CHAPTER 1

INTRODUCTION

1.1. Organ Transplantation

Transplantation of organ is a promptly advancing field and provides a potential treatment for patients with end-stage organ disease or failure. Considering the fact, that need for organs persist to exceed the supply, however just distribution of available organs is a highly important issue within the transplant community. End-stage organ failure occurs when the functional capacity of an organ system completely diminishes and finally it requires the affected individual to start a treatment regimen. The most frequently affected organs are kidney, liver, heart and lungs; among the various organs transplanted, kidney is of prior importance owing to its transplant recipient, donor blood type match, antibody barriers and its success rate. The first successful kidney transplantation in India was done in 1971 by the group of doctors led by Dr. Mohan Rao and Dr. K.V Johny at Christian Medical College, Vellore (Singh and Kumar 2016). Since then, transplantation of kidney has come a long way with huge development in the technology being used. Thus the objective of the present study is to address the basic challenges such as post surgical infection and its associated antimicrobial treatment that are encountered during transplantation, thus by making unique prophylaxis treatment recommendations for a successful kidney transplantation program.

Transplantation of Human Organs Act (1994) in India has been authorized the donation and transplantation of organs in the country. In accordance with the THO Act, organ donation are done only from the live related and deceased or cadaveric donors. The chief source of donation of organs is from Deceased Donors (DD) who has died recently in the critical care unit, though their essential organs other than brain are being preserved by medical support. In the case of cadaveric donor, the factors such as cause (disease), location and time of death determine the organ donation (Segev et al. 2005).
Table 1.1 Total number of transplantation done in Tamil Nadu in sequential year

Source: Data from Indian Transplant Registry (2016)

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Year</th>
<th>No. of transplantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1984</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>1985</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>1986</td>
<td>141</td>
</tr>
<tr>
<td>4</td>
<td>1987</td>
<td>224</td>
</tr>
<tr>
<td>5</td>
<td>1988</td>
<td>270</td>
</tr>
<tr>
<td>6</td>
<td>1989</td>
<td>334</td>
</tr>
<tr>
<td>7</td>
<td>1990</td>
<td>330</td>
</tr>
<tr>
<td>8</td>
<td>1991</td>
<td>366</td>
</tr>
<tr>
<td>9</td>
<td>1992</td>
<td>385</td>
</tr>
<tr>
<td>10</td>
<td>1993</td>
<td>358</td>
</tr>
<tr>
<td>11</td>
<td>1994</td>
<td>458</td>
</tr>
<tr>
<td>12</td>
<td>1995</td>
<td>283</td>
</tr>
<tr>
<td>13</td>
<td>1996</td>
<td>291</td>
</tr>
<tr>
<td>14</td>
<td>1997</td>
<td>342</td>
</tr>
<tr>
<td>15</td>
<td>1998</td>
<td>303</td>
</tr>
<tr>
<td>16</td>
<td>1999</td>
<td>291</td>
</tr>
<tr>
<td>17</td>
<td>2000</td>
<td>355</td>
</tr>
<tr>
<td>18</td>
<td>2001</td>
<td>356</td>
</tr>
<tr>
<td>19</td>
<td>2002</td>
<td>326</td>
</tr>
<tr>
<td>20</td>
<td>2003</td>
<td>366</td>
</tr>
<tr>
<td>21</td>
<td>2004</td>
<td>357</td>
</tr>
<tr>
<td>22</td>
<td>2005</td>
<td>253</td>
</tr>
<tr>
<td>23</td>
<td>2006</td>
<td>141</td>
</tr>
<tr>
<td>24</td>
<td>2007</td>
<td>61</td>
</tr>
<tr>
<td>25</td>
<td>2008</td>
<td>29</td>
</tr>
<tr>
<td>26</td>
<td>2010</td>
<td>24</td>
</tr>
<tr>
<td>27</td>
<td>2011</td>
<td>33</td>
</tr>
<tr>
<td>28</td>
<td>2012</td>
<td>35</td>
</tr>
<tr>
<td>29</td>
<td>2015</td>
<td>44</td>
</tr>
</tbody>
</table>
Globally, kidney transplantation is associated with high morbidity and mortality rate with approximately 735,000 deaths annually and in India its ranging from 4% to 17.2%. Thus, transplantation is the 12<sup>th</sup> most common cause of death and the 17<sup>th</sup> most common cause of disability around worldwide (Singh and Anish 2016). Based on the available data from the Indian Network for Organ Sharing (INOS 2016), over 21,513 patients in India have been underwent kidney and liver transplants since 1970, and this count is expected to increase substantially due to advancements in technological developments recently. Organ donation rate in India is 1.2% per million populations in 2016 which is very lesser number when compared to other developed countries such as United States 46, China’s 38.2, and Germany’s 34.7 per million respectively (2016).

In India, there are around 500,000 patients die annually because of availability of fewer donors; 200,000 patients die because of liver disease and 50,000 from cardiac disease. Nearly 150,000 patients await kidney transplantation, but only 5,000 patients receives kidney each year. Although, the number of transplantation done has been substantially increasing, but only around 5,000 kidneys, 1000 livers and around 15 heart transplantation are done annually. With a one per million-donation rate, India would have 1,100 organ donors or 2,200 kidneys, 1,000 hearts, 1,100 livers, 1,100 pancreas and 2,200 eyes. This should take care of almost all current demands for organs. At a 2 per million-donation rate there would be 2,200 organ donors and the above figures would double. Then there would be no necessity to undertake living kidney donations (Barret et al. 2003). There is approximately 200,000 kidneys, 50,000 hearts and 50,000 livers are needed for transplantation each year (2016). Recent data suggests that globally around 2.6 million people were on dialysis in 2010, with 93% in developed countries. However, the number of people undergo transplantation was estimated at 4.9-9 million, suggesting that about 2.3 million died prematurely because of lack of access to proper medical supervision. By 2030, the number of people receiving transplantation around the world is projected to increase to 5.5 million and this numbers will be in the developing countries of Asia and Africa. In India, today, more than 200 medical centers are undergoing kidney transplantation and it depends only on the living donors. Throughout India, kidney transplantation are done with far more potential compared to liver, heart, and lung transplants and therefore complications of pre and post kidney transplantation are documented elaborately throughout this study.
1.2. Terminology of Kidney

Kidney is considered as one of the essential organs in the vertebrates for the purification of blood which excretes the metabolic waste and also performs the homeostatic function. Currently, kidney and its related disorders are the leading cause of major nephrological problems throughout the country. The main process that happens in kidney is:

- Filtration of blood plasma
- Re-absorption of major discriminating substances such as sugar, fatty acids, amino acids and low substances such as water and salt
- Secretion of uric acid, organic ions, creatinine etc.

1.2.1. End Stage Renal Disease (ESRD)

Kidney failure is a condition, that has caused permanent damage to both or either one of the kidneys in which the damaged kidney fails to remove metabolic end-products from the blood and regulates the fluid, electrolyte and pH balance of the extracellular fluids and it is also the final stage of Chronic Kidney Disease (CKD) and kidney dysfunctions (Jad and Daniel 2005). Depending on the causes, the renal failure can be classified into two types:

- Acute renal failure
- Chronic renal failure

1.2.1.1. Acute renal failure

Acute renal failure is a condition in which kidney functions stop rapidly which subsequently results in low volume of urine and electrolyte imbalances and less accumulation of fluids in the body.

1.2.1.2. Chronic renal failure

Chronic kidney failure is a progressively worse condition in which the kidney fails to maintain the metabolic, fluid and electrolyte balance resulting in uremia, which forces the patient to rely on hemodialysis for the internal body metabolism and also to avoid uremia. In the initial stage of kidney failure, symptoms would be reduced through hemodialysis and formulation of controlled diet, regulation of fluid intake and use of
proper medication, as kidneys function becomes deteriorate, these treatment would become ineffective. Chronic kidney failure is the outcome of long term disease or deterioration to both of the kidneys and it is the final result of renal damage caused by diseases such as diabetes, high cholesterol and blood pressure etc.

1.3. Treatments for kidney failure

Treatment of renal failure commonly comprises of various measures to assist symptoms, lowers consequences and reduces progression of the severity. There are two types of treatment usually followed:

- Hemodialysis
- Transplantation of Kidney

1.3.1. Hemodialysis

Hemodialysis is considered as a life supporting medical treatment for the acute and chronic kidney failure and it impose special type membrane (dialyzer) that acts as an alternative kidney, which assists in getting temporarily free of additional salt, water and harmful wastes in the patient’s blood. It also helps to stabilize the blood pressure and also to keep the proper balance of electrolytes in the blood plasma. It involves in separating the accumulated metabolic waste products from the blood plasma using an artificial kidney
dialyzer. During the treatment period, patient’s blood flows through the tubes in the
dialyzer, where the wastes and salts, additional water were filtered and finally purified
blood flows through the set of tubes back into the patient’s body. The treatment of chronic
kidney failure with hemodialysis includes fluid and diet therapy, medication, caring of
urinary catheter site and mental support etc.

1.3.2. Transplantation of Kidney

Kidney transplantation is the final treatment of option for patients with Chronic
Kidney Disease (CKD) and subsequent infection is a major problem concerning the
transplanted patients. In kidney transplantation, the aseptic surgical replacement of healthy
kidney from the donor is carried out, regardless of the type of donor, special blood tests are
necessary to determine the blood and tissue type to be match with the recipient.

Kidney transplantation possesses the intense risk of developing bacterial, viral and
fungal infections as a result of immunosuppressive therapy and medications. Bacterial
infection is one of the most frequent infections that occur during the initial six months of
the post transplantation recovery period; and it is also estimated that nearly 140 million of
patients encounter infections during their recovery process, resulting in more than six
billion dollars in direct healthcare expenditure. Therefore, more focus is usually being
placed on kidney transplantation worldwide especially in India and therefore specific
immunological investigation can improve the long-term success of transplantation in
individual cases of end stage kidney failure patients.

Several investigative studies have been done around worldwide showing the
etiological sequence of bacterial infection among kidney transplanted patients. Besides
these studies, the major problem of life-threatening infection strikes during the first three to
six months of post-transplant period, during which patients are facilitated with urethral,
intra-vascular catheter access and invasive instruments (Burroughs and Moscona 2000).
Urinary Tract Infection (UTI) coupled with vascular catheterization is generally a common
infection in transplanted patients and it is a major source of nosocomial infection and
septicemia. Treatment of bacterial infection also becomes more complex and it influence
the various factors such as age, sex, transplant source, infected donor, allograft rejection,
duration on dialysis and medical condition set (Kalaivani et al. 2016). Over the past years,
patients are exposed to a vast range of medications over the certain drugs in the perspective of resistance to the causative pathogens in the transplanted individuals.

1.3.2.1. Pre-transplant Screening

Pre-transplant screening of organ donors and recipients is a key factor in the success of organ transplantation. The prime focus of pre-transplant screening is to identify the donor organ conditions, which may disqualify either donor or recipient; recognize and define the risk of infection and develop strategies for preventing and mitigating post-transplant infection; and implement preventative measures, including immunizations. Women with prior pregnancies are liable to be sensitized, but the antibodies may have been vanished after few years (Khan and Zaman 2006).

1.3.2.2. Post-Transplant Complications

The major complication that follows kidney transplantation includes:

- Graft rejection
- Diabetes mellitus
- Post-transplant infections

1.3.2.2a. Graft rejection

Since the donor kidney is foreign, the recipient body’s immune system tries to decline the kidney by destroying it. Hence, the recipients will take immunosuppressive treatment till the rest of their life supporting process to prevent their body from rejecting transplanted kidney.

Graft rejection can occur in several forms:

- **Hyper acute rejection** – It occurs immediately after transplantation due to cross-matching. This type of rejection is very rare.
- **Acute rejection** – It occurs in the initial few months after transplantation which can be treated by increasing medication doses suppress the immune system.
- **Chronic rejection** – Usually it occurs in the post-operative transplantation period and has no treatment to stop this form of rejection. It can be characterized by terminating the functions of kidney slowly.
1.3.2.2b Diabetes mellitus

Diabetes is the condition in which the body imbalances normal blood sugar levels and few medications that are taking after kidney transplantation might cause diabetes.

Table 1.2 Complications of Graft Rejection

<table>
<thead>
<tr>
<th>Transplant Rejection</th>
<th>Type</th>
<th>Duration</th>
<th>Function &amp; Vessel Histology</th>
<th>Type of Hypersensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hyper acute</td>
<td>Immediate</td>
<td>Preformed antibodies directed against the donor tissue. Caused by accidental ABO blood type incompatibility which is very rare. Presents while still in surgery with thrombosis and occlusion of graft vessels.</td>
<td>II</td>
</tr>
<tr>
<td>&quot;Host vs. Graft&quot;</td>
<td>Acute</td>
<td>Week to Months</td>
<td>T-Cell mediated immune response directed against the foreign MHC. Inflammation and leukocyte infiltration of graft vessels results. Most common type.</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Chronic</td>
<td>Months to years</td>
<td>T-Cell mediated process resulting from the foreign MHC &quot;looking like&quot; a self MHC carrying an antigen. Results in intimal thickening and fibrosis of graft vessels as well as graft atrophy.</td>
<td>III &amp; IV</td>
</tr>
<tr>
<td></td>
<td>Graft vs. Host</td>
<td>Varies</td>
<td>Donor T-Cells in the graft proliferate and attack the recipient tissue. Most commonly seen in bone marrow transplants. Presents with diarrhea rash and jaundice. (Barkholt et al. 2003)</td>
<td>IV</td>
</tr>
</tbody>
</table>

Hence, the non-diabetic transplants may begin using insulin and its dosage should be increased to help control the blood sugar level.
1.3.2.2c. Post-transplant infection

Even though, improved surgical techniques and more potent immunosuppressive therapies are available, pathogenic bacterial infections still plays a predominant problem among the kidney transplanted patient. With suitable pre-transplant assessment, effective immunization, post-transplant medical checkups and prophylaxis, the severity of infections would be reduced. Among various post-transplant pathological infections that exist, Urinary Tract Infections (UTIs) becomes the most frequent bacterial infection in post transplanted patients. Viruses are also among the most common causes of opportunistic infection after transplantation. The expectation for viral infection is a function of the specific virus encountered, the intensity of immune suppression used to prevent graft rejection, and other host factors governing susceptibility. The most common opportunistic pathogen is CMV which is seen in transplant recipients. Apart from this virus numerous other viruses, herpes simplex virus (HSV), Epstein-Barr virus (EBV), parainfluenza viruses types 1, 2, and 3, measles and rubella are found in renal transplants. have also affected outcomes. In some cases, preventive measures such as pretransplant screening, prophylactic antiviral therapy, or posttransplant viral monitoring may limit the impact of these infections. Recent advances many more theropys are practised. Studies of viral latency, reactivation, and the cellular effects of viral infection will provide ideas for future strategies in prevention and treatment of viral infections.

1.4. Epidemiology of Infections

The majority of pathological infections that occurs in the transplant patients is opportunistic and is a significant cause of death in the immunosuppressive patient. Immediate infections are caused by microorganisms that are mostly predominant in the environment, but they would scanty cause infection in the immune-competent host. Standard protection from opportunistic organisms in the human is from innate and acquired immune mechanisms and normal flora. Gram negative and gram positive bacteria are persistent in the environment are the most common organisms implicated in infection in the immune suppressed host (Bush and Fisher 2011).

Bacterial infections are the frequent infections in kidney transplant recipients, followed by fungal and viral infections. Women are at huge risk for bacterial infections; other risk factors include deceased donor kidney transplant, kidney with severe bladder
drainage, prolonged catheterization, uretero-vesical stents, and increased immunocompromised state (Lorenz and Cosio 2010). Escherichia coli are the most frequent occurring uropathogens in the renal transplant recipients. All levels of bacterial infections in renal transplant recipients are treated as complicated, and thus regular treatment specifically involves 1 to 2 weeks of antibiotic therapy (Rice and Safdar 2009). Treatment of bacterial infections may be problematic due to the interaction of drugs between antimicrobial agents and immunocompromised medications.

Effective usage of immunocompromised drugs in a kidney transplanted patient requires an attentive balancing act. Availability of advanced, effective and specific immunocompromised agents has reduced the occurrence of acute rejection to about 10-15% in most medical centers. Even though, refinements in diagnostic techniques and discovery of new antibacterial drugs, has not lowered the severity of infection amongst transplanted patients. About 60% of all transplanted patients encounter at least one time of infection for the initial one year. Bacterial infections are responsible for 48% of all deaths with functioning allograft in India, and are the major reason of death in the developed nations.

Infection control after transplantation combines different strategies such as screening patients for past and latent infections, treating active infections, and prevention of community-acquired and nosocomial infections with immunization, chemoprophylaxis, and appropriate infection control measures. Careful attention should be given to good hand washing practices, central line care, and practices to decrease the risk of pneumonia. Specialized air filters and negative pressure rooms should prevent the infectious droplets of measles, varicella and tuberculosis from entering the general air supply and infecting others. Contact precautions must be applied to limit the spread of organisms such as methicillin-resistant staphylococcus aureus, vancomycin-resistant enterococci, clostridium difficile, respiratory syncytial virus, and other multi-drug resistant gram-negative bacilli.

With the recent improvements in technology and development in medicine, there has been a huge deal of achievement in immunocompetent therapy and this has brought about a considerable impact on the changing sequence of infection and diseases in kidney transplant individuals. In the view of prevention of infection, everyone should understand the standard type of infection in that specific group of patients. Hence, in this study, an
effort was made to investigate the present pattern of infections and their occurrence in renal transplant patients.

1.5. Antibiotic Resistance

Development of effective antibiotics is one of the greatest medical attainments in human race (Yoneyama and Katsumata 2006). Antibiotics are group of chemical substances that are simply defined as against microbes and it is secreted by actinomycetes, fungi and bacteria; it would also interact with structure of bacterial species causing no damage to the human host cells (Rubin 2002).

Antibiotics are generally classified according to their antimicrobial classification structure (toxicity, structural pattern and potency), but the most effective antibiotics are the one that depends on the chemical structure and their mode of action. Based on the activity of chemical substance present in the antibiotics, they are classified as β-lactams (penicillin and cephalosporin), macrolides, fluoroquinolones, tetracycline and aminoglycosides (Alos et al. 2005). Among the different group of antibiotics, β-lactam is known as the largest group of antibiotics which includes penicillin (Sulphur which containing penam (carbapenem and oxapenam) and its related compounds), cephalosporin (sulphur which containing cephem (carbacephem, oxacephem) and its related compounds) and natural & synthetic monocyclic β-lactams. Penicillin is one of the first naturally secreting β-lactam antibiotics to be used in the treatment of pathogenic bacteria and its related infections (Canbaz et al. 2002). In addition to penicillin, cephalosporin was developed and it has a novel structural substance in its base nucleus unique to that in penicillin with only exception in the thiazolidine ring which experiences a unique ring amplification to form dihydrothiazine ring. Cephalosporin itself is not alone a potent antimicrobial agent in treating pathogens; its efficacy depends on the structural substance and chemical modification of the acetyl group in its nucleus (Pfeifer et al. 2010).

Extended Spectrum β-Lactamases (ESBL) are one of the rapidly developing group of β-lactamases that is discovered in 1982, which has the capacity to break down the third generation cephalosporin and aztreonam that are impeded by Clavulanic acid (Naas et al. 2008 & Tankhiwale et al. 2004). Unfavorably the arrival of new group of antibiotics β-lactams and plasmid associated enzymes TEM-1 and TEM-2 in E.coli experience diverse
levels of modification near the active center that potentially increased their capability to break down extensively variable substance. Almost there are more than eighty five ESBL’s with twenty five in SHV lineage group and sixty in TEM group and that have been established with extensive alteration. Therefore, these variations extremely increase the hydrolytic energy of the enzyme against vital antibiotics of prime significance, such as ceftazidime and ceftriaxone (Romanus et al. 2009).

The complication of antibiotic resistance is experienced with one or more infections namely, Hospital-Acquired Infection (HAI) and Urinary Tract Bacterial (UTI) infections. UTI is one of the most common bacterial infection and it contains a variety of fluids, bile salts and excess waste products and it has been reported to cause infection around 180 million individuals in India every year and approximately 160 million individuals availing medical treatment annually and more than 22.5 million individuals undergoing hospitalized administration per year in our country (Sahm et al. 2001 & Sharma et al. 2007). The organism responsible for causing this infection is basically associated with Escherichia coli and it is one of the principal investigative agents in all type of patients (Zhang et al. 2009).

Despite the ease availability of these effective antimicrobial agents, urinary tract infection becomes the most significant bacterial infection in kidney transplanted patients (Stamm and Norrby 2001). Antibiotic agents are administered empirically in prior to the medical examination of urine culture and appropriate knowledge of medication in treating the organisms that cause infections and their antimicrobial susceptibility is required (Green et al. 2011).

Antibiotic resistance strives to emerge and grows rapidly at new position due to the conflict of one bacterium with other for the unique biological niche (Bean et al. 2008). As a result there always sustain a strong urge for the requirement of new antibiotics; certainly transmission of contact infection must be limited by the use of different organisms such as methicillin-resistant staphylococcus aureus, vancomycin-resistant enterococci, clostridium difficile, respiratory syncytial virus, and other multi-drug resistant gram-negative bacilli.

Transplant patients should remain in medical regime for a period of time, which differs according to the type of allograft, previous existence of co-morbidities, and also the underlying disease responsible for transplantation. During medical treatment, most patients
receive broad-spectrum of antibiotics and some patient develops infections with multidrug-resistant (MDR) bacteria. The use of implantable devices (urinary catheters), parental nutrition and prolonged intubation and the need for renal replacement therapy all increase the risk of this complication. Multidrug resistant (MDR) was defined as lack of susceptibility to one or more agents in three or more antimicrobial categories active against the isolated bacteria. In additional to these bacteria that are non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e., bacterial isolates remain susceptible to only one or two antimicrobial categories) are defined as extensively drug resistant (XDR).

Therefore now it is clearly evident that the medical significant resistance to all of the antimicrobial agents has been appeared only in few months to years except for the antibiotic vancomycin which took 20 years (Hryniewicz et al. 2001 & Babini and Livermore. 2000). Health care associated bacterial infections due to resistance of gram negative bacilli are the most significant menace all over the world (Pinheiro et al. 2010).

Transplantation in Indian scenario is certainly unapproachable and there is little control in the use of antimicrobial agents. In the lack of national or state level monitoring agency, the scenario in our country is still hypothetical and unfavorable, most of the drugs prescribed by the physicians are far more widespread and the community precaution of these problems are involved in poor antibiotic therapy and thus they are over countered (Pfeifer et al. 2010). Ongoing surveillance for antimicrobial resistance at all levels of the health care centers in all regions in India should be introduced and those progressions are in the incubation stage in the developing countries.

Thus the aim of the present study is to record the common clinical presentation, risk factors of bacterial infection and their distribution of bacterial strains isolated from pre and post-transplant patients of either sex different age group from Government hospital, Chennai, Tamil Nadu, India and their antimicrobial resistance pattern against commonly used antibiotics and also to analyze the β-lactamase enzyme & its associated genes with the following objectives:

- To isolate, identify and characterize the bacterial isolates associated with transplanted patients.
- To ascertain the antibiotic sensitivity pattern of the bacterial isolates.
• To select the drug resistant bacteria isolates based on their antimicrobial resistance pattern in kidney transplant patients.
• To determine the kinetic parameters of the partially purified β-lactamase of certain antimicrobial resistant bacteria isolates.

Not much of work has been done on Pre and Post Kidney transplantation patients with bacterial infections, so I have taken this topic to work.
CHAPTER 2

LITERATURE REVIEW

The development of multi-drug resistance among bacterial pathogens is a problem of increasing incidence since the early 1960s and is currently viewed as a major threat to clinical practice in the therapy towards various infectious diseases. Multi-drug resistant bacteria were capable of causing life-threatening infections (Mammeri and Nordmann 2007). World Health Organization (WHO) have recognized the multi-drug resistant bacteria as a serious global threat.

2.1. Renal failure

Renal failure is a condition in which kidney stopped to function less than 10% of normal activity; hypertension, diabetes and chronic glomerular disorders are the major causes of renal failure. More than 85% of kidney failure patients require transplantation (Hemalatha et al. 2007). People in Africa and America have a greater risk of hypertensive and diabetic kidney failure when compared to Europeans (Barkholt et al. 2003).

In India, the exact number of people with kidney failure is not known; however, the conservative estimates have calculated that around 0.1 million patients with kidney failure are added each year to the existing pool of patients (Aalten et al. 2008). There are two different types of renal failure and they are acute and chronic renal failure. Acute failure can be potentially reversible but chronic renal failure progresses can lead to permanently failure. In India only less than 5% of all patients with renal failure undergo transplantation though it has been in practice in India for more than 3 decades, it has been inadequate for Indian patients in terms of actual numbers, due to an acute shortage of resources and donor organs (Tanriover et al. 2014).

The number of kidney transplantations is increasing worldwide. Similarly the number of patients waiting to get a kidney has also increased tremendously. The number of patients on waiting list for kidney in July 2016 was 99,413 in USA as per data of Organ
Procurement and Transplantation Network (OPTN, 2015) and it was 0.72 million in India (Indian Network for Organ Sharing (INOS, 2014). Unfortunately only 17,878 patients could get a kidney, despite the recent relaxation of rules to accept expanded criteria donors the gap between demand and supply is still huge (Gandolfini et al. 2014).

Transplant rate is very low in countries with average HDI (Human Development Index) and a large spread of transplant rates are seen among the developed nations. Transplant rates of more than 30 per million populations (pmp) in 2010 were restricted to Western Europe, USA, and Australia, with a slightly broader spread of countries achieving between 20 and 30 pmp (Jad Khoury and Daniel Brennan 2005).

In Canada, all minority groups have significantly lower transplant rates; compared to Whites, the rates in native and African Canadians, Indo Asians, and East Asians were 46%, 34%, and 31% lower, respectively. In US, transplantation rates are significantly lower among African Americans, women, and the poor, compared to Caucasians, men, and the more affluent populations. The situation is similar in Australia where Aboriginal Australians fare worse than non-indigenous Australians (12% vs. 45%) and in New Zealand where Maori/Pacific Islanders are disadvantaged (14% vs. 53%). In Mexico, the transplant rate among uninsured patients is 7 pmp compared with 72 pmp among those with health insurance (Frutos et al. 2012 & Rizvi et al. 2011)

Gupta and Stamm (2009) have found that the chronic diseases have become a major cause of morbidity and mortality worldwide. About 800 per million populations possess chronic kidney disease and 150-200 per million populations possess end-stage renal disease. Diabetic nephropathy is found to be the most common cause of chronic kidney disease. In India, approximately 3500 organ transplants are done annually which indicates only 10% of the kidney failure patients get kidney replacement.

Foxman (2010) studied that only less than thousands of all patients in India undergo renal transplants, the reasons for our cadaveric transplant program not taking off effectively in India are many and they are complex. The main reasons are due to social and religious beliefs, non-existence of networked organ tracking and retrieval organizations and the non-eligibility of transplantation between willing, related donors with the ESRD.
patients. A few published literatures were available to forecast the waiting list of transplant patients.

2.2. Transplantation

Transplantation can be defined as the process in which an organ is transferred from a donor to a recipient (e.g. kidney, liver, heart, lung or pancreas). Our immune system has built up elaborate and efficient mechanisms to combat foreign agents. These mechanisms are too affected in the rejection of transplanted organs, which are picked out as alien by the immune system of the recipient. The understanding of mechanisms is important for interpreting the clinical characteristics of rejection and creating an early diagnosis for delivering appropriate treatment. Knowledge of these mechanisms is important in preparing schemes for minimizing rejection, and developing novel drugs and treatments for the effectiveness of immune system of transplanted organs, hence ensuring longer survival of these organs.

A study by Green et al. (1999) suggests that same outcomes are seen for younger recipients in comparison to older one. In such case, Medawar first of all in 1944 showed the skin allograft rejection. The first successful kidney transplant was done by Joseph E. Murray in 1954 and liver transplant by Dr. Thomas E. Starzl in 1967 followed by heart transplantation by Christian Barnard in 1967 and bone marrow transplantation by E. Donnall Thomas in 1968 (Klintmalm 2004). Schwartz and Dameshek (1959) showed the immunosuppressive effect of 6-mercaptopurine in rats. After which, many selective immunosuppressive agents have been developed by various researchers which enabled the improved survival of transplanted recipients.

2.3. Post transplantation bacterial infections

The occurrence of bacterial infection in post transplantation is a significant; type of infections after solid-organ transplantation is dependent on various factors including immunosuppression, organ type, surgical complications, environmental exposures and the time frame after transplantation (Hooper 2000). The infection usually falls from the first month to 6 months after the transplantation (Saemann and Horl 2008).
During the first decades of the renal transplantation era, a serious infectious complication developed in up to 70% of patients following transplantation, resulting in fatal outcome in as many as 11% to 40% of cases. In the late 90’s, the incidence of infections had declined to 15%-44% with a mortality rate of less than 5%. Immediate infections after transplantation are due to nosocomial infections include bacterial, candida and UTI, wound/surgical site infections, catheter-related infections, and pneumonia (Fiorante et al. 2011).

After the first 6 months from transplantation, the kidney transplant function becomes the main predictor of risk for opportunistic infections “classically” associated with transplantation (Mohammed et al. 2007 & Midtvedt et al. 2008). The post-transplantation period has traditionally been divided into three time frames in relation to the incidence and type of infectious complications: the first month, the second through the sixth months, and the late post-transplantation period beyond the sixth month. Infections in the first month post-transplantation are mostly similar in pattern to postsurgical infections in non-immunocompromised individuals. These include urinary tract infections, wound infections, pneumonia and line sepsis and are mostly caused by bacteria and Candida species (Sanderson and Alshafi 2005).

The source of infection can be divided into three groups: de novo infections which may arise from organisms colonizing the recipient or from a nosocomial origin, reactivation of latent infections present in the recipient or transmitted with the donor allograft, or contamination of the allograft during the procurement or preservation process. De novo infections occur mostly in the first month and these include UTI, line sepsis, wound infections, and pneumonia. Reactivation of latent infections in the recipient accounts for a number of infections and this includes CMV, tuberculosis, and histoplasmosis. Another cause of infection is contamination of the allograft during procurement or preservation. The incidence of bacterial contamination of donor kidneys varies widely, ranging from 2.1% to 23.4%. The majority of cultures indicate that the organisms are skin contaminants or organisms of low virulence (Sahm et al. 2001). Colonization might result the recurrent infections. Vidal et al. 2012 states infections in transplant might occur through four reasons

- Infections due to surgical or technical complications
• Infections related to prolonged hospitalization (nosocomial infections)
• Infections associated with immunosuppression (opportunistic infections)
• Infections occurring when the transplant recipient resumes normal activity (community-acquired infections)

The immunosuppressed state of the recipient after transplantation predisposes the patient to bacterial pathogens not commonly observed in the normal host. These opportunistic pathogens include *Legionella* spp. *Nocardia* spp. *Rhodococcus* spp. and *Mycobacteria* spp. After transplantation, the bacterial infection might disrupt the anatomic barriers and the upper airway is usually colonized with bacteria where as the lower respiratory tract is sterile. Endotracheal intubation might create a passage between the upper and lower respiratory tract, introducing bacteria to the lower part and resulting disease in the lung parenchyma (Thoung et al. 2003).

2.3.1. Antibiotic resistance pattern in bacterial infections

Chuang et al. (2005) study demonstrated that the incidence of UTI rate was 33.56% among the kidney transplants, whereas the study from Djamali et al. (2006) and George et al. (2001) showed about 37% of patients developed UTI. (Kahlmeter 2003). *Escherichia coli* and *Enterobacter* is the most prevalent causative organisms in kidney transplant patients in their study. The literature survey on etiology of UTI in kidney transplant patients revealed the occurrence of bacteria the transplantation are *Escherichia coli*, *Enterococcus* spp., *Staphylococcus* and Klebsiella (Chuang et al. 2005); *Enterococcus faecium* (33%) and *Escherichia coli* (31%) (Valera et al. 2006); *Escherichia coli* (51%) and *Pseudomonas* (18%) (Manges et al. 2006); *Escherichia coli* (31.5%), *Candida albicans* (21.0%) and *Enterococcus* spp. (10.5%) (Djamali et al. 2006); *Escherichia coli* (18.4%) and *Klebsiella pneumonia* (14.6%) (Barbouch et al. 2012). Dantas et al. (2006) & Enright et al. (2002) have found in their study that *Escherichia coli*, *Enterobacter* and *Klebsiella* spp. were responsible for 30.4% and 40.0% of infections in post-transplant patients respectively.

Shaheen et al. (2005) evaluated the clinical and epidemiologic data of 175 community-acquired UTI patients. They have observed complicated UTI was the most
common (39%) followed by bacteremia (18%), acute uncomplicated pyelonephritis (12%). *Escherichia coli* were present in most of the patients with asymptomatic bacteriuria (60%) and uncomplicated cystitis (94%). Of the bacteria isolated, 92% were sensitive to ciprofloxacin and most of them were resistant towards ampicillin, cefuroxime, cefazolin, and co-trimoxazole.

Most patients after kidney transplantation require immunosuppressive medications to prevent rejection which will intuitively increase the rate of infections including UTI. Incidence of UTI in kidney transplants is important as they may be associated with congenital abnormality or an error in management. If not treated, might lead to recurrent infections causing damage to the urinary tract. Infection may occur at many places along the genitourinary tract: urethra, bladder, urethra, renal pelvis, or renal parenchyma (De Souza and Olsburgh 2008 & Senger et al. 2007). The prevalence of UTI generally varies with gender and age. Patients who are not receiving antimicrobial prophylaxis has been reported to vary from 5%-36%. Beyond three months after transplantation, the incidence of UTI decreases progressively. Risk factors include pre-transplantation UTI, prolonged period of HD, polycystic kidney disease, DM, prolonged postoperative bladder catheterization, immunosuppression, allograft trauma, and technical complications associated with urethral anastomosis. Female recipients had significantly more UTI than males and that the majority of the organisms were Gram-negative bacilli with *Escherichia coli* being the most common (Anis-ur-Rehman et al. 2008 & Shasikala 2004).

In the present investigation, a high incidence of bacterial infection with multi-drug resistance was observed which might be due to two factors: prolonged use of antibiotics and a population with a high percentage of young population, as UTI is common in the early years of life (Moland et al. 2008).

Current scenario in antibiotic resistance makes it difficult to suggest treatments for community-acquired UTI. After child birth, the pre-urethral area is usually colonized with enterobacteriaceae and enterococci to function as a defense barrier against uropathogens (Anis-ur-Rehman et al. 2008). In young girls, *E.coli* is found to be dominant, whereas *E.coli* and *Proteus* spp. were predominant in boys. Children of 5 years old are prone to have UTI due to the pre-urethral colonization of above said bacteria (Yagi et al. 2000; Anis-ur-Rehman et al. 2008; Nasim et al. 2010 & Mitra and Alangaden 2011). These
potential uropathogens were rarely found in children older than 5 years (Wright and Eiland 2008).

Sanderson and Alshafi (2005) have examined the UTI in 33 patients with the help of their used objects such as bed sheets and bedside chairs. Of them, 10 patients were found to possess UTI by the occurrence of *E.coli*, *E.cloacae*, *K.pneumoniae* and *E.faecalis* in bed sheets, streptococci and *C. tropicalis* in bedside chairs. Catheter Associated Urinary Tract Infections (CAUTI) is the most common nosocomial infections as they are asymptomatic and are major reservoirs of multi-drug resistant pathogens. Of various strategies devised to reduce their incidence, a very few were found to be effective (Green et al. 2011).

The prescription of antibiotics most notably penicillin was thought to end the bacterial infections. Unfortunately the rapid recognition of penicillin resistance within a year of its introduction disabused the physician of this notion (Hirsch and Randhawa 2009). Multiple antibiotic resistances to useful antibiotics including the beta-lactams, aminoglycosides and quinolones have generally emerged and this has been increasingly observed among gram-negative pathogens like Enterobacteriaceae bacteria (Yildiz et al. 2007). The infections caused by multi-drug resistant bacteria are most prevalent in hospitals, where the usage of antibiotics is extensive. Thus bacteria carrying antimicrobial resistance genes had survival advantage that facilitated dissemination in this setting (Drawz and Bonomo 2010). However, Hirsch and Randhawa (2009) observed a disturbing trend on the spread of antimicrobial resistant bacteria within the community. The major reason is the increasing dosage of antibiotics and studies indicate correlation between usage and the extent of antimicrobial resistance (Ganguly et al. 2011).

The bacteria develop antibiotic resistance by mutation or acquiring of new genes by horizontal gene transfer. The process involves the transfer of antibiotic resistance genes among pathogens (Anbumani and Mallika 2007). Numerous reports were available to prove the resistance genes could be transferred from one bacterium to the other. Transfer of such resistance genes between *Shigella* spp. and *Escherichia coli* has been observed (Hooper 2000 & Anbumani and Mallika 2007). Kathleen Head (2008) reported *in vitro* gene transfer among enteric bacteria of diarrhea origin. Subha et al. (2003) observed an
extensive transfer of tetracycline resistant determinants among bacteroids and other enteric bacteria.

Stapleton (2003) has studied that, the common uropathogens *E. coli* are characterized by their properties, products or structure called virulence factors which is responsible for causing disease in host. Several virulence factors are importance in UTI pathogenesis include adhesion, the aerobactin system, hemolysin, K capsule and resistant to serum killing are most common in genetically related strains of *E. coli*. The strains which express more virulence factors were more severe in causing infection. These virulence factors are mostly causing pyelonephritis and also develop cystitis and asymptomatic bacteriuria. Though these factors are found in wild-type of strains can’t able to spread into non-virulence organism. Use of biochemical and immunological anti-virulence factors are effective in animal models of UTI and this will help in human in future.

The high incidence of Recurrent UTI (RUTI) caused by multi-drug resistant *Escherichia coli* is consistent with the high prevalence of antibiotic resistance. For the clinical diagnosis, predictive prognosis and treatment of Recurrent UTI, the correlations between virulence factors, resistance spectrums, and recurrence rates are essential (Taneja et al. 2008). Moland et al. (2008) have conducted a cross-sectional study to assess the appropriateness of antibiotic prescription for UTI and to evaluate the variability of antibiotic prescription. The patients diagnosed with acute urinary infection, aged less than 14 years were selected and the main variables observed were: type of urinary infection, urine culture request, antibiotic prescription, hospital admission and co-morbidity (Khawar Abbas et al. 2015).

Around 3797 patients with acute urinary tract infections was studied and found 89% were possessing lower UTI. Ciprofloxacin and amoxicillin are the most commonly used antibiotics. The researchers observed a high variability in antibiotic prescription among hospitals. Rubin (2002) reported that in healthy women, occurrence of urogenital micro flora is depending upon reproductive stages. In premenopausal the vaginal flora are lactobacilli which protects against colonization of uropathogens and properties of lactobacilli such as adhesive ability and production of bacteriocins, acids, hydrogen
peroxide, appear to confer host protection. With the use of probiotics, the unbalanced flora has been restored and which can prevents the usage of antibiotics in the future.

Studies by Kathleen Head (2008) showed that lower UTIs are common in younger women, during pregnancy and in pre and post-menopausal women and were often prescribed with long-term antibiotics results antibiotic resistance. Dantas et al. (2006) obtained data from NAUTI in hospitalized urological patients: NAUTI was diagnosed according to the Centers for Disease Control and Prevention (CDC) criteria in 727 of the 6033 patients hospitalized on study days in urological departments. The most commonly reported pathogen was *E. coli*, followed by species of *Pseudomonas*, *Enterococcus*, *Klebsiella*, *Enterobacter* and *Proteus*. The resistance of *E. coli*, *Klebsiella* and *Proteus* spp. was below 45% against the common antibiotics. *Enterococcus* spp. and *Pseudomonas* spp. had resistance rates above 70% to most antibiotics. About 56% of the hospitalized patients receive antibiotic therapy; 26% for UTI, 21% for only clinically suspected UTI and 7% for other infections. Fluoroquinolones, cephalosporins, penicillins, aminoglycosides, and co-trimoxazole were he commonly used antibiotics. Differences between countries were highly significant.

A repeated course of antibiotics is often prescribed for the treatment and prevention of RUTIs, which selects for resistant strains of *Escherichia coli*. Blango and Mulvey (2010) investigated the virulence factors, resistance, and their correlations with bacterial strains isolated separately from patients. Most of these strains were resistant to multiple antibiotics: 159 strains were resistant to three antibiotics (multidrug resistant) and 114 were resistant to five antibiotics (pan drug resistant). Piperacillin showed the highest resistance rate, followed by gentamicin, levofloxacin, and cefazolin (Bush and Jacoby 2010).

Mekki et al. (2010) reported on the activity of tigecycline against multi-drug resistant enterobacteriaceae as part of the tigecycline evaluation and surveillance trial. The Genomic DNA from 70 urinary isolates of antimicrobial-susceptible and multi-drug resistant *Escherichia coli* was restricted using XbaI and analyzed by pulsed-field gel electrophoresis. Antimicrobial susceptible bacterial isolates showed limited genetic relatedness, and two epidemiologic clusters containing 40 isolates were identified among the multidrug resistant isolates (James Karlowskya et al. 2007).
From the urine samples collected from 3 hospitals in Mubi, 187 urine samples consisting 68.9% female and 31.1% male yielded Enterobacteriaceae bacteria growth. The other isolates include *E. coli* (51.5%), *K. pneumonia* (24.4%), *K. oxytoca* (3.1%), *E. aerogenes* (9.7%) and *C. freundii* (10.9%). The study also revealed the high resistance rates of isolates to ampicillin (37.5%), ciprofloxacin (36.4%) and coamoxyclav (21.3%). Streptomycin, nalidixic acid, cephalaxin and gentamicin highly inhibited growth of the organisms tested. Transfer rates of 34.8% and 41.1% were obtained for inter generic and intra species transfer of gentamicin resistance genes (Gmr) among the Enterobacteriaceae isolates. Evidence of transferability of Gmr *in vitro* concurs to the assertion that under favorable conditions conjugal transfers of gentamicin resistance determinants and hence R plasmid occur only *in vivo* (Jain and Mondal 2008).

Transfer of virulence and resistance genes was demonstrated between *Salmonella* spp. inside epithelial cells. This transfer of antibiotic resistance genes has led to rapid emergence and spread of antibiotic resistance among bacteria populations and thus pathogens involved in UTI. Gentamicin resistance genes aac (3)-IIa and aac (3)-VIa have been reportedly detected in gram-negative bacteria in clinical settings. Sequencing and PCR experiments have confirmed that these genes are present on mobile genetic element that can facilitate their horizontal transfer among bacteria (Ariza-Heredia et al. 2013).

Manges et al. (2008) reported that *E.coli* strains resistant to ciprofloxacin were isolated from the digestive tracts of villagers from rain forest community in Guyana, despite that they had never been given the drug. Most of the villagers however had been given chloroquine, a drug closely related to ciprofloxacin. In view of the wide usage of ciprofloxacin, resistance to this antibiotic and other fluoroquinolones could be an important public health problem in area where malaria is endemic. Therefore there is need to prevent malaria using integrated approach coupled with development of effective vaccine so that humans will not end up creating more problems. Another possibility of resistance development is by horizontal transfers that have been shown to be common among members of Enterobacteriaceae (Bonadio et al. 2006; Mashouf et al. 2009 & Fiorante et al. 2011).

Hunstad and Justice (2010) in their work, highlights the current and future therapy options for UTIs. There are two aims preferred in treatment of UTI. One is rapid and
effective treatment with care to prevent the infection. Second aim is to prevent the development of resistance towards chemotherapy. In recurrent UTIs low dose of antibiotic is effective and suggested vaccination may be important in future. Hooton (2003) revealed that, uncomplicated UTI can be treated by drugs like co-trimoxazole, nitrofurantoin and cephalosporins. But the treatment period may be depending on, where the infection occurs and selection of antibiotic depends on proper diagnosis of UTI. Collection of urine sample from mid stream catch and microbial count should be $10^5$ mg/ml is essential for diagnosis of UTI.

Fiorante et al. (2011) reported that, due to antibiotic resistance bacterial infection becomes complicated. Antibiotics act on microbes by some specific actions including interfering cell wall synthesis, inhibiting of protein synthesis, interfering with nucleic acid synthesis and inhibition of metabolic pathway. Susceptible bacteria might acquire resistance gene from resistance bacteria through various genetic transformation and get ability to produce an enzyme which destroy or prevent the antibiotic from action. *E. coli* contained bla-TEM-1 indicated low level of ceftazidime resistance whereas the *E. coli* contained the second bla-SHV-1 indicated high level resistance towards third-generation cephalosporins.

The correlation between virulence factors and antibiotic resistance was evaluated by Aboderin et al. (2009) in Enterococcus strains of inpatient. Ninety one Enterococcus strains (59 were *E. faecalis*, 31 were *E. faecium* & 1 was *E. gallinarum*) were isolated from urine cultures on inpatients. Presence of the genes for some virulence factors such as aggregation substance (asal), hyaluronidase (hyl) and enterococcal surface protein (esp) were studied by molecular method. In Vancomycin resistance genes (VanA and VanB) test result showed 8 (25.8%) *E. faecium* isolates were glycopeptides resistance, among this 7 had VanA and in one it was neither VanA nor VanB. In antibiotic sensitivity test *E. faecium* and *E. faecalis* showed high level of resistance against gentamicin (*E. faecium*= 74.2%; *E. faecalis*=22%) and streptomycin (*E. faecium*=61.3%; *E. faecalis*=27.1%). hyl gene was higher in *E. faecium*, esp and asl genes productivity, haemolysin production and gelatinase activity were higher in *E. faecalis*. Enterococcal surface protein (25.6%) and Aggregation substance (26.7%) were the frequent virulence factors. Asal gene positive strains were resistant towards ciprofloxacin, norfloxacin and levofloxacin than Asal
negative strains. esp positive *E. faecalis* strains were resistant towards doxycycline than esp negatives and hyl gene positive *E. faecium* were more resistance to nitrofurantoin.

Health Protection Agency (2006) reported that, *E. coli* is a common gut flora and lives harmlessly. ESBL producing *E. coli* was more likely to cause UTIs. ESBL bacteria spread to non-pathogenic organism by fecal contaminated hands or unclean equipments used for the treatment of UTI. There are many way to stop the spreading of ESBL producing bacteria. In both hospital and community acquired infection the spread of ESBL is successful due to mobilization and evolution of the genetic elements. Antibiotic resistance test were observed in 285 isolates includes 172 - *E. coli*; 84 - *Klebsiella* spp. 20 - *Enterobacter* spp. 5 - *Salmonella* spp. & 4 - *Citrobacter* spp (Shamila Karuthu & Emily Blumberg 2012).

### 2.3.2. Treatment of bacterial infection

The choice of antibiotic in the treatment after renal transplantation is largely dependent on the susceptibility of the bacteria identified in the urine, blood, or wound culture, and is very important due to increasing bacterial resistance to commonly used antimicrobials. Fluoroquinolones, cephalosporins, or penicillin’s are commonly used against UTIs. For infections due to staphylococci or ampicillin-resistant enterococci, vancomycin is utilized. Critically sick patients require initial broad spectrum antimicrobials, which should then be narrowed as culture results become available. Nephrotoxic agents (aminoglycosides) should be avoided whenever possible, relying on effective non-nephrotoxic alternatives instead. Treatment duration may vary depends on the severity of infection.

Wound infections and most UTIs require treatment for 5 to 7 days, whereas pyelonephritis usually requires 2 weeks of therapy or longer. Imaging to overcome obstruction or anatomic abnormalities should be remembered in cases of recurrent UTIs (Trullas et al. 2011). In addition, wound infections may require debridement with an adjunctive antibiotic regimen. Patients with neutropenic fever may receive granulocyte colony stimulating growth factors, which have been shown not to increase the risk of acute rejection (Wyner 2004). Depending on the infection, immunosuppression, with close monitoring of graft function, may also play a major role in clearing the infection.
CHAPTER 3

EXPERIMENTAL METHODOLOGY

3.1. Chemicals & Reagents

Chemicals, reagents, solvents and media ingredients used in this investigation were of scientific grade and were procured from Hi-Media, India.

3.2. Sterilization and media preparation

All the petri plates, test tubes, glassware and the media used for this retrospective study were at sterilized at 12-15 lbs pressure for 15-20 minutes at 121˚C in autoclave.

3.3. Study Design

The study was a retrospective observational and descriptive study conducted in Stanley Government Medical College and Hospital, Chennai, India.

3.3.1. Study population

Patients included in this study were classified according to their age, sex, medical conditions and various other factors. Whether the kidney transplanted patients received a living or deceased donor kidneys were also taken into consideration for this study. Both the demographic and clinical data were collected using a questionnaire. There were around 77 (23.9%) Female and 259 (77.1%) Male volunteers between the ages 18 and 60 were selected for the present retrospective study.

3.3.2. Sampling

Biological samples including midstream urine, pus, sputum, Cerebrospinal Fluid (CSF), catheter tips and blood were collected from the selected volunteers. Single sample from each individual patient was collected and no repetition was done. The subjects who
underwent kidney transplantation and fulfilled the successful inclusion criteria were included in the study after proper consent.

3.3.2.1. Inclusion criteria

- Kidney transplanted patients above 18 years old and who are willing to participate in this study.
- Patients with chronic renal failure and sustaining through hemodialysis.
- Patients who can understand Tamil, English or either one of the language.
- Patients who are accessible, at the time of data collection process.

3.3.2.2. Exclusion criteria

- Kidney recipient below 18 years old or who declined the consent
- Patients who are not willing to participate and not accessible throughout the study.
- Patients with chronic renal failure and sustaining through hemodialysis who are critically ill.
- Patients who cannot understand Tamil and English language or either one of the language.

3.3.3. Sample size determination

Using the Daniel’s formula below, the minimal sample needed was calculated to 336 patients.

\[ n = \frac{Nz^2Pq}{E^2(N - 1) + (z^2Pq)} \]

Where

- \( N \) = Minimum sample size
- \( N \) = Total population of kidney recipients on follow up
- \( Z \) = Normal standard deviation 95% confidence interval
- \( P \) = Prevalence of the disease
- \( Q = 1 - \text{Prevalence} \)
- \( E = \text{Margin of error (0.05)} \)
3.3.4. Data management and analysis

Data’s were collected using medical observations and questionnaire. Data entry, checking and validation were done, cleaned and transferred into MS Excel and finally analyzed using SPSS software version 20.0 (IBM, India). Continuous data e.g. age, duration of dialysis and time after transplantation was summarized into means, standard deviation, modes, median, and range. Categorical data e.g. gender, education, immunosuppressive therapy was summarized into proportions and percentage. Prevalence was calculated as percentage of the whole study sample and results were documented as tables, bar charts, line graphs and pie charts.

3.3.5. Ethical Approval

The study was initiated after approval by the Institutional Ethical Committee, Stanley Government Medical College and Hospital, Chennai, India. Enrolment into the study was voluntary after obtaining written informed consent. The study did not involve the performance of invasive procedures that would expose the participant to severe risks. Information documented from the subjects was kept confidential. Those patients diagnosed to have bacterial infection were informed and a copy of their results were attached to the file after informing the primary clinician working in the transplantation centre for appropriate care. Participants were free to withdraw from the study at any time without jeopardizing their care.

3.3.6. Collection of Processing of Samples

Approximately 10 ml of clean catch specimen of samples were collected in a sterile screw capped bottles containing transport media. The samples thus collected were immediately transferred to the laboratory for further processing. The entire kidney transplanted patients received cefazolin 2g as preoperative and norfloxacin 200 mg as a postoperative prophylaxis. In the presence of a bacterial infection, the subjects were excluded during analysis of the incidence of that infection in all the patients during the post-transplant period (Kalaivani et al. 2016).
3.4. Isolation of Microorganism

Samples collected from the patients were analyzed for the presence of bacterial infection. A standard calibrated loop of 1μl or 10μl urine sample with specific turbidity was immersed vertically into a stably mixed sample and was cautiously streaked in the centre of a nutrient agar plate. Without heating the calibrated loop quadrant streaks were made at an angle of 90° i.e. perpendicular to the original streak angle. Inter cross streaks were made closer and stretched towards the corner of the plate and the plates were incubated at 37°C for 24 h. The specimen was considered positive for infection if a single organism was cultured at a concentration of $>10^5$cfu/ml. The primary identification of the bacterial isolates was confirmed based on morphology of colonial appearance and pigmentation. Subcultures were then incorporated into nutrient agar plates and banked till further use. The gram’s staining and biochemical characterization procedures (Motility, Catalase, Citrate utilization, Oxidase, Methyl Red (MR), Voges-Proskauer (VP), Indole production, Urease, Triple Sugar Iron (TSI), Carbohydrate fermentation were further performed according to Bergey's Manual of Systematic Bacteriology.

3.4.1. Cultural characterization

The cultures were then allowed to grow on MacConkey Agar, Eosin Methylene Blue Agar, Blood agar Mannitol salt agar and Nutrient agar (Hi media, India).

3.4.1.1. MacConkey agar

The urine samples were streaked on the sterilized MacConkey agar plates and the plates were incubated at 37°C for 24 hours. The colonies formed were counted and the lactose fermenting organisms were characterized by the formation of dry pink colonies.

3.4.1.2. Eosin Methylene Blue agar

Eosin Methylene Blue (EMB) agar was prepared and sterilized at 121°C for 15 minutes at 15 lbs pressure. The lactose fermenting colonies developed in MacConkey agar were inoculated into EMB agar and incubated at 37°C for 24 hours.
3.4.1.3. Blood agar

All the blood agar compositions except blood were mixed and sterilized at 121°C for 15 minutes at 15 lbs pressure. The medium were cooled to 45 to 50°C and 5% defibrinated blood was added. The flask was agitated to mix thoroughly, preventing the formation of bubbles and poured into sterile petri dishes. Bacterial culture was streaked on the prepared plates and the plates were incubated at 37°C for 24-48 hours.

3.4.1.4. Mannitol Salt agar

Mannitol agar plates were prepared and the culture was streaked aseptically. The bacterial growth was observed after 48 hrs incubation at 37°C. Mannitol fermentation was indicated by the formation of yellow colour in the medium.

3.4.2. Characterization of Isolates (Mackie and McCartney 1998)

Conventional methods for bacterial identification depend on phenotypic identification of the isolated strains using gram staining, culture and biochemical methods.

