INTRODUCTION

Antibiotics - the ‘miracle drugs’ of the 20th century - continue to play a major role in curing infectious diseases. The discovery of ‘Salvarsan’, an arsenic based drug for syphilis in 1909, development of natural antibiotic penicillin in 1928 and synthesis of a sulpha drug, Prontosil in 1935, opened a golden era of novel antibiotic inventions. Alexander Fleming - the discoverer of the first natural antibiotic, penicillin - was also among the first who warned about the potential resistance to penicillin if used too little or for too short a period during treatment (Aminov, 2010). The ability of microbes to grow or survive in the presence of a concentration of antibiotic that is usually sufficient to kill them or inhibit their growth is termed as antibiotic resistance. Although antibiotic resistance can be considered to be a natural phenomenon driven by the selection pressure of antimicrobial agent, indiscriminate use of antibiotics is a major factor facilitating the emergence of resistance worldwide (Sabtu et al., 2015). Emergence of multidrug resistance, which is defined as non-susceptibility of bacteria towards at least one agent in three or more antimicrobial categories, has become a significant public health threat (Allcock et al., 2017).

1.1 Molecular mechanisms of antibiotic resistance

Bacteria can attain antibiotic resistance through intrinsic or acquired mechanisms. Intrinsic mechanisms are those specified by naturally occurring genes found on the host’s chromosome and such resistance can be a result of impaired permeability of the bacterial envelope, efficient drug export systems, the absence or presence of low affinity antimicrobial target or the presence of enzymes which inhibit or destroy the antibiotics (Galán et al., 2013). Acquired resistance, can either be a consequence of de novo mutations or may arise due to acquisition of resistance determinants borne on plasmids, bacteriophages, transposons, and other mobile genetic elements by horizontal transfer. Acquisition of new genetic material is accomplished through the processes of conjugation, transformation, or transduction (Alekshun and Levy, 2007).
Mutations resulting in antibiotic resistance alter the antibiotic action via one of the following mechanisms, such as (i) antibiotic inactivation, (ii) changes in target site, (iii) active efflux and (iv) down regulation of outer membrane protein channel are all transferrable to subsequent generations - a phenomenon known as vertical evolution (Munita and Arias, 2016).

1.2 Role of porins and efflux pumps in multidrug resistant (MDR) gram-negative bacteria

Antibiotic resistance in gram-negative bacteria is attributable to a synergetic interplay between reduced drug intake due to low permeability of outer membrane and an active drug export consequent to upregulation of efflux pumps. These two aspects reportedly play a critical role in the development of the MDR phenotype (Fernández and Hancock, 2012).

Gram-negative bacteria are intrinsically resistant to many antibiotics due to the permeability barrier provided by their multifaceted cell envelope comprising of an outer membrane embedded with channels of both specific and non-specific types. Porins are nonspecific, water-filled open channels which act as molecular filters for hydrophilic substances, mediate transport of nutrients and ions including antibiotics across the membrane into the periplasm (Fernández and Hancock, 2012; Galdiero et al., 2012).

Bacterial efflux pumps are transporting systems lodged in cell membrane which drive out various compounds such as physiological substrates, non-antibiotic substrates as well as antibiotics from the cell. Multidrug efflux pumps are encoded by bacterial genomes and commonly belong to various families on the basis of their sequence similarity, substrate specificity, number of components (single or multiple), number of transmembrane-spanning regions and energy source. Currently, these have been categorized into six families: (i) the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, (ii) the major facilitator superfamily (MFS), (iii) the small multidrug resistance (SMR) family, (iv) the multidrug and toxic compound extrusion (MATE) family (v) the resistance-nodulation-cell division (RND) superfamily and (vi) proteobacterial antimicrobial
compound efflux (PACE) family (Masi et al., 2017). The RND efflux pumps play a key role in the development of both intrinsic and acquired multidrug resistance in gram-negative bacteria (Blair and Piddock, 2009; Vargiu et al., 2016; Alibert et al., 2017). These pumps are comprised of a characteristic tripartite complex formed of a cytoplasmic membrane transporter, a membrane fusion protein (MFP) and an outer membrane protein (OMP) channel (Symmons et al., 2009; Daury et al., 2016; Puzari and Chetia, 2017).

