Study on membrane-mediated antibiotic resistance mechanisms and pathogenic potential of clinical and environmental multidrug resistant *Enterobacter* isolates

By

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Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by **Mitali Mishra** entitled **"Study on membrane-mediated antibiotic resistance mechanisms and pathogenic potential in clinical and environmental *Enterobacter* isolates"** and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

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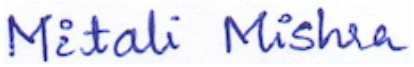

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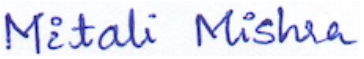

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List of Publications arising from the thesis

A) Published:

1. **Mishra M**, Patole S, Mohapatra H. Draft genome sequences non-clinical and clinical *Enterobacter cloacae* isolates exhibiting multiple antibiotic resistance and virulence factors. *Genome announcement*, 2017. https://doi.org/10.1128/genomeA.01218-17.
2. **Mishra M,** Kumar S, Majhi RK, Goswami L, Goswami C, Mohapatra H. Antibacterial efficacy of polysaccharide capped silver nanoparticles is not compromised by AcrAB-TolC efflux pump. *Frontiers in Microbiology*. 2018. 9:823. doi: 10.3389/fmicb.2018.00823.

B) Manuscript under preparation (pertaining to thesis):

1. **Mishra M**, Mohapatra DP, Singh SK, Panda S, Sahu S, Debata N, Singh DV, Mohapatra H. Outer-membrane proteins (OMPs) association with antibiotic resistance and virulence in clinical and environmental *Enterobacter* Spp.
2. **Mishra M**, Mohapatra DP, Singh SK, Debata N, Mohapatra H. pH and cephalosporin drugs differentially affect AcrAB-TolC expression in MDR *Enterobacter* isolates.

C) Other Publications:

1. Sanyasi S, Majhi RK, Kumar S, [#]**Mishra M**, Ghosh A, Suar M, Satyam PV, Mohapatra H, Goswami C, Goswami L. 2016. Polysaccharide-capped silver Nanoparticles inhibit biofilm formation and eliminate multi-drug-resistant bacteria by disrupting bacterial cytoskeleton with reduced cytotoxicity towards mammalian cells. *Scientific Reports* 6, Article number: 24929.
2. Singh SK, Ekka R, [#]**Mishra M**, Mohapatra H. 2017. Association study of Multiple Antibiotic Resistance and Virulence: A strategy to assess extent of risk posed by bacterial population in aquatic environment. *Environmental monitoring and assessment* 189:320.
3. Mohapatra DP, Singh SK, Sahoo M, Patole S, **Mishra M**, Debata NK and Mohapatra H. 2018. Retrospective study on clonal relationship of multidrug resistant *Klebsiella* spp. indicates closed circulation and initiation of clonal divergence. *Journal of Medical Microbiology*. DOI 10.1099/jmm.0.000715

4. Patole S, **Mishra M,** Mohapatra H. 2017**.** Draft genome sequences of clinical and non-clinical isolates of *Klebsiella* spp. exhibiting non-heritable tolerance towards antimicrobial compounds. *Genome announcement*.**.** https://doi.org/10.1128/genomeA.01217-17.
5. Singh SK, **Mishra M**, Sahoo M, Patole S, Sahu S, Mishra SR, Mohapatra H. Antibiotic resistance determinants and clonal relationships among multidrug-resistant isolates of *Klebsiella pneumoniae. Microbial pathogenesis* 110, 31-36.
6. Singh SK, **Mishra M**, Sahoo M, Patole S, Mohapatra H. 2017. Efflux mediated colistin resistance in diverse clones of *Klebsiella pneumoniae* from aquatic environment. *Microbial pathogenesis* 102, 109-112.
7. Kumar S, Majhi R, Singh A, **Mishra M**, Chawla S, Guha P, Satpati B, Mohapatra H, Goswami C, Goswami L. Carbohydrate-coated Gold-Silver nanoparticles for efficient elimination of multi drug resistant bacteria and in vivo wound healing. (Submitted to *ACS Applied Materials & Interfaces*.)

#-Part of the work belonged to this thesis.

D. <u>Oral and poster presentation in international conferences</u>

- Oral presentation on "Studies on acrAB-tolC multi drug efflux pump expression in response to pH and antibiotic stress in *Enterobacter* isolates" as a speaker at 1st Gordon research seminar (GRS) on "Multidrug efflux systems" held at Tuscany, Italy in 25-26th April 2015.
- Presented poster on "Studies on acrAB-tolC multi drug efflux pump expression in response to pH and antibiotic stress in *Enterobacter* isolates" at 6th Gordon research conference (GRC) on "Multidrug efflux systems" held at Tuscany, Italy in 27th April-1st May 2015.
- Participated in an abstract entitled " A study on pH dependent modulation of carbonyl cyanide m-chlorophenylhydrazone (CCCP) activity and efflux pump functioning in *Enterobacter* isolates" at 6th Gordon research conference (GRC) on "Multidrug efflux systems" held at Tuscany, Italy in 27th April-1st May 2015.

- Presented poster on "Outer membrane proteins of *Enterobacter* spp: Association with antibiotic resistance and virulence factors" at 15th International congress of Bacteriology and Applied Microbiology (BAM) during International Union of Microbiological Societies (IUMS-2017) conference held at Singapore in 17th-21st July 2017.

E. <u>Poster presentation/Participation in national conferences</u>

- Participated in 99th Indian Science congress held at KIIT University, Bhubaneswar from 3rd-7th January 2012.
- Presented poster on "Diversity and function of multidrug efflux pumps in *Enterobacter* Spp." at 53rd Annual meeting of Association of microbiologists of INDIA (AMI) held at KIIT university, Bhubaneswar from 22nd-25th November 2012.
- Presented poster on "Response of AcrAB-TolC multidrug efflux pump to physiological stimuli in *Enterobacter* spp." at 38th All India Cell Biology Conference (AICBC) held at Central Drug research Institute (CDRI), Lucknow held during Dec 2014.
- Presented poster on "Polysaccharide-capped silver Nanoparticles inhibit biofilm formation and eliminate Multi-drug resistant bacteria by disrupting bacterial cytoskeleton with reduced cytotoxicity towards mammalian cells" at International Interdisciplinary conference on Humanitarian technology (IICHT) 2016 held at KIIT University during 15-17th December 2016.
- Presented e-poster on "Significance of bacterial membrane proteins in mediating antibiotic resistance and virulence in *Enterobacter* spp." at 59th Annual meeting of Association of microbiologists (AMI), INDIA held at University of Hyderabad from 9th-12th December 2018.

F. Workshops/symposiums attended

- Participated in workshop on "Hands on training in Flow Cytometry course" Held at C-CAMP, NCBS campus, Bangalore from 4th-7th March 2014.
- Participated in Science Communication workshop on scientific writing, organized by The Wellcome Trust/DBT India Alliance held on 10th April 2014 at ILS, Bhubaneswar.
- Participated in the Orientation workshop on Laboratory Animal Sciences held on 13-15th July 2015 at Institute of Life Sciences (ILS), jointly organized by ILS and NISER.
- Participated in workshop and hands-on training on the topic of "Advanced Techniques in Protein Design and Engineering" at the Indian Institute of Science Education and Research (IISER) Mohali during 15-19th March 2016.
- Participated in the hands-on workshop on "Course on advanced microscopy and imaging techniques" jointly organized by Olympus medical systems India Pvt. Ltd. And DSS ImageTech Pvt. Ltd., held at NISER, Jatni campus from 4-6th August 2016.
- Participated in Indian immunological society- Odisha chapter II jointly organized by NISER and ILS at NISER, Jatni campus on 6th January 2017.

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Dedication

I dedicate this thesis to my grandparents, parents and brother, without whom none of my achievements would have been possible and for whom my doctoral degree holds even more significance and glory.

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perseverance to finish my PhD came looking up to you, your enormous help and support throughout these 7 years can't be put into words. My best friends Manoj, Rakesh, Shrikant, Subhranshu, Sony, Tapas, for patiently handling my frustrations and always being supportive. My heartfelt thanks to my colleagues and friends at NISER for enjoyable company and beautiful memories to cherish forever.

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CONTENTS

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SYNOPSIS OF Ph. D. THESIS

1. **Name of the Student: Mitali Mishra**
2. **Name of the Constituent Institution: National Institute of Science Education & Research**
3. **Enrolment No. : LIFE11201104007**
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5. **Board of Studies: Life Sciences**

SYNOPSIS

(Limited to 10 pages in double spacing)

Gram-negative bacteria have unique cellular architecture consisting of an outer membrane and inner cytoplasmic membrane. While the plasma membrane is a phospholipid bilayer forming a highly selective barrier against ionic compounds, the outer membrane is comparatively more permeable to ionic solutes and small hydrophilic antibiotics such as β-lactams (Delcour, 2009). Traditional knowledge attributed the transport activities to be regulated by outer membrane proteins (also referred to as Porins) and the efflux pumps proteins (Fernández and Hancock, 2012, Li *et al*., 2016). Investigations done in multidrug resistant *E. coli* isolates revealed involvement of the efflux pump proteins and porins in facilitating the resistant phenotype (Ma *et al*., 1995, Viveiros *et al*., 2007). This work presented in this thesis explores the diversity of multidrug efflux pumps and outer membrane proteins (OMPs) in clinical and environmental

Enterobacter isolates. The study explores the association of outer membrane proteins with antibiotic resistance and virulence and, delves deeper to understand the effect of various physical and chemical parameters on expression of efflux pump proteins. The results of the study enhance our understanding functional significance of efflux pump proteins and OMPs beyond antibiotic resistance.

The outer membrane contains water filled open channels that facilitate passive penetration of hydrophilic drugs restricted to <600 kDa. The proteins that constitute these pores are generally referred to as porins (Fernández and Hancock, 2012). Based on their function and architecture, the porins or outer-membrane proteins (OMPs) are categorized into small β-barrel membrane anchors (e.g. OmpA, OmpX), general non-specific porins (e.g. OmpF, OmpC), substrate specific porins (e.g. PhoE, LamB) and TonB-dependent receptors (e.g. FhuA, FepA) (Koebnik *et al*., 2000). Besides their roles as solute carriers, OMPs have diverse physiological roles in bacteria (Koebnik *et al*., 2000).

Due to this substrate non-specificity the efflux pumps aid in conferring resistance towards dyes, toxic compounds and multiple drugs of diverse structures (Sun *et al*., 2014). Based upon their substrate specificity and energy requirements, bacterial multidrug efflux pumps are categorized into five different super families namely: RND (Resistance-Nodulation-Division), MATE (Multiple antibiotic and toxic extrusion), MFS (Major facilitator Superfamily), SMR (Small Multidrug resistance) and ABC (ATP-Binding Cassette) transporters, (Piddock, 2006; Padilla *et al*., 2010). However, AcrAB-TolC type tripartite efflux pumps belonging to RND superfamily are widely distributed in many gram-negative pathogens including *Escherichia coli*, *Klebsiella* Spp., & *Enterobacter* spp., significantly contribute towards MDR phenotype (Padilla *et al*., 2010; Pereze *et al*., 2007; Boucher *et al*., 2009).

Enterobacter spp. belonging to family Enterobacteriaceae is facultative anaerobic gram-negative bacilli. *Enterobacter* spp. has been listed as one of the top six emerging opportunistic pathogens belonging to 'ESKAPE' group; as they escaped killing by current antimicrobial agents used in clinics (Sanders and Sanders, 1997). *E. aerogenes* and *E. cloacae*, two predominant species of *Enterobacter*, are associated with plethora of diseases including nosocomial infections like UTI's (specifically catheter related), abdominal cavity/intestinal infections, wound infections, pneumonia, and septicemia (Davin-Regli and Pages, 2015). They are known to tolerate wide pH variations as evident from their ubiquitous presence in terrestrial, aquatic environments and inside human gastrointestinal tract. (Grimont and Grimont, 2006).

Realizing the significance of OMPs and AcrAB-TolC efflux pump proteins on contributing resistance phenotype, many reports exists that elucidate the protein structure and mechanistic aspect of their function. With this background, my thesis work focused on "Study of membrane-mediated antibiotic resistance mechanisms and pathogenic potential in clinical and environmental multidrug resistant *Enterobacter* isolates". To achieve this, following objectives were laid down for the study:

1. To determine the occurrence and diversity of efflux pumps in multidrug resistant clinical and environmental *Enterobacter* isolates.
2. To perform comparative study on association of outer membrane proteins in multidrug resistant environmental and clinical *Enterobacter* isolates.
3. To study the effect of physico-chemical environment on expression of AcrAB-TolC multidrug efflux pump in *Enterobacter* isolates.

This thesis has been organized into five chapters and contents of the same have been discussed in detail as follows:

Chapter 1– Introduction: This chapter introduces to the reader to bacterial membrane structure and functions. It goes on describing the transport proteins including the Outer Membrane Proteins and Efflux pumps.

Chapter 2 – Review of Literature: The second chapter reviews the current literature on Outer Membrane Proteins and Efflux pumps. It concisely presents the updated available information on structure function studies and mechanistic aspect of efflux pump function. It also reads out the available information from the literature on association of OMPs with antibiotic resistance and virulence. The chapter concludes with details of the aim and objectives laid down for the study.

Chapter 3 – Materials and Methodology: This chapter discusses in details, the materials used and methodology followed to attain the objectives. It includes basic microbiological assay, molecular biology protocols, cloning and expression, imaging techniques, cell culture techniques and general proteomics experiments.

Chapter 4- Results and Discussion: This section reports the results obtained from the experiments, analyses the observations and discusses it in light of the current literature. This chapter has three sections described as follows:

Section 4.1: To determine the occurrence and diversity of efflux pumps in multidrug resistant clinical and environmental *Enterobacter* isolates.: This section of the chapter describes the results that provide a comparative data on resistance profile and characterizes the isolates for virulence factors among. Antibiotic resistance profiling of the isolates suggested that most of the environmental isolates exhibited multi-drug resistance phenotype where as clinical isolates exhibited pan-drug (n=3) and extensive-drug resistance (n=6) phenotype. MIC in presence of CCCP resulted in more than two fold decrease towards multiple classes of antibiotics, indicating role of efflux pumps in MDR phenotypes. PCR based screening, slot blot

hybridization and sequence confirmation revealed predominance of *acrAB-tolC* type of RND tripartite efflux pump genes in both clinical and environmental isolates, followed by MATE superfamily of efflux pumps. Further SMR and MFS families of efflux pumps were only present in clinical isolates but not in environmental isolates.

Section 4.2: Comparative study on association of outer membrane proteins (OMPs) in multidrug resistant environmental and clinical *Enterobacter* isolates: This section reports the distribution of OMPs in environmental and clinical isolates and explores the association of OMPs with antibiotic resistance and *in-vitro* cell adhesive/invasive properties. Multiplex PCR followed by slot blot hybridization and sequence confirmation revealed, *OmpA* and *OmpX* predominate in both clinical (30% and 24%) and environmental strains (36% and 35%), with higher occurrence in environmental isolates. Similarly, *LamB* and *OmpF* were found in 24% and 22% of clinical isolates, whereas only 14-15% of environmental isolates were positive for the same. Among the environmental isolates, we observed an association between *OmpA* and *OmpX* positive isolates and β-lactam, cephalosporins resistance. Association analysis of OMPs with cell adhesion and invasion phenotype utilizing Pearson correlation matrix revealed that *E. cloacae* ATCC 13047 which was positive for *OmpA,OmpX*, *LamB, OmpF* exhibited highest adhesion-invasion frequency, indicating greater pathogenic ability. Further, clinical and environmental isolates positive for *OmpA*, *OmpX* and *LamB* showed moderate and weak adhesion-invasion frequency respectively. In addition, environmental isolate SR4.9 positive for *OmpA* and *OmpX* could show only strong cell-adhesive property. Interestingly, EspAH4 that was positive only for *OmpA* could only display weaker cell adhesive quality, whereas isolates like SR5.7, EcIMS21, EspAH3 which were devoid of any OMPs, did not have in-vitro adhesion or invasion features. These results suggested that presence of OmpF and LamB facilitate *Enterobacter* spp. in establishing infection in host cells.

Section 4.3: To study the effect of physico-chemical environment on expression of AcrAB-TolC multidrug efflux pump in *Enterobacter* isolates: This section has further been divided into three sub-sections, presenting results of detailed study on the expression of AcrAB-TolC efflux pump proteins in response to various physical and chemical stimulus.

4.3.1: To determine effect of pH and/or antibiotics on AcrAB-tolC efflux pump expression in *Enterobacter* isolates: This part of the section describes the effect of pH and cephalosporin antibiotics on expression MDR efflux pump proteins AcrAB-TolC. Effect of pH on efflux activity could be attributed from tolerance to higher concentration of antibiotics and growth of the organisms. Further stability of antibiotics exposed to varied pH was tested by HPLC, which suggested that there is no degradation. Immunoblotting results revealed the distinct differences in constitutive expression of AcrAB-TolC proteins in clinical and environmental isolates, with the former being relatively higher than later. Cephalosporin drugs were found to induce AcrAB-TolC protein expression. Expression of TolC was higher at acidic pH than at alkaline pH. Increase in TolC expression at alkaline pH showed positive association with alkaline survival of isolates. Results obtained with wild type isolates were further confirmed in prototype isolates and *E.coli* MDR isolates.

4.3.2: To determine effect of silver nanoparticles on AcrAB-TolC expression in multidrug resistant Enterobacter isolates: This second sub-section focused on the effect of two nanoparticles namely, silver nanoparticle (AgNP) and bimetallic carbohydrate conjugated nanoparticle (Ag-MCNP), on AcrAB-TolC expression. Both these nanoparticles inhibited bacterial growth in a dose dependent manner against multiple gram-negative and gram-positive MDR pathogens. We observed that neither of the nanoparticles tested had any significantly effect on the expression of AcrAB-TolC protein, suggesting the feasibility of these being utilized as antibacterial compounds.

4.3.3. Whole genome sequence analysis of MDR environmental *Enterobacter cloacae* isolate DL4.3 and clinical isolate EspIMS6

This last section reports analysis of the whole-genome sequences of two MDR *Enterobacter cloacae* isolates DL4.3 and EspIMS6. Draft genome sequence analysis revealed that EspIMS6 and DL4.3 were of 5,296,869 and 4,820,048 bp in size, with 54.7% and 54.9% G+C content respectively. Both of them harbor multiple drug resistance genes and MDR efflux pumps. Both the organisms possessed resistance towards beta-lactams, fluoroquinolones, fosfomycin and heavy metals such as copper, cobalt-zinc-cadmium. This is a prerequisite for understanding the molecular basis of antibiotic resistance, the repertoire of efflux genes along with their regulatory proteins encoded by the organisms, and the contribution of efflux proteins in physiological functions in the cell, particularly in the context of the source of isolation.

Chapter 5 - Summary and conclusion: This chapter summarizes the salient findings of this study with concluding remarks and future prospects.

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- Participated in workshop and hands-on training on the topic of "Advanced Techniques in Protein Design and Engineering" at the Indian Institute of Science Education and Research (IISER) Mohali during 15-19th March 2016.
- Participated in the hands-on workshop on "Course on advanced microscopy and imaging techniques" jointly organized by Olympus medical systems India Pvt. Ltd. And DSS ImageTech Pvt. Ltd., held at NISER, Jatni campus from 4-6th August 2016.
- Participated in Indian immunological society- Odisha chapter II jointly organized by NISER and ILS at NISER, Jatni campus on 6th January 2017.

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Date: 25.04.2018.

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2.	Dr. Harapriya Mohapatra	Guide/ Convene		25.04.2018
3.		Co-guide (if any)		
4.	Dr. Asima Bhattacharyya	Member	Asima Bhattacharyya	25.04.2018
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List of Abbreviations

AR	Antibiotic resistance
ATCC	American type culture collection
BHI	Brain Heart infusion
BLAST	Basic Local Alignment Search Tool
CDC	Centers for Disease Control and Prevention
CFDA	Carboxy fluorescein diacetate
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
EMBL agar	Eosin methylene blue agar
ESBL	Extended spectrum β-lactamase
HGT	Horizontal gene transfer
ICU	Intensive care unit
LB	Luria-Bertani
LPS	Lipopolysaccharides
MAR index	Multiple antibiotic resistances index
MDR	Multi-drug-resistant
MHA	Muller-Hinton agar
MHB	Muller-Hinton broth
MIC	Minimum inhibitory concentration
MLST	Multi locus sequence typing
NDM	New Delhi metallo-β-lactamase
OD	Optical density
ORF	Open reading frames
PCR	Polymerase chain reaction
QRDR	Quinolone-resistance determining region
SDS	Sodium dodecyl sulfate
ST	Sequence type
WHO	World health organization

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Chapter 1

Introduction

Antibiotic resistance has now become a pandemic health problem, fostering the development of multi-drug, pan-drug and extreme-drug resistant bacteria [1]. Eventually such drug resistance has managed to disseminate to environmental and aquaculture ecosystems in a direct or indirect way; by virtue of irrational use of antibiotics in clinics, animals and plants, unregulated hospital waste disposal and other anthropogenic activities. Despite such adverse situation, non-pathogenic bacteria survive in environment by two of its paralleled strategy; drug resistance (inherent or acquired) and virulence factors [2]. Certainly, antibiotic resistance can trigger virulence or fitness properties in bacteria to adapt to their ecological niches. This is more prevalent in hospitals, where opportunistic pathogens, if multi-drug resistant, are found to be rapid in its infection process and persist in chronic infections amidst antimicrobial therapy.

Such is the case of *Enterobacter* species that have now emerged as one of the top six opportunistic pathogens globally belonging to 'ESKAPE' group [3]; as they escaped killing by current antimicrobial agents used in clinics, thus limiting therapeutic options. This gram-negative, facultative anaerobic, rod-shaped bacteria belonging to *Enterobacteriaceae* family, is ubiquitously found in the terrestrial and aquatic environments (water, sewage, soil), diary products nd hospital settings, is an inhabitant of human gastrointestinal tract too. Since *Enterobacter* spp. play an important role as a pathogen of plants, insects, and humans, acquisition and spread of multidrug resistance in such versatile organism is more challenging [4].

Enterobacter cloacae and *Enterobacter aerogenes* two predominant and well-studied species of *Enterobacter*, are associated with a plethora of diseases such as lower respiratory tract infections, pneumonia, urinary tract infections, skin/soft-tissue

infections, septicemia, wound infections, meningitis and nosocomial infections in intensive care units [5], [6]. *E. cloacae* and *E. aerogenes* are responsible for the majority of *Enterobacter* infections, accounting to 65-75% and 15-25% respectively; both have emerged as important nosocomial pathogens, especially in immunocompromised patients. *Enterobacter sakazakii*, which is later acronymed as *Cronobacter sakazakii*, is an important causative agent of sepsis, meningitis, brain abscess and necrotizing enterocolitis in neonates, immune-compromised patients and children below age 3-4 years. However, very few cases of *E. sakazakii* infections have been reported in adults, who are considered as a low- risk group [7].

Enterobacter spp. are commonly found in intensive care units and are responsible for 8.6% of nosocomial infections according to the US Centers for Disease Control and Prevention (CDC). Although community acquired infection is less reported, but *Enterobacter* infections are more prevalent in patients admitted in ICU for prolonged periods, or prior use of antimicrobial agents, use of foreign devices such as intravenous catheters, and serious underlying conditions such as burns, mechanical ventilation, and immunosuppression [8]. Crude mortality rates associated with *Enterobacter* infections range from 15-87%, *E. cloacae* infection is associated with the highest mortality rate of all *Enterobacter* infections. Major groups of antibiotics that are commonly used to treat *Enterobacter* infections include second and third generation cephalosporins, carbapenems, aminoglycosides, fluoroquinolones. However, it is noteworthy that most of the *Enterobacter* spp. has developed resistance to antimicrobial therapy, more likely towards cephalosporins, aminoglycosides, fluoroquinolones, trimethoprim-sulfamethoxazole etc. Though infrequent, but few cases of carbapenem-resistant *Enterobacter* spp. has also been reported [9]. Further, recent reports reveal that almost

50% of the isolates have even developed resistance to carbapenems and colistin, the last-line of antibiotics used for treatment.

Most of the *Enterobacter* spp. naturally expresses chromosomal AmpC β-lactamase at low level conferring resistance to first generation cephalosporins. However, presence of third generation cephalosporin or mutation in AmpR repressor, induces overproduction of AmpC β-lactamases, resulting in resistance to almost all β-lactams. Several reports have indicated the presence of β-lactamases to be the prominent cause of β-lactam resistance in *Enterobacter* spp. Several studies have highlighted regarding enzymatic resistance to drugs in *Enterobacter* spp. globally; elucidated by presence of various ESBLs (TEM, SHV, CTX type conferring resistance to β-lactams and cephalosporins), carbapenemases (including metallo β-lactamases like NDM, VIM and serine carbapenemase KPC type exhibiting resistance to carbapenems) and enzymes inactivating/modifying aminoglycosides and fluoroquinolones [8]. Apart from enzymatic barrier, presence of target mutations also contributed greatly to quinolone resistance and subsequent higher MIC values in *Enterobacter* spp. Besides such classical and intrinsic resistance strategies, basal efflux pump expression in bacteria is certainly the first mechanism along with membrane impermeability that reduces the effect of antimicrobials on the bacteria [10]. Such membrane-mediated resistance mechanism is novel and clinically significant in MDR *Enterobacter* spp., and hence need to be investigated in detail. It is believed that these membrane embedded transporters, porins and efflux pumps, constitute 60-70% of the total bacterial membrane proteins, indicative of their probable involvement in fitness and survivability of the pathogens.

The activity of efflux pump in multiple resistant clinical *Enterobacter* isolates was genetically and functionally characterized, where occurrence of active efflux

contributing to MDR phenotype was found in approximately 40% of MDR clinical strains [11]. Several studies on *E. cloacae* and *E. aerogenes* have reported the presence of MDR efflux pumps belonging to RND (primarily AcrAB-TolC type) and MATE super families [12],[13]. In both these *Enterobacter* spp., the sequence similarities and biological activity are particularly high in the context of AcrAB–TolC [12]. Moreover, various chemicals such as salicylate, chloramphenicol, and imipenem were found to induce the genetic cascade controlling the expression of *Enterobacter* AcrAB–TolC pump [14]. Several studies have reported the loss of porins (particularly OmpF and OmpC) or altered expression of OMPs in carbapenem-resistant *Enterobacter* isolates [9],[5].

Observationally, it is well understood that controlling membrane permeability, comprising of both influx and efflux of antimicrobials, have a larger role in antibiotic resistance in *Enterobacter* spp., helping the pathogen to adapt to adverse situations. Despite several studies reporting involvement of efflux pump and outer membrane proteins in antibiotic resistance, population level studies on role of membrane-mediated antibiotic resistance in *Enterobacter* spp, belonging to both clinical and environmental sources is still unexplored. The present study explores the association of outer membrane proteins (OMPs) with antibiotic resistance and virulence and, delves deeper to understand the effect of various physicochemical parameters on expression of efflux pump proteins. The results of this study compares and contrasts the significant differences observed in antibiotic resistance mechanisms in *Enterobacter* isolates from clinical and environmental sources. Lastly, this work broadens our understanding on functional significance of efflux pump proteins and OMPs beyond antibiotic resistance.

CHAPTER-2

Review of Literature

2.1. Antimicrobial resistance in ESKAPE Pathogens

Antimicrobial resistance (AMR) has emergedis currently as one of the principal leading public health problems of 21st century worldwide that challenge the efficacy of antimicrobial therapy. Undoubtedly we are now in the "post-antibiotic era" and are exposed to a global health crisis [1]. The most prevalent pathogens termed as "ESKAPE", is the acromym of a group of pathogens including *Enterococcus faecium, Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp; which are the principal cause of nosocomial infections and thus exhibit utmost threat to antimicrobial therapies worldwide over the last decade [3]. Resistance to antibiotics is known to be developed by five classical methodsthe following ways; 1] limited membrane permeability restricting the entry ofaccess of the antibiotic insideto the target, 2] alteration of antibiotic target site, 3] modification and/or inactivation of drugsantibiotic inactivation by various hydrolyzing enzymes, 4] modification of antibiotic by enzymes and 4] active efflux pumps that expels antibiotics out of the cell [15].

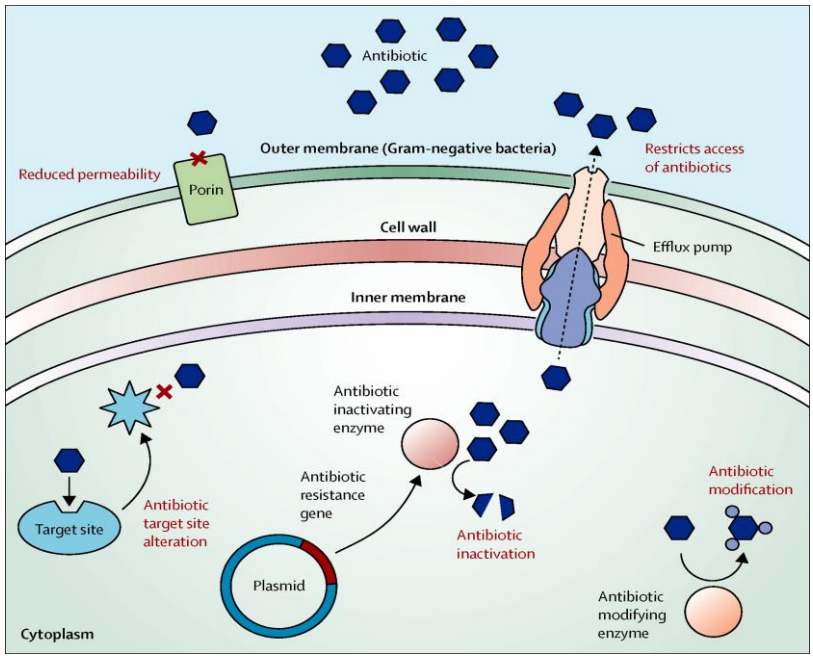


Figure no. 2.1.1: Major mechanisms of antimicrobial resistance in Gram-negative bacteria. It summarizes the resistance strategies followed by bacteria (Ref: Sherrard et al., 2014).

2.1.1. Molecular mechanisms and origins of antibiotic resistance

On the whole, ESKAPE pathogens exhibit numerous antibiotic resistance mechanisms that resulted in higher morbidity and mortality worldwide. The molecular mechanisms of resistance to antibiotics have been extensively investigated over the past half century and well described in many pathogens and commensals. However, mechanisms involved in ESKAPE pathogens mostly included drug inactivation/alteration and modification of drug binding sites/targets, which are briefly discussed below.

2.1.1.1 Drug inactivation or Alteration

The most important genetic mechanisms involved is production of enzymes that modify or inactivate antibiotics, such as β-lactamase, aminoglycoside-modifying enzymes etc. β-lactamase enzymes are ancient, widely distributed in bacterial genera with a wide range of types, usually acts by hydrolyzing the β-lactam rings [16]. Random mutations of the genes encoding such enzymes gives rise to extended spectra of antibiotic resistance to β-lactams as well as cephalosporins. β-lactamase enzymes were classified as Ambler class-A, B, C and D [17]. The Ambler class A group of β-lactamase enzymes included Extended spectrum β-lactamases (ESBLs), mainly TEM, SHV, CTX-M and KPC types which were are prevalent in Enterobacteriaceae. Ambler class B group included Metallo β-lactamases (MBLs) such as IMP, VIM and NDM-1; whereas Ambler class D group included many Oxacillin hydrolyzing enzymes (OXA). However, Ambler class C group consists of AmpC β-lactamases, which are usually chromosomally encoded in *Enterobacter* spp., and resulted in low-level resistance to narrow-spectrum cephalosporin drugs, but gets induced in the presence of antibiotics [18].

Another important antibiotic resistance mechanism is resistance to fluoroquinolones, caused by Qnr (Quinolone resistance) determinants, which are a family of DNA-binding proteins and are frequently responsible for low levels of quinolone resistance [19].The dissemination of antibiotic-resistant genes among pathogens is associated with horizontal gene transfer (HGT), exchange of plasmids bearing antibiotic resistance determinants and mutations. Integrons are another major mobile genetic elements responsible for transfer and insertion of resistance genes in Gram-negative pathogens [20].

2.1.1.2 Modification of Drug binding sites

Resistant pathogens avoid recognition by antimicrobial agents by modifying their target sites. The mutation of gene encoding for penicillin-binding proteins (PBPs), which are enzymes typically anchored on the cytoplasmic membrane, resulted in low affinity for β-lactam antibiotics and thus the pathogens escapes the antimicrobial action [21].

2.1.2. Membrane-mediated mechanisms of antibiotic resistance

Rapid emergence of resistant bacteria involves different mechanisms as illustrated above. Certainly, the uptake and removal of antibiotics determines the susceptibility of a pathogen to a particular drug. This membrane-mediated mechanism of antibiotic resistance is achieved through: decreased membrane permeability by porins and active efflux of antimicrobials by efflux pumps [22]. By regulating the import/export of antibiotics, these transport proteins, hence thus forms the primary mechanical barrier, to antimicrobial therapy against gram-negative pathogens [23].

Gram-negative bacterial outer membrane (OM) consists of two electron dense leaflets: OM is highly asymmetric in nature and is composed of lipopolysaccharides whereas inner membrane is composed of mainly phospholipids [24]. The OM often contains

proteins embedded in it that form a channel for in/out flow of nutrients, enzymes, water molecules, simple sugar molecules etc, as elucidated by outer membrane proteins (OMPs). Slightly different from this are the proteins embedded in the cytoplasmic membrane, which are either single component or multicomponent efflux pump, as depicted in figure no. 2.1.2.

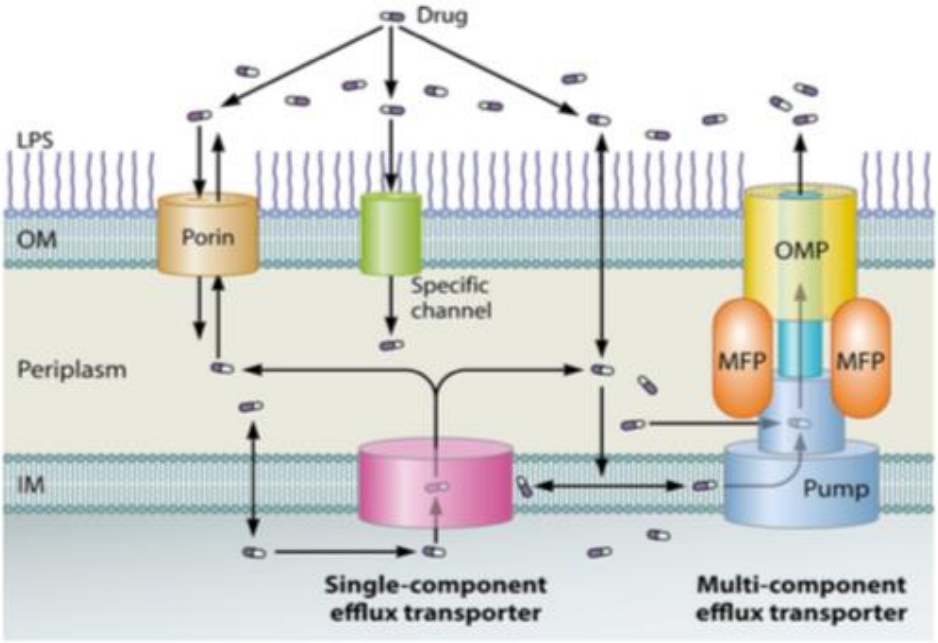


Figure no. 2.1.2: Distribution of porins and drug efflux pumps in Gram-negative bacteria. Influx of drugs (shown as pills) occurs through porin/specific protein channels present in outer membrane. Extrusion of drugs occurs via single-component or multicomponent tripartite efflux pumps. (Ref: Li et al., 2015)

The single component efflux pump extrudes antibiotics form the cytoplasm to periplasm, which then gets degraded by various hydrolyzing enzymes present in the periplasm, or get effluxed out by other tripartite efflux system. On the contrary, the multicomponent efflux pumps constituted of outer membrane proteins, the cytoplasmic transporter protein joined together by periplasmic adaptor proteins; to form a functional tripartite complex, which is capable of extruding molecules from the cytoplasm as well as the periplasm [10].

2.2. Bacterial Multidrug efflux pumps

Certainly, efflux pumps today constitute the most prominent resistance determinant, which is ubiquitous in bacteria to mammals, signifying their importance in both clinical and environmental settings. While most of the pathogens harbor chromosomally encoded efflux pumps, few others also acquired acquire the genes though plasmids (e.g. *tet* or *qac* genes) or via horizontal gene transfer [25]. The chromosomally encoded efflux pumps display wide substrate specificity, hence affects virtually all classes of antibiotics and subsequently results in multi-drug resistance phenotypes [26]. Efflux pumps can contribute to antibiotic resistance at three different levels: a) Constitutively basal level expression resulting in susceptibility of a pathogen towards an antibiotic (*Intrinsic*), b) Increased level of expression of chromosomally encoded efflux pumps by mutation/HGT (*Acquired*) and c) Transient, non-inheritable increased expression of an efflux pump in the presence of an effector (*Phenotypic*) [27]. In either of the case, the intracellular concentration of the antibiotic is lowered making ineffective, and the organism escapes the antimicrobial effect and become susceptible resistant to that particular agent.

This review highlights the discovery, types and distribution of efflux pumps (in gram-negative bacteria), efflux pump regulation and expression, advances in understanding the structure of efflux pumps, mechanism of drug extrusion and efflux pump inhibition in detail.

2.2.1. Discovery of bacterial efflux pumps

The resistance to antibiotics arises to 1940s, where mostly the resistance was believed to be acquired by horizontal gene transfer and/or via plasmids or inherent in nature (chromosomally induced). Antibiotic efflux leading to bacterial resistance was first reported in *E. coli*, expelling tetracycline by plasmid-encoded proteins and conferring

resistance to the same [28]. Though this resistance mechanism was novel, but it was believed to occur via acquisition of resistance genes, contributing resistance to structurally similar group of antibiotics. Later on, discovery of chromosomally encoded efflux pump conferring resistance to multiple structurally diverse drugs challenged this paradigm [29]. However, in 1998, the first efflux mechanism involved in extrusion of multiple antibiotics in *Enterobacter* spp. was elucidated; and AcrAB-TolC type RND efflux pumps were found to be predominant in most of the multidrug resistant isolates of *Enterobacter aerogenes* [30].

2.2.2. Classification of bacterial efflux pump families & their distribution

Bacterial efflux pumps responsible for multidrug resistance, (MDR) efflux transporters can be categorized braodly into three evolutionary distinct and diverse efflux super families based on their bioenergetics, structure and transport mechanisms: 1) ATP-binding cassette-ABC type (driven by ATP-hydrolysis), 2) Major Facilitator Superfamily-MFS type (Drug/Proton or Cation antiporters) and 3) Resistance Nodulation division-RND type, (Drug/Proton antiporters) are tripartite efflux system [31].

Moreover, few members of MDR transporters form smaller families of a superfamily: for instance: the small multidrug resistance (SMR) family (belonging to drug/metabolite transporter [DMT] superfamily) and the multidrug and toxic compound extrusion (MATE) family (constituting the multidrug/oligosaccharidyl-lipid/polysaccharide [MOP] superfamily) [32]. Recently, there was a new addition to the family of efflux transporters, namely 'Proteobacterial antimicrobial compound efflux' (PACE) transporters, which was reportedly responsible for extrusion of chlorhexidine in *Acinetobacter* spp. [33]. Hence, in bacteria there are six distinct super

families of efflux transporters, namely SMR, PACE, MFS, MATE, RND and ABC efflux pumps, are discussed in detail below.

Broadly, MDR efflux transporters can be categorized into two major groups on the basis of their energy requirements: *Primary active transporters*, for eg. ABC efflux pumps, hydrolyze ATP to translocate which couple substrate. translocation with ATP hydrolysis and *Secondary active transporters* including multidrug transporters of all other superfamilies, transport structurally different substrates by which utilizinge electro-chemical gradients of ions (protons/sodium) to transport structurally diverse substrates [34]. Both primary and secondary transporters are ubiquitous in bacteria; where single component efflux transporters are prevalent in both gram-positive and gram-negative bacteria. But, multicomponent trans envelope efflux transporters are exclusively present in gram-negative bacteria owing to their membrane architecture as shown in the figure below.

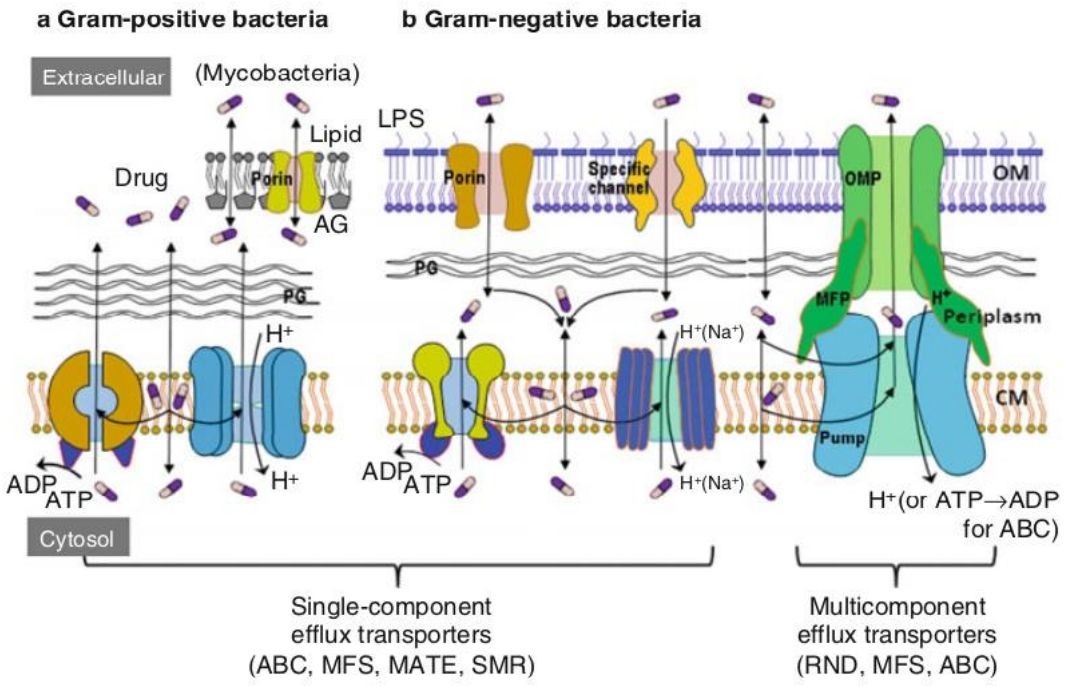


Figure no. 2.2.1: Diversity of bacterial MDR efflux pumps. Both single component and multicomponent efflux transporters prevalent in gram-negative and gram-positive bacteria are shown here. (Ref- Li et al., 2015).

It is noteworthy that a single organism may have multiple efflux pumps and even variant of a single efflux pump. Also, a given efflux pump may extrude different antibiotics belonging to same/different classes of antibiotics [35].

2.2.2.1: SMR and PACE efflux pumps

The SMR secondary multidrug transporters are the smallest known efflux pumps, belonging to the DMT superfamily. They are 100-120 aminoacids length and contain four trans membrane (TM) helices [36]. Present in both gram-positive and gram-negative bacteria, these efflux pumps utilize proton motive force (PMF) to extrude multiple antibiotics and noxious compounds. Well-studied model is EmrE in *Escherichia coli* (consisting of 110 residues), functioning as an antiparallel homodimer, extrudes erythromycin, tetracycline, ethidium and proflavine out of the cell [37]. Other representatives of SMR families are QacC and SepA in *Staphylococcus aureus* [38] and KpnEF in *Klebsiella pneumoniae* [39]. The newly described PACE efflux transporters exemplified by AceI (*Acinetobacter* chlorhexidine efflux) in *A. baumannii*, is similar to SMR family, as they are 150 amino acids long with two tandem TM domains [33].

2.2.2.2: MFS efflux pumps

Among the all efflux pump families discussed above, members of the MFS super families, belong to the largest superfamily of secondary transporters, widely distributed in bacteria (both gram-positive and gram-negative), archaea and eukaryotes. These transporters utilize proton motive force (PMF) to operate in symport, uniport and antiport of various substrates such as ions, sugars, oligosaccharides and antibiotics such as fluoroquinolones and tetracycline [38]. For instances, NorA (chromosomally encoded), QacA and QacB (Plasmid acquired) in *S.*

aureus and PmrA in *Streptococcus pneumoniae,* are the extensively studied MFS efflux pumps in gram-positive bacteria [26]. However in gram-negative bacteria, these pumps are prevalent as tripartite structure with an adaptor and outer membrane protein, for ex. EmrAB-TolC (in *E. coli*), that efflux cotrimoxazole and other hydrophobic uncouplers and FarAB-MtrE (in *Neisseria gonorrhoeae*), that efflux fatty acids. Nevertheless, there are other well-studied MFS transporters, which are single component in nature, for e.g., MdtM, QepA and MdfA (in *E. coli*); which extrude bile salts, fluoroquinolones and chloramphenicol, tetracyclines, trimethoprim and fluoroquinolones respectively [27]. MFS pumps consist of 12-14 transmembrane domains and are of 400-600 amino acids in length [40].

2.2.2.3: ABC efflux pumps

The ATP-binding cassette (ABC) transporters are the only efflux family that is primary active transporter, which utilizes ATP hydrolysis to extrude wide range of substrates. Well distributed in both gram-positive and gram-negative bacteria, fungi, and eukaryotes; these efflux proteins are multi-domain in nature, with two nucleotide-binding domains (NBDs) and two transmembrane (TM) domains. The NBDs are the cytoplasmic ATPase subunit that binds and hydrolyzes ATP, whereas the TM domains (usually six α-helices) are associated with substrate recognition and extrusion [38]. The most extensively studied ABC transporters are MacAB-TolC (in *E. coli*) responsible for macrolides efflux [27], LmrA in *L. lactis* [40] that is a homologous of the human MDR transporter p-glycoprotein.

2.2.2.4: MATE efflux pumps

Efflux transporters belonging to MATE families have been described for both gram-positive bacteria and gram-negative bacteria, such as *Vibrio parahaemolyticus* (NorM)

Table no. 2.2.1: Overview of super families of multidrug efflux pumps in bacteria

	The ATP binding cassette (ABC) superfamily	The major facilitator superfamily (MFS)	The small multidrug resistance (SMR) family	The Resistance/nodulation/division (RND) super family
Energy source	Drug/ATP antiporter driven by ATP hydrolysis	Proton/monovalent ions based drug symporters/antiporters/uniporters	Drug/Proton antiporter uses (PMF)	Drug/proton antiporter uses Proton motive force (PMF)
Components composition	Mulicomponent, multidomain system; Total size C1000 residues, 6 transmembrane spanners, approximately 500 sequenced members	Size C400-600 residues; 12 or 14 transmembrane spanners, approximately 500 sequenced members	Homodimers, Subunit size: C100 residues with 4 transmembrane helices, 10 sequenced members	Multicomponent, Subunit size: C1000 residues, 12 transmembrane spanners, 16 sequenced members
Distribution	Bacteria, Archaea, and Eukaryotes	Bacteria, Archaea, and Eukaryotes	Only in prokaryotes	Prokaryotes (mainly gram-negative bacteria)
Substrates exported	Antibiotics, sugars, amino acids, ions, vitamins, iron complexes, peptides, proteins, complex carbohydrates	Antibiotics, anions, sugars, metabolites and other substrates	Drugs and other substrates	Multiple antibiotics, toxic dyes, detergents, bile salts etc.
Sub Family	Twenty eight families out of which only 3-4 of them are drug efflux pumps	Seventeen families where, MDR efflux pumps are found in 3 of the 17 recognized families.	Two subfamilies; one for drugs and other for other substrates	Seven families including three specific for drugs, metal ions and lipopolysaccharides respectively
Examples	LmrA, and MacB	NorA, PmrA, MdtM	Qac and EmrE	AcrB and MexB

(Adapted from Saier et al., 1998 [41] with few modifications).

[42], *Haemophilus influenzae* (HmrM), *Clostridium difficile* (CdeA), *Pseudomonas aeruginosa* (PmpM) [10], *Enterobacter cloacae* (EmmDr) [43] and *S. aureus* (MepA).

MATE secondary efflux transporters consist of 12 alpha-helical TM regions and range from 400-700 amino acids in length. They are capable of extruding structurally diverse substrates *viz.* antibiotics - such as fluoroquinolones, chloramphenicol, ampicillin and other compounds such as ethidium, triethylammonium, metformin, cimetidine etc [38]. MATE efflux pumps can utilize two energy sources: the proton motive force (PMF) and the sodium ion gradient. Though MATE transporters extrudes similar substrates as of RND; but the distinguished difference amongst the two is, MATE transporters are single component, unlike tripartite RND efflux systems [26].

2.2.2.5: RND efflux pumps

Efflux pumps belonging to Resistance-Nodulation-cell division (RND) superfamily, function as drug/proton antiporter and unlike other efflux transporters are believed to be exclusively present in gram-negative pathogens,. However, recent studies have revealed RND type monomers in gram-positive bacteria as well, such as in *S. aureus*, *B. subtilis* and *Clostridium difficile*, to name a few [10]. RND superfamily of transporters consisted of seven families including the hydrophobic/amphiphilic efflux (HAE) family the SecDF protein-secretion accessory protein (SecDF) family the heavy metal efflux (HME) family [32] Transporters of the HAE subfamily in *E. coli* included five efflux transporters, AcrAB, AcrAD, AcrEF, MdtAB and MdtEF [44]. AcrAB-TolC efflux pumps in *E. coli* and MexAB-OprM from *P. aeruginosa* are the two extensively studied RND efflux systems. Overexpression of RND efflux pumps has been associated with MDR phenotype in many clinically significant opportunistic pathogens [26]. AcrAB-TolC efflux pumps are also known to have functional

significance in both clinical and non-clinical set up, conferring intrinsic and/or acquired resistance to multiple antibiotics, bile salts, toxins, detergents, dyes, free fatty acids, solvents etc.[45].

2.2.3. AcrAB-TolC: Frontier of RND efflux pumps

As mentioned previously, AcrAB-TolC efflux pumps belong to the HAE subfamily of the RND superfamily. This efflux pump is organized as a tripartite complex consisting of three major components: a trans membrane RND transporter (AcrB) in the inner membrane involved in substrate recognition and transfer, an outer membrane protein (TolC) providing an exit-channel for substrate out of cell, and a periplasmic membrane fusion adaptor protein (AcrA) that acts as a connecting link between both AcrB and TolC and three of these proteins together form an effective efflux unit [24], [46].

This type of efflux pumps is highly efficient in extrusion of substrates both from cytoplasmic and periplasmic space. AcrAB-TolC efflux pumps utilize proton motive force (PMF) gradient as energy source and are drug/proton antiporters. The proton dislocation in these proteins helps in substrate transport across the membranes. This PMF is generated by hydrolysis of ATP, catalyzed by membrane bound ATPases and by oxidative metabolism, and hence forms the driving force for the activity of RND efflux pump [47]. AcrAB-TolC efflux pumps are primarily associated with multiple antibiotic resistance in many clinically significant gram-negative pathogens including *Enterobacter* spp., *E. coli*, *P. aeruginosa*, *Salmonella* spp. and *Klebsiella* spp. [48], [49], [50].

2.2.3.1.Structure and assembly of AcrAB-TolC efflux complex

Knowledge regarding structure of these RND transporters is of paramount importance to understand their function. This has been hindered due to the difficulty associated

with the purification and crystallization of bacterial membrane transporters. However, the first transporter whose structure was described was mammalian p-glycoprotein, an ABC type pump. The first crystal structure of a RND transporter was described by Murakami and groups [51], for the AcrB pump of the *E. coli* AcrAB-TolC efflux system at 3.5 Å resolutions. The same research group had later revealed crystal structures of AcrB bound to substrates like doxorubicin and minocycline, to demonstrate different substrates may bind to different residues, indicative of wide substrate specificity of AcrB transporters [52]. AcrAB-TolC of *E. coli* representing prototype member of RND transporters, are one of those few efflux pumps, for which complete structure is available [53]. It was strongly proposed that this tripartite complex displays a 3:6:3 (AcrB:AcrA:TolC) stoichiometry [53], [54], though there were other type of complexes reported [55], and the actual arrangement of the complex is still a matter of debate.

Structural analysis revealed that AcrB is a homo-trimeric complex with monomer of 1049 amino acids; where each of its protomer exhibits a pseudo-symmetrical 2×6 transmembrane symmetry, derived possibly form a gene duplication event [56]. Each AcrB protomer contained three major parts comprising of ~10 Å cytoplasmic domain, ~40 Å transmembrane domain and ~70 Å periplasmic domain (figure no. 2.2.2).

The periplasmic domain, which is protruding the periplasmic space, is again divided into porter domain and TolC docking domain. The central pore in the base of the periplasmic domain may represent a potential substrate-binding pocket and allow export of drugs from periplasm, which became evident later from other data obtained for RND efflux pumps [57], [58]. Outer membrane protein, TolC belonging to type-I secretion systems, is the major outer membrane factor (OMF) family of proteins in E.

coli. TolC exhibits central and promiscuous role in efflux of structurally diverse substrates, toxic products, colicins (got its name as 'tolerance to colicin') [10]. The crystal structure of TolC revealed that TolC has a 140-Å long homotrimer canon like assembly as represented in figure no. 2.2.2c, with each protomer has a 40 Å long β-barrel domain anchored to the outer membrane and a 100 Å long α-helical domain consisting of 12 coiled coils extended into the periplasmic space [59]; [49].

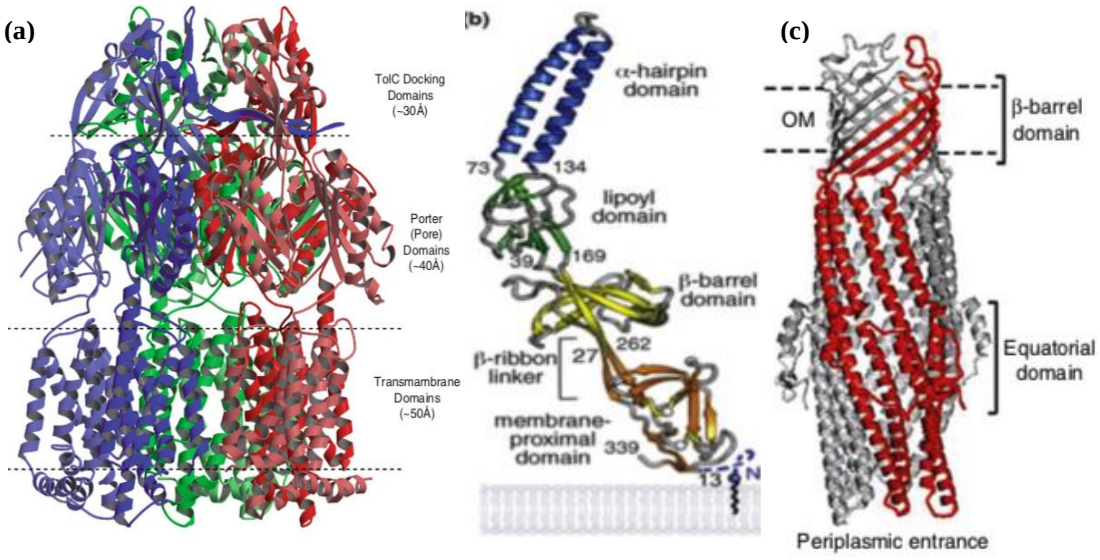


Figure no. 2.2.2: Crystal structure of AcrB (a), AcrA(b) and TolC(c) efflux system (Ref- Du et al., 2014)

Unlike other outer membrane protein channels, the β-barrels in TolC were unique as they contributed four β-strands to build a 12-stranded barrel [32]. The complete assembled structure has an outer membrane pore along with a periplasmic tunnel projected into the periplasm for substrate translocation.

Membrane fusion proteins (MFPs) or periplasmic adaptor proteins (PAPs) are highly elongated proteins, anchored from the inner membrane through palmitoylization of an N-terminal cysteine and extended towards the periplasmic space as shown in figure no. 2.2.2b. Partial crystal structures of MFPs from diverse species revealed three

characteristic domains: β-barrel, central lipoyl domain and α-helical coiled hairpin domain, which are linearly arranged [58]. Some of the MFPs also contained fourth domain that is a membrane-proximal domain, while others have a longer or shorter α-helical hairpin domain [24]. Nevertheless, the common feature of such MFPs is their conformational flexibility, which was accounted to the hinges between these domains, which are flexible unstructured regions [47].

2.2.3.2.Mechanism of drug extrusion by AcrAB-TolC efflux pump

Structural information along with genetic and biochemical data on individual tripartite AcrAB-TolC pump components has developed our understanding regarding the mechanism of the drug expulsion by the complete functional assembly. Considered as the housekeeping efflux system in *E. coli*, AcrAB-TolC efflux had gathered much attention owing to its high efficiency of drug extrusion along with broad substrate specificities [57]. The range of substrates included cationic dyes such as-acriflavine, ethidium bromide, crystal violet and rhodamine 6G; antibiotics belonging to different classes such as-penicillins, cephalosporins, macrolides, chloramphenicol, fluoroquinolones tetracyclines, fusidic acid, novobiocin, trimethoprim, rifampicin; detergents such as sodium dodecylsulphate (SDS), bile acids and Triton X-100 along with other solvents, toxins etc. It is evident from all those structurally dissimilar compounds that, they contained, to some extent, lipophilic domains, that might be the reason for them being a probable substrate for AcrB transporter [60].

Most of the investigations done on trans-envelope drug efflux systems like RND transporters, emphasized on the importance of membrane fusion protein (MFP) such as AcrA, in functional communication between the transporter and outer membrane protein channel [61]. Reconstitution studies have shown AcrA to undergo

conformational switch along with the transporter AcrB to increase the rates of substrate transport and energy consumption [62]. Again, the interaction between AcrA and AcrB was found to be more stable when AcrB is in its protonated stage, suggesting that these interactions are related to the "binding" state of the transporter itself. Moreover, interaction of AcrA with TolC seemed dynamic, which lead to the opening of the TolC channel [63].

AcrB is an asymetric homotrimer of 1049 amino acids, embeded circularly into the membrane. Thus three of such bundles (each consisting of 12 α-helices) created a central spatial hole of approximately 40 Å in diameter. Each of this protomer displays distinct conformational state designated as: "access" ("loose/**L**"), "binding" ("tight/**T**"), and "extrusion" ("open/**O**") conformations, constituting the three major functional consecutive states, where conformational cycling lead to substrate translocation [64], [56] as depicted below in figure no. 2.2.3.

The LLL confirmation of the AcrB trimer indicated "resting stage", where the protein is depleted from substrate (figure no. 2.2.3). Binding of substrate to the L (access) monomer triggered the conversion from L (access) to T (binding) figure no. 2.2.3. Once substrate is recognized and bound to the binding pocket in the T monomer, conformational transitions from the T (binding) to the O (extrusion) occurred. The T to O transition is energy dependent managed by the binding of protons in the transmembrane domain and this subsequently caused a drastic conformational change in the periplasmic domain of AcrB [58], [57]. This remote conformational change at the periplasmic domain, which facilitates substrate translocation at the periplasmic region. Eventually, the bound substrates get squeezed out from the central funnel of AcrB and expelled from TolC under the action of a peristaltic pump [64], [56].

However, it was noteworthy that binding of second substrate to adjacent monomer (L) will enable the first substrate transfer to the periplasm and so on.

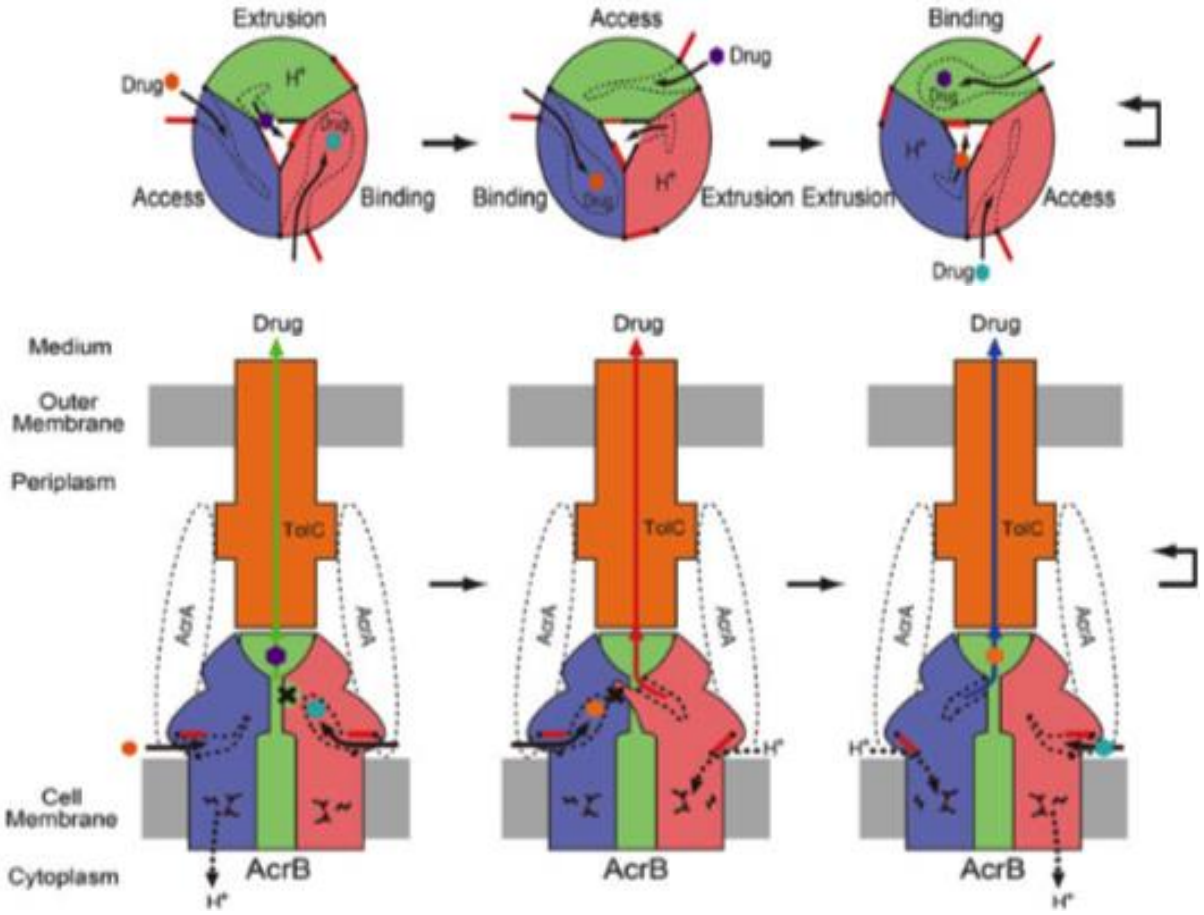


Figure no. 2.2.3: Mechanism of drug efflux by AcrB type RND transporter. Structure of AcrB transporter and the functional rotation mechanism of drug efflux by the RND efflux system AcrAB-TolC. The three protomers are colored as blue, green and red respectively. The route of drug access, binding and extrusion is shown.
(Ref- Sun et al., 2014).

However, recent studies have indicated that, AcrB activity may to be modulated by another fourth component, AcrZ, a small protein, which is a 49 amino acids monotropic membrane protein and entirely α-helical in nature [53]. This small accessory protein is thought to regulate extrusion of some substrates and is only present in some gram-negative bacteria, which indicates the possible presence of other similar accessory proteins responsible for efflux activity of other RND transporters.

The helical hairpins of AcrA connect towards the ends of the helical portion of TolC leading to open state of the assembly. While interacting with AcrB, the membrane-proximal domain and β-barrel domain of AcrA plays significant role (figure no. 2.2.1b). Site-specific cross-linking based reports suggested the AcrA-TolC interaction is most favored, when TolC remain in its open state [49]. It was proposed that once AcrA is bound to AcrB, TolC is recruited next and remain constitutively in open state, allowing transport of substrates directly from transporter AcrB to outer membrane channel TolC without leaking into periplasm; and AcrA, being the connecting link, stabilized this interaction [58]. However, the parameters affecting opening of TolC channel still remain elusive till date.

Overall, the broad specificity of RND transporters could be explained by the presence of large, hydrophobic, flexible central cavity; capable of accommodating different structures within the periplasm [64]. The ligands interacted with multiple hydrophobic residues via hydrogen-bonding and charged residues for electrostatic interactions with anionic or cationic substrates. Proton binding and release takes place in the transmembrane domain of the RND transporters [65]. Irrespective of such vast structural and functional studies on all components of AcrAB-TolC efflux pump, there are still many basic facts not fully understood; regarding drug/proton coupling, assembly mechanism, energy transduction, stoichiometry and transport kinetics.

2.2.3.3. Genetic organization of AcrAB-TolC efflux systems

The Acriflavine (Acr) RND efflux system is one of the best-studied efflux pump and presents wide range of substrate specificity. AcrAB (both the RND transporter and MFP component) are encoded in a single operon under the regulatory control of

transcriptional repressor AcrR [44]. But the outer membrane component of this tripartite system, TolC is coded elsewhere on the chromosome flanked by nudF and ygiA (as shown in figure no. 2.2.4).

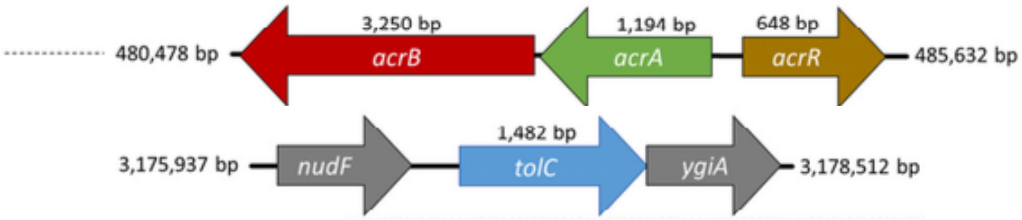


Figure no. 2.2.4: Schematic representation of position and size of efflux genes Architecture of AcrAB-TolC efflux genes: RND transporter *acrB* is in red, MFP *acrA* is in green and OMP *tolC* is shown in blue, whereas regulator gene *acrR* is shown in yellow (Ref-Anes et al., 2015).

TolC was previously shown to involve with majority of MFP-dependent transporters encoded in the genome of *E. coli* (apart from AcrAB RND transporters); for instance MFS, ABC and other RND transporters. This flexible multi-functionality of TolC is reflected by the fact that the *tolC* gene is transcribed independently from their inner membrane components. Expression of TolC, being a part of *marA*/*soxS*/*rob* regulon too, remains unaffected by the expression of AcrAB. Again genetic makeup of TolC is conserved exclusively in the closely related species (i.e., all Enterobacteriaceae) [66]..

AcrD, another acriflavine RND transporter, consists of 1037 amino acids and share 66.1% homology with AcrB. But, unlike AcrB, AcrD doesn't come in a single operon with AcrA, but is under the regulatory control of BaeR, SdiA and CpxRA [44]. AcrAD-TolC has preference for hydrophilic substrates such as aminoglycosides and negatively charged β-lactams [67]. Chimeric study involving replacement of the two large external loops of AcrD with the equivalent loops of AcrB, was shown to broaden its substrate range; indicating the importance of those large periplasmic loops in the substrate selection for such RND transporters [60].[44].

AcrEF, another acriflavine efflux system share close homology with AcrAB; AcrF being of 1034 amino acids long share 77.6% similarity with AcrB and AcrE, the AcrF of 385 amino acids share 69.3% similarity with AcrA. Hence AcrEF was proposed to have similar substrates profile as of AcrAB; known substrates for such system were quinolones, tigecycline, solvents [44]. AcrEF was shown to be less expressed under laboratory conditions; however the exact physiological conditions affecting their expression is still unclear.

2.2.4. Regulators of expression of AcrAB-TolC efflux pumps

Expression of AcrAB-TolC efflux system is regulated at multiple levels, including transcriptional (local and global) and post-transcriptional regulations [68]. In *E. coli*, MarA is known to regulate AcrAB-TolC expression, whereas in *Salmonella enterica*, RamA controlled these genes [69]. Despite such divergence, different bacteria in *Enterobacteriaceae*, still share certain common molecules in regulating the expression of AcrAB-TolC. Broadly, the regulators of AcrAB-TolC efflux pumps can be categorized into different levels:

(i) Global transcriptional activators mediated positive regulation,

(ii) Local repressors of efflux pump components leading to negative regulation

(iii) Response to chemical or pharmaceutical or other environmental factors

2.2.4.1.Positive regulation by global transcriptional activators

Many global response regulators regulate expression of AcrAB-TolC efflux pump, in the presence/absence of a local repressor. Such are XylS/AraC family of regulators (*mar/sox/rob* regulon) that show high homology to each other; possess 100 amino acids long ‘α-helix-turn-α-helix’ (HTH) DNA-binding motifs, called as ‘marbox’ [70].

Global response regulators bind to these marbox sequences on the *acrAB* promoter and activate their expression in response to various stimuli [68].

A) Mar regulation

The multiple antibiotic resistance (*mar*) locus encode the key regulator MarA, in *E. coli*, that was shown to simultaneously regulate porin and efflux pump expression. The *marRAB* operon encoding *marR*, *marA* and *marB* genes responsible for antibiotic resistance is genetically conserved among the *Enterobacteriaceae*, except for the transmembrane protein *marC* [68]. Expression of *marRAB* operon is constitutively repressed under normal conditions by specific repressor MarR, by binding to two palindromic sequences between its operator DNA sequence (*marO*) and promoter. De-repression of MarR is caused by the presence of certain ligands (e.g. phenolic compounds like salicyliate), antibiotics, oxidative stress, or as a consequence of mutations in the binding sites of *marR* or modification of MarR at the protein level [10]. MarA, being the key regulator, was shown to activate the *acr* operon by binding to the intergenic region between *acrR* and *acrA*, which triggered synchronous limited influx and enhanced efflux of antimicrobials [71]. *marB*, located downstream of *marA*, is believed to increase the levels of *marA*, but the mechanisms involved is unknown.

B) SoxRS regulation

Superoxide-generating agents including Nitric oxide, Hydrogen peroxide and Methyl viologen activated the oxidative stress regulon, *soxRS*, represented by its effector protein SoxS. SoxS consists of 107 amino acids, and shared ~50% homology with MarA [32]. In the absence stress signals, the repressor SoxR binds to the *soxS* promoter and represses the transcription of SoxS [68].. Under oxidative stress conditions, SoxR becomes oxidized from reduced state and triggered the transcription

of *soxS*, (as shown in figure no.2.2.5) thereby activating *acrAB*. Studies suggested SoxS to upregulate *acrAB* expression and thereby triggered MDR phenotype in *E. cloacae* isolates [72].

C) Rob regulation

Rob is another prominent member of the AraC family, of 289 amino acid protein. N-terminal domain of Rob shares 71% similarity with MarA, whereas its C-terminal domain differs from MarA and SoxS, as it is involved in ligand binding. Rob is constitutively expressed as inactive form, and gets activated after binding of effector molecules, for eg. decanoate or bile salts, that cause conformational changes [29]. Once activated, Rob induces *acrAB* expression in resistance to antibiotics, organic solvents [73].

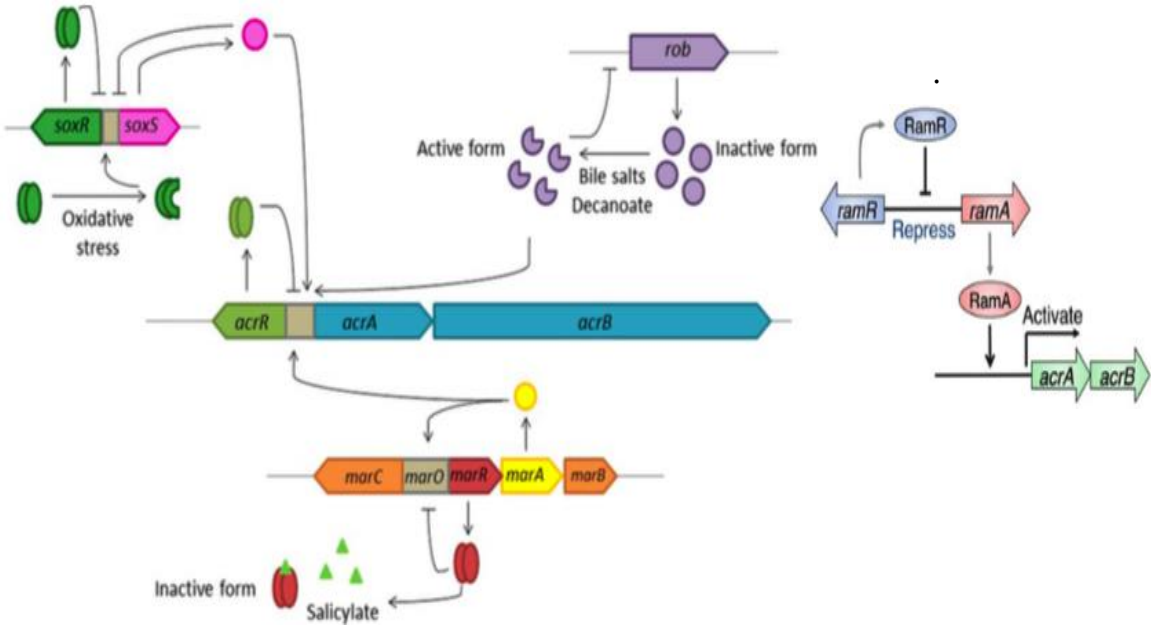


Figure no. 2.2.5: Regulatory network of RND efflux gene (*acrAB*) expression. Schematic representation of regulation of *acrAB* multidrug efflux pumps expression in *E. coli*. MarA encoded by marRAB operon, gets induced in the presence of salicylate, and activated acrAB expression. Similarly, oxidative stress induced SoxSR system and bile salt induced Rob, upon activation induces *acrAB* expression. Induction of RamA occurs though bacterial metabolites such as indole, which activates *acrAB* expression, and is the leading regulator conferring multiple antibiotic resistances in many pathogens. (Ref- Blanco et al., 2016).

D) Ram regulation

RamA homologue of MarA, belongs to AraC/XylS transcription activator family and, regulate expression of AcrAB-TolC efflux pumps in *Salmonella* spp. [74], *Klebsiella pneumoniae*, *Enterobacter cloacae* [75] and *Enterobacter aerogenes* [76]. RamA has not been reported in *E. coli* or *Shigella* spp. Though MarA, SoxS and Rob play an important role in expression of *acrAB* in these organisms; but RamA acts as the primary regulator conferring multidrug resistance, by binding to the upstream sequence of *acrAB* and *tolC* loci known as 'rambox' [68]. Therefore, constitutive higher expression of RamA resulted in MDR bacteria by enhanced expression of AcrAB-TolC efflux pump, similarly its inactivation lead to reduced expression of *acrAB*. RamR, located upstream of *ramA*, acts as the local repressor of *ramA* transcription.

Expression of *ramA* is controlled at multiple levels, as several factors were shown to stimulate the expression of this transcription factor. In *E. aerogenes* RamA conferred multidrug resistance to structurally diverse antimicrobials including fluoroquinolones, chloramphenicol, trimethoprim, tetracycline, tigecycline, etc., as a consequence of reduced expression of OmpF and enhanced efflux by AcrAB-TolC [76]. It was reported that bile inhibited binding of RamR to the promoter region of *ramA*, resulting in de-repression of *ramA*, that induces *acrAB* expression [77]. Reports also displayed bacterial metabolite such as indole, to increase the promoter activity of *ramA* and enhance expression of *ramA*, [78]. Further, in *Salmonella*, exposure to several biocides and antibiotics including ciprofloxacin, chloramphenicol, cefamandole, rifampicin and cloxacillin was shown to increase *ramA* expression [79]. On the whole, reports suggested a probable mechanism where RamA can switch between "activated state" and "overexpressed state" in response to various environmental signals, thereby

regulating the expression of AcrAB-TolC system.

2.2.4.2. Regulation by local transcriptional repressors

Besides global regulation, the RND efflux pump components also encode local repressor, a physically linked regulatory gene that inhibits overexpression of that pump [26]. AcrR belonging to TetR family of transcriptional repressors is one such example of local regulation. Located upstream of the *acrAB* operon, *acrR* is known to repress transcription of self and *acrAB* [80]. However, its transcription is increased under presence of 0.5 M NaCl, 4% ethanol and the onset of stationary phase in Luria-Bertani medium [68]. Mutations in *acrR* have also been shown to derepress *acrB* in clinical isolates of *S. Typhimurium*, *E. coli* and *E. aerogenes* [71].

Other proteins involved in local regulation of *acrAB* and *tolC* expressions are AcrS/EnvR, the histone-like nucleoid structuring protein (H-NS). AcrS/EnvR, previously thought to repress *acrEF* efflux pump genes; also repress *acrAB* in *E. coli* in response to increased activity in *acrEF*, suggesting cross-regulation mechanism of RND efflux pumps [81]. In response to osmotic stress, H-NS proteins were observed to regulate expression of porins and efflux pumps in *E. coli* and *E. aerogenes*. Reports revealed H-NS system to regulate expression of the cation-selective outer membrane protein OmpX [32]. In *S. enterica* and *E. coli*, H-NS is believed to downregulate expression of *acrEF*, and not *acrAB,* in response to environmental signals such as osmolarity, pH and temperature [68].

The expression of efflux pumps can also be under the control of quorum-sensing signals. For instance, Suppressor of division inhibition (Sdi)A, a LuxR protein, is another positive regulator of *acrAB* that regulates cell division genes in a manner dependent upon quorum sensing [82]. However, these SdiA regulators seem to have

minor role in *E. coli* and *S. enterica,* in regulating expression of *acrAB* and *tolC,* as deletion of these genes resulted in minimal effect in efflux activity via AcrAB [68].

2.2.4.3. Regulation at post-transcriptional and post-translational level

The Lon protease, an ATP-dependent protease of the AAA (ATPases associated with a variety of cellular activities) superfamily, was shown to contribute in post-transcription and translation regulation of AcrAB-TolC [83]. In *E. coli,* Lon protease gets involved in post-translational regulation of MarA and SoxS by proteolytic degradation. It was also demonstrated that mutations in Lon triggered *acrAB* efflux resulting in MDR phenotypes, but greater extent of MDR was noticed when coupled with a *marR* mutation [84]. Performing in a similar fashion, in *Salmonella,* the Lon protease recognizes and binds to the N-terminal region of RamA, and degrade the protein. This in turn reset the levels of RamA to basal level when the protein is no longer in use or there is absence of a stimulus [68].. Another recently described global regulator of AcrAB is, carbon storage regulator A (CsrA), which is a RNA binding protein, and can bind directly to the 5' end of the *acrAB* transcript. This modified RNA secondary structure, and inhibited the development of a repressive RNA structure that hinders binding to ribosome, resulting in efficient translation of AcrAB proteins [85].

2.2.5. Response to chemical/pharmaceutical/other environmental factors-Effect on efflux pump expression

2.2.5.1. Bile-induced efflux system

Bile salts usually are detergent-like molecules and lipid solubilizer in nature disturbing bacterial membrane, and hence trigger expression of bile-induced efflux mechanisms. Organisms belonging to *Enterobacteriaceae,* inhabit gastrointestinal tract of the host and encounter different environmental factors, such as bile (deoxycholate) that induces

the expression of *acrAB-tolC* [32]. In *E. coli*, binding of bile to Rob produces conformational changes, which resulted in increased expression of *acrAB*. Similarly in *Salmonella* spp., bile-dependent RamA-mediated induction of *acrAB* was also discussed [73], [78].

2.2.5.2. Oxidative stress

Oxidative stress, in the form of reactive oxygen species (ROS) including superperoxide and hydrogen peroxide, contributes significantly towards stress response in bacteria [32]. Oxidative stress has been shown to activate expression of efflux genes followed by increased resistance in many organisms including *E. coli*, *Salmonella* spp., *Klebsiella* spp. and *Enterobacter* spp., facilitating the organism's survival [86]. In *E. coli* and *Salmonella* spp., SoxRS-dependent regulation of *acrAB* induction under oxidative stress was reported. Similarly SoxS-mediated and paraquat induced *acrAB* expression was reported in *Salmonella enterica serovar* Typhimurium [87] and *Enterobacter cloacae* [72], in the latter promoting a MDR phenotype. Presence of phenolic compounds such as salicylate lso triggered expression of *acrAB*, through *marRAB* promoter [29] that was implicated in plant defence against bacterial pathogens.

2.2.5.3. Chemical factors

Larger number of chemically heterogeneous compounds, for instance antibiotics (chloramphenicol, tigecycline and tetracycline), biocides (triclosan, disinfectants), Triton X-100, the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), cyclohexane, acetylsalicylate (aspirin), acetaminophen, sodium benzoate, menadione, dinitro-phenol and methyl viologen, were also observed as the transcriptional regulators of AcrAB-TolC efflux pump [32]. This ultimately resulted in MDR

phenotype, though the exact mechanism of induction of *acrAB,* by each of these compounds is not explored.

2.2.5.4. pH-mediated efflux

It is speculated that proton-driven transporters could utilize the protons for extrusion, and thus have a role to play in maintaining cellular homeostasis in the event of external pH stress [88]. pH is known to differentially regulate a wide array of membrane and periplasmic proteins in *E. coli* K12 cells; where low external pH (<pH 5.0) has shown to favor proton export and high external pH (≥ pH 8.0) shown to enhance protein import [89]. In another study in 2013, on *emh*ABC efflux pump belonging to the RND superfamily from *Pseudomonas fluorescens*, [90] have reported 6% decrease in chloramphenicol efflux at pH 5.8. In contrast, Zagurskaya and Nikaido and Martins *et.al,*, in *E. coli*, had reported enhanced efflux of fluorescent lipid and ethidium bromide respectively, at low pH *via* AcrAB-TolC efflux proteins of RND superfamily [91],[92]. In *E. coli*, MFS multidrug efflux system MdtM (homologue of MdfA) was found responsible for their alkali tolerance, as mutants of these pumps failed to grow at alkaline pH [93]. The above-mentioned reports indicate pH to affect activities of RND efflux pump proteins and different types of efflux proteins are affected differentially by pH. However whether pH affects expression of *acrAB-tolC,* remains still elusive [32].

2.2.5.5. Antibiotics induction

Antimicrobial induced resistance mechanisms involving efflux has been well reported in many gram-positive as well as gram-negative bacteria. In MDR gram-positive isolates of *Enterococcus faecium* and *Enterococcus faecalis*, aminoglycosides and chloramphenicol induced expression of *efrAB* efflux genes [94]. The *mex*XY

multidrug efflux system in *Pseudomonas aeruginosa*, gets induced by antibiotics such as macrolides, aminoglycosides and also by polymyxin B, colistin (polymyxin E), mediated by ParRS two-component regulatory system [95], [96].

In *Enterobacter aerogenes*, elevated RamA expression induced MDR phenotype in drug-susceptible *E. aerogenes* ATCC 13048 and increased expression of *acrAB*, as evident from 2- to 16-fold-increased resistance towards β-lactams, chloramphenicol, tetracycline and quinolones [76]. Reduced susceptibility to tigecycline in *Enterobacter cloacae* was correlated with RamA-dependent overexpression of the *AcrAB* efflux pump [75]. Chloramphenicol- and imipenem-selected resistant mutants in *E. aerogenes* showed enhanced *AcrAB* expression, which was related to resistance observed towards quinolones and tetracyclines [96].

2.2.5.6. Fatty-acids

Antibacterial fatty acids are known to positively regulate efflux genes expression in *E. coli* and *S. aureus*. NorB in *S. aureus*, excludes fattyacids, which are membrane-damaging molecules, and contributed towards bacterial survival [32]. However in *E. coli*, fattyacids induced *acrAB* expression promoting MDR phenotype [73].

2.2.5.7. Two-component system

The two-component systems (TCS), the signaling cascades involved in bacterial stress response, can act as both local and global response regulator in controlling the expression of drug efflux genes [97]. Widely distributed in prokaryotes, TCS generally consists of a histidine kinase (HK) sensor protein and a response regulator (RR) effector protein. Where, the sensing HK transduce dicerse external signals via phosphorylation of the RR, and this transcriptional regulator regulates the expression

of downstream genes. Several RND efflux pumps, get induced by myriad of envelope stress-responsive TCSs and sigma factors. For instance, in *E. coli*, BaeSR system developed resistance to novobiocin and β-lactams, by activating the expression of *mdtABC* and *acrD* MDR efflux systems [98]. Similarly, the TCS BaeSR system in *Samonella*, in response to copper or zinc elevated the expression of *mdtABC* and *acrD* [99]. The CpxRA TCS system of *E. coli* and other gram-negative enteric bacteria activated the expression of *mdtABC* and *acrD*, in response to envelope stress, and conferred resistance to several β-lactams, aminoglycosides and novobiocin [32]. However, in *Salmonella*, PhoPQ TCS in response to low magnesium level and low pH conditions, becomes the key regulator involved in expression of MacAB, RND type efflux pump and virulence [57]. The AmgRS TCS of *P. aeruginosa*, gets activated by aminoglycoside exposure and triggered the expression of *mexXY* multidrug efflux operon, ultimately plays an integral role in intrinsic aminoglycoside resistance in this organism [100].

Put together, it is evident that the regulation cascade of antibiotic resistance in gram-negative organisms like *Enterobacter* spp. is quite complex and redundant (as presented in figure no. 2.2.6), resulting in MDR by simultaneous limited influx (via decreased OmpF porin) and elevated efflux (via AcrAB-TolC) activity [8]. Both activators (MarA, SoxS, RamA) and repressors (MarR, RamR, and AcrR) could be conjointly associated at global and local level; in response to various inducers, chemicals and metabolites, which in turn modulate efflux pump expression. Such an integrated model, that involved various mechanisms (e.g., influx and efflux) and their respective regulation pathways (global as well as local regulators) has been proposed (as presented in figure no. 2.2.6), for *Enterobacter* spp. in particular [32], which ultimately decides the intracellular accumulation of the antimicrobial agents.

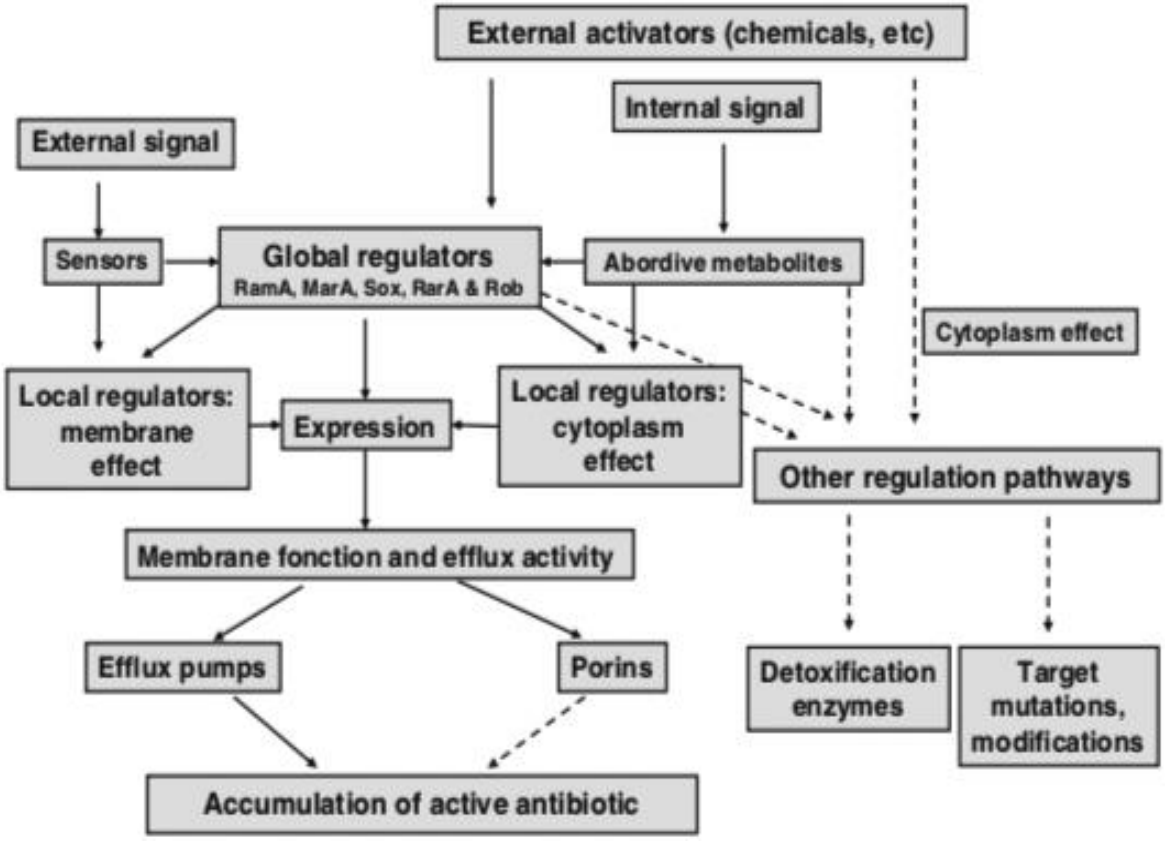


Figure no. 2.2.6: Regulation cascade of efflux pump expression in *Enterobacter* spp. The genetic control of efflux pump expression includes various sensors, such as two-component system (TCS), global regulators (RamA, MarA, Sox, RobA) and local regulators (AcrR for efflux pump and OmpX for porins). Global regulators can also be triggered by external stimuli and metabolites, which ultimately affects expression of porins and/or efflux pumps, and thereby regulating membrane permeability (Reference-Li et al., 2016).

2.2.6. Inhibitors of efflux pumps

Rapid emergence of multidrug resistant pathogens was significantly related to overexpression of multidrug efflux pumps in clinical isolates limiting the antimicrobial action. Several studies on the structure/function aspect of multidrug efflux pumps presented essential cues for the discovery of efflux pump inhibitors (EPIs) [101]). In principle, EPIs on RND efflux pumps, can act in different ways [102]): (also represented in the figure. no. 2.2.7)

i) Suppression of efflux pump's expression to be controlled by targeting activators and repressors and other regulatory network

ii) Alteration of antibiotic structure so that they fail to get recognized and extruded by the efflux pump.

iii) Prvention of the assembly of a functional tripartite complex by inhibiting the individual protein component's interactions

iv) Disruption of the interaction between RND transporter (e.g. AcrB) and their small accessory protein (e.g. AcrZ).

v) Blocking of MFP by competitive substrate binding or trapping the MFP in an inactive conformation.

vi) Blocking of OMP channel/exit duct.

vii) Depletion of proton motive force (PMF) required for substrate transfer

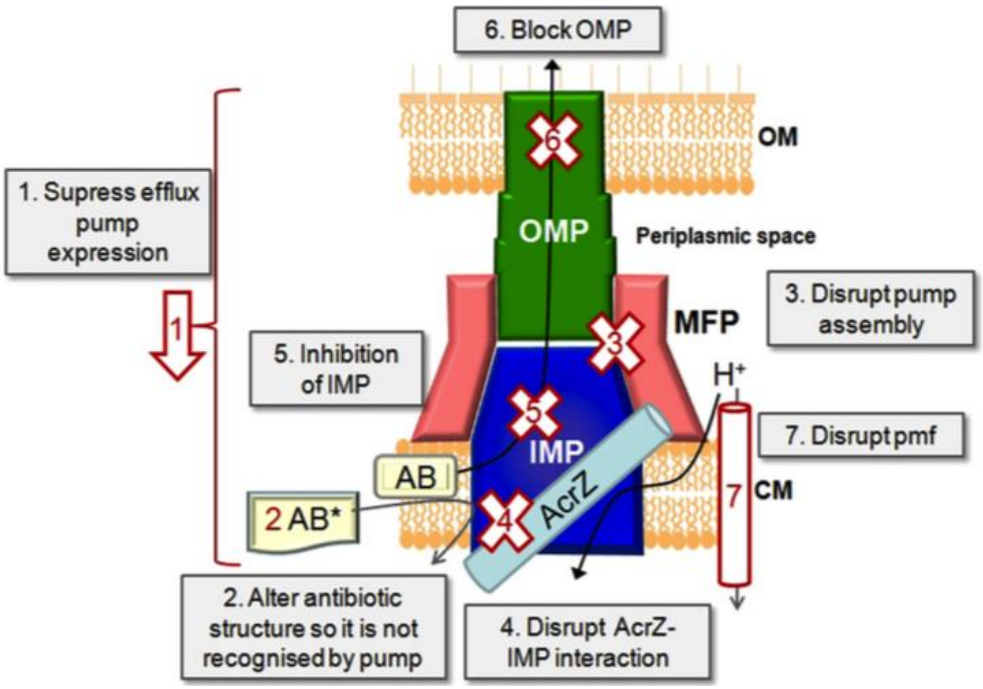


Figure no. 2.2.7: Probable mechanisms of efflux inhibition. Different inhibition strategies have been depicted for inhibiting RND efflux pump activity by efflux pump inhibitors. (Ref-Venter et al., 2015)

However, many of above approaches for development of EPIs are limited to laboratory studies and are yet to be approved in clinical trials for their efficiency and application.

Though, many potent inhibitors of RND efflux pumps, from both synthetic and natural sources, have been reported in literature, none of them have been approved in clinic [103]. The most common problem faced in current screening methodology is the synergisms observed with their antibiotics, with similar cytotoxic activity against mammalian cells [102]. Therefore, there has been substantial interest gathered fordeveloping EPIs for the RND family efflux pumps.

To date, two classes of broad-spectrum EPI have been extensively characterized, such as peptidomimetics and pyridopyrimidines,. A family of peptidomimetics, for example: Phenyl-arginine beta-naphthylamide PAβN (MC-207 110) was amongst the first identified EPIs that exhibited potent inhibition of RND efflux pumps (MexAB–OprM, MexCD–OprJ, MexEF–OprN, and MexXY–OprM) in *P. aeruginosa* [57]. Initially PAβN was developed as an adjunctive therapy and was also observed to have inhibitory role against the AcrAB-TolC efflux pumps in several gram-negative pathogens, including *E. coli*, *S. Typhimurium* and *K. pneumoniae.* It was proposed to be competitive inhibitors of the efflux pumps and thought to act by binding to the large substrate-binding pocket of the RND transporters. Despite being specific to RND efflux pumps, unfortunately PAβN was found to permealize the outer membrane with toxicity issues, making them inappropriate for use as EPIs [104].

Another interesting group of EPIs are structurally similar to quinolones, consisting of chloroquinolone, alkoxyquinolone, alkylaminoquinolone, pyrridoquinolone, and thioalkoxyquinolone. These derivatives have shown significant activities against many laboratory susceptible and clinically multi-drug resistant isolates of *Enterobacter aerogenes* and *K. pneumoniae* [10]. Though, this series of derivatives have shown negligible intrinsic activity with limited side effect on membrane permeability, but their pharmacodynamics and toxicity studies are yet to be done [32].

2.2.7. Role of efflux pumps in multiple antibiotic resistance

Considering the limited options available for therapeutic treatment, antibiotic resistance is of deep concern specifically in context of ESKAPE pathogens. In the last decade, significant observations were made regarding the involvement of efflux pumps in developing MDR phenotypes in such ESKAPE pathogens. It was believed that MDR efflux pumps contribute to antibiotic resistance at three different levels, as shown in the figure no. 2.2.8 below.

i) *Intrinsic resistance*- where efflux pumps are expressed at a basal level under any conditions and contributed to inherent resistance towards antibiotics.
ii) *Acquired resistance*- where mutants with high-level expression of the efflux pumps (de-repression) are selected that conferred stable acquired resistance.
iii) *Phenotypic resistance*- where presence of specific inducers/growth conditions triggered the expression of efflux pumps contributing to transient non-inheritable phenotypic resistance.

Being considered as a housekeeping gene, AcrAB-TolC are found to be constitutively expressed in *E. coli* and *E. cloacae*. Nevertheless, the first involvement of efflux mechanism in extruding antibiotics in *Enterobacter* spp. was described in 1998 [30]. Also, such AcrAB-TolC efflux pump was found to be highly efficient, since 80–90 % of the norfloxacin was extruded during the first 10–15 min. Later on, approximately 40% of MDR clinical strains were reported to have an active efflux pump contributing to observed MDR phenotype [32].

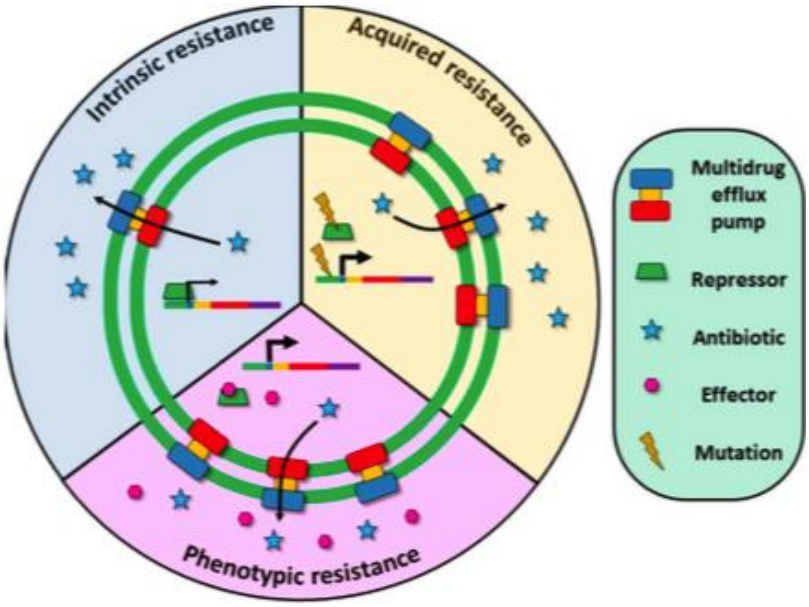


Figure no. 2.2.8: Involvement of MDR efflux pumps in antibiotic resistance. Contribution of MDR efflux pumps in antibiotic resistance (intrinsic/acquired/phenotypic). (Ref- Blanco et al., 2016).

Primarily, EefABC and AcrAB-TolC efflux pump genes in *E. aerogenes* have been widely studied and various reports suggested their involvement in antimicrobial resistance. Studies on *E. cloacae* have emphasized on predominance of efflux pumps belonging to RND superfamily such as AcrAB-TolC and OqxAB. Between *E. aerogenes* and *E. cloacae*, AcrAB-TolC efflux pumps share greater sequence similarities and biological activity [12]. Deletion of *acrA* in *E. cloacae* isolates presumably decreased the MICs to multiple antibiotics including oxacillin, clindamycin erythromycin, chloramphenicol, linezolid, ciprofloxacin, tetracycline, and tigecycline [72]. Greater number of fluoroquinolone-resistant clinical isolates of *E. cloacae* appeared to overproduce multidrug efflux pump, presumably AcrB (Nikaido 1998). In *E. coli* a strong correlation between *acrAB* over-expression and elevated levels of fluoroquinolone resistance has been reported [105]. Similar observation on higher *acrAB* transcript expression in ciprofloxacin resistant *K. pneumoniae* isolates from burn patients in Tehran was reported [106]. Clinical isolates *E. cloacae* during ciprofloxacin treatment, was observed to develop resistance to tigecycline through

RamA-dependent overproduction of AcrAB [107].

In comparison to the conventional knowledge about resistance genes harbored by pathogens, MDR efflux pumps confer certain added features, which enable them as favorite candidates for antibiotic resistance amongst ESKAPE group.

i) MDR efflux pumps are ubiquitous in nature, present in both prokaryotes and eukaryotes, irrespective of their distribution and pathogenic index. Several reports on diverse pathogens have emphasized on the contribution of MDR efflux pumps to the acquisition of multidrug resistance.
ii) Such efflux systems are not substrate-specific, and are able to extrude antibiotics belonging to different class and other structurally dissimilar compounds, solvents etc. Therefore, efflux pumps significantly differ from other modes of drug resistance (e.g. β-lactamases) that acts on a specific group of antibiotics. Any single efflux pump can extrude a broad range of structurally diverse substrates; hence its inhibition will increase the bacterial susceptibility to several antimicrobials.
iii) Distribution of efflux pumps are complicated in nature: a single organism can possess 10 different MDR efflux pumps belonging to different families; or a single family of efflux pump is conserved through out different species of specific bacterial genera. Although expression of a single efflux pump can confer MDR phenotype, simultaneous overexpression of more than one MDR efflux system in pathogen could be alarming.

2.2.8. Role of efflux pumps in bacterial pathogenicity

Though most studies focused on the contribution of MDR efflux pumps in antibiotic

resistance; however, their wide distribution and overlapping functions in bacteria suggest that these efflux pumps have physiological roles beyond merely drug resistance [29]. Of late, there has been increase in our understanding of alternative, important functions, of multidrug pumps in virulence. Some of these include bacterial quorum sensing, stress response, survival fitness, colonization, intracellular survival, and biofilm formation, which are briefly discussed below.

2.2.8.1. Efflux pumps and cell to cell communication

The capability to sense the environment and adapt to the niche is critical for bacteria to survive. One such essential inter-cellular signaling mechanism is called as quorum sensing (or cell to cell communication). This phenomenon involves low-molecular weight compounds produced (called as autoinducers) are sensed by the molecular receptors present in other cells, promoting a specific response to the stimuli [108], [109]. Further, quorum sensing (QS) can act upon population level resulting in physiological changes that enhanced better survivability.

Best suited example is *P. aeruginosa*, where RND efflux pump MexAB-OprM was found to extrude quorum sensing signal molecules (homoserine lactones containing different acyl chain modifications-AHLs); thus resistant mutants overexpressing *mexAB* accumulated lower quantities of such quorum sensing signal, and became less virulent in nature [110], [111]. Moreover, deletion of efflux pump MexGHI in *P. aeruginosa*, was shown to reduce the production of different AHLs [29]. MexGHI-OpmD and MexEF-OprN system in *P. aeruginosa*, was found to efflux out the QS signaling precursor molecules, anthranilate and kynurenine, respectively [112].

This phenomenon was observed in other organisms too, in *E. coli* increased AcrA and AcrB protein levels was reportedly induced by overexpression of the QS regulator

SdiA, revealing potential role of the AcrAB efflux pump in QS [82]. Further, mutation of such pump components affected the survival ability of the organism at its stationary growth. This highlighted the diversity of QS compounds being extruded by RND efflux pumps that provided an advantage for the bacteria to adapt to their habitats.

2.2.8.2. Role of efflux pumps in pathogen virulence

Following the recognition of MDR efflux pumps as relevant antibiotic resistance determinants, it was later suggested to have significant role in bacterial pathogenicity [26]. During the course of microbial infection, the first step is colonization in the host, which gets hindered by various host-derived compounds such as bile salts, long-chain fatty acids and antimicrobial peptides. Several MDR efflux systems in gram-negative bacteria confer resistance to bile salts *in vitro* [113]. The best-studied example is AcrAB-TolC efflux pump in *E. coli*. The AcrAB efflux system in *E. coli*, exhibited resistance to both bile salts and long-chain fatty acids, hence prevents *E. coli* from from colonizing mucosal surfaces.

Contribution of AcrAB-TolC efflux pumps on virulence of *S. Typhimurium* was studied using efflux pump defect mutants in a chicken model [69]. The authors observed poor colonizing ability of mutants deficient in either *acrB* or *tolC* genes, even these mutants fail to persist in the avian gut, indicating that AcrAB-TolC efflux system is crucial for the colonization of *S. Typhimurium* in chickens. This suggested AcrB to be critical for gastrointestinal persistence, with insignificant roles in gut colonization, whereas TolC was found important for both colonization and survival, but with less frequency [114]. Even, in *Salmonella enterica* serovar Typhimurium, inactivation of *acrA, acrB* and *tolC* decreased expression of numerous genes encoding proteins involved in bacterial pathogenicity, indicating a crosstalk between resistance

and virulence [112]. Disruption of *acrB* or *tolC* reduced the invasion followed by survival in non-phagocytic intestinal epithelial cells, whereas inactivation of *acrA*, repressed the survival and multiplication in phagocytic cells [69]. Recent studies have elucidated that Δ*acrB* in *K. pneumoniae* and Δ*acrA* or Δ*tolC* in *Enterobacter cloacae,* reduced the ability of the pathogens to cause infection in a mouse model, indicating that AcrAB-TolC efflux pump is vital for the virulence of *K. pneumoniae* and *E. cloacae* [48], [72]. Similarly, a mutant of *Vibrio cholerae* deficient in RND efflux pumps, produced significantly less cholera toxin and fewer toxin-coregulated pili [115].

Connection between MDR efflux pumps and expression of the type III secretion system (T3SS) has also been investigated. T3SS system enables bacteria to inject a group of effector molecules directly into the cytoplasm of eukaryotic host cells. It has been shown in *P. aeruginosa* that constitutive overexpression of MexCD-OprJ or MexEF-OprN type RND efflux pumps reduced the expression of the T3SS system [116].

Biofilm formation is another crucial virulence factor associated with most of the chronic and persistent bacterial infections that accelerated the emergence and rapid spread of multidrug resistant bacteria. Studies revealed that inhibition of efflux activities by efflux pump inhibitors (EPIs) in both *E. coli* and *Klebsiella* strains reduced biofilm formation, and concurrent treatment with different EPIs completely abolished biofilm formation [117]. Such impaired biofilm formation resulting from efflux pump inactivation was also reported in *E. coli*, *S. aureus* and *P. aeruginosa* [118], [57], The results indicate a promising anti-biofilm strategy via inhibition of efflux activity. Put together, review of literature suggested the significance of the presence of functional MDR efflux pumps in bacterial fitness and virulence.

2.2.8.3. Role of efflux pumps in biocide resistance

Biocides are chemically diverse range of antimicrobials, used as antiseptics, disinfectants and preservatives primarily in the food industry, veterinary, household cleaning and other applications [119]. Biocide resistance is frequently observed in gram-negative Enterobacteriaceae. This resistance has been implicated to the presence of multidrug efflux pumps because of their redundancy and substrate non-specificity nature [120]. Various reports demonstrated that expression of efflux pumps reduced the efficiency of distinct classes of biocides, including chlorhexidine digluconate, benzalkonium chloride, hydrogen peroxide, iodine compounds, chloroxylenol, quaternary ammonium compounds, triclosan, phenolic parabens and DNA intercalating agents [29]. Efflux pump-dependent biocide resistance has been elucidated in many environmental and clinically relevant bacteria. The best-studied organisms for efflux-mediated biocides resistance include: *P. aeruginosa* (MexAB-OprM, MexCD-OprJ, and MexEF-OprN), *E. coli* (AcrAB-TolC, AcrEF-TolC, and EmrE) and *Stenotrophomonas maltophilia (*SmeDEF) [10].

However, there have been increasing reports on efflux dependent biocide resistance driven by TolC-independent single component secondary efflux pumps belonging to SMR, MATE, MFS or PACE superfamily [120]. For instance, SugE belonging to SMR family of transporters was reported in *Enterobacter cloacae* that conferred resistance to cetylpyridinium chloride, cetyltrimethylammonium bromide, benzalkonium chloride, tetraphenylphosphonium, ethidium bromide and sodium dodecyl sulfate [121]

2.2.8.4. Role of efflux pumps in non-clinical environments

It is very interesting to note that numbers of genes encoding efflux pumps are plentiful

in natural environment. The highest numbers of predicted multidrug efflux systems are generally present in soil- or plant-associated organisms such as *Streptomyces* spp., *Pseudomonas* spp., *Agrobacterium tumefaciens, Sinorhizobium meliloti* and *Bacillus* spp. [122].

Apart from being facilitators of antibiotic resistance in clinical pathogens, the efflux pump activity may be physiologically relevant in environmental ecosystems. Indeed, efflux pumps discovered in many plant pathogens or epiphytes have divulged role of efflux systems in plant-bacteria or bacteria-bacteria interactions. One such efflux system is the flavonoid-responsive RND family of efflux pumps, which includes AcrAB from *Erwinia amylovora*, MexAB-OprM from *Pseudomonas syringae*, EmrAB in *Sinorhizobium meliloti* and BjG30 from *Bradyrhizobium japonicum*, to name a few [112]. Certain efflux pumps participate in plant colonization, while others are implicated in bacteria/plant symbiosis processes. For instance, the plant pathogen *S. maltophilia* possess the SmeDEF efflux pump, that confers quinolone resistance. It was observed that a mutant lacking smeE was unable to colonize the roots of the plants, indicating it's involvement in bacteria/plant interactions [45]. Flavonoid-induced AcrAB-TolC efflux pump in *E. amylovora* conferred resistance to plant compounds (phytoalexins and flavonoids) and antibiotics, leading to successful colonization in plants [29].

Further, efflux pumps have additional functions in modulating bacteria/plant and intermicrobial interactions. For example, a *tolC* mutant of *E. chrysanthemi*, defective in extruding the plant antimicrobial compound berberine, was unsuccessful in plant tissue maceration *in planta* [123]. Moreover, the AcrAB-TolC efflux system in *E. chrysanthemi* was found to be inducible by salicylic acid, an important plant hormone implicated in both local and systemic plant resistance [112]. Apart from

antimicrobials, there are other toxic elements including heavy metals, xenobiotic compounds, to which the organisms get exposed in non-clinical environments. Bacterial MDR efflux pumps are believed to regulate the intracellular metal concentrations that are required as cofactors for bacterial proteins, and thus maintain metal homeostasis. The expression of efflux-encoded genes in *P. aeruginosa* was upregulated in response to increasing levels of heavy metals. For instance, levels of heavy metal resistance was correlated with overexpression of the efflux pump CzcCBA in *P. aeruginosa*, which was under the regulatory control of a TCS, sensed by heavy metals [124].

On the contrary, unlike heavy metal efflux pumps, solvent-extruding efflux pumps are not substrate specific and can accommodate antibiotics too. Organic solvents and xenobiotic compounds was effluxed by many RND efflux pumps abundant in nature, including AcrAB-TolC in *E. coli* and MexAB-OprM in *P. aeruginosa* [45]. Unlike clinical settings, where antimicrobials serve as the predominant substrate for MDR efflux pumps, in non-clinical environment a lot of toxic elements, besides antimicrobials, were encountered and gets effluxed out by MDR efflux pumps. Accordingly MDR efflux pumps correspond to 10% of the transporter genes present in bacterial species, and very aptly referred to as detoxification element [50]. Undoubtedly these ancient molecules were evolutionary preserved due to their physiologically relevant roles. MDR efflux pumps have critical role in bacterial physiology and ecological behavior (as depicted in figure no. 2.2.9), hence are equally abundant in both clinical as well as non-clinical environments [125].

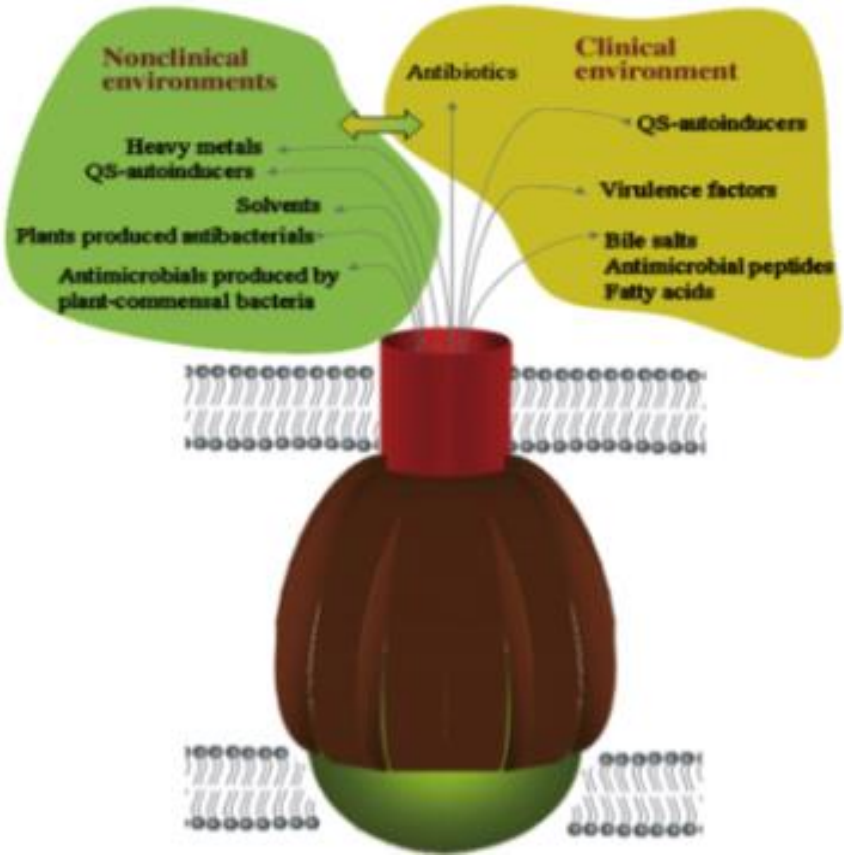


Figure no. 2.2.9: Significance of bacterial efflux pumps in clinical and non-clinical environmental setup. This figureNote: depicts Comparative illustration of functional role of bacterial efflux pumps in clinical and non-clinical environment, in terms of virulence, physiology, colonization to host and resistance to multiple antibiotics, toxic elements and antimicrobial peptides. (Ref: Martinez et al., 2009).

2.3. The Outer Membrane Proteins:

The escalation in antibiotic resistance in ESKAPE pathogens had necessitated understanding the vital mechanisms of antibiotic entry. The limited outer membrane (OM) permeability in gram-negative bacteria has been identified as a challenging barrier. Passing through the OM barricade and to achieve inhibitory concentration levels of intracellular antibiotic in the vicinity of their target is a key step for antibiotics to be effective. In this review, the organization of OM, classification of OMPs, their regulation and association with antibiotic resistance and virulence will be briefly discussed.

2.3.1. Organization of Outer membrane

Bacterial outer membranes constitute a selective permeability barrier, providing sufficient nutrients and solutes into the cell and simultaneously protecting from toxic compounds in the extracellular millieu [14]. In gram-negative bacteria, the OM exists as an asymmetric bilayer of phospholipid and lipopolysaccharides (LPS), both differ in their composition and function. LPS is a hydrophobic, fatty acid chain consisting of three parts: a) Lipid A, a glucosamine-based phospholipid, b) A short core oligosaccharide, c) a distal polysaccharide (O-antigen), exclusively found in the outer leaflet. Albeit, the phospholipid composition of the inner leaflet of the OM contain of about 80% of phosphatidylethanolamine, 15% of phosphatidylglycerol and 5% of cardiolipin, similar to the cytoplasmic membrane [126]. The outer membrane constitutes of large number of proteins, some are abundant (OmpA and general diffusion porins), and few are induced (LamB, PhoE). Generally, OMPs contain water-filled pores extending across the membrane to facilitate influx of hydrophilic compounds up to a certain size exclusion limit. The proteins that constitute these pores are generally referred to as porins [127] and were first characterized in 1997 for *E. coli* [128]. Presence of pore forming proteins (porins) makes the outer membrane more leaky in nature as compared to the inner membrane. Later on, several studies reported the presence of different porins in gram-negative bacteria, and their association with wide array of cellular functions.

2.3.2. Structural and functional properties of OMPs

OMPs are distinct β-barrels structure, made up of transmembrane antiparallel β-strands with alternating hydrophobic amino acids (facing outwards) and hydrophilic amino acids (facing inwards); assembled into instead of hydrophobic α-helices often found in

cytoplasmic membrane proteins [127]. . Such β-barrel configuration generate a central hydrophilic pore in each β-barrel, which determines the size exclusion limit and permeation properties of the porin [67]. The amphipathic β-barrels of porins are connected by short periplasmic turns and by extracellular (usually longer) surface exposed loops. Such protruding extracellular domain serves as the interacting sites for specific colicins and phages.

The functional properties of OMPs in terms of their permeation ability, conductance, voltage sensitivity and ionic selectivity were investigated subsequently. Molecular sieving properties of OMPs was determined to be about 600 Daltons (for OmpF), which implied that influx of ions, small sugars and amino acids to the periplasm occurs thorugh general diffusion porins.

The table below (table no. 2.3.1) summarizes key information about five families of prototype integral OMPs from *E. coli*.

2.3.2.1. OmpA

The OmpA protein is one of the most abundant OMPs of *E. coli* that occurs at > 10^5 copies per cell, like murein lipoprotein (Lpp) and other general diffusion porins. OmpA consisted of two domains: membrane-embedded N-terminal domain of 170 amino acid residues, acting as an anchor; and periplasmic C-terminal domain made up of 155 amino acid residues, thought to interact specifically with the peptidoglycan layer [129]. The physiological role of OmpA protein is to serve as a physical linkage connecting the outer membrane and the underlying peptidoglycan layer. This maintenance of structural integrity of the bacterial cell surface was validated, when absence of OmpA and Lpp was found to compromise the cell shape [67].

Table no. 2.3.1: Characteristic features of prototype OMPs from *E. coli*

Families of OMPs	**Small β-barrel membrane anchors**		**General non-specific porins**	**Substrate specific porins**	**TonB-dependent receptors**
Prototype protein	**OmpA**	**OmpX**	**OmpF**	**LamB**	**FhuA**
Function	Physical linkage between OM and peptidoglycan layer	Neutralizing host-defense mechanisms	Diffusion pores for ions and other small molecules	Maltose and maltodextrin uptake	Uptake of iron-siderophore complexes; Signal transduction
Oligomeric state	Monomer	Monomer	Homotrimer	Homotrimer	Monomer
Domain structure	Two co-linear domains	One domain	One domain	One domain	Two inter-connected domains
Size of the membrane domain	171 residues	148 residues	340 residues	421 residues	714 residues
No. of transmembrane β-strands (*n*) Shear number (*S*)	*n*=8 *S*=10	*n*=8 *S*=8	*n*=16 *S*=20	*n*=18 *S*=22	*n*=22 *S*=24

Reference: Adpated from Koebnik et al., 2000

Owing to its relatively small size and monomeric nature, OmpA is best studied as a model for understanding folding of β-structured membrane proteins *in vivo* as well as *in vitro* [130]. They also serve as receptors for various bacteriophages (K3, M1, Ox2) and colicins (Colicin K, Colicin L) [131], [10].

2.3.2.2. OmpX

Being structural homologs of OmpA, OmpX protein is composed of the basic β-barrel architecture, as mentioned above (eight antiparallel amphipathic β-strands with hydrophilic surface-exposed loops and periplasmic turns, cluster of internal salt bridges and internal cavities) [129]. Besides such structural similarity, OmpX differed from OmpA by three major ways [132]:

i) OmpX has a shear number (*S*) of 8 unlike OmpA (*S*=10), resulting in a less tilted arrangement of its β-strands (35° versus 42°).
ii) OmpX has more ellipsoidal cross-section with an axes ratio of 1.6:1.0, whereas in case of OmpA, it is 1.25:1.0.
iii) OmpX protein has a structural motif, which is extended elongation of four of its β-strands beyond the polar head groups of outer leaflet, reminiscent of a 'fishing rod' appearance. This protruding β–sheet presumably binds to external proteins promoting cell adhesion, invasion and inhibition of the complement system.

OmpX, first characterized in *Enterobacter cloacae* [133], belongs to a family of highly conserved proteins, with functional significance in virulence by neutralizing host defence mechanisms [134]. For instance, OmpX protein (Ail) in *Yersinia enterocolitica*, contributes to its virulence, as OmpX is essential for the organism's adhesion to and internalization into mammalian cells [135].

2.3.2.3. General non-specific porins (OmpF, OmpC, PhoE)

The general diffusion porins including OmpF, OmpC and PhoE are homotrimers of 16-stranded β–barrels forming a size-selective defined channel [129]. Predominantly, such general porins allows the diffusion of hydrophilic charged molecules (>600 Da) including antibiotics such as β-lactams and fluoroquinolones, with no particular substrate specificity, despite some selectivity for either cations or anions [127]. These porins are conserved through out the phylum of γ-proteobacteria [136]. OmpF porin was the first to be crystallized followed by the PhoE porin, in *E. coli* [137]. OmpF and OmpC show a slight preference for cations, whereas PhoE selects inorganic phosphate and anions.

Unlike other OMPs, the pore in the general diffusion porins is constricted by the inwardly folded extracellular loop, the third loop 'L3', which gives it an hourglass-like shape. This loop, together with the opposite barrel wall, forms the constriction zone, that contributes significantly to the size exclusion limit, ion selectivity and permeability properties of the pore. It was noteworthy that this loop contained a sequence motif, PEFGG, which is highly conserved among Enterobacterial porins [129].

2.3.2.4. Substrate specific porins (LamB)

Besides naturally occurring general diffusion pores that selectively allows solutes based on their size and charge, there are certain OMPs with substrate specificity.The best-studied examples include the maltoporin LamB in *E. coli* and the sucrose-specific porin ScrY in *S. Typhimurium*, both are homotrimers consisting of 18-stranded antiparallel β-barrels [129]. The *lamB* gene in *E. coli* is expressed as part of the *mal* regulon, and gets induced in the presence of maltose or maltodextrins [138]**.**

2.3.2.5. Other OMPs

Apart from the prototype OMPs listed above, all gram-negative bacteria contained several high-molecular-weight OMPs such as TonB-dependent receptors (e.g. FhuA and FepA), responsible for binding and subsequent transport of large substrates, such as iron-siderophore complexes or vitamin B12 respectively [139]. Phospholipase A (OMPLA), another OMP is involved in colicin release from *E. coli* and is implicated in the virulence of *Campylobacter* and *Helicobacter* strains [140]. OMPLA represents the only outer membrane enzyme whose three-dimensional structure is available. Although OMPLA is known to hydrolyze phospholipids, but it's physiological relevance in the outer membrane is still not completely understood [129].

2.3.3. Porin-mediated antibiotic permeability

Translocation of the antibiotics through porin channel is the first essential step in its journey towards target site. Several physico-chemical experiments were performed to characterize the permeation ability of substrates across the lipid layer or through a particular membrane channel. It was believed that the chemical properties of antibiotics affect their transport process through interactions with the porin channels. The first evidence of such facilitated diffusion via binding was observed in maltoporin in *E. coli*, a maltose-specific channel present in their outer membrane [138].

Antibiotic permeability by porin channel has been well described for both β-lactams and fluoroquinolones. Large β-lactams molecules, with bulky side chains, for eg. piperacillin and azlocillin have been found to have low permeation rates, whereas ampicillin and amoxicillin had higher diffusion rates. The constriction zone present in OmpF appeared to facilitate drug translocation by interactions that largely depend on the nature and position of specific charges on the antibiotic molecule [67].

Studies suggested that the uncharged quinolones cross the OM through the lipid bilayer, whereas the negatively charged molecules mostly pass through porin channels as magnesium chelates [32]. In a similar fashion, tetracylines too uses both porin and lipid-mediated influx pathways [67]. It is evident that the preference for porin-mediated and lipid-mediated translocation of antibiotics depends on the protonation/deprotonation states of the drug, which is further influenced by pH.

Presumably, taking into considerations of all experimental evidences, it is evident that uptake of small antimicrobial agents (β-lactams, chloramphenicol, tetracycline and fluoroquinolones) primarily occur via porin channels (in Enterobacteriaceae, to be particular), which have high-permeability porins. On the contrary, large lipophilic agents, such as rifamycins, macrolides, novobiocin, and fusidic acid, face difficulties in diffusing through the porin channels, and therefore utilize the lipid bilayer [24].

2.3.4. Regulation of porin expression

The regulation cascade of porin expression in Enterobacteriaceae is complex involving both local and global regulators and other external stimuli. Expression of porin is usually assessed in terms of type of porin expressed, level of expression, and regulation and association with a resistant phenotype [141]. The regulation of expression of nonspecific porins in *E. coli* is briefly summarized belowin figure no. 2.3.1. As discussed earlier several global transcriptional regulons (*mar-sox-rob*), belonging to XylS-AraC family of regulatory proteins, positive regulators contributing to the multidrug resistance (MDR) response, can strongly downregulate OmpF expression by activating the transcription of *micF* antisense RNA, which binds to the 5'-region of the *ompF* mRNA and inhibits its translation [127].

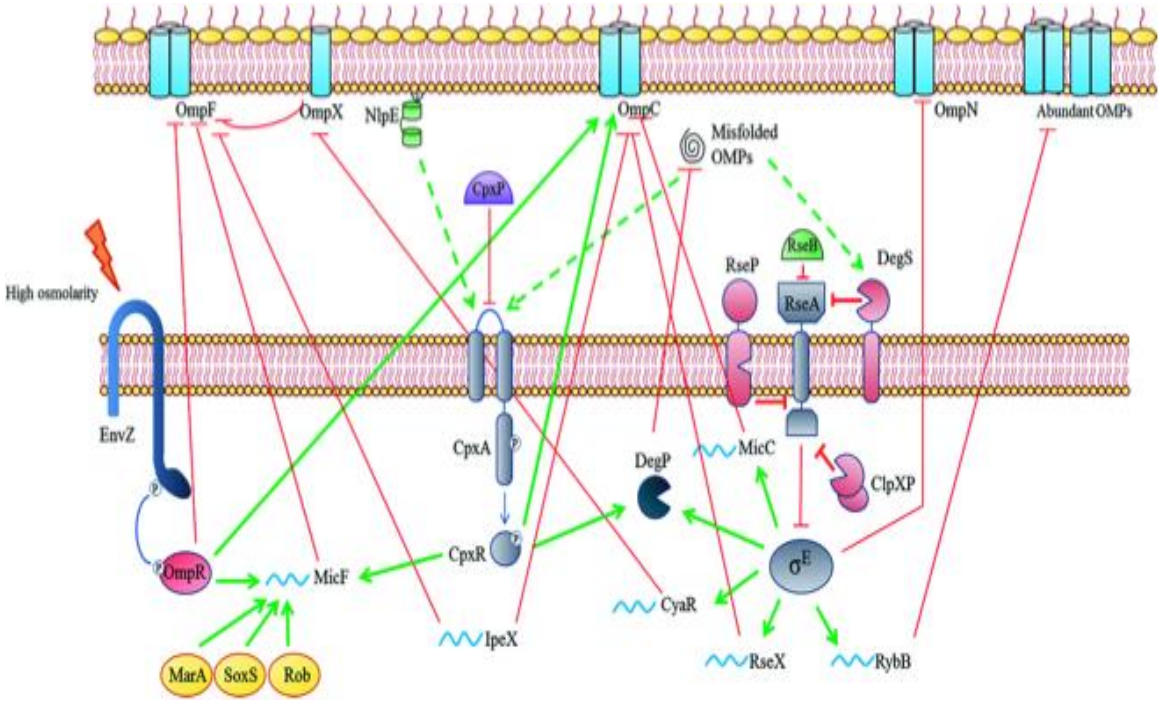


Figure no. 2.3.1: Regulatory mechanisms responsible for expression of OMPs. It is representing major regulatory cascade involved in porin regulation in *E. coli* involving Two-component system (EnvZ/OmpR), Envelope stress response (CpxAR), and other global and local (OmpR and OmpX) transcriptional regulators. (Ref: Dam et al., 2018)

Negative regulation by repressors of porins also plays a major role, where overproduction of LamB and OmpX was associated with major porin loss [142]. In addition, OmpX expression was affected by various environmental factors, for instance- salicylate was shown to induce MarA-dependent upregulation of *ompX* [143].

In *E. coli*, a complex regulation cascade involving the TCS EnvZ-OmpR that gets activated under osmotic stress, controls the OmpC/OmpF balance. The sensor kinase Envz sensed osmolarity and, signal was transferred to response regulator OmpR; high osmolarity resulted in the phosphorylation of OmpR. Phosphorylated OmpR (OmpR~P) differentially modulated the expression of *ompF* and *ompC* porin genes

[144]. At low osmolarity (nutrient-scarce conditions), high levels of OmpR~P resulted in higher expression of *ompF*, whereas at high osmolarity (nutrient-rich conditions), low levels of OmpR~P repressed *ompF* and activated *ompC* transcription [14]. EnvZ/OmpR dependent transcriptional regulation of OmpF/OmpC was also triggered by pH. It was noticed that at acidic pH 5.2, the *ompF* porin becomes strongly repressed and expression of *ompC* was upregulated [24].

Another environmental stress response encoded by CpxAR, primarly induced by alkaline pH and accumulation of misfolded transporters; can directly affect the expression of both OmpF and OmpC, including miCF. On the contrary, the stress responsive sigma factor σ^E , important for OM biogenesis, is induced by membrane abnormalities, also regulated the OMPs expression following a stringent regulatory cascade [144]. Interestingly, the σ^E-dependent repression of porin synthesis only occurs at the post-transcriptional level, whereas base-paring small non-coding RNAs (sRNAs) inhibited translation of *omp* mRNA. Few examples of well-conserved σ^E-regulated sRNAs and their associated porins include RybB (*ompC* and *lamB* in *E. coli*; *ompN* and *ompW* in *Salmonella*), MicA (*ompA*), RseX (*ompC* and *ompA*), CyaR (*ompX*) [145].

2.3.5. Role of porins in antibiotic resistance

Development of multidrug resistance phenotype in gram-negative pathogens has been associated with porin modification in three major ways: (i) alterations in porin expression, (ii) decreased porin expression, and (iii) mutation in porins. All of the above aspects, individually or in combination affect bacterial susceptibility towards antibiotics, particularly the β-lactams [14]. A well-coordinated interplay between outer membrane protein expression and subsequent folding, increased efflux activity and

controlled outer membrane permeability, have been associated with multidrug resistant (MDR) phenotype in *E. coli* ([146]. In *Acinetobacter baumannii*, OmpA disruption lead to severe reduction in minimum inhibitory concentration for multiple antibiotics, suggesting its contribution towards MDR phenotype [147].

i) Alterations in porin expression

In most of the clinical isolates of *K. pneumoniae*, undergoing antibiotic therapy, exhibited altered outer-membrane permeability; where porin OmpK35 (belonging to OmpF porin group) with larger channel size, was exchanged with OmpK36 (belonging to OmpC porin group) bearing a narrow channel size. Even, *K. pneumoniae* strains expressing OmpK35 showed differential β-lactam susceptibility as compared to strains possessing OmpK36. Susceptibility to β-lactams, including Cefepime, Cefotaxime and Cefpirome, was observed 4–8 times higher in the former than the latter [148].

Moreover, modification in porin expression in bacterial outer membrane is regulated by environmental stimuli. For example: under low osmolarity or nutrient-scarce conditions, there is a considerable increased expression of OmpF (with higher pore size) in the outer membrane, facilitating influx of nutrients. On the contrary, expression of the OmpC porin with its restrictive channel is generally favoured, under high osmolarity conditions in patients [127]. Complete impermeability to β-lactams in resistant isolates was accounted to total loss of OmpC porin, representing an 'extreme step' in the porin adaptive response.

ii) Decreased porin expression

Reduced expression of porins in *E. coli* upon exposure to chlortetracycline, tetracycline and to the biocide benzalkonium chloride has been well documented

[149],[150]. Similarly, sub inhibitory concentrations of the benzodiazepine drug diazepam, resulted in adaptive multi-resistance in *E. coli* and *K. pneumoniae* by reducing porin expression and inducing efflux systems [151]. Pages and groups also examined for expression of porins in large number of β-lactam-resistant clinical strains of Enterobacteriaceae; which revealed loss of porins in 6% of resistant strains of *E. cloacae* (1 out of 17 resistant isolates) and 44% of resistant strains of *E. aerogenes* (20 out of 44 resistant strains) [72].

The loss of major Omp36 porin (OmpC homologue) was associated with β-lactam (cephalosporin and imipenem) resistance and imipenem susceptibility [152]. This highlighted that *in vivo* antibiotic treatment can result in a resistant phenotype that is correlated with porin loss from an original susceptible isolate. For instance, in a patient suffering from bacteraemia due to *E. cloacae,* was treated with imipenem and amikacin over 3 weeks, resulting in development of a resistant strain after antibiotic treatment. Further characterization of the susceptible and resistant *E. cloacae* isolates revealed decreased production of major porins and increased expression of efflux pumps [153].

iii) Mutation in porins

Mutations in porins, modulating the expression and/or function of porins, have a direct impact on the antimicrobial susceptibility in bacteria. Acquisition of mutational resistance has three major effects including porin loss, modification of the size or conductance of the porin channel (restricted channel) or decreased expression of porin. However, all of these mutations resulted in limited, substantially slower diffusion of the antibiotics and affect resistance to β-lactams, tetracycline, fluoroquinolones and chloramphenicol [14].

Best-studied examples include, the OmpC and Omp36 porin mutations detected in clinical strains of *E. coli* and *E. aerogenes* respectively, and isolated after antibiotic treatment [152]. Ertapenem resistant *E. cloacae* isolate lacking porin OmpF as observed from SDS-PAGE analysis, was found to carry a mutation in the promoter that led to a 20-fold reduction in the expression level of the *ompF* gene [9]. Another key porin mutation, a G112D mutation in loop 3 of the OmpF/OmpC-like protein, was detected in *E. aerogenes* clinical isolates. This mutation caused strong hindrance in the channel and hence severely impaired β-lactam susceptibility [14]. Subsequently, this Omp36 G112D mutant of *E. aerogenes* resulted in 3-fold decrease in ion conductance and significant reduction in cephalosporin sensitivity (e.g. MICs of cefotaxime and cefepime were 7- to 9-fold higher in clinical isolate as compared to reference strain) [154].

2.3.6. Role of porins in bacterial virulence

Besides facilitating antibiotic resistance, OMPs are also believed to play a pivotal role in bacterial pathogenesis. They serve as receptors for bacteriocins, hemolysin, other toxins and antibodies [127]. As surface exposed structures, OMPs are potential candidates for interfacing bacteria with their mammalian host and its defenses against bacteriophages, other bacteria.

OmpA family of proteins are involved in multiple mechanisms of bacterial pathogenesis, including bacterial adhesion to mucosal surfaces, subsequent invasion, evasion of host defenses, serum resistance and antimicrobial peptide resistance [155]. Being highly immunogenic in nature, the role of OmpA in both innate and acquired immunity was investigated, which suggested OmpA to be a pathogen-associated molecular pattern (PAMP) molecule interacting with antigen presenting cells (APCs),

which opened up prospects of it being a potential target for vaccine development [156]. On the other hand, OmpX was shown to be involved in neutralizing host defense mechanisms, invasion of host cells and bacterial defense against the host complement systems [157].

Reports revealed that deletion mutants of *ompA* in *E. coli* K1 reduced the expression of type 1 fimbriae, which further decreased the abilities of *E. coli* K1 to adhere and invade human brain microvascular endothelial cells-HBMEC [158]. In *Cronobacter sakazakii*, compared to the wild type isolates, deletion mutants of *ompA* and *ompX* isolates exhibited reduced adhesion and invasion to human epithelial cell INT-407 and human enterocyte like epithelial CaCo-2 cells [159]. Further, the authors have observed drastic reduction of invasion in case of double mutants suggesting synergistic/additive effect of OmpA and OmpX in INT-407 invasion by *Cronobacter sakazakii*. Similar observations were also made in avian pathogenic *E. coli*, where inactivation of *ompF* and *ompC* were shown to significantly hamper its adhesive, invasive and colonization abilities [160]. Previous report in clinical *E. aerogenes* isolates, suggested the significance of OMPs in modulating membrane permeability, which in turn affected its susceptibility to antibiotic and colonization abilities in nematodes [161]. Subjected to imipenem treatment, imipenem resistant clinical *E. aerogenes* isolates exhibited higher virulent properties in *Caenorhabditis elegans* model as compared to the imipenem susceptible strains. Another study suggested that OmpA in *Enterobacter sakazakii* to interact with host cytoskeleton, thus aiding in the bacterial invasion of the human epithelial cells INT-407. Moreover, deletion mutant of *ompA* in *E. sakazakii* was significantly attenuated in its invasive features, which was restored by complementation experiments. [162].

From a detailed review of literature it is evident that OMP's and AcrAB-TolC efflux pump proteins are significant players mediating antibiotic resistance. Also, it is understood that perhaps there exists a synergy in their actions that contribute to multidrug resistance phenotype. In view of their increasing association with clinically relevant MDR pathogens, several studies have been done on structure-function aspect of these proteins and their regulatory aspects. However, there remained a lot to explore on the role of physic-chemical parameters affecting expression of the efflux proteins.

With this background, aim of the present investigation was focused on "Study of membrane-mediated antibiotic resistance mechanisms and pathogenic potential in clinical and environmental multidrug resistant *Enterobacter* isolates". To achieve this, following objectives were laid down for the study:

1. To determine the occurrence and diversity of efflux pumps in multidrug resistant clinical and environmental *Enterobacter* isolates.
2. To perform comparative study on association of outer membrane proteins in multidrug resistant environmental and clinical *Enterobacter* isolates.
3. To study the effect of physico-chemical environment on expression of AcrAB-TolC multidrug efflux pump in *Enterobacter* isolates.

Chapter 3

Materials & Methodology

3. 1. MATERIALS:

3. 1. 1. Bacterial strains used in the study

Enterobacter isolates of clinical and environmental origin were used in this study as listed in table no. 4.1.1.1. Twenty non-duplicate *Enterobacter* strains were isolated from water sources, near Jamshedpur area, Jharkhand, India and twenty-two *Enterobacter* strains isolated from patients admitted in tertiary care hospitals in and around Bhubaneswar, Odisha were included in the study. Clinical isolates were from urine (n=6), pus (n=6), wound swab (n=4), blood (n=2), tracheal aspirate (n=2) and other sources (n=2) culture. Clinical isolates of *Enterobacter* spp. were identified using automated identification system (Vitek 2.0) at the hospitals. All the bacterial isolates were given a unique laboratory identification code.

3. 1. 2. Media, chemicals and kits used

A] Microbiological Media:

Table 3.1.1: Bacterial growth media and differential media

Medium	Use	Compositions	Gram/liter	Reference
Nutrient Agar (Difco, USA) pH 6.8 ± 0.2	Routine growth and maintenance of non-fastidious bacteria	Beef Extract Peptone Agar	3.0 5.0 15.0	
Luria-Bertani, Miller (Difco, USA)	Growth and maintenance of pure	Tryptone Yeast Extract Sodium Chloride	10.0 5.0 10.0	[163]

pH 7.5 ± 0.2	cultures as well as recombinant strains	Agar	15.0	
Brain Heart infusion Agar (Difco, USA) pH 7.4 ± 0.2	Aerobic bacteriology and specific test	Beef heart infusion Tryptose Sodium chloride Agar	10.0 10.0 5.0 13.5	
Muller-Hinton Agar (Difco, USA) pH 7.3 ± 0.2	Antimicrobial susceptibility testing	Beef extract Acid digest of casein Starch Agar	2.0 17.5 1.5 17.0	
Tryptic Soy Broth (Difco, USA) pH 7.3 ± 0.2	Enrichment medium used in qualitative procedures	Bacto Tryptone Bacto Soyatone Glucose Sodium chloride K_2HPO_4 Agar	17.0 3.0 2.5 5.0 2.5 17.0	
Mac-Conkey agar (Difco, USA) pH 7.1 ± 0.2	Differential media	Pancreatic Digest of Gelatin Peptones Lactose Bile salt Sodium chloride Neutral red Crystal violet Agar	17.0 3.0 10.0 1.5 5.0 0.03 0.001 13.5	
Eosin methylene blue agar (Difco, USA) pH 7.2 ±	Differential media	Pancreatic Digest of Gelatin Lactose Sucrose K_2HPO_4	10.0 5.0 5.0 2.0	[164]

0.2		Eosin Y	0.4
		Methylene Blue	0.065
		Agar	13.5
Columbia blood agar base (Difco, USA) pH 7.3 ± 0.2	Base media used for preparation of blood agar media for growth of fastidious organisms	Pancreatic Digest of casein	10.0
		Proteose peptones No-3	5.0
		Yeast extract	5.0
		Beef heart infusion from 500g	3.0
		Corn starch	1.0
		Sodium chloride	5.0
		Agar	13.5
Decarboxylase broth base (Himedia, India, pH 5.80-6.20)	Differentiate bacteria on the basis of their ability to decarboxylate amino acid.	Peptic digest of animal tissue	5.0
		Beef extract	5.0
		Dextrose	0.5
		Bromocresol purple	0.01
		Cresol red	0.005
		Pyridoxal	0.005
		L-aminoacids	10 .000
Malonate Broth (Himedia, India, pH 6.7±0.2)	Differentiate *Enterobacter* and *Escherichia* on the basis of malonate utilization	Ammonium sulphate	2.0
		Dipotassium phosphate	0.6
		Monopotassium phosphate	0.4
		Sodium chloride	2.0
		Sodium malonate	3.0
		Bromothymol blue	0.025

B] Chemicals & Reagents:

The antibiotics (as presented in table no. 3.1.2.) were supplied in powder form and stored at 4°C for further use. Stock solutions (10 mg/ml or 5 mg/ml) were prepared by dissolving appropriate amounts of these antibiotics in respective solvent in 15 ml sterile beaker and mixed until the antibiotic had dissolved. Dissolved antibiotic solutions were filter sterilized using 0.22µm syringe filter (Milipore, USA) and aliquots were stored at 4°C for immediate use. Appropriate precautions were taken while handling light and temperature sensitive antibiotic powders. The antibiotic disks included in the disc diffusion study along with their potencies and zone diameter break points are listed in table no. 3.1.3.

Table 3.1.2: Antibiotic powders used in this study

Antibiotics	Solvent	Supplier	Catalog No.
Amikacin	Water	Sigma, USA	A2324-5G
Ampicillin	Water	Sigma, USA	A0166-25G
Cefepime	Water	Lupine	
Cefuroxime sodium	Water	GSK	
Cefotaxime sodium	Water	Sigma, USA	C7912-5G
Chloramphenicol	Water	Sigma, USA	C3175-100MG
Ciprofloxacin	0.01N HCl	Fluka, Germany	17850-25G-F
Erythromycin	50% methanol	Sigma, USA	E6376-25G
Gentamycin	Water	USB, USA	16051
Levofloxacin	Water	Sigma, USA	28266-10G-F
Norfloxacin	33% acetic acid	MP biomedical	155949
Tetracycline	Water	USB, USA	22105
Trimethoprim	50% methanol	Fluka, Germany	92131-25G

Table 3.1.3: Details of the antibiotics discs (Hi-Media, India) used in this study

Sl No.	Antibiotics	Code	Conc. Mg	Resistant	Intermediate	Sensitive
1	Ampicillin	A	10	13	14-16	17
2	Amoxyclav (Amoxicillin clavulanic acid)	AMC	30	13	14-17	18
3	Azithromycin	AT/AZM	15	13	14-17	18
4	Azlocillin	AZ	75	17		18
5	Colistin	CL	50	11		12
6	Ceftazidime	CAZ	30	14	15-17	18
7	Chloramphenicol	C	30	12	13-17	18
8	Cefoxitin	CX	30	14	15-17	18
9	Cinoxacin	CIN	100	14	15-18	19
10	Co-trimoxazole	CO	25	10	11 to 15	16
11	Cefuroxime	CXM	30	14	15-17	18
12	Cephalothin	CH/CEP	30	14	15-17	18
13	Ciprofloxacin	CIP	5	15	16-20	21
14	Cefpirome	CFP	30	14	16-17	18
15	Cefepime	CPM	30	14	15-17	18
16	Cloxacillin	CX	10			
17	Cefalexin	CN	30	18	19-20	21
18	Carbenicillin	CB	100	19	20-22	23
19	Ceftizoxime	CZX	30	14	15-19	20
20	Cefotaxime	CTX	30	14	15-22	23
21	Ceftriaxone	CTR	30	13	14-20	21
22	Enoxacin	EN	10	14	15-17	18

23	Furazolidone	FR	50	20	21-22	23
24	Gentamycin	G	120	12	13-14	15
25	Gemifloxacin	GM	5	15	16-19	20
26	Gatifloxacin	GAT	5	14	15-17	18
27	Levofloxacin	LE	5	15	16-18	19
28	Lomefloxacin	LO	10	18	19-21	22
29	Methicillin	M	5	9	10- 13	14
30	Moxifloxacin	MO	5	20	21-23	24
31	Mezlocillin	MZ	75	17	18-20	21
32	Neomycin	N	30	12	13-16	17
33	Nafcillin	NAF	1	10	11-12	13
34	Nalidixic Acid	NA	30	13	14-18	19
35	Norfloxacin	NX	10	12	13-16	17
36	Ofloxacine	OF	5	12	13-15	16
37	Oxacillin	OX	1	10	11-12	13
38	Penicillin G	P	10	28		29
39	Polymyxin B	PB	300	11		12
40	Pefloxacin	PF	5	<=15		<=19
41	Piperacillin	PI	100	17		18
42	Rifampicin* (for 5 mcg)	R	30	16	17-18	20
43	Sparfloxacin	SC	5	15	16-18	19
44	Streptomycin* (for 100 mcg)	S	300	<=11	12.14	>=15
45	Ticarcillin	TI	75	14	15-19	20
46	Tetracycline	T	30	14	15-18	19
47	Trimethoprim	TR	5	10	11 to 15	16

48	Imepenem	IPM	10	13	14-15	16
49	Meropenem	MRP	10	13	14-15	16

Table 3.1.4: List of Chemicals used

Sl. No	Chemical name	Supplier	Catalog No.
1	Acrylamide	Sigma-Aldrich, USA	A8887-500G
2	Agar	Hi-Media, India	GRM666-500G
3	Agarose powder (Mol. Bio grade)	Hi-Media, India	MBOO2-500G
4	Agarose, Low melting EEO	Sigma-Aldrich, USA	A9539-100G
5	Ammonium Persulphate (APS)	Sigma-Aldrich, USA	A3678-25G
6	Bis-Acrylamide	Sigma-Aldrich, USA	M7279-250G
7	ß-Mercaptoethanol	Sigma-Aldrich, USA	M3148-25 ml
8	Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	MP Biomedical, USA	195094
10	Coomassie Brilliant Blue (CBB) R-250	MP Biomedicals, USA	190682
11	Concentrated HCL (6N)	Himedia, India	AS004-500ML
12	Crystal Violet	Fisher Scientific, India	39222
13	Ethanol	Merck, Germany	100983
14	Ethidium bromide	Sigma-Aldrich, USA	E7637-25G
15	Ethylene diamine tetraacetic acid (EDTA)	Affimetrix, USA	4177731
16	Formamide	Sigma-Aldrich, USA	47671-1L-F

17	Formal dehyde	Himedia, India	AS017-500ML
18	FM 4-64-Fx Membrane dye	Invitrogen, USA	F34653
19	Glacial Acetic acid	Fisher scientific, USA	11007
20	Ultrapure Glycerol	Invitrogen, USA	15514-011
21	Glycine	USB chemicals, USA	16407
22	Hi-Di formamide	Applied Biosystems, USA	4311320
23	Lysozyme	USB, USA	18645
24	Methanol	Hi-Media, India	AS061-2.5L
25	Pop7 polymer	Applied Biosystems, USA	4363786
26	Ponceau stain	Sigma-Aldrich, USA	P1710-1L
27	Proteinase-K	USB chemicals, USA	78468
28	PIPES	MP Biomedicals, USA	190257
29	Propan-2-ol/Isopropanol	Hi-Media, India	MB063-1L
30	Sodium chloride (NaCl)	MP Biomedicals, USA	194848
31	Sodium dodecyl sulphate (SDS)	MP Biomedicals, USA	194831
32	TEMED	Sigma-Aldrich, USA	T7024-100ML
33	Tris base	Affimetrix, USA	75825
34	Triton-x 100, cell culture grade	Hi-Media, India	TC286-100ml
35	Tween-20	Sigma-Aldrich, USA	P9416
36	Xylene	Hi-Media, India	AS078

Table 3.1.5: Reagents and buffers used

Sl. No	Reagents	Components	Supplier	Catalog. No.
1	PCR reagent	Go Taq flexi Buffer (5X) $MgCl_2$ (25mM) Go Taq DNA polymerase (500 units/µl)	Promega, USA	M8295
2	DNA sequencing consumables	Sequencing buffer	Applied Biosystems, USA	402824
		Ready reaction mix		4337455
3	DNA ladder	100 base pair	NEB, USA	#N3231L
		1 Kilo base		#N3232L
4	DNA gel loading dyes (6X)		Promega, USA	G1881
5	DNA sequencer Anode/cathode buffer (10X)	Buffer with EDTA	Applied Biosystems, USA	402824
6	Western Blot reprobing buffer		Thermo Fisher Scientific, USA	
9	Prestained Protein Ladder (10-250 kDa)		Thermo Fisher Scientific, USA	26619
10	Supersignal west femto maximum sensitivity substrate (100 ml Kit)		Thermo Fisher Scientific, USA	34095
11	Pierce ECL western blotting substrate		Thermo Fisher Scientific,USA	32109

Table 3.1.6: Cell lines and Cell culture consumables used

Sl. No.	Description	Supplier	Catalog. No.
1	RAW 264.7 cell line	Courtesy: Subhashis Chattopadhyay Lab, NISER	
2	RPMI-1640 w/ L-Glutamine and Sodium bicarbonate 1X Liquid Cell Culture Medium	Himedia, India	AL028A
3	Fetal Bovine serum (FBS Good Forte filtrated bovine serum US origin), 500 ml	PAN Biotech	P40-48500
4	Fetal Bovine serum (FBS Good Forte filtrated bovine serum Australia origin), 500 ml	PAN Biotech	P40-49500
5	Antibiotic Solution 100X Liquid (w/ 10,000 U Penicillin and 10 mg Streptomycin per ml in 0.9% normal saline) Sterile filtered	Himedia, India	A001
6	Amphotericin B Solution (250µg/ml) Cell Culture Tested	Himedia, India	A011
7	Trypsin - EDTA Solution 1X (w/ 0.025% Trypsin and 0.01% EDTA in Dulbecco's Phosphate Buffered Saline) Sterile filtered	Himedia, India	TCL099

Table no. 3.1.7: Antibodies used in the study

Sl. No.	Antibodies	Company	Catalog no.
1	Anti-AcrA Rabbit Polyclonal antibody (1mg/ml)	Genscript, USA	49558-1Custom synthesized
2	Anti-AcrB Rabbit Polyclonal antibody (1mg/ml)	Genscript, USA	Custom synthesized49558-4
3	Anti-TolC Rabbit Polyclonal antibody (1mg/ml)	Genscript, USA	Custom synthesized49558-7
4	Goat Anti-Rabbit Polyclonal IgG secondary Antibody (1mg/ml)	Abcam, USA	ab97051
5	Anti-GroEL Rabbit Polyclonal Antibody (1mg/ml)	Abcam, USA	ab90522
6	Alexa fluor 488,Goat Anti-Rabbit IgG (H+L), (2mg/ml)	Life Technologies	A11008
7	Blocking peptide (custom synthesized for AcrA-AcrB-TolC)	Genscript, USA	U8878-BF130

3. 2. METHODOLOGY:

3. 2. 1. Identification of the isolates

Initial identification of all the bacterial isolates obtained from clinical and non-clinical sources was made by routine microbiological methods. This allow the cultures to grow on various media, based on their growth and phenotype, or morphology, they are categorized into *Enterobacter* isolates. Following biochemical identification, further confirmation was done by 16srRNA sequencing.

3. 2. 1. 1. Enrichment and differential media

Differential media are widely used for isolation and tentative identification of closely related organisms or groups of organisms. Few of them are listed below:

- Eosin methylene blue agar (EMBA) (Difco, USA) is a selective and differential medium for the isolation and differentiation of gram-negative enteric bacilli, in which *Enterobacter* spp. appear as large, mucoid, blue-purple colored colonies. Columbia blood agar (Difco. USA) was Prepared by adding 5% sheep blood in Columbia blood agar base. It is a highly nutritious, general-purpose medium for the isolation and cultivation of non-fastidious and fastidious microorganisms from a variety of sources**.** Colonies can appear α-hemolytic (colony is dark and greenish due to partial hemolysis), or β-hemolytic (complete hemolytic, transparent in nature) and γ-hemolytic (no hemolytic).
- Mac-Conkey agar (Difco, USA) is selective and differential plating media mainly used for the detection and isolation of lactose fermenting and non-lactose fermenting gram-negative organisms. The lactose fermenter will appear as light pink to dark pink, whereas the non-lactose fermenter will be colorless in nature.

3. 2. 1. 2. Decarboxylase media

Decarboxylase broth (Himedia, India) with the addition of appropriate L-amino acids like, L- Lysine, L-Arginine or L-Ornithine, is useful for differentiating gram-negative *Enterobacteriaceae* on the basis of their ability to decarboxylate amino acids. Decarboxylase broth also contains nutrients, dextrose (a fermentable carbohydrate), pyridoxal (an enzyme cofactor for decarboxylase), and the pH indicators bromcresol purple and cresol red. If an organism is able to decarboxylate the amino acid present in the medium, alkaline byproducts are then produced, as a result of which bromocresol purple turns purple at an alkaline pH. If the inoculated medium is yellow, or if there is no color change, the organism is decarboxylase-negative for that amino acid.

3. 2. 1. 3. Malonate broth utilization

Malonate Broth is recommended for the differentiation of *Enterobacter* and *Escherichia* on the basis of malonate utilization., where While *Enterobacter* utilizes malonate and *Escherichia coli* does not. An organism that can simultaneously utilize sodium malonate as its carbon source and ammonium sulfate as its nitrogen source produces alkalinity due to the formation of sodium hydroxide. The alkali changes the color of the bromothymol blue indicator in the medium to light blue and finally to prussian blue. The color of the medium remains unchanged in the presence of an organism that cannot utilize these substances.

3. 2. 2. 16S rRNA sequencing and analysis

Following phenotypic characterization, the genus level identification of isolates was done by phenotypic and subsequent 16S rRNA sequencing. A 1500 bp region of the 16SrRNA gene was PCR amplified using universal bacterial 16S primers [165], Bact_63f_62C 5'–CAGGCCTAACACATGCAAGTC-3' and Bact_1389r_63C 5'–ACGGGCGGTