CHAPTER 1

INTRODUCTION

1.1 Emerging Multidrug Resistance Bacteria

*Klebsiella pneumoniae* is an opportunistic pathogen that causes a significant proportion of hospital-acquired urinary tract infections, pneumonia, septicemias and soft tissue infections (Ullmann U & Podschun R 1998). *K. pneumoniae* has increasingly become resistant to the commonly used cephalosporin antibiotics due to the plasmid encoded Extended-Spectrum Beta-Lactamase (ESβL) genes, further resistance to cephalosporin-inhibitor combinations and even carbapenem antibiotics is on the rise and pose a serious threat for health care-associated infections (Ullmann U & Podschun R 1998; Chen et al 2012). For such resistant bacteria quinolone antibiotics may be a feasible approach. However, these resistant *K. pneumoniae* frequently co-harbour quinolone resistance determinants making them difficult to treat. Persistant infections of *K. pneumoniae* are also associated with biofilm formation.

Antibacterial resistance occurs by numerous mechanisms, including enzymatic inactivation or modification of drugs, drug target alteration or protection, and due to the increased active efflux of the drugs. To overcome the antibiotic resistance new therapeutic strategies are needed to address this challenge. The first step is to have a collection of bacterial strains with a known mechanism of action against antibiotics so that they may be used for screening purposes.

In this Ph.D. Thesis, we have investigated the mechanisms of plasmid mediated resistance to quinolones. Further bacterial strains with high biofilm formation or high efflux have been identified. These MDR bacterial strains have been used to screen for natural compounds which can inhibit biofilm formation and inhibit antibiotic efflux.
1.2 Fluoroquinolone Resistance

Quinolones constitute an important group of antimicrobials active against Gram-negative and Gram positive bacteria (Lesher et al 1962). Fluoroquinolones are the third largest selling drug class with sales of US$ 7.1 billion, accounting for 17% of the world antibiotic market in 2009 (Hamad, 2010). Since the introduction of fluoroquinolones for therapy in 1962, resistance of the Enterobacteriaceae to these agents has become common, widespread and generally non-clonal (Strahilevitz et al 2009). Quinolone resistance can be due to mutations in the chromosomal genes for the DNA gyrase and topoisomerase IV, the targets of quinolone action; and by changes in expression of efflux pumps and porins that control the accumulation of these agents inside the bacterial cell. Plasmid-mediated quinolone resistance (PMQR) was first reported in 1988 in a fluoroquinolone resistant strain of K. pneumoniae in Alabama, USA (Martínez-Martínez et al 1998). This gene encoding quinolone resistance, named qnrA1, belongs to a pentapeptide repeat family. Two other transferable quinolone resistance determinants have also been described, aac(6')-Ib-cr which encodes a variant aminoglycoside acetyltransferase with two amino acid alterations allowing it to inactivate ciprofloxacin (Robicsek et al 2006) and qepA genes which encode efflux pumps that extrude quinolones (Park et al 2009).

The qnr genes show a high level of diversity. Five main types of the qnr genes, qnrA (Martínez-Martínez et al 1998), qnrB (Jacoby et al 2006), qnrC (Wang et al 2009), qnrS (Hata et al 2005) and qnrD (Cavaco et al 2009) have been identified. The qnrB gene was identified first in an isolate of K. pneumoniae from South India, and subsequently has been found in isolates in the USA, Korea, Kuwait, France and Taiwan (Jacoby et al 2006; Strahilevitz et al 2009). The qnrA genes were also reported in K. pneumoniae from South Indian isolates in the same paper (Jacoby et al 2006). After the initial report by Jacoby et al., there has not been any screening for the prevalence of the qnr genes in K. pneumoniae in India. In a study from our laboratory, we analysed 23 isolates of K. pneumoniae collected in October 2009 and found them to be Extended-Spectrum Beta-Lactamase (ESBL) isolates with plasmids containing different combinations of blaSHV, blaOXA-1 and blaCTX-M genes. Since ESBL isolates are frequently resistant to quinolones, and the plasmids containing ESBL genes also have the genes conferring quinolone resistance, we undertook this study to screen for qnr genes in these 23 ESBL isolates of K. pneumoniae collected in October 2009. This is the first report from India demonstrating plasmid
mediated quinolone resistance (PMQR) mediated by qnr genes and the aac(6′)-Ib-cr allele in K. pneumoniae.

1.3 Biofilm Inhibition

Persistent infections are a global challenge for human beings, claiming millions of lives every year and demanding huge medical and social resources. One common survival strategy employed by bacteria pathogens is to form a biofilm, an amorphous and dynamic structure that is not only resistant to antibiotics, but also resistant to host immune clearance (Chen & Wen, 2011). Biofilm formation is a two-stage biological process controlled by surface adhesins and cell-to-cell communication pathways. Aggregated bacterial cells protected and/or coated by extracellular matrix, are insensitive to both nutritional stimulation and hostile attacks. In the human body, biofilms may trigger persistent infections with chronic inflammation.

Factors that are implicated in the virulence of K. pneumoniae strains include the capsular serotype, lipopolysaccharide, iron-scavenging systems, fimbrial and non-fimbrial adhesions. The ability of bacteria to form a biofilm on host tissue surfaces is an important step in the development of infection. As a correlation between biofilm formation and bacterial persistence has been established (Balaban et al 2004), the possibility of using drugs targeting biofilm formation in combination with the current antibiotics is emerging as a potential therapeutic approach for this type of bacterial persistent infection. With an increase in reports of multi drug resistant infections, and the importance of biofilm formation resulting in decreasing susceptibility to antibiotics, there is an urgency to discover molecules targeting the inhibition of biofilm formation of bacteria. The objective of the present study was to identify natural compounds for their potential to inhibit biofilm formation. Natural products chitosan, eugenol, curcumin and linoleic acid which are known antibacterials (Martinez et al 2010 ; Soni et al 2008) and efflux pump inhibitors reserpine and berberine (Kvist et al 2008) were analysed for their potential to suppress biofilm formation in high biofilm forming strains of K. pneumoniae. For each compound, we quantitatively compared the potencies for bacterial growth inhibition versus biofilm inhibition. Such data would enable selection of natural compounds which could synergize with established anti-microbials, and increase their therapeutic efficacy.
With the objective of identifying natural compounds that have biofilm inhibition activity, high biofilm producing clinical isolates of multi drug resistant (MDR) *K. pneumoniae* were first screened and subsequently the effects of the 6 test compounds were analyzed on biofilm formation. The analysis showed a dose dependent biofilm inhibitory activity for each of the 6 test compounds. The results from this analysis will be useful in designing combinatorial treatment of antibiotics and biofilm inhibitors.

### 1.4 Bacterial efflux pumps inhibitors

Plants have been a source of antimicrobial agents in many forms of alternative medicine. However since the plant extracts are complex mixtures, the mode of action is frequently not known clearly. The search of the sole bio-active compound from the plant extract has not been successful in most cases since the pure compound is less effective than the whole extract. It has been suggested that plant extracts might have other compounds which might be synergistic effect.

One of the mechanisms of bacterial antibiotic resistance is the increase in efflux pump activity leading to lower intracellular accumulation of an antibiotic leading to increase in its minimal inhibitory concentration (Zechini & Versace, 2009). Efflux-mediated resistance is clinically relevant and can render antibacterial therapy ineffective (Li & Nikaido, 2009). Various compounds with efflux plump inhibitory activity have been identified but none of them are in use clinically. Development of compounds able to modulate efflux systems [Efflux Pump Inhibitors (EPIs)] and thus restore the activity of antibiotics when used in combination has been considered a promising approach to overcome bacterial resistance (Stavri et al 2007). Plant-derived antibacterials are always a source of novel therapeutics having terpenoids, glycososteroids, flavonoids and polyphenols which are highly potent and fight against the MDR bacterial infection by synergistic interaction with antibiotics (Hemaiswarya et al 2008).

We undertook a study of a few commonly used antimicrobial agents and investigated if they had a synergistic effect with the antibiotic ciprofloxacin. Then we analysed the extracts for the inhibition of the antibiotic efflux. The combinational use of an EPI with antibacterial agents should potentiate the activity of antibacterials, and it would also reduce the frequency of emergence of resistant mutants.
Bacterial multidrug efflux transporters (Webber & Piddock, 2003) are divided into five major structural families: (1) resistance nodulation cell division (RND), (2) major facilitator superfamily (MFS), (3) small multidrug resistance (SMR), (4) multidrug and toxic compound extrusion (MATE), and (5) ATP-binding cassette (ABC). Energetically, RND, MFS, and SMR are H+/drug antiporters, MATE pumps are Na+/drug antiporters, while ATP hydrolysis is linked to drug transport in ABC transporter family. In particular, drug exporters belonging to RND family play a key role in clinically relevant resistance in Gram-negative bacteria. The RND efflux pump systems are tripartite structures consisting of a transporter, linker and an outer membrane pore. The AcrAB-TolC complex of E.coli and K. pneumoniae (Morgan-Linnell et al 2009) and the MexAB-OprM of Pseudomonas aeruginosa (Mahamoud A 2006) have been well characterized.

1.5 Strategies for altering the Efflux pump

The alteration of efflux pump can be obtained by targeting the driving force of the mechanism, or by competitive or non-competitive inhibition. Compounds that affect the energy level of the bacterial membrane such as carbonylcyanide m-chlorophenylhydrazone (CCCP), valinomycin and dinitrophenol (DNP) are used to abolish completely the efflux of different molecules. These compounds are recognized as highly cytotoxic. To date, no molecules belonging to this family have been developed for clinical use.