3.4.2.1. Gram staining

A small quantity of bacterial culture from agar was transferred to a drop of sterile water on a glass slide and mixed and it was move out evenly over the glass slide. The slide was allowed to air dry at room temperature. After the smear was dried, the next step was to attach the bacteria to the slide by heat-fixing. This was accomplished by gently heating, passing the slide two to three times through the hot portion of the flame of a bunsen burner.

Heat fixed smear of the isolates were stained with basic crystal violet dye and kept for two minute followed by treatment with Gram’s iodine. It was also kept for 1 minute which increased the interaction between the bacterial cell and the dye. Then the slide was washed under the running water and the smear was decolorized by washing with an agent such as 95% ethanol. Gram-positive bacteria retained the crystal violet-iodine complex when washed with the decolorizing agent, whereas gram-negative bacteria lost their crystal violet-iodine complex and became colorless. Finally, the smear was counter stained with a basic dye safranin. The end result was that gram-positive bacteria were deep purple in color and gram-negative bacteria were pinkish to red in color.
• **Crystalviolet**
  Crystal violet : 2.0 g in 20 ml 95% ethanol  
  Ammonium Oxalate : 8.0 g in 80 ml distilled water.

• **Gram's iodine**
  Iodine : 1.0 g  
  Potassium Iodine : 2.0 g  
  Distilled Water : 300.0 ml

• **Acetone alcohol**
  95% ethanol : 70.0 ml  
  Distilled water : 30.0 ml

• **Gram's safranin**
  Safranin : 0.25 g  
  95% ethanol : 10 ml  
  Distilled Water : 100 ml

### 3.4.2.2. Motility test

A hollow slide was cleaned thoroughly under running tap water, such that water droplets do not remain on its surface. A hollow slide is a rectangular glass slide with a small cavity at the centre of the slide into which bacterial culture was suspended and hanged and the slide is dried by swabbing with bibulous paper and subsequently, slightly heating it over flame by applying petroleum jelly around the slide. A loopful of bacterial culture is taken out from the 24 hour old broth culture aseptically and placed at the center of a cover slip. The slide is then inverted and mounted on the cover slip by pressing together gently and it is inverted slowly, such that the drop hangs into the hollow cavity without touching it and then the slide is observed under objective oil immersion microscope.

### 3.4.2.3. Catalase test

The catalase test was done by transferring a loop full of 24 hrs culture of test isolate on to a glass slide with an equal volume of 3% (v/v) hydrogen peroxide (H₂O₂) and mixed; production of gas bubbles indicates the positive result for catalase test. This test was performed in a sterile place with precautions to avoid aerosols. If the bacteria produces
catalase enzyme, then it will decompose the hydrogen peroxide into oxygen and water and the effervescence of gas bubbles will be evolved.

3.4.2.4. Oxidase test

The oxidase test is a basic experiment used for the characterization and identification of bacteria and they will use oxygen as the final electron acceptor in the aerobic respiration process. A filter paper was immersed with a substrate tetramethyl-p-phenylenediamine dihydrochloride and then the paper was air dried and cleaned with sterile distilled water. A loopful of bacterial culture was placed on the filter paper and then the presence of the bacterial enzyme it gets oxidized into a deep blue or purple color within 10-20 seconds.

3.4.2.5. Coagulase Test

The bacterial suspension when mixed with the plasma (human) forms clots indicating the production of the enzyme coagulase. The clot is formed due to a prothrombin activator, which converts fibrinogen to a fibrin. The test is performed by two methods

3.4.2.5a. Slide coagulase test

The bound coagulase on the surface of bacteria directly acts on fibrinogen to produce an insoluble fibrin clot within 10 seconds. A dense suspension of the test culture was emulsified in a drop of saline on a clean glass slide. If auto-agglutination occurred, further step is skipped and the test organism was subjected to the tube coagulase test. If the emulsification is complete, then a straight wire dipped in undiluted plasma is stirred into the bacterial emulsion. Development of the precipitate (coarse clumping) visible to the naked eye within 5-10 seconds is read as positive. Negative or delayed results appearing after more than 10 seconds are confirmed by tube test.

3.4.2.5b. Tube test

Compared to the bound coagulase, extracellular (free) coagulase first reacts with Coagulase Reacting Factor (CRF) to produce coagulase-CRF complex, a substance that is
clinically indistinguishable from thrombin. This complex then acts on fibrinogen to produce an insoluble clot.

Plasma is tenfold diluted with saline was prepared and 0.5 ml of the diluted plasma was placed in narrow test tubes, 0.1 ml of the overnight grown broth culture of testing isolates was added. The tubes were incubated at 37°C in a water bath, and examined at 1, 3 and 6 hours for coagulation, which appeared as a stiff gel that remained in place even if the tube is inverted for a moment. If negative, then the tubes were kept at room temperature overnight and then again observed for coagulation.

3.5. Biochemical Characterization tests (Prescott et al. 2002)

Bacteria isolated from infected urine samples were identified by various standard biochemical tests as follows:

3.5.1. Indole test

Bacteria that contain the enzyme tryptophanase can hydrolyze tryptophan to its metabolic products, namely indole, pyruvic acid and ammonia. The presence of indole can be detected by the addition of Kovacs’ reagent. Kovacs’ reagent reacts with indole, producing a bright red compound on the surface of the medium. Tryptophan broth was prepared and sterilized at 121°C for 15 minutes at 15 lbs pressure. The isolate was inoculated in the broth under aseptic condition and incubated at 37°C for 24 hours. After incubation few drops of Kovac’s reagent was added. The formation of cherry red colored ring in the surface of the medium was recorded as positive result

- **Tryptone broth**
  
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1000.0 ml</td>
</tr>
</tbody>
</table>

- **Kovac's reagent**
  
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Para-dimethyl amino benzaldehyde</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Butyl alcohol</td>
<td>75.0 ml</td>
</tr>
<tr>
<td>Conc. hydrochloric acid</td>
<td>25.0 ml</td>
</tr>
</tbody>
</table>
3.5.2. MR-VP Test

Methyl Red (MR) and Voges-Proskauer (VP) test is done by two methods. They are the following

3.5.2.1. Methyl Red test

This test is to find whether acid or acetoin is the end product of glucose metabolism in specified bacteria. It is measured on the basis of pH change in the medium with the help of indicator methyl red which turns red at pH 4 and yellow at alkaline pH. MR-VP broth was prepared and sterilized at 121°C for 15 minutes at 15 lbs pressure. The isolates to be tested were inoculated in the broth under aseptic condition and incubated at 37°C for 24 hours. The indicator methyl red was added and the formation of red colour was recorded as positive result.

- Glucose - phosphate broth (MR-VP)
  
<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>7.0 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Di-Potassium phosphate</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1000.0 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>

3.5.2.2. Voges - Proskauer test

This test is to find the accumulation of 2-3 butene-di-ol in the medium released from glucose metabolism. The addition of Barritt’s reagent will detect the presence of acetoin, a precursor in the synthesis of 2, 3-butanediol. MR-VP broth was prepared and sterilized at 121°C for 15 minutes at 15 lbs pressure. Culture was inoculated with MR-VP medium and incubated at 37°C for 24-48 hrs. After incubation, 3 ml of Barritt’s reagent A and one ml of Barritt’s reagent B were added. The tubes were shaken and allowed to stand for 15 minutes and observed for colour change. The development of pink colour was considered as positive result.
• **Barritt’s Reagent A**
  
  5% alpha naphthol : 5.0 g  
  Absolute ethanol : 95.0 ml

• **Reagent B**
  
  Potassium hydroxide : 40.0 g  
  Creatine : 3.0 g  
  Distilled Water : 1000.0 ml

**3.5.3. Test for H₂S production and glucose utilization**

Culture was inoculated with triple sugar iron agar slants and incubated at 37°C for 24 hrs. The change in colour of the medium from red to yellow indicated the production of acid from glucose. A blackening of the medium indicated the production of H₂S. Breaks in the medium showed the production of gas from glucose.

**3.5.4. Triple Sugar Iron agar (TSI test)**

Triple sugar iron agar was prepared and sterilized at 121°C for 12 minutes at 15 lbs pressure. The slant with butt was prepared in tubes and inoculated by streaking the slant surface using a zig-zag pattern and then stabbing the agar deep with a straight inoculating needle. The tubes were incubated for 18 to 24 hours in order to detect the presence of sugar fermentation, gas production and H₂S production.

- Sterilize the inoculating needle in the blue flame of the bunsen burner till red hot and then allowed to cool.
- From the rack, take the tryptic soy broth tube containing the 24-48 hour culture, remove the cap and flame the neck of the tube.
- Using aseptic technique, take the culture of the organism from the TSB (tryptic soy broth) tube with the needle.
- Again flame the neck of the tube and replace the tube in the test tube rack.
- Take a sterile TSI slant tube from the rack, remove the cap and flame the neck of the tube.
- Stab the needle containing the pure culture into the medium, up to the butt of the TSI tube, and then streak the needle back and forth along the surface of the slant.
- Again flame the neck of the TSI tube, cap it and place it in the test tube rack.
• Incubate at 37°C for 18 to 24 hours.

**Indication and Interpretations**

- Alkaline slant-acid butt (red/yellow) - fermentation of dextrose
- Acid slant-acid butt (yellow/yellow) - fermentation of dextrose, lactose and/or sucrose
- Alkaline slant-alkaline butt (red/red) - dextrose or lactose were not fermented (non-fermenter)
- Cracks, splits, or bubbles in medium - gas production
- Black precipitate in butt - hydrogen sulfide production

**3.5.5. Urease test**

Suspend the ingredients in distilled water, boil to dissolve completely, and autoclave at 121°C and 15 lbs for 15 minutes. Cool the medium to 50 to 55°C. Aseptically add 100 ml of filter-sterilized urea base to the cooled agar solution and mix thoroughly. Distribute 4 to 5 ml of medium to sterile test tubes and allowed to solidify.

Cultures were inoculated with urease medium and incubated at 37°C for 24 hours. Urease is the enzyme possessed by the bacterium which hydrolysis urea and releases ammonia and carbon-di-oxide, ammonia reacts in solution to form ammonium carbonate which is alkaline leading to increase in the pH. Phenol red indicator which was incorporated in the medium changes its colour from yellow to red in alkaline pH, thus indicating the presence of urease activity.

**Urea agar**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Di-Potassium phosphate</td>
<td>1.20 g</td>
</tr>
<tr>
<td>Mono-Potassium phosphate</td>
<td>0.8 g</td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.012 g</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
</tr>
</tbody>
</table>
3.5.6. Haemolysis test

This test demonstrates the ability of isolate to lyse the Red Blood Cells (RBC). 5 ml of defibrinated human blood was added to sterilized 100 ml of cooled (40°C) nutrient agar medium and poured into sterile petri plates. 5% blood agar plates were streaked with test isolates and incubated at 37°C for 24 hrs. The haemolysis of RBCs was observed as clear zone around the isolated colonies and test isolate was considered as positive for haemolysis (Mackie and McCartney 1998).

3.5.7. Citrate Test

This test is based on the utilization of citrate as carbon source by the bacterial isolates. Citrate on utilization liberates carbon dioxide which combines with sodium in the medium to give alkaline end product which is detected with the presence of bromothymol indicator. Simmon's media contains bromothymol blue, a pH indicator with a range of 6.0 to 7.6 and it turns yellow at acidic pH, and gradually changes to blue at more alkaline pH. Un-inoculated Simmon's citrate agar has a pH of 6.9, so it is an intermediate green color. Growth of bacteria in the media leads to development of a prussian blue color (positive citrate).

3.6. Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) is the minimum concentration of the antimicrobial agent which inhibits growth of the organisms. This procedure quantitatively determines the concentration of an antibiotic that is effective in preventing the growth of the pathogen and gives an indication of the dosage of the antibiotic that should be effective in controlling the infection in the patient.

Broth dilution method as per guidelines was employed to determine MIC of confirmed isolates against the drug oxacillin. According to the guidelines, the MIC range was taken to prepare the highest dilution factor and two-fold dilution was made in a series of fifteen 5 ml test tubes.

Preparation of oxacillin dilutions for MIC: The stock solution was prepared and further dilutions were made as follows.
The weight of antibiotic in mg required is calculated using the following formula.

\[ W = \frac{1000}{P \times V \times C} \]

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 \( V \).

\[ W = \frac{1000}{899 \times 2 \times 10} \]
\[ W = 22.246 \text{ mg} \]

In this case the potency of oxacillin used was 899\( \mu \text{g/mg} \). For 2 ml of stock solution with concentration 10,000 mg/l, 22.246 mg of oxacillin was added.

- Working standard solution: Working standard solution of oxacillin was prepared and when required by adding 9 ml of distilled water to a flask containing 1 ml of stock solution of oxacillin.
- Preparation of inoculum: Each clinical isolate was inoculated in 5 ml of sterile nutrient broth held in different test tubes and incubated at 37ºC for 6 hrs till moderate turbidity was developed. The turbidity was matched with 0.5 McFarland’s standard.
- Inoculation: One ml of Mueller-Hinton broth was added in 15 test tubes of 5 ml volume. 2 ml working antibiotic solution was added in the first tube. From the first tube 1 ml was transferred into the second tube further. In this manner it was serially diluted in two folds till 14th test tube. 1 ml was discarded from 14th test tube. The last tube receives no antibiotic and serves as growth control. And 1 ml of the inoculum was added to each tube.
- Incubation and interpretation of results: After inoculation, tubes were incubated at 35ºC for 24 hrs. The incubated tubes were observed for the lowest concentration of oxacillin at which there is no visible growth.

3.7. Antimicrobial Susceptibility Testing (AST)

Antibiogram is one of the most useful and important quality control measure for the verification of results generated on an isolate. Antimicrobial susceptibility testing of all isolates identified was performed qualitatively by Kirby-Bauer’s disc diffusion test and
quantitatively by Minimum Inhibitory Concentration (MIC). Antimicrobial susceptibility test was performed for aerobic bacteria by Kirby-Bauer disk diffusion method, for anaerobic bacteria agar diffusion assay were performed and for fungi disk diffusion and broth micro-dilution method were used

3.7.1. Kirby-Bauer’s disc diffusion method

Antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion method on MHA plates (NCLSI 2008). Disc diffusion is one of the most commonly used Antimicrobial Susceptibility Testing (AST) methods used in the diagnostic laboratories. This is a well-recognized and mostly used technique and also possesses accepted standards including from National Committee for Clinical Laboratory Standards (NCLSI 2008). Methods and interpretive criteria for the disc diffusion methodology are published by NCLSI in Performance Standards for Antimicrobial Disc Susceptibility Tests and are revised and updated as needed.

3.7.1a. Antibiotic discs

The various antibiotic discs and the Mueller-Hinton agar employed in the test for antibiogram were purchased from Hi Media Laboratories, India, they are as follows; Imipenem (10 μg), Aztreonam (10 μg), Ertapenem (10 μg), Cefepime (30 μg), Ceftazidime (30 μg), Ceftazidime/Clavulanic acid (30/10 μg), Amoxicillin (20 μg), Penicillin (20 μg), Cefotaxime (30 μg) and Ceftriaxone (30 μg).

3.7.1b. Media Preparation

MHA was autoclaved and poured into sterile petri-dishes on a level surface to give a uniform depth of 4 mm. The prepared plates were stored in inverted position at 4°C.

3.7.1c. Procedure

A sterile swab was dipped into the overnight grown bacterial inoculum and the excess was removed by rotation of swab against the side of tube above the fluid level. The swab was streaked over the surface of medium. Antibiotic disks were applied on the surface of inoculated plate using sterile forceps and were gently pressed down to ensure even contact with the medium. Plates were incubated at 37°C for 24 hours.
3.7.1d. Interpretation

Diameter of each zone including the diameter of the disk was measured and recorded in mm with the help of zone scale (Hi-Media).

3.8. β-Lactamase Enzyme Studies

Phenotypic methods may not precisely detect all classes of β-lactamases and tends to be consuming more time and this leads to medical therapeutic failure. These types of studies can assist healthcare centers to assess their β-lactamase resistance patterns, thereby providing the use of appropriate bacterial infection control measures.

3.8.1. ESBL detection by phenotypic method

Phenotypic detection of Extended Spectrum β-Lactamase (ESBL) was done by monitoring phenotypically by their susceptibility to β-lactamase inhibitor, clavulinic acid. If the zone size is greater than 5 mm in the presence of inhibitor, it confirms the presence of ESBL. The procedure was similar to the antibiogram test using standard antibiotic discs with the exception of the usage of combined disc with a β-lactam antibiotic and β-lactamase inhibitor, clavulinic acid. i.e. Mueller-Hinton agar plates with and without 10 μg of amoxyclav. (30/10) were used. Susceptibility test results were interpreted according to the criteria established by the NCCLS. A 5 mm increase in the zone diameter of amoxicillin and third generation cephalosporin, with that tested in combination with amoxyclav versus its zone when tested alone was considered indicative of ESBL producing organism (NCLSI, 2008).

3.8.2. AmpC β-Lactamase detection

Cephalosporinase (AmpC β-lactamase) was phenotypically detected by double disc potentiation method based on their resistance pattern to majority of cephalosporin group of β-lactam antibiotics and clavulinic acid.

Ten disc procedure was used to detect AmpC β-lactamase. Mueller Hinton agar plates were prepared and set to room temperature. 0.5 McFarland standard of the organism to be tested was prepared in nutrient broth and evenly spread on to the surface of Muller Hinton agar plate using a cotton swab followed by dispensing the appropriate disks:
Aztreonam (30), Ceftazidime (30), Ceftazidime + clavulante (30/10), Cefotaxime (30), Cefotaxime + clavulante (30/10), Cefoxitin (30), Ceftriaxone (30), Cefepime (30), Ertapenem (10), Imipenem (10) in specified order and incubated the plate overnight at 37ºC and zone of inhibition was measured and recorded. Susceptibility test results were interpreted according to the criteria established by the NCCLS. AmpC β-lactamase strains are resistant to the Cephamycins (i.e., Cefoxitin and Cefotetan) and susceptible to cefepime. High level AmpCβ lactamase producers cause resistance to all I, II and III generation Cephalosporins, the β-lactam-inhibitor drugs and the monobactams (i.e., aztreonam) (Yan et al. 2006).

3.8.3. Extraction of β-Lactamase

β-lactamase enzyme present in the periplasmic space of gram negative bacteria was extracted through centrifugation and sonication as devised by Bonfiglio et al. (1998) with some modifications. Cultures were grown overnight at 37ºC in 50 ml of nutrient broth (1% inoculum) 10% inoculum was diluted in fresh nutrient broth and kept in shaker for 5 hours. The cells were harvested in 6000g, washed twice and suspended in 2 ml of 100 mM phosphate buffer (pH 7.0) and sonicated twice for 30 seconds. The supernatant was centrifuged at 12000g at 4ºC for 20 min served as enzyme source (Bonfigilio et al. 1998).

3.8.4. Partial purification of β-Lactamase

β-lactamase extraction was performed as previously described by Bonfigilio et al. (1998). The clarified supernatant containing the β-lactamase was extensively dialyzed again 50 mM Tris HCl buffer (pH 5.5) and loaded onto a Sepharose S FF column (2.5 × 30 cm) equilibrated with the phosphate buffer. The enzyme was eluted with a linear gradient (0-1M NaCl) in the same buffer; the fractions containing β-lactamase activity were concentrated and the hydrolytic activity against β-lactams was evaluated spectrophotometry.

3.8.5. β-Lactam hydrolysis assay

Partially purified β-lactamase (0.5 ml) was taken and added to 0.5 ml of 0.1 mM β-lactam solution in 100 mM phosphate buffer, (pH 7.0) followed by dilution with 1 ml of 100mM phosphate buffer and the reactions were followed in UV spectrophotometer at an
interval of 1 minute at different wavelengths such as at 265 nm for benzyl penicillin, at 295 nm for ceftazidime. Initially 1.5 ml of 1mM β-lactam solution and phosphate buffer was measured at their respective wavelength before the addition of enzyme. 100 mM phosphate buffer served as blank. The time which shows a greater difference in absorbance between β-lactam solution alone and β-lactam solution with enzyme was recorded. The β-lactam hydrolysis activity per ml per minute was measured using the formula:

\[
\text{Difference in absorbance (O.D)/ Time taken (minutes)} \times 0.5 \text{ ml of enzyme}
\]

One unit of β-lactamase was defined as the change in 0.1 O.D (difference in absorbance) at their respective wavelengths activity per minute per ml of partially purified enzyme extract.

### 3.8.6. Estimation of Protein (Lowry et al. 1951)

It is the most commonly used method for determination of protein because of its high sensitivity and quantities as low as 20 micro gram proteins can be measured. The peptide bonds react with copper sulphate to give a blue colored complex. In addition, tyrosine and tryptophan residues of protein cause reduction of the phosphomolybdate and phosphotungstate components of the folin-ciocalteau reagent to give bluish products. Different aliquots (0.2, 0.4, 0.6, 0.8, 1.0 ml) of BSA were made up to 1 ml with distilled water. 5 ml of alkaline copper sulphate reagent was added to all tubes. The contents were mixed properly, and after 10 minutes 0.5 ml of Folin’s reagent were added. All the tubes were incubated for 30 min at room temperature. The absorbance was read at 660 nm against blank. A standard curve of absorbance at 660nm versus micro gram of BSA was plotted. From this standard curve (mg/g of protein) the amount of protein in the sample was calculated.

### 3.8.7. Kinetic measurements

Kinetic measurements for the β-lactamase activity was carried out by measuring the β-lactam hydrolysis assay at different concentration of β-lactam substrate benzyl penicillin and ceftazidime (0.1mM - 0.6mM) at 15 minutes incubation for a reaction at 37°C and enzyme activity was measured in units (Kwon, 2006). The β-lactam hydrolysis activity per ml per minute was measured using the same formula used to calculate enzyme activity.
Michaelis-Menten kinetic constants $K_m$ and $V_{max}$ were measured using Graph Pad Prism software version 6.0

3.9. Statistical methods

All continuous data were expressed as means ±SE. Data were analysed and compared between different patient groups using the $\chi^2$ test. Correlation coefficient was used to quantify the degree of agreement between the estimated probabilities obtained via logistic regression and via the scoring system for each individual. Data were analyzed using SPSS Statistical Software, version 20.0 (IBM, USA). The level of significance was set at 0.05 for all comparisons.
CHAPTER 4

RESULTS

4.1. Sample Classification

The results of the present retrospective study were recorded as follows:

- Sample classification based on different parameters
- Isolation and identification of bacteria from urine sample
- Antibiotic resistance studies
- β-lactamase enzyme studies

4.1.1. Sample classification based on sex and mode of infection

Among three hundred and thirty six samples collected, 77 samples were from female and 259 samples were collected from male patients, out of the total 336 samples, 156 samples were from hospitalized patients and 180 samples were collected from patients with community acquired infections (Table 4.1).

<table>
<thead>
<tr>
<th>Type of infection</th>
<th>Female</th>
<th>Male</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospitalized infection</td>
<td>32</td>
<td>124</td>
<td>156</td>
</tr>
<tr>
<td>Community acquired infection</td>
<td>45</td>
<td>135</td>
<td>180</td>
</tr>
<tr>
<td>Total patients</td>
<td>77</td>
<td>259</td>
<td>336</td>
</tr>
</tbody>
</table>

Based on this categorization, the current investigation was obvious with more repeated cases of bacterial infection in both hospital and community acquired male
patients. Among the 336 samples collected, 77% were collected from male patients and 23% were collected from female in both hospitalized and community acquired patients.

Among 156 hospitalized patient’s samples, 79% were from male and 21% were from female gender. In this investigation, patients from community infection was exceeds the hospitalized patients with 53% of samples from community category. In all of the above cases, male gender samples associated with both hospitalized and community acquired infection comprise the large number (>70%) and contribute to the overall cases of the bacterial infection. The correlation study analysis was carried out based on gender and type of bacterial infection using SPSS version 20.0 (IBM, USA). The SPSS analysis revealed that the number of male and female; hospitalized and community acquired samples varied significantly at 0.01 level (one tailed test). In the present study, most of the samples were collected from male patients.

4.1.2. Subject classification based on sex and age group

The subjects were further classified into five different groups based on their age, gender and incidence of infection as follows: Children and adolescents (18-20 years), younger adults (20-30 years), adults (30-40 years), mature adults (40-50 years) and older adults (greater than 50). The details of classification of the sample based on gender and age group are presented in Table.4.2. The age group 30-40 and 40-50 years were reported with greater frequency and with lesser prevalence in the age group of 20-30 years. In all the age groups male patients exceeds the female gender and the difference based on gender was fewer in the age group of 18-20 years. In the scene of male gender, more prevalence was seen in the age group of 30-40 (27%) and 40-50 (34%) and in male, higher samples were reported in the age group of 30-40 (28%) and 40-50 (36%).

<table>
<thead>
<tr>
<th>Age/Sex</th>
<th>18-20 years</th>
<th>20-30 years</th>
<th>30-40 years</th>
<th>40-50 years</th>
<th>&gt; 50 years</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>05</td>
<td>11</td>
<td>21</td>
<td>26</td>
<td>14</td>
<td>77</td>
</tr>
<tr>
<td>Male</td>
<td>17</td>
<td>35</td>
<td>72</td>
<td>94</td>
<td>41</td>
<td>259</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>46</td>
<td>93</td>
<td>120</td>
<td>55</td>
<td>336</td>
</tr>
</tbody>
</table>

Table 4.2 Sample classification based on gender and age group
The current investigative study has reported less number of samples from the age group of 18-20 (6%) and 30-40 (27-29%) in the case of both male and female patients. In the male patients the sample size was increased with decrease in age groups and for the females, the samples size is decrease with increased in age group between 30-50 years. In males, the number of samples were consistently retained at standard level with regards to age group (20-40) and in females, the numbers were highly variable (20-100) and they revealed no significant difference.

Statistical assessment of samples based on the gender and age groups revealed no potential variation among gender, but considerable difference was recorded in the age groups at 0.01 level. By interpreting these groups through one-way analysis of variance (ANOVA) in SPSS, age 20-30 and 30-40 years exceptionally varied \((p=0.0197)\) at 0.001 level.

**4.1.3. Sample classification based on age groups and mode of infection**

Collected samples were also compared on the basis of age groups and mode of infection (hospitalized or community acquired). As previously reported, the majority of age groups in community acquired infection patients was more prevalent. Number of samples classified on the basis of five different age groups and mode of infection as reported in Table 4.3. The age group 40-50 years was found with more number of hospitalized infection and the age group 30-50 years in the case of community acquired infections. Very few cases were observed in the age group of 18-20 years in both type of infections.

**Table 4.3 Sample classification based on the age group and mode of infection**

<table>
<thead>
<tr>
<th>Age/Type of Infection</th>
<th>18-20 years</th>
<th>20-30 years</th>
<th>30-40 years</th>
<th>40-50 years</th>
<th>&gt; 50 years</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospital acquired</td>
<td>12</td>
<td>21</td>
<td>42</td>
<td>54</td>
<td>34</td>
<td>156</td>
</tr>
<tr>
<td>Community acquired</td>
<td>10</td>
<td>25</td>
<td>51</td>
<td>66</td>
<td>21</td>
<td>180</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>46</td>
<td>93</td>
<td>120</td>
<td>55</td>
<td>336</td>
</tr>
</tbody>
</table>
Almost all the age groups in community infected patients outnumbered the hospitalized patients with an abnormality in the age group between 50 years which was documented with 21 patients in community infected and 27 samples in hospitalized patients. This would be due to the fact, that above 40 years of age people would tend to have reduced immune level and were more prone to bacterial infectious diseases with frequent hospitalization.

In the age group of 30-50 years, a huge difference was experienced between the hospital and community infected patients and also the hospital infected patients were distributed in the range of 14%-50% and 20-60% in the case of community infected patients.

By examining numerically through multivariate correlation, the age group depends on mode of bacterial infection that varied considerably at 0.001 level and no significant difference was seen between both the hospital and community infected patients based on the age groups.

**4.2. Isolation and characterization of microorganisms from urine sample**

The collected samples were examined for the presence of microbial bacteria within twenty four hours of collection period using calibrated loop method. Only the culture plates showing the bacterial growth were picked for further analysis of antibiotic sensitivity studies. Selected isolated bacterial colonies were further identified based on the morphological and biochemical characterization. Out of the three hundred and thirty six samples collected, there were only two hundred and forty three samples were found to be positive for bacterial growth around 72.3% of collected samples were positive for further identification.

**4.2.1. Morphological and cultural characterization**

MacConkey agar was used to detect the presence of gram negative organisms like *Escherichia coli*, *Klebsiella* spp. *Proteus* spp. and *Pseudomonas* spp. The formation of mucoid with pink color colonies in MacConkey agar plates indicated the presence of lactose positive *Klebsiella* spp. (Fig. 4.1). The formation of colonies of swarming motility indicated the presence of *Proteus* spp. The dry, flat, rough lactose positive rose colony
surrounded by a zone of precipitated bile indicated the presence of *Escherichia coli*. Strong fermentation of lactose with high levels of acid production by the bacteria caused the colonies to develop steadily and showed a color change from bright pink to red (Fig.4.2).

Fig 4.1 *Klebsiella* spp. grown in MacConkey agar plate

Fig 4.2 *Escherichia coli* grown in Blood agar plate
Fig 4.3 *Enterococcus* spp. grown in Mannitol salt agar

Fig 4.4 *Escherichia coli* in EMB agar plate showing metallic sheen colonies
*Pseudomonas* spp. was recorded with absence of lactose fermentation with opaque bacterial colonies in MacConkey agar. *Enterobacter* spp. produced pink colonies in MacConkey agar indicating lactose fermentation and it produced pink mucoid colonies in Eosin Methylene Blue agar (EMB agar).

Bacterial isolates that are collected from the infected sample in the blood agar plate was reported in Fig 4.2. Mannitol salt agar was used to identify the gram positive *Enterococcus* spp. (Fig 4.3). Selective agar medium like EMB agar was further used to confirm the presence of *E. coli* which appeared as greenish metallic sheen colonies (Fig. 4.4).

### 4.2.2. Microscopical characterization

Bacterial isolates obtained in differential media and selective media were further confirmed microscopically through gram staining. Thin smear of the bacterial isolates were stained with crystal violet, Gram’s iodine and safranin. The bacterial isolates that possessed the purple colour of the crystal violet were recorded as gram positive bacterium and that stained the pink colour of safranin were confirmed as gram negative. The morphology of the selected isolates was observed under oil immersion microscope. The majority of the bacterial isolates belong to gram negative followed by small numbers of gram positive isolates in the collected samples (Fig 4.5 & Fig 4.6).

### 4.2.3. Biochemical characterization

The biochemical characterization tests were used to identify the genera of the above confirmed organisms and it utilized the tests including, Indole test, Methyl Red & Voges Proskauer (MR-VP), Glucose fermentation & gas production, Triple Sugar Iron (TSI), Urease, *Haemolysis test*, Citrate Utilization, Oxidase and Catalase identification tests. Almost all the organisms were confirmed at genera level except *Escherichia coli* which was confirmed only in MacConkey agar and EMB agar plates. Results of different biochemical tests confirmed the presence of isolated microorganisms from various sample and are summarized in the Table.4.4.
The results of IMVIC test were reported as follows; *E. coli* and *Morgella* spp. was revealed with positive result for indole and methyl red test, *Klebsiella* spp. and *Enterobacter* spp. reported positive results for all test except the methyl red, *Proteus* spp. and *Providencia* spp. were positive to indole, methyl red and citrate test. *Pseudomonas* spp. was positive only to citrate utilization test. *Enterococcus* spp. was positive for methyl red test. The organisms were identified and speculated based on colony morphology and biochemical reactions.

Fig 4.5 Gram positive coccid colonies under 100X oil immersion

Fig 4.6 Gram negative rod under 100X oil immersion
Table 4.4 Biochemical Characterization of bacterial isolates

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose fermentation</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indole</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Methyl red</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Voges Proskauer</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>+</td>
<td>A/G</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Triple Sugar Iron test</td>
<td>+/-</td>
<td>+/-</td>
<td>-/+</td>
<td>-/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>No H₂S</td>
<td>No H₂S</td>
<td>H₂S</td>
<td>NO H₂S</td>
<td>No H₂S</td>
<td>No H₂S</td>
<td>No H₂S</td>
<td>No H₂S</td>
</tr>
<tr>
<td>Urease test</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Haemolysis test</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

NA - Not Applicable
A/G - Acid and Gas production
+ - Positive result for majority of isolates
- - Negative result for majority of isolates
+/- - Acid butt and slant
-/+- - Alkaline butt and slant
-/+- - Alkaline butt and acidic slant
From the morphological, microscopical and biochemical analysis, two hundred and forty three bacterial isolates were identified and reported. The class of enterobacteriaceae family is the majority (78%) source of the infection in the kidney transplanted patients. Among the enterobacteriaceae, *Escherichia coli* (52%) was the common microbe which forms one-third of the infection, which was followed by *Pseudomonas* spp. (19% of positive samples), *Klebsiella* spp. (12 % of positive samples) and *Proteus* spp. (13% of positive samples). *Enterobacter* spp. were also isolated from four samples (0.8 % of positive sample) and gram positive cocci, *Enterococcus* spp. (2.5%) were also reported, then there was no incidence of *Staphylococcus* infection among the patients. In addition to the previous confirmed organisms *Morganella* spp. and *Providencia* spp. were found to be seen in very few patients; 0.7% and 0.3% respectively.

### 4.3. Antibiotic Susceptibility Test (AST)

Gram negative organisms play a vital role in antibiotic resistance and examining the latter fact, among 243 isolates, 195 isolates were selected including: *Escherichia coli* (86), *Pseudomonas* spp. (43), *Klebsiella* spp. (31) and *Proteus* spp. (25) and gram positive *Enterococcus* spp. (10) for further analysis. *Enterobacter* spp. *Providencia* spp. and *Morganella* spp. were excluded from the susceptibility testing as they were documented only in very only few transplanted patients. Details of antibiotic sensitivity pattern of the selected bacterial isolates are reported in Table 4.5.

Since the present investigative study was centered on the examination of Beta-lactamase enzymes, the following Beta-lactam antimicrobial drugs were picked up for the antibiotic sensitivity studies with the following groups

<table>
<thead>
<tr>
<th>Name of the organism</th>
<th>Number of the organism (n = 195)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>86</td>
</tr>
<tr>
<td><em>Pseudomonas</em> spp.</td>
<td>43</td>
</tr>
<tr>
<td><em>Klebsiella</em> spp.</td>
<td>31</td>
</tr>
<tr>
<td><em>Proteus</em> spp.</td>
<td>25</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp.</td>
<td>10</td>
</tr>
</tbody>
</table>
**Penam:** Penicillin (Carbapenem, Imipenem and Ertapenem and Oxapenams); **Cephem:** third generation Cephalosporins, (Carbacephems, Oxacephams, Ceftriaxone, Cefotaxime, Ceftazidime, Cefixime; fourth generation: Cefepime; **Monobactam:** Aztreonam.

Furthermore homogenous antibiotic discs like Amoxicillin, Imipenem, EDTA, Ceftazidime and Clavulanic Acid (CAC) were used to identify the Beta-Lactamases, Metallo Beta-lactamase and AmpCase enzymes characteristically. Thus, ten standard and common antibiotics were used to report the susceptibility testing of the selected urinary isolates. Table 4.6 provides the details of the regular antibiotics, their significance and the system of susceptibility pattern employed for the antimicrobial testing studies.

**4.3.1. Overall resistance rate of urinary isolates**

All the selected bacterial isolates were showed resistant to penicillin, except *Enterococcus* spp. all the other isolates were reported with 100% of resistance to aztreonam, which was Monobactam group of antibiotics. All the bacterial isolates were showed susceptible to Carbapenem group of antibiotics in which *E. coli* alone shows a zone of intermediate inhibition in Imipenem in fewer samples (<1%). On the other side *Pseudomonas* spp. reported with 100% resistance to Cefixime and Cefepime in all the patients and Amoxicillin shows only 30%; 45% for *Klebsiella* spp. and 35% for *Pseudomonas* spp. and other two antibiotics. *Pseudomonas* spp. recorded overall high resistance with both 3rd and 4th generation antibiotics (Cephem) at exceptional rates compared to the other four isolates.

All the *Escherichia coli* isolates was reported with resistance (>40%) to both 3rd and 4th generation antibiotics with highest range in cefixime. *Klebsiella* spp. (60%) and *Enterococcus* spp. (55%) was recorded with lesser resistance rates to 4th generation antibiotics. In addition, *Klebsiella* spp. encounters an average pattern of resistance to amoxicillin (45%) and lesser to Ceftriaxone (15%) and Cefepime (25%). In compare to this *Proteus* spp. showed higher resistance to 4th generation antibiotics (65%) and lesser to 3rd generation antibiotics except Ceftazidime. *Enterococcus* spp. reported two third of the higher resistance to 3rd generation cefepime (78%) than that of fourth generation cefixime (45%).
Table 4.6 Standard Antibiotic Chart with susceptibility pattern as recommended by National committee for Clinical laboratory standards

<table>
<thead>
<tr>
<th>S. No</th>
<th>Name of the antibiotic disc</th>
<th>Symbol</th>
<th>Disc Concentration (mcg)</th>
<th>Zone Diameter in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Resistance Less than</td>
</tr>
<tr>
<td>1</td>
<td>Amoxicillin</td>
<td>AMX</td>
<td>30</td>
<td>&lt;13</td>
</tr>
<tr>
<td>2</td>
<td>Penicillin</td>
<td>P</td>
<td>10</td>
<td>&lt;16-19</td>
</tr>
<tr>
<td>3</td>
<td>Ceftriaxone</td>
<td>CTR</td>
<td>30</td>
<td>&lt;13</td>
</tr>
<tr>
<td>4</td>
<td>Cefotaxime</td>
<td>CTX</td>
<td>30</td>
<td>&lt;14</td>
</tr>
<tr>
<td>5</td>
<td>Ceftazidine</td>
<td>CAZ</td>
<td>30</td>
<td>&lt;14</td>
</tr>
<tr>
<td>6</td>
<td>Cefixime</td>
<td>CFM</td>
<td>5</td>
<td>&lt;15</td>
</tr>
<tr>
<td>7</td>
<td>Cefepime</td>
<td>CPM</td>
<td>30</td>
<td>&lt;14</td>
</tr>
<tr>
<td>8</td>
<td>Ertapenem</td>
<td>ETP</td>
<td>10</td>
<td>&lt;15</td>
</tr>
<tr>
<td>9</td>
<td>Aztreonam</td>
<td>AT</td>
<td>30</td>
<td>&lt;15</td>
</tr>
<tr>
<td>10</td>
<td>Imipenem</td>
<td>IPM</td>
<td>10</td>
<td>&lt;13</td>
</tr>
</tbody>
</table>

4.3.2. Multidrug resistant pattern of uropathogens

Among all the isolates, *Pseudomonas* spp. was recorded with elevated range of resistance (>70%) for all antibiotics except imipenem. *E. coli* was in the next range with greater than 60% for all antibiotics except imipenem. *Klebsiella* spp. showed lesser rate of resistance to the selected antibiotics. All the selected Gram negative urinary isolates showed resistance at differential rates to all cephalosporins. Antimicrobial resistance to cefepime falls in the range of 40% for *Klebsiella* spp. to 65%, 73% and 79% for *Proteus* spp., *E.coli* and *Pseudomonas* spp. respectively.

*Klebsiella* spp. was more sensitive to third generation Cephalosporin particularly to Ceftriaxone and Ceftazidine than fourth generation Cefepime. Sixteen (24%) isolates of *Klebsiella* spp. and eighteen (25%) isolates of *E. coli* were resistant to all the antimicrobial agents studied except for Imipenem. Seventy seven isolates (66.6%) of *Klebsiella* spp. and twenty six isolates (81.1%) of *E. coli* isolates were resistant to all 3rd generation Cephalosporin antibiotics tested.
Resistance to Etapenem was recorded variably among lactose fermentative and non-fermentative groups of isolates, 51% and 45% of resistance were observed for *E. coli* and *Klebsiella* spp. and higher resistance were noted for *Pseudomonas* spp. (72%), *Proteus* spp. (63%) and gram positive *Enterococcus* spp. (72%). Since, all the bacterial isolates isolated in this study showed complete resistance to penicillin and Aztreonam, except *Enterococcus* spp. which showed less resistance to Aztreonam, these antibiotics can in no way be used to remove these gram negative pathogens. Antibiogram results are presented in Fig.4.7 (sensitive), Fig.4.8 (highly resistant) and Fig.4.9 (moderately resistant).

### 4.3.3. Minimal Inhibitory Concentration

Since the present study was directed towards β-lactamase activity, 3rd generation cephalosporin antibiotics, ceftazidime and cefotaxime were selected for the determination of MIC against multidrug resistant bacterial isolates (n=33). Minimal inhibitory concentration of ceftazidime and cefotaxime on *E.coli* (n=10), *Klebsiella* spp. (n=9), *Pseudomonas* spp. (n=9), *Proteus* spp. (n=3) and *Enterococcus* spp. (n=2) were measured using MIC strips of concentration in the range of 0.001 to 30 μg (0.001, 0.01, 0.1, 1.0, 3.0, 7.5, 15, 30) and 0.01 μg to 240 μg (0.01, 0.1, 5, 10, 30, 60, 120, 240).

Three *E. coli* and eight *Pseudomonas* spp. showed MIC ≥100μg/ml for ceftazidime and four *E. coli*, six *Pseudomonas* spp. and one *Enterococcus* spp. were reported with MIC≥100μg/ml for cefotaxime. Minimal inhibitory concentration for ceftazidime in the range of 50-100 μg/ml was observed in five *E. coli*, one *Pseudomonas* spp. one *Klebsiella* spp. and one *Proteus* spp. MIC values ≥50, ≤100 μg/ml for cefotaxime were reported in five *E. coli*, three *Pseudomonas* spp. six *Klebsiella* spp. two *Enterococcus* spp. MIC values less than 20 μg/ml were recorded in one *E. coli*, eight *Klebsiella* spp. three *Enterococcus* spp. for ceftazidime and three *Klebsiella* spp. and three *Proteus* spp. for cefotaxime, respectively. Table 4.7 reports the level of MIC of Ceftazidime and Cefotaxime on selected bacterial isolates and MIC of bacterial isolate measured in disc is also reported (Fig.4.10).
Fig 4.7 Antibiogram of a urinary isolate sensitive to Clavulinic Acid (CAC)

Details of antibiotics: 1- Clavulinic Acid (CAC) surrounded by 2-CZX, 3-CAZ, 4-IPM & 5-CTX

Fig 4.8 Antibiotic sensitivity pattern of urinary bacterial sample against selected nine β-lactam antibiotics.
Left Plate: 1-AMC, 2-ceftazidime, 3-ertapenem, 4- cefixime & 5-CXM
Right plate: 1-AMC, 2-amoxicillin, 3-cefotaxime & 4- ceftriaxone
The isolate was resistant to all antibiotics except ertapenem
Fig 4.9 Antibiotic sensitivity pattern of urinary bacterial sample against selected nine β-lactam antibiotics using AMC.
Left plate: 1- Clavulinic Acid (CAC), 2- ceftazidime, 3-ceftriaxone, 4-cefepime & 5-cefixime.
Right plate: 1- Amoxycillin/Clavulinic Acid, 2-Cefotaxime, 3-Ertapenem, 4-imipenem & 5- Aztreonam.
The isolate was resistant to all except CAC, CTR and IPM.

Fig 4.10 Minimal inhibitory concentration of ceftazidime on urinary bacterial isolate
Table 4.7 Minimal inhibitory concentration of Ceftazidime and Cefotaxime against urinary pathogens

<table>
<thead>
<tr>
<th>Name of the Isolate</th>
<th>Name of the antibiotic</th>
<th>MIC (µg)/ml (no of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (n=10)</td>
<td>Ceftazidime</td>
<td>15-20 (n=1); 50-100 (n=5); 100-240 (n=3)</td>
</tr>
<tr>
<td></td>
<td>Ceftazidime</td>
<td>15-20 (n=0); 50-100 (n=5); 100-240 (n=4)</td>
</tr>
<tr>
<td><em>Pseudomonas</em> spp.(n=9)</td>
<td>Ceftazidime</td>
<td>15-20 (n=0); 50-100 (n=1); 100-240 (n=8)</td>
</tr>
<tr>
<td></td>
<td>Ceftazidime</td>
<td>15-20 (n=0); 50-100 (n=3); 100-240 (n=6)</td>
</tr>
<tr>
<td><em>Klebsiella</em> spp. (n=9)</td>
<td>Ceftazidime</td>
<td>15-20 (n=8); 50-100 (n=1); 100-240 (n=0)</td>
</tr>
<tr>
<td></td>
<td>Ceftazidime</td>
<td>15-20 (n=3); 50-100 (n=6); 100-240 (n=0)</td>
</tr>
<tr>
<td><em>Proteus</em> spp. (n=3)</td>
<td>Ceftazidime</td>
<td>15-20 (n=1); 50-100 (n=1); 100-240 (n=0)</td>
</tr>
<tr>
<td></td>
<td>Ceftazidime</td>
<td>15-20 (n=3); 50-100 (n=0); 100-240 (n=0)</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp.(n=3)</td>
<td>Ceftazidime</td>
<td>15-20 (n=3); 50-100 (n=0); 100-240 (n=0)</td>
</tr>
<tr>
<td></td>
<td>Ceftazidime</td>
<td>15-20 (n=0); 50-100 (n=2); 100-240 (n=1)</td>
</tr>
</tbody>
</table>

4.4. β-lactamase enzyme studies

The second part of the research focused on the prevalence of antibiotic resistance enzymes: Extended Spectrum β-Lactamases (ESBL) and Cephalosporinase (AmpCase) in selected gram negative bacterial isolates (n=31).

4.4.1. Prevalence of β-lactamase enzymes in bacterial isolates

Prevalence of β-lactamase enzymes among the selected urinary isolates is reported in Figure 4.6.

All the isolates were reported with the enzymes. Among the two enzymes ESBL seemed to be more prevalent than AmpC β-lactamase. Presence of ESBL and AmpC β-lactamase are shown in separate plates. Among the ESBL enzymes recorded, 53% was
occupied by *E. coli* 19% by *Klebsiella* spp. 16% by *Pseudomonas* spp. and 12% by *Proteus* spp. AmpC β-lactamase enzyme was reported in 51% of *E. coli* followed by 23% of *Klebsiella* spp. 20% of *Pseudomonas* spp. and 7% of *Proteus* spp. *E. coli* was reported with the increased incidence of both ESBL and AmpC β-lactamase.

In all the bacterial isolates, ESBL exceeded the AmpC β-lactamase. AmpC β-lactamase showed nearly one third of the ESBL. *Proteus* spp. showed the least rate of the occurrence of both the enzyme. Presence of ESBL and AmpC β-lactamase decreased in the order of *E. coli*, *Klebsiella* spp. *Pseudomonas* spp. and *Proteus* spp. ESBL in *E. coli* was the most prevalent β-lactamase enzyme. The most potent AmpC β-lactamase enzyme was reported in lower levels. *E. coli* was reported with the highest level of β-lactamase enzymes.

### 4.4.2. Biochemical detection of extended spectrum β-lactamase in urinary isolates

From the bacterial isolates reported with the β-lactamase enzymes, seven *E. coli*, four *Pseudomonas* species, three *Klebsiella* species and two *Proteus* species were further selected (n=16) for the detection of ESBL activity. Table 4.8 reports the β-lactamase units of the selected isolates. The isolates showed reduction in O.D value after the addition of enzyme which implies the breakdown of the β-lactam ring structure by β-lactamase. Bacterial isolates showing absence of ESBL and AmpCase and sensitive to most of the antibiotics were used as negative control for enzyme assay.

In *E. coli*, the strain N1R showed the highest activity (23.33 U/ml/min) and the strain N76R showed the least activity (7.4 U/ml/min). The negative control *E. coli* previously reported with absence of ESBL through phenotypic detection, showed traces of β-lactam hydrolysis with activity of 0.07 U/ml/min. Other *E.coli* strains showed moderate activity of ESBL in the range of 10-17 U/ml/min. Among four *Pseudomonas* spp. N1S was reported with increased activity and others were in the range of 12-14 U/ml/min. *Klebsiella* spp. was observed with less activity than the other three species. The topmost activity in *Klebsiella* spp. was 16.15 U/ml/min measured in N12B strain. Maximum rate of activity was 22.82 U/ml/min in N14R strain of *Proteus* spp. Negative control species selected for measuring enzyme activity showed activity less than 1 U/ml/min.
# Table 4.8 β-Lactamase activity of urinary bacterial samples

<table>
<thead>
<tr>
<th>Name of the isolate</th>
<th>β-lactamase units/ml/min</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC <em>E. coli</em> (N87R)</td>
<td>0.57 ± 0.001</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> (N1R)</td>
<td>23.33 ± 0.01****</td>
<td>162.01</td>
</tr>
<tr>
<td><em>E. coli</em> (N9R)</td>
<td>13.07 ± 0.013****</td>
<td>136.15</td>
</tr>
<tr>
<td><em>E. coli</em> (N15R)</td>
<td>11.05 ± 0.002****</td>
<td>32.89</td>
</tr>
<tr>
<td><em>E. coli</em> (N19R)</td>
<td>17.24 ± 0.014****</td>
<td>67.87</td>
</tr>
<tr>
<td><em>E. coli</em> (N34R)</td>
<td>10.02 ± 0.021****</td>
<td>63.4</td>
</tr>
<tr>
<td><em>E. coli</em> (N76R)</td>
<td>7.4 ± 0.32****</td>
<td>30.33</td>
</tr>
<tr>
<td><em>E. coli</em> (N121R)</td>
<td>12.27 ± 0.16****</td>
<td>82.9</td>
</tr>
<tr>
<td>NC <em>Pseudomonas</em> spp. (N69S)</td>
<td>0.04 ± 0.0015</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas</em> spp.(N5S)</td>
<td>14.42 ± 0.031****</td>
<td>62.15</td>
</tr>
<tr>
<td><em>Pseudomonas</em> spp.(N15S)</td>
<td>17.97 ± 0.011****</td>
<td>67.05</td>
</tr>
<tr>
<td><em>Pseudomonas</em> spp.(N35S)</td>
<td>12.86 ± 0.14****</td>
<td>68.4</td>
</tr>
<tr>
<td><em>Pseudomonas</em> spp.(N67S)</td>
<td>12.19 ± 0.05****</td>
<td>66.25</td>
</tr>
<tr>
<td>NC <em>Klebsiella</em> spp. (N302B)</td>
<td>0.6 ± 0.02</td>
<td>-</td>
</tr>
<tr>
<td><em>Klebsiella</em> spp. (N12B)</td>
<td>16.15 ± 0.16**</td>
<td>68.43</td>
</tr>
<tr>
<td><em>Klebsiella</em> spp. (N17B)</td>
<td>11.78 ± 0.012**</td>
<td>74.55</td>
</tr>
<tr>
<td><em>Klebsiella</em> spp. (N32B)</td>
<td>10.66 ± 0.01**</td>
<td>74.02</td>
</tr>
<tr>
<td>NC <em>Proteus</em> spp.(N300T)</td>
<td>0.4 ± 0.01</td>
<td>-</td>
</tr>
<tr>
<td><em>Proteus</em> spp.(N5T)</td>
<td>21.7 ± 0.002***</td>
<td>63.82</td>
</tr>
<tr>
<td><em>Proteus</em> spp.(N14T)</td>
<td>22.82 ± 0.019***</td>
<td>64.83</td>
</tr>
</tbody>
</table>
Assay of β-lactamase at 265 nm using benzyl penicillin as substrate resulted in the activity at the range from 7.0 to 23.33 U/ml per min. Highest level of ESBL activity was reported in *E. coli* and *Proteus* spp. *Pseudomonas* spp. showed a moderate activity of ESBL. Negative control for enzyme activity was taken from the respective isolates which showed the absence of β-lactamase enzyme (ESBL and AmpCase) through phenotypic detection [*E. coli* (N₈₇R), *Klebsiella* spp. (N₉₀S), *Pseudomonas* spp. (N₃₀₂B) and *Proteus* spp. (N₃₀₀T)].

Specific activity of the ESBL enzyme was found to be highly variable with 162.01 in *E. coli* N₁₁R and 30.33 in *E. coli* N₇₆R. Other isolates (*Pseudomonas* spp., *Klebsiella* spp. and *Proteus* spp.) were reported with specific activity in the range of 60-70 with highest values of 74.55 in *Klebsiella* spp., 68.4 in *Pseudomonas* spp. and 64.83 in *Proteus* spp.

Statistical comparison of the ESBL enzyme activity in seven positive isolates of *E. coli* with that of control strain *E. coli* (N₈₇R), using ANOVA: Design 1 between Subject Factor in ezanova software reported with the following results, ESBL F (1, 12) = 41.8 \( p < 0.000031 \) SS=583.60 MSe=13.97[Control] vs [Test] t (12) = 6.46 \( p < 0.0001 \). Thus there was a significant difference in the ESBL enzyme activity in the selected positive strains.

Statistical comparison of the ESBL enzyme activity in four positive isolates of *Pseudomonas* spp. with that of control strain *Pseudomonas* spp. (N₆₀S), using ANOVA: Design 1 between Subject Factor in ezanova software reported with the following results, ESBL in *Pseudomonas* spp. F (1, 6) = 115 \( p < 0.000039 \) SS=384.20 MSe=3.33. [Control] vs [Test] t (6) =10.74 \( p < 0.0001 \).

Statistical comparison of the ESBL enzyme activity in four positive isolates of *Pseudomonas* spp. with that of control strain *Pseudomonas* spp. (N₆₀ S), using ANOVA: Design 1 between Subject Factor in ezanova software reported with the following results,
ESBL in *Pseudomonas* spp. $F (1, 6) = 115 \ p<0.00039 \ SS=384.20 \ MSe=3.33. \ \text{[Control]} \ vs \ [Test] \ t (6) =10.74 \ p<0.0001.$

Statistical comparison of the ESBL enzyme activity in three positive isolates of *Klebsiella* spp. with that of control strain *Klebsiella* spp. (N302 B), using ANOVA: Design 1 between Subject Factor in ezanova software reported with the following results: ESBL in *Klebsiella* spp. $F (1, 4) = 53.6 \ p<0.001851 \ SS=225.58 \ MSe=4.21. \ \text{[Control]} \ vs \ [Test] \ t (4) =7.32 \ p<0.0019.$

Statistical comparison of the ESBL enzyme activity in two positive isolates of *Proteus* spp. with that of control strain *Proteus* spp. (N306 T), using ANOVA: Design 1 between Subject Factor in ezanova software reported with the following results, ESBL in *Proteus* spp. $F (1,2) = 1524 \ p<0.000656 \ SS=477.86 \ MSe=0.31. \ \text{[Control]} \ vs \ [Test] \ t (2) =39.04, \ p<0.0007.$

Thus through statistical tests, it was confirmed that the ESBL hydrolytic activity in all the phenotypically positive strains of differed significantly. From the ESBL studies, it can be concluded that *E.coli* N1R was found to have higher activity and *E. coli* N76R with lowest activity.

