Infectious diseases represent the significant percent of most serious health issues resulting in huge number of deaths worldwide (Barreto et al., 2006). Antimicrobial agents have helped in curing many such infections. However the unprecedented increase in antimicrobial resistance phenotype in clinically significant microbial pathogens especially nosocomial super bugs has severely challenged the treatment and prevention of severe infections. The extensive use of antimicrobial agents has contributed to the continued emergence and widespread dissemination of multidrug resistant (MDR) bacteria worldwide. Resistance to at least two or more classes of antibiotics is considered as the "multidrug resistance" behaviour displayed by that pathogen (Magiorakos et al., 2012). Gram-negative pathogens of "ESKAPE" group are becoming an increasing menace globally especially in health care settings. They can withstand any adverse environmental conditions and has great tendency to resist almost all classes of antimicrobials. Among several bacterial pathogens, the *Acinetobacters* mainly the strains of *A. baumannii* has emerged as a global threat among the nosocomial pathogens from past few decades (Peleg et al., 2008).It was about 40 years ago when they were susceptible to most of the antimicrobial agents (Bergogne-Berezin and Towner, 1996) but now the situation is very worse. These pathogen are now considered as one of the most annoying human pathogen, frequently found in hospital environment and from diseased patients mainly those who are critically-ill (Fournier and Richet, 2006). In current scenario, there are very few treatment options left for the treatment of many infections caused by MDR *A. baumannii* and designing novel strategies to solve this problem is the demand of present situation and current priorities of research. The infections caused by the MDR *A. baumannii* are characterized by increase in morbidity and mortality rates and a consequent increase in their treatment costs (Kim et al., 2012) leaving therapeutic options very limited. The MDR strains are on a steep rise everywhere in the hospitals settings as well as outside hospitals and are hugely associated with large number of epidemic outbreaks (Howard et al., 2012; Eveillard et al., 2013). Despite the huge incidences of such infections, unfortunately very few studies have been done on decoding the mechanisms of resistance. ***In this thesis, we performed epidemiological studies that aided in the identification and characterization of A. baumannii strains from Indian scenario and we also demonstrated that A. baumannii employ versatile array of mechanisms to confer the multidrug resistance phenomenon. Besides, the study was expanded by decoding the additional mechanisms of antimicrobial resistance with a special emphasis on decoding the role of OmpR-EnvZ signaling system in mediating antimicrobial resistance in A. baumannii for the first time***.

In this study, we collected the *Acinetobacter* isolates from medical centres in India and investigated their general physiology and their tolerance to different classes of antibiotics. The isolates were initially screened for their antimicrobial susceptibility behaviour and the most of the isolates (42%) were found to be highly drug resistant showing resistance to more than 20 antibiotics among the tested drugs. The isolates displayed resistance to all groups of antibiotics including β-lactams, aminoglycosides and fluoroquinolones, including polymyxins (colistin and polymyxin B) which are considered the last drug resort for treatment of severe *A. baumannii* infections. As demonstrated in many reports, a number of factors are responsible for causing antimicrobial resistance (Peleg et al., 2008). Initially, when we screened for the presence of resistance genes encoding various antibiotic degrading enzymes, we found several β-lactamases belonging to ESBLs, MBLs and OXAs families in our collection. The percentage of ESBLs,MBLs and OXAs identified differed in various isolates. Importantly, the prevalence of genes encoding for ESBLs and oxacillinases were high as compared to other β-lactamases screened. Among the AMEs, which confers resistance to aminoglycosides, the isolates showed the prevalence of *aadA1* (25%) and *aadB* (15.8%) and *aacA4* (6.6%) in this collection. Since many of the isolates did not harbour any of these resistance genes, presence of other resistance determinants were to be explored. The role of mobile genetic elements such as plasmids and class 1 integrons, which play an important role in gene transfer and acquisition of resistance genes were also investigated. The presence of class 1 integrons were screened and we found that the conserved 5'CS region and variable regions elements were detected in strains used in this study, however the 3'CS could not be identified which indicated that possibly the 3'CS were modified, further extension of work to elucidate their modification is highly warranted. The plasmids, often responsible for drug resistance in bacteria were abundantly found in the isolates. Analysis of all the clinical isolates revealed that plasmids were present in maximum isolates (~85%) and among them 36.9% harboured multiple plasmids. Majority of the isolates harbouring multiple plasmids were found to be highly MDR, interestingly, some of the isolates that had no plasmids were also MDR. Plasmids from selected strains were transformed into a sensitive heterologous *E.coli* host. The colonies obtained on different antibiotics plates confirmed the role of plasmid in mediating multidrug resistance. As reported, *Acinetobacter* utilizes both the intrinsic and acquired resistance determinants to confer MDR phenotype. Alteration in the outer membrane (OM) permeability is considered one of the major broad spectrum resistance determinants. Alteration in size of porins or over-expressed efflux pump facilitates the drug resistance behaviour in these pathogens. The OMP

profile in some of the MDR isolates on testing showed the expression of some over-expressed bands in these isolates, their further sequencing confirmed them to be metabolic enzymes. Apart from these; the role of active efflux in mediating antimicrobial resistance was evidenced by performing various standardized growth inactivation assays and fluorimetric assays. The general physiological behaviour of these isolates was tested by determining their colony morphology, growth under different pH stress and oxidative stress. Biofilm formation represents a strong survival capability of these pathogens and so the isolates were tested for their biofilm forming ability. They were able to form biofilm and majority of those biofilm forming strains were highly MDR exhibiting resistance profile of 18 antibiotics or more. The results overall imply that extent of drug resistance to correlate with biofilm formation ability of clinical MDR isolates. The biofilm formation capacity was also slightly enhanced in osmotic stress condition (0.015M and 0.15 M NaCl). *A. baumannii* has a tremendous propensity to cause outbreaks in hospitals. It can easily survive desiccation and disinfectants manifested in the hospital settings thereby staying for longer time on the surfaces (Jawad et al., 1998; Rajamohan et al., 2010). The biocides popularly used as hospital-based disinfectants serve to prevent the dissemination of various pathogens in the hospitals environments (Rutala and Weber, 1999). Upon testing the tolerance of these isolates to biocides through MIC assay it was deduced that isolates exhibited medium level of tolerance to these biocides. It was interesting to observe that majority of them were MDR which indicate that the isolates can not only resists antibiotics but also hospital disinfectants. The growth inactivation assay performed for these biocides showed that active efflux can extrude out these biocides imparting broad level of resistance to antimicrobial agents. Overall, the strains were found to be harbour diverse resistome leading to its MDR phenotype and also exhibited different tolerance capacity to various stress conditions.

The behaviour of these isolates towards various stress condition including antimicrobial stress basically depends on some of the signaling proteins acting as a transcriptional regulators, functioning as an activators or repressors for several genes. The signaling systems of bacteria help them to sense the changes in external environment in order to make necessary modulations in expression of certain genes. Out of wide array of sensing mechanisms, TCS play a central role in sensing and acclimatization of bacterial pathogen including *A. baumannii*. These system acts as an important regulator for the expression of various resistance determinants. The *in silico* analysis of the signaling proteins in the MDR *A. baumannii* strains revealed ~290 signaling proteins of which ~30 proteins on an average

belongs to TCS. Very few TCS functions have been elucidated so far. The OmpR-EnvZ was one of the TCS found in the genome of *A. baumannii* strains which shared homology to previously characterised OmpR-EnvZ signaling system in other bacteria. The OmpR in *A. baumannii* is a 254 aminoacids residue long protein and has two domains, the receiver domain and the output domain. The protein shares ~67% identity with OmpR found in *S. oneidensis*, *S. marcescens*, *S. enterica* serovar Typhi, *K. pneumoniae*, *S. flexneri*, and *E. coli*. The EnvZ which is a 485 amino acids long protein and was found sharing maximum identity of 35.14% with other bacterial homologs. The expression of the putative TCS, *ompR-envZ* was later analysed in some of the clinical MDR isolates through RT-PCR analysis which showed its expression level to be >3-fold higher relative to other TCS. PCR based detection of *ompR-envZ* and its prevalence in multiple clonally unrelated strains strongly revealed that *ompR-envZ* may have a prominent role in regulating drug resistance. The PCR detection coupled with Southern blot analysis confirmed this signaling system to be conserved in maximum number of the isolates in our collection. The kinase domain of *envZ* was cloned and expressed and the autophosphorylation activity was checked using *in vitro* kinase assay. The protein was able to get autophosphorylated and interestingly we observed that the autophosphorylation of the sensor protein was more calcium dependent compared to other divalent cations.

