Mechanism of resistance and genetic relatedness among fluoroquinolone resistant *Escherichia coli* causing urinary tract infections

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Certificate

This is to certify that the thesis entitled **"Mechanism of resistance and genetic relatedness among fluoroquinolone resistant *Escherichia coli* causing urinary tract infections"** is the bonafide work done by **Dr. P.Padmaja** in partial fulfillment of the rules and regulations for MD Branch IV (Microbiology) examination of **The Tamilnadu Dr. M.G.R. Medical University, Chennai,** to be held in March 2007. Her work was carried out under the guidance of **Dr. Elizabeth Mathai, Professor and Head in Clinical Microbiology.**

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INTRODUCTION

Resistance to antimicrobials among bacterial pathogens is a rapidly emerging global problem [1]. Antimicrobial resistance (AMR) is not just a problem in the hospital setting but has already spread to the community. Multidrug resistance has become a reality in the management of many important infections. This limits the choice of therapy, increases mortality, morbidity and the cost. Although the exact magnitude of economic burden and social issues related to AMR are not known, especially from developing countries, it is also likely to be significant. Hence it becomes important to understand the issues related to emergence and spread of AMR in bacteria.

The evolution of resistance has two key steps i.e., emergence and dissemination. Emergence results from mutations in housekeeping structural or regulatory genes or from acquiring new genetic information. Dissemination can occur at the level of the bacteria (Clonal spread) or at the genetic level (plasmids and transposons). In order to control the increasing prevalence of AMR it is probably easier for us to interfere with dissemination than with emergence. Understanding the mechanisms of AMR will help us to elucidate the most likely method of emergence and dissemination and therefore develop methods for preventing it [2].

Urinary tract infections (UTI) are considered to be the third most common cause of hospital visits in India [3]. Many different microorganisms can infect the urinary tract, but by far the most common agents are gram-negative bacilli of which *Escherichia coli* is the commonest, accounting for 85% of community acquired UTI and 50% of hospital acquired UTI [4-6]. For UTI, drugs like amoxicillin and cotrimoxazole used to be the mainstay of oral therapy. These agents are no longer recommended as reliable because over 50% of isolates from community acquired UTI are now resistant to these agents [7]. Resistance to oral cephalosporins is also increasing. This would mean that there might be many clinical and microbiological failures, if these drugs are used [8].

Therefore fluoroquinolones became the most widely used antibiotics for the treatment of UTI. The expanded spectrum quinolones such as norfloxacin and ciprofloxacin were highly active against gram-negative bacilli and eradicated bacteruria in more than 90% cases of UTI [9]. However, this scenario is also changing with rapid development of resistance among gram-negative bacilli to these drugs [10].

Multiple studies have demonstrated increasing resistance to fluoroquinolones. A study of *E. coli* isolates from women in America with uncomplicated cystitis has shown a 3.5 fold increase in ciprofloxacin resistance from 1995 – 2001. According to a Dutch surveillance study *E. coli* resistance to norfloxacin has increased from 1.3% in 1989 to 5.8% in 1998. In South Korea, UTI caused by quinolone resistant *E.coli* has increased from 14.4% in 1996 to 21.3% in 2000 [11]. According to our data, 20-22% of *E.coli* isolated from antenatal women with UTI are resistant to fluoroquinolones. The resistance rates are as high as 70-90%in hospital strains causing UTI [5].

Therefore it was considered important to understand the mechanisms of fluoroquinolone resistance and its association with resistance to other antimicrobials in current use. A study was therefore undertaken for this purpose and also to understand the clonal spread of fluoroquinolone resistant (FQR) *E.coli* in our hospital and in the community.

AIMS AND OBJECTIVES

2.1 Aim:

To describe possible mechanisms for fluoroquinolone résistance (FQR), to document the susceptibility patterns of FQR *E.coli* causing Urinary tract infections (UTI) and to ascertain whether these organisms belong to a single clone.

2.2 Objectives:

1) To determine the prevalence of efflux pump mediated FQR among *E.coli* causing UTI.

2) To determine the prevalence of high-level resistance to nalidixic acid among FQR *E.coli* as a phenotypic marker of *gyr A* mutations.

3) To type FQR *E.coli* using Random Amplified Polymorphic DNA pattern.

4) To determine the prevalence of resistance to other antimicrobials like aminoglycosides and cephalosporins among the FQR *E.coli.*

5) To determine the prevalence of extended spectrum beta lactamase (ESBL) production among FQR *E.coli.*

REVIEW OF LITERATURE

Quinolones have been the center of considerable scientific and clinical interest since their discovery in the early 1960s. This is because they potentially offer many of the attributes of an ideal antibiotic, combining high potency, a broad spectrum of activity, good bioavailability, high serum levels, wide distribution, oral and intravenous formulations and a potentially low incidence of side effects. They target the bacterial DNA replication and maintenance [12].

3.1 History:

The first member of the quinolone class of anti microbial agents is nalidixic acid which is a 1,8 naphthyridine structure [8]. It was identified by George Lesher and his associates in 1962 among the by products of chloroquine synthesis [13]. In 1975 Smith et al demonstrated that nalidixic acid inhibits a critical enzyme for bacterial multiplication. Subsequently Gellert et al purified the enzyme and named it as DNA gyrase (topoisomerase II) [14]. The therapeutic utility of nalidixic acid was limited to the treatment of gram-negative infections of the urinary tract [13].

Newer quinolones like pipemidic acid, oxolinic acid and cinoxacin were introduced in the late 1970s [13]. These were somewhat more potent but the real breakthrough was achieved in the early 1980s by the addition of fluorine atom at the C6 position and piperazine substituents at C7 position of the basic quinolone structure. This was the beginning of the era of "fluoroquinolones"[15]. They had high potency, expanded spectrum, slow development of resistance, better tissue penetration and good tolerability. These agents were not only potent against gram-negative bacteria but also had limited activity against Gram positives and anaerobes [11]. In the 1990s further alterations of the quinolones resulted in the discovery of novel compounds that had better activity against gram-positive aerobic bacteria and anaerobes with some loss of gram-negative coverage [11, 14]. Newer compounds such as trovafloxacin have also shown promising activity

against anaerobes [11, 16]. Recently, non-fluorinated quinolones have been developed further opening novel avenues in the development of quinolones [8].

3.2 Structure:

All quinolone derivatives have a dual ring structure (fig: 3.1a & b) with nitrogen at position 1, a carboxyl group attached to the carbon at position 3 of the first ring and a carbonyl group at position 4 [8].

Fig: 3.1(a) – Nalidixic acid
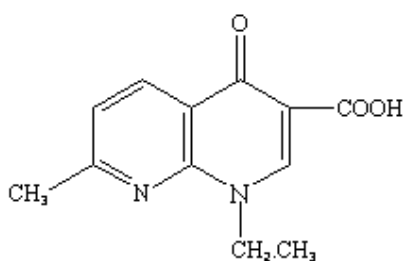
Fig: 3.1(b) – Norfloxacin
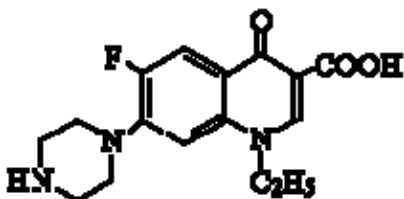
Nalidixic acid is a 1,8 naphthyridine with 1 ethyl and 7 methyl substituents. In case of fluoroquinolones fluorine is added at position 6 to improve the potency. Potency against gram-negative bacteria is further enhanced by the addition of piperazenyl (norfloxacin, ciprofloxacin), methylpiperazenyl (pefloxacin, ofloxacin, lomefloxacin) and dimethyl piperazenyl groups (sparfloxacin). Pyrrolidinyl substitution improves bactericidal activity against gram-positive organisms [8].

3.3 Classification:

This class of antimicrobials has undergone several decades of structural refinements (table 3.1). They can be categorized as follows [16].

I - Nalidixic acid and two variants - oxolinic acid and cinoxacin.

Their use is limited only to gram-negative infections.

II - First group of fluoroquinolones. They are further divided in to IIa and IIb.

IIa: Ciprofloxacin, norfloxacin, ofloxacin, levofloxacin

These are effective against gram-negative infections. Their main disadvantage is, limited activity against Gram-positive pathogens.

IIb: Temofloxacin and grepafloxacin.

Consists of compounds, with a broader spectrum of activity encompassing the Gram-positive organisms. They have a longer half-life, which permits for a once daily dosing.

III - They are further divided in to IIIa and IIIb.

IIIa: Gatifloxacin, moxifloxacin, trovafloxacin

This group has a broader antimicrobial spectrum particularly against *Streptococcus pneumoniae*, *Mycoplasma spp* and *Chlamydia spp* at the loss of some gram-negative coverage. Trovafloxacin has activity against anaerobes also.

IIIb: Gemifloxacin is currently the only generation IIIb quinolone in phase III development. This has good activity against ciprofloxacin resistant and penicillin resistant strains of *S.pneumoniae.*

IV - Desfluoroquinolones:

These are novel compounds that lack the fluorine atom at the C6 position of the quinolone structure. The mechanism of action is similar to other fluoroquinolones with the primary target being DNA gyrase. They have a broad spectrum of antibacterial

activity, which includes anaerobes, gram-positive bacteria and quinolone resistant

pathogens. These agents are claimed to have lesser incidence of joint toxicity [17].

Table 3.1: Antibacterial spectrum and adverse effects of some quinolones [16]:

S.NO.	GENERATION	NAME OF THE DRUG	ANTIMICROBIAL SPECTRUM	ADVERSE EFFECTS
1	I	Nalidixic acid*	Active against common enterobacteriaceae	GI upset, rashes, CNS effects
2	II	Ciprofloxacin* Norfloxacin* Ofloxacin* Sparfloxacin Levofloxacin*	Enhanced activity, mainly against Gram-negative pathogens; limited potency against Gram-positive pathogens and methicillin-resistant *Staphylococcus aureus,* ciprofloxacin most active against *P. aeruginosa*	Cartilage damage in children, gastrointestinal, skin rashes and allergic reactions.
3	III	Trovafloxacin Gatifloxacin* Moxifloxacin	Enhanced activity against Gram-positive pathogens; retained activity against ciprofloxacin-resistant pneumococci; highly active against atypical respiratory tract infection pathogens; reduced activity versus Gram-negative pathogens	CNS effects, hepatic damage, phototoxicity and allergic reactions

* Agents currently in clinical use.

3.4 Mechanism of action:

Quinolones rapidly inhibit DNA synthesis bringing about cell death. They inhibit enzymatic activities of the topoisomerase class of enzymes. The topoisomerases consist of four enzymes namely topoisomerase – I, topoisomerase – II or DNA gyrase, topoisomerase – III, and topoisomerase – IV. The main targets of fluoroquinolones are DNA gyrase and topoisomerase – IV encoded by *gyrA, gyr B* and *parC, parE* genes respectively [18].

DNA gyrase:

It is a bacterial enzyme composed of two A and two B subunits, which catalyses the introduction of negative supercoils into the linear DNA double helix. This process is initiated by, binding of the tetrameric enzyme to the double stranded DNA helix leading to cleavage of the DNA strands at staggered sites. This is followed by the passage of another segment of the DNA through the break and resealing. Supercoiling is essential for the well being of the bacteria, as it enables them to accommodate their chromosome (1300μ long) with in their cell envelope (2μ x 1μ) and also affects the initiation of DNA replication and transcription of many genes [19, 20].

Topoisomerase IV:

It is structurally related to DNA gyrase. It separates the daughter DNA after replication [21].

3.4.1 Inhibition by quinolones:

Quinolones inhibit the action of DNA gyrase by binding to the enzyme-DNA complex after strand breakage and before the resealing of DNA .The drug-DNA-enzyme complex generates a permanent DNA break that the cell is unable to repair. This leads to irreversible damage to the DNA followed by cell death [8].

For many gram-negative bacteria DNA gyrase is the primary quinolone target. On the other hand topoisomerase IV is the primary target for many gram-positive bacteria. Other enzymes serve as secondary targets in both cases [18, 19].

In addition to the initial interaction of fluoroquinolones with the DNA topoisomerase complex bacterial killing may need the synthesis of certain new gene products. This explains the fact that certain drugs, which inhibit RNA and protein synthesis, reduce the bactericidal activity of quinolones but don't affect their ability to inhibit bacterial DNA synthesis. A similar situation may arise at high concentrations of quinolones where there is secondary inhibition of protein synthesis and reduced bacterial killing. The nature of the gene product that contributes to the killing is yet to be defined [8].

3.5 Pharmacokinetics:

3.5.1 Absorption:

Quinolones are well absorbed from the upper GIT with the bioavailability exceeding 50 % for all compounds. Peak concentrations are reached in the serum usually within 1 to 3 hours of administering dose. Concentrations in prostate, stool, bile, lungs, neutrophils and macrophages usually exceed serum concentration. Concentrations in urine and kidney are high for quinolones with major renal route of elimination. Concentrations of quinolones in saliva, prostatic fluid, bone and CSF are lower than concentration in serum.

Aluminium, magnesium and calcium containing antacids lower the oral bioavailability of quinolones due to the formation of cation-quinolone complex, which is poorly absorbed. Ferrous sulphate, multi-vitamins, Zinc and buffered formulations of dideoxyionose also reduce quinolone absorption. Ranitidine reduces enoxacin absorption by 60% and omeprazole reduces trovafloxacin absorption by 17%. Intravenous

formulations of ciprofloxacin and pefloxacin get precipitated when they are infused with aminophylline, flucloxacillin or amoxicillin with or without clavulanic acid [8, 12].

3.5.2 Elimination:

The terminal half lives of elimination from the serum range from 3 hours for norfloxacin and ciprofloxacin to 20 hours for sparfloxacin. Principal routes of elimination differ for different quinolones. They are renal and non-renal as shown in table 3.2.

Table 3.2: Routes of elimination of fluoroquinolones:

Renal	Non renal (Hepatobiliary)
Ofloxacin	Pefloxacin
Lomefloxacin	Sparfloxacin
Ciprofloxacin	Trovafloxacin

Transintestinal secretion has been identified for the intravenous administration of ciprofloxacin and accounted for about 10 to 15 % of drug excretion [8, 12].

3.6 Resistance to Antimicrobials:

3.6.1 General information:

Antimicrobial resistance (AMR) is a natural biological response of microbes to the selective pressure of an antimicrobial drug. Drug resistance can be described as a state of insensitivity or of decreased susceptibility of microorganisms to drugs that ordinarily cause growth inhibition or cell death [22].

The discovery of an antimicrobial agent by Paul Ehrlich was one of the most remarkable discoveries that changed the face of medical practice. The increased global flow of antimicrobials brought with it the threat of increased AMR [22]. Resistance can result from modification or functional by passing of an antibacterial's target or can be contingent on impermeability, efflux or enzymatic inactivation. All these could be either inherent or acquired [23]. Most bacteria have multiple modes of AMR to any drug

and once resistance develops they can rapidly give rise to vast numbers of resistant progeny [23].

3.6.2 Types of resistance:

1) Physiological resistance to antibiotics:

Resistance can be physiological, meaning that resistance is only expressed during certain growth conditions. The most discussed type of physiological resistance is that seen in bacterial biofilms. Bacteria growing in the biofilms are difficult to eradicate with antibiotic treatment. The actual mechanism behind this physiological resistance is far from clear. Various reasons have been discussed. One reason is that the organisms are in a balanced state of growth and death. In the stationary phase and during biofilm mode of growth, persisters might occur in large numbers. Many antibiotics do not directly kill the microbe but trigger a program within the organism itself that leads to death. This mode of action may not be possible with metabolically inactive cells like persisters [24].

The location of the microbe during infection may in some instances prevent the drug from reaching appropriate concentrations where the microbe is growing. This could be another reason for physiological resistance. For example, *E.coli* expressing type I fimbriae may be internalized by uroepithelial cells during a bladder infection. It is therefore possible that recurrent UTI in women by *E.coli*, despite antibiotic treatment, could be the result of an outgrowth from a small number of intracellularly located organisms surviving the treatment [25].

2) Intrinsic antibiotic resistance:

It is the natural resistance possessed by bacteria to certain antibiotics and not associated with any additional genetic alteration. For example, *Mycoplasma spp* are always resistant to beta lactam antibiotics as they lack peptidoglycan as a cell wall component [24].

Many enteric bacterial species including *Pseudomonas aeruginosa* exhibit a very low susceptibility to hydrophobic antibiotics like macrolides because they are unable to penetrate the outer membrane of these organisms. Their susceptibility to hydrophilic antibiotics is determined by the rate of permeation of the antibiotic through water filled protein channels (porins) in the outer membrane. In *P. aeruginosa* the low total surface area of the porins in the outer membrane confers high resistance to hydrophilic antibiotics also [26, 27].

3) Acquired antibiotic resistance:

Acquired antibiotic resistance occurs either by mutations or by horizontal gene transfer. For each class of antibiotics there are usually a number of mechanisms that can cause resistance. These mechanisms may also differ depending on the bacterial species and its genetic make-up [24].

The main mechanisms of resistance include:

a) Decreased uptake and increased elimination of drug:

Alterations in porins are usually associated with up regulated efflux and elimination of drugs. This is the type of resistance, seen in many carbapenem resistant *P.aeruginosa* [28].

b) Trapping:

Trapping is an alternative mechanism for lowering the intracellular concentrations of antibiotics. Binding of the antibiotic to enzymes prevents the binding to target proteins even in the absence of drug destruction. This phenomenon has been observed in AMR to β-lactam antibiotics that are resistant to hydrolysis by β-lactamases. This mechanism has also been reported in aminoglycoside resistance and in low-level resistance to glycopeptides among *Staphylococcus spp* [29].

c) Modification of the drug target:

Mutations in the 16S rRNA, limits the tetracycline binding to its target site at the 30S subunit of the ribosome in *Helicobacter pylori* thereby disrupting its ability to inhibit protein synthesis and cell growth [30].

d) Introduction of new drug resistant targets:

A penicillin binding protein (PBP) that has a low affinity to βlactam antibiotics mediates methicillin resistance in *Staphylococcus aureus* [31].

e) Enzymatic hydrolysis of the antibiotic:

β-lactamases produced by bacteria inactivate the βlactam antibiotics by splitting the amide bond of the β-lactam ring [30].

f) Modification of the antibiotic:

Resistance to aminoglycosides is mediated by drug modifying enzymes like acetyltransferases (AAC), nucleotidyl transferases (ANT) and phosphotransferases (APH) [32].

g) Bypass of metabolic pathways:

Sulfonamides exert their antibacterial action by disrupting bacterial folic acid synthesis from para amino benzoic acid (PABA) by a competitive inhibition of the enzyme dihydropteroate synthase. AMR to sulfonamides can result from the synthesis of a new dihydropteroate synthase that has poor affinity for sulfonamides [33, 34].

3.7 Mechanism of Fluoroquinolone resistance:

The two principal mechanisms by which bacteria acquire resistance to fluoroquinolones are

(1) Alterations in the drug targets.

(2) Decreased accumulation of the drug inside the bacteria due to impermeability of the membrane and /or over expression of efflux pump systems [35].

These mechanisms are due to mutations in the chromosomal genes encoding for the targets or those controlling the expression of outer membrane porin proteins and endogenous multidrug efflux pumps. Majority of studies on the mechanism of action and resistance to quinolones have been done on enterobacteriaceae especially *E.coli* [35].

3.7.1 Target alterations:

a) Alterations in DNA gyrase:

gyrA and *gyr B* genes encode the A and B subunits of the DNA gyrase respectively. Among resistant strains obtained from clinical isolates there is a significant preponderance of mutations in *gyrA*. However mutations in *gyrA* and *gyrB* are found in equal proportions among resistant *E.coli* strains obtained from other sources [35]. The point mutations responsible for quinolone resistance in *E.coli* result in changes within the region between the amino acids 67 and 106 of the GyrA protein. This region is known as the quinolone resistance-determining region (QRDR) and is located in the N- terminal region of the GyrA protein close to the tyrosine 122, which is the binding site of the cleaved DNA [35-37].

Mutations affecting codons 67,81, 82, 83, 84, 87 and 106 of *gyrA* have been observed to be responsible for quinolone resistance in *E. coli*. A part of these mutations can even occur in position 51, a region outside the QRDR, which would result in decreased susceptibility to quinolones. The most frequent mutation associated with quinolone resistance in clinical isolates of *E.coli* affects codon 83 of *gyrA*. The second most common mutation affects codon 87 [35].

Resistance levels are dependent on the site and number of mutations. Quinolone minimum inhibitory concentrations (MIC) are highest for mutants with mutations at codon 83 followed by those with mutations at codons 87,81,84,67 and 106 in decreasing order [36] . Resistance levels conferred by mutations at both sites (83 and 87) can be two

to three folds higher than when only one position is mutated. Additional mutations in the *parC* gene further increase the level of resistance [38]. The level of resistance and there by the MIC is also determined by the specific amino acid substitution. For example, point mutations in the codon for serine 83 can induce substitution by leucine, tryptophan, proline or threonine. Serine 83 to leucine substitution is the commonest and confers the greatest reduction in susceptibility to quinolones [35, 36].

Quinolone resistance determining aminoacid substitutions have been described at positions 426 (Asp 426 to Asn) and 447 (lysine 447 to Glu) of the Gyr B protein of *E.coli*. Substitutions at position 426 confer resistance to all quinolones, whereas those at position 447 result in an increased level of resistance to nalidixic acid, but greater susceptibility to fluorinated quinolones [35].

b) Alterations in Topoisomerase IV:

parC and *parE* genes encode the A and B subunits of the topoisomerase IV respectively. Though *gyrA* mutations play a major role in the development of fluoroquinolone resistance in *E.coli, parC* mutations are additionally associated with resistance. Resistance mutations in *parC* gene of *E.coli* most commonly occur at positions 80 and 84 leading to substitution of serine 80 and glutamic acid 84 by hydrophobic and positively charged amino acids [35, 38]. Another substitution, glycine 78 to aspartate has also been described in quinolone resistant *E.coli*.Aminoacid substitutions in *parE* do not contribute to the development of quinolone resistance [35].

c) Sequential mutations:

Stepwise increase in AMR to fluoroquinolones is brought about by sequential mutations in the *gyrA* (or *gyrB*) and *parC* (or *parE*) genes. The first step mutation occurs in a gene for the more sensitive target enzyme (*gyrA* in *E.coli*). For example, mutation at codon 83 of the *gyrA* normally leads to moderate level resistance to quinolones like

nalidixic acid. Resistance is increased by the addition of one or two *parC* mutations. Three mutations (two *gyrA* and one *parC*) lead to high-level resistance and four mutations (two *gyrA* and two *parC*) are associated with very high levels of resistance and FQR [8, 38].

3.7.2 Decreased accumulation of the drug:

Quinolone accumulation with in the bacterial cell can be reduced by two mechanisms:

1. Increase in the bacterial cell wall impermeability
2. Over expression of efflux pumps

The two mechanisms operate synergistically and can be induced.

Transport of quinolones across the outer membrane is either through specific porins or by diffusion through the phospholipid bilayer. Thus alterations in the composition of porins or in the lipopolysaccharides can lead to alterations in the susceptibility to quinolones [35].

The outer membrane of *E.coli* consists of three main porins namely ompA, ompC and ompF. Decreased expression of ompF is associated with reduced susceptibility to certain quinolones and also to other antibacterial agents such as βlactams, tetracyclines and chloramphenicol [35].

Around 37 different putative efflux pump systems have been described in *E.coli* [39]. The most important fluoroquinolone efflux system is the AcrABTolC (encoded by the *acrABtolC* gene). This system is expressed in wild strains under normal laboratory growth conditions and contributes to intrinsic resistance. Another efflux system known as the AcrEF (encoded by the *acrEF* genes) is not expressed in wild type strains but has the same substrate specificity as that of the former. The most striking feature of the fluoroquinolone efflux systems is their broad substrate specificity encompassing a variety

of structurally unrelated antimicrobial agents, including clinically relevant antibiotics, dyes, detergents, disinfectants, organic solvents, inhibitors of fatty acid synthesis and homoserine lactones involved in bacterial cell-to-cell signaling [40].

The expression of outer membrane porins as well as efflux pumps is regulated by chromosomal loci. Two such loci are the marRAB operon or the mar locus and the soxRS operon. The mar locus consists of three genes namely *marR* (encoding a repressor protein MarR), *marA* (encoding a transcriptional activator MarA) and *marB* (encoding a protein with an unknown function). The soxRS operon encodes for two proteins namely SoxR (a regulator protein) and SoxS (a transcriptional activator) [35] .

Expression of MarA produces an increase in the expression of micF, an antisense regulator that induces a posttranscriptional repression of the synthesis of OmpF. The soxRS operon also regulates the expression of micF [35].

AcrR is a repressor protein of *acrAB tolC* encoded by the *acrR* gene, which is located immediately adjacent to the efflux genes. The mar locus is the most important site of mutations in *E.coli* that lead to multiple antibiotic resistance. MarR is a repressor of MarA, which in turn is a transcriptional activator of *acrAB tolC*. Therefore mutations leading to inactivation of *marR* or *acrR* result in up regulation of the efflux activity of the AcrAB TolC multidrug efflux pump [35, 40].

3.7.3 Transferable quinolone resistance:

In 1994 a novel gene named *qnr* located within an integron on the plasmid pMG252 was identified in a clinical isolate of *Klebsiella pneumoniae* [41-43]. This gene is associated with transferable multidrug resistance in gram-negative bacteria. The gene encodes for a protein (Qnr) of 218 amino acids belonging to the pentapeptide repeat family. Qnr confers low-level quinolone resistance by protecting the DNA gyrase from quinolone action. The exact mechanism is yet to be established. Recent studies have

shown that there is a family of qnr proteins, all of which can cause low-level quinolone resistance. qnrA, qnrB and qnrS are proteins belonging to the family and possibly there are many other proteins yet to be discovered. The qnr gene has also been linked to the presence of extended spectrum or AmpC β-lactamases. The significance of qnr lies in its ability to increase the frequency of selection of chromosomal mutations leading to high-level quinolone resistance [44].

3.8. Bacterial efflux systems:

During the process of evolution bacteria have been exposed to a number of toxic products. As a protective mechanism they have developed unidirectional efflux systems, which catalyze the active extrusion of a number of structurally and functionally unrelated compounds from the cytoplasm to the exterior. This natural phenomenon often leads to multidrug resistance (MDR) [45] .

Bacterial MDR pumps belong to four major families namely,

1. The major facilitator super family (MFS)

2. The small multi drug resistance (SMR) protein

3. The ATP binding cassette and

4. The resistance – nodulation - division (RND) family

Recently a fifth family, the multi drug and toxic compound extrusion (MATE) family has been identified. The RND pumps are unique to the gram-negative bacteria. They are energy dependent and work in conjunction with a periplasmic membrane fusion protein (MFP) and an outer membrane protein (OMP). This organization facilitates the efflux of antibiotics across both membranes of the typical gram-negative cell wall [40, 45].

The most important efflux system in *E.coli* is the AcrAB TolC system in which TolC protein is the OMP and AcrA is the MFP. It has been demonstrated that mutant

strains, which manifest the organic solvent tolerance phenotype, are associated with enhanced transcriptional activity of the *acrAB* genes [40].

3.9 Epidemiology of FQR *E.coli:*

The advent of FQ was an important milestone in the history of antimicrobial therapy for UTI caused by gram-negative pathogens especially *E.coli*. Fluoroquinolone resistance among *E.coli* was an uncommon phenomenon until a decade ago. Reports from all over the world suggest that the emergence of resistance to this important class of antibiotics has already begun and is increasing steadily. FQR *E.coli* have been reported in equal proportions from both hospital and community acquired infections. FQR is also frequently associated with multiple antibiotic resistance. This is a cause for great concern because it might ultimately limit the therapeutic utility of these agents.

3.9.1 Prevalence of FQR *E.coli:*

FQR *E.coli* are being reported with increasing frequency from all parts of the world. Reports on FQR *E.coli* from various centers across United States and Canada have shown variable prevalence rates, with some centers reporting up to 25% [46, 47] .

A study on FQR *E.coli* from North America showed that the isolates were frequently associated with multidrug resistance. These isolates showed high rates of resistance to ampicillin (79.8%) and cotrimoxazole (66.5%). Resistance to nitrofurantoin (4%) was less frequent. All the isolates were susceptible to parenteral carbapenems [48].

According to the Euro surveillance report 2006 the proportion of FQR *E.coli* isolates from Ireland increased from 5% in 2002 to 13% in 2004 and 17% in 2005 [49]. A study from Netherlands showed that norfloxacin resistance in *E.coli* increased from 1.3% in 1989 to 5.8% in 1998 with a concurrent increase in multidrug resistance from 0.5% in 1989 to 4% in 1998 [50]. Susceptibility data of *E.coli* isolates from community acquired UTI in Greece showed a 36% resistance to ciprofloxacin [51].

A Latin American study on antimicrobial resistance of *E.coli* isolated from patients with UTI reported 24.5% resistance to ciprofloxacin [52]. A survey from hospitals in Taiwan revealed that 11.3% of *E.coli* isolates were resistant to FQ and another 21.7% had reduced susceptibility [53].

3.9.2 In India:

Reports on FQR *E.coli* from India are scanty. Data from our hospital show that 80 to 90% of *E.coli* causing nosocomial UTI are resistant to ciprofloxacin while only 20% of *E.coli* causing community acquired UTI are similarly resistant. These isolates showed high rates of resistance to cotrimoxazole also [5].

A study from Ludhiana during the year 1997-1998 showed that around 69-75% of *E.coli* isolated from UTI were resistant to ciprofloxacin and norfloxacin. More than 80% of these isolates were resistant to ampicillin and cotrimoxazole [54]. Another study from Bangalore during the year 1999 recorded 65.7%resistance to norfloxacin among *E.coli* isolated from UTI. The isolates also showed high level of resistance to ampicillin and cotrimoxazole [3].

A study published in December 2001 reported 70-95% resistance to amoxicillin, cotrimoxazole, nalidixic acid, norfloxacin and ciprofloxacin among *E.coli* isolated from urine cultures [4].

3.9.3 Risk factors for FQR:

FQ consumption, besides the underlying disease appears to be one of the most important risk factors. The highest resistance rates were found in nursing home residents where risk factors such as frequent use of quinolones, complicated infections and use of urinary catheters were commonly present [55, 56].

Data from a study in Netherlands indicated that gender strongly influenced FQR probably on account of the different anatomic nature of the urinary tract in males and

females. In women uncomplicated cystitis is the most common whereas in males complicated cystitis is more common for which they are likely to receive prolonged therapy with FQ, which may explain the relatively high resistance rates. Increased resistance is more common in older age groups because of increased cumulative exposure to the drug [50].

3.10 Extended spectrum β-lactamases:

β-lactamase production is the predominant cause of resistance to β lactam antibiotics in gram-negative bacteria [57]. The common β-lactamases are TEM1, TEM2 and SHV1. Extended-spectrum β-lactamases (ESBLs) are a group of rapidly evolving β-lactamases that have the ability to hydrolyze the oxyimino- cephalosporins and aztreonam [57, 58]. These enzymes are inhibited by clavulanic acid and majority are variants that are derived through point mutations in the genes encoding for the common β-lactamases. The mutations lead to amino acid substitutions in the active sites of the TEM1, TEM2 or SHV1group of enzymes. Currently more than 150 different ESBLs have been described [59].

The genes encoding ESBL production may be chromosomal or extra chromosomal. Extra chromosomal propagation is most commonly through plasmids but can also occur through transposons. Plasmids carrying genes encoding ESBLs may also carry genes encoding resistance to many of the aminoglycosides and cotrimoxazole [60, 61].

The various phenotypic methods used for detection of ESBL are based on Kirby Bauer disc diffusion test methodology. The common techniques that are in use are the double disc approximation test [62], three-dimensional test described by Thomson and Sanders [63], Etest ESBL strips [64], disc diffusion test using commercially available antibiotic disc containing an expanded spectrum cephalosporin plus clavulanate and MIC performed with expanded spectrum cephalosporins with and with out the addition of

clavulanic acid. ESBLs can be characterized by molecular detection techniques including DNA probes, PCR, oligotyping, PCR - RFLP, PCR - SSCP, LCR and nucleotide sequencing [59]. Analytical isoelectric focusing (IEF) is a rapid method to assess the relative nature of β-lactamases present in a particular organism and a means for comparison of β-lactamases present in different organisms. However IEF alone cannot identify specific β-lactamases [65, 66].

Infections caused by ESBL producing organisms are prone for treatment failures with expanded spectrum β-lactam antibiotics. According to NCCLS criteria any organism that is confirmed for ESBL production should be reported as resistant to all expanded spectrum beta lactam antibiotics regardless of the susceptibility test result [67].

3.10.1 ESBLs and Fluoroquinolone resistance:

Studies have shown that ESBL producing strains are more frequently associated with FQ resistance. In a study from Turkey it was demonstrated that ESBL producers were significantly more frequent among ciprofloxacin resistant *E.coli* strains than among ciprofloxacin susceptible *E.coli* [61]. Another Turkish study on intensive care and renal transplant patients reported that up to 40% of *E.coli* strains were ESBL producers and that the incidence of ciprofloxacin resistance among these strains was as high as 56% [68]. Possible explanations for FQR among ESBL producing *E.coli* are mutations in the mar locus and alterations in the outer membrane proteins. Further epidemiological and molecular studies are needed to understand the mechanisms involved in cross-resistance [61].

3.11 Typing systems:

Microbial epidemiologists monitor the spread of viruses, bacteria, fungi and protozoan parasites associated with human or animal infectious diseases at levels ranging from a single host or ecosystem to the worldwide environment. On the basis of

epidemiological investigations, public health risks can be determined and interventions in the spread of diseases can be designed and their efficacy can be assessed [69].

Epidemiological markers provide a means of distinguishing between different subgroups within the species and hence of addressing specific questions about the epidemiology of the diseases [70]. The methods used for typing the organisms can be classified as phenotypic and genotypic methods. Phenotyping procedures take advantage of biochemical, physiological and biological phenomena whereas genotyping aims to detect polymorphisms at the nucleic acid level.

3.11.1 Genotyping:

Typing provides the means to discriminate between and catalogue microbial nucleic acids. The specific purpose of epidemiological typing includes study of bacterial population genetics, pathogenesis of infection, surveillance of infectious diseases and out break investigations [69].

In order to obtain meaningful epidemiological information from DNA finger printing methods, a genetic marker must give different patterns for epidemiologically unrelated strains and identical patterns for strains from a common source.

For molecular typing of *E.coli* several different typing methods have been employed. These include ribotyping, ERIC PCR, RAPD and PFGE [71-73]. A comparative analysis of various typing method have shown that RAPD has the highest discriminatory capacity for typing *E.coli* isolates [71, 74].

3.11.2 RAPD:

RAPD is an amplification based DNA fingerprinting technique, which amplifies multiple targets on the genomic DNA to reveal polymorphisms [75]. It was first described by Williams et al in 1990 [76]. It is based on the use of short random sequence primers of 9 to 10 base pairs in length. The rationale behind using such primers is that they are likely

to be complementary to many sites on the bacterial genome and many loci can be identified with a single primer [77]. The primer binds in an inverted orientation to two different sites on opposite strands of the DNA template at low annealing temperatures (Fig 3.2). If the sites of binding of the primers are close enough to each other, the intervening DNA is amplified during the cycles of PCR. The number and location of these random primer sites vary for different strains of bacterial species. Separation of the amplification products by agarose gel electrophoresis results in a pattern of bands characteristic of the particular strain [78, 79].

Fig.3.2: Principle of RAPD

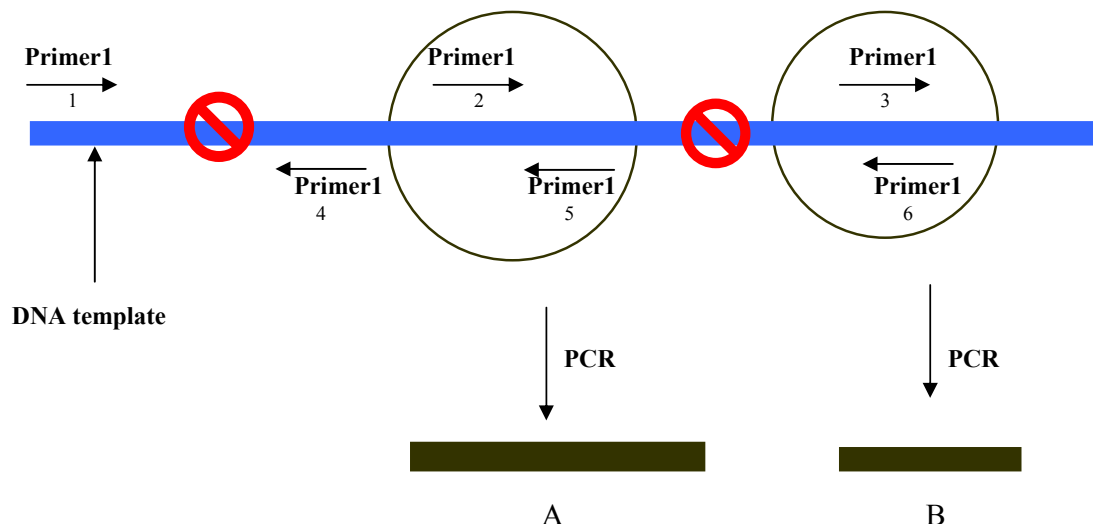
Fig 3.2 depicts the principle of RAPD. The arrows represent multiple copies of the same primer (same sequence). The direction of the arrow indicates the direction in which DNA synthesis will occur. The numbers represent locations on the DNA template to which the primers anneal. Primers anneal to sites 1, 2 and 3 on the top strand and to sites 4,5 and 6 on the bottom strand of the DNA template.

Product A is produced by PCR amplification of the DNA sequence which lies in between the primers bound at positions 2 and 5. Product B is produced by PCR amplification of the DNA sequence, which lies in between the primers, bound at positions 3 and 6.Therefore the same primer will produce multiple products of different molecular weight. No PCR product is produced by the primers bound at positions 1 and 4 because these primers are too far away to allow the completion of the PCR reaction. Similarly no PCR product is produced by the primers bound at positions 5 and 3 because these primers are not oriented towards each other.

The relatedness of the isolates is assessed based on the genetic between each other. This is done using computer programme like RAPDistance programme. This programme generates data based on the sizes, and the presence or absence of shared bands. The primary data is then used to calculate the pair wise distance between the samples using one of the various metrics like the coefficient by Jaccard or Dice [80] . Using the distance data thus obtained phylogenetic trees can be constructed. Some of the methods that are used for tree construction are the unweighted pair group method of analysis, Farris's method, Sattath and Tversky's method, Li's method, Tateno et al.'s, modified Farris method and the neighbour joining method. The neighbour joining (NJ) method, which was first described by Saitou and Nei (1987) and later modified by Studier and Kepler (1988), has been shown to be an efficient and reliable method for analyzing bands obtained in RAPD of strains within a species. This method seeks to build a tree that minimizes the sum of all edge lengths, i.e., it adopts the minimum evolution criterion. It is applicable to any type of evolutionary distance data [81, 82].

RAPD is easy to use and interpret. The sample preparation is much less laborious because only a very small amount of DNA is required. The procedure can be performed with a universal set of primers without the need for probe isolation, filter preparation or

nucleotide sequencing. Studies have shown that the polymorphisms with in a species can be identified using even a small number of primers [76]. Mulcahy et al has reported that a single primer is often sufficient to study the polymorphism [83]. The presence of single point mutations in the genome can also be identified by this method. The assay can be automated [84]. It has a high discriminatory power and the cost per test is low. The result can be obtained in a day and is reliable [78]. The method has the potential for analyzing phylogenetic relationship among closely related species and can distinguish between strains within a species [85]. It is also a valuable tool in the genetic analysis of organisms whose genome has not been described completely [76]. The availability of RAPD has provided a valuable approach in genotyping *E. coli* [71].

MATERIALS AND METHODS

E.coli isolated from routine urine cultures were further evaluated to understand more details about Fluoroquinolone resistance (FQR), like its association with other antimicrobial resistance (AMR) and the possible mechanisms involved in causing FQR. The clonal relatedness of the isolates were also studied. The protocol followed is summarized in figure 4.1.Detailed methodology is given below.

4.1 Sample size:

The expected prevalence of strains with up regulated efflux pumps was taken, as 60% based on data published from Sweden [9] since there is no data from India. The sample size for the organic solvent tolerance test was calculated as 343 by using the formula,

$n = (Z_\alpha + Z_{1} -\beta)^2 \; 2PQ \setminus d^2$

$n = 10.3 \times 2 \times 60 \times 40 \setminus 12 \times 12 = 343$

$Z_\alpha = 1.96$, $Z1 -\beta = 2.58$

P = Expected prevalence of strains with efflux pumps.

d = Absolute difference (12%).

Q = 1- P.

The same isolates were used to study other AMR in *E.coli,* including ESBL.

A sample size of 120 strains was chosen for RAPD typing, which included a minimum sample size of 30 strains in each category. A sample size of 30 would give us enough information to conclude whether the strains belonged to single or multiple clones. It would also enable us to obtain information on the predominant genotypes in the hospital setting and in the community. This method of an acceptable sample size in each category was followed since there was no data on the clonal characteristics of FQR *E.coli* from India.

Fig.4.1: Flow chart showing selection of samples and methods followed.

Routine urine cultures

E.coli $\geq 10^4$ CFU /ml and pure growth (Pathogens)

E.coli along with contaminants and $<10^3$ CFU/ml (Commensal)

Susceptibility to Norfloxacin determined by disc diffusion

Resistant n = 343

Susceptibility to Norfloxacin determined by disc diffusion

Susceptible n = 30

Nalidixic acid resistant n = 30

Resistant n = 30

MIC

RAPD

Urology in-patients (hospital acquired) n = 30

Antenatal women and medicine out patients (community acquired) n = 30

RAPD

Analysis of susceptibility to other antimicrobials

MIC for nalidixic acid To understand the nature of gyrA mutations

RAPD

Efflux mediated resistance detected by Organic solvent tolerance test

Analysis of susceptibility to other antibiotics

Double disc diffusion for those with cefotaxime <27mm for ESBL

4.2 Bacterial isolates:

4.2.1 Collection:

E.coli obtained from routine urine cultures during the period from September 2004 to December 2005 in the Department of Clinical Microbiology, Christian Medical College Hospital, Vellore were included in the study.

Pure growth of *E.coli* obtained in counts of $\geq 10^4$ CFU /ml were considered as those causing urinary tract infections (UTI). Those isolated in counts of < 1000 CFU/ ml along with other organisms were considered as commensals. The *E.coli* were identified based on their ability to grow on Maconkey agar, oxidase negativity, biochemical reactions in triple sugar iron agar and mannitol motility medium, ability to produce indole, ferment sorbitol and inability to utilize citrate.

Antimicrobial susceptibility testing was done routinely by Kirby Bauer method by following CLSI criteria strictly. Those *E.coli* that were resistant to norfloxacin (10μg/ml Span diagnostics, Surat, India) were selected for further study. The organic solvent tolerance test was done on all 343 isolates thus selected to detect the presence of efflux-mediated resistance. MIC of nalidixic acid (Pure substance, Himedia, Mumbai, India) was determined for 60 strains consisting 30 each of community acquired and hospital acquired strains. MIC of nalidixic acid was also determined for 30 fluoroquinolone susceptible but nalidixic acid (30μg/ml, Span Diagnostics, Surat India) resistant strains. To understand the prevalence of resistance to other antimicrobials among FQR strains, data on susceptibility to other antibiotics were collected. Those isolates with <27mm zone size for cefotaxime (30μg/ml, in-house preparation, pure substance from Sigma USA) were subjected to double disk diffusion to detect ESBL production.

To understand the clonal relatedness, a subset of thirty resistant strains each isolated from urology in-patients with hospital acquired UTI and from antenatal women

and medicine outpatients with community acquired UTI were subjected to RAPD analysis. Thirty isolates of *E.coli*, which were susceptible to norfloxacin and thirty isolates of commensal *E.coli*, which were resistant to norfloxacin, were also subjected to the same test.

4.3 Organic solvent tolerance test for identifying isolates with up-regulated efflux:

The organic solvent tolerance test is a phenotypic test that correlates positively with the presence of an efflux mediated antimicrobial resistance. The test was performed as follows using hexane and cyclohexane as the organic solvents [9, 86, 87].

4.3.1 Preparation of media:

The LBGMg agar, which consists of Luria agar (Appendix 1), 0.1%glucose and 10mM of MgSO4 prepared in glass petri dishes, was used for the test.

4.3.2 Preparation of bacterial suspension:

E.coli were subcultured on blood agar to obtain pure growth and then suspended in 0.5 ml of 0.9% sterile NaCl to match 0.5 McFarland approximately.

E.coli MG1655 was used as a control strain that grew on hexane but not on cyclohexane. Two in house controls, one positive and one negative for efflux were also chosen from the test strains, to be included in each batch of test done.

4.3.3 Procedure:

Five plates of LBGMg medium were prepared and 5µl each of the bacterial suspensions were spot inoculated on all the plates using a template as for MIC testing. The first plate was used as a control plate. The second plate was overlaid with pure hexane. The other three plates were overlaid with a mixture of hexane and cyclohexane in the ratio of 3:1, 1:3 and 1:1 respectively. The solvents were overlaid to a thickness of 3mm uniformly. The plates were sealed and incubated at 37ºC for 16 to 18 hours.

4.3.4 Reading and interpretation:

  The plates were read after 18 hours of incubation. The control plate was read first to ensure that there is adequate growth of all the isolates. Then, the readings for the control strains were taken from the organic solvent overlaid plates. Confluent growth occurring in the presence of hexane and cyclohexane in 1:1 ratio was taken as organic solvent tolerance. This is indicative of an up-regulated AcrAB-TolC efflux pump mechanism. The readings from the other plates (1:3 and 3:1) were used to correlate with the readings on the 1:1 plate.

4.4 Antimicrobial resistance:

4.4.1 Minimum Inhibitory Concentration (MIC) of nalidixic acid:

  The degree of resistance to nalidixic acid can be used to understand the mutations in *gyrA*. A high MIC of $\geq$ 256 mg/ml is usually indicative of a mutation at codon 83 or multiple stepwise mutations [9].

a) *Preparation of antibiotic solution:*

  This is described in Appendix 2.

b) *Preparation of media:*

  Mueller-Hinton Agar (MHA) was used for agar dilution. MHA medium (18ml) was prepared in tubes, autoclaved and allowed to cool to 50ºC. Diluted (to obtain concentrations of 256 –0.025µg/ml) antibiotic solution (2ml) was added to the molten and cooled medium in each tube. The contents were mixed well and poured into petri dishes. A control plate of the medium without the antibiotic was prepared for each day of testing.

 c) *Preparation of the inoculum:*

  The inoculum was an actively growing culture of about 10^4 microorganisms per ml. In order to achieve this 3 to 5 well-isolated colonies of the test strain were touched with a loop and inoculated into 0.5 ml of nutrient broth and incubated at 37ºC for 2 hours.

The turbidity of the actively growing broth culture was adjusted to 0.5 McFarland standard and then diluted 1 in 10 in sterile normal saline.

d) *Inoculation of the test plates:*

A platinum loop calibrated to deliver 0.001 ml of the inoculum was used to spot inoculate the plates. Each spot was about 5 to 8mm in size. After the inoculum had dried the plates were inverted and incubated at 37ºC for 16 to 18 hours. *E.coli* ATCC 25922 was used as the control strain.

e) *Reading and Interpretation:*

The control plate was read first to ensure adequate growth of all the test strains. It was verified whether the MIC of the control strain was in the expected range. The lowest concentration of the antimicrobial that completely inhibited the growth was considered the end point. A barely visible haziness or a single colony was disregarded. Results were reported as µg/ml. CLSI interpretative criteria for MIC determination was used to define susceptibility categories [67].

4.4.2 Double disk diffusion:

Of the 343 isolates of *E.coli*, those which showed <27mm zone size for cefotaxime (30µg/ml), were subjected to double disk diffusion.

a) *Preparation of the inoculum:*

Well-isolated colonies (3-5 no.) of the test strain were touched with a loop and inoculated into 0.5 ml of nutrient broth and incubated at 37ºC for 2 hours. The turbidity of the actively growing broth culture was adjusted to 0.5 McFarland standard with sterile normal saline.

b) *Control strains:*

Klebsiella pneumoniae ATCC 700603 and *E.coli* ATCC 25922 were used as positive and negative controls respectively.

c) *Inoculation of the test plates and application of the antibiotic discs:*

The standardized inoculum was streaked on Mueller-Hinton Agar (MHA) to obtain a lawn culture. Two discs namely Cefotaxime-clavulanic acid (30μg/ml and 10μg/ml Becton and Dickson, USA) and Cefotaxime (30μg/ml,in house preparation) were applied to the culture at an approximate distance of 2mm from each other and incubated at 37ºC for 16 to 18 hours.

d) *Reading and Interpretation*:

A relative increase in the cefotaxime-clavulanic acid zone diameter of $\geq$ 3mm in comparison with cefotaxime alone was considered to be indicative of ESBL production in the test strains. Readings of test strains were taken if the control strains gave acceptable results.

4.4.3 AMR to other antibiotics:

Data on susceptibility to a panel of antibiotics including cotrimoxazole, nitrofurantoin, cephalosporins and aminoglycosides were collected, in order to understand the prevalence of resistance to other antimicrobials among FQR strains.

4.5 Genotyping by RAPD:

The protocol of Pacheco et al was used for RAPD typing [88]. A total of 120 isolates of *E.coli* were typed by this method and analyzed. NU14 and ATCC *E.coli* 25922 were used as control strains.

4.5.1 Extraction of chromosomal DNA:

The isolates were grown on sheep blood agar for 16 to 18 hours and the cells were suspended in 100μl of milliQ water. The cell suspension was boiled for 2 minutes at 100ºC in a water bath and centrifuged at 12,000 rpm for 1 minute. The supernatant was used as the DNA template.

4.5.2 Procedure:

The PCR was carried out with two primers separately. The primers used were 1254 (5' – CCGCAGCCA – 3') and 1290 (5' – GTGGATGCGA – 3'). The reactants were constituted to 30µl volumes with 20mM Tris HCl (pH 8.4), 50mM KCl, 3mM MgSO4, 250 µM each of dNTPs, 30pmol of primer, 1unit of Taq polymerase and 3µl of bacterial lysate.

Temperature cycling was controlled in a thermal cycler (PTC – 100 DNA Peltier thermal cycler, MJ Research, USA.). The thermal cycler was programmed for denaturation at 94ºC for 5 minutes, annealing at 37ºC for 5minutes,extension at 72ºC for 5 minutes followed by 30 cycles of 94ºC for 1 minute, 37ºC for 1 minute, 72ºC for 2 minutes and a final extension step at 72ºC for 10 minutes. Amplified products were subjected to electrophoresis at 100volts for 90 minutes in a 1.2% agarose gel containing 0.5µg/ml of ethidium bromide. DNA ladder (gene ruler – 100bp) was used as molecular weight marker. Gel pictures were made.

4.5.3 Analysis of RAPD data:

From the gel pictures the number of bands and their respective positions were recorded for both primers. This information was entered into the RAPDistance program version 1.04. The relatedness of the isolates was calculated by the program based on the number of shared bands and the number of unique bands in each isolate. A matrix of pair wise differences was prepared using the Jaccard coefficient (**S = a/(a+b+c)** where "**a**" is the number of shared bands between samples 1 and 2, "**b**" is the number of bands present in sample 1 but not in 2 and "**c**" is the number of bands present in sample 2 but not in 1) in the same program. This matrix was used to construct a dendrogram using the NJTREE program [81, 82].

RESULTS

Three hundred and forty three strains of *E.coli* resistant to norfloxacin isolated from urine samples were used for the study. All the strains were resistant to nalidixic acid and all except two were resistant to ciprofloxacin.

5.1 Antimicrobial resistance among fluoroquinolone resistant (FQR) *E.coli*:

The antibiotic susceptibility profile of the strains to a panel of antibiotics including nitrofurantoin, cotrimoxazole, gentamicin, amikacin, netilmicin, cefuroxime and cefotaxime were analyzed. The results are summarized in Table-1.

Table - 5.1: Antibiotic susceptibility profile of FQR *E.coli*.

Antibiotic	Resistant		Sensitive	
	No.	%	No.	%
Nitrofurantoin	104	30.3	239	69.7
Cotrimoxazole	287	83.7	56	16.3
Gentamicin	228	66.5	115	33.5
Amikacin	125	36.4	218	63.6
Netilmicin	114	33.2	229	66.8
Cefuroxime	242	70.6	101	29.4
Cefotaxime	219	63.8	124	36.2

Among the antimicrobials tested, maximum susceptibility was to nitrofurantoin (69.7%). This was higher than that to netilmicin (66.8%) and amikacin (63.6%). A high percentage of isolates were resistant to cotrimoxazole (83.7%) followed by cefuroxime (70.6%) and gentamicin (66.5%). In comparison only 43.3% of the fluoroquinolone susceptible strains were resistant to cotrimoxazole (Table – 5.2) and majority of the strains were susceptible to nitrofurantoin, gentamicin and cefuroxime. Forty percent of fluoroquinolone susceptible strains were resistant to nalidixic acid. The difference in the prevalence of AMR to nitrofurantoin, cotrimoxazole, gentamicin and cefuroxime between FQR and fluoroquinolone susceptible *E.coli* is statistically significant (p value was calculated using the Statcalc programme of the Epi info version – 3.2.2).

Table – 5.2 Antibiotic susceptibility profile of Fluoroquinolone susceptible *E.coli* (n=30).

Antibiotic	Resistant		Sensitive		*P value
	No.	%	No.	%	
Nitrofurantoin	1	3.3	29	96.7	0.002
Cotrimoxazole	13	43.3	17	56.7	<0.001
Gentamicin	2	6.7	28	93.3	<0.001
Cefuroxime	1	3.3	29	96.7	<0.001
Nalidixic acid	12	40	18	60	<0.001

* Compared to FQR *E.coli*

5.1.1 Aminoglycoside resistance among FQR *E.coli*

A total of 228 isolates were resistant to gentamicin of which 54.9% and 50% were resistant to amikacin and netilmicin respectively (Table –5.3).

Table – 5.3: Resistance to aminoglycosides.

Isolates resistant to gentamicin n=228				
Antibiotic	Resistant		Susceptible	
	No.	%	No.	%
Amikacin	125	54.9	103	45.1
Netilmicin	114	50	114	50

5.1.2 Extended spectrum β – lactamases (ESBL) among FQR *E.coli*

Out of the 343 isolates analyzed, 237 strains (69.1%) showed a zone size of $\leq$ 27mm to cefotaxime. Among the 237 strains 219 (63.9%) showed a zone size of < 23mm and were designated as resistant to cefotaxime according to NCCLS criteria. All the resistant strains except two (99.1%) were positive for ESBL by double disc diffusion. None of the strains with zone sizes between 23 and 27mm were ESBL producers (Table 5.4). In the FQ susceptible group only one isolate showed cephalosporins resistance and ESBL production. (p= < 0.001).

Table – 5.4 ESBL among FQR *E.coli*:

Cefotaxime Zone size (mm)	ESBL			
	Positive		Negative	
	No.	%	No.	%
< 23	217	99.1	2	0.9
23 - 27	-	-	18	100

5.2 Efflux mediated resistance among FQR *E.coli*:

Among the 343 isolates subjected to the organic solvent test, 137 (39.9%) isolates were positive, indicating up-regulated efflux pumps (fig.5.1).

Fig.5.1 Efflux mediated resistance among FQR *E.coli*
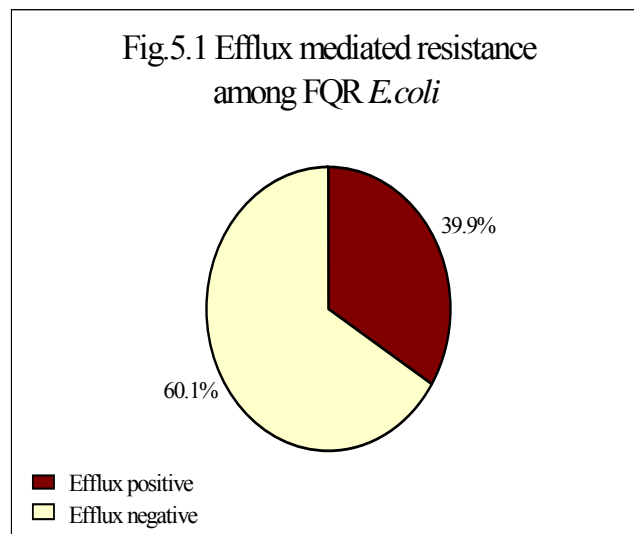
The tolerance shown by the FQR *E.coli* to various proportions of hexane and cyclohexane in the organic solvent tolerance test (fig. 5.2, 5.3, 5.4, 5.5, 5.6) is shown in Table – 5.5. Only those strains, which were able to grow in the presence of hexane and cyclohexane in the ratio of 1:1, were considered as efflux positive. Among the efflux positive strains, all grew in the presence of pure hexane and hexane/cyclohexane in the

ratio of 3:1. Growth was found in 14.6% of the strains even when the concentration of cyclohexane was increased to 1:3 ratio. None of the efflux negative strains grew in the presence of hexane/cyclohexane in 1:1 ratio.

Table – 5.5 Organic solvent tolerance

Organic solvent tolerance	Hexane: Cyclohexane ratio			
	Pure hexane	3:1	1:1	1:3
Positive (n=137)	137	137	137	20
Negative(n=206)	50	18	0	0

5.2.1 Association of efflux pumps with resistance to other antibiotics

The association of efflux pumps with other AMR is shown in Table – 5.6.

Table 5.6

Antibiotic		Efflux				P value
		Positive		Negative		
		No.	%	No.	%	
Nitrofurantoin	Susceptible	83	60.6	156	75.7	0.002
	Resistant	54	39.4	50	24.3	
Cotrimoxazole	Susceptible	23	16.8	33	16	0.85
	Resistant	114	83.2	173	84	
Gentamicin	Susceptible	39	28.5	76	36.9	0.1
	Resistant	98	71.5	130	63.1	
Amikacin	Susceptible	79	57.7	126	61.2	0.51
	Resistant	58	42.3	80	38.8	
Netilmicin	Susceptible	87	63.5	133	64.6	0.84
	Resistant	50	36.5	73	35.4	
Cefuroxime	Susceptible	26	18.9	75	36.4	<0.001
	Resistant	111	81.1	131	63.6	
Cefotaxime	Susceptible	37	27	87	42.2	0.004
	Resistant	100	73	119	57.8	

AMR to nitrofurantoin, cefuroxime and cefotaxime was more among the efflux positive strains when compared to the efflux negative strains and the difference was statistically significant. Aminoglycoside resistance was not significantly different in efflux positive and efflux negative groups.

5.2.2 Susceptibility patterns of efflux positive and efflux negative FQR *E.coli*

Out of the 343 strains only 14 strains were resistant to fluoroquinolones alone of which 12 strains (86%) were efflux negative (p=0.006) (Table - 5.7). However multiple AMR i.e. resistance to three or more different groups of antibiotics was found to be more among the efflux positive strains (p <0.001). Among the efflux positive strains 119 of the 137 strains (86.9%) showed multiple antibiotic resistance. In comparison, 71.4% of the efflux negative strains showed multidrug resistance. The commonest MDR was to cephalosporins, cotrimoxazole and aminoglycosides amongst both efflux positive and efflux negative isolates. MDR, which includes cephalosporins, was found in 107 (78.1%) of the efflux positive strains compared to 127(61.7%) in the efflux negative group.

The incidence of MDR among FQR isolates was significantly high when compared to the fluoroquinolone susceptible strains (Table 5.8). The difference was statistically significant (p value <0.001).

Table – 5.7

Antibiotics	Efflux positive (n=137)		Efflux negative (n=206)	
	No.	%	No.	%
Fluoroquinolones (FQ) alone	2	1.5	12	5.8
FQ + Cephalosporins	2	1.5	4	1.9
FQ + Aminoglycosides	4	2.9	3	1.5
FQ + Cotrimoxazole	8	5.8	38	18.5
FQ + Nitrofurantoin	2	1.5	2	1
FQ + Cephalosporins + Aminoglycosides	9	6.6	10	4.9
FQ + Cephalosporins + Cotrimoxazole	12	8.8	14	6.8
FQ + Cephalosporins+ Nitrofurantoin	1	0.7	0	0
FQ + Cephalosporins + Cotrimoxazole + Nitrofurantoin	6	4.4	2	1
FQ + Cephalosporins + Cotrimoxazole+ Aminoglycosides	51	37.2	62	30
FQ + Cephalosporins+ Nitrofurantoin+ Aminoglycosides	0	0	2	1
FQ + Cephalosporins + Cotrimoxazole+ Aminoglycosides + Nitrofurantoin	28	20.4	37	18
FQ + Cotrimoxazole+ Nitrofurantoin	4	2.9	4	1.5
FQ + Cotrimoxazole+ Aminoglycosides	4	2.9	13	6.3
FQ + Cotrimoxazole+ Nitrofurantoin+ Aminoglycosides	4	2.9	3	1.5

Table 5.8

Strains		Multi drug resistance		P value
		No.	%	
FQR (n=343)	Efflux positive	119	86.9	<0.001*
	Efflux negative	147	71.4	
FQ susceptible (n=30)		1	3.3	<0.001**

* Efflux positive Vs Efflux negative

** FQR Vs FQ susceptible

5.2.3 Efflux in different populations:

Presence of efflux pumps in two groups of isolates viz. one from hospital-acquired infections and the other from community-acquired infections were compared (Table – 5.9).

Table – 5.9

Strains	Efflux positive		Efflux negative	
	No.	%	No.	%
Community acquired (n = 30)	12	40	18	60
Hospital acquired (n = 30)	13	43.3	17	56.7

Efflux positivity was found in 40% of community acquired and 43.3% of the hospital-acquired strains. The difference is not statistically significant (p= 0.79).

5.3 Minimum inhibitory concentration for nalidixic acid as a phenotypic marker of *gyrA* mutations:

MIC for nalidixic acid was determined for 60 FQR strains 30, each from community acquired infections and hospital-acquired infections respectively. Fifty-nine of them had MIC above 256µg/ml. MIC was also done on 30 nalidixic acid resistant and fluoroquinolone susceptible isolates (Table 5.10).

Table – 5.10

Isolates		No. of isolates tested	MICµg/ml			
			32	64	128	≥256
FQR	Community acquired	30	-	-	-	30
	Hospital acquired	30	-	-	1	29
FQ susceptible		30	1	2	15	12

5.4 Relationship between different groups of isolates by RAPD:

A total of 120 isolates consisting of four populations of 30 each were typed by RAPD to determine the genetic relatedness among the strains. The four populations consisted of 30 fluoroquinolone susceptible *E.coli* and 30 each of fluoroquinolone resistant *E.coli* from community acquired and hospital acquired infections and commensals. The RAPD profile of each isolate was obtained using two PCRs with primers 1254 and 1290. The protocol produced discrete well-resolved bands in all runs included for analysis (fig. 5.7, 5.8, 5.9, 5.10). The controls ATCC *E.coli* 25922 and *E.coli* NU14 gave reproducible results in each run. When the primer 1254 was used, the bands generated by the amplified products consisted of 15 bands overall with a minimum of 1 and a maximum of 9 bands. With the primer 1290, 16 unique bands were obtained with a minimum of 1 and a maximum of 10 bands. Therefore relatedness was calculated based on 31 bands. The molecular weights of the bands ranged from 200bp to 5000bp.
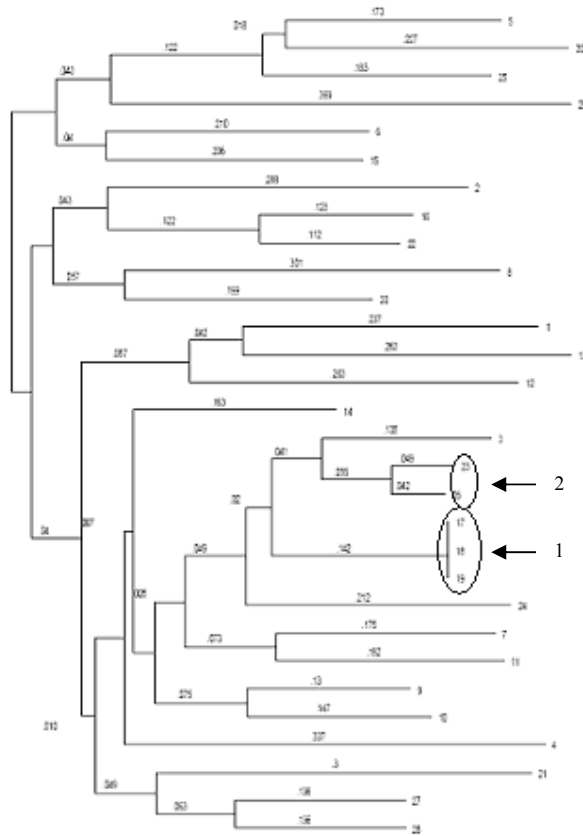
5.4.1 Analysis of genotypes:

Dendrograms constructed using the NJTREE (neighbor- joining tree) software (fig. 5.11, 5.12, 5.13, 5.14) showed that many different RAPD types existed among the isolates. There was very little homology between the strains with only two or three isolates being identical with each other.

a) Fluoroquinolone susceptible *E.coli*:

Twenty-seven RAPD types were identified among the fluoroquinolone susceptible *E.coli*. Of the final right and left neighbors the right had 24 (80%) isolates. In this group 19 isolates belonged to a single sub branch. Three isolates in this branch were identical. The minimum distance from the root was 0.281 and the maximum distance was 0.448.

Fig. 5.11: Dendrogram of fluoroquinolone susceptible *E.coli*

b) Commensal FQR *E.coli*:

Among the commensal *E.coli* 29 RAPD types were found. Here again the right branch had 24 (80%) isolates of the 30. Twenty isolates (73 %) belonged to a single sub branch in the right branch. Two isolates in the smaller group were identical. The minimum distance from the root was 0.319 and the maximum distance was 0.512.

Fig. 5.12: Dendrogram of commensal FQR

c) Community acquired FQR *E.coli*

All 30 isolates in the community-acquired group gave unique RAPD patterns. The right and the left branches in the NJTree had 22 and 8 isolates each. Within these two groups all isolates were at distances of >0.121. The minimum distance from the root was 0.338 and the maximum distance was 0.501.

Fig. 5.13: Dendrogram of community acquired FQR *E.coli*
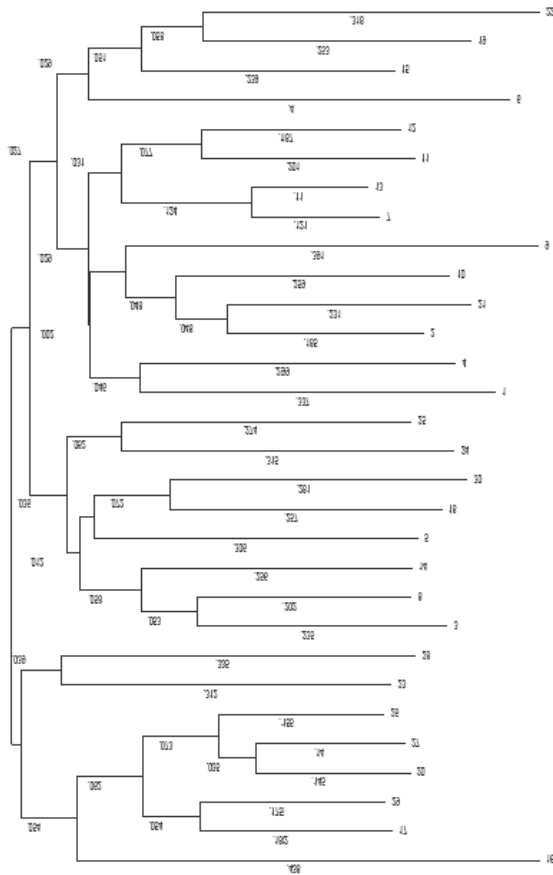
d) Hospital acquired FQR *E.coli*:

Twenty-nine RAPD types were identified in this group. In this group the right and the left branches had 13 and 12 isolates each. There were two identical isolates and a third at a distance of 4% from them. Another two isolates were at a distance of 0.06 (1 and 16). Apart from these, all the isolates were at a distance of 10% or further from each other. With the exception of these two clusters with a total of 5 isolates all the other isolates were different from each other. The minimum distance from the root was 0.316 and the maximum distance was 0.469.

Fig. 5.14 Dendrogram of hospital acquired FQR *E.coli*
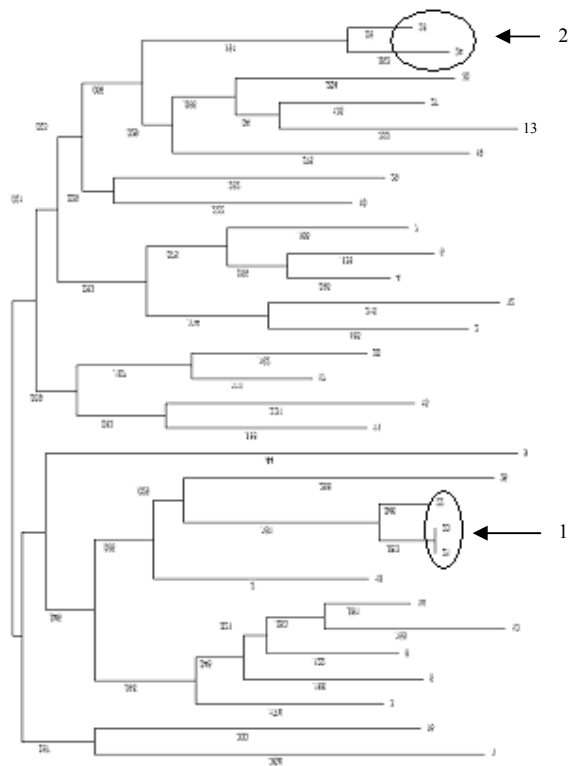
Comparison of isolates belonging to different categories showed that the isolates from commensal and community acquired infections did not belong to separate groups. The isolates were scattered throughout the tree. A similar pattern was observed with commensal and hospital acquired *E.coli.*

Distinctive groups were seen on comparing the FQR commensal *E.coli* with the fluoroquinolone susceptible *E.coli.* Twenty of the 30 fluoroquinolone susceptible *E.coli* were at the right branch. The same difference was seen on comparing the fluoroquinolone susceptible *E.coli* and the FQR hospital acquired *E.coli.* All the fluoroquinolone susceptible isolates fell into one arm, while the FQR hospital acquired *E.coli* fell into the other arm. Similarly the distribution of fluoroquinolone susceptible *E.coli* and the community acquired FQR *E.coli* in the dendrogram showed that they were genetically different.

DISCUSSION

Fluoroquinolones are used for various indications and in large quantities worldwide. Their introduction in to clinical use marked the beginning of a new era in the treatment of urinary tract infections and other infections caused by gram-negative bacteria [89]. Excellent therapeutic efficacy, broad spectrum of activity, good bioavailability, low cost and fewer side effects led to their widespread use both in the hospitals and in the community [11, 12]. Ongoing research and better understanding of the chemical structure and mechanisms of action have led to the development of newer compounds with wider spectrum of activity, improved pharmacokinetic properties and favorable side effect profile. In the current scenario, fluoroquinolones are being used increasingly in the treatment of almost all types of infections including those caused by gram-negative and gram-positive bacteria, anaerobes and even mycobacteria.

At the time of their introduction it was believed that the potential for developing resistance to these agents among enterobaceriaceae was very low. Rampant use and misuse of these antimicrobial agents over the last decade has led to the emergence of resistance [56]. Fluoroquinolone resistant *E.coli* are being reported from all parts of the world [48-53]. Data from our hospital have shown 20 to 22% resistance among *E.coli* causing community acquired UTI and 70 to 90% resistance among those causing hospital-acquired UTI. [5].

Emergence of FQR *E.coli* is a potential threat to the therapeutic utility of this important class of antimicrobials in the treatment of UTI. Knowledge on the prevalence and distribution of FQR *E.coli* and the mechanisms involved in the development of resistance are indispensable to design specific interventions to limit dissemination.

The principal mechanisms involved in FQR are alterations in the target enzymes and decreased intracellular concentration of the drug due to impermeability of the outer membrane and/or up regulated efflux pumps. Both are primarily mediated through

chromosomal mutations [35]. The change from susceptible phenotype to resistant phenotype requires several stepwise mutations [8, 38]. Single mutations most often do not produce resistance [9].

A high level of resistance (MIC $\geq$256 μg/ml) to nalidixic acid is predictive of mutations at codon 83 of *gyrA*. Ninety seven percent of high nalidixic acid resistant isolates have been found to possess this mutation [9]. All 60 FQR *E.coli* tested in the study showed high-level resistance to nalidixic acid. Twenty seven (90%) of fluoroquinolone susceptible *E.coli* also showed high-level resistance to nalidixic acid. FQR *E.coli* commonly have additional mutations in *gyrA* and probably *parC*. A Swedish study has reported that 87% of FQR *E.coli* had mutations at two loci responsible for codon 83 and 87 of *gyrA* and that mutations in both *gyrA* and *parC* were found in 77% of resistant isolates [9]. Since 30% of community acquired infections and 90% of hospital-acquired infections are FQR it can be assumed that a good proportion of our *E.coli* causing UTI carry several mutations. This may be applicable to *E.coli* causing other infections also. The presence of high MIC to nalidixic acid in 90% of fluoroquinolone susceptible but nalidixic acid resistant strains indicates that these strains may probably carry gyrA mutations at codon 83. Treatment of infections caused by such "susceptible" strain with fluoroquinolones can lead to more mutations and rapid evolution and selection of FQR strains. It is therefore prudent to test for nalidixic acid resistance and limit the use of fluoroquinolones if the *E.coli* are found to be resistant.

The second mechanism, which leads to decreased intracellular concentration of the antibiotic, is also mediated by chromosomal mutations, leading to up-regulation of some efflux pumps and decreased expression of outer membrane proteins like the ompF [35]. Of the 343 isolates 137(40%) were positive for an up-

regulated efflux. This shows that at least 40% of the strains have both mechanisms of resistance for FQR. This again is an indication of multiple chromosomal mutations [9].

Efflux pumps are transport proteins involved in the extrusion of toxic substrates from within cells into the external environment [45]. Several such efflux pumps have been described in *E.coli* but the most important among them is the AcrAB-TolC [40]. Over expression of this efflux pump is usually the result of mutations in the *marR* locus, which is a repressor gene [35]. Increased efflux activity results in the ability to extrude organic solvents and thus tolerance to these chemicals. Hence this method was used as a phenotypic marker for up-regulated efflux pumps [9]. Organic solvent tolerance test is a simple test, which is easy to perform even in small laboratories and the cost per test is low. The test is usually performed using glass petri dishes because the solvents used in the test can dissolve plastic. Efflux positive *E.coli* have shown the ability survive under such adverse conditions. Besides hexane and cyclohexane the test can also be performed using other organic solvents like diphenyl ether, and p-xylene [86]. The importance of detecting efflux-mediated resistance is that it is often associated with multiple antibiotic resistance [35, 40].

Studies have reported increased mutational rates in FQR *E.coli*. FQR *E.coli* are mostly intermediate mutators, which show a mutational rate of about 10 times more than that of susceptible isolates [9]. Strong mutators with *mutS,* which have 100 times more mutating potential, have also been described [90, 91]. The mutator allele can be co-selected by antibiotic treatment [92]. Even though strong mutators promote the rapid evolution of resistance they have a low survival rate due to accumulation of other deleterious mutations also. Strains with intermediate mutational rates, which are strongly associated with FQ resistance, probably have better survival chances. This is in agreement with the mathematical analysis of mutator dynamics in fluctuating environment [93].

The practical implication is that antimicrobial treatment of strains with high mutation rate can lead to selection of resistant strains rapidly. Strains, which are likely to mutate, can be identified by nalidixic acid MIC. Presence of high-level resistance to nalidixic acid indicates that mutations already exist and exposure to antimicrobials can lead to rapid development of further resistance by mutations [9, 35]. Ninety eight percent of FQR *E.coli* and 90% of fluoroquinolone susceptible *E.coli* showed a MIC of $\geq$ 128µg/ml with nalidixic acid.

In our study 329 (96%) of the 343 FQR isolates had resistance to other antibiotics. This is significantly different from the AMR seen in fluoroquinolone susceptible strains. On analyzing the presence of other AMR in efflux positive isolates it was found that these strains were more frequently associated with resistance to multiple antimicrobials like cephalosporins, aminoglycosides, cotrimoxazole and nitrofurantoin. On considering individual drug groups resistance to cephalosporins and nitrofurantoin were significantly associated with a positive efflux. A similar association was not found with aminoglycoside resistance. Even though multiple antimicrobial resistance was found among both efflux positive and efflux negative FQR *E.coli* the percentage was higher in efflux positive strains (pvalue = 0.001). Among the efflux positive strains 87% showed resistance to multiple antibiotics where as only 71% of the efflux negative strains were multidrug resistant. In contrast among the 30 FQ susceptible strains tested in our study, only one strain exhibited multidrug resistance. It appears as though FQR strains have the potential for acquiring other resistances. This in agreement with previous published papers, which have shown that resistance to antibiotics like ampicillin and nalidixic acid, may be the starting point for acquiring multidrug resistance [60, 94]. Although efflux pumps appear to play a role, this is not the only mechanism for MDR in our area.

Tests revealed that 64% of FQR *E.coli* were resistant to cefotaxime and 99% percent of them were ESBL producers. The evolution of ESBL is usually through mutations in the plasmid genes encoding for the earlier broad-spectrum beta lactamases like TEM –1, TEM-2 and SHV-1. ESBL production was significantly more in FQR *E.coli* when compared to the susceptible strains in other studies also [61]. Since ESBL genes are carried on plasmids the reason for this association is not clear. There are reports showing that ESBL may also be chromosomally mediated [61]. The association between FQR *E.coli* and ESBL production has been previously reported. A Turkish study has reported that 5.1% of ciprofloxacin resistant *E.coli* produced ESBL while none of the susceptible strains were ESBL producers (66). This association was found to be more consistent in *E.coli* when compared to *Klebsiella pneumoniae* in which ESBL production was found almost in equal proportions among FQ resistant and susceptible strains [61]. Earlier, Paterson et al have also reported a similar association [60]. Although the authors have suggested that mutations in the *mar* locus might be responsible for ESBL production and multiple antibiotic resistance [61, 95] it is unlikely since according to our data we found equal proportions of ESBL producers among efflux positive and efflux negative strains. It is more likely that bacteria that are able to produce ESBL have additional survival advantages in the environment created by excessive fluoroquinolone use.

This study identified ESBL in only 217 of the 219 cefotaxime resistant *E.coli*. Two strains did not have ESBL and neither tazobactam nor clavulanic acid improved the susceptibility to ßlactams. These two isolates were positive for efflux by the organic solvent tolerance test. Therefore it is possible that in a small number of isolates efflux alone is responsible for resistance to third generation cephalosporins. The therapeutic implication of this is that these infections may not respond to ß-lactam and ß-lactamase

inhibitor combinations and may require therapy with antibiotics belonging to other groups like imipenem.

It is well known that plasmids encoding ESBL can also carry genes coding for resistance to aminoglycosides and cotrimoxazole [60]. We found that 114 cefotaxime resistant strains were also resistant to aminoglycosides and cotrimoxazole in addition to fluoroquinolones.

On comparing FQR from community acquired and hospital acquired infections there was no difference in the prevalence of high-level resistance to nalidixic acid and also in efflux-mediated resistance between the two groups. Although the overall prevalence of FQR is significantly different in these two populations, the proportion of different mechanisms of resistance appears to be similar in both groups. Therefore it is possible that FQR arising in the hospital is being transported to the community and propagated there.

In the study of the epidemiology of *E.coli,* phenotypic characterization methods like antibiogram, serotyping and biotyping alone are not sufficient for coming to a conclusion regarding strain diversity or similarity [96]. Genotyping methods are required for subtle discrimination of various subgroups with in a bacterial population. Molecular epidemiology is essential for the understanding of the origin and spread of infective agents.

FQR *E.coli* are known to have high mutational rates and therefore require a sensitive technique to study the clonal relatedness. In this study we adopted the RAPD typing method, which is sensitive enough to detect even a single point mutation anywhere in the genome. It is a rapid method and easy to perform. It has high discriminatory capacity and can detect subtle intraspecies variations [73, 84].

We found that there were great genetic variations among the FQR *E.coli*. Performing the RAPD typing with two primers, there were only two or three isolates, which were identical with each other. In the fluoroquinolone susceptible population nearly one third of the isolates belonged to a single group implying a common origin from where they mutate and diversify. The commensal *E.coli* also showed a large, related group of isolates suggesting a common ancestry. However there were differences between the individual isolates. Hospital acquired strains showed 2 clusters with 3 and 2 isolates each. These types of clusters were not seen in the community-acquired infecting group. Other than the two clusters that were identified, the other isolates were unrelated. This shows a multiclonal origin of drug resistant *E.coli* in the hospital environment. The clustering indicates the presence of cross infection in the hospital.

The fluoroquinolone susceptible and resistant *E.coli* belonged to two different groups and were genetically unrelated. This type of grouping was not found within the FQR *E.coli.*

A comparison of commensal and infecting strains showed that they were different genetically. This high degree of variability probably reflects the high mutation rates and therefore the random changes in the nucleotide that occurs at a high rate in these *E.coli*. These changes cannot be identified by any existing biotyping method. In this group there were several isolates with similar antibiogram but all of them were different by genetic typing. Therefore antibiogram alone is not useful method for identifying genetic relatedness especially in populations where multidrug resistance is common. RAPD proved to have high discriminatory power and identified many different types.

CONCLUSION

The study was done to determine the mechanisms of fluoroquinolone resistance (FQR) among the *E.coli* causing urinary tract infections (UTI), its association with other antimicrobial resistance and genetic relatedness among them. The summary of the findings are as follows,

1) Antimicrobial resistance was significantly more among FQR *E.coli* when compared to fluoroquinolone susceptible *E.coli*.

2) A significantly high number of FQR *E.coli* produced ESBL when compared to fluoroquinolone susceptible isolates.

3) Multidrug resistance i.e. resistance to three or more groups of antimicrobials was significantly high among FQR *E.coli*. This might limit the choice of therapy and lead to therapeutic failures in the management of important infections. Further studies are required to understand why FQR, which is chromosomally mediated is significantly associated with plasmid mediated resistance like ESBL production.

4) All the FQR *E.coli* had the phenotypic evidence of mutations in the genes encoding for the target enzymes like *gyrA*. Similar evidence was also found among 90% of fluoroquinolone susceptible *E.coli,* which were resistant to nalidixic acid. Limiting the use of fluoroquinolones in the treatment of infections caused by nalidixic resistant *E.coli* is necessary to prevent further evolution of resistant strains.

5) In addition to target alterations 40% of FQR *E.coli* had up-regulated efflux pumps contributing to resistance.

6) A significant number of the efflux positive isolates were multidrug resistant compared to efflux negative isolates. However 71% of efflux negative strains also exhibited MDR suggesting that factors other than efflux play a role in MDR.

7) Up regulated efflux was significantly associated with nitrofurantoin resistance and also with cephalosporin resistance when compared to efflux negative FQR *E.coli*.

8) Organic solvent tolerance test is simple and an easy test to perform even in small laboratories.

9) Ninety nine percent of cefotaxime resistant FQR *E.coli* were ESBL producers. The remaining 1% were positive for efflux only.

10) Although the prevalence of FQR is more among the hospital acquired *E.coli* when compared to the community acquired *E.coli*, the mechanisms of resistance were similar in both the groups.

11) Genotyping by RAPD revealed that FQR *E.coli* belonged to many different clones. There were 115 RAPD types among the 120 isolates. Genetic similarity was not common among the isolates tested. Limited clustering among the hospital acquired strains was seen, indicating the presence of cross infection.

12) Grouping suggestive of a common origin was observed among the fluoroquinolone susceptible *E.coli* and also among FQR commensal *E.coli*.

13) There was no evidence of genetic relatedness between fluoroquinolone susceptible and FQR *E.coli*. These two formed two separate groups in the NJTREE.

14) RAPD is a sensitive technique with a high discriminatory power for determining genetic relatedness.

APPENDIX

Appendix I:

Preparation of Luria agar with magnesium sulphate and glucose (LBMg agar):

Ingredients:

Bacto tryptone	1.0gm
Bacto yeast extract	0.5gms
Sodium chloride	1.5gms
Magnesium sulphate	0.25gms
Glucose	0.001gms
Agar	1.5gms
Distilled water	100ml

The ingredients were mixed in water by heating. The pH was adjusted to 7.0 ± 0.2.

Sterilized by autoclaving for 10 minutes at 121°C. About 20 ml was poured into each of the glass petri dishes.

Appendix II:

Preparation of antibiotic solution:

Concentration of the pure substance of nalidixic acid was 1mg =1000µgms.

To obtain a final concentration of 2560µg /ml of nalidixic acid - 20mgs of nalidixic acid was diluted in 7.5 ml of distilled water and 0.3 ml of 1M NaOH was added

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