Apisarnthanarak et al. (2007) & Pullukcu et al. 2007 demonstrated the efficient hydrolysis of the amide bond of the β-lactam ring yielding biologically inactive product. Serine β-lactamases acylate β-lactam antibiotics, much like PBPs, and then use strategically positioned water molecules to hydrolyze the acylated β-lactam. In this manner, the β-lactamase is regenerated and can inactivate additional β-lactam molecules. Hassan et al. (2007) and Popescu and Doyle (1996) reported that most of the ESBL strains (n=43) were recovered from urine (24 strains) or sputum samples (19 strains) and all of them were resistant to cefotaxime, ceftazidime or ceftriaxone. Jacoby and Munozprice (2005) reported that new extended spectrum cephalosporins with an oxyimino side chain, carbapenems, cephamycins, and monobactams were introduced due to the growing number of β-lactamases in *E. coli* and *K. pneumoniae*. The present study recorded the enzyme activity in the sequence of *E.coli, Proteus* spp., *Pseudomonas* spp., and *Klebsiella* spp.
## Table 4.9 β-Lactamase (AmpC β-lactamase) activity of urinary bacterial pathogens

<table>
<thead>
<tr>
<th>Name of the isolate</th>
<th>β-lactamase units/ml/min</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC E. coli (N87R)</td>
<td>0.91 ± 0.03</td>
<td>-</td>
</tr>
<tr>
<td>E. coli (N12R)</td>
<td>22.8 ± 0.12***</td>
<td>132.51</td>
</tr>
<tr>
<td>E. coli (N54R)</td>
<td>16.69 ± 1.5***</td>
<td>59.61</td>
</tr>
<tr>
<td>E. coli (N91R)</td>
<td>21.74 ± 0.13***</td>
<td>89.09</td>
</tr>
<tr>
<td>NC Pseudomonas spp. (N69S)</td>
<td>0.83 ± 0.09</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas spp.(N11S)</td>
<td>22.88 ± 0.26****</td>
<td>133.02</td>
</tr>
<tr>
<td>Pseudomonas spp.(N12S)</td>
<td>17.65 ± 0.11****</td>
<td>44.125</td>
</tr>
<tr>
<td>Pseudomonas spp.(N18S)</td>
<td>21.06 ± 2.3****</td>
<td>65.81</td>
</tr>
<tr>
<td>Pseudomonas spp.(N45S)</td>
<td>21.71 ± 0.012****</td>
<td>67.84</td>
</tr>
<tr>
<td>Pseudomonas spp.(N96S)</td>
<td>25.24 ± 0.23****</td>
<td>75.12</td>
</tr>
<tr>
<td>NC Klebsiella spp. (N302B)</td>
<td>0.07 ± 0.001</td>
<td>-</td>
</tr>
<tr>
<td>Klebsiella spp. (N8B)</td>
<td>21.73 ± 1.2****</td>
<td>57.18</td>
</tr>
<tr>
<td>Klebsiella spp. (N20B)</td>
<td>17.92 ± 0.01****</td>
<td>56.7</td>
</tr>
<tr>
<td>Klebsiella spp. (N24B)</td>
<td>18.07 ± 0.05****</td>
<td>53.14</td>
</tr>
<tr>
<td>Klebsiella spp. (N30B)</td>
<td>18.83 ± 0.15****</td>
<td>54.74</td>
</tr>
<tr>
<td>Klebsiella spp. (N32B)</td>
<td>19.39 ± 0.14****</td>
<td>49.46</td>
</tr>
<tr>
<td>Klebsiella spp. (N34B)</td>
<td>18.13 ± 0.25****</td>
<td>51.5</td>
</tr>
<tr>
<td>NC Proteus spp.(N30T)</td>
<td>0.99 ± 0.01</td>
<td>-</td>
</tr>
<tr>
<td>Proteus spp.(N1T)</td>
<td>12.14 ± 0.15</td>
<td>43.36</td>
</tr>
</tbody>
</table>
4.4.3. Biochemical detection of Cephalosporinase

For detection of AmpC β-lactamase, three *E.coli*, five *Pseudomonas* spp., six *Klebsiella* spp. and one *Proteus* spp. were selected (n=15). Table.4.9 reported the activity of AmpC β-lactamase with ceftazidime as substrate.

Activity of AmpCase was in the range of 12-25 U/ml/min, with the lowest activity being observed in *Proteus* spp. (12.14 U/ml/min) and highest activity being reported in *Pseudomonas* spp. (25.24 U/ml/min). *E. coli*, strain N12R (22.8 U/ml/min); *Pseudomonas* spp. N98S (25.24 U/ml/min); *Klebsiella* spp. N3B (21.73 U/ml/min) being reported with highest activity among the selected fifteen bacterial isolates. Specific activity of the enzyme helps to measure the purity of the enzyme. Since the enzyme was partially purified, the specific activity was highly variable among different species. *E.coli* strain N12R (132.51) and the *Pseudomonas* spp. N11S (133.01) were found to be with highest specific activity. The least specific activity for AmpC β-lactamase with 43.36 and 44.125 was reported in the *Proteus* spp. N1T and *Pseudomonas* spp. N12S, respectively.

For the enzyme activity, negative control was taken from the respective isolates which showed the absence of β-lactamase enzyme (ESBL and AmpC β-lactamase) through phenotypic detection [*E. coli* (N87R), *Klebsiella* spp. (N69S), *Pseudomonas* spp. (N302B) and *Proteus* spp. (N300T)].

Statistical comparison of the AmpC β-lactamase enzyme activity in three positive isolates of *E.coli* with that of control strain *E.coli* (N87), using ANOVA: Design 1 between Subject Factor in ezanova software reported with the following results, AmpC β-lactamase in *E.coli* F(1,4) = 107 p<0.000493 SS=570.38 MSe=5.33.[control] vs [Test] t(4)=10.34 p<0.0005.
Thus there was a significant difference in the AmpC β-lactamase enzyme activity in the selected positive E.coli strains. Statistical comparison of the AmpC β-lactamase enzyme active in three positive isolates of Pseudomonas spp. with that of control strain Pseudomonas spp. (N67), using ANOVA: Design 1 between Subject Factor in ezanova software reported with the following results, AmpCase in Pseudomonas spp. F(1,8)=284 p<0.000001 SS=1089.73 MSe=3.84 [control] vs [Test] t(8)=16.84, p<0.0001. Statistical comparison of the AmpC β-lactamase activity in three positive isolates of Klebsiella spp. with that of control strain Klebsiella spp. (N67), using ANOVA: Design 1 between Subject Factor in ezanova software reported with the following results, AmpCase F(1,10)=1033 p<0.000001 SS=1076.36 MSe=1.04 [Control] vs [Test] t(10)=32.14, p<0.0001.

Alangaden (2007) defined AmpC β-lactamases as Group I cephalosporinases that confer resistance to a wide variety of β-lactam antibiotics including alpha methoxy β-lactams such as cefoxitin, narrow and broad spectrum cephalosporins (cefotetan, ceftiraxone, and cefotaxime) aztreonam, and are poorly inhibited by β-lactamase inhibitors such as clavulanic acid.

Biochemical characterization performed with penicillin and ceftazidime to quantify the substrate specificity and hydrolyzing efficiency of the β-lactamase showed that more amount of enzyme is detected in E.coli and Proteus spp. for ESBL and Pseudomonas spp. for AmpC β-lactamases. In Proteus spp. higher units for ESBL and lower units for AmpC β-lactamases infer that Proteus species can resist β-lactam inhibitor only to a certain extent. Klebsiella spp. was phenotypically and biochemically reported with less rate of ceftazidime hydrolysis.

4.4.4. Michaelis-Menten kinetics of ESBL

Kinetic parameters (maximal hydrolysis rates) and $K_m$ (binding affinity) (Table 4.10) were further calculated by measuring the velocity of enzyme ESBL at different concentrations of substrate (0.1mM - 0.6mM of benzylpenicillin). Graph-pad Prism version 6 software was utilized to calculate and $V_{max}$. E. coli N1R was reported with increased $V_{max}$ (60.64) followed by Klebsiella spp. N12B (54.08), Proteus spp. (52.67). The highest value of ESBL $V_{max}$ in Pseudomonas spp. was reported to be 47.62. $K_m$ was low in
Proteus spp. N₅T (0.12) and N₁₄T (0.128) followed by *E. coli* N₀R (0.1308). *Klebsiella* spp. was reported with highest values of $K_m$ than the other three species.

### 4.4.5. Michaelis-Menten kinetics of Cephalosporinase

Kinetic parameters $K_m$ and $V_{max}$ of AmpC β-lactamase (Table 4.11) were further calculated by measuring the velocity of enzyme AmpC β-lactamase at variable concentrations of substrate (0.1mM- 0.6mM of ceftazidime). Graph-pad Prism software version 6 was utilized to calculate $K_m$ and $V_{max}$. Maximum velocity of cephalosporinase (AmpCβ-lactamase) was observed in *Pseudomonas* spp. N₁₂S (67.34) and N₉₆S (60.1). *Klebsiella* spp. formed the next level with 59.54 in N₃₈B. Maximal velocity of AmpCase was observed in *E.coli* strain N₁₂R and Proteus spp. N₁T with 56.21 and 54.65, respectively. *E.coli* strain N₁₉R was reported with lowest $K_m$ (0.1198) and *Proteus* spp. N₁T was reported with highest $K_m$ (0.2747).

Bush and Fisher (2011) & Bush (1986) reported that a complete set of $V_{max}$ and $K_m$ values should be available for every β-lactamase and substrate profiles indicating the range of β-lactam antibiotics hydrolyzed and hydrolytic parameters of β-lactamases provide some of the most definitive characteristics of β-lactamases. Benzyl penicillin has been used as the historical reference for studying comparisons in β-lactam hydrolysis. The hydrolysis profile of novel β-lactamases can be studied by utilizing the extended-spectrum cephalosporins: cefotaxime and ceftazidime (Charfeddine et al. 2005).

Studies on two important β-lactamases ESBL and AmpC β-lactamase in selected isolates with phenotypic detection and hydrolytic efficiency on β-lactam antibiotics: benzyl penicillin and ceftazidime led to a conclusion that both the enzymes are prevalent in Gram negative urinary isolates at different rates with increased incidence of ESBL in *E. coli* followed by *Proteus* spp. Specific activity of ESBL and AmpC β-lactamase was elevated in *E.coli* spp. which differed from the enzyme activity report. Maximal velocity for ESBL was reported in *E. coli* and maximal velocity of AmpC β-lactamase was found in *Pseudomonas* spp. which was similar to that of enzymes specific activity.
Table 4.10 Michaelis-Menten Kinetics ($K_m$ and $V_{max}$) of ESBL by Urinary bacterial Pathogens

<table>
<thead>
<tr>
<th>Name of the isolate</th>
<th>Kinetic parameters</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (mM)</td>
<td>$V_{max}$ (umol/min)</td>
<td></td>
</tr>
<tr>
<td>$E. coli$ (N$_{1R}$)</td>
<td>0.1456</td>
<td>60.64</td>
<td></td>
</tr>
<tr>
<td>$E. coli$ (N$_{9R}$)</td>
<td>0.1308</td>
<td>32.5</td>
<td></td>
</tr>
<tr>
<td>$E. coli$ (N$_{12R}$)</td>
<td>0.1832</td>
<td>33.8</td>
<td></td>
</tr>
<tr>
<td>$E. coli$ (N19R)</td>
<td>0.171</td>
<td>48.2</td>
<td></td>
</tr>
<tr>
<td>$E. coli$ (N$_{34R}$)</td>
<td>0.2215</td>
<td>40.66</td>
<td></td>
</tr>
<tr>
<td>$E. coli$ (N$_{76R}$)</td>
<td>0.2847</td>
<td>37.91</td>
<td></td>
</tr>
<tr>
<td>$E. coli$ (N$_{121R}$)</td>
<td>0.1562</td>
<td>35.4</td>
<td></td>
</tr>
<tr>
<td>$Pseudomonas$ spp.(N$_{5S}$)</td>
<td>0.1596</td>
<td>42.58</td>
<td></td>
</tr>
<tr>
<td>$Pseudomonas$ spp.(N$_{15S}$)</td>
<td>0.142</td>
<td>44.97</td>
<td></td>
</tr>
<tr>
<td>$Pseudomonas$ spp.(N$_{35S}$)</td>
<td>0.1651</td>
<td>36.76</td>
<td></td>
</tr>
<tr>
<td>$Pseudomonas$ spp.(N$_{67S}$)</td>
<td>0.2182</td>
<td>47.62</td>
<td></td>
</tr>
<tr>
<td>$Klebsiella$ spp. (N$_{12B}$)</td>
<td>0.1843</td>
<td>54.08</td>
<td></td>
</tr>
<tr>
<td>$Klebsiella$ spp. (N$_{17B}$)</td>
<td>0.2653</td>
<td>51.95</td>
<td></td>
</tr>
<tr>
<td>$Klebsiella$ spp. (N$_{32B}$)</td>
<td>0.2415</td>
<td>42.55</td>
<td></td>
</tr>
<tr>
<td>$Proteus$ spp.(N$_{5T}$)</td>
<td>0.128</td>
<td>51.42</td>
<td></td>
</tr>
<tr>
<td>$Proteus$ spp.(N$_{15T}$)</td>
<td>0.1286</td>
<td>52.67</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.11 Michaelis-Menten Kinetics ($K_m$ and $V_{max}$) of AmpC β-lactamase by Urinary bacterial pathogens

<table>
<thead>
<tr>
<th>Name of the isolate</th>
<th>Kinetic parameters (Enzyme only)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (mM)</td>
<td>$V_{max}$ (umol/min)</td>
</tr>
<tr>
<td>E. coli (N\textsubscript{12}R)</td>
<td>0.1442</td>
<td>56.21</td>
</tr>
<tr>
<td>E. coli (N\textsubscript{54}R)</td>
<td>0.1401</td>
<td>41.17</td>
</tr>
<tr>
<td>E. coli (N\textsubscript{91}R)</td>
<td>0.1198</td>
<td>49.3</td>
</tr>
<tr>
<td>Pseudomonas spp.(N\textsubscript{11}S)</td>
<td>0.1587</td>
<td>59.74</td>
</tr>
<tr>
<td>Pseudomonas spp.(N\textsubscript{13}S)</td>
<td>0.2305</td>
<td>67.34</td>
</tr>
<tr>
<td>Pseudomonas spp.(N\textsubscript{18}S)</td>
<td>0.1292</td>
<td>53</td>
</tr>
<tr>
<td>Pseudomonas spp.(N\textsubscript{43}S)</td>
<td>0.1133</td>
<td>48.95</td>
</tr>
<tr>
<td>Pseudomonas spp.(N\textsubscript{96}S)</td>
<td>0.1392</td>
<td>60.1</td>
</tr>
<tr>
<td>Klebsiella spp. (N\textsubscript{8}B)</td>
<td>0.1542</td>
<td>56.78</td>
</tr>
<tr>
<td>Klebsiella spp. (N\textsubscript{20}B)</td>
<td>0.1682</td>
<td>50.18</td>
</tr>
<tr>
<td>Klebsiella spp. (N\textsubscript{24}B)</td>
<td>0.1502</td>
<td>44.85</td>
</tr>
<tr>
<td>Klebsiella spp. (N\textsubscript{30}B)</td>
<td>0.1701</td>
<td>53.7</td>
</tr>
<tr>
<td>Klebsiella spp. (N\textsubscript{32}B)</td>
<td>0.1623</td>
<td>53.34</td>
</tr>
<tr>
<td>Klebsiella spp. (N\textsubscript{34}B)</td>
<td>0.1865</td>
<td>59.54</td>
</tr>
<tr>
<td>Proteus spp.(N\textsubscript{1}T)</td>
<td>0.2747</td>
<td>54.65</td>
</tr>
</tbody>
</table>
CHAPTER 5

DISCUSSION

This study presents a comprehensive bacterial profile of pre and post kidney transplantation of the transplant recipients and their effective management strategies. With the increased incidence of diabetes, and other reasons globally, there is increasing problem of failure in kidney functions of the individuals. Organ transplantation is considered as the standard therapy for the failure of solid organs including liver, heart, lungs and kidney (Barkholt et al. 2003). Several advanced surgical techniques and therapies were available to improve the quality and survival rate of the transplanted patients. The immunosuppressive therapy used after transplantation usually results in the complications including graft rejections and infections (Hooton 2000).

Bacterial infections are the most common infections in the immunosuppressive therapy. The kidney transplant recipients possess higher incidence of bacterial infections followed by lung transplant recipients, liver transplant recipients and heart transplant recipients (Burroughs and Moscona 2004). Another problem is with the development of multi-drug resistance among the bacteria because of frequent use of antibiotics by the physicians, efforts were made to study the profile of infection post transplantation and to see the associations of different study characteristics.

Among the isolated bacteria, *Escherichia coli* is found to be prevalent followed by *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* which are commonly isolated from body fluids collected from general population all around the world (Hirsch and Randhawa 2009). Klieg et al. (2008) also reported *Escherichia coli* (31%), followed by species of *Pseudomonas, Klebsiella* and *Enterobacter* as most prevalent among organ transplanted patients. It may be attributed by the life style, poor healthcare system, lack of education, and also may be due to geographical variations.
Antibiogram studies are employed for the analysis of resistance pattern of microbial pathogens against standard antibiotics and make a suitable antibiotic prescription for pathogens. Antibiotic susceptibility and resistance are highly variable according to genus and species and to certain extent within species. This test should be done to individual species isolated from different samples and it will be more precise to have a unique standard profile for each laboratory. The following general patterns of susceptibility hold well for most of the hospitals, although individual modifications may occur. For example, *Escherichia coli* is found to be resistant towards ampicillin and cephalothin; *Klebsiella pneumoniae* is resistant to ampicillin and carbenicillin whereas *Klebsiella oxytoca* is sensitive to carbenicillin *etc*.

