: *bla*CTX-M, Lane 8: *bla* CTX-M+OXA-2, Lane 9: +ve control (*bla*CTX-M), Lane 10: −ve control.

Table 2. Distribution of Extended-Spectrum β-Lactamase Gene Among Uropathogens

	Microorganism			
bla*gene*	Escherichia coli	Klebsiella pneumoniae	Proteus mirabilis	*Total frequency*
CTX-M-15	24	8	0	32
OXA-2	4	1	0	5
TEM	0	1	0	1
CTX-M-15+ OXA-2	9	6	0	15
CTX-M-15+TEM	2	0	0	2
TEM+SHV-76	1	0	0	1
CTX-M-15+ OXA-2+TEM	4	2	1	6
TEM+OXA-2+ SHV-76	1	0	0	2

Table 3. Resistance Pattern of Isolated Uropathogens Against Commonly Used Antibiotics

	Antibiotics												
Microorganisms	*AMP (%)*	*GEN (%)*	*TZP (%)*	*NET (%)*	*NOR (%)*	*CEP (%)*	*FOX (%)*	*CXM (%)*	*CIP (%)*	*CAZ (%)*	*SXT (%)*	*NIT (%)*	*IMI (%)*
E. coli	84.7	25.14	25.88	20.7	87.88	52.9	43.27	66.76	57.89	66.37	71.6	44	10.1
K. pneumoniae	88	4.67	23.91	16.30	20.65	44.56	31.52	48.91	35.86	60.86	70	67	13.4
P. mirabilis	66.67	11.12	11.12	22.23	22.23	44.45	33.34	44.45	36.37	55.56	81.5	100	10.4
Pseudomonas aeruginosa	90	9	9.09	54.5	27.2	64.6	54.54	72.7	11.12	81.81	100	100	15.8

AMP, ampicillin; GEN, gentamicin; TZP, piperacillin/tazobactam; NET, netillin; CEP, cephalothin; FOX, cefoxitin; CXM, cefuroxime; CIP, ciprofloxacin; CAZ, ceftazidime; SXT, trimethoprim/sulfamethoxazole; NIT, nitrofurantoin; IMP, imipenem.

cephalosporin resistance in clinical settings.[10] Similarly, in this study, most of the Gram-negative bacilli produced *bla*CTX-M-15 in concomitant with *bla*OXA-2. From our study, we documented that *bla*CTX-M-15 in combination with *bla*OXA-2 is the dominant ESBLs in Sikkim and Darjeeling hills of India. On the contrary, Bajpai *et al.* observed *bla*TEM as the dominant type followed by *bla*CTX-M and *bla*SHV in central India among male and female UTI patients of the study area.[15]

Moreover, we also found that some uropathogens harbored a combination of three ESBL genes. None of the isolates produced GES, VEB, or PER β-lactamase. We tried to check the medical history of patients harboring the resistance gene and we found that most of the patients were suffering from pyelonephritis and recurrent UTI with multidrug-resistant bacteria. In the stability analysis, the different incompatibility types harboring β-lactamase genes showed progressive plasmid loss after 28 days, unlike Maurya *et al.*, who showed a complete plasmid loss after 40 passages in P Inc type harboring *bla*CTX-M-15.[10] Through this kind of study that has been conducted in this region, we can understand the local distribution of these ESBL resistant genes and their movement, adaptability, and propagation under antibiotic exposure in different clinical environmental conditions. Interestingly, in this study, it was observed that β-lactamase genes were horizontally transferred through multiple incompatible types of plasmids. This proves their diverse source of origin and adaptation in both hospital and community settings. ESBL genes are known to be pandemic and are often reported to be carried within Inc FII plasmid.[15]

In our study, we found the majority of plasmids belonged to an incompatibility group of FIA+FIB ($n=9$), followed by Y ($n=9$), HI1 ($n=5$), FIA ($n=4$), and I1 ($n=3$). There are some reports of plasmids belonging to incompatibility groups of I1, A/C, L/M, and N types as well. On the contrary, Maurya *et al.*, reported their carriage through I1, F (FIA = 6; FIC = 3; FrepB = 3), W, and P Inc groups 15. The presence of this resistance gene in uropathogens in hospital and community might be due to the extensive use of CTX and ceftriaxone in this setting.

Conclusion

In this study, *E. coli* was found to be the main etiological agent of UTI with ESBL production among the patients of Sikkim and Darjeeling. The *bla*CTX-M-I5 group was the most prevalent ESBL type, followed by *bla*OXA-2 and *bla*CTX-M-I5 enzymes together. Imipenem and gentamicin were found to be the drug of choice against commonly isolated uropathogens. This study was conducted for the first time in this part of India. This study advocates further investigation of community-acquired UTIs to identify the cause and sources of such infections. Therefore, continuous surveillance for ESBL-producing uropathogens needs to be carried out specifically among women residing in higher altitude areas in different parts of this country.

Disclosure Statement

No competing financial interests exist.

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Prevalence and Antibiogram Profile of Uropathogens Isolated from Symptomatic and Asymptomatic Female Patients with Urinary Tract Infections and its Associated Risk Factors: Focus on Cephalosporin

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Abstract

Urinary tract infections (UTIs) are common among women. Here, the present study probes the prevalence of urinary tract infection (UTI), assess the adequacy of empirical therapy, susceptibility of antibiotics and resistance pattern of bacteria responsible for UTI and its associated risk factors. A total of 1309 urine samples were collected from UTI suspected female patients of tertiary care hospitals between the ages of 18-49. Moreover, a total of 207 urine samples were collected randomly from female population of same group without the symptoms of UTI by direct interview using comprehensive pre-structured English questionnaire. The standard microbiological techniques were used for isolation and identification of the samples. Antibiotic susceptibility test was performed by disk diffusion and agar dilution method. Relative risk analyses were carried out using online software ''MEDCALC''. The total prevalence of UTI among females was 29.44%, with prevalence rate of asymptomatic UTI and symptomatic UTI as 4.34% and 25.1% respectively. Additionally, we show that Escherichia coli as predominant uropathogen (74.3%) followed by Klebsiella pneumoniae (20.1%), Pseudomonas aeruginosa (2.4%) and Proteus mirabilis. Imipenem and piperacillin/tazobactam were more effective. MIC value of most of the uropathogen against cephalosporin group of antibiotics is on rise with MIC value of most of the uropathogen as 256 uglml. The main risk factor associated with asymptomatic UTI was frequency of sexual activity and the quantity of water consumption with a relative risk factor of 1.3913 and 1.0531 respectively. Increasing resistance against commonly used antibiotics is a matter of great concern for treating physician and health policy makers.

Keywords: *Asymptomatic UTI, females, risk factors*

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INTRODUCTION

Urinary tract infections (UTIs) are some of the most common bacterial infections, which affect 150 million people each year worldwide. UTI with its diverse clinical spectrum ranging from Asymptomatic bacteriuria (ABU) to acute cystitis to pyelonephritis remains one of the most frequent bacterial infections in women encountered by general practitioners [1]. Asymptomatic bacteriuria is defined by the occurrence of at least 10^5 colony-forming units of a urinary tract pathogen per milliliter in a culture of a midstream urine specimen obtained from an asymptomatic woman on a routinely programmed visit [2]. A woman is considered to have symptomatic urinary tract infection if she complains of dysuria, frequency in urination, or urgency in urinary together with at least 10^5 urinary tract pathogens per milliliter.

Regarding treatment, it is known that antibiotics are recommended for the treatment of symptomatic urinary infections. Since the last few decades, various questions have been raised against the treatment of asymptomatic bacteriuria. However, after the randomized controlled trials the use of antibiotics has been suggested in asymptomatic bacteriuria in pregnancy [3]. It has been reported that the screening for asymptomatic bacteriuria is not supported for children, and it seems that of little value in adults [4]. The prevalence of asymptomatic bacteriuria in healthy women of age group 18 to 40 years of age is found to be approximately 5 percent, and it rises with age to 20 percent or more in ambulatory elderly women [5, 6].

Since it is a known fact that gram-negative organisms are mostly responsible for hospital-acquired urinary tract infections [7] only gram-negative uropathogens were included in the study. The pathogens causing UTI are constant across the globe. The pathogenesis of UTI involves ascending infection with coliform bacteria colonizing the perineum in susceptible women (80–90% *Escherichia coli*, 5–10% Staphylococcus saprophyticus with the remainder caused by Proteus and other Gram-negative rods) [8]. UTI represents an important cause of morbidity and is not generally considered as a cause of significant mortality [8].

Typical symptoms of UTI include dysuria (painful urination), urgency (the enhanced desire to void the bladder) and frequency (increased frequency of urination) [9]. Over the last decades, uropathogens have shown a slow but steady increase in resistance to several groups of antibiotics [10]. In India, only a few studies on UTI have been reported, though UTI is the third most common infection found in this country [11]. In Eastern India, UTI has been a common infection found among all age groups ranging from infants to elderly persons. However, conclusive studies on UTI and the sensitivity pattern of antibiotics in Eastern India are still underway, and there is an extensive dispute on the selection of antibiotics due to the requirement of clear guidelines [12].

Here, we conduct a study to reveal the prevalence of symptomatic UTI, asymptomatic UTI, risk factors associated with UTI among female and antibiotic susceptibility profile of common uropathogens in Sikkim and Darjeeling areas of North Eastern India. To our knowledge, this is first detailed study analyzing UTI and antibiotic susceptibility test in this part of India. This study is important because the hilly area is quite different weather-wise, and it has been reported that UTI is more common in high altitude regions.

MATERIALS AND METHODS

The present study was conducted at the Department of Microbiology, Sikkim University, Sikkim India from May 2014 to July 2016.

Sample Collection from the Hospital: A total of 1309 clean-catch midstream urine samples were collected from hospitalized UTI suspected female patients of age group 18 to 48 manifesting symptoms of UTI. The samples were collected from the tertiary care hospitals of North Eastern Region of India.

Sample and Data Collection from the Community: The study was carried out on a randomly selected female population of same age group by direct interview using comprehensive pre-structured English questionnaire (explained in local languages) based feedback model system for data collection (Appendix A). Consent was obtained from the participants before the collection of the sample (Appendix B). Random locations including villages, markets, panchayats, and health centers were selected for sampling. Participants were well informed about the purpose and outcome of the study. To check the presence of asymptomatic bacteriuria, after proper instruction, a total of 207 urine samples were collected from the participants who did not show the symptoms of UTI.

Ethical Considerations: Ethical clearance for this work was obtained from the Institutional Ethical Committee, Sikkim University and written informed consent from community participants samples were collected.

Isolation and Identification of Uropathogen: Urine samples were cultured by semi-quantitative method onto Cysteine Lactose Electrolyte Deficient Agar (CLED), Hi chrome UTI agar and MacConkey agar plates and the

plates were incubated at 37˚C aerobically for 18–24 hrs. After incubation, the plates were examined and growth characteristics were recorded. Bacteria were identified using standard biochemical procedures and Gram staining [13]. Isolates were identified on the basis of Gram staining, colony morphology and standard biochemical tests. The isolates were further identified by Vitek 2 System (VITEK 2 compact, Biomerieux).

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed by Kirby-Bauer Disk Diffusion Method [14] on the Muller Hinton Agar (MHA) as per Clinical Laboratory Standards Institute guidelines 2011 to determine the drug resistance pattern of different bacterial isolates [15]. Briefly, 3 to 4 pure colonies of test organism were inoculated into 5 ml of nutrient broth. The test suspensions of 0.5 Mc Farland was spread with the help of sterile cotton swabs on the Muller Hinton agar plates. Aseptically the antibiotic discs were placed onto the dried MHA plate containing test inoculums. The plates were incubated for 24hrs at 37ºC. Test interpretation was done by measuring the zone diameter as per standard procedure [15].

The antibiotics ampicillin (10 µg), gentamicin (120 µg), piperacillin/tazobactam (100/10 µg), netillin (30µg), norfloxacin (10 µg), cephalothin (30 µg), cefoxitin (30 µg), cefuroxime (30 µg), ciprofloxacin (30 µg), ceftazidime (30 µg), Trimethoprim/Sulfamethoxazole (1.25 + 23.75 µg), Nitrofurantoin (300 µg), and Imipenem (10 µg) were used. MTCC *S. aureus* 7443 and MTCC *E. coli* 1089 and was used as positive and negative control organism respectively.

Determination of Minimum Inhibitory Concentration

Minimum inhibitory concentrations (MICs) were determined using the agar dilution method [13]. The MIC interpretive standards for the susceptibility categories were categorised as per the recommended breakpoints by the CLSI for cefotaxime, ceftriaxone, cefepime, ceftazidime, cefpodoxime, aztreonam and imipenem [13]. The antibiotics were obtained from a pharmaceutical company Alkem laboratories Ltd. (Kumrek Rangpo, East Sikkim, India). The different concentrations of the drug analyzed were 0.5 to 256 µg/ml [13]. MTCC E. coli 1089 was inoculated on each plate as the growth control. The growth control was read first followed by the MICs of the test strains.

Statistical Analysis

The questionnaire was checked for errors and data was entered into MS-excel and it was converted into Microsoft Excel format (.xls) and statistically analyzed using Graph pad prism V5.01.exe software (San Diego, USA). Relative Risk Calculation Relative Risk analyses were carried out using online software ‘‘MEDCALC’’ (Version 12.2.1- 1993–2012, MedCalc Software, Broekstraat 52, 9030 Mariakerke, Belgium). Relative risk = [a/ (a ? b)]/[c/(c ? d)] (Table 1).

Criteria for Significance of Relative Risks

RR = 1 means the association between exposure and disease unlikely to exist;
RR >1 means the increased risk of disease among those that have been exposed.
RR <1 means the decreased risk of disease among those that have been exposed (Table 1).

Table 1: *Relative Risk Calculation.*

Exposed group		
Number with positive outcome:	a=	
Number with negative outcome:	b=	
Control group		
Number with positive outcome:	c=	
Number with negative outcome:	d=	

RESULTS

Out of a total of 1516 urine samples, 454 showed significant growth (significant bacteriuria) of a single type of microorganism with the prevalence rate of 29.44 %. The prevalence of symptomatic and a symptomatic bacteriuria were found to be 25.1% and 4.34% respectively. As it is shown in Figure 1, *Escherichia coli* were found to be the most predominant uropathogen with a percentage of 74.3% followed by *Klebsiella pneumoniae*

(20.1%), *Pseudomonas aeruginosa* (2.4%) and *Proteus mirabilis* (1.98%). In general, imipenem, gentamicin and piperacillin/tazobactam was found to be effective against all the uropathogen while ampicillin being the least effective. Interestingly, *Pseudomanos aeruginosa* showed 100% resistance to ampicillin. A rise in the antibiotic resistance pattern of uropathogens against commonly used antibiotic like ciprofloxacin was observed (Table 2).

MIC result showed that 55.28% (n=251), 25.11% (n=114), 19.82% (n=90), 17.62% (n=80) and 16.74% (n=76) isolates had MIC value of 256 µg/ml against cefpodoxime, ceftrixone, cefotaxime, ceftazidime and cefepime respectively.

On the contrary, 22.69% (n=103) and 24.2% (n=110) of isolates were intermediate against cefepime and ceftazidime with their MIC values of 16µg/ml and 8µg/ml respectively. In case of aztreonam higher number of isolates (22%, n=75) showed MIC value of 128µg/ml. Imipenem showed good sensitivity against a maximum number of uropathogens with the MIC value as 2µg/ml for 26.43 % of isolates. Interestingly, 14.4% of *E. coli* and 15.2% of *Klebsiella pneumoniae* has a MIC value of 4µg/ml which if increases its MIC by two-fold will fall into the category of resistance (Table 3). Involvement of sexual activity and frequency of water consumption may be one of the risk factors for asymptomatic bacteriuria since they have a relative risk factor of more than 1 that is 1.3913 and 1.0531 respectively (Table 4).

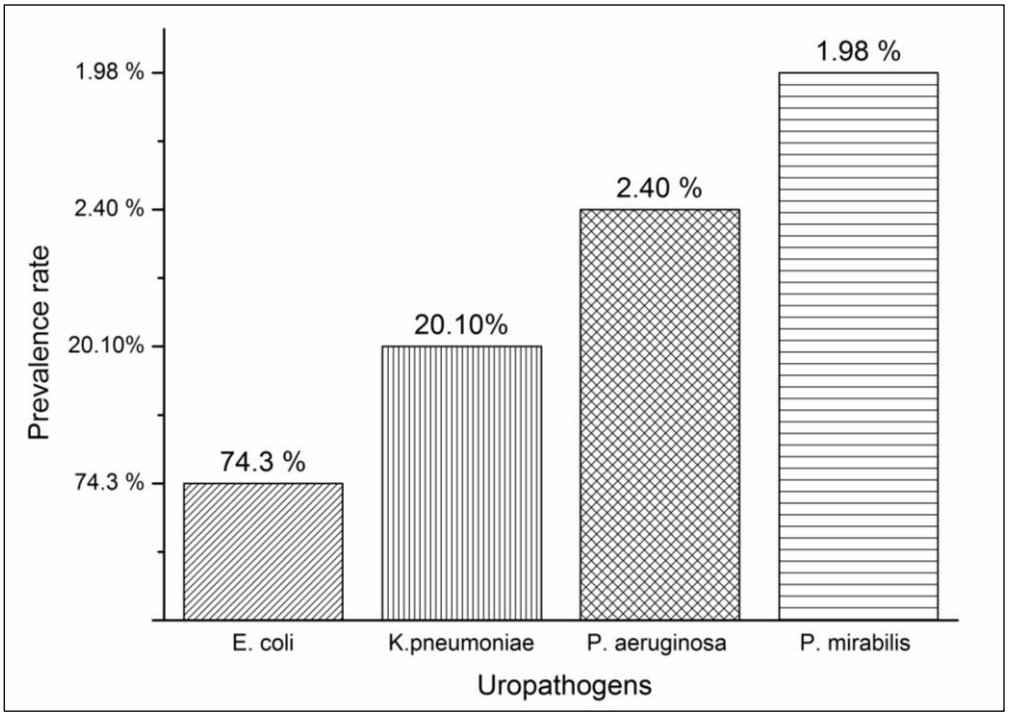

Fig. 1: *The Prevalence of uropathogens in North Eastern Region of India.*

Table 2: *Resistance Pattern of Isolated Uropathogens against Commonly Used Antibiotics.*

Microorganisms	***Antibiotics***												
	AMP (%)	**GEN (%)**	**TZP (%)**	**NET (%)**	**NOR (%)**	**CEP (%)**	**FOX (%)**	**CXM (%)**	**CIP (%)**	**CAZ (%)**	**SXT (%)**	**NIT (%)**	**IMI (%)**
E. coli	84.7	25.14	25.88	20.7	87.88	52.9	43.27	66.76	57.89	66.37	71.6	44	10.1
K. pneumoniae	88	4.67	23.91	16.30	20.65	44.56	31.52	48.91	35.86	60.86	70	67	13.4
P. mirabilis	66.67	11.12	11.12	22.23	22.23	44.45	33.34	44.45	36.37	55.56	81.5	100	10.4
P. aeruginosa	90	9	9.09	54.5	27.2	64.6	54.54	72.7	11.12	81.81	100	100	15.8

(*AMP, Ampicillin, GEN Gentamicin; TZP, Pipercillin/Tazobactum; NET, Netillin; CEP, Cephalothin; FOX, Cefoxitin; CXM, Cefuroxime; CIP, Ciprofloxacin; CAZ, Ceftazidime SXT Trimethoprim/Sulfamethoxazole; NIT, Nitrofurantoin; IMP, Imipenem)*

Table 3: MIC pattern of different uropathogens against common antibiotics

Sensitive | Intermediate | Resistant

SL no.	Microorganism	Antibiotic concentration (Cefotaxime)									
		0.5 µg/ml	1 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	256 µg/ml
1	*E.coli* (n=340)	45	16	17	48	15	23	20	48	37	71
2	*K.pneumoniae*(n=92)	16	2	4	6	10	4	3	18	12	17
3	*P.mirabilis* (n=9)	2	0	0	2	0	0	1	0	2	2
4	*P.aeruginosa* (n=9)	1	0	0	0	0	2	1	3	1	3
Ceftriaxone											
1	*E.coli* (n=340)	21	10	31	36	44	12	21	33	47	85
2	*K.pneumoniae* (n=92)	6	6	7	8	12	2	10	3	15	23
3	*P.aeruginosa* (n=11)	0	0	3	0	0	0	0	0	4	4
4	*P.mirabilis* (n=9)	2	1	1	1	2	0	0	0	0	2
Cefepime											
1	*E.coli* (n=340)	60	6	7	45	15	77	10	36	32	52
2	*K.pneumoniae* (n=92)	15	2	0	4	4	22	3	10	6	26
3	*P.aeruginosa* (n=11)	0	0	0	1	0	3	0	3	0	4
4	*P.mirabilis* (n=9)	3	0	0	0	1	1	0	2	0	2
Ceftazidime											
1	*E.coli* (n=340)	16	14	14	35	83	31	46	18	28	55
2	*K.pneumoniae*(n=92)	2	2	4	5	22	14	15	3	7	18
3	*P.aeruginosa* (n=11)	1	0	2	0	2	2	0	0	3	1
4	*P.mirabilis* (n=9)	1	0	0	0	3	2	0	0	0	5
Cefpodoxime											
1	*E.coli* (n=340)	8	15	0	1	0	13	8	78	61	151
2	*K.pneumoniae* (n=92)	9	3	0	0	0	1	0	20	18	40
3	*P.aeruginosa* (n=11)	3	0	0	0	0	0	0	3	0	5
4	*P.mirabilis* (n=9)	*0*	1	0	0	0	0	0	0	3	5
Aztreonam											
1	*E.coli* (n=340)	18	25	22	55	53	23	16	31	65	32
2	*K.pneumoniae* (n=92)	13	5	2	12	12	12	7	5	7	17
3	*P.aeruginosa* (n=11)	0	2	0	0	1	0	0	3	0	5
4	*P.mirabilis* (n=9)	0	1	0	0	0	0	0	2	3	5
Imipenem											
1	*E.coli* (n=340)	41	51	76	48	16	27	12	20	22	27
2	*K.pneumoniae* (n=92)	20	7	20	14	5	7	3	2	7	6
3	*P.aeruginosa* (n=11)	3	0	5	1	0	1	0	1	0	3
4	*P.mirabilis* (n=9)	2	2	1	2	0	0	0	2	0	0

Table 4: *Risk Factors Associated with Asymptomatic UTI.*

Risk factor of asymptomatic UTI	Status	Total	Total no. of respondents	Statistics			
				RR	95% CI	Z-test	P-value
Sexual activity	Sexual activity (Yes)	144	207	1.3913	1.2194 to 1.5875	4.907	P < 0.0001
	Sexual activity (No)	63	207				
Frequency of water consumption	Less than 1litre	109	207	1.0531	0.8964 to 1.2373	0.630	0.5288
	More than 1 litre	98	207				

DISCUSSION

In the present study, the prevalence of symptomatic bacteriuria was found to be low as compared to the findings of Pratap R *et al.* 2013 (prevalence rate =32%) [16] and slightly high as compared to the study conducted in North Bengal (prevalence rate =21.8%) [17]. The rates of asymptomatic bacteriuria found in the present study are similar to those described in sexually active women that are 5%, non-pregnant [18, 19] and pregnant [18, 20] women, but higher than those healthy schoolgirls [21]. On the contrary, Ankur et al., 2015 found the prevalence of asymptomatic UTI slightly high (8.8%) as compared to the present study [22].

As reported by Saha *et al.*, 2014, the most prevalent UTI pathogen was found to be *E. coli* which is followed by *Klebsiella pneumonia*e [23]. The finding is also in concistent with the study conducted in Pakistan [24] and in contrast with findings reported in Mangalore India where only 44% of *E. coli* was isolated from UTI patients [25]. Similar to the findings of a study conducted in Bihar, India, only 2.4% *Pseudomonas aeruginosa* and 1.98% P*roteus mirabilis* were isolated [16]. The high prevalence of resistance to commonly used antibiotics such as ampicillin, ciprofloxacin, and cefuroxime has caused considerable alarm [25]. In case of ampicillin 100% resistance is shown by *Pseudomonas aeruginosa* followed by *Klebsiella pneumoniae* (88%), *E. coli* (85.9%) and *Proteus mirabilis* (66.66%) which is consistent with the findings of Mandal *et al.* 2012 where 80% of the *E.co*li were resistant to ampicillin [26]. The present study is similar to the findings of Saha S. et al 2014 who reported that *Klebsiella spp* isolated from UTI samples of Eastern India were maximally resistant to penicillin group of antibiotics, followed by aminoglycosides and third generation cephalosporin [23], being second highest resistance against different classes of antibiotics followed by *E. coli* in this study. Ciprofloxacin which still remains the first drug of choice among the fluoroquinolones group in community-acquired UTI was found to be less effective against uropathogens. It was found that 58.2% of *Escherichia coli* showed resistance to ciprofloxacin followed by *Klebsiella pneumoni*ae (35.82%), *Proteus mirabilis* (11.11%) and *Pseudomonas aeruginosa* (9%).

High MICs for third-generation cephalosporins make it inappropriate for empirical therapy. Therefore, MIC test of uropathogens was performed against cephalosporin group of antibiotics, aztreonam, and imipenem for 10 different concentrations which ranged from 0.5 to 256ug µg/ ml. Maximum number of *E. coli* had a MIC value of 256ug/ml in case of cefotaxime, ceftriaxone, and cefpodoxime. In case of cefepime and ceftazidime, highest number of isolates falls into the category of an intermediate which is expected to increase its MIC in due course of time. Similar to our present study, a study conducted in China by Jean et al., 2015 maximum number of *E. coli* had a MIC value of more than 128ug/ml in case of cefotaxime. On the contrary to our study maximum isolates had a MIC value of 16ug/ml against ceftazidime and cefepime.

In the present study, the maximum number of uropathogens had a MIC value more than 256ug/ml on the contrary Jean et al 2015 found the maximum number of uropathogens had a MIC value of 32ug/ml [27]. Nineteen percent isolate of *E.coli* showed MIC value of 128ug/ml against aztreonam. Imipenem which is known as the last line of the drugs showed a good sensitivity against a maximum number of isolates. However, 14.4% of *E. coli* and 15.2% of *Klebsiella pneumoniae* has a MIC value of 4ug/ml which if increases its MIC by two-fold will fall into the category of resistance (Table 3).

There are various known risk factors associated with urinary tract infection. Various known and unknown risk factors were observed during the study. One of the unknown risk factors which may aid to urinary tract infection was frequency of water consumption. It was observed that the respondents having a frequency of diet less than one litre a day is susceptible to urinary tract infection. Amongst all risk factors sexual activity represents one of the risk factor for symptomatic urinary tract infection.

It has been reported that sexual intercourse increases the risk of symptomatic urinary tract infections in young women [28]. However, the present study reveals that sexual activity is also one of the risk factor for asymptomatic UTI, as all the participants with asymptomatic

bacteriuria was sexually active with a relative risk factor of 1.3913. The present study has been conducted first time in the study area. Till now no study has been conducted in the asymptomaic UTI. The present study is preliminary in nature and it provides the basic data about the prevalence of asymptomatic bacteriuria risk factors associated with it in North Eastern Region of India. The present study shows the rise in antibiotic resistance pattern and increases in the MIC value of third generation cephalosporin which is a matter of concern.

As right from sexual activity, amount of drinking water and lack of information can be attributed to the problem, it requires a comprehensive strategy to deal with it.

It is expected that the medical services of the state will be highly benefited by the findings of the study. The antibiotic resistance pattern will help the medicinal practitioner to provide suitable medical treatment to the people. Moreover, the study can be valuable for them in designing the effective remedial measures to decrease the risk factors of urinary tract infection and enlighten the common and will help them to bring necessary changes in it to improve the quality of life of people.

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APPENDIX A

Questionnaire based feedback form for the characterization of common bacterial pathogens from patients of urinary tract infection from Sikkim and Darjeeling

Demographics

- Name-
- Gender-
- Age (Yrs)-(18-49) _____
- Population type- Rural/Urban
- Name of the place _____
- Sikkim/ Darjeeling

Risk Factors

- Water source - Government provided /Natural spring/river /Not known
- Water consumed- Raw /Boiled /Filtered /Purified
- Amount of water intake
- Frequency of water consumption in a day- 1 lite/ 2 litres / more than 2 litres
- Sanitary condition-Western style/Indian style
- Process of cleaning their private part after excretion of feces- Water/ toilet paper/stone/ stick
- Do you use cleansing agent to clean their private part after excretion of feces- Yes/No
- Distance of toilet from their work place or home- nearby/ far/very far
- Do u wash your hands after toilet- Yes/No
- Sexual activity- Yes/ No
- Are you consuming antibiotics? Yes/ No

Symptoms of Acute Urinary Tract Infection

- Fever (high grade)
- Dysuria (burning pain on passing urine)
- Frequency in urination
- Urgency in urination
- Malaise
- Shivering
- Pain in abdomen

Symptoms of Chronic UTI

- Similar symptoms as of acute UTI with a low-grade fever
- Tenderness in pubic region
- History of any Infection/ diseases
- Heart diseases
- Diabetes
- High blood pressure
- Cancer
- Spinal cord injury
- Any specific observation.

APPENDIX B

Consent Form
(To be filled by the participants/parents and Researcher)
I along with my son/daughter do hereby agree to be a participant in the research work as titled:
"Characterization of common bacterial pathogens from patients of Urinary Tract Infection from Sikkim and Darjeeling"
In this study the participant's personal information and the urine sample will be provided with. I have been explained and have understood that this type of study has been conducted in several parts of the world and has no adverse effects on the health of the respondent.

Varsha Rani Gajamer will be contacted if additional information is required in this context. (Mobile no. 9647775465)

Name and signature of the participant

Researchers name,
Varsha Rani Gajamer
Signature
Date:

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Escherichia coli encoding bla_{NDM-5} associated with community-acquired urinary tract infections with unusual MIC creep-like phenomenon against imipenem

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ABSTRACT

Objectives: Carbapenemase-producing *Escherichia coli* are a major clinical concern. The current study aimed to identify NDM-5-producing *E. coli* associated with community-acquired urinary tract infections (UTIs) co-harbouring extended-spectrum β-lactamases (ESBLs) and showing a phenomenon of imipenem minimum inhibitory concentration (MIC) creep.

Methods: A total of 973 urine samples were collected from females aged between 18–49 years diagnosed with UTI in Northeast India (June 2014–July 2016). Isolates were identified by standard microbiological methods. The presence of bla_{NDM} and ESBL genes was determined by PCR and sequencing. PCR-based replicon typing was performed. Plasmid stability of all β-lactamase-producers and their transformants was analysed by serial passage, and the MIC creep phenomenon was analysed by studying revertants.

Results: Among 34 bla_{NDM-5}-positive *E. coli* isolates, 21 (61.8%) co harboured $bla_{CTX-M-15}$, followed by multiple combinations of genes. This study revealed diverse plasmid types (HI1, I1, FIA + FIB, FIA and Y). The strains showed progressive plasmid loss after 31 passages. Most if the isolates had MICs of 0.5 μg/mL and 1 μg/mL to imipenem, ertapenem and meropenem. However, on studying the MIC creep phenomenon, the MIC was found to be elevated from 0.5 μg/mL to 64 μg/mL and from 1 μg/mL to 128 μg/mL. Analysis of revertants shows that the MIC of most NDM-positive isolates was reduced to 16 μg/mL after the 30th serial passage.

Conclusion: This study observed a unique phenotype of NDM-producers that has not been reported previously. The observed phenomenon poses a global threat as these pathogens may evade phenotypic screening by routine laboratories.

1. Introduction

Carbapenemase-producing Enterobacteriaceae (CPE) causing bacteraemia are of great clinical concern. Carbapenemases are a versatile group of β-lactamases that are characterised by their ability to hydrolyse virtually all β-lactam antibiotics, including cephalosporins and carbapenems, consequently complicating therapy and limiting treatment options [1]. CPE infections are also associated with high mortality rates [2]. Asia is a known epicentre of antimicrobial drug resistance due to extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae, with CTX-M being recognised as the most common β-lactamase [3,4]. In the past 10 years, there has been a marked increase in isolation rates of multidrug-resistant Enterobacteriaceae producing ESBLs, AmpC β-lactamases or carbapenemases in community settings [5–7]. The prevalence of ESBL- and carbapenemase-producing Enterobacteriaceae varies remarkably across the Asia-Pacific region [8].

In the last decade, New Delhi metallo-β-lactamase (NDM)- and *Klebsiella pneumoniae* carbapenemase (KPC)-producing Enterobacteriaceae have become endemic on the Indian subcontinent as well as in China [5,7,8]. There are also reports of the occurrence of bla_{NDM-1} and bla_{NDM-5} in a tertiary referral hospital in North India [9]. In the current study, the prevalence of the bla_{NDM} gene among uropathogens isolated from females in Northeast India was

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determined. No such studies have been previously undertaken to ascertain the magnitude of carbapenemase-producers in healthcare or community settings in this region. This study was performed to determine the prevalence of carbapenemases, carbapenemase-encoding genes and co-existing ESBL genes among uropathogens. Since carbapenems are not commonly used for the treatment of community-acquired infections in this region, expression of resistance genes was detected by determining minimum inhibitory concentrations (MICs) under carbapenem pressure.

2. Materials and methods

2.1. Collection of bacterial isolates

From June 2014 to July 2016, a total of 973 urine samples were collected from females aged 18–49 years diagnosed with community-acquired UTI in Northeast India. Standard microbiological techniques were used for collection, transportation and processing of the samples [10]. Uropathogens were isolated on cystine–lactose–electrolyte-deficient (CLED) agar, HiChrome UTI agar and MacConkey agar (HiMedia, Mumbai, India) plates by a semiquantitative method [11]. Specimens yielding $\geq 10^5$ CFU/mL were interpreted as significant bacteriuria. All of the isolates were identified on the basis of Gram staining, colony morphology and standard biochemical tests [11], and representative strains were identified using a VITEK®2 Compact instrument (bioMérieux, Marcy-l'Étoile, France).

2.2. Molecular detection of the bla$_{NDM}$ gene

The presence of *bla*$_{NDM}$ was determined by PCR using the primers NDM-F (5′-GGGCAGTCGCTTCCAACGGT-3′) and NDM-R (5′-GTAGTGCTCAGTGTCGGCAT-3′) [9]. PCR was performed under the following conditions: initial denaturation at 94 °C for 10 min; 30 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 40 s and extension at 72 °C for 1 min; and a final elongation step at 72 °C for 7 min. Amplified products were purified using a MinElute® PCR Purification Kit (QIAGEN, Hilden, Germany) and were sequenced to confirm the *bla*$_{NDM}$ variant.

2.3. Plasmid preparation and transformation

NDM-positive bacterial isolates were grown overnight in Luria–Bertani (LB) broth (HiMedia) containing 0.25 μg/mL imipenem. Plasmid DNA was extracted from the overnight cultures using a QIAprep Spin Miniprep Kit (QIAGEN) and the extracted plasmids were then subjected to transformation by the heat-shock method using *Escherichia coli* JM107 as the recipient strain. Transformants were selected on LB agar supplemented with 2 μg/mL imipenem and were further confirmed both by phenotypic and PCR analysis.

2.4. PCR-based replicon typing (PBRT)

PBRT was carried out targeting 18 different replicon types, including FIA, FIB, FIC, HI1, HI2, I1/Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA, targeted in five multiplex and three simplex PCRs [12].

2.5. Plasmid stability testing

Plasmid stability of all β-lactamase-producers as well as their transformants was analysed by serial passage for 110 consecutive days at 1:1000 dilutions in LB broth without antimicrobial pressure [13]. After each passage, PCR was carried out to detect the presence of *bla* genes in the isolates.

2.6. Detection of co-existence of extended-spectrum β-lactamase genes

PCR was performed to determine the presence of ESBL genes. The genes were detected by multiplex PCR targeting *bla*$_{TEM}$, *bla*$_{PER}$, *bla*$_{OXA-2}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{VEB}$ and *bla*$_{GES}$. PCR was performed under the following conditions: initial denaturation at 94 °C for 5 min; 33 cycles at 94 °C for 35 s, 51 °C for 1 min and 72 °C for 1 min; and final extension at 72 °C for 7 min. Amplified products were further sequenced to confirm the co-existence of ESBL genes [14].

2.7. Sanger sequencing

Amplified products were selected for sequencing to identify the *bla*$_{NDM}$ variant. DNA was sequenced at SciGenom Labs (Cochin, India) using the dideoxynucleotide chain termination method. The ABI sequence files were assembled and contigs were prepared using Codon Code Aligner software v.7.0.1.

Nucleotide sequence similarity searches were performed using the National Center for Biotechnology Information (NCBI) BLAST (Basic Local Alignment Search Tool) server on GenBank database release 138.0 12.

2.8. Antimicrobial susceptibility testing

The antimicrobial susceptibility pattern of the isolates was determined by the Kirby–Bauer disk diffusion method against ampicillin (10 μg), gentamicin (120 μg), piperacillin/tazobactam (TZP) (100/10 μg), netilmicin (30 μg), norfloxacin (10 μg), cefalotin (30 μg), cefoxitin (30 μg), cefuroxime (30 μg), ciprofloxacin (30 μg), ceftazidime (30 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), nitrofurantoin (300 μg) and imipenem (10 μg) on Muller–Hinton agar (HiMedia) plates. *E. coli* ATCC 25922 was used as a control and the results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [15].

2.9. Minimum inhibitory concentration determination of NDM-positive isolates

MICs of isolates harbouring the *bla*$_{NDM}$ gene were determined by the agar dilution method against imipenem (Lupin India, Mumbai, India), ertapenem (INVANZ®; MSD, India) and meropenem (Meronem®; AstraZeneca, Bangalore, India). MIC interpretive standards for the susceptibility categories were categorised according to the breakpoints recommended by the CLSI [15]. The concentrations of drugs analysed were 0.5–256 μg/mL. *E. coli* ATCC 25922 was inoculated on each plate as a growth control.

2.10. Observation of MIC creep

NDM-positive isolates having an MIC of ≤4 μg/mL were subjected to serial passage in LB broth with a gradually increasing concentration of imipenem from 0.25 μg/mL to 8 μg/mL for six consecutive days.

2.11. Analysis of revertants

The isolates tested for MIC creep with an elevated MIC against imipenem were subjected to serial passage in LB broth at 1:1000 dilutions in the absence of imipenem for 30 consecutive days [16]. The MIC of each strain was subsequently determined on the 10th, 20th and 30th day of passage.

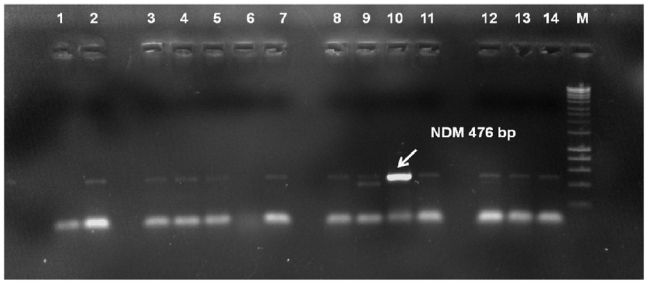

Fig. 1. Gel picture of amplified products of the bla_{NDM} gene. Lane 1, negative control; lane 2, positive control; lanes 3–5, bla_{NDM} gene; lane 6, no band; lanes 7–14, bla_{NDM} gene; lane M, 1-kb ladder.

2.12. Ethical considerations

Ethical approval for this work was obtained from the Institutional Ethical Committee of Sikkim University (Gangtok, Sikkim, India), and written consent was obtained from community participants.

3. Results and discussion

A total of 241 uropathogens were isolated among 973 urine samples collected from females, giving a prevalence rate of UTI of 24.8%. *E. coli* was the predominant uropathogen (75.5%), followed by *K. pneumoniae* (19.9%), *Pseudomonas aeruginosa* (2.5%) and *Proteus mirabilis* (2.1%). Among the total of 182 *E. coli* isolates, 34 isolates harboured the bla_{NDM} gene (Fig. 1). Sequencing results revealed that the isolates harboured the bla_{NDM-5} variant, giving a prevalence rate of bla_{NDM-5} among *E. coli* isolates in female patients with community-acquired UTI of 18.7% (34/182). The antibiotic susceptibility profile of the uropathogens showed that imipenem, gentamicin and TZP were found to be more effective against the uropathogens (Table 1).

In contrast, the prevalence rate of the carbapenemase gene was found to be 28.6% in a study conducted on clinical isolates from Mulago National Referral Hospital in Kampala, Uganda [17]. However, the prevalence rate in the current study is much higher than that obtained in studies from Morocco, China and Germany [18–20] as well as in a surveillance study in Spain that reported a carbapenemase-encoding gene prevalence of only 0.04% [21].

Plasmid analysis showed that bla_{NDM-5} was located on a plasmid of ca. 65 kb in size. All transformants carrying the bla_{NDM-5} gene were selected on screening agar and the resistance determinant was found to be carried on plasmids of diverse incompatibility (Inc) groups, namely HI1, I1, FIA + FIB, FIA and Y types. In the stability analysis, the studied strains showed progressive plasmid loss after 31 passages.

Owing to acquisition of genes by ESBL-producers, resistance to the most commonly used antibiotics has greatly increased among Gram-negative bacteria, especially in the Enterobacteriaceae family [22]. Carbapenems have been the treatment of choice for infections caused by ESBL-producing bacteria [23]. However, increased production of β-lactamases hydrolysing all β-lactam antibiotics including carbapenems has been reported worldwide [4]. Therefore, co-carriage of ESBL genes among NDM-5-producing *E. coli* was analysed. It was found that 61.8% of bla_{NDM-5} positive *E.coli* isolates co-harboured $bla_{CTX-M-15}$ ($n = 21$), followed by 17.6% co-harbouring $bla_{CTX-M-15} + bla_{OXA-2}$ ($n = 6$), 11.8% co-harbouring $bla_{CTX-M-15} + bla_{TEM} + bla_{OXA-2}$ ($n = 4$), and 2.9% co-harbouring bla_{OXA-2} ($n = 1$), $bla_{CTX-M-15} + bla_{TEM} + bla_{SHV-76}$ ($n = 1$) and $bla_{CTX-M-15} + bla_{TEM}$ ($n = 1$). All NDM-5-harbouring *E. coli* isolates were subjected to MIC determination against carbapenems (ertapenem, meropenem and imipenem). Most of the isolates had MICs of 0.5 μg/mL or 1 μg/mL (Table 2).

On studying the MIC creep phenomenon, NDM-positive isolates with MICs of ≤2 μg/mL and ≤4 μg/mL when exposed to gradually increasing imipenem stress for six consecutive days in LB broth, the MIC was found to be elevated to 64 μg/mL and 128 μg/mL, respectively (Table 2; Fig. 2). Analysis of the revertants showed that after 10 consecutive serial passages without imipenem stress the MIC of most of the NDM-positive isolates had reduced to 64 μg/mL and 32 μg/mL, whilst the MIC remained at 128 μg/mL for the rest (Table 2). The MIC was further found to be reduced to 16 μg/mL for most of the isolates after the 20th and 30th serial passages, respectively (Table 2).

The rising trend in MICs of antibiotics against clinical isolates is worrisome. This study identified the presence of NDM-5-mediated carbapenem resistance in community-acquired UTI cases, although with routine antibiogram profiling most of them resulted susceptible. Carbapenems are not commonly used for the treatment of community-acquired infections in this region, therefore we anticipate that this gene might have been acquired along with other resistance determinants through a common plasmid and maintained within the host. Because of the absence of carbapenem pressure, the resistance gene was not expressed and showed an MIC below the breakpoint. Most of the bla_{NDM-5}-harbouring *E. coli* had MICs of 1 μg/mL and 0.5 μg/mL against carbapenems, masquerading as susceptible. Isolates with MICs of ≤2 μg/mL and ≤4 μg/mL when exposed to gradually increasing imipenem, meropenem and ertapenem stress for six consecutive days in LB broth developed elevated MICs to 64 μg/mL and 128 μg/mL, respectively, showing MIC creep activity. This is the first report showing the MIC creep phenomenon in NDM-producers. Interestingly, to date there is no report suggesting carbapenem MIC creep in NDM-producers.

This observed phenomenon poses a global threat as these pathogens may evade phenotypic screening in routine laboratories. On increasing the exposure to imipenem and ertapenem, the MIC of the NDM-producers increased from 0.5 μg/mL and 1 μg/mL to 64 μg/mL and 128 μg/mL, respectively, suggesting that continuous exposure or usage of carbapenems, which is also known as a drug of last resort, may be associated with an increased probability of treatment failure, reducing therapeutic choices Therefore,

Table 1
Antimicrobial susceptibility pattern of uropathogens ($n = 454$) against commonly used antimicrobial agents.

Isolate	% susceptible												
	AMP	GEN	TZP	NET	NOR	CEP	FOX	CXM	CIP	CAZ	SXT	NIT	IPM
Escherichia coli	15.3	74.86	74.12	79.3	87.88	47.1	56.73	33.24	42.11	33.33	28.4	56	89.9
Klebsiella pneumoniae	12	95.33	76.09	83.7	20.65	55.44	68.48	51.09	64.14	39.14	30	33	86.6
Proteus mirabilis	33.33	88.88	88.88	77.77	77.77	55.55	66.66	55.55	63.63	44.44	18.5	0	89.6
Pseudomonas aeruginosa	10	91	90.91	45.5	72.8	35.4	45.46	27.3	88.88	18.19	0	0	84.2

AMP, ampicillin; GEN, gentamicin; TZP, piperacillin/tazobactam; NET, netilmicin; NOR, norfloxacin; CEP, cefalotin; FOX, cefoxitin; CXM, cefuroxime; CIP, ciprofloxacin; CAZ, ceftazidime; SXT, trimethoprim/sulfamethoxazole; NIT, nitrofurantoin; IPM, imipenem.

Table 2
Escherichia coli isolates harbouring the bla_{NDM-5} gene and showing the minimum inhibitory concentration (MIC) creep phenomenon against imipenem.

Sl no.	Sample code	Initial MIC (μg/mL)			IPM MIC creep after exposure (μg/mL)	IPM MIC of revertants (μg/mL)		
		ETP	IPM	MEM		After 10th passage	After 20th passage	After 30th passage
1	SK:053	1	1	0.5	128	64	16	16
2	D:064	0.5	16	1	128	64	16	16
3	SK:001	0.5	1	1	128	64	16	16
4	D:067	0.5	0.5	1	64	64	16	16
5	D:065	1	4	0.5	128	64	16	16
6	SK:057	2	4	0.5	128	64	16	16
7	D:105	64	128	0.5	128	128	128	128
8	SK:125	1	4	1	128	64	16	16
9	SK:017	0.5	4	1	128	64	16	16
10	D:024	1	16	1	128	32	16	16
11	SK:016	1	1	0.5	64	32	16	16
12	SK:130	32	64	1	128	128	16	16
13	SK:014	0.5	128	1	128	128	128	128
14	SK:043	16	64	0.5	128	128	32	16
15	SK:031	8	64	1	128	64	32	16
16	SK:135	1	0.5	1	64	32	16	16
17	D:074	1	1	0.5	128	64	16	16
18	D:017	1	16	0.5	128	64	16	16
19	SK:056	0.5	1	1	128	128	64	16
20	D:089	0.5	2	1	64	64	16	16
21	D:014	1	0.5	1	64	64	32	32
22	SK:049	0.5	2	0.5	64	64	16	16
23	SK:131	0.5	4	1	128	128	64	16
24	D:057	1	0.5	0.5	64	64	16	16
25	D:024	1	0.5	1	64	64	16	16
26	D:091	2	1	32	64	64	16	16
27	SK:047	0.5	1	4	128	64	16	16
28	SK:021	1	1	64	128	64	32	16
29	D:011	1	0.5	16	128	64	16	16
30	SK:012	1	4	0.5	128	64	32	16
31	SK:003	16	0.5	64	64	32	16	16
32	SK:041	8	0.5	64	64	64	16	16
33	D:068	1	0.5	1	64	64	16	16
34	D:101	1	0.5	1	64	32	16	16

ETP, ertapenem; IPM, imipenem MEM, meropenem.

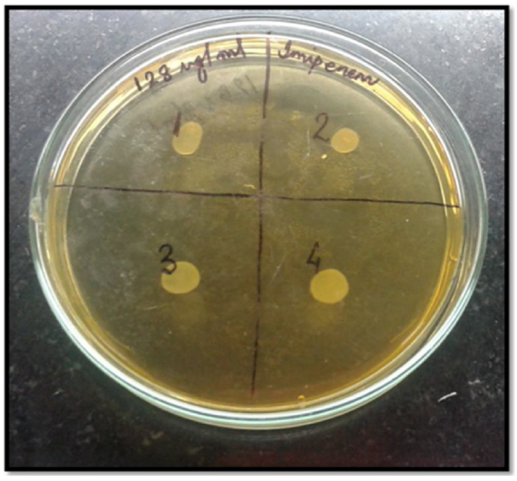

Fig. 2. *Escherichia coli* growth showing the minimum inhibitory concentration (MIC) creep phenomenon. The imipenem MIC increased from 1 μg/mL to 256 μg/mL on Muller–Hinton agar (imipenem at 128 μg/mL).

carbapenem antibiotics should be used cautiously with proper molecular level analysis of carbapenemases. The bla_{NDM} gene among the other carbapenemases has gained particular attention owing to its global dissemination and multidrug resistance phenotype. In summary, this study has demonstrated a high prevalence of the bla_{NDM} gene in *E. coli* isolated from female UTI patients in Northeast India. The main finding of this study was established using the 2017 MIC breakpoints; we found evidence for imipenem MIC creep in NDM-producers and evidence of a decreased rate of susceptibility to carbapenem antibiotics among *E. coli*. Even a slight but steady increase in MICs is important long-term and thus trends in MICs must continue to be monitored. This is especially important since ESBL-producers appear to be increasingly significant in India, both in hospital and community-associated infections. Monitoring the susceptibility profiles of Enterobacteriaceae against important antibiotics is still warranted.

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Competing interests

None declared.

Ethical approval

Ethical approval for this work was obtained from the Institutional Ethical Committee of Sikkim University. Written consent was obtained from community participants.

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Poster/ Oral presentations at National and International conferences/ seminars

1. Presented paper entitled ***"Molecular detection of extended spectrum ß-lactamase genes from uropathogens of Sikkim and Darjeeling"*** as an oral presentation in International conference on Contemporary and Antimicrobial Research 2016" at Assam University on 17th November, 2016.

2. Presented paper entitled ***"Occurrence of Carbapenemase and Extended Spectrum ß-lactamase genes in isolates associated with Urinary Tract Infection in females of Sikkim and Darjeeling "*** as an oral presentation in an Annual Conference of association of Microbiologists of India (AMI 2017) Lucknow on 18th November, 2017.

ICCAR 2016
Towards Intelligent Antimicrobials

International Conference on
Contemporary Antimicrobial Research 2016
Assam University, Silchar, India
November 14-17, 2016

Certificate

We have great pleasure in awarding this certificate to

Varsha Rani Gajamer

for attending ICCAR 2016 as a delegate and presenting an oral paper/poster entitled

Molecular detection of extended Sepectrum β-lactamase genes from Uropathogens of Sikkim and Darjeeling

Prof. M. Dutta Choudhury
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CERTIFICATE

This is to certify that Mr./Ms./Mrs./Dr./Prof. Varsha Rani Gajamer

participated/presented a Poster/Oral/Invited/Lead Lecture titled Occurrence of Carbapenemase & Extended Spectrum β lactamase (ESBL) genes in isolates associated with Urinary Tract infection in females of Sikkim & Darjeeling in this International Conference.

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