In another mechanism of inhibition, EPIs can bind directly to the pump and block it, either in a competitive or a non-competitive manner with the substrates. The prototype EPI inhibitor is the MC-207 110 or phenylalanine arginyl β-naphthylamide (PaβN). They act by competitively binding the substrate binding sites of RND-family efflux pumps and are exported by RND pumps (Askoura et al 2011). Other classes of efflux pumps that have been reported against RND pumps include arylpiperazines, arylpiperidines and quinoline compounds (Pages et al 2010). Two compounds from the Berberis plant were found to be synergistic to the antibacterial activity of the berberine alkaloid. These compounds porphyrin pheophorbide A and the flavoligan 5´- methoxyhydnocarpin (5´-MHC) were found to be the inhibitors of the S.aureus NorA efflux pump (Stermitz et al 2000; Zechini & Versace 2009).

The strategy for EPI identification starts with the screening of a synergistic activity with an antibiotic. The checkerboard assay identifies synergistic combinations of antimicrobial agents and the potential EPIs. In this assay, 2-fold serial dilutions of a pump substrate,
such as norfloxacin or berberine, and 2-fold serial dilutions of a fraction or test compound result in microtitre wells with a different combination of pump substrate and test compound concentration. The presence of synergy can be identified by this method. Further, to find out if the synergy is due to an inhibitor of efflux, the effect of these compounds on norfloxacin accumulation is performed.

In this study, we use 5 representative MDR bacterial isolates for screening for the synergy of ciprofloxacin with plant extracts. The alcoholic extracts from the following plants with reported antimicrobial activity: *Tectona grandis* (Purushotham et al 2010; Mahesh & Jayakumaran, 2010), *Acacia nilotica* (Kavitha et al 2013), *Hemidesmus indicus* (Gayathri & Kannabiran, 2009), white papery rind of *Punica granatum* (Prashanth et al 2001) and *Syzygium cumini* (Ramana & Prakash, 2012). These plants were chosen as they all have a use in various systems of traditional medicine and some are used as dietary supplements. The plant extracts which showed synergy with the ciprofloxacin antibiotic were further screened for their effect on the inhibition of efflux by the norfloxacin accumulation assay. The extracts which displayed inhibition of efflux were further characterised by sequential fractionation to identify the fractions with EPI activity. The plant extracts were further analyzed by TLC and bioautography. The extract and antibiotic combination were analysed by a time kill assay.

1.6 Motivation of the Study

Emerging antimicrobial resistance is a serious global problem due to the escalating time, costs in controlling bacterial diseases and its spread across continents. As a result of the increased rates of antimicrobial use, resistance to antibiotics has increased in the last 20 years. Regular monitoring of the Antimicrobial susceptibility patterns of clinical bacterial isolates and the molecular characterization of the mechanisms of antibiotic resistance is a very important study in devising methods to control the spread of antibiotic resistance. Therefore we undertook this study to screen and characterize the emerging resistance of ESβL producing *K. pneumoniae* isolates to fluoroquinolones drug groups and antibiotic resistance efflux mechanisms. The genes conferring these resistance pattern and plasmid mediated resistance to other species were also characterized. However, bacteria in natural environments usually form communities of surface-adherent organisms embedded in an extracellular matrix, called
biofilms. Current antimicrobial strategies often fail to control bacteria in the biofilm mode of growth. Treatment failure is particularly frequent in bacterial biofilms formed on medical devices and compromised host immunity. The rising prevalence of these risk factors over the last decades has paralleled the increase in biofilm infections. To overcome the biofilm mediated treatment failure, we had analysed some natural products against clinically isolated biofilm forming *Klebsiella pneumoniae*. And also antibacterial and efflux pumps inhibitory (EPI’s) activity had been demonstrated in the MDR *Klebsiella pneumoniae, E.coli* and *Pseudomonas aeruginosa*.

**Aim** - To determine the Fluoroquinolone drug resistance mechanism of clinically important gram negative MDR bacteria and to identify the resistance modifying agents from natural products against the bacterial biofilms and antibiotic efflux mechanisms

**1.7 Objectives of the Study**

- To determine the Antimicrobial susceptibility profile of clinical isolates of *Klebsiella pneumoniae*.
- Fluoroquinolones, *qnr* genotyping by PCR, Identification of the *qnr* alleles by sequencing and bioinformatic analysis.
- To characterize the plasmid mediated nature of fluoroquinolones resistance by electroporation.
- To determine the biofilm forming capacity of MDR *Klebsiella pneumoniae*.
- Screening for concentration dependent MDR *Klebsiella pneumoniae* biofilm inhibition potency of some natural products.
- Identification of antibacterial property of 5 medicinal plant extracts and their synergism with ciprofloxacin against MDR bacteria.
- Determination of Efflux pump inhibition (EPI’s) activity of crude and the sequential fraction of some medicinal plant extracts against MDR bacteria.