STUDIES ON MULTI-DRUG RESISTANCE IN URINARY TRACT INFECTION PATIENTS

ABSTRACT

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

BIOTECHNOLOGY

BY

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2009
Abstract
Abstract

Urinary tract infections (UTIs) remain the common infections diagnosed in outpatients as well as hospitalized patients. A major concern to the medical community is the increasing occurrence of multi-drug resistant (MDR) E. coli, presenting challenges to the empirical treatment of UTI. Therefore, current knowledge on antimicrobial susceptibility pattern is essential for appropriate therapy. ESBL producing bacteria may not be detectable by routine disk diffusion susceptibility test, leading to inappropriate use of antibiotics and treatment failure. The aim of this study was to determine the distribution and antibiotic susceptibility patterns of bacterial strains isolated from patients with community acquired urinary tract infections (UTIs) at Aligarh hospital in India as well as identification of ESBL producers in the population of E. coli and Klebsiella spp. isolates. Urinary isolates from symptomatic UTI cases attending to the J.N. Medical College and hospital at Aligarh were identified by conventional methods. Antimicrobial susceptibility testing was done by Kirby Bauer’s disc diffusion method. Isolates resistant to third generation cephalosporin were tested for ESBL production by double disk synergy test method. Of the 920 tested samples, 100 samples showed growth of pathogens among which the most prevalent were E. coli (61%) followed by Klebsiella sp (22%). The majority (66.66%) of the isolates were from women while the remaining were from men. Among the gram-negative enteric bacilli high prevalence of resistance was observed against ampicillin and co-trimoxazole. Most of the isolates were resistant to 4 or more number of antibiotics. 42% of isolates were detected to produce ESBL among which 34.42 % were E. coli isolates. This study revealed that E. coli was the predominant
bacterial pathogen of community acquired UTIs in Aligarh, India. This study also demonstrated an increasing resistance to ampicillin and Co-trimoxazole and production of extended spectrum β-lactamase among UTI pathogens in the community.

India is not excluded from the problems encountered world-wide in the treatment of community acquired urinary tract infections, commonly caused by different members of family Enterobacteriaceae. These pathogenic bacteria produces enzymes that include the β-lactamases and extended-spectrum β-lactamases (ESBLs) capable of hydrolysing the β-lactam antibiotics and in particular the expanded-spectrum cephalosporins which are frequently used. These bacteria are often multiple resistant. Transmission of these bacterial strains between patients is a serious problem in community settings. Antimicrobial resistance genes are often clustered in integrons, genetic elements capable of recombination, which are responsible for the spread of varied resistant markers in the community.

We have also determined the prevalence of integrons among ESBLs producing *Escherichia coli* causing community acquired urinary tract infections (UTIs). All ESBLs producing *E. coli* isolates were tested for integrons by amplifying 5'CS and 3'CS region, the class of integrons was further confirmed by specifically amplifying Int I and SulI genes. Of the twenty two isolates tested, seven were found to have class 1 integrons. DNA sequencing of these isolates showed the presence of multiple gene cassettes i.e. aadA1, aadA5, dfr7, dfrI7, aacA4, and CmlA1. This study demonstrated that class 1 integrons are widely prevalent in Indian isolates and hence they might play
a role in multiple drug resistance in *E. coli* of community acquired urinary tract infections.

We also examined the role of β-lactamases in resistance development in commonly encountered pathogens implicated in urinary tract infections. Resistance to the β-lactam agents, ceftriaxone, ceftazidime, cefotaxime, cefoxitin and penicillins was suspected to involve the presence of one or more β-lactamases in the isolates from patients attending OPD at J.N.M.C. hospital. 34% of all the *E. coli* isolates were found positive for ESBLs production. These β-lactamase producers were characterized by genetic analysis (DNA amplification by PCR) to investigate the presence of possible genes responsible for resistance development. β-lactam resistance could be attributed to the presence and action of β-lactamases such as the TEM, SHV and CTX-M type enzymes. The most common group of ESBLs not belonging to the bla*TEM* or bla*SHV* families were termed bla*CTX-M*. The designation “CTX-M” refers to potent activity against cefotaxime, an aminotiazolil cephalosporin with a methoximino group; these enzymes have only remnant activity toward ceftazidime, another aminotiazolil cephalosporin with a 2-carboxy-2-oxypropaneimino group instead of the methoximino group. These enzymes are inhibited by clavulanic acid, sulbactam and tazobactam. There are only few reports available on prevalence of CTX-M beta-lactamases in some parts of India. Therefore we determined the prevalence of these enzymes in our region by using PCR. Among all isolates, only three isolates were found to be positive for presence of CTX-M gene. As reported earlier by many groups that bla*CTX-M* enzymes were plasmid mediated, in our study we found that all three isolates harbor bla*CTX-M* genes on chromosomal DNA. This is first report from India showing the
presence of bla \textit{CTX-M} gene on bacterial chromosome. Nucleotide sequence analysis of these PCR products revealed that all the three amplicons belong to CTX-15 type beta-lactamases.

To evaluate the role of plasmids in spread of drug resistance we carried the conjugation experiments. We found that eighteen of the twenty two strains showed transfer of multiple resistance markers from donor to recipient susceptible strain EJ-53. Transfer frequency of these resistance markers varied between $3.17 \times 10^{-6}$ and $5.2 \times 10^{3}$. We further investigate the genotypes of the twenty two ESBLs producing \textit{E. coli} strains by PFGE. Pulsed-field gel electrophoresis (PFGE) of XbaI digests was performed to determine the genetic similarity amongst isolates showing multidrug resistance. The isolates investigated were genotypically diverse and divided in various types and subtypes by applying criteria of Tenover.

It is quite alarming to note that almost all of the isolates included in this study were found resistant to four or more antibiotics. This study concludes that \textit{E. coli} and other isolates were more sensitive to imipenem and amikacin compared to the other antibiotics tested and therefore these may be the drugs of choice for the treatment of community-acquired UTIs in our region. This study also shows that the integrons are one of the means of antibiotic resistant marker transfer among members of \textit{Enterobacteriaceae}. Our findings unveil the increasing role of the bla-\textit{CTX-M} \textbeta-lactamases in antibiotic resistance and emphasize on the significance of appropriate empirical treatment for infections caused by coliforms, especially in urinary tract infection patients. This study also shows that the horizontal gene transfer of
Abstract

antimicrobial resistance genes can occur very efficiently and at a high rate among *Enterobacteraceae* in a community setting.
Certificate

I certify that the work embodied in the thesis entitled “Studies on multi-drug resistance in urinary tract infection patients”, is an original work, unless otherwise stated, carried out by Mr. Mohammad Akram under my supervision and is suitable for submission in fulfillment of the requirements for the award of Doctor of Philosophy in Biotechnology.

Asad Ullah Khan, PhD
Assoc. Professor
Declaration

I, hereby, declare that the thesis entitled "Studies on multi-drug resistance in urinary tract infection patients" embodies the work carried out by me.

Mohammad Akram
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To my family, who offered me unconditional love and support.
Acknowledgements
I bow in utmost reverence to the “Almighty Allah” (Most beneficent and Most Merciful) whose benevolent sanction led me to produce this “oeuvre” in its present façade and giving me the strength to pass through difficult periods and complete this task. Completing a PhD is truly a marathon event, and I would not have been able to complete this journey without the aid and support of countless people over the past four years.

I must first express my gratitude towards my supervisor, Dr. Asad Ullah Khan, whose invaluable guidance, support, attention to detail, hard work, expertise, understanding, and patience, added considerably to my research experience. Dr. Asad is the one teacher who truly made a difference in my life. It was under his tutelage that I developed a focus and became interested in research. I also appreciate his vast knowledge and skill in many areas (e.g., molecular and microbiological techniques), and his assistance in writing reports (i.e., grant proposals, research papers and this thesis), which have on occasion made me “GREEN” with envy. He provided me with direction, moral support and became more of a friend than a mentor. His persistence, enthusiasm and kindness have helped me to complete this work. I doubt that I will ever be able to convey my appreciation fully, but I owe him my eternal gratitude.

I thank Prof. M. Saleemuddin, Coordinator, Interdisciplinary Biotechnology Unit, for his constant support in providing me all the facilities for this study. I also thank other faculty members in the department, Dr. R. H. Khan, Dr. M. Owais, and Dr. H. Yunus for the assistance they provided at all levels of the research work.

I would like to thank Dr. M. Shahid from the Faculty of Medical Microbiology, J. N. M. C. H for taking time out from his busy schedule to serve as my Co-supervisor. He provided timely and instructive comments and insights that guided and challenged my thinking, substantially improving the finished work.
Acknowledgements

Appreciation also goes out to Mr. Lal Mohammad, Mr. Faisal Maqbool, Mr. Iqtedar, and Mr. Ramesh Singh, for all of their computer and technical assistance throughout my research program, and to the Mr. Amir, Mr. Isham Khan, Mr. Chanderpal Singh, Mr. Nasir, Mr. Aslam, Mr. Mashqoor, Mr. Rajinder, Mr. Rajesh, and Mr. Zakir, office staff for all the instances in which their assistance helped me along the way.

Special thanks to those postgraduate students who have worked with me along the way: Shariq, Afroz, Ayesha, Shazi, Bilal and Aanisa.

I also thank some of my fellow Ph.D. students: Tahseen, Iram, Rubab, Anees, Sayeed, Danish, Faraz, Mairaj, Hafeez, Nazia, Sadaf, Sahar, Zainab, Nishat, Ankita, Munzsa, Sana, Rosina, Priyankar, Ejaz, Eizaj, Maroof, Varun, Azmat, Arun, Qamar, Javed, Rabban and Badar.

I am really at loss of words in thanking Barira and Shahper for their whole hearted co-operation and frequent suggestions to me in the lab at various stages during the course of my work. The present work bears at every stage the impress of their collaborative efforts. I shall ever remain indebted to them. They each helped make my time in the PhD program more fun and interesting. I appreciate you both and would not have made it without you!

Deepest thanks to one of my best friend Atif. He has been through the thick and thin of my life and I don't have right words to describe his love, support and sacrifice. May Allah give him what he wants in life.

I cannot thank enough Zakir for your advice and guidance and for your willingness to support me through the writing process—your help make the Ph.D. journey more enjoyable while redefining the meaning of "format" at the same time!

To the rest of my colleagues and juniors — thanks for their insightful feedback and words of encouragement.

I would also like to thank my friends in the hostel, particularly Ahmad Raza Faruqi, Ameer Ahmad, Hasan, Kaleem and Tanseer for our philosophical
Acknowledgements

debates, exchanges of knowledge, skills, and venting of frustration during my research program, which helped enrich the experience.

I would also like to thank my family for the support they provided me through my entire life and in particular, I must acknowledge my brother and best friend, Aslam Wali, without whose love, encouragement and moral support, I would not have finished this thesis.

Finally, I would be remiss without mentioning Dr. Jacoby and Dr. M. Grape, whose extreme generosity will be remembered always.

In conclusion, I recognize that this research would not have been possible without the financial assistance of CSIR, Human Resource and Development Group, India, and express my gratitude to this agency.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>%</td>
<td>Percent</td>
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<tr>
<td>μg/ml</td>
<td>Microgram per milli litre</td>
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<tr>
<td>&lt;</td>
<td>Less than</td>
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<tr>
<td>&gt;</td>
<td>Greater than</td>
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<tr>
<td>≤</td>
<td>Less than or equal to</td>
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<tr>
<td>≥</td>
<td>Greater than or equal to</td>
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<tr>
<td>μg</td>
<td>Microgram</td>
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<td>μl</td>
<td>Microlitre</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<td>et al</td>
<td>and others</td>
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<td>hr</td>
<td>Hour</td>
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<tr>
<td>μg/l</td>
<td>Microgram per litre</td>
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<td>min</td>
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<td>sec</td>
<td>Second</td>
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<td>ml</td>
<td>Millilitre</td>
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<td>Millimolar</td>
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<td>μM</td>
<td>Micromolar</td>
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<tr>
<td>nM</td>
<td>Nano Molar</td>
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<tr>
<td>ng</td>
<td>Nano gram</td>
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<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>pH</td>
<td>The Negative logarithm of H⁺ concentration</td>
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<tr>
<td>rpm</td>
<td>Revolution per minute</td>
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<td>gm</td>
<td>Gram</td>
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<td>Sp. gr.</td>
<td>Specific gravity</td>
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<td>Kb</td>
<td>Kilobase</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
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<tr>
<td>CFU</td>
<td>Colony Forming Unit.</td>
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<tr>
<td>F</td>
<td>Forward</td>
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<tr>
<td>R</td>
<td>Reverse</td>
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<tr>
<td>mA</td>
<td>Milli Ampere</td>
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<tr>
<td>RE</td>
<td>Restriction enzyme</td>
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<td>M</td>
<td>Molar</td>
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<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<td>UTIs</td>
<td>Urinary tract infections</td>
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<tr>
<td>SMX</td>
<td>Trimethoprim and sulfamethoxazole</td>
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<tr>
<td>SXT</td>
<td>Co-trimoxazole</td>
</tr>
<tr>
<td>3'CS</td>
<td>3' Conserved region</td>
</tr>
<tr>
<td>5'CS</td>
<td>5' Conserved region</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>LGT</td>
<td>Lateral gene transfer</td>
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<tr>
<td>MRE</td>
<td>Multidrug-resistant Enterobacteriaceae</td>
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<tr>
<td>ESBLs</td>
<td>Extended spectrum beta-lactamases</td>
</tr>
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<td>VRE</td>
<td>Vancomycin resistant Enterobacteriaceae</td>
</tr>
<tr>
<td>JNMCH</td>
<td>Jawahar Lal Nehro Medical College</td>
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<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
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<tr>
<td>PFGE</td>
<td>Pulsed field gel electrophoresis</td>
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<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>EMB</td>
<td>Eosin methylene blue</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
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<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis (β-Aminoethyl Ether) N,N,N',N'- tetra acetic acid</td>
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<tr>
<td>NB</td>
<td>New born</td>
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<tr>
<td>TEM</td>
<td>Temoniera</td>
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<tr>
<td>SHV</td>
<td>Sulphhydryl variable</td>
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<tr>
<td>ExPEC</td>
<td>Extra intestinal pathogenic <em>E. coli</em></td>
</tr>
<tr>
<td>UPEC</td>
<td>Uropathogenic <em>E. coli</em></td>
</tr>
<tr>
<td>PBP's</td>
<td>Penicillin binding proteins</td>
</tr>
<tr>
<td>PABA</td>
<td>Para amino benzoic acid</td>
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<tr>
<td>DHPS</td>
<td>Dihydropteroate synthetase</td>
</tr>
<tr>
<td>DHF</td>
<td>Dihydrifolic acid</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrifolic acid reductase</td>
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<tr>
<td>MRSA</td>
<td>Methicillin resistant <em>Staphylococcus aureus</em></td>
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<tr>
<td>VRSA</td>
<td>Vancomycin resistant <em>Staphylococcus aureus</em></td>
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<tr>
<td>VISA</td>
<td>Vancomycin intermediate <em>Staphylococcus aureus</em></td>
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<tr>
<td>CDC</td>
<td>Centre for disease control and prevention</td>
</tr>
<tr>
<td>Cep²</td>
<td>Second generation cephalosporins</td>
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<tr>
<td>Cep³</td>
<td>Third generation cephalosporins</td>
</tr>
<tr>
<td>Cep⁴</td>
<td>Fourth generation cephalosporins</td>
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<tr>
<td>Amn</td>
<td>Aminoglycosides</td>
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<td>Mon</td>
<td>Monobactam</td>
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<td>Co</td>
<td>Cotrimoxazole</td>
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<td>T</td>
<td>Tetracycline</td>
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<tr>
<td>Nf</td>
<td>Nitrofurantoin</td>
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<td>Car</td>
<td>Carbapenems</td>
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Figure 1.1. Common causes of urinary tract infections. The percentage of infections caused by different bacteria in outpatients is shown (Taken from Mims et al., 1998).

Figure 1.2. Flow chart showing the LOGIC system for identification of Gram negative bacilli isolated from urine (Taken from Collee et al., 1996).

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Figure 4.2(B). PCR amplification of 5'-CS-3'-CS conserved sequences of integron. Lane M: 1 Kb DNA ladder, Lane P: Positive control, Lane 1-3: E. coli clinical isolates (UE-61, UE-90, UE-152) and Lane 4: Negative control.

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Figure 4.3(B). Integron mapping by PCR amplification of IntI gene. Lane M: 1Kb DNA ladder, Lane P: Positive control, Lane 1-5: E. coli clinical isolates (UE-137, UE-152, UE-170, UE-144, UE-103) and Lane 6: Negative control.

Figure 4.4 (A). Integron mapping by PCR amplification of SulI gene. Lane M: 1Kb DNA ladder, Lane P: Positive control, Lane 1-5: E. coli clinical isolates (UE-61, UE-90, UE-112, UE-128, UE-133) and Lane 6: Negative control.

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Abstract
Abstract

Urinary tract infections (UTIs) remain the common infections diagnosed in outpatients as well as hospitalized patients. A major concern to the medical community is the increasing occurrence of multi-drug resistant (MDR) *E. coli*, presenting challenges to the empirical treatment of UTI. Therefore, current knowledge on antimicrobial susceptibility pattern is essential for appropriate therapy. ESBL producing bacteria may not be detectable by routine disk diffusion susceptibility test, leading to inappropriate use of antibiotics and treatment failure. The aim of this study was to determine the distribution and antibiotic susceptibility patterns of bacterial strains isolated from patients with community acquired urinary tract infections (UTIs) at Aligarh hospital in India as well as identification of ESBL producers in the population of *E. coli* and *Klebsiella spp.* isolates. Urinary isolates from symptomatic UTI cases attending to the J.N. Medical College and hospital at Aligarh were identified by conventional methods. Antimicrobial susceptibility testing was done by Kirby Bauer's disc diffusion method. Isolates resistant to third generation cephalosporin were tested for ESBL production by double disk synergy test method. Of the 920 tested samples, 100 samples showed growth of pathogens among which the most prevalent were *E. coli* (61%) followed by *Klebsiella sp* (22%). The majority (66.66%) of the isolates were from women while the remaining were from men. Among the gram-negative enteric bacilli high prevalence of resistance was observed against ampicillin and co-trimoxazole. Most of the isolates were resistant to 4 or more number of antibiotics. 42% of isolates were detected to produce ESBL among which 34.42% were *E. coli* isolates. This study revealed that *E. coli* was the predominant
bacterial pathogen of community acquired UTIs in Aligarh, India. This study also
demonstrated an increasing resistance to ampicillin and Co-trimoxazole and
production of extended spectrum β-lactamase among UTI pathogens in the
community.

India is not excluded from the problems encountered world-wide in the
treatment of community acquired urinary tract infections, commonly caused by
different members of family Enterobacteriaceae. These pathogenic bacteria produces
enzymes that include the β-lactamases and extended-spectrum β-lactamases (ESBLs)
capable of hydrolysing the β-lactam antibiotics and in particular the expanded-
spectrum cephalosporins which are frequently used. These bacteria are often multiple
resistant. Transmission of these bacterial strains between patients is a serious problem
in community settings. Antimicrobial resistance genes are often clustered in integrons,
genetic elements capable of recombination, which are responsible for the spread of
varied resistant markers in the community.

We have also determined the prevalence of integrons among ESBLs producing
*Escherichia coli* causing community acquired urinary tract infections (UTIs). All
ESBLs producing *E. coli* isolates were tested for integrons by amplifying 5'CS and
3'CS region, the class of integrons was further confirmed by specifically amplifying
Int 1 and Sul1 genes. Of the twenty two isolates tested, seven were found to have class
1 integrons. DNA sequencing of these isolates showed the presence of multiple gene
cassettes i.e. aadA1, aadA5, dfr7, dfr17, aacA4, and CmlA1. This study demonstrated
that class 1 integrons are widely prevalent in Indian isolates and hence they might play
a role in multiple drug resistance in *E. coli* of community acquired urinary tract infections.

We also examined the role of β-lactamases in resistance development in commonly encountered pathogens implicated in urinary tract infections. Resistance to the β-lactam agents, ceftriaxone, ceftazidime, cefotaxime, cefoxitin and penicillins was suspected to involve the presence of one or more β-lactamases in the isolates from patients attending OPD at J.N.M.C. hospital. 34% of all the *E. coli* isolates were found positive for ESBLs production. These β-lactamase producers were characterized by genetic analysis (DNA amplification by PCR) to investigate the presence of possible genes responsible for resistance development. β-lactam resistance could be attributed to the presence and action of β-lactamases such as the TEM, SHV and CTX-M type enzymes. The most common group of ESBLs not belonging to the bla<sub>TEM</sub> or bla<sub>SHV</sub> families were termed bla<sub>CTX-M</sub>. The designation “CTX-M” refers to potent activity against cefotaxime, an aminotiazolil cephalosporin with a methoximino group; these enzymes have only remnant activity toward ceftazidime, another aminotiazolil cephalosporin with a 2-carboxy-2-oxypropaneimino group instead of the methoximino group. These enzymes are inhibited by clavulanic acid, sulbactam and tazobactam. There are only few reports available on prevalence of CTX-M beta-lactamases in some parts of India. Therefore we determined the prevalence of these enzymes in our region by using PCR. Among all isolates, only three isolates were found to be positive for presence of CTX-M gene. As reported earlier by many groups that bla<sub>CTX-M</sub> enzymes were plasmid mediated, in our study we found that all three isolates harbor bla<sub>CTX-M</sub> genes on chromosomal DNA. This is first report from India showing the
presence of \textit{bla}_{CTX-M} gene on bacterial chromosome. Nucleotide sequence analysis of these PCR products revealed that all the three amplicons belong to CTX-15 type beta-lactamases.

To evaluate the role of plasmids in spread of drug resistance we carried the conjugation experiments. We found that eighteen of the twenty two strains showed transfer of multiple resistance markers from donor to recipient susceptible strain EJ-53. Transfer frequency of these resistance markers varied between $3.17 \times 10^6$ and $5.2 \times 10^8$. We further investigate the genotypes of the twenty two ESBLs producing \textit{E. coli} strains by PFGE. Pulsed-field gel electrophoresis (PFGE) of Xbal digests was performed to determine the genetic similarity amongst isolates showing multidrug resistance. The isolates investigated were genotypically diverse and divided in various types and subtypes by applying criteria of Tenover.

It is quite alarming to note that almost all of the isolates included in this study were found resistant to four or more antibiotics. This study concludes that \textit{E. coli} and other isolates were more sensitive to imipenem and amikacin compared to the other antibiotics tested and therefore these may be the drugs of choice for the treatment of community-acquired UTIs in our region. This study also shows that the integrons are one of the means of antibiotic resistant marker transfer among members of \textit{Enterobacteriaceae}. Our findings unveil the increasing role of the \textit{bla}_{CTX-M} \beta-lactamases in antibiotic resistance and emphasize on the significance of appropriate empirical treatment for infections caused by coliforms, especially in urinary tract infection patients. This study also shows that the horizontal gene transfer of
antimicrobial resistance genes can occur very efficiently and at a high rate among *Enterobacteraceae* in a community setting.
Chapter 1

Review of Literature
1.1 Introduction

*Escherichia coli* is a very diverse species of bacteria found naturally in the intestinal tract of all humans and many other animal species. A subset of *E. coli* are capable of causing enteric/diarrhoeal disease, and *Escherichia coli* strains capable of causing disease outside the gastrointestinal tract belong to a diverse group of isolates referred to as extra intestinal pathogenic *E. coli* (ExPEC) [Russo et al., 2000; Johnson et al., 2005]. ExPEC strains are responsible for a variety of diseases, including urinary tract infections (UTIs), newborn meningitis, septicemia, nosocomial pneumonia, intra-abdominal infections, osteomyelitis and wound infections [Eisenstein et al., 1988; de Louvois et al., 1994; Russo et al., 2000; Johnson et al., 2002]. Uropathogenic *E. coli* (UPEC), a prominent member of the ExPEC family, is responsible for up to 90% of uncomplicated UTIs in otherwise healthy individuals [Zhang et al., 2003].

1.2 Urinary Tract Infections (UTIs)

Urinary tract infections (UTIs) are among the most common human bacterial infections, with global infection rates nearing 180 million per year [Russo & Johnson, 2003]. UTIs are clinically defined as the bacterial colonization of any tissue along the urinary tract, from the urethral opening to the kidneys. The term urinary tract infection encompasses a broad range of clinical entities that are associated with a common finding of a significant amount of bacteria in urine and a positive urine culture [Dusé & Klugman, 1993]. The infection may be community acquired or nosocomial, often as a consequence of urethral catheterization and is more prevalent in women [Neu, 1992; Begg & Gillespie, 2000; Bannister, Hooton, 2001].
1.2.1 Aetiology of UTIs

Enterobacteriaceae are endogenous to the gastrointestinal tract and are often implicated in ± 80% of all UTIs. In Figure 1.1 it can be seen that Escherichia coli causes 50% - 80% of cystitis cases and community acquired UTIs [Finkelstein et al., 1998; Sanders et al., 1988] while Klebsiella pneumoniae is responsible for another 8% to 13%. Staphylococcus saprophyticus cause between 5% and 20% [Neu, 1997] and together with group D Streptococci (Enterococci) are the only Gram positive enteric bacteria that commonly cause UTIs [Rebuck et al., 2000]. Infrequent causative agents of UTIs include bacteria such as Staphylococcus aureus, Gardnerella vaginalis, Corynebacterium and Lactobacilli, yeasts such as Candida (in diabetics or patients with indwelling urinary catheters) and viruses such as Adenovirus type 2 (associated with acute haemorraghic cystitis in children) [Dusé & Klugman, 1993]. Non-specific urethritis is frequently caused by sepsis of Chlamydia ureaplasma and Mycoplasma, mainly introduced by sexual contact. Gonococci may also invade the urinary tract as well as the reproductive system, by entering the bladder via the urethra with an interim phase or periurethral and distal urethral colonization [Hooton, 2000].
Figure 1.1 Common causes of urinary tract infections. The percentage of infections caused by different bacteria in outpatients is shown. *Escherichia coli* is by far the most common isolate in both groups of patients. (Taken from Medical Microbiology by Mims, Playfair, Roitt, Wakelin and Williams; 1998)
1.2.2 Clinical manifestations of UTIs

Infections of the lower urinary tract may be confined to the urethra (urethritis) or the bladder (cystitis), with symptoms including frequent, painful, and urgent urination. Diagnosis is typically made based upon pyuria of at least 10 leukocytes per high power microscopic field and the presence of at least $10^5$ colony forming units of infecting organisms per mL of clean catch urine, though infections with lower numbers of organisms are not infrequent. Upper tract infections frequently occur in the kidneys (pyelonephritis), often accompanied by flank pain and tenderness, fever, chills, nausea, and vomiting and may also include symptoms of lower UTI [Sobel & Kaye, 2004]. Many urinary infections can, however, occur without specific symptoms [Sanders et al., 1988]. Based on the presence of physiological or anatomical abnormalities, including obstruction of the bladder outlet, vesicoureteral reflux, enlarged prostate in men, diabetes and pregnancy, UTIs are referred to as complicated, whereas those in an uncompromised host are termed uncomplicated [Kunin, 1987; Sobel & Kaye, 2004].

1.2.3 Microbiological causes of UTIs

There are several organisms known to cause UTIs, including *Pseudomonas aeruginosa*, *Staphylococcus saprophyticus*, *Enterococcus spp.*, *Proteus mirabilis*, *Klebsiella spp.*, *Citrobacter spp.*, and most importantly *Escherichia coli*, the most common infecting agent [Nicolle, 2002; Zhanel et al., 2005]. *E. coli* is an enteric Gram-negative bacillus found in the large intestine of all healthy individuals, and is responsible for 70-95% of episodes of cystitis and more than 90% of cases of pyelonephritis [Hooton, 2003; Russo & Johnson, 2003]. Certain serogroups of *E. coli*,
based on their ability to adhere to and colonize the urinary tract, are known as uropathogenic \textit{E. coli} (UPEC). UPEC are distinguished from commensals by the presence of special virulence factors, including adhesins and toxins, which enhance their ability to both cause infection and evade host responses. Adhesins are chromosomally encoded, non-filamentous outer-membrane proteins known as pili or fimbriae [Sobel & Kaye, 2004]. UPEC express many types of fimbriae differentiated by their receptor affinity. Type-1 fimbriae, characterized by their ability to bind mannose-containing oligosaccharides on host glycoproteins, are encoded by the \textit{fim} gene cluster, composed of nine genes [Emody, 2000]. These are especially well known as important factors for colonization of the bladder. The FimH adhesin, located on the tip of the pilus, recognizes terminal D-mannose moieties on secreted and cell-bound glycoproteins and is responsible for UPEC cell binding to host cells [Bergan, 1997]. Single nucleotide differences in the \textit{fimH} gene produce FimH proteins with the UTI-associated mono-mannose binding phenotype as opposed to the commensal type that binds to tri-mannose receptors [Johnson & Russo, 2005]. P-fimbriae are known for their enhanced ability to bind to the colon and remain in the gastrointestinal tract for an extended period. Following this, they confer the ability to spread to the perineum and urinary tract, causing ascending infections [Sobel & Kaye, 2004]. The allelic variation in the \textit{papG} gene, which encodes for the adhesin at the tip of these fimbriae, produces variants that preferentially bind to different Gal (β 1-4) Gal-containing glycolipids. Because these glycolipids are distributed differentially throughout the body, only certain pap-G variants contribute to disease in specific anatomical sites [Johnson & Russo, 2005]. Two of the four pap-G adhesin classes are associated with
UTIs, with class II mainly in pyelonephritis and class III in strains causing cystitis [Marrs et al., 2005]. In addition to molecules that aid in adherence, UPEC produce toxins that enhance infectivity. Cytotoxic necrotizing factor I (CNF-1) is a protein that induces host cell cytoskeleton rearrangement by permanently stimulating the Rho protein. By modifying the permeability of uroepithelium, bacteria are able to invade these cells, avoiding host defenses and antibiotics [Emody, 2000]. In addition to CNF-1, UPEC may also produce β-hemolysin, a toxin that facilitates invasion of host tissues and causes damage to renal tubules and epithelial and parenchymal cells [Marrs et al., 2005].

1.2.4 Routes of UTIs

The incidence of UTIs differs greatly between the sexes, with infections occurring fourteen times more often in females than males [Prescott et al., 2002]. The means through which infection begins greatly influences the differing rates between women and men. UTIs can occur in one of three ways: via the ascending route, the hematogenous route, or the lymphatic route. The ascending route is undoubtedly the most common route of infection. Bacteria from the normal flora of the colon are regularly excreted in fecal matter, and pathogenic strains may travel to the nearby periurethral area where they enter the urinary tract and cause ascending infection. The close proximity of the urethral meatus to the anus puts females at greater risk for UTIs. Sexual intercourse is indicated as a predisposing factor for ascending UTIs, as coliforms that colonize the vaginal introitus may be transferred to the urethral opening during sexual activity [Sobel & Kaye, 2004]. The male anatomy greatly reduces the risk of UTI among men; however, infections are seen in small numbers. Rectal
intercourse puts men at increased risk for a UTI, while a lack of circumcision may also play a role [Bergan, 1997]. The hematogenous route of infection is far less common and limited only to infection of the kidneys, where blood-borne pathogens enter and contribute to the formation of abscesses. These infections are almost exclusively caused by bacteremic *Staphylococcus species*, with *E. coli* infections being virtually non-existent. There is little evidence to indicate the importance of infection via the lymphatic route, however due to the presence of direct lymphatic channels between the intestines and the kidneys, their role in producing upper tract infections cannot be ruled out entirely [Kunin, 1987; Sobel & Kaye, 2004].

1.2.5 Pathogenic bacteria implicated

1.2.5.1 *Escherichia coli*

*E. coli* can remain in the urinary tract by adhering to the epithelial receptor cells lining the UT and are able to avoid elimination by the flushing action of voided urine. They possess various virulence factors such as (1) fimbriae or pilli (2) haemolysin (3) aerobactin and (4) exotoxins and other features. Fimbriae or pilli are highly specialized surface glycoprotein projections that serve as ligands for glycoprotein and glycolipid receptors on uro-epithelial cells in the bladder, urethra and the vagina [Bannister et al., 2000]. Haemolysin degrades renal tubular red cells [Dusé & Klugman, 1993; McClane & Mietzner, 1999]. Aerobactin is a sidophore that enhances iron acquisition and chelation [Dusé & Klugman, 1993; McClane & Mietzner, 1999; Hooton, 2000]. Other virulence features include the capsular acid polysaccharide antigen that appear, together with exotoxins, to assist in localization of organisms in the kidney to cause renal damage and are associated with pyelonephritis and inhibition
of phagocytosis [Jarlier et al., 1988; McClane & Mietzner, 1999]. Although most \textit{E. coli} can cause UTIs, the disease is most commonly associated with specific serotypes other than those associated with gastrointestinal tract [Mims et al., 1993].

1.2.5.2 \textit{Klebsiella pneumoniae}

\textit{K. pneumoniae} are opportunistic pathogens frequently associated with community acquired infections such as primary lobar pneumonia in immunocompromised and other high risk patient groups and 8-13\% of cases of UTIs are attributed to infection by \textit{K. pneumoniae} [Murray et al., 1998]. It is also known to be a major cause of nosocomial infections and infections of wound and soft tissues [Vaara & Vaara, 1983; Murray et al., 1998]. Alcoholics, people with compromised pulmonary function, and other patients in high-risk groups (patients with central venous and urinary tract catheters, patients on mechanical ventilation, etc.) are also at risk of being infected with \textit{K. pneumoniae} [Jarlier et al., 1988; Weiner et al., 1999; Rebuck et al., 2000; Lautenbach et al., 2001]. Several factors contribute to the virulence of \textit{K. pneumoniae}: (1) the presence of cell wall receptors enable the organism to attach to host cells and protect the bacteria from phagocytosis and intracellular killing by polymorphonuclear leukocytes, (2) an extensive polysaccharide capsule (K antigen) protects the bacterial cell from phagocytosis and directly suppresses the immune response [Mims et al., 1993; Hooton, 2000], (3) production of long chain-O-antigen polysaccharide in the endotoxin of the outer membrane that may contribute to resistance by inhibiting complement-mediated serum killing, and (4) the possession of a large plasmid (180 kb) that encodes aerobactin (a protein involved in iron acquisition and regulation of the mucoid phenotype).
1.2.6 Isolation and identification of UTIs

The urinary tract as well as the urine secreted in the kidneys and the bladder is bacteriologically sterile. However, the urethral meatus and surrounding perineum are colonized with a mixture of skin and bowel flora so that normal voided urine may contain small numbers of bacteria [McClane & Mietzner, 1999; Bannister et al., 2000]. A number of screening techniques have been developed for the rapid detection of UTI with mid-stream urine as the most suitable specimen. These techniques involve application of dipsticks to detect glucose, blood, protein, nitrite and leucocyte esterase. Other tests include semi-quantitative bacterial culture, where infection is usually indicated if colony counts >10⁵/L. The diagnosis of a UTI is confirmed by demonstrating the presence of an etiologic agent, which usually is a bacterium in the urine [Virella, 1997]. The purity of the culture is also important as a mixed culture is likely to indicate a contaminated specimen. For most clinical situations, simple or presumptive identification is sufficient, e.g. a lactose fermenting organism capable of indole production with a characteristic colonial morphology may be labeled a coliform. Checkerboard matrices may also provide simple presumptive and accurate species identification [Collee et al., 1996]. Commercial identification systems, semi-automated and automated, produce a species or genus identification by use of a computer database [Bannister et al., 2000]. An illustration of flow chart method for manual identification of genera and species of Enterobacteriaceae is given in Figure 1.2 [Taken from Collee et al., 1996].
1.2.7 Recommended treatment regimens of UTIs

The morbidity associated with UTIs makes treatment of these infections a necessity. When choosing an appropriate agent to combat these infections, there are several factors for clinicians to consider. First, the drug of choice should have good in vitro and in vivo activity against many of the organisms known to cause UTIs, not just against *E. coli*. Additionally, the agent should be able to achieve high and prolonged concentrations in the urine and surrounding urinary tract tissues without loss of activity [Bergan, 1997; Jancel & Dudas, 2002]. In terms of the patient’s well being, drugs should have the fewest and mildest side effects possible, with minimal effects on normal colonic and vaginal flora [Sobel & Kaye, 2004]. Treatment of uncomplicated UTIs have in the past included aminopenicillins (amoxicillin, ampicillin), first generation cephalosporins (cephalothin, cefazolin), second generation cephalosporins (cefoxitin, cefuroxime), third generation cephalosporins (cephem, ceftazidime), sulfamethaxazole-trimethoprim combinations (cotrimoxazole), amoxicillin/ clavulanic acid (augmentin), and recently, the fluoroquinolones such as ciprofloxacin [Dusé & Klugman, 1993; Hindler et al., 1994; Abdel-Rahman & Kearns, 1998; Bannister et al., 2000].

1.2.7.1 Penicillins

In 1929, the discovery of penicillin by Alexander Fleming revolutionized the world of infectious diseases. By 1949, penicillin was isolated in a relatively pure form, in sufficient quantities to treat a number of bacterial infections. The crucial point in the
Figure 1.2. Flow chart showing the LOGIC system for identification of Gram negative bacilli isolated from urine (Taken from Collee et al., 1996).

The LOGIC scheme includes tests for lysine and ornithine decarboxylation, indole production, glucose and cellobiose fermentation and urease production. (*) Salmonella and Serratia species are distinguished by additional biochemical and serological tests.
history of this drug class came in 1945, when the basic chemical structure of all penicillins was revealed as a bicyclic β-lactam-thiazolidine nucleus with variable side chains. The β-lactam ring holds the key to the drug’s antibacterial activity, while the side chain determines the spectrum of activity for each particular penicillin compound [Preston & Drusano, 1999]. Penicillins exert their antibacterial properties by interfering with peptidoglycan synthesis, which compromises cell wall integrity, leading to cell death. Penicillins inhibit the cross-linking of N-acetylmuramic acid residues of adjacent peptidoglycan strands during the final step of peptidoglycan synthesis by acting as structural analogues of acyl-D-alanyl-D-alanine and competing for the active site of the enzyme transpeptidase. In doing so, they prevent the cross-linking of peptidoglycan precursors, thereby disrupting cell wall stability [Preston & Drusano, 1999; Mascaretti, 2003]. In addition to transpeptidases, penicillins bind to a variety of other bacterial enzymes, collectively known as penicillin-binding proteins (PBPs). In E. coli, eight PBPs have been described, including carboxypeptidases and endopeptidases, all of which are important in cell wall formation and maintenance [White, 2000]. While the original, natural penicillins demonstrated excellent activity against Gram-positive organisms, they had very limited activity against Gram-negative pathogens, including E. coli. The aminopenicillins were synthesized to overcome this problem. With the addition of an amino group to the fused β-lactam-thiazolidine nucleus, aminopenicillins exhibited enhanced penetration into Gram-negative bacteria, such as E. coli, while retaining activity against gram-positive UTI pathogens, such as Enterococcus spp. and Staphylococcus saprophyticus, making them ideal agents for the treatment of UTIs [Yao et al., 1999].
1.2.7.2 Sulfonamides and trimethoprim

When coadministered, trimethoprim and sulfamethoxazole (TMP/SMX) are bactericidal agents with excellent antibacterial activity. Used in combination to treat UTI since 1968, TMP/SMX interferes with bacterial folate metabolism, by sequentially blocking the formation of tetrahydrofolic acid (THF). SMX is a structural analogue of paminobenzoic acid (PABA), the substrate for the enzyme dihydropteroate synthetase (DHPS), used to make dihydropterico acid in the first step of THF synthesis. SMX preferentially binds to DHPS over PABA, thereby blocking the formation of dihydropterico acid (DHP), which is needed to form dihydrofolic acid (DHF) in a later step along the THF synthesis pathway. TMP is a structural analogue of the pteridine moiety of DHF and preferentially binds to dihydrofolic acid reductase (DHFR), the enzyme normally responsible for the conversion of DHF to THF. By creating a sequential blockade in the formation of THF, TMP/SMX eliminates the thymidine pool required for DNA synthesis, leading to bacterial cell death [Goldstein & Stein, 1999; Mascaretti, 2003]. Its low cost, high absorption, oral availability, extended half-life, and activity against Gram-positive and Gram-negative organisms has made TMP/SMX the first choice for empiric treatment of upper and lower UTIs [Goldstein & Stein, 1999; Gupta & Stamm, 2002].

1.2.7.3 Fluoroquinolones

A relatively modern class of drugs, fluoroquinolones are becoming increasingly popular in the treatment of UTI due to their broad spectrum of activity and rapid bactericidal effects. The prototype for modern day quinolones, nalidixic acid, was discovered in 1962 as a byproduct of the formation of the anti-malarial drug
chloroquine. The 1980s brought about the synthesis of the second-generation quinolones, the fluoroquinolones, including ciprofloxacin and levofloxacin, which have the broad spectrum of Gram-negative activity of the first generation quinolones as well as improved activity against Gram-positive organisms [Zhanel et al., 2002]. The fluoroquinolones exhibit their action on bacterial cells by interacting with DNA gyrase and topoisomerase IV, inhibiting their ability to regulate the topological state of DNA during replication, transcription, and repair. The tetrameric DNA gyrase protein is composed of two “A” subunits, which produce and re-seal strand breaks in the chromosome during replication, and two “B” subunits, which introduce negative supercoils into the strand of DNA. Second-generation quinolones act primarily by binding to the DNA-DNA gyrase complex formed during replication, preventing the A subunits from resealing strand breaks in the bacterial chromosome. Topoisomerase IV plays an integral role in chromosome segregation during bacterial cell division in addition to its role in relieving the DNA molecule of torsional strain. While the details of quinolone interactions with Topoisomerase IV are not as well understood, the importance of its loss of function must not be overlooked [Mulholland, 1996; Zhanel et al., 2002]. With their excellent bioavailability when orally administrated, good tissue penetration, low incidence of side effects and broad spectrum of activity, fluoroquinolones are prescribed to combat a variety of clinical infections and are especially favored in combating complicated UTIs [Mulholland, 1996; Mascaretti, 2003].
1.2.7.4 β-Lactam antibiotics

β-Lactam antibiotics form a large group of different compounds containing a β-lactam nucleus. Different groups within the family are distinguished by the structure of the ring and the side chain attached to the β-lactam nucleus (Figure 1.3). Penicillins differ from cephalosporins in having a 5-membered thiazolidine ring complex and cephalosporins contain a 6-membered dihydrothiazine β-lactam ring complex [Dever & Dermody, 1991] (Figure 1.3). Since the discovery of β-lactam antibiotics in 1928 by Alexander Fleming [Rolinson, 1998], developments have led to the synthesis of semi-synthetic compounds that can be divided into bicyclic penicillins, cephalosporins, monocyclic monobactams, and β-lactamase inhibitor combinations [Hamilton-Miller, 1999] (Figure 1.3). β-Lactam antibiotics are commonly prescribed and have a wide spectrum clinical use because of low toxicity and strong bactericidal activity [Gruneberg, 1994; Petrosino et al., 1998]. They can be used in higher dosages for treatment of more severe infections and UTIs [Sanders & Sanders, 1992]. The primary targets for β-lactam antibiotics are the penicillin-binding proteins (PBPs), consisting of transpeptidases and carboxypeptidases responsible for creating cross-linkages between peptide chains. Following penetration of the bacterial cell surface, β-lactam antibiotics attach to the PBPs, to form a β-lactam- PBP complex. Catalytic activity is lost and cell wall synthesis and division interrupted [Richmond, 1981; Essack, Alexander & Pillay, 1994; Prober, 1998; Wright, 1999]. The anti-bacterial effect of all β-lactam antibiotics depends on the capacity of the antibiotic to diffuse through the cell membrane of the bacterial cell, the affinity of the antibiotic for its target proteins (the PBPs anchored in the cytoplasmic membrane of the bacterium) and the stability of
the antibiotic against bacterial degradation complex system [Dever & Dermody, 1991; Pitout et al., 1997]. β-Lactam antibiotics inhibit the cross linking (final stage) of peptidoglycan or murein synthesis of actively dividing bacterial cells (Figure 1.4) [Gould & Mackenzie, 1997]. Three PBPs of E. coli have been shown to be targeted by β-lactam agents: PBP-1, PBP-2 and PBP-3. Inhibition of PBP-1A and 1B is associated with rapid killing and lysis of bacterial cell, inhibition of PBP-2 results in cell wall deficient spherical non-growing cells, and inhibition of PBP-3 cause lysis as well as filamentous changes [Bryan & Godfrey, 1991; Georgepapadakou, 1993; Katsanis et al., 1994; Gould & Mackenzie, 1997]. β-lactam antibiotics can also inhibit bacterial growth by mechanisms that do not solely involve the inhibition of cell wall synthesis. Inhibition of the formation of cell wall precursor by these agents can result in autolysis through the unsuppressed activity of murein hydrolases [Dever & Dermody, 1991]. Murein hydrolases are autolytic enzymes that cause nicks in the cell wall to provide sites for new peptidoglycan synthesis during cell wall enlargement. The inhibition of cell wall synthesis by β-lactam antibiotics does not alter the activity of these enzymes; therefore, bacterial autolysis can result from the effects of osmotic pressure on the cell wall damaged by murein hydrolases [Wright, 1999].

Introduced in the 1960s cephalosporins are widely used routinely in many preoperative procedures, because of their broad-spectrum effectiveness and low toxicity (SAML supplement). Cephalosporins are classified according to the route of administration and their in vitro anti-bacterial spectra [Bannister et al., 2000]. Currently there are four generations of agents, each succeeding a generation possessing a greater spectrum of activity. The antibacterial activity of cephalosporins
depends on their ability to penetrate the bacterial cell wall, resist inactivation by β-lactamases and bind to and inactivate PBPs. Resistance can, however, develop at each of these steps [Prober, 1998]. Expanded-spectrum cephalosporins were specifically designed and introduced into clinical practice in the early 1980s, being resistant to hydrolysis by the older broad-spectrum β-lactamases commonly encountered at that time, i.e. β-lactamases such as TEM-1, TEM-2 and SHV-1 [Pitout et al., 1998]. Some of the well-known agents include cefotaxime, ceftazidime and ceftriaxone [Heritage et al., 1999].

1.3 History of antibiotic development

In order to comprehend the problem of antibiotic resistance as it exists today, it is useful to understand the history and development of both antibiotics and antibiotic resistance. Antimicrobial drugs have generally been classified into two categories; one includes the synthetic drugs, such as the sulfonamides and the quinolones, and the second, antibiotics, synthesized by microorganisms. In recent years, increasing numbers of semi-synthetic drugs have been developed which are chemical derivatives of antibiotics, thereby blurring the distinction between synthetic and natural antibiotics. Interest in antimicrobial chemotherapy was kindled as soon as microorganisms were understood to be agents of infectious disease. In earlier times, plant products were sometimes used successfully in the treatment of disease, but neither doctors nor patients knew the basis for the action of these therapeutic agents. Many early medicines were used to cure protozoan diseases, rather than bacterial
Figure 1.3: Structure of β-lactam antibiotics with sites for enzymatic degradation of penicillins/cephalosporins.

A=Classical penam and cepham ring respectively [also known as thiazolidine ring in basic structure], B= Four membered β-lactam ring [azetidinone], shared by all compounds; it remains unmodified (Hamilton-Miller, 1999), R= Acyl side chain, varies according to specific agent. 
1=β-lactamases, 2= acylases, 3= esterases.
Figure 1.4: The consequences of the interruption of peptidoglycan synthesis, such as caused by penicillins and cephalosporins (Richmond, 1981).

1. Normal rod-shaped bacterium.
2. Inhibition of peptidoglycan biosynthesis leads to a weakening of the cell walls. This is often first seen at the point where the next division furrow would become apparent were growth to continue normally.
3. The cell bursts.
diseases. As early as 1619, it was known that malaria could be treated with the extract of cinchona bark (quinine) and that amoebic dysentery could be treated with ipecacuanha root (emetine) [Garrod et al., 1971; Greenwood et al., 2000]. Only a few antibacterials, such as mercury, which was used to treat syphilis, were in use when the era of true chemotherapy began. It was in the early 1900's when Paul Ehrlich first hypothesized that dyes could be used as antimicrobial drugs, based on their differential affinities for various tissues. In 1904, Ehrlich and Shiga discovered that a red dye called trypanrot was effective against trypanosomes [Mitsuhashi et al., 1993]. It was around this time that arsenicals drew Ehrlich's interest. Ehrlich, along with Sahachiro Hata in 1909, found that arsphenamine (named Salvarsan) was active against spirochetes and, therefore, was an effective cure for syphilis [Greenwood et al., 2000].

The first truly effective class of antimicrobial drugs were the sulfonamides, discovered by Gerhard Domagk [Domagk et al., 1935]. In 1932, two scientists at the Bayer company, Mietzsch and Klarer, synthesized Prontosil red, a red dye bound to a sulfonamide group. Domagk [Domagk et al., 1935] showed, in 1935, that infections in mice caused by hemolytic streptococci were cured by Prontosil red [Garrod et al., 1971; Greenwood et al., 2000]. Unfortunately for Bayer, Prontosil red was shown to have no antibacterial activity in vitro. This lack of activity was explained by Trefouël et al. [Trefouel et al., 1935] when they showed that Prontosil red is split in vivo into its component dye and sulfanilamide, the active antibacterial agent and a previously described molecule that was already in the public domain. From that point, sulfanilamide was manufactured by a number of companies and work was begun to modify the molecule to enhance performance, leading to decreased side effects and a
broader spectrum of action. Although penicillin was the first natural antibiotic to be discovered, the idea of using microorganisms therapeutically was not new. Fungi had been used in poultices for many years, and by 1899, a product called pyocyanase, which was an extract from *Pseudomonas aeruginosa*, was used in the treatment of wounds [Garrod et al., 1971]. Penicillin was first isolated from *Penicillium notatum* in 1928 by Alexander Fleming [Fleming et al., 1929], but he was unable to isolate and purify enough drug to be of any use. After a worldwide search for *Penicillium* strains that could produce more penicillin, Raper and Fennel [Raper et al., 1946] found a strain of *Penicillium chrysogenum* on a moldy cantaloupe at a local market that was capable of even higher yields of penicillin [Demain et al., 1999].

A series of different antibiotics were quickly discovered after penicillin came into use. In 1940, Selman Waksman began searching for antibiotic compounds produced by soil microorganisms [Greenwood et al., 2000]. In 1943, one of Waksman's students discovered streptomycin [Schatz et al., 1944], leading to a flood of researchers combing the world for new drugs. It was in this same period that Rene Dubos [Hotchkiss et al., 1941] discovered gramicidin, the first antibiotic active against gram-positive bacteria. Chlorotetracycline, chloramphenicol, and others were discovered shortly thereafter [Garrod et al., 1971]. Many discoveries were of drugs that were too toxic for human use, or that had already been discovered. Nevertheless, this work did lead to many new drugs and within only 10 years, drugs comprising the major classes of antibiotics were found [Greenwood et al., 2000]. In addition to soil, many of these drugs were discovered by isolating the producing microorganisms from interesting and unusual sources. For example, some antibiotic-producing bacteria were isolated from a
wound infection and others from sewage, a chicken’s throat, and a wet patch of wall in Paris [Garrod et al., 1971]. In 1962, one of the later discoveries was a synthetic drug, nalidixic acid, the first of the quinolones to be described, and although not therapeutically important by itself, modification of nalidixic acid led to the production of the highly effective fluoro-quinolones. Members of this class, such as ciprofloxacin, norfloxacin, enrofloxacin, and ofloxacin, have become very important in the treatment of diseases in both humans and animals [Mitsuhashi et al., 1993]. Since the 1960’s, there have been few discoveries of new antibiotic drugs. The drugs developed since have mostly been chemical modifications of existing drugs. These modifications have been very useful in treating infectious diseases, leading to enhanced killing of pathogens, increased spectrum of action, reduced toxicity, and reduced side effects. Unfortunately, since the 1970’s, only one new class of antibiotics has been introduced [Lipsitch et al., 2002] and a recent trend in antibiotic therapy has been to employ combinations of drugs with different mechanisms of action, in order to increase their effectiveness and to overcome the problem of drug resistance.

1.4 Mechanisms of antibiotic action and resistance

There are several major classes of antibiotics that can be categorized based on their mode of antibacterial action. In general, antibiotics can be defined as those that inhibit cell wall synthesis, those that inhibit protein synthesis, and those that inhibit nucleic acid synthesis. See table 1.1 for a summary of the major antibiotic classes. The selective toxicity of antibiotics lies in the differences in cellular structures between eukaryotic and prokaryotic cells. However, differences in cellular structure among bacterial species can lead to resistance to certain antibiotics. The definition of bacteria
# Table 1.1. Major classes and examples of antibiotics.

<table>
<thead>
<tr>
<th>Cell wall synthesis</th>
<th>B-lactams</th>
<th>Glycopeptides</th>
<th>Protein synthesis</th>
<th>Aminoglycosides</th>
<th>Chloramphenicol</th>
<th>Tetracyclines</th>
<th>Macrolides</th>
<th>Streptogramins</th>
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<td>Penicillins</td>
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<td>Streptomycin</td>
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<td>Cephalosporins</td>
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<td>Tetracyclines</td>
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<td>Carbapenems</td>
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<td>Kanamycin</td>
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<td>Tetracyclines</td>
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<td>Gentamicin</td>
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<tr>
<td>Nucleic acid synthesis</td>
<td>Sulphonamides (diaminopyrimidines)</td>
<td>Quinolones</td>
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<td>Sulfamethoxazole-trimethoprim</td>
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as resistant or susceptible is critical for clinicians. It is also very important to note the difference between intrinsic and acquired resistance to an antibiotic. **Intrinsic resistance** can best be described as resistance of an entire species to an antibiotic, based on inherent (and inherited) characteristics requiring no genetic alteration. This is usually due to the absence of a target for the action of a given antibiotic or the inability of a specific drug to reach its target. For example, mycoplasmas are always resistant to N-lactam antibiotics since they lack peptidoglycan (which the N-lactams act upon). Similarly, the outer membrane of gram negative cells can prevent an antibiotic from reaching its target. For example, *Pseudomonas aeruginosa* exhibits high intrinsic resistance to many antibiotics due to its drug efflux pumps and restricted outer membrane permeability. **Acquired resistance** can arise either through mutation or horizontal gene transfer. Presence of the antibiotic in question leads to selection for resistant organisms, thereby shifting the population towards resistance.

Mechanisms of antibiotic resistance and the over coming methods are thus an intense area of research. Several mechanisms of antimicrobial resistance are readily spread to a variety of bacterial genera. First, the organism may acquire genes encoding enzymes, such as β-lactamases, that destroy the antibacterial agent before it can have an effect. Second, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect. Third, bacteria may acquire several genes for a metabolic pathway which ultimately produces altered bacterial cell walls that no longer contain the binding site of the antimicrobial agent. Fourth, bacteria may acquire mutations that limit access of antimicrobial agents to the intracellular target site via down regulation of porin genes [Tenover et al., 2006].
Thus, normally susceptible populations of bacteria may become resistant to antimicrobial agents through mutation and selection, or by acquiring from other bacteria the genetic information that encodes resistance. Acquired resistance that develops due to chromosomal mutation and selection is termed vertical evolution.

1.4.1 Inhibitors of cell wall synthesis

There are two major groups of cell wall synthesis inhibitors, the N-lactams and the glycopeptides. As bacterial cell walls are wholly unlike the membranes of eukaryotes, they are an obvious target for selectively toxic antibiotics. The N-lactams include the penicillins, cephalosporins, and the carbapenems. These agents bind to the penicillin binding proteins (PBP's) that cross-link strands of peptidoglycan in the cell wall. In gram negative cells, this leads to the formation of fragile spheroplasts that are easily ruptured. In gram positive cells, autolysis is triggered by the release of lipoteichoic acid [Greenwood et al., 2000].

1.4.1.1 Mechanism of N-lactam resistance

N-lactamases play a major role in the development of N-lactam resistance. These enzymes catalyze hydrolysis of the N-lactam ring and, thereby, inactivate these antibiotics. Many bacteria contain chromosomally encoded N-lactamases necessary for cell wall production and it is only through over-production of these enzymes that resistance occurs [Greenwood et al., 2000]. N-lactamases encoded on plasmids or other transmissible elements can lead to such overproduction and, therefore, to resistance [Normark et al., 2002]. There are also some bacteria that possess altered PBP's that result in reduced penicillin binding [Greenwood et al., 2000]. Since the discovery of penicillin and resistant bacteria, various new versions of the N-lactams
have been used that have different spectrums of activity and different susceptibility to N-lactamases. Since the 1970s, several compounds, such as clavulanic acid, have been discovered that have the ability to bind irreversibly to N-lactamases and, thereby, inhibit their action. Combinations of these compounds with N-lactam drugs have been very successful in treatment of disease [Bryan et al., 1984]. The glycopeptides are a group of antibiotics that include vancomycin, avoparcin, and others that bind to acyl-D-alanyl-D-alanine. Binding of this compound prevents the addition of new subunits to the growing peptidoglycan cell wall. These drugs are large molecules that are excluded from gram negative cells by the outer membrane, thus limiting their action to gram positive organisms.

1.4.1.2 Glycopeptide resistance

Glycopeptide resistance was long thought to be rare, but has recently been shown to be quite common [Bryan et al., 1984]. Resistance in enterococci has developed through newly discovered enzymes that use D-alanyl-D-lactate in place of acyl-D-alanyl-D-alanine, allowing cell wall synthesis to continue. Other mechanisms of resistance involve the over-production of peptidoglycan precursors which overwhelm the drug [Greenwood et al., 2000].

1.4.2 Inhibitors of protein synthesis

There are many types of antibiotics that inhibit bacterial protein synthesis. These drugs take advantage of structural differences between bacterial ribosomes and eukaryotic ribosomes. The aminoglycoside antibiotics are a group whose mechanism of action is not completely understood. The three major groups of aminoglycosides are the streptomycins, neomycins, and kanamycins. These drugs enter bacterial cells by an
active transport that involves quinones that are absent in anaerobes and streptococci, thus excluding these organisms from the spectrum of action. Streptomycins act by binding to the 30S ribosomal subunit. Kanamycins and neomycins bind to both the 50S subunit and to a site on the 30S subunit different from that of streptomycin [Greenwood et al., 2000]. Activity involving initiation complexes and cell membrane proteins that contribute to cell death plays a role in the action of these antibiotics, but this is poorly understood [Bryan et al., 1984; Greenwood et al., 2000]. There are three mechanisms of aminoglycoside resistance that have been identified to date. The first involves only streptomycin. Since streptomycin binds to one particular protein on the ribosome, alteration of this protein, even by a single amino acid in its structure, confers high-level resistance to the drug [Bryan et al., 1984]. The other mechanisms involve decreased uptake of the antibiotic and in one of these the cell membrane is altered, preventing active transport of the drug. In the other, one of many enzymes alters the antibiotic as it enters the cell, causing a block in further active transport [Bryan et al., 1984]. Chloramphenicol is a broad-spectrum antibiotic that, although naturally occurring, is produced by chemical synthesis. Chloramphenicol inhibits peptide bond formation on 70S ribosomes [Bryan et al., 1984]. This drug is especially useful in that it can penetrate eukaryotic cells and cerebrospinal fluid, making it a drug of choice for treatment of meningitis and intracellular bacterial infections such as those caused by chlamydia. It is not in widespread use, however, because of potentially fatal side-effects, namely, aplastic anemia [Greenwood et al., 2000]. Resistance to chloramphenicol is conferred by the enzyme chloramphenicol acetyltransferase. A number of these enzymes have been discovered, each altering the
chloramphenicol molecule to prevent binding to the bacterial ribosome. Chloramphenicol resistance in gram negative cells can also arise from alteration in outer membrane permeability that prevents the drug from entering the cell [Bryan et al., 1984].

The tetracyclines are another group of broad-spectrum antibiotics that inhibit bacterial protein synthesis. They are brought into the cell by active transport and, once there, bind to the 30S subunit to prevent binding of aminoacyl tRNA [Roberts et al., 1996]. Resistance to the tetracyclines occurs via three mechanisms. First, production of a membrane efflux pump removes the drug as rapidly as it enters and there are several genes encoding these pumps. Second, several ribosome protection proteins act to prevent tetracycline from binding to the ribosome, thus conferring resistance. Third, a protein found only in Bacteroides spp. enzymatically inactivates tetracycline [Roberts et al., 1996]. Interestingly, efflux pump inhibitors have recently been discovered that may allow combinations of these inhibitors and tetracyclines to be used against previously resistant strains [Chopra et al., 2002]. The macrolides are a group of antibiotics commonly used to treat gram positive and intracellular bacterial pathogens. Erythromycin was the first of these, and several other important macrolides have been discovered since, including clarithromycin and azithromycin. Azithromycin has a longer plasma half-life which allows treatment with a single dose for some pathogens or a once daily dose for others. Clarithromycin has enhanced absorption and causes less gastrointestinal discomfort [Gaynor et al., 2003]. It was originally believed that erythromycin inhibited protein synthesis by competing with amino acids for ribosomal binding sites, but newer research shows several mechanisms are involved
[Garrod et al., 1971]. The macrolides are now believed to promote dissociation of tRNA from the ribosome, inhibit peptide bond formation, inhibit ribosome assembly, and prevent amino acid chain elongation [Gaynor et al., 2003]. There are two major mechanisms of macrolide resistance. First, an efflux pump has been found that removes the drug from the cell. Second, modification of the ribosome can confer resistance. Mutations at several sites of the ribosome can allosterically prevent macrolide binding and a common alteration is dimethylation of one nucleotide on the 23S rRNA. This dimethylation not only prevents macrolide binding, but also confers resistance to lincosamide and streptogramin antibiotics [Gaynor et al., 2003]. The streptogramins are another class of antibiotic that inhibits bacterial protein synthesis, mostly in gram positive organisms (due to decreased permeability of the gram negative outer membrane). These antibiotics are actually combinations of structurally different drugs, types A and B that act synergistically. These compounds bind to separate sites on the 50S subunit. Type A drugs block attachment of substrates at two sites on the 50S subunit, whereas type B drugs cause release of incomplete protein chains. The synergistic effect arises from a conformational change induced by the binding of a type A drug which significantly increases affinity of type B drugs [Johnston et al., 2002]. Streptogramins currently in use include virginiamycin, pristinamycin, and quinupristin/dalfopristin. Resistance to streptogramin antibiotics can be found in several forms. Efflux pumps for both type A and B streptogramins have been identified. Type A streptogramins can be inactivated by one of the virginiamycin acetyl-transferases, and several enzymes have been identified that can inactivate type B streptogramins. Alteration of bacterial ribosomal proteins or RNA
can also confer resistance. A common mutation is the dimethylation of one nucleotide on the 23S rRNA, mentioned previously, that gives rise to resistance to type B drugs, as well as macrolides and lincosamides [Johnston et al., 2002].

1.4.3 Inhibitors of nucleic acid synthesis

The sulfonamides and the diaminopyrimidines should be discussed together, in that both only indirectly inhibit nucleic acid synthesis by inhibiting folate synthesis. Folate is a coenzyme necessary for the synthesis of purines and pyrimidines. Although both types of drugs are useful on their own, they exhibit a synergistic effect when combined. Sulfonamides are currently not used commonly in medicine, but the combination drug trimethoprim-sulfamethoxazole is sometimes used in the treatment of urinary tract infections. Sulfonamides serve as an analog of p-aminobenzoic acid. Therefore, they competitively inhibit an early step in folate synthesis.

Diaminopyrimidines, of which trimethoprim is the most common, inhibit dihydrofolate reductase, the enzyme that catalyzes the final step in folate synthesis [Greenwood et al., 2000]. There are several resistance mechanisms microorganisms employ against each of the anti-folate drugs. For example, sulfonamides are rendered ineffective by over-production of p-aminobenzoic acid or production of an altered dihydropteroate synthetase. The substrate for dihydropteroate synthetase is p-aminobenzoic acid, and the altered form has a much lower affinity for sulfonamides than for p-aminobenzoic acid [Then et al., 1982]. Trimethoprim resistance can also result from several mechanisms, e.g., over-production of dihydrofolate reductase or production of an altered, drug-resistant form can lead to resistance [Bryan et al.,
1984]. In addition, both drugs can be enzymatically inactivated, resulting in resistance [Then et al., 1982].

The quinolones are a chemically varied class of broad-spectrum antibiotics widely used to treat many diseases, including gonorrhea and anthrax. Drugs in this class include nalidixic acid, norfloxacin, and ciprofloxacin. These drugs are commonly used and, worldwide, more ciprofloxacin is consumed than any other antibacterial agent [Acar et al., 1997]. Quinolones inhibit bacterial growth by acting on DNA gyrase and topoisomerase IV, which are necessary for correct functioning of supercoiled DNA [Greenwood et al., 2000]. Although quinolones target both enzymes, in gram negative organisms the primary target is DNA gyrase and, in gram positive organisms, the primary target is topoisomerase IV [Ruiz et al., 2003]. There are three main mechanisms of resistance to quinolones. Resistance to some quinolones occurs with decreased expression of membrane porins. Cross-resistance to other drugs requiring these porins for activity also results from these changes. A second mechanism of resistance is expression of efflux pumps in both gram negative and gram positive organisms [Novak et al., 1999] and the third is alteration of the target enzymes. Several mutations have been described in both quinolone target proteins that result in reduced binding affinities [Ruiz et al., 2003]. It is believed that high-level quinolone resistance is brought about by a series of successive mutations in the target genes, rather than a single mutation [Novak et al., 1999].