With an objective to study the antibiotic susceptibility pattern of the bacterial pathogens existing in the kidney transplant recipients, the present research work was initiated with the isolation of bacteria from the different biological samples. Samples were collected from kidney transplant patients who had the symptoms of infection like frequent micturition, or pain during urination. Three hundred and thirty six samples of Mid-Stream Urine (MSU), pus, sputum, Cerebrospinal Fluid (CSF), catheter tips and blood from the kidney transplant recipients were collected. The samples collected for the present analysis were classified based on their gender, age and infection type (Hospitalized & Community acquired).

Hooton (2000); Memikoglu et al. (2007) & Barkholt et al. (1999) reasons out that the bacterial infection was more prevalent in female patients due to the shorter urethra near the anus. The findings was supported by Badaruddin and Memon (2007), who stated that among females UTI are common and the majority of the infections are observed in outpatients and many are treated empirically by symptoms without analyzing their susceptibility pattern (Alangaden et al. 2006).

In contrast to above said reports in this research male patients are more in number then comparative. It may be due to the bad habits of common men inherit all sorts of disease, that includes kidney problem also, in India prevalence of diabetic is very very normal in our population.
Similar result was observed by Gonzalez and Schaeffer (1999) and Asher et al. (1986). In contrast, around 50% of samples from outpatients were found to possess bacterial infections as reported by Grossi et al. 2002; Jiang et al. (2006) & Lockhart et al. (2007). Aboderin et al. (2009) also reported that high incidence of bacterial infection was found in female hospitalized patients and male outpatients. Colodner et al. (2004) reported that from the lower urinary tract, through the bloodstream, bacteria can reach the kidney through the bloodstream or from the lower urinary tract. Bacterial infection becomes established when the body’s natural defense mechanism is overcome by bacterial antigenic substance or intrinsic (urinary calculi) or extrinsic factors (catheterization). Once the bladder was infected, the ascending infection was based on microbial virulence factor (Eshwarappa et al. 2011 & Bashir et al. 2008). The major risk factors identified for infection includes, sexual intercourse, spermicide contraception and history of UTIs (Manges et al. 2008). Very few reports were available to study the risk factors associated with E. coli infection based on demographic and clinical data (Akram et al. 2008; Muthuselvam et al. 2008 & Hafeez et al. 2009). Hanssen et al. (2004) stated that large scale and local studies of surveillance are required for understanding the drug resistance in the selected community.

Bacterial infections in rural areas are most often associated with community acquired infections. As already stated in various reports, the present investigation also noted with an increased number bacterial infection from male patients social behavior of men in public life. From the present study, it can be concluded that bacterial infections are more prevalent in both hospital acquired and community acquired male patients. These studies carried along with the details of gender and source of infection may help to identify their prevalence at different geographical locations (Bashir et al. 2008).

Mulvey and Simor (2009) had reported that UTI are the second most common bacterial infection in young children, with few signs or symptoms other than fever. Kader and Angamuthu (2005) had reported that 77.5% of bacterial pathogens were from females and 1.6% was from patients less than 25 years, 25.3% were from patients with 20 to 50 years, and 73.1% were from patients above 50 years with the mean age of 47.7 years (Aboderin et al. 2009). From the results, it can be concluded that for bacterial infections, the mean age group of female falls in the range of 30-50 years. In male it is not confined to any particular age group.
Akram et al. (2008) have reported that the bacterial infections are more in patients with 30-49 years (51.04 %), principally women (66.66%) than in pediatric patients (36.45%). According to Marshall and Wilmoth (2009), the infection rate was high in the male patients (77.8%) compared to female (22.2%) and the group between 25 years and 45 years were at high risk (38.7%). Mekki et al. (2010) had reported that the rate of prevalence of bacterial infection were 49.6% in mature adults (40 to 58 years), 26.8% in adults (25 to 40 years), 15.0% in younger adults (15 to 25 years) and 8.6% in children’s (5 to 15 years). Thus from the results, it can be concluded that bacterial infections were more prevalent in females with community acquired infections in the age group of 40-50 years. Thus concluding that the bacterial infection is common nowadays in all group of people irrespective of their gender and age. The infection should be properly treated or else the bacteria may become more potent biologically threatening organisms (Jarvis and Martone 2012)

Eryilmazi et al. (2010) suggest that community acquired bacterial infections caused significant illness in the mid age group and are considered as a common disease in mature adults and older adults, particularly in India. Csete (2008) and Crouzet et al. (2007) had reported that catheter associated UTI comprises the largest reservoir of antibiotic resistance bacterial pathogens. Akram et al. (2008) have confirmed that UTI was the most common infections diagnosed in outpatients and hospitalized patients. In contrast to our study, many previous studies are reported with lesser percentages of positive cultures. Akram et al. (2008) have reported that only 10.86% (n=100) urine samples gave significant growth of pathogens and Eswarappa et al. (2011) reported with 9.1% (n=501) positive samples. In the present study, majority of the collected samples showed positive results. The bacterial isolates developed were further characterized by morphological, cultural and biochemical characterization.

Rajaduraipandi et al. (2006); Schwalbe et al. (2007) & Salembekhit et al. (2012) reported that 73.56% of Pseudomonas strains isolated from clinical isolates collected at Government Medical College Hospital, Nagur had MIC as low as 2 μg/ml and 5.71% of the Pseudomonas strains were having MIC as high as 1024 μg/ml. From the results reported for MIC, E. coli and Pseudomonas spp. were associated with higher values of MIC and hence supporting their multidrug resistant property.
Gram negative organisms develop antimicrobial resistance compared to that of gram positive isolates because of the nature of its cell wall. Among the gram negative, *E. coli*, *Klebsiella* species, *Pseudomonas* species and *Proteus* species are frequently encountered in urinary tract infections. β-lactam are the major antibiotics group with sub classes. So the work was restricted to Gram negative isolate’s resistance towards β-lactam antibiotics through β-lactamase enzymes. Previous reports were focused towards resistance on 2nd and 3rd generation cephalosporins and the present work additionally included 4th generation cephalosporin antibiotics.

Extended Spectrum β-Lactamase and AmpCase were detected based on their inhibition towards clavulanic acid. The urinary isolates which are resistant to amoxicillin and increase their zone size in the presence of Amoxicillin and Clavulanic Acid (AMC) are considered as ESBL producers and the urinary isolates resistant to majority of second, third and fourth generation cephalosporins as well as their combined disc with clavulanic acid are considered as AmpCase (Nzeako 2006). Lazinska et al. (2005) reported the emergence of novel ESBLs in *E. coli* and *Klebsiella pneumoniae* after prolonged use of the extended-spectrum cephalosporins cefotaxime and ceftazidime. Nazareth and King (2009) reported that the major mechanism of bacteria develops resistance to β-lactam antibiotics by the production of β-lactamases which inactivate the β-lactam antibiotics by hydrolyzing the β-lactam ring.

Anthony and Hill (1988) defined cephalosporinases as enzymes capable of hydrolyzing all β-lactams and they act as an important mediator of antimicrobial resistance in gram negative bacilli. Dharnidharka et al. (2004) reported the occurrence of AmpCβ-lactamase in 47.3% of clinical isolates and four-fifths of them occurred in combination with ESBL’s. Khadri et al. (2007) reported that the AmpC β-lactamases are frequently observed in isolates of *Klebsiella pneumoniae* and *Klebsiella oxytoca* (72%), *Escherichia coli* (62.5%) and *Salmonella* spp., *Proteus mirabilis* etc., and the higher incidence of AmpC producing isolates may reflect two modes of production: hyper production of chromosome mediated AmpC and plasmid mediated AmpC β-lactamases.

Thomson et al. (2001) reported that the Gram negative bacteria require more control measures against the concern of their resistance. Trouillhet et al. (2005) suggested that the ESBL production was observed in 41% of *E. coli*. But Valdez-Ortiz et al (2011)
proposed that the high ratio of ESBL production is noted more in *Klebsiella* species (67.4%) than the *E. coli* (62.3%) and added that ESBL producers were susceptible towards meropenem (99.8%) and cefaperazone (93.3%). The results of the present investigation reported with higher incidence of ESBL in *E. coli* and it coincides with the result of Agrawal et al. (2008) & Nitin et al. (2011) who reported ESBL in the rate of 49, 38 and 13% for *E. coli*, *K. pneumoniae* and *K. oxytoca* respectively and higher resistance rate was recorded for 3rd generation cephalosporins. β-lactamase enzymes become as an important hurdle in the antibiotic therapy. Hence, routine screening techniques are essential to monitor β-lactamases for suitable antibiotic therapy. Continuous monitoring methods followed with strict antibiotic policy can reduce the prevalence of these enzymes resulting in a better therapeutic regimen.

Thus, the bacteria isolated from the biological samples were initially characterized, based on their morphological behavior in differential and selective medium. Both lactose fermenters and non-lactose fermenters were distributed in the samples with high proportion of lactose positive isolates (Kanj et al. 2008).

Bacterial infection was more commonly caused by members of the family enterobacteriaceae in the sequence of *E. coli* (70-80%), *Proteus mirabilis* (10%), *Klebsiella* spp. (5%), *Staphylococcus arophyticus* (10%), *Staphylococcus epidermis*, *Enterococcus* (5%), *Pseudomonas aeruginosa* (2%) and other coliforms (<1%) (Green et al. 2011). Bashir et al. (2008) reported that *E.coli* was the most prevalent bacterial pathogen followed by *Enterococcus* spp., *Candida* spp. and *Pseudomonas* spp., *Klebsiella* spp. and *Enterobacter* spp. In a similar study carried out in Iran reported with *Escherichia coli* (57.4%), *Klebsiella pneumoniae* (9.7%), *Enterobacter aerogenes* (7.0%), *Staphylococcus aureus* (5.8%), *C. fruendii* (5.1%), *Proteus mirabilis* (4.5%), *Pseudomonas aeruginosa* (3.2%), *Acetobacter baumannii* (2.2%), coagulase-negative *Staphylococcus* (3.2%) and *Enterococcus fecalis* (1.9%) (Bean et al., 2008; Mashouf et al., 2009). Babic et al (2006) also reported higher incidence of gram negative bacteria (87%) than gram positive bacteria (13%).

*E.coli* was reported as the common and major bacterial pathogen by many reports (Maragakis and Perl 2008; Gephart et al. 1981; Aruna and Mobashshera 2012 & Baduruddin and Memon 2007). Alsahli and Abdulkhair (2011) stated that the dominance
of *E.coli* might be due to the reason that bacterial infections were derived from patients’ own fecal flora. *Escherichia coli* was followed by *Klebsiella* spp. as reported by Dharnidharka et al. 2006 & Yan et al. (2006). From the results, it can be concluded that majority of the bacteria isolated were gram negative, belonging to enterobacteriaceae family and only very few cases were reported with gram positive isolates (*Enterococcus* spp.). Identification of bacteria was followed by antibiotic susceptibility test to identify their resistance pattern (Jabar and Mossawi 2007 & Khanfar et al. 2009).

The increased resistance is the result of many factors, but the foremost cause is the overall volume of antibiotic consumption (Waldvogel 2000; Bush 2012 & Whiting et al. 2006). Diekema (2001) suggested that the usage of antibiotics like fluoroquinolones (35%), cephalosporins (27%), amionoglycosides (15%) and Cotrimoxazole (9%).

Similar to the present study, Mashouf et al. (2009) also reported that among gram negative bacteria *Pseudomonas aeruginosa* was the most resistant isolates and among gram positive urinary isolates, *Enterococcus faecalis* showed highest resistance, followed by *Staphylococcus* spp. Jiang et al. (2006) reported that 48% (n=34) of *Pseudomonas* spp. showed resistance to extended spectrum β-lactam group including aztreonam. Hafeez et al. (2009) suggested carbapenems as alternative source for those isolates resistant towards cephalosporins. Velasco et al. (2007) concluded that resistance for β-lactam agents shows variability among different species. In contrast to the present study, resistance to imipenem was reported with a rate of 8.05% in *Pseudomonas* spp. by Tomasz (1994). Yuksel et al. (2006) suggested that ertapenem retains good activity towards Multidrug resistance bacteria but the limitation is it requires higher concentration than the imipenem.

Antibiogram studies were further analyzed by the comparison of overall resistance percentage observed in selected urinary isolates against the ten β-lactam antibiotics. It shows the overall resistance percentage by selected bacterial isolates towards β-lactam antibiotics. *Pseudomonas* spp. showed the highest resistance followed by *E.coli.* *Enterococcus* spp. and *Proteus* spp. showed resistance at a similar rate. *Klebsiella* spp. showed the least percent of resistance towards β-lactam antibiotics (Hryniewicz et al. 2001). When combined together, all the isolates showed increased resistance, thus giving a stress that majority of urinary isolates were multidrug resistant and care should be taken during the prescription of antibiotics.
Rossolini and Mantengoli (2005) & Lockhart et al. (2007) have reported that *P. aeruginosa* is the major bacterium with the problem of Multi-drug resistance. Neelam Taneja (2009) & Jiang et al. (2006) isolated strains of *K. pneumoniae* from different clinical samples which is resistant towards cephalosporins, aminoglycosides, carbapenems, fluoroquinolones together with the Tri-Methoprim (TMP) and cotrimoxazole and other antibiotics. Kahlmeter et al. (2003) stressed that the resistance has increased over the years highly variable from country to country and can be controlled with the help of surveillance data. As bacterial resistance has many of the features of an epidemic disease, strict rules should be formulated to prevent the antibiotic resistant organisms. Periodic change of antimicrobials may also help to avoid the development of antibiotic resistant microorganisms.

Multidrug resistance was recorded to study the antibiotic resistance pattern in different bacterial isolates and gives the multidrug resistance pattern observed. Only a very few bacterial isolates were found to be resistant towards nine antibiotics with an exception to *Pseudomonas* spp. *Pseudomonas* spp. is sensitive to at least two antibiotics whereas, *E. coli* was found to be resistant to six antibiotics (Nicolle 2008; Osazuwa et al. 2011 & Olowe et al. 2008). *Proteus* spp. were resistant to four antibiotics and in *Enterococcus* spp. were resistant to five antibiotics. Among the patterns observed, *Klebsiella* spp. forms the least with approximately 1% resistant to nine antibiotics and 29% resistant towards three antibiotics.

Rao and Chandrakanth (2008) recorded the co resistance pattern in *Pseudomonas* spp. that ceftazidime resistant strains were found to be resistant to cefuroxime, cefotaxime and ticaricillin. Similar to the present findings, higher frequency of Multi-drug resistance was recorded with *Pseudomonas* spp. against β- lactam antibiotics. Compared with many earlier reports, the present investigation is notable with high resistance towards aztreonam, ertapenem and cefepime. Based on the Multidrug resistance pattern and phenotypic detection of β-lactamase enzyme, 33 uropathogens were selected for further analysis, which included *E. coli* (n=10), *Klebsiella* spp. (n=9), *Pseudomonas* spp. (n=9), *Proteus* spp. (n=3) and *Enterococcus* spp. (n=2).
5.1 Conclusion

From the present retrospective analysis it is concluded that majority of the urinary tract bacterial strains were gram negative organism, belonging to enterobacteriaceae family and only very few patients were observed in \textit{Enterococcus} spp. of gram positive isolates. Only selected Gram negative bacterial isolates were analyzed for their resistance pattern towards various antibiotics and selected β-lactam antibiotics. All the selected bacterial strains were resistant to penicillin, with an exception to \textit{Enterococcus} spp. all the bacterial isolates were recorded with 100% of resistance to monobactam group of antibiotics aztreonam. All the isolates were susceptible to carbapenems group: Imipenem with an exception to \textit{E. coli} which showed zone of intermediate in lesser cases (<1%).

Since all the bacterial isolates in this study showed complete resistance to penicillin and aztreonam, except \textit{Enterococcus} spp. which showed less resistance to aztreonam, these antibiotics would not be able to use to remove these gram negative microbes. Based on the drug resistance pattern and phenotypic detection of β-lactamase enzyme 33 bacterial isolates were selected for further analysis; \textit{E. coli} (12), \textit{Klebsiella} spp. (10), \textit{Pseudomonas} spp. (8), \textit{Proteus} spp. (4) and \textit{Enterococcus} spp. (2).

At the same time more than 99.9% of the selected isolates were sensitive to imipenem which helps to make a conclusion that the geographical region where the work was carried out is free from metallo β-lactamase, which is showing an alarming rise in some parts of Tamil Nadu. \textit{E. coli} and \textit{Pseudomonas} spp. were associated with higher values of MIC thus supporting their antibiotic resistant property.

Samples from the transplanted patients were analyzed for the presence of single type of bacteria with greater than $10^5$ colonies. 79.2% of the samples were positive, thus reporting that the urinary tract infection is more frequent nowadays in all groups of people irrespective of their gender, age and geographical location. Based on the morphological, microscopical and biochemical analysis, more than hundred bacteria were identified at genus level. The members of enterobacteriaceae family are responsible for the majority of the infection. Among the enterobacteriaceae, \textit{Escherichia coli} was the common pathogen which caused one third of the infection, followed by \textit{Pseudomonas} spp. \textit{Klebsiella} spp. \textit{Proteus} spp. \textit{Enterobacter} were isolated from only three to five patients and among gram positive cocci, \textit{Enterococcus} spp. (2.3%) are report and there was no incidence of
Staphylococci infection in the present study. In addition to the above said organisms Morgenella spp. (0.6%) and Providencia spp. (0.4%) were reported in very few cases.

The antibiotic resistance rates of bacterial isolates detected from culture was found to be resistant to most of the antibiotics. The development of extensive use of aminoglycosides, cephalosporins and fluoroquinolones in the management of bacterial infections might led to development of multi-resistance among the bacteria. It is found that, Ampicillin is continued to lose their sensitivity so it is better to avoid it in empiric treatment of bacterial infection management post transplantation. Thus, the present study analyzed, common risk factors associated with post transplant bacterial infection and also implemented a new prophylaxis antimicrobial regimen for the treatment of post-operative infection against commonly used antibiotics and also analyzed the β-lactamase enzyme activity and its associated genes pattern.