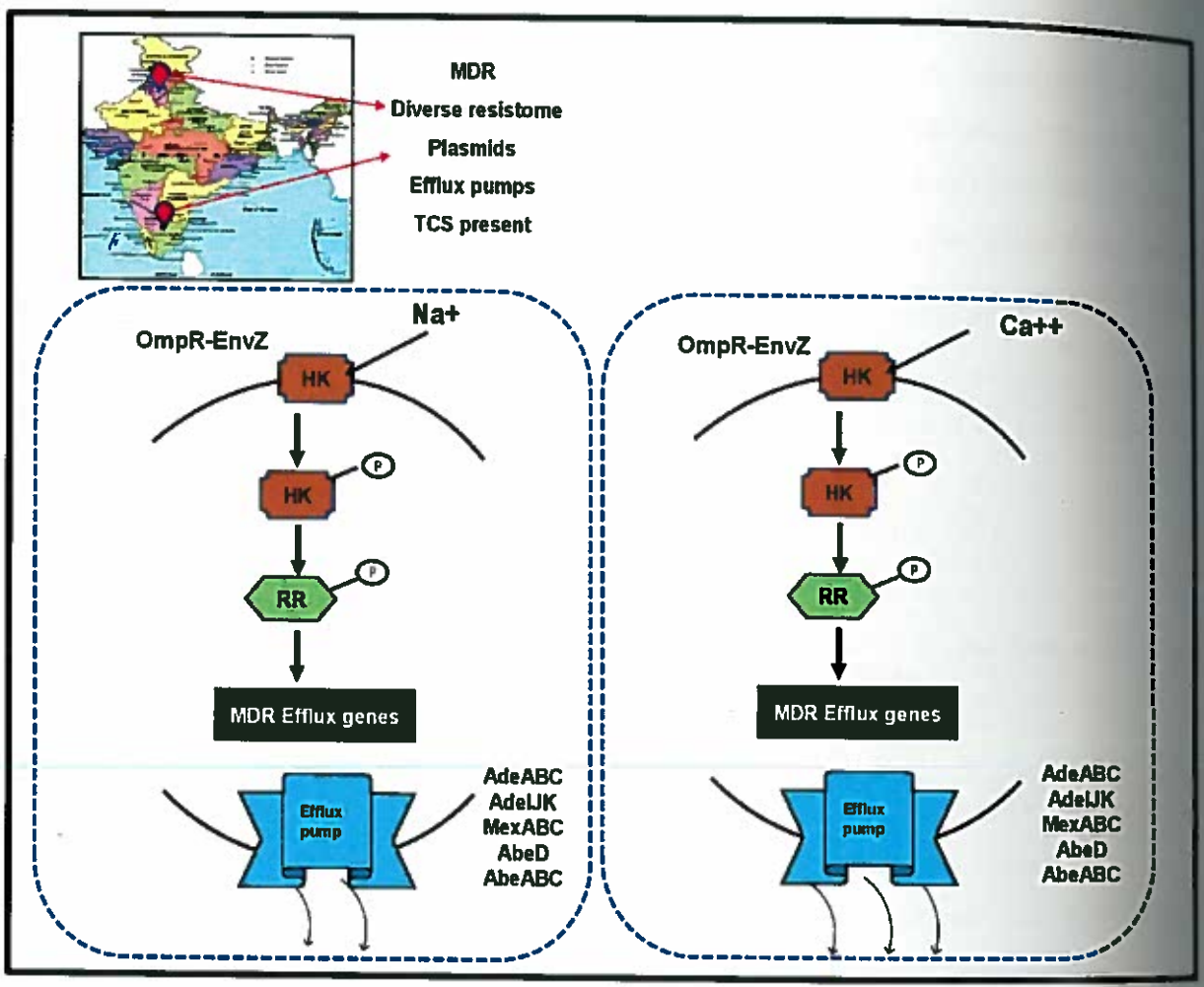
The OmpR-EnvZ is primarily an osmoresponsive TCS extensively characterized in *E. coli* as well as in other Gram-negative bacteria. Various studies have shown its regulatory scope beyond osmolarity response such as regulation of virulence, motility, biofilm formation as well as antibiotic resistance in many Gram-negative pathogens (Lee et al., 2000; Lin et al., 2008; Brzostek et al., 2007; Cameron and Dorman, 2012). Therefore, it is a considered a multifunctional TCS with varied functions and in our study, we have elucidated its role in *Acinetobacter* physiology and drug resistance through deletion of *ompR-envZ* in strain *A. baumannii* AYE by homologous recombination. Disruption of *ompR-envZ* altered the bacterial growth and oxidative stress tolerance capability. The *ompR-envZ* mutants also displayed increased sensitivity to antimicrobials and also showed lower biofilm forming ability. Since *ompR-envZ* primarily respond to osmolarity, as expected deletion of operon resulted in reduction of osmotic stress tolerance. By it was very provoking to see what would be the impact of osmotic stress on antibiotic susceptibility pattern of the clinical isolates. Alarmingly, analysis of data revealed that few of the isolates displayed increased resistance to different classes of antibiotics compared to the control. As the kinase domain was found to be

sensing calcium and getting autophosphorylated strongly, it was intriguing to know the impact of calcium exposure on the antibiogram profiles. Therefore, we tested the impact of calcium exposure on to the susceptibility profiles of clinical strains. Results displayed a significant increase in the resistance pattern of all the isolates tested. From the results it was anticipated that calcium might be involved strongly in influencing the drug resistance phenotype in this pathogen.

The efflux pumps are an important intrinsic resistance determinant in mediating broad range of antimicrobial resistance in *A. baumannii* whose expressions are basically under the regulations of TCS such as AdeRS, BaeSR (Marchand et al., 2004; Lin et al., 2014). The regulatory aspects of OmpR-EnvZ of *A. baumannii* were tested by various gel shift assays with different RND efflux promoters. OmpR which is DNA-binding protein has the binding sites on various target genes related to osmolarity, virulence, motility, biofilm, acid tolerance as well as antibiotic resistance. The promoters of various RND efflux pumps of *A. baumannii* were analysed for putative binding sites of OmpR by *in silico* approach. The OmpR binding with all the RND efflux promoters tested confirmed the result of *in silico* analysis. On analysis of experiments, a highly evident nucleoprotein complex was found in the autorad of gel shift assays. Interestingly, the calcium influenced the binding of OmpR with these promoters as observed from strong intensity of binding complex compared to its contemporary magnesium ion. Thus, overall the calcium in our study was found to be a strong player in modulating the *ompR-envZ* expression in mediating antimicrobial resistance in this pathogen. Our RT-PCR analysis identified a very novel uncharacterized efflux pump (designated as *abeABC*) whose expression in *ompR-envZ* deletion mutant was decreased. This efflux pump gene was identified as RND efflux pump and its expression level was >3-fold decreased compared to other characterised membrane transporters. The biological characterization of this efflux pump deciphered its role in antimicrobial resistance and physiology for the very first time.

Overall, this thesis presented a brief epidemiological study which delineated the diverse antibiotic resistome in Indian scenario, evidence for the physiological functions of uncharacterized OmpR-EnvZ signaling system for first time and its involvement in regulating the expression of efflux pump abeABC to modulate antimicrobial resistance for the very first time in the nosocomial pathogen A. baumannii.

Schematic representation of the novel results obtained under different objectives



- ***Strains collected across clinical centres in India were highly MDR and harboured diverse resistome such as antibiotic inactivation enzymes, mobile genetic elements (plasmids) and active efflux pumps.***
- ***The osmoresponsive OmpR-EnvZ TCS was highly prevalent in one-third of the clinical isolates.***
- ***This TCS has a substantial role in Acinetobacter physiology and drug resistance as deletion of ompR-envZ altered the normal physiology of bacteria such as growth, biofilm formation ability, tolerance to oxidative and osmotic stress including antimicrobial susceptibility.***
- ***The OmpR-EnvZ TCS regulates multidrug efflux pumps including abeABC in mediating antimicrobial resistance in A. baumannii.***
- ***The OmpR-EnvZ was investigated in strongly responding to calcium in mediating MDR for the very first time in A. baumannii.***