1.5 Genetic mechanisms of resistance

Bacteria also develop resistance through the acquisition of new genetic material from other resistant organisms. This is termed horizontal evolution, and may occur
between strains of the same species or between different bacterial species or genera. Mechanisms of genetic exchange include conjugation, transduction, and transformation [McManus et al., 1997].

**Transformation** (Fig. 1.5) involves the passage of DNA to a recipient through a specific medium. This process of transferring genetic material is mostly observed in vitro in the molecular biology laboratory.

**Transduction** (Fig. 1.5) is a process whereby DNA is transferred from a donor to a recipient by way of a host, a phage. It is still unknown whether this process causes clinically observed resistance to antibiotics. Because this process is highly dependent on specific phages, it may occur only within certain bacterial species. Only a limited amount of DNA that can be packed into the head of a phage can normally be transferred. Transduction therefore cannot be responsible for multiple drug resistance.

**Conjugation** (Fig. 1.5) is the process when DNA transfers from a donor to a recipient by simple, direct cell-to-cell contact. The process allows for the passage of more than one functional gene at a time, so that multiple resistance could occur within a single step. Furthermore, many different organisms can act as recipients, allowing DNA (resistant genes) to be donated freely from different sources. Resistance can be passed from commensals in the gut to a pathogens existing in the same environment. Conjugation is thus an important and highly efficient process for transferring genes, and the acquisition of resistance by most pathogens is probably a result of this process.

Other mobile genetic elements include transposons and integrons.

**Transposons** are sequences of DNA that are capable of transposing or moving from one region to another or between plasmids and chromosomes. Transposons can
contain one or more genes, including those necessary for translocation, and often contain either resistance genes or genes necessary for degradative enzymes [Scott et al., 2002]. These genes are flanked by insertion sequences that allow insertion of the DNA into the chromosome or plasmid.

**Integrons** are mobile elements that consist of conserved sequences of DNA bordering "cassettes" of genes. The conserved sequences contain the genes necessary for integration, promotion, and capture of cassettes. Gene cassettes are promoter-less units that contain the genes for antibiotic resistance or for virulence factors [Roe et al., 2003]. Integrons are capable of carrying several gene cassettes and are associated with multiple antibiotic resistance and pathogenicity islands. In recent years, through the study of genomics, we have come to learn that horizontal gene transfer occurs far more widely and often then we ever imagined. As complete genomes have been sequenced, evidence is mounting that not only do bacteria share genes with one another, but transfer occurs between all three domains of life, the Archaea, Eubacteria, and Eukaryotes. In 1998, Lawrence and Ochman [Lawrence et al., 1998] showed that 18% of the *E. coli* genome had been acquired through horizontal transfer. It has been shown that archaeal genes comprise 24% of the *Thermatoga maritima* genome. This is particularly surprising since *T. maritima* is thought to be among the slowest evolving, and on the deepest branch, of the Eubacteria [Nelson et al., 1999]. Eukaryotes apparently trade genes with bacteria as well. In an investigation of *Deinococcus radiodurans*, Makarova et al. [Makarova et al., 2001] found several eukaryotic genes including human, yeast, and fly genes, some of which have only ever been found in
Figure 1.5. Mechanisms of genetic exchange: Horizontal gene transfer

(Courtesy by Kenneth Todar, University of Wisconsin-Madison, Department of Bacteriology)
Plasmids are defined as a double stranded, circular or linear DNA molecule capable of autonomous replication. They are mostly a phenomenon of the prokaryotic world, although they exist in some groups of primitive eukaryotes as yeast, fungi and cellular slime moulds [Rush et al., 1985]. Plasmids are not organisms. They belong to a special biological category of extra-chromosomal, accessory DNA elements [Reanney et al., 1976, Campbell et al., 1981]. By definition, plasmids do not carry genes essential for growth of their host under no stressed conditions. Plasmids are a fashionable field of scientific research, since they are still studied as excellent models of DNA replication as well as in biotechnology. Natural plasmids have systems guarantying their autonomous replication but also mechanisms controlling the copy-number and ensuring stable inheritance during cell division. Many plasmids can promote their horizontal transfer among bacteria of different genera and kingdoms, through the conjugation process. However, the presence of plasmids must have some biological cost for the bacterial host. It is therefore plausible that a selfish element that does not benefit its host can be eliminated from the bacterial population. The plasmid-encoded functions have been extensively investigated and there is ample evidence that natural plasmids have evolved as an integral part of the bacterial genome, providing additional functions to their host [Thomas et al., 2005]. In many cases the selective advantage of a plasmid appears obvious, because it carries genes that confer a selectable phenotypic character under specific niche conditions [Lenski et al., 1998]. The best example of phenotypic advantage associated to a plasmid is given by the
antimicrobial resistance. Resistance genes located on plasmids offer immediate advantage to their host under antimicrobial pressure. However, the real advantage of the plasmid location of resistance genes is not evident: plasmid-located resistance genes can be successfully transferred to the bacterial chromosome, therefore disfavoring the plasmid maintenance. An advantage of plasmid location for selectable genes has been hypothesized in the relative higher copy number of plasmids, influencing the expression levels by increasing the gene dosage. However, there are numerous other factors influencing the chromosome/plasmid harmony and evolution. Plasmids must be thought like autonomous selfish molecules, encoding extra genetic information, establishing complex relationships with the recipient host and playing multiple roles in the bacterial population. Bacterial plasmid genome sequence comparisons provided the historical events through which plasmids are assembled. Their evolution seems to proceed by the assemblage of modular components by transposition, homologous recombination, and illegitimate recombinational events [Bennett et al., 2004]. This is particularly evident when the phenomenon of antimicrobial resistance is analyzed. Antimicrobial resistance arises from a complex multi-factorial process supported by panoply of mobile genetic elements that contain and transfer resistance determinants. Resistance genes located on plasmids move from one bacterium to another, conferring phenotypic characteristics. Several resistance plasmids have been described to carry virulence factors, such as bacteriocins, siderophores, cytotoxins, or adhesion factors [Martinez et al., 2002] and virulence plasmids have been described to carry resistance genes [Guerra et al., 2002, Villa et al., 2005, Herrero et al., 2006]. For plasmids carrying virulence and resistance linked
determinants, an infective population will be selected for antimicrobial resistance, and antimicrobial resistance pressure will select the virulence traits. However, once those determinants have been selected in the bacterial host, they can evolve further and eventually be transferred to other bacterial population. The acquisition of antimicrobial resistance genes on virulence plasmids, could represent a novel tool in bacterial evolution, implementing adaptive strategies to explore and colonize novel hosts and environments [Martinez et al., 2002].

Plasmid-mediated resistance to β-lactam antibiotics in *E. coli* is of major concern in hospitals. In South Africa this has resulted in widespread resistance of *E. coli* to ampicillin and co-trimoxazole, preventing their usage in urinary tract infections [Klugman, 1993]. Studies show that for both nosocomial and community acquired infections (such as UTIs), the mortality and morbidity in prolonged hospitalisation, are twice as high in patients infected with antibiotic-resistant strains than with susceptible strains [Pitout et al., 1997; Silva et al., 1999; Lautenbach et al., 2001]. Bacteria can possess plasmids that can code for more than one β-lactamase in addition to their expression of chromosomal enzyme. Due to carriage of plasmids and promiscuous exchange of such material between bacteria, these resistance genes have spread widely and are also subject to mutation [Lee, Yuen & Kumana, 2001]. Plasmid mediated β-lactamases were first recognized in Gram-negative bacteria in the early 1960s, shortly after the introduction of ampicillin [Livermore, 1993].
1.6 Antibiotic resistance in UTIs

While a number of drugs are available for UTI management, increasing resistance rates among infecting organisms have limited the use of a number of antibiotics, leading to greater difficulty in treating these infections.

1.6.1 Resistance to aminopenicillins

The aminopenicillins were the longstanding favorites in the treatment of UTI. However due to extraordinary resistance rates in uropathogens their usefulness is diminishing [Mulholland, 1997]. In Gram-negative organisms, aminopenicillin resistance is largely due to the production of β-lactamases, secreted enzymes that destroy the β-lactam nucleus of penicillins [Preston & Drusano, 1999]. In contrast, modifications in PBPs play a major role in Gram-positive penicillin resistance, though in Gram-negative organisms such as E. coli, this is not a common occurrence [Quintilliani et al., 1999]. Presently, more than 35% of E. coli isolates in North America are resistant to aminopenicillins, causing these once first-line antibiotics to be demoted to second- or third-line therapy for UTIs [Zhanel et al., 2005].

1.6.2 Resistance to TMP/SMX

TMP/SMX remains the drug of choice for empirical treatment of uncomplicated UTIs in the U.S. as it has for over 20 years [Hooton, 2003; Burman et al., 2003]. However, increases in resistance to this drug combination in recent years may soon render it unsuitable for empiric treatment of UTI [Carrie et al., 2004]. In the Enterobacteriaceae, mechanisms of acquired resistance to SMX include a) hyperproduction of PABA, which competes with SMX for the active site of the enzyme DHPS; b) altered DHPS with decreased affinity for SMX; c) hyperproduction
of DHPS; and d) decreased membrane permeability to SMX. Similarly, resistance to TMP is attributed to either altered or hyperproduction of the enzyme DHFR. The widespread use of these compounds has led to the development of resistance in most bacterial species on a global scale [Goldstein & Stein, 1999]. Guidelines set by the Infectious Disease Society of America recommend fluoroquinolones for empiric therapy of UTI once TMP/SMX resistance levels exceed 20%, and such rates have been reported [Burman et al., 2003; Hooton, 2003; Zhanel et al., 2005]. This has led to a shift in prescribing for UTI, with TMP/SMX prescriptions dropping from 48 to 24% between 1989 and 1998, and fluoroquinolone use increasing from 19 to 29% over the same period [Hooton, 2003].

1.6.3 Fluoroquinolone resistance

While quinolone resistance rates in North America are relatively low, their increased popularity in recent years has led to a rise in resistance. Currently ciprofloxacin resistance rates in North American urinary E. coli isolates sit slightly above 5% [Zhanel et al., 2005]. The ability of Gram-negative organisms to prevent the cellular accumulation of fluoroquinolones has been documented. Resistant isolates utilize energy-dependent efflux pumps to remove fluoroquinolones from the cell while reducing porin channels to decrease diffusion into the cell [Mascaretti, 2003]. Additionally, chromosomal mutations in gyrA and parC result in altered DNA gyrase and topoisomerase IV, respectively, leading to decreased fluoroquinolone affinity [Zhanel et al., 2002; Mascaretti, 2003].

1.6.4 β-Lactam resistance

There are three major ways bacteria avoid the bactericidal effect of β-lactams:
(a) Production of β-lactamases. β-lactamases are bacterial enzymes that hydrolyze the β-lactam ring and render the antibiotic inactive before it reaches the PBP target. The underlying structural kinship that β-lactamases share with PBPs allows these enzymes to bind, acylate, and use a strategically located water molecule to hydrolyze and thereby inactivate the β-lactam [Massova et al., 1998].

(b) Altered PBPs that exhibit low affinity for β-lactam antibiotics. Examples are PBP 2x of Streptococcus pneumoniae and PBP 2’ (PBP2α) of Staphylococcus aureus [Chambers et al., 1997]. These PBPs are relatively resistant to inactivation by penicillins and are able to assume the functions of other PBPs when the latter are inactivated.

(c) Lack or diminished expression of outer membrane proteins (OMPs) in gram-negative bacteria. The loss of OMPs restricts the entry of certain β-lactams into the periplasmic space of gram-negative bacteria and hence access to PBPs on the inner membrane. Imipenem resistance in Pseudomonas aeruginosa and K. pneumoniae can arise from the loss of OMP D2 and of OmpK36, respectively [Livermore et al., 2001; Gootz et al., 2004; Jacoby et al., 2004]. The destruction of β-lactams by β-lactamases is the most important resistance mechanism in gram-negative bacteria.

1.6.5 Multi-drug resistance

A current phenomenon of great concern in the medical community is the rise in multi-drug resistant organisms, defined as bacteria with simultaneous resistance to three or more different classes of antibiotics [Guyot et al., 1999]. In a recent survey of North American fluoroquinolone resistant uropathogens, more than 55% of E. coli
isolates exhibited additional resistance to ampicillin as well as TMP/SMX [Zhanel, Unpublished data]. Patients infected with such organisms experience significantly higher degrees of treatment failure, prolonging antibiotic usage and morbidity associated with infection [Gupta & Stamm, 2002; Sobel & Kaye, 2004].

1.7 β-Lactamase classification and properties

β-lactamases are globular proteins that characteristically have an α-helices, β-pleated sheets, and share similar structural features. β-lactamases are categorized based on similarity in amino acid sequence (Ambler classes A through D) or on substrate and inhibitor profile (Bush-Jacoby-Medeiros Groups 1 through 4) (Table 1.2) [Ambler et al., 1980, Jaurin et al., 1981; Ouellette et al., 1987; Huovinen et al., 1991; Bush et al., 1995]. Ambler Class A enzymes (Bush Group 2) are penicillinases that are susceptible to β-lactamase inhibitors. The TEM-1 and SHV-1 β-lactamases, Group 2b, are the β-lactamases usually found in E. coli and K. pneumoniae that confer resistance to penicillins (ampicillin and piperacillin) [Bush et al., 1995]. Ambler Class B enzymes (Bush Group 3) are MBLs that use one of two zinc (Zn2+) atoms for inactivating penicillins and cephalosporins. In bacteria, MBLs confer resistance to carbapenems, cephalosporins and penicillins. Bacteria possessing MBLs are among the most resistant phenotypes encountered by clinicians. MBLs are inhibited by chelating agents (EDTA), but not by clavulanic acid or sulfones. The bla genes encoding MBLs are found on a variety of genetic elements (chromosome, plasmid, integrons, etc.) [Walsh et al., 2005]. Clinically important MBLs that are widespread are the IMP-type and VIM-type of P. aeruginosa. Nearly 20% of imipenem-resistant P. aeruginosa strains possess a MBL. Ambler class C enzymes (Bush Group 1)
<table>
<thead>
<tr>
<th>Ambler Classification System</th>
<th>Bush-Jacoby-Medeiros Classification System</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Class A</strong> Penicillinases</td>
<td><strong>Group 1</strong> Cephalosporinases, hydrolyz extended spectrum cephalosporinases, Clavulanic acid resistant, AmpCs, CMY-2, ACT-1, MIR-1</td>
</tr>
<tr>
<td><strong>Class B</strong> Metallo-beta-lactamases</td>
<td><strong>Group 2</strong> All clavulanic acid susceptible, PC-1 from <em>S. aureus</em></td>
</tr>
<tr>
<td><strong>Class C</strong> Cephalosporinases</td>
<td><strong>2a</strong> Penicillinase,</td>
</tr>
<tr>
<td><strong>Class D</strong> Oxacillinases</td>
<td><strong>2b</strong> broad spectrum penicillinase, TEM-1, SHV-1, TEM-2</td>
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<tr>
<td></td>
<td><strong>2be</strong> ESBLs, SHV-2, TEM-10, CTX-Ms</td>
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<td></td>
<td><strong>2br</strong> inhibitor resistant, TEMs, IRTs, TEM-30, TEM-31</td>
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<td></td>
<td><strong>2c</strong> Carbenicillin hydrolyzing, PSE-1</td>
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<td></td>
<td><strong>2d</strong> Oxacillin hydrolyzing, OXA-1, OXA-10,</td>
</tr>
<tr>
<td></td>
<td><strong>2e</strong> Cephalosporinases inhibited by clavulanate, FEC-1</td>
</tr>
<tr>
<td></td>
<td><strong>2f</strong> Carbapenemases, KPC-1, SME-1</td>
</tr>
<tr>
<td></td>
<td><strong>Group 3</strong> metallo-β-lactamases, Hydrolyze imipenem, inhibited by EDTA, resistant to clavulanate IMP-1, VIM-1, Ccr-A</td>
</tr>
<tr>
<td></td>
<td><strong>Group 4</strong> Miscellaneous</td>
</tr>
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</table>
include the chromosomally encoded AmpC type β-lactamases found in *Citrobacter freundii, Enterobacter aerogenes* and *Enterobacter cloacae, Morganella morganii, P. aeruginosa* and *Serratia marcescens*. Bacteria possessing AmpC β-lactamases are resistant to penicillins, β-lactamase inhibitors, cefoxitin, cefotetan, ceftazidime, ceftriaxone, and cefotaxime. Aztreonam and cefepime are usually more active against bacteria possessing class C β-lactamases. Historically, it was thought that ampC genes encoding class C β-lactamases were located exclusively on the chromosome but, within the last 17 years, an increasing number of ampC genes have been found on plasmids [Walther-Rasmussen et al., 2002]. These have mostly been acquired by AmpC-deficient pathogenic bacteria, which consequently are supplied with new and additional resistance phenotypes. Ambler classD enzymes (Bush group 2f) are serine β-lactamases that are able to hydrolyze oxacillin (hence oxacillinases or OXA β-lactamases). The OXA enzymes of *Acinetobacter baumannii* and *P. aeruginosa* represent the most structurally diverse and rapidly growing group of β-lactamases [Brown et al., 2006, Walther-Rasmussen et al., 2006]. Depending on the OXA enzyme, these β-lactamases confer resistance to penicillins, cephalosporins, extended spectrum cephalosporins (OXA-type ESBLs) or carbapenems (OXA-type carbapenemases). OXA enzymes are relatively resistant to clavulanic acid inactivation, but are inhibited by sodium chloride [Naas et al., 1998, Poirel et al., 2002].
1.7.1 β-Lactamases implicated in resistance to β-lactam antibiotics used in urinary tract infections

TEM-1, TEM-2 and SHV-1 are widespread enzymes that attack the narrow spectrum cephalosporins, cefamandole and cefoperazone and most of the penicillins. The name "TEM" is a contraction of Temoniera, the name of the patient from whom resistant bacteria were isolated, whereas SHV is a contraction of sulfhydryl variable; a description of the biochemical properties of this β- lactamase (Heritage et al., 1999). Epidemiological studies have shown the plasmid-mediated enzymes TEM-1, TEM-2 and SHV-1 to be the most commonly encountered with TEM-1 predominant and responsible for 90% of ampicillin resistance in *E. coli* (Baker, 1999). The degree of resistance depends on the amount of TEM and SHV enzymes, which can vary 150-fold among isolates, reflecting gene dosage and promoter efficiency (Reguera et al., 1991; Heritage et al., 1999). These enzymes can be distinguished by biochemical criteria (substrate profiles, kinetic properties and reaction with inhibitors), physical properties (molecular size and isoelectric point), or by genetic criteria, such as inducibility and location of their genes on plasmids or on the chromosome (Philippon et al., 1989; Jacoby, 1994; Livermore & Williams, 1996). Within the bacterial cell, β-lactamases contribute to antibiotic resistance in several ways. In *E. coli* (TEM-1 producers) the β-lactamases remain localised in the periplasmic space and can destroy antibiotic molecules as they make their way through the outer membrane. Consequently, high level of resistance occurs within a single bacterial cell (Medeiros, 1984; Zhou et al., 1994). Several parameters contribute to the level of antibiotic resistance mediated by a particular β- Lactamase in a population of bacteria. These
include rate of hydrolysis ($V_{\text{max}}$), affinity for the antibiotic ($K_m$), and the amount of β-lactamase produced (Pitout et al., 1998).

By mid-1980s, resistance to expanded-spectrum cephalosporins had appeared in clinically significant Gram-negative bacteria, caused by the production of β-lactamases. Among the first of the extended-spectrum β-lactamases to cause significant clinical problems, were mutants derived from SHV-1 or TEM-1 β-lactamases. The genes encoding these mutants are present on mobile genetic elements, facilitating their spread in nosocomial pathogens (Heritage et al., 1999).

(a) TEM-1

TEM-1 is the most common plasmid encoded β-lactamase and was first recognized in *E. coli* in 1965 (Bryan & Godfrey, 1991; Ho et al., 1998). It has since spread to 20 – 60% of isolates of *Enterobacteriaceae*, with varying frequency in species and location. Expression of TEM-1 is constitutive but the amount varies amongst strains. Low level TEM-1 causes resistance to ampicillin, amoxicillin and ticarcillin, while higher levels can result in resistance to piperacillin, mezlocillin, cephalothin, cefamandole and cefoperazone (Livermore, 1993).

(b) SHV-1

SHV-1 is a narrow spectrum β-lactamase with activity against penicillins. It was first described as a chromosomally encoded β-lactamase in *Klebsiella* (Heritage, 1999). It is classified in the Bush group 2b enzymes (Sanders & Sanders, 1992) and is encoded by blaSHV-1 gene, commonly encountered in clinical isolates on Self-transmissible plasmids. The amount of enzyme produced and expressed vary more than one hundred fold amongst strains, depending on the number of plasmid copies per organism, the
efficiency of the promoter and the degree of gene amplification (Jacoby & Carreras, 1990; Abdel-Rahman & Kearns, 1998; Petrosino et al., 1998).