1.3 *Pseudomonas aeruginosa*

*P. aeruginosa*, a ‘priority pathogen’ has now been included in a list of 12 families of bacteria which pose a serious health threat to man (WHO, 2017a). This opportunistic microbe is also held responsible for nosocomial infections worldwide. Known to possess a large number of virulence factors, it causes severe infections with high morbidity and mortality rate, particularly in immune-compromised patients or those with underlying disease (Poole, 2001; Strateva and Yordanov, 2009; Askoura et al., 2011; Porras-Gómez et al., 2012; Chatterjee et al., 2016). *P. aeruginosa* has now achieved a superbug status by acquiring multidrug-resistant phenotypes through (i) intrinsic resistance mechanisms such as those mediated by constitutive expression of AmpC beta-lactamase, efflux pumps and porin down-regulation and (ii) acquired resistance caused by mutational changes or acquisition of resistance mechanisms via horizontal gene transfer. Together, these processes contribute toward development of overwhelming resistance against a variety of structurally unrelated antibiotics leading to difficulties or failure in therapy (Poole, 2011). Efflux pumps of clinical relevance in *P. aeruginosa* belong to the RND family, of which MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY represent the predominant sets of efflux systems, with a broad range of drug specificities. *P. aeruginosa* infections are commonly treated with carbapenems which belong to the beta-lactam class of antibiotics. Carbapenem-uptake mainly occurs through OprD porin proteins. However, the occurrence of carbapenem-resistant strains is gradually increasing and recent studies indicate involvement of OprD down-regulation in combination with overexpression of efflux systems in the
development of resistance (Lee and Ko, 2012; Ocampo-Sosa et al., 2012; Fang et al., 2014; Zeng et al., 2014; Kim et al., 2016).

1.4 Selection pressure and dissemination of antibiotic resistance as a global phenomenon

Under the selective pressure of antibiotics, bacteria which are either resistant intrinsically or have acquired antibiotic resistant determinants possess a greater chance to survive and multiply. Overuse, inappropriate choices, inadequate dosing and poor adherence to treatment guidelines of antibiotics are the underlying causes of the widespread increased antibiotic resistance observed at a global level (Prestinaci et al., 2015). Antibiotics used in livestock and aquaculture for growth promotion, disease treatment and prophylaxis are considered to be the major contributors to the overall problem of resistance (Marshall and Levy, 2011). The majority of consumed antibiotics released into the environment through waste streams and wastewater treatment plants, are thought to be the evolutionary hotspots for antimicrobial resistance dissemination, since resistance genes, mobile genetic elements and antibiotic selection pressure from various sources are introduced to commensals and pathogens (von Wintersdorff et al., 2016).

1.5 Indian scenario of antibiotic resistance

Antibiotic resistance is a stark reality across the world including the Indian subcontinent. In the country the challenges associated with controlling antibiotic resistance are various and multifaceted. Disease burden in India is among the highest in the world due to the inappropriate and irrational use of antimicrobial agents and reports in 2010 showed India as the world’s largest consumer of antibiotics for human health. Multiple factors, such as high disease burden, poor public health infrastructure, rising incomes, unregulated sales of cheap antibiotics and poverty among the low-income strata of society result in large-scale selection and dissemination of resistance genes in India (Laxminarayan and Chaudhury, 2016). Health sector in India suffers from gross inadequacy of public finance leading to favourable conditions for development of drug resistance. Also, systematic nationwide surveillance programme of antibiotic resistant pathogens in
various sectors is inadequate or lacking in India. Paucity of national repository of resistant pathogens makes it difficult to understand the severity of the problem and the involvement of various factors responsible for emergence of antimicrobial resistance (Kumar et al., 2013). Several new initiatives have been launched by various agencies to address this problem including, i) IndiaClen - Indian Clinical Epidemiology Network, which has generated data on resistance in pathogens like *Pneumococcus, Haemophilus influenzae* across the country, ii) IIMAR - Indian Initiative for Management of Antibiotic Resistance launched in March 2008, with WHO support, by a consortium of NGOs to promote prudent use of antimicrobials, iii) INSAR - Indian Network for Surveillance of Antimicrobial Resistance, a network of 20 laboratories in the private as well as public sectors across the country to generate quality data on drug resistance, iv) organization by the ICMR of an expert group meeting in December 2009 and v) an Indo-Swedish workshop held at New Delhi on 2nd February, 2010 to discuss on a joint strategy for containment of antimicrobial resistance (WHO, 2010).

Studies and reports on the emergence of drug resistant bacteria including MDR in both hospital-based and other environments from Kerala State are sparse with very few reports available in published literature (Manjusha and Sarita, 2011; Ahmed et al., 2012; Krishna et al., 2014) including the reports from the laboratory where the present study was carried out (Narayanan et al., 2016 and Nithya et al., 2017). Recently, Kerala government has formulated a new antibiotic stewardship programme to control antibiotic resistance, which not only takes stock of the antibiotic resistance in the state but also builds awareness among doctors, hospital staff and patients as well as the larger community on this critical health issue of antibiotic resistance (IIMR, 2016). A detailed survey of available literature including internet resources failed to reveal a clear picture on the extent or prevalence of antibiotic resistance mediated by efflux pump-based mechanisms and role of porin down-regulation despite several studies carried out across the world and a few from within the Indian subcontinent. Hence, the doctoral work presented in this Thesis, though limited, focuses on the above mentioned aspects of antimicrobial resistance mechanisms.
1.6 About the Thesis

1.6.1 Aims and objectives

The present study was undertaken

(i) to determine the prevalence of MDR gram-negative clinical bacterial isolates in Kerala

(ii) to gain deeper molecular insights into efflux pump and porin related genes in *P. aeruginosa* selected from clinical isolates of MDR gram-negative bacteria.

Objectives of this study included:

1. Collection of MDR gram-negative bacterial isolates from various clinical laboratories in Kerala

2. Identification of bacteria, antibiotic profiling and phenotypic detection of efflux pump - mediated drug resistance therein

3. Phenotypic detection of various beta-lactamases in *P. aeruginosa* selected from clinical isolates of MDR gram-negative bacteria and their RAPD profiling

4. Expression analysis of efflux pump and porin genes in the selected isolates of MDR *P. aeruginosa*

5. Mutational variations and phylogenetic analyses of efflux pump regulatory genes from MDR *P. aeruginosa*

6. Mutational variations and phylogenetic analysis of porin *oprD* gene, molecular modelling of porin protein and

7. Restriction mapping of *oprD*-derived amplicons for potential diagnostics.
1.6.2 Work plan and parameters evaluated in the present study

A flow chart summarizing the work plan and the parameters evaluated in the study is given below (Fig. 1.1):

![Flowchart of Work Plan and Parameters](image)

**Fig. 1.1. Work plan and the parameters (flowchart) evaluated in the study.**
1.6.3 Thesis layout

The Thesis includes six major chapters. The first gives a brief introduction on the major theme of the doctoral research work. The second chapter deals with review of literature which covers the discovery and classification of antibiotics, antibiotic resistance mechanisms, factors contributing to the spread of antibiotic resistance along with a general account on the present global and Indian scenario of antibiotic resistance. Details of the ‘Materials and Methods’ used in the study form the third chapter whilst the Fourth chapter incorporates the experimental results (presented in tables and figures), their analyses followed by relevant and appropriate discussions. The final and the fifth chapter consists of ‘Conclusions’ and ‘Future prospective’. This chapter summarizes the highlights of the entire research work and also provides a brief perspective on future directions and implications of the present study. The ‘References’ section gives full bibliographic information of all citations quoted in the text. An ‘Appendix’ given at the end contains information on reagents, solutions and buffers used in the study. Pre-pages (v - x) show lists of all abbreviations, figures and tables included in the Thesis.