(c) Extended spectrum beta-lactamases (ESBLs)

Members of the family Enterobacteriaceae commonly express plasmid-encoded β-lactamases (e.g., TEM-1, TEM-2, and SHV-1) which confer resistance to penicillin but not to expanded-spectrum cephalosporins [Harbak; 2007]. In the mid-1980s a new group of enzymes, the extended-spectrum β-lactamases (ESBLs), was detected. ESBLs are beta-lactamases that hydrolyze extended-spectrum cephalosporins with an oxyimino side chain, e.g., CTX-M [http://en.wikipedia.org/wiki/Beta-Lactamase]. These cephalosporins include cefotaxime, ceftriaxone, and ceftazidime, as well as the oxyimino-monobactam aztreonam. ESBLs confer resistance to expanded spectrum cephalosporins (e.g. ceftriaxime, cefotaxime, and ceftazidime), aztreonam, and related oxyimino-beta lactamas [http://en.wikipedia.org/wiki/Beta-Lactamase; Rossolini et al., 2006]. Typically, they drive from genes for TEM-1, TEM-2, or SHV-1 by mutations that alter the amino acid composition around the active site of these β-lactamases. This extends the spectrum of β-lactam antibiotics susceptible to hydrolysis by these enzymes [Harbak; 2007; http://en.wikipedia.org/wiki/Beta-lactamase]. The ESBLs are frequently plasmid encoded. Plasmids responsible for ESBL production frequently carry genes encoding resistance to other drug classes (for example, aminoglycosides) [Bradford; 2001]. Therefore, antibiotic options in the treatment of ESBL-producing organisms are extremely limited. Carbapenems are the treatment of choice for serious infections due to ESBL-producing organisms, yet carbapenem-resistant isolates have recently been reported [Bradford; 2001; Rossolini et al., 2006]. ESBL-producing
organisms may appear susceptible to some extended-spectrum cephalosporins. However, treatment which such antibiotics have been associated with high failure rates [http://en.wikipedia.org/wiki/Beta-Lactamase]

(d) CTX-M-beta-lactamases

In gram-negative pathogens, beta-lactamase production remains the most important contributing factor to beta-lactam resistance [Medeiros; 1991]. Penicillins, cephalosporins, monobactams and carbapenems can all be hydrolyzed by multiple members of the beta-lactamase family of enzymes, which results in microbiologically ineffective compounds [Bush et al., 1998]. Although numerous new beta-lactams have been developed during the past 40 years in attempts to circumvent the activity of beta-lactamases, it appears that the prime result has been the selection of more diverse and potentially more deleterious beta-lactamases.

CTX-M is a recently described family of the extended-spectrum beta-lactamases [Tzouvelekis et al., 2000]. The name CTX reflects the potent hydrolytic activity of these beta-lactamases against cefotaxime [Bonnet; 2004]. These enzymes hydrolyze cephalothin better than benzylpenicillin and they preferentially hydrolyze cefotaxime over ceftazidime. They are inhibited better by the beta-lactamase inhibitor tazobactam than by sulbactam and clavulanate [Bush et al., 1993]. Rather than arising by mutation, they represent examples of plasmid acquisition of beta-lactamase genes that are normally found on the chromosome of Kluyvera species [Humeniuk et al., 2002]. CTX-M beta-lactamases were predominantly found in three geographic regions: South America, the Far East and Eastern Europe [Tzouvelekis et al., 2000; Baraniak et al., 2002; Cao et al., 2002; Radice et al., 2002]. However, in recent years, CTX-M type
beta-lactamases have been reported in Western Europe, North America, China, Japan and India [Babini et al., 2000; Karim et al., 2001; Moland et al., 2003; Wang et al., 2003; Yamasaki et al., 2003]. CTX-M type beta-lactamases may be the most frequent type of ESBLs worldwide. The number of CTX-M type beta-lactamases is rapidly expanding. More than 40 CTX-M variants are currently known [Tzouvelekis et al., 2000]. They have been found in different Enterobacteriaceae including Salmonella spp [Bradford et al., 1998].

CTX-M enzymes can be sub classified by amino acid sequence similarities [Bonnet et al., 2000]. The phylogenetic study reveals five major groups of acquired CTX-M enzymes. (i) The CTX-M-1 group includes six plasmid-mediated enzymes (CTX-M-1, CTX-M-3, CTX-M-10, CTX-M-12, CTX-M-15, and FEC-1) [Matsumoto et al., 1988; Barthelemy et al., 1992; Gniadkowski et al., 1998; Karim et al., 2001; Kariuki et al., 2001; Oliver et al., 2001] and the unpublished enzymes CTX-M-22, CTX-M-23, and CTX-M-28 (Gene Bank accession numbers AY080894, AF488377, and AJ549244, respectively). (ii) The CTX-M-2 group includes eight plasmid-mediated CTX-M enzymes (CTX-M-2, CTX-M-4, CTX-M-4L, CTX-M-5, CTX-M-6, CTX-M-7, CTX-M-20, and Toho-1) [Ishii et al., 1995; Bauernfeind et al., 1996; Bradford et al., 1998; Gazouli et al., 1998; Gazouli et al., 1998; Tassios et al., 1999; Saladin et al., 2002]. (iii) The CTX-M-8 group includes one plasmid-mediated member [Bonnet et al., 2000]. (iv) The CTX-M-9 group includes nine plasmid-mediated enzymes (CTX-M-9, CTX-M-13, CTX-M-14, CTX-M-16, CTX-M-17, CTX-M-19, CTX-M-21, CTX-M-27, and Toho-2) [Ma et al., 1998; Labia; 1999; Sabate et al., 2000; Bonnet et al., 2001; Pai et al., 2001; Poirel et al., 2001; Cao et al.
2002; Chanawong et al., 2002; Saladin et al., 2002; Bonnet et al., 2003] and two unpublished enzymes (CTX-M-24[Gene Bank accession number AY143430] and CTX-M corresponding to Gene Bank accession number JP0074). (v) The CTX-M-25 group includes the CTX-M-25 and CTX-M-26 enzymes (Gene Bank accession numbers AY157676 and AF518567, respectively).

1.8 Objectives of the study

In view of the present background we initiated our study with the following objectives:

1. To determine the distribution and antibiotic susceptibility patterns of bacterial strains isolated from patients with community acquired urinary tract infections (UTIs) at Aligarh hospital in India as well as identification of ESBL producers in the population of different uropathogens.

2. To investigate the role of integrons as vehicles for antibiotic resistance genes.

3. To detect which transferable extended spectrum beta-lactamase circulated in the community.

4. To study role of horizontal gene transfer in multidrug resistant *E. coli* strains.

5. To investigate and identify the outbreak strain among ESBLs producing *E. coli* strains by molecular genotyping.
Chapter 2

Materials and Methods
2.1. Materials

Materials used during the study were listed below

2.1.1. Antibiotics

The number of antibiotic discs used to carry out the study is listed below. All the antibiotic discs were purchased from Hi-Media, Pvt. Ltd, Mumbai, India.

<table>
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<tr>
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<th>Symbol</th>
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</tr>
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<td>Tobramycin</td>
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</tr>
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</table>

2.1.2. Culture media

List of the culture media used for the isolation and identification of the organism was purchased from Hi-Media, Pvt. Ltd. Mumbai, India.

Agar agar

Brain-Heart Infusion Broth.

EMB-Broth

Luria-Bertani broth

Mac-Conkey agar

Mueller-Hinton agar

Mueller-Hinton broth

Nutrient agar

Nutrient broth
Tryptic Soy Broth

2.1.3. Chemicals and reagents used:

<table>
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<tr>
<th>Chemical/Reagent</th>
<th>Supplier</th>
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</table>
2.2. Methods

2.2.1. Bacterial isolates

The study was conducted on patients attending outpatient clinics at the J.N.M.C.H, A.M.U., Aligarh between August 2004 and July 2005. Freshly voided midstream specimens of urine (n= 920) were submitted to the clinical microbiology laboratory of J.N.M.C.H, Aligarh for processing. Semi quantitative urine culture using a calibrated loop was used to inoculate blood agar and Mac-Conkey agar plates [Claridge et al., 1987]. Following the recommendations of Kass [Kass, 1957] in distinguishing genuine infection from contamination, significant monomicrobic bacteriuria was defined as culture of a single bacterial species from the urine sample at a concentration of >10^5 cfu/ml. Only a single positive culture per patient was included in the analysis. The significant pathogens were identified by standard biochemical procedures [Baron et al., 1994]. UTI patients included in this study were classified as young and middle aged (20-49 years), pediatrics (New born to 19 years) and elderly patients (50-80 years). Isolates were screened for antibiotic resistance by routine disc susceptibility and those reported to be resistant to more than one drug were stored at −70°C in a solution containing 7% glucose, 7% peptone and 30% glycerol.

2.2.2. Bacterial identification

Each sample after overnight incubation at 37°C was transferred to selective media. Set of biochemical tests (Gram staining, motility test, capsule staining, carbohydrate fermentation test, Indole test, methyl red test, Voges Proskauer test, citrate test, urease test, oxidase test and nitratase test) was used for all the isolates. E. coli, was
grown on Eosin Methylene Blue (EMB) agar and also confirmed by Hi E. coli Identification Kit (Hi Media). A typical E. coli gave colonies with metallic sheen on EMB agar. Moreover E. coli isolates gave Indole and Methyl Red test as positive; and Voges-proskauer, Citrate, and Urease test as negative. On Mac Conkey Agar, E. coli appeared as non-capsulated, motile, circular, convex, and smooth colonies. Klebsiella spp. was confirmed by growing on Hi Crome Klebsiella Selection Agar Base with Klebsiella selective supplement. It appeared as small, dark brown colonies. On Mac Conkey agar Klebsiella appeared as large pink colonies, very mucoid, capsulated and non-motile. A typical Klebsiella pneumoniae gives Indole and Methyl Red test as negative; and Voges-proskauer, Citrate, and Urease test as positive.

2.2.3. Antimicrobial susceptibility testing

2.2.3.1. Disc diffusion test

2.2.3.1.1. Storage of antimicrobial discs

Cartridges containing commercially prepared paper disks specifically for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. Discs were stored at 8°C or below or froze at -14°C or below, until needed. The unopened disk containers were removed from the refrigerator 1 to 2 hr before use to equilibrate these to room temperature. Once a cartridge of discs has been removed from its sealed package, it was placed in tightly sealed, desiccated box.

2.2.3.1.2. Turbidity standard for inoculum preparation

To standardize the inoculum density for a susceptibility test, the BaSO₄ turbidity standard equivalent to a 0.5 McFarland standard was used (Andrews et al., 2004). BaSO₄ 0.5 McFarland standard was prepared as follows;
A 0.5 ml aliquot of 0.048 M BaCl₂ (1.175% w/v BaCl₂·2H₂O) was added to 99 ml of 0.18 M H₂SO₄ (1% v/v) with constant stirring. The correct density of the 0.5 McFarland standard was verified by determination of absorbance at 625 nm with a 1 cm path length and was found to be 0.08 to 0.10. The turbidity suspension was transferred in 4 to 6 ml aliquots into screw-cap tubes of the same size or those used in growing or diluting the bacterial inoculum. The tubes were tightly sealed and stored in the dark at room temperature. The turbidity standard was vigorously agitated on a mechanical vortex mixer before each use and inspected for a uniformly turbid appearance. Standards may be stored for up to six months after which time they should be discarded.

2.2.3.1.3. Inoculum preparation

Three to five isolated colonies of the same morphological types were selected from an agar plate culture. The top of each colony was touched with a loop and the growth was transferred into a tube containing 5 ml of a suitable medium such as tryptic soy broth. The broth culture was incubated at 37°C until it achieved or exceeded the turbidity of the 0.5 McFarland standard (usually 2 to 6 hours). This results in a suspension containing approximately 1 to 2x10⁸ CFU/ml. The turbidity of the actively growing broth culture was adjusted with sterile saline or broth to obtain turbidity optically comparable to that of the 0.5 McFarland standard. This results in a suspension containing approximately 1.5x10⁸ CFU/ml.

2.2.3.1.4. Inoculation of test plates

Optimally, within 15 min after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab
was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step the rim of the jar was swabbed. The lid was kept ajar (slightly open) for 3 to 5 minutes to allow for any excess surface moisture to be absorbed before applying the drug impregnated discs.

2.2.3.1.5. Application of discs to inoculated agar plates

Within 15 min after the inoculation of plates, the antibiotic disks were applied so that diffusion and growth could proceed simultaneously. The discs were applied with the help of sterile forceps. Four discs were applied on each agar plate arranged at least 20 mm from the edge of the plate and apart from each other by a distance of 15 to 20 mm. The plates were inverted and placed in an incubator set at 37°C within 15 minutes after the discs were applied. The incubation period was maintained for 18 to 24 hrs.

2.2.3.1.6. Reading plates and interpreting results

After 18 to 24 hrs of incubation, each plate was examined. The zones of inhibition were uniformly circular due to a confluent lawn of growth. The diameters of the zones of complete inhibition were measured, including the diameter of the disc. The zones were measured to the nearest whole millimeter, using a ruler, which was held on the back of the inverted petri plate. The petri plates were held a few inches above a black, non reflecting background and illuminated with reflecting light. Discrete colonies
growing within a clear zone of inhibition were subcultured, re-identified, and retested. The sizes of the zones of inhibition were interpreted by referring to standard tables in NCCLS documents (NCCLS, 7th Edt. 2000). *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212, *E. coli* BCC 2132 (ESBL producer), and *E. coli* ATCC 35218 (non-ESBL producer) were used as quality control strains. Interpretative criteria for each antimicrobial tested were those recommended by the CLSI-2000 [CLSI, 2000].

### 2.2.4. ESBL detection by CLSI phenotypic method

Production of ESBLs was studied by the double disc synergy test as described by Jarlier et al., (1988). Isolates were inoculated on MH-agar plates. Discs containing respectively ceftazidime (30μg), cefazolin (30μg), cefoxitin (30μg) and aztreonam were placed 25mm (centre to centre of the discs), from a disc containing amoxicillin/clavulanic acid (10/20μg). After overnight incubation at 37°C, the diameters of inhibition zones around the antibiotic discs were measured using a antibiotic inhibition zone reader scale. A clear extension of the edges of the inhibition zone of any of the antibiotics towards the disc containing clavulanic acid, was regarded as a phenotypic confirmation of the presence of ESBL (Jarlier et al., 1988; Acar and Goldstein, 1996; Collee et al., 1996). Susceptibility test results were interpreted according to the criteria established by the CLSI [CLSI, 2001]. A ≥ 5-mm increase in the zone of diameter of third generation cephalosporins, tested in combination with amoxyclav versus its zone when tested alone was considered indicative of ESBL production. *E. coli* ATCC 25922 and *K. pneumoniae* 700603 were used as control strains.
2.2.5. Statistical analysis

To analyze the data it was reported in the form of diameter of inhibition zone during susceptibility testing of all bacterial isolates by disc diffusion test against different classes of antimicrobial agents. One-way ANOVA was performed to check the significant difference among the different groups. A difference was considered significant if the probability that chance would explain the results was reduced to less than 5% (p<0.05). The normality and homogeneity was also checked.

2.2.6. Transfer of resistance through conjugation

To determine if the genes are located on the chromosome or on a plasmid, conjugation was done by using selected resistant strains as donors and *Escherichia coli (EJ-53)* (resistant to 250 μg of sodium azide), as selective recipient in all conjugation studies. Multi-drug resistant donor strains and recipient strain EJ-53 were inoculated into nutrient broth separately and incubated at 37°C until the cells reached logarithmic growth phase. 200 μl of recipient and 50μl of donor were then added to 5 ml of fresh nutrient broth and incubated overnight at 37°C. A loop-ful of the mixture was then spread on the surface of MacConkey agar containing ampicillin (25 μg/ml) plus sodium azide (250μg/ml) as selective antibiotics. The plates were incubated overnight at 37°C. Colonies of *E. coli (EJ-53)* that grew on the sodium azide /ampicillin selective plates and again on subculture on ampicillin plates were regarded as trans-conjugants.

2.2.7. Plasmid DNA preparation

The plasmid DNAs were extracted by QIAprep Spin Miniprep Kit. In brief, one colony from each strain was inoculated in 5 ml of LB (Luria-Bertani broth) containing
a suitable concentration of antibiotic for selection, for 18 hours at 37 °C. The
overnight cultures were centrifuged at 8,000 rpm for 15 minutes at 4 °C to pellet the
cells. The pellets were resuspended in 250 μl of Buffer P1 (50 mM TRIS-HCl, 10 mM
EDTA, pH 8.0 containing 100 μg/ml RNase A). 250 μl of Buffer P2 [200 mM NaOH,
1% SDS (w/v)] was added to the samples, mixed thoroughly by vigorously inverting
4-6 times and incubated at room temperature for 5 minutes. Then 350 μl of chilled
Buffer N3 (3.0 M potassium acetate, pH 5.5) was added to the cell lysates, mixed
thoroughly by vigorously inverting 4-6 times and incubated on ice for 15 minutes. The
samples were centrifuged for 10 minutes at 13,000 rpm at 4 °C. The supernatants
containing the plasmids were applied to the QIAprep spin columns. The columns were
washed twice with 0.75 ml of Buffer PE [70% ethanol]. Discard the flow-through, and
centrifuge for an additional 1 min to remove residual wash buffer. The plasmid DNAs
were eluted with 50 μl of Buffer EB [10 mM Tris-Cl, pH 8.5] or distilled water.

2.2.8. Total genomic DNA preparation

Total DNAs from strains containing studied plasmids were extracted by QIAamp
DNA Mini Kit, by the following procedure: one colony from each strain was
inoculated in 5 ml of LB (Luria-Bertani broth) containing a suitable concentration of
antibiotic for selection, for 18 hours at 37 °C. 1 ml of overnight cultures were
transferred in a 1.5 ml micro-centrifuge tube and centrifuged at 7500 rpm for 10
minutes to pellet the cells. To lyse the cells, the pellets were resuspended in 180 μl of
Buffer ATL, 20 μl of proteinase K was added, mix by vortexing, and incubated at 56
°C until the cells were completely lysed (lysis is usually complete in 1-3 hours).
Vortex occasionally during incubation to disperse the sample. 4 μl of RNase Solution
(100 mg/ml) were added to cell lysates, mix by pulse vortexing for 15s, and incubated at 37 °C for 2 minutes. 200 μl Buffer AL was added to the RNase-treated cell lysates, vortexed vigorously for 15s and incubated at 70 °C for 10 minutes. Then 200 μl ethanol (96-100%) was added to the sample, and mix by pulse vortexing. This mixture was then applied to the QIAamp Mini spin column with 2 ml collection tube and centrifuge at 8000 rpm for 5 minutes. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate. 500 μl Buffer AW1 was added and centrifuge at 8000 rpm for 2 minutes. The columns were washed again with 500 μl Buffer AW2 and centrifuge at 8000 rpm for 3 minutes. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer. The genomic DNAs were eluted with 200 μl of Buffer AE [10 mM Tris-Cl, pH 8.5] or distilled water.

2.2.9. PCR amplification

Isolates positive for ESBL production were subjected to polymerase chain reaction (PCR) amplification using primers designed for the detection of integrons, blaTEM, blaSHV and blaCTX-M genes. These samples were screened by PCR with 11 sets of primers for the detection of integrons; TEM-and SHV- derived extended spectrum beta-lactamases; CTX-M-1, CTX-M-2, CTX-M-3, CTX-M-8, CTX-M-9 and CTX-M-15 beta-lactamases (Table 2.1). The sets of PCR primers and the amplification conditions used to detect beta-lactamases and integrons have been reported previously (Levesque et al, 1995, Mugnaoli et al, 2006, Kim et al, 2005, and Shibata et al, 2006.). The PCR amplicons were electrophoresed on a 1% agarose gel. Oligonucleotide primers used for PCR amplification are listed in table (2.1). Standard PCRs were
carried out in 50 µL volumes containing 5 µL of 10 x PCR buffer (100 mM Tris-HCl (pH 8.8), 500 mM KCl, 15 mM MgCl₂, 1% Triton X-100), 5 µL of 10 x deoxynucleotide triphosphate mix (2 mM each dATP, dCTP, dGTP and dTTP) (Sigma, USA.), 2 µL of each primer stock solution (25 pmol/µL), 35 µL sterile distilled water and 1 µL Taq- polymerase (1.5 U/µL diluted solution) (Sigma, USA), and 2-5 µL (100ng-1μg) of template DNA. Amplification was performed in a thermocycler (Applied Biosystem, veriti, 96-well thermal cycler 9902, Connecticut, USA).

2.2.9.1. Agarose gel electrophoresis

PCR products (25 µl) were mixed with 2-8 µl of 10 x TAE buffer and 1-4 µl of bromophenol blue (0.25%). PCR mixtures were applied to 1-1.5% agarose gels (Sigma, USA) and products separated by electrophoresis for 2-4 h at 90 V using 1 x TAE running buffer (4.84 g/L Tris, 0.37 g/L EDTA, pH 8). The products were visualized by placing gel on a UV transilluminator (254-360 nm) and photographed using Gel Documentation (Bio-Rad Laboratories). A 100 bp DNA ladder, 1kb DNA ladder and DNA mix ruler were included in this study.

2.2.9.2. DNA extraction

PCR products (100 µl) were separated by agarose gel electrophoresis (section 2.4.2) on 1% gels and bands of predicted sizes cut from the gels with minimum exposure to UV illumination. The DNA was extracted from the gel slices using a DNA extraction kit (Qiagen, USA) by incubation in sodium perchlorate (for extracting fragments <500 bp) or sodium iodide (for fragments >500 bp) at 55°C for 5 min. Resin (15 µl) was then added and the samples incubated at room temperature for 1 min. After centrifugation at 16 000 x g for 30 sec, the pellet was resuspended in 1 ml
### Table 2.1. Primers used in the study.

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Annealing temperature (°C)/Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHV-A: 5'-CAC TCA AGG ATG TAT TGT G-3'</td>
<td>58 / AY223863</td>
</tr>
<tr>
<td>SHV-B: 5'-TTA GCG TTG CCA GTG CTC G-3'</td>
<td></td>
</tr>
<tr>
<td>TEM-S: 5'-ATAAAATTCTTGGAAGACGAAA-3'</td>
<td>55 / AB103506</td>
</tr>
<tr>
<td>TEM-AS: 5'-GACAGTTACCAATGCTTAATC-3'</td>
<td></td>
</tr>
<tr>
<td>5'CS: 5'-GGCATCCAAGCAGCAAG-3'</td>
<td>55 / M73819</td>
</tr>
<tr>
<td>3'CS: 5'-AAGCAGACTTGACCTGA-3'</td>
<td></td>
</tr>
<tr>
<td>IntI-F: 5'-CTACCTCTCACTAGTGAGGGGCGG-3'</td>
<td>55 / U12338</td>
</tr>
<tr>
<td>IntI-B: 5'-GGGACGCAAGCAGAAGTCGAGGC-3'</td>
<td></td>
</tr>
<tr>
<td>Sull: 5'-ATGGTGACCGGTGTCCGCGAT3'</td>
<td>58</td>
</tr>
<tr>
<td>Sull: 5'-CTAGGCATGATCTAACCTC 3'</td>
<td></td>
</tr>
<tr>
<td>CTX-MU2: 5'-TGGGTRAARTARGTSAACCAGA-3'</td>
<td></td>
</tr>
<tr>
<td>CTX-M-1-S: 5'-CGTCACCTCAGGTTAGGAA-3'</td>
<td>50 / AJ632119.1</td>
</tr>
<tr>
<td>CTX-M-1-AS: 5'-ACGGCTTTCTGCTTAGTT-3'</td>
<td></td>
</tr>
<tr>
<td>CT-M-2-S: 5'-TTATGAGCAGCAGCAGATTC-3'</td>
<td>50 / X92507</td>
</tr>
<tr>
<td>CT-M-2-AS: 5'-GATACCTCGCTTATTTATT-3'</td>
<td></td>
</tr>
<tr>
<td>CTX-M3G-B: 5'-ACGGAAATGAGTTCCCCCATT-3'</td>
<td></td>
</tr>
<tr>
<td>CTX-M-8-F: 5'-CGGATGATGCTATGACAAC-3'</td>
<td>50 / AAC, FEB.2006, P-791-795.</td>
</tr>
<tr>
<td>CTX-M-8-R: 5'-GTCAGATTGCGAAGCGTC-3'</td>
<td></td>
</tr>
<tr>
<td>CTX-M-9-S: 5'-TATGAGGAGGTTGGAAGATG-3'</td>
<td>50 / AAC, FEB.2006, P-791-795.</td>
</tr>
<tr>
<td>CTX-M-9-AS: 5'-TCCTTCAACTCAGAAAAAGT-3'</td>
<td></td>
</tr>
<tr>
<td>CTX-M15R: 5'-ACGGTCGTTGACGATTTTAG-3'</td>
<td></td>
</tr>
</tbody>
</table>
washing solution, followed by a 1:1 dilution with 99% ethanol. The sample was again centrifuged at top speed for 30 sec and the supernatant discarded. The pellet was incubated at 55°C (tube with open lid) until all the alcohol had evaporated. Sterile distilled H₂O (10 µl) was added to the pellet after 1 min incubation at room temperature. After centrifugation for 30 s the supernatant containing the DNA was carefully removed to a clean tube. The final step was repeated to yield approximately 20 µl of DNA sample.

2.2.10. Genotyping using PFGE

PFGE typing of clinical Escherichia coli strains isolated during the study was performed as described by Liu, et al. (1995).

2.2.10.1. Preparation of agarose plugs and treatment with lysis buffer

The test strains were grown in 5 ml nutrient broth. 200 µl of an overnight grown culture was taken in a sterile eppendorf tube and centrifuge at 10,000 rpm for 5 min and aspirated the supernatant. The pellet was resuspended in 200 µl of EET buffer (100 mM EDTA, 10 mM EGTA, 10 mM Tris-Hcl, pH 8.0) and was adjusted to a concentration of 10⁹ CFU/ml with a colorimeter. This bacterial suspension was equilibrated at 37°C in water bath for 10 minutes, mixed with an equal volume of 2% low-melting-point agarose and allowed to solidify an a 100 µl plug mold (Bio-Rad Laboratories). This was done quickly and carefully to avoid the shearing of DNA and creation of bubbles in the tube or subsequently in the plug mold. The plug molds were kept on ice for 30 min for the agarose to set. For lysis, the DNA blocks were incubated overnight at 37°C in a 2ml lysis buffer (10mM Tris-Hcl [pH 7.6], 100mM EDTA, 100 mM Nacl, 0.5% Brij58, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine,
and lysozyme [0.5mg/ml]). Following this step, the lysis buffer was replaced with 2ml of proteolysis buffer (1% sodium lauryl sarcosine, 0.5M EDTA [pH 9.5], and 500μg of proteinase K [Sigma Chemical Co., St. Louis, Mo] per ml). and this solution was incubated with gentle shaking at 56°C for 2 days, the proteolysis buffer was changed after 24 hr. To eliminate the lysed bacterial material and inactivate proteinase K activity, the DNA blocks were washed once for 1h at room temperature in 10ml of TE buffer (10mM Tris-Hcl [pH7.5] and 10mM EDTA) and once for 1h at 37°C in TE buffer containing 1mM phenylmethylsulfonyl fluoride (Sigma), To remove phenylmethylsulfonyl fluoride, the DNA blocks were washed once in 2ml of TE buffer at 4°C for 1h. The DNA blocks were washed once in 1ml of 0.1XTE buffer at 4°C for 30 min. to avoid the activity of restriction endonuclease being decreased by TE buffer.

2.2.10.2. Restriction digestion with XbaI

A slice of each block (3.5 by 5mm) was cut and washed in 100 μl of XbaI reaction buffer (Fermentas) at 4°C for 30 min. Then, XbaI reaction buffer was replaced with 100 μl of fresh reaction buffer containing 50U of the restriction endonuclease XbaI (Fermentas). The DNA blocks were incubated at 37°C for 24h.

2.2.10.3. Pulsed field gel electrophoresis

After DNA digestion, the agarose plugs were incubated with 1 ml of TE buffer at 37°C for 1 hr. The plugs were then loaded into 1.2% agarose gel in 0.5X TBE buffer. After loading the wells were sealed with 1.5% low melting point agarose. Restriction fragments of the DNA were separated by PFGE with a CHER-DRII apparatus (Bio-Rad Laboratories) through 1.2% PFGE agarose (Sigma) at a field strength of 6V/cm
for 22 h at 14 °C, with the pulse time being increased from 5 to 20s. A lambda ladder (Sigma) was used as the molecular size marker.

2.2.10.4. Staining and visualizing gel

To stain the gel after a run, the gel was slid off the platform in to a 0.5μg/ml ethidium bromide solution in water and was let to be stained for 20-30 minutes. The gel was destained in distilled water for 1-2 hours. The DNA was visualized by placing gel on a UV transilluminator (254-360nm) and photographed using Gel Documentation (Bio-Rad Laboratories).
Chapter 3

Etiology and antibiotic susceptibility patterns of uropathogens
3.1. Introduction

Community-acquired urinary tract infection (UTI) is one of the most common infectious diseases and a frequent cause of presentation for outpatient treatment. Worldwide, about 150 million people are diagnosed with UTI each year, costing the global economy in excess of 6 billion US dollars [Gonzalez et al., 1999]. UTI may involve only the lower urinary tract or may involve both the upper and lower tract. The term cystitis has been used to describe lower UTI, which is characterized by a syndrome involving dysuria, frequency, urgency and occasionally suprapubic tenderness. However, the presence of symptoms of lower tract without upper tract symptoms does not exclude upper tract infection, which is also often present [Sobel et al., 2000].

UTIs are often treated with different broad-spectrum antibiotics when one with a narrow spectrum of activity may be appropriate because of concerns about infection with resistant organisms. Fluoroquinolone are preferred as initial agents for empiric therapy of UTI in area where resistance is likely to be of concern [Schaeffer et al., 2002]. This is because they have high bacteriological and clinical cure rates, as well as low rates of resistance, among most common uropathogens [Goldstein et al., 2000]. The extensive uses of antimicrobial agents have invariably resulted in the development of antibiotic resistance, which, in recent years, has become a major problem worldwide [Biswas et al., 2006]. Epidemiological studies to better manage infections such as UTIs generally involve surveys of isolates from a defined location or locations for the purpose of identifying common resistance profiles [Farrell et al., 2003; Kurutepe et al., 2005; Turnidge et al., 2002]. These studies attempt to survey
resistance to clinically important antibiotics generally or focus on particular families of antibiotics and the corresponding genes that encode resistance to them. Such surveys can lead to treatment strategies that make use of antibiotics to which strains are likely to be sensitive and can be useful in the short term for the development of detection and management strategies [Gupta et al., 2001; Turnidge et al., 2007]. The resistance pattern of community acquired UTI pathogens has not been studied extensively [Goldstein et al., 2000]. The etiology of UTI and the antibiotic resistance of uropathogenes have been changing over the past years, both in community and nosocomial infection [Gupta et al., 2002, and Tankhiwale et al., 2004]. However, there are not much information on etiology and resistance pattern of community acquired UTIs in India is available. This retrospective study was conducted to compare the frequency and drug resistance pattern in uropathogenes isolated from patients with community acquired UTIs in Aligarh, India as well as identification of ESBL producer strains among the uropathogens. This study is important for clinician in order to facilitate the empiric treatment of patients and management of patients with symptoms of UTIs. Moreover, the data would also help authorities to formulate antibiotic prescription policies.

3.2. Experimental overview

Urinary isolates from symptomatic UTI cases attending to the JN Medical College and hospital at Aligarh were identified by conventional biochemical methods by using Biochemical test kits from Hi-media laboratories, Mumbai, India. Antimicrobial susceptibility testing was performed by Kirby Bauer's disc diffusion method outlined in section 3.2. Isolates resistant to third generation cephalosporin
were tested for ESBL production by double disk synergy test method as discussed in section 3.3.

### 3.3. Results

Of the 920 urine samples processed 100 (10.86%) gave significant growth of pathogens. The patients were between new born and 80 years of age. More cases of UTIs were recorded among young and middle age patients (20-49 years, 51.04%). Pediatric patients (new born to 19 years) comprised 36.45% and elderly (50-80 years) constituted 16.66 % of the total number. More organisms were isolated from women (66.66 %) than from men (33.34 %).

Age and gender wise data of prevalence of uropathogenes revealed that *E. coli* (87%) *Klebsiella spp.* (89%) *Psueodomonas spp.* (100%) and *Acinetobacter spp.* (67%) infection was found to be more prevalent among middle aged female candidates as shown in table 3.1. Moreover, none of the *Psueodomonas* and *Acinetobacter spp.* were found in pediatric patients (NB to 19 years) whereas *Acinetobacter spp* were not observed among elderly patients (50-80 years) in our study (table 3.1).

Of the 100 significant isolates, gram-negative aerobic rods accounted for 92 % while gram-positive cocci accounted for the remaining 8 % of the total pathogens. The frequency and distribution of the different microorganisms is summarized in Table 3.2. *E. coli* (61%), *Klebsiella spp.* (22%), *Pseudomonas spp.* (4.0%), *Staphylococcus aureus* (7.0%), *Acinetobacter spp.* (3.0%), *Citrobacter spp.* (2.0%) and *E. faecalis* (1.0%) were the most prevalent microorganisms in UTI patients. Frequency of UTIs was found more in elderly patients (51.04 %), principally women (66.66 %) than in pediatric patients.
Table 3.1. Age and gender wise distribution of urinary tract infection (UTI) patients.

<table>
<thead>
<tr>
<th>Patient’s age group (Years)</th>
<th>No. of UTI Patients (%)</th>
<th>Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Neonate to teenagers)</td>
<td>30 (31.25)</td>
<td>M (33.33%)</td>
</tr>
<tr>
<td>(20-50)</td>
<td>50 (52.08)</td>
<td></td>
</tr>
<tr>
<td>(50-80)</td>
<td>16 (16.66)</td>
<td>F (66.6%)</td>
</tr>
<tr>
<td>Total patients</td>
<td>96.00</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2. Frequency and distribution of urinary pathogens isolated from community acquired infection patients.

<table>
<thead>
<tr>
<th>Uropathogen</th>
<th>Numbers</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>61</td>
<td>61</td>
</tr>
<tr>
<td><em>Klebsiella spp.</em></td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>07</td>
<td>07</td>
</tr>
<tr>
<td><em>Pseudomonas spp.</em></td>
<td>04</td>
<td>04</td>
</tr>
<tr>
<td><em>Acinetobacter spp.</em></td>
<td>03</td>
<td>03</td>
</tr>
<tr>
<td><em>Citrobacter spp.</em></td>
<td>02</td>
<td>02</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>01</td>
<td>01</td>
</tr>
</tbody>
</table>
The antimicrobial potency and spectrum for 27 selected antimicrobial agents of different classes against the five most frequent UTI pathogens are summarized in table 3.3. Among the β-lactum antibiotics, imipenem had the widest coverage against *E. coli* isolates (100%), followed by amikacin (49%), and extended spectrum cephalosporins (15-45%). Moreover, a high potency of the fluoroquinolones against *E. coli* was observed.

Our *Klebsiella* isolates showed high percent susceptibility against imipenem (88%) followed by amikacin and cephotaxime (59%) and ceftriaxone (53%). Nitrofurantoin, tetracycline, co-trimoxazole, and cefpodoxime were found to be highly resistant (100%) against *Pseudomonas* isolates. Among the beta lactam antibiotics, imipenem had the widest coverage against gram-negative isolates (100%). This was followed by the amikacin, ciprofloxacin and norfloxacin (67%).

Imipenem, amikacin, ciprofloxacin and norfloxacin showed highest percent susceptibility (100%) against *Acinetobacter spp.* in this study. Third and fourth generation cephalosporins were found to be more effective with significant percent susceptibility (67%) against *Acinetobacter spp.* Aminoglycosides and macrolides also had the same percent susceptibility (67%). Whereas, all *Staphylococcus aureus* isolates were found to be susceptible against imipenem, ceftriaxone and cephotaxime. The high resistance rate (60%) against cefpodoxime, nalidixic acid and aztreonem was also observed among these isolates.

Age wise distribution of antibiotic resistance pattern revealed that uropathogens isolated from the patients of different age groups showed slight variation in percent resistance against different antibiotics. It was found that percent resistance
### Table 3.3. Antimicrobial potency and spectrum for 27 selected antimicrobial agents tested against most frequently occurring UTI pathogens and % ESBL production.

<table>
<thead>
<tr>
<th>Class of Antimicrobial agents</th>
<th>Percent resistance (%) against antimicrobial agents for</th>
<th>E. coli</th>
<th>E. asp.</th>
<th>Pseudomonas spp.</th>
<th>S. aureus</th>
<th>Acinetobacter spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; gen. Cephalosporins</td>
<td>Cephalothin</td>
<td>85</td>
<td>71</td>
<td>67</td>
<td>20</td>
<td>67</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>71</td>
<td>53</td>
<td>67</td>
<td>20</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; gen. Cephalosporins</td>
<td>Cefuroxime</td>
<td>78</td>
<td>53</td>
<td>100</td>
<td>40</td>
<td>67</td>
</tr>
<tr>
<td>Cephoxitin</td>
<td>69</td>
<td>53</td>
<td>67</td>
<td>40</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; gen. Cephalosporins</td>
<td>Cefotaxime</td>
<td>56</td>
<td>41</td>
<td>67</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>55</td>
<td>47</td>
<td>67</td>
<td>0</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Cefpodoxime</td>
<td>85</td>
<td>65</td>
<td>100</td>
<td>60</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt; gen. Cephalosporins</td>
<td>Cefepime</td>
<td>67</td>
<td>53</td>
<td>67</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Gentamicin</td>
<td>64</td>
<td>53</td>
<td>67</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td>Amikacin</td>
<td>51</td>
<td>35</td>
<td>33</td>
<td>20</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Tobramycin</td>
<td>73</td>
<td>53</td>
<td>67</td>
<td>20</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Penicillins</td>
<td>Ampicillin</td>
<td>75</td>
<td>76</td>
<td>100</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td>Penicillin-G</td>
<td>87</td>
<td>88</td>
<td>100</td>
<td>40</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>Piperacillin</td>
<td>84</td>
<td>82</td>
<td>67</td>
<td>40</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>Carbapenem</td>
<td>Imipenem</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Monobactum</td>
<td>Aztreonam</td>
<td>75</td>
<td>59</td>
<td>67</td>
<td>60</td>
<td>67</td>
</tr>
<tr>
<td>Macrolides</td>
<td>Erythromycin</td>
<td>89</td>
<td>82</td>
<td>100</td>
<td>40</td>
<td>67</td>
</tr>
<tr>
<td>Lincosamides</td>
<td>Clindamycin</td>
<td>91</td>
<td>94</td>
<td>100</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td>Quinolones</td>
<td>Nalidixic acid</td>
<td>87</td>
<td>59</td>
<td>100</td>
<td>60</td>
<td>67</td>
</tr>
<tr>
<td>Fluroquinolones</td>
<td>Ciprofloxacin</td>
<td>69</td>
<td>47</td>
<td>33</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>69</td>
<td>47</td>
<td>33</td>
<td>40</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Phenicols</td>
<td>Chloramphenicol</td>
<td>53</td>
<td>35</td>
<td>100</td>
<td>40</td>
<td>33</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Tetracycline</td>
<td>76</td>
<td>53</td>
<td>100</td>
<td>40</td>
<td>33</td>
</tr>
<tr>
<td>Beta-lactum Inhibitors</td>
<td>Amoxycyclav</td>
<td>75</td>
<td>53</td>
<td>100</td>
<td>40</td>
<td>33</td>
</tr>
<tr>
<td>Others</td>
<td>Co-trimoxazole</td>
<td>76</td>
<td>53</td>
<td>100</td>
<td>40</td>
<td>33</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>80</td>
<td>76</td>
<td>100</td>
<td>20</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>% ESBL producers</td>
<td>34.42</td>
<td>27.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Numbers of isolates are significant.
against different generation of cephalosporin was found to be 60-80% in pediatric patients whereas 40-70% was observed among elderly and middle aged patients. Moreover, 2-3% isolates among middle aged and pediatric patients were resistant against imipenim while no resistance against imipenim was seen against elderly patients. Percent resistance against norfloxacin was quite high (74%) among pediatric patients compare to middle aged and elderly patients (55%). Rate of resistance against gentamycin was higher (75%) among pediatric patients than elderly and middle aged patients (50%). Moreover, almost all of the isolates included in this study were found resistant to four or more antibiotics as shown in table 3.4.

A total of 42% isolates were found to produce ESBL detected by the double disc diffusion test (Figure 3.1). Table 3.3 shows the frequency of ESBL producers for different UTI pathogens. Among the five most frequent UTI pathogens, E. coli (34.42%) and Klebsiella spp. (27.3%) were most prevalent ESBL producers. Other isolates were also ESBL producers but their numbers were insignificant as shown in table 3.3.

Statistical analysis revealed that the data obtained was obeying the normality as well as principle of homogeneity throughout; p-values were also calculated as indicated in Table 3.3.
Figure 3.1 ESBL detection by double disc synergy test. A: Ceftazidime (30μg), B: Cefixime (30μg), C: Cephtaxime (30μg), D: Ceftriaxone (30μg) and E: Amoxyclav (20/10 μg).
Table 3.4. Resistance patterns of various uropathogens.

<table>
<thead>
<tr>
<th>Uropathogens</th>
<th>No. of Patterns</th>
<th>Resistance Patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E.coli.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Cep(^1), Cep(^2), Cep(^3), Cep(^4), Amn, Mon, Pen, Qun, Fqn, Mac, Lin, T, Nf, Bli,</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Cep(^1), Cep(^2), Cep(^3), Cep(^4), Amn, Mon, Pen, Qun, Fqn, Mac, Lin, T, Nf, Bli, Co and (\text{C}'.)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Cep(^1), Cep(^2), Cep(^3), Cep(^4), Amn, Mon, Pen, Qun, Fqn, Mac, Lin, T, Nf, Bli and C.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Cep(^3), Cep(^4), Cep(^1), Pen, Qun, Lin.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Cep(^5), Mon, Mac, Pen, Qun.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Cep, Mac, Lin, Pen, Amn.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Cep(^1), Cep(^2), Amn, Pen, Qun, Fqn, Mac, Lin, T, Nf, Bli and Co.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Cep(^1), Cep(^2), Amn, Pen, Qun, Fqn, Mac, Lin, Mon and Co.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Cep(^1), Cep(^2), Cep(^4), Mon, Pen, Mac, Lin, T, Nf, Bli.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Cep(^1), T, Nf, Co, C, Amn, Pen, Mon, Qun, , Mac, Lin.</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Cep(^1), Nf, Bli, Co, Amn, Mon, Pen, Qun, Fqn, Mac, Lin.</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Cep(^1), T, Nf, Co, Amn, Pen, Mac, Lin.</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Cep(^1), Cep(^2), Cep(^3), Cep(^4), Amn, Mon, Pen, Qun, Fqn, Mac, Lin, T, Nf, Bli, Co.</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Cep(^1), Cep(^2), Cep(^3), Cep(^4), Amn, Mon, Pen, Qun, Fqn, Lin, Nf, and C.</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Cep(^1), Cep(^2), Cep(^3), Cep(^4), Amn, Mon, Pen, Qun, Fqn, Mac, Lin, T, Nf, Bli and Co.</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Cep(^1), Cep(^2), Cep(^3), Qun, Mac, Lin, and Nf.</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Cep(^1), Cep(^2), Cep(^3), Cep(^4), Amn, Mon, Pen, Qun, Mac, Lin, T, Bli, Co and C.</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Cep(^1), Pen, Mac, Lin, T, Nf, Bli, Co and C.</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Mac, Lin, and Nf</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Cep(^1), Cep(^2), Amn, Mon, Pen, Qun, Fqn, Mac, Lin, T, Nf, Bli, Co and C.</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Cep(^1), Cep(^4), Qun, Bli, Nf, T, and Co.</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Cep(^1), Cep(^2), Amn, Pen, Qun, Fqn, Mac, Lin, T, and Bli.</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Cep(^1), Cep(^2), Pen, Qun, Fqn, Mac, Lin, T, Bli and Co.</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Cep(^1), Cep(^2), Cep(^3), Amn, Mon, Pen, Qun, Fqn, Mac, Lin, T, Bli and Co.</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Cep(^1), Mon, Qun and Nf.</td>
<td></td>
</tr>
<tr>
<td><strong>Klebsiella spp</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Cep(^1), Cep(^2), Cep(^3), Cep(^4), Amn, Mon, Pen, Qun, Fqn, Lin, Bli, T, Nf and C.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Cep(^1), Cep(^2), Cep(^3), Cep(^4), Mon, Pen, Qun, Mac and Lin.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Cep(^1), Cep(^2), Amn, Mon, Pen, Qun, Mac, Lin, T and Nf.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Cep(^1), Pen, Fqn, Lin, Mac, T and Co.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Pen, Nf, Mac and Lin.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Cep(^1), Cep(^2), Amn, Mon, Pen, Qun, Fqn, Mac, Lin, T, Nf and Co.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Cep(^2), Pen, Mac, Lin, T and C.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Resistant to all the groups.</td>
<td></td>
</tr>
</tbody>
</table>

**S.aureus.**

|               |                |                     |
| 1              | Mon, Cep\(^1\), Amp, Qun, fqn,Mac,and Co. |
| 2              | Cep\(^2\), Cep\(^3\), Pen, Qun, Fqn, Bli, C, T, Mon. |
| 3              | Cep\(^3\), Mon, Qun. |
| 4              | Cep\(^1\), Cep\(^2\), Cep\(^3\),Pen, Fqn, Amn, Mac, Lin, Bli, C, T, Nf, and Co. |
3.4. Discussion

This study shows the distribution and antibiotic susceptibility pattern of microbial species isolated from patients with community acquired UTIs in J.N.M.C.H, Aligarh. These organisms cause a variety of infections including UTIs [Schaeffer et al., 2002]. In this study urinary samples of children were also included. A majority of pathogens were isolated from adult patients (51.04 %), principally women (66.66 %). It has been extensively reported that adult women have a higher prevalence of UTI than men, principally owing to anatomic and physical factors [Biswas et al., 2006 and CLSI, 2000].

Antibiotic resistance is a major clinical problem in treating infections caused by these microorganisms. The resistance to the antimicrobials has increased over the years. Resistance rates vary from country to country [CLSI, 2001]. Overall, isolates from Latin American countries show the lowest susceptibility rates to all antimicrobial agents followed by Asian-Pacific isolates and European strains. Strains from Canada exhibit the best global susceptibility testing results. (SENTRY Antimicrobial Surveillance Program, SASP) [CLSI, 2001]. In our study, it accounted for approximately 61% of all clinically significant urinary isolates and 63% of all Enterobacteriaceae. This is consistent with the findings of previous studies in which E.coli was the predominant pathogen isolated from patients with community acquired UTIs [Gupta et al., 2002, and Meharwal et al., 2002]. However, Klebsiella spp. are rarely encountered in cases of community-acquired UTI [Biswas et al., 2006, Gupta et al., 2002, and Khan et al., 2004]. In the present study 22% of Klebsiella isolates were found to be present among all uropathogens studied. These isolates shows resistance
against first generation cephalosporin, cephalothin, aminoglycosides, macrolides and lincosamides which is consistent with the previous data of other community-based studies [Gales et al., 2001].

Our *E. coli* and *Klebsiella* isolates are equally resistant to ampicillin (76% and 75% respectively) while for Co-trimoxazole, *E. coli* is more resistant (75%) than *Klebsiella* (53%) in this region. Indian isolates showed higher resistance against ampicillin and co-trimoxazole than the isolates from USA (39.1% and 18.6 % respectively) [Philippon et al., 1996] and Europe (29.8% and 14.1% respectively) [Kunin et al., 1994]. On the other hand, rate of resistance against these antibiotics in countries like Senegal (77% and 55%), Spain, (65% and 33%), Taiwan (80% and 56%), and Israel (66% and 26%) is comparable with Indian isolates [Dimitrov et al., 2003, Vromen et al., 1999, Kahlmeter et al., 2003, and Dromigny et al., 2002]. Our findings with regard to the overall high resistance of urinary *E. coli* strains to antibiotics such as Co-trimoxazole (76%), ampicillin (75%), and any beta-lactams studied (55-85%) are in agreement with those of other recent studies [Anatoliotaki et al; 2007, Garcia et al; 2007, Karki et al; 2001, Prelog et al; 2008]. This shows the limited possibility of using these antibiotics in the empirical treatment of UTIs.

In this study *E. coli* and *Klebsiella* isolates are highly resistant against nitrofurantoin (80% and 76% resistant respectively). Whereas, this drug exhibited low resistance rate in the major part of the world (0-5.4%), despite of it's being used for many years [Daza et al., 2001]. This is probably due to the fact that this antibiotic has been widely used in treating community-acquired UTIs over the past decade in this region (Lau et al., 2004, Colodner et al., 2001, and Honderlick et al., 2006).
resistance rate of *E. coli* to extended spectrum cephalosporins ranges from 55% to 85%, which is contrary to other community-acquired UTI studies in Europe, Israel and the US [Dromigny et al., 2002, Mudur et al., 2000]. Higher resistance rate to all antibiotics used in this study with the exception of imipenem and amikacin may be explained as uncontrolled consumption of these antibiotics during the past decade in our region (Lau et al., 2004, Colodner et al., 2001, and Honderlick et al., 2006).

In the present study overall imipenem resistance was 12% for *Klebsiella spp.*, whereas, other isolates of uropathogens were found to be sensitive to imipenem. It is highly stable against β-lactamase and has an unusual property of causing a post antibiotic effect on gram-negative bacteria [Svetlansky et al., 2001]. Due to its small molecular size it can overcome the poor permeability of β-lactams for *Pseudomonas* by efficient penetration through the porin, OMP D [Hillier et al., 2002]. Extended spectrum cephalosporins showed remarkable rates of resistance against *E. coli*, *Klebsiella spp*, *S. aureus*, *Acinetobacter spp*, and *Pseudomonas spp*. All *S. aureus* isolates were susceptible to third generation cephalosporins i.e. cephotaxime and ceftriaxone. Whereas, among *Pseudomonas spp* all isolates showed resistance to cepodoxime and cefuroxime. All *Acinetobacter* isolates were susceptible to amikacin and fluoroquinolones. The reported resistance varies from 10 to 30% in *Pseudomonas spp* and 3 to 10.3% in *Acinetobacter spp*.

In this study, 42 out of 100 isolates of UTI pathogens (42%) were found to produce ESBL. High prevalence rate of ESBL producing strains have also been reported earlier in *Klebsiella spp* [Farrel et al 2003, and Neu et al., 1992]. This is consistent with other drug resistance groups in India (48.3%) [Amin et al., 2005].
34.42% of our *E. coli* isolates were ESBL producers, followed by 27.3% of *Klebsiella spp.* It might be possible that the high level of multi-drug resistance was most probably due to production of extended spectrum beta lactamases in these isolates [Grover et al., 2006, Mathai et al., 2002, and Mohanty et al. 2005]. More studies are required to know the exact magnitude of the problem in India.

### 3.5. Conclusion

It is quite alarming to note that almost all of the isolates included in this study were found resistant to four or more antibiotics. Antibiotic resistance is becoming a big problem for the public health which threatens the lives of hospitalized individuals as well as those with chronic conditions and adds considerably to health care cost. Therefore, it is an important issue to be addressed by the policy makers to formulate a strict antibiotics prescription policy in our country. Moreover, this study concludes that *E. coli* and other isolates were more sensitive to imipenem and amikacin compared to the other antibiotics tested and therefore these may be the drugs of choice for the treatment of community-acquired UTIs in our region.
Chapter 4

Molecular characterization of ESBLs producing $E. \text{coli}$ strains of UTIs
4.1. Introduction

Urinary tract infection (UTI) is an extremely common condition requiring antimicrobial therapy [Sefton et al., 2000]. Increasing antimicrobial resistance among bacteria causing UTI is therefore of great concern. Patterns of increased antimicrobial usage are the main driving force in generating and maintaining resistant bacteria [WHO, Geneva, Switzerland, 2001]. Studies also show that, once evolved, resistance genes can spread through the world’s bacterial populations irrespective of the pattern of antimicrobial use in an area [O’Brien et al., 2002]. Therefore, mechanisms other than selection pressure might exist for maintaining a resistant bacterial pool.

Epidemiological studies to better manage infections such as UTIs generally involve surveys of isolates from a defined location or locations for the purpose of identifying common resistance profiles [Turnidge et al., 2002; Farrel et al., 2003; Kurutepe et al., 2005]. These studies attempt to survey resistance to clinically important antibiotics generally or focus on particular families of antibiotics and their corresponding genes that encode resistance to them. Such surveys can lead to treatment strategies that make use of antibiotics to which strains are likely to be sensitive and can be useful in the short term for the development of detection and management strategies [Gupta et al., 2001; Turnidge et al., 2007]. However, they do not usually take into account the genetic contexts in which resistance genes are found. Presently several methods of bacterial strain typing are used such as bacteriophage typing, serotyping, plasmid fingerprinting, ribotyping, PCR-based methods. Most of these techniques are not sensitive enough to distinguish different strains or affected by
physiological factors. Hence, the most recommended technique used for genotyping of
the strains is PFGE [Swaminathan et al., 1993; Arbeit et al., 1995].

One group of elements that has been an increasing focus of study in the context of
UTIs is integrons. In particular, integrons belonging to classes 1, 2, and 3 are most
commonly associated with the spread of antibiotic resistance among pathogens
[Marquez et al., 2008]. Integrons are a problem generally in managing the spread of
resistance, they are especially common in UTIs [Blahna et al., 2006]. Surveys that
examine the prevalence of various genetic elements in multidrug-resistant UTI-
causing strains invariably show a high correlation between the presence of a class 1
integron and particular antimicrobial resistance profiles. Most notable among these are
resistance to ampicillin (AMP) and streptomycin but especially trimethoprim and sul-
famethoxazole (SMX) [Blahna et al., 2006; Solberg et al., 2006; Smith et al., 2008]. In
case of class 1 integrons, SMX resistance is normally derived from the sul1 gene that
is contained within a region downstream of the integron called the 3' conserved
segment (3'-CS), and this region is present in the majority of class 1 integrons from
clinical environments. Consequently, SMX resistance is a common feature of strains
that carry class 1 integrons. In addition, the use of co-trimoxazole (SXT) to treat UTIs
and many other community acquired infections is most probable reason why a variety
of dfrA gene cassettes are found in class 1 integrons from UTI mediating organisms,
thus leading to SXT resistance being very common [Grape et al., 2005; Solberg et al.,
2006]. The ongoing use of antibiotics is associated with more complex multi-
resistance patterns attributable to the influx of more resistance genes into clinical
isolates [Yu et al., 2003].
The class 1 integrons that carry drug resistance genes in clinical isolates have a relatively conserved structure. This structure commonly comprises two conserved DNA sequences—the 5′-CS and 3′-CS—separating a variable region where mobile gene cassettes are located [Stokes et al., 1989]. This arrangement (5′-CS-variable region-3′-CS) has been used as a PCR tool for the simple recovery of cassette arrays irrespective of knowledge of the many and varied cassettes that may be present [Le’vesque et al., 1995; Recchia et al., 1995]. As well as being able to recover and analyze cassettes from specific isolates, this method has also been used as a tool in broader epidemiological studies [Jones et al., 2003; Severino et al., 2004]. Among Enterobacteriaceae, prevalence of integrons varies and has been reported up to 59% [Jones et al., 1997; Martinez et al., 1998; Fluit et al., 1999; White et al., 2001; Lee et al., 2001; Schmitz et al., 2001; Leverstein et al., 2002]. There is, however, a paucity of data from community-acquired infections. In one such study from Netherlands 19% of *E. coli* had integron class 1 [Leverstein et al., 2002]. No such data were obtained from India although prevalence of co-trimoxazole resistance is high among *E. coli*.

Multidrug-resistant Enterobacteriaceae (MRE) strains are being isolated at an increasing rate in hospital settings and are having a significant impact on clinical practice and overall treatment costs [Cross et al., 1983; Holmberg et al., 1987; Cohen et al., 1992; Hobson et al., 1996; Leverstein et al., 2001]. This multi-resistance may be mediated by chromosomally located resistance determinants or mutations in a resident gene; however, it may also develop through the acquisition of resistance genes or an array of resistance genes by horizontal gene transfer. This latter phenomenon is currently thought to play an important role in developing multi-drug resistance in
Enterobacteriaceae [Rowe et al., 1996]. Plasmids and transposons are known to be involved in the transfer of resistance genes from one cell to another. These elements can hunt as a pack by interacting with each other in a variety of ways that enhance their collective ability to transfer resistance genes. Some of the plasmids which carry multiple resistance genes also have transfer systems that enable them to transfer DNA between unrelated species (promiscuous or broad host range plasmids) [Hall et al., 1997]. Several studies have investigated the prevalence of integrons in clinical isolates and have found them to be widespread [Sallen et al., 1995; Gonzalez et al., 1998; Martinez et al., 1998]. However, the kinds of gene cassettes inserted into the integrons were not identified, except in the work of Sallen et al., [Sallen et al., 1995].

The incidence of nosocomial infection caused by members of the family Enterobacteriaceae that produce ESBLs and other enzymes capable of hydrolyzing cefotaxime, ceftriaxone, ceftazidime and aztreonam is increasing worldwide [Tenover et al., 1999]. Extended spectrum beta-lactamases (ESBL)-producing organisms pose a therapeutic challenge as they do not only mediate resistance to oxyiminocephalosporins, but as they are also frequently resistant to other kinds of antimicrobial drugs, including aminoglycosides, fluoroquinolones, tetracycline, gentamicin and trimethoprim-sulphamethoxazole [Martinez et al., 1998; Paterson et al., 2000; Gniadkowski et al., 2001; Winokur et al., 2001; Edelstein et al., 2003; Eckert et al., 2004; Rodriguez et al., 2004]. Most ESBLs are mutants of TEM and SHV enzymes, but CTX-M enzymes are increasingly important as a major source of resistance. Over 40 CTX-M beta-lactamases have been described [Woodford et al., 2004]. CTX-M type ESBLs are mainly found in *E. coli* strains. CTX-M producers are most frequently
found in urinary tract infections [Johnson et al., 2002; Bonnet et al., 2004; Pitout et al., 2005; Lavigne et al., 2006]. Most known CTX-M enzymes are CTX-M-1 and CTX-M-15 [Bonnet et al., 2004; Canton et al., 2006]. The present study was aimed to investigate the prevalence of CTX-M type enzymes in community acquired \textit{E. coli} isolates. PCR based methods have been proved extremely effective in detecting antimicrobial resistance genes of complex mechanisms found in Gram negative and Gram positive bacteria, including among others, those encoding β-lactam resistance in \textit{Enterobacteriaceae} [Pfaller et al., 2001]. It has the advantage of being speedy, sensitive and specific [Mims et al., 1998; Schwarts et al., 2002] and can produce extremely large number of relevant genes [Eglinton et al., 1996].

4.2. Experimental overview

Extended spectrum beta-lactamases producing \textit{E. coli} isolates were selected for the screening of CTX-M β-lactamases by using PCR methods as discussed in section 3.7.3. Integron screening was also performed. Integron positive isolates were sequenced. These ESBLs producing \textit{E. coli} strains were typed by pulsed field gel electrophoresis technique described in section 4.1. Conjugation studies for marker transfer were also carried out on these strains by broth mating method as discussed in section 3.5.

4.3. Results

4.3.1. PFGE-analysis

Of twenty two ESBLs producing \textit{E. coli} isolates, eight strains were subjected to PFGE to investigate their clonality. Banding patterns and clusters were interpreted using the guidelines suggested by Tenover [Tenover et al., 1995]. These strains were
grouped into different subtypes. The results are shown in Table 4.1. Considering the criteria by Tenover [Tenover et al., 1995], we found that six isolates had 2-5 fragment differences and were grouped under different subtypes; designated as A, A1, A2, A3, A4, and A5. Because the restriction pattern was unique for the other two isolates, we could not include these isolates in any of the six subtypes, so they were classified into two separate PFGE type B and C. (Figure 4.1).

4.3.2. Integron screening

In total, seven isolates were evidenced for the class 1 integrons among the twenty two ESBLs producing *E. coli* isolates. The amplification products of class 1 integrons varied in size between 0.9 and 2.3 kbp (Figure 4.2 A & B; Table 4.2). These integron harboring isolates were further screened for the presence of Intl and Sul1 genes, which are characteristic features of class1 integrons. All the seven integrons were found to be positive for these genes. The amplification products of Intl and Sul1 genes were found to be 845 bp and 840 bp respectively (Figures 4.3 and 4.4 A & B).
Table 4.1. Genotyping of ESBL producing *E. coli* isolates.

<table>
<thead>
<tr>
<th>Strains</th>
<th>PFGE types</th>
</tr>
</thead>
<tbody>
<tr>
<td>UE-61</td>
<td>A</td>
</tr>
<tr>
<td>UE-90</td>
<td>A1</td>
</tr>
<tr>
<td>UE-133</td>
<td>A2</td>
</tr>
<tr>
<td>UE-112</td>
<td>A3</td>
</tr>
<tr>
<td>UE-152</td>
<td>A4</td>
</tr>
<tr>
<td>UE-170</td>
<td>A5</td>
</tr>
<tr>
<td>UE-64</td>
<td>B</td>
</tr>
<tr>
<td>UE-128</td>
<td>C</td>
</tr>
</tbody>
</table>
Figure 4.1. PFGE profiles of ESBLs producing *E. coli* strains isolated from different UTI patients. Lateral lane contains multi-mers of phage lambda DNA (48.5 kb) molecular mass markers. Sizes of lambda DNA molecular mass markers are indicated on the left of the panel. Different PFGE types and subtypes identified are indicated on the top of each lane.
Table 4.2. Association of integrons with multiple drug resistance in *E. coli* isolates.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Integron content</th>
<th>Antibiogram</th>
<th>Gene cassettes</th>
</tr>
</thead>
<tbody>
<tr>
<td>UE-61</td>
<td>0.97kb</td>
<td>1GC,2GC,3GC,4GC,Amn,Pen,Quin,Fquin,Ac</td>
<td>aadA1</td>
</tr>
<tr>
<td>UE-90</td>
<td>1.99kb</td>
<td>1GC,2GC,3GC,4GC,Amn,Pen,Quin,Fquin,Nf,Ac, Co</td>
<td>aadA5, dfrA17</td>
</tr>
<tr>
<td>UE-112</td>
<td>2.21kb</td>
<td>1GC,2GC,3GC,4GC,Amn,Co,C,Quin,Fquin,Nf,Ac</td>
<td>aacA4, dfrA1, CmlA1</td>
</tr>
<tr>
<td>UE-128</td>
<td>2.31kb</td>
<td>1GC,2GC,3GC, Pen,Quin,Fquin,Nf,Ac</td>
<td>None</td>
</tr>
<tr>
<td>UE-133</td>
<td>1.95kb</td>
<td>1GC,2GC,3GC,4GC,G,Tb,Pen,Quin,Fquin,Ac, Co</td>
<td>aadA5, dfrA17, dfrA7</td>
</tr>
<tr>
<td>UE-152</td>
<td>2.04kb</td>
<td>1GC,2GC,3GC,4GC,Amn,Co,Quin,Fquin,Nf,Ac</td>
<td>aadA5, dhfrA17, dfrA7</td>
</tr>
<tr>
<td>UE-170</td>
<td>0.90kb</td>
<td>1GC,2GC,3GC,4GC,G,Ak,Co,Pen,Quin,Fquin,Nf,Ac</td>
<td>aadA2, dhfrA12</td>
</tr>
</tbody>
</table>
Figure 4.2 (A). PCR amplification of 5'-CS-3'-CS conserved sequences of integron. Lane M: 1 Kb DNA ladder, Lane P: Positive control, Lane 1-5: *E. coli* clinical isolates (UE-128, UE-133, UE-137, UE-112, UE-170) and Lane 6: Negative control.

<table>
<thead>
<tr>
<th>Kb</th>
<th>10</th>
<th>2.5</th>
<th>1.5</th>
<th>1.0</th>
<th>0.5</th>
</tr>
</thead>
</table>

Figure 4.2 (B). PCR amplification of 5'-CS-3'-CS conserved sequences of integron. Lane M: 1 Kb DNA ladder, Lane P: Positive control, Lane 1-3: *E. coli* clinical isolates (UE-61, UE-90, UE-152) and Lane 4: Negative control.

<table>
<thead>
<tr>
<th>Kb</th>
<th>10</th>
<th>2.5</th>
<th>1.5</th>
<th>1.0</th>
<th>0.5</th>
</tr>
</thead>
</table>
Figure 4.3 (A). Integron mapping by PCR amplification of Int1 gene. Lane M: 1 Kb DNA ladder, Lane P: Positive control, Lane 1-5: *E. coli* clinical isolates (UE-61, UE-90, UE-112, UE-128, UE-133) and Lane 6: Negative control.

Figure 4.3 (B). Integron mapping by PCR amplification of Int1 gene. Lane M: 1 Kb DNA ladder, Lane P: Positive control, Lane 1-5: *E. coli* clinical isolates (UE-137, UE-152, UE-170, UE-144, UE-103) and Lane 6: Negative control.
Figure 4.4 (A). Integron mapping by PCR amplification of Sul1 gene. Lane M: 1 Kb DNA ladder, Lane P: Positive control, Lane 1-5: *E. coli* clinical isolates (UE-61, UE-90, UE-112, UE-128, UE-133) and Lane 6: Negative control.

Figure 4.4 (B). Integron mapping by PCR amplification of Sul1 gene. Lane M: 1 Kb DNA ladder, Lane 1-2: *E. coli* clinical isolates (UE-152, UE-170) and Lane 3: Negative control.
4.3.3. Integron sequence analysis

Purified PCR products were subjected to DNA sequencing by using the commercial facility at Macrogen, South Korea, to look for the resistance gene cassettes present in these strains. Sequencing data obtained in an electronic format were compared to the GenBank database of the National Centre for Biotechnology Information BLAST network [Altschul et al., 1990]. DNA sequencing of class 1 integrons showed the presence of one to three gene cassettes and the combinations of these (Table 4.2). Most of the gene cassettes found within the variable region of class 1 integrons in our *E. coli* isolates corresponded to different variants of dfrA and aadA genes. These genes are associated with trimethoprim and streptomycin resistance respectively, and aadA5-dfrA17-dfrA7 was the combination of gene most prevalent in our study. One PCR product of about the same size, derived from *E. coli* isolate UE-112, contained a aacA4-dfrAl-CmlA1 array. CmlA cassette encodes chloramphenicol transporter known as chloramphenicol resistance gene. A 2 kbp amplicon amplified from *E. coli* UE-90 genomic DNA was found to contain aadA5, dhfr17 gene cassettes. The aadA1 gene cassette encoding aminoglycoside 3'(9)-O-adenyltransferase associated with streptomycin and spectinomycin resistance was found only in one amplicon of 0.97 kbp while other amplicon of same size was found to have aadA2 and dhfr12 gene cassettes which encode resistance to both aminoglycosides and trimethoprim-sulfamethoxazole respectively. No such gene cassettes were found in 2.3 kbp amplicon.
4.3.4. Screening of ESBLs coding genes

Of 65 *E. coli* isolates, only 22 *E. coli* strains had a phenotype consistent with production of an ESBL. These 22 *E. coli* isolates were screened for the presence of cefotaxime hydrolyzing β-lactamases (CTX-M) on both plasmid as well as genomic DNA by PCR using universal primers. Only 3 strains exclusively demonstrated alleles encoding CTX-M enzymes belonging to phylogenetic group 1 (CTX-M-1-related enzymes). One strain was found to contain CTX-M gene both on plasmid as well as genomic DNA while other two were on Chromosomal DNA only. No alleles encoding for CTX-M-2-related enzymes or CTX-M-9-related enzymes were found. The size of PCR product was found to be 604 bp (Figure 4.5). These CTX-M positive isolates were further screened for the presence of TEM and SHV beta-lactamases by PCR. Out of three CTX-M positive isolates two were found positive for TEM, while all the three were found negative for SHV (Figure 4.7). The PCR amplicons of CTX-M gene were subjected to digestion with HinfI and it was found that the three fragments of 304, 200 and 100 bp were observed in all the 3 CTX-M positive isolates (Figure 4.6). All the three purified PCR products of CTX-M gene and two of TEM, were sequenced by using commercial facility at Chromous Biotech, New Delhi, India. Sequencing data obtained in an electronic format were compared to the GenBank database of the National Centre for Biotechnology Information BLAST network [Altschul et al., 1990]. The nucleotide sequence analysis showed that all the three CTX-M gene amplicons belong to bla<sub>CTX-M-15</sub> gene while that of TEM were bla<sub>TEM-1</sub>.
Figure 4.5. PCR amplification of CTX-M gene. Lane M: 1 Kb DNA ladder, Lane P: Positive control. Lane 1-4: *E. coli* clinical isolates (UE-90, UE-112, UE-112P, UE-152) and Lane 5: Negative control.
Figure 4.6. Hinfl restriction fragment analysis of amplified fragment of CTX-M gene in different strains. Lane M: 1 Kb DNA ladder, Lane 1 and 6: Negative control (undigested), Lane 2-4: *E. coli* clinical isolates (UE-90, UE-112, and UE-112P) and Lane 5: pBR322 as positive control.
Figure 4.7. PCR amplification of TEM-1 gene. Lane M: 1 Kb DNA ladder, Lane 1-3: *E. coli* clinical isolates (UE-90, UE-112, UE-112P) and Lane 4: Negative control.
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Microorganism</th>
<th>No. of Resistance markers</th>
<th>No. of TransferredResistance markers</th>
<th>No. of EJ-53 receiving T resistance</th>
<th>Total no. of EJ-53</th>
<th>Transfer frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UE-59</td>
<td><em>E. coli</em></td>
<td>25</td>
<td>13</td>
<td>5.04x10^7</td>
<td>1.02x10^9</td>
<td>0.049</td>
</tr>
<tr>
<td>UE-61</td>
<td><em>E. coli</em></td>
<td>23</td>
<td>09</td>
<td>2.6x10^6</td>
<td>2.12x10^8</td>
<td>0.012</td>
</tr>
<tr>
<td>UE-64</td>
<td><em>E. coli</em></td>
<td>07</td>
<td>04</td>
<td>1.12x10^4</td>
<td>4.04x10^7</td>
<td>0.0027</td>
</tr>
<tr>
<td>UE-67</td>
<td><em>E. coli</em></td>
<td>11</td>
<td>10</td>
<td>2.6x10^6</td>
<td>2.12x10^8</td>
<td>0.012</td>
</tr>
<tr>
<td>UE-68</td>
<td><em>E. coli</em></td>
<td>13</td>
<td>07</td>
<td>5.04x10^7</td>
<td>2.12x10^8</td>
<td>0.237</td>
</tr>
<tr>
<td>UE-70</td>
<td><em>E. coli</em></td>
<td>08</td>
<td>05</td>
<td>1.12x10^4</td>
<td>2.12x10^8</td>
<td>0.00052</td>
</tr>
<tr>
<td>UE-92</td>
<td><em>E. coli</em></td>
<td>24</td>
<td>07</td>
<td>3.24x10^3</td>
<td>1.02x10^9</td>
<td>3.17x10^6</td>
</tr>
<tr>
<td>UE-93</td>
<td><em>E. coli</em></td>
<td>15</td>
<td>13</td>
<td>3.24x10^3</td>
<td>4.04x10^7</td>
<td>0.80x10^4</td>
</tr>
<tr>
<td>UE-95</td>
<td><em>E. coli</em></td>
<td>08</td>
<td>02</td>
<td>2.6x10^6</td>
<td>2.12x10^8</td>
<td>0.012</td>
</tr>
<tr>
<td>UE-96</td>
<td><em>E. coli</em></td>
<td>11</td>
<td>05</td>
<td>3.24x10^3</td>
<td>2.12x10^8</td>
<td>1.52x10^5</td>
</tr>
<tr>
<td>UE-98</td>
<td><em>E. coli</em></td>
<td>24</td>
<td>18</td>
<td>2.6x10^6</td>
<td>4.04x10^7</td>
<td>0.064</td>
</tr>
<tr>
<td>UE-102</td>
<td><em>E. coli</em></td>
<td>13</td>
<td>09</td>
<td>3.24x10^3</td>
<td>4.04x10^7</td>
<td>0.80x10^4</td>
</tr>
<tr>
<td>UE-103</td>
<td><em>E. coli</em></td>
<td>23</td>
<td>15</td>
<td>1.12x10^4</td>
<td>4.04x10^7</td>
<td>0.0027</td>
</tr>
<tr>
<td>UE-112</td>
<td><em>E. coli</em></td>
<td>23</td>
<td>04</td>
<td>1.12x10^4</td>
<td>2.12x10^8</td>
<td>0.00052</td>
</tr>
<tr>
<td>UE-128</td>
<td><em>E. coli</em></td>
<td>01</td>
<td>01</td>
<td>5.04x10^7</td>
<td>1.02x10^9</td>
<td>0.049</td>
</tr>
<tr>
<td>UE-133</td>
<td><em>E. coli</em></td>
<td>08</td>
<td>01</td>
<td>4.03x10^2</td>
<td>4.04x10^7</td>
<td>0.997x10^5</td>
</tr>
<tr>
<td>UE-137</td>
<td><em>E. coli</em></td>
<td>02</td>
<td>01</td>
<td>5.04x10^7</td>
<td>4.04x10^7</td>
<td>1.24</td>
</tr>
<tr>
<td>UE-144</td>
<td><em>E. coli</em></td>
<td>14</td>
<td>12</td>
<td>1.12x10^4</td>
<td>2.12x10^8</td>
<td>0.52x10^4</td>
</tr>
</tbody>
</table>
4.3.5. Horizontal gene transfer

Eighteen of the twenty-two *E. coli* cultures transferred their drug resistance through conjugation to EJ-53 as recipient susceptible strain. Table 4.2 illustrates the characteristics of the 18 *E. coli* strains which transferred drug resistance and the frequency of transfer of resistance to EJ-53. Transfer frequency varied between $3.17 \times 10^6$ and $5.2 \times 10^9$. All of the 18 strains of *E. coli* which showed transfer of resistance markers to the susceptible strain having at least 1-25 antimicrobial resistant markers (Table 4.3). In all the cases, 01-17 resistance markers were found to be transferred to EJ-53 strain. Plasmid DNAs were isolated from both donor as well as trans-conjugants and electrophoresed on 0.7 % agarose gel (Figure 4.8 A and B). Sizes of plasmids were calculated by quantity one software and were found to be 18-19 kb.
Figure 4.8 (A). Agarose gel electrophoresis of plasmid DNA isolated from donor and transconjugants of clinical isolates of *E. coli*. Lane M: 1 Kb DNA ladder, Lane 1-4: donor and transconjugants (UE-128, UET-128, UE-133, UET-133).

Figure 4.8 (B) Agarose gel electrophoresis of plasmid DNA isolated from donor and transconjugants of clinical isolates of *E. coli*. Lane M: 1 Kb DNA ladder, Lane 1-6: donor and transconjugants (UE-61, UET-61, UE-67, UET-67,UE-70, UET-70).
4.4. Discussion

The present study was performed on the isolates from community-acquired urinary tract infections. Resistance to more than three antibiotics was common among these isolates. It shows that integrons are widespread among these community-acquired \textit{E. coli} UTI in one part of the India and play a significant role in the prevailing multidrug resistance situation. Integron prevalence among \textit{E. coli} in low-income countries seems to be similar to have in many European countries.

\textit{Escherichia coli} is the most common uropathogen and accounts for more than 80\% of UTI cases. Twenty-two of all the resistant \textit{E. coli} isolates that were ESBL-producers were selected for this study. Genotyping of these isolates could reveal whether isolates are clonally related, i.e. have common origins in suspected nosocomial/community outbreaks or possible cross-transmission events [Adamsson et al., 2002]. PFGE has been used in many studies to show the international spread of Methicillin-Resistant \textit{Staphylococcus aureus} (MRSA), and the criteria used for micro epidemiological analyses have frequently been employed [Cookson et al., 2007].

Tenover criteria [Tenover et al., 1995] are simple and helpful for genotyping a group of bacteria based on PFGE, which are closely related. The increased development of resistant strains causing severe nosocomial or community infections has been a major driving force for the development of new antibiotics but more importantly to find a way of preventing the spread. Epidemiological studies regarding dissemination pathways of potential pathogens in the community and hospital wards are of great concern.
Integrons play an important role in the antimicrobial resistance of clinical *Escherichia coli* strains because they are able to capture, integrate and express gene cassettes encoding proteins associated with antimicrobial resistance. The presence of integrons in clinical multi-resistant *E. coli* isolates recovered from the community as well as hospital environment is frequently reported [Schmitz et al., 2001; Hall et al., 2002; Mathai et al., 2004; Fonseca et al., 2005; Solberg et al., 2006; Machado et al., 2007]. Our data revealed the prevalence of integrons in uropathogenic *E. coli*, whereas earlier studies in Asia and Europe also supports our findings [Chang et al., 1997; Yu et al., 2003; Mathai et al., 2004; Yu et al., 2004; Grape et al., 2005; Solberg et al., 2006]. Other reports have shown the prevalence of class 1 integrons in gram-negative clinical isolates to be 43% in Western and Central Europe [Martinez et al., 1998], >50% in the Netherlands [Jones et al., 1997] and 59% in France [Sallen et al., 1995]. These results, together with those obtained in the present study, indicate that the class 1 integrons are widespread in clinical isolates. This study has undertaken to examine the role of integrons in prevailing antimicrobial resistance situation in India, showed that integrons are widespread and that they play a major role in producing MDR among *E. coli* isolates. The prevalence of integrons (33%) in northern Indian isolates tested was similar to that found in the study of Mathai et al., from south India [Mathai et al., 2004]. As previously noted, integrons of class 1 were more prevalent than integrons of class 2 [Conway et al., 2007; Foxman et al., 2000]. *E. coli* with integrons were significantly more likely to exhibit MDR and resistance to cephalosporins, gentamicin, chloramphenicol, ampicillin, nalidixic acid and amoxyclav. The screening for integrons has been performed using three different pairs
of primer. One primer pair gives products of the entire class 1 integron including the different gene cassettes. This is very useful for estimation of size and gene cassette content of the class 1 integron. The second pair of primer used in this study consisted therefore of two primers specific for the integrase class 1 gene only. Since this gene is essential for the function of the integron, all isolates positive for the integrase class 1 gene can hence also be considered positive for integrons of class 1. The third primer pair used was specific for Sull1 gene which is located at 3'-CS. Most of the gene cassettes found within the variable region of class 1 integrons in our E. coli isolates corresponded to different variants of dfrA and aadA genes. These genes are associated with trimethoprim and streptomycin resistance, respectively, and dfrA17- aadA5-dfrA7 was in combination most frequently detected not only in our study but also in E. coli isolates recovered from healthy and sick humans, animals and foods in other studies [Eisenach et al., 1995; AAPC., 1999; Beetz et al., 2002; Blahna et al., 2006; CLSI., 2006; Anatoliotaki et al., 2007; Conway et al., 2007]. The reason for the wide distribution of some successful integrons with a specific arrangement is not known, although the possible inclusion of these integrons in transposons and/or plasmids could explain their wide dissemination in different environments.

The prevalence of class 1 integrons (33%) is in agreement with the prevalence found in similar Indian (36% [Mathai et al., 2004]), Korean (55% [Yu et al., 2003]), and Australian (46% [White et al., 2001]) studies. A Swedish study on urinary E. coli isolates in a general population showed the higher rate of prevalence of class 1 integrons [Grape et al., 2005].
Usually sulfamethoxazole resistance is encoded by the Sul genes. We found that seven *E. coli* strains possessed Sul1 genes, and all showed sulfamethoxazole resistance phenotype. Contrary to our data earlier studies showed that the Sul2 gene was more predominant in *E. coli* strains isolated in UTI episodes [Kern et al., 2002; Blahna et al., 2006]. In our study, SXT treatment was associated with the occurrence of Sul genes and with increased phenotypic resistance to SXT. The association between resistance to nalidixic acid and integrons is interesting since resistance to quinolones is primarily chromosomal and we have all the seven integrons located on *E. coli* chromosomal DNA.

ESBL production is the major emerging mechanism of resistance to expanded spectrum cephalosporins and mono-bactams among *Enterobacteriaceae* and is a matter of major concern in the field of microbial drug resistance. In the European scenario, where the TEM- and SHV-type ESBLs were first detected [Knoth et al., 1983; Sougakoff et al., 1988] and are widespread [Perilli et al., 2002; Morris et al., 2003; De Champs et al., 2004; Paterson et al., 2005; Rodriguez et al., 2006], recent reports have shown a rapid and alarming dissemination of *Enterobacteriaceae*, producing ESBLs of the CTX-M type in some countries, with notable changes in the epidemiologies of these resistance determinants [Eckert et al., 2004; Woodford et al., 2004; Livermore et al., 2005]. This work has provided insights of molecular epidemiology of emerging problem in India. Unlike in other countries (e.g., Spain, France), where members of multiple CTX-M lineages have been reported [Eckert et al., 2004; Valverde et al., 2004], a virtually absolute prevalence of the members of the CTX-M-1 lineage was observed in this study.
Our findings were contrary to an Indian study, in which a variant of the \textit{bla}_{\text{CTX-M-3}} enzyme, designated as \textit{bla}_{\text{CTX-M-15}}, was reported from 6 unrelated members of \textit{Enterobacteriaceae} isolated between April and May 2000 [Sekar et al., 2006]. The authors reported that 44.4\% of \textit{E. coli} and 35.29\% of \textit{K. pneumoniae} strains were found to be positive for \textit{bla}_{\text{CTX-M}} gene. However, our results were consistent with the findings of Shahid et al., 2006, with lower frequency of \textit{E. coli} isolates being found to be CTX-M group-1 positive by PCR in the northern Indian isolates.

The results of the conjugation experiments were supportive of the hypothesis that horizontal transfer of some of the resistance markers took place in vivo. [Maurine et al., 2002]. It was interesting to note that the complete resistance patterns (except for known chromosomally located resistance determinants), were transferred in their entirety in most of the conjugation experiments, suggesting a very efficient mechanism of transferring packages of resistance genes.

\textbf{4.5. Conclusion}

This study concludes that the integrons are one of the means of antibiotic resistant marker transfer among members of \textit{Enterobacteriaceae}. However, their prevalence in different areas appeared to be highly variable, which could reflect the scenario of a relatively early stage of dissemination of these resistance determinants in the community setting. Continuing surveillance will be necessary to monitor the evolution of extended spectrum beta-lactamases and to verify whether the CTX-M type ESBLs will eventually prevail over the TEM- and SHV-type ESBLs, which are still widespread, especially in some areas. It will also be interesting to investigate if these epidemiological differences could reflect differences in regional antimicrobial
policies. Our findings unveil the increasing role of the bla-CTX-M β-lactamases in antibiotic resistance and emphasize on the significance of appropriate empirical treatment for infections caused by coliforms, especially in urinary tract infection patients. In conclusion, the data presented here illustrates the complexity and extent of the spread of ESBL-producing Enterobacteriaceae among the tertiary care hospital patients. This study also shows that the horizontal gene transfer of antimicrobial resistance genes can occur very efficiently and at a high rate among Enterobacteriaceae in a community setting. The phenotypic resistance patterns in the investigated isolates do not directly reflect the antibiotic usage for urinary tract infections in geographic location and at the time of isolation. Part of the explanation is the possibility of co-selection, because of the conjugative plasmids contains multiple resistance determinants. Another part of the explanation may be the overall antimicrobial agents used in the community. All antimicrobial agents used may provide a selection pressure on other enteric bacteria, which may transfer resistance genes to Escherichiae coli. Further studies are needed, however, to determine whether antibiotic policies or other measures can halt or lower the level of horizontal transfer that occurs in a hospital or community setting.
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Appendix
Appendix

(A) Composition of EET buffer for PFGE

100 mM EDTA
10 mM EGTA
10 mM Tris-HCl, pH 8.0

(B) Composition of proteolysis buffer for PFGE

EET buffer
20% SDS
Proteinase K (500 μg/ml)

(C) Composition of wash buffer with or without PMSF for PFGE

10 mM Tris-HCl, pH 7.5
10 mm EDTA
1 mM PMSF

(D) Composition of equilibration buffer for PFGE

10X restriction enzyme assay buffer
Autoclaved double distilled water

(E) Composition of 10X TBE buffer

45 mM Tris
45 mM Borate
1.0 mM EDTA, pH 8.3
Composition of different growth media

The composition of brain heart infusion broth is as follows:

Component | gms/l |
--- | --- |
Beef infusion from | 250.0 |
Calf brain infusion from | 200.0 |
Proteose peptone | 10.0 |
Sodium chloride | 5.0 |
Disodium phosphate | 2.5 |
Dextrose | 2.0 |

Dissolve 37gms in 1000 ml water, pH 7.4± 0.2 at 25°C.

The composition of tryptone soya broth is as follows:

Component | gms/l |
--- | --- |
Casein enzymic hydrolysate | 17.00 |
Papaic digest of soyabean meal | 3.00 |
Sodium chloride | 5.00 |
Dopotasium phosphate | 2.50 |
Dextrose | 2.50 |

Dissolve 30gms in 1000 ml water, pH 7.3± 0.2 at 25°C.
List of publications/Conference presentations

Journal Publications


9. Naeem A, Akram M, Khan RH. Conformational states of trifluoroacetic acid-
treated cytochrome c in the presence of salts and alcohols. Protein J. 2004 Apr;
23(3):185-95. (Impact factor- 0.96).

Conferences/Workshops /Seminars attended:

1. Attended National conference on" Antimicrobial Resistance: From emerging threat
to reality” held during March 23rd to 25th, 2009 at Allahabad Agricultural Institute-
Deemed University, Allahabad, India.
2. Attended International symposium on “The predictive, preventive and mechanistic
mutagenesis” and XXXIII EMSI annual meeting held during January 01-03 2008 at
Aligarh Muslim University, Aligarh.
3. Attended DBT sponsored Workshop on “In Silico Molecular Modeling and Drug
Design” held at Distributed information sub -centre, Biotechnology Building at
Aligarh Muslim University, Aligarh, India during March 18 to 19, 2008.
4. Attended 47th Annual Conference of Association of Microbiologist of India held at
Barkatullah University, Bhopal, during December 06-08, 2006 and presented a poster
paper entitled “Etiology and antibiotic resistance patterns of community acquired
urinary tract infections in the aligarh hospital.”
5. Attended National Symposium on Stability and stabilization of biomolecules held on
March 13-14, 2001 at Interdisciplinary Biotechnology Unit, Aligarh Muslim
University, Aligarh, India.
6. Attended National Science Day sponsored by the Department of Biotechnology,
Ministry of Science and Technology, Govt. of India, New Delhi and organized by
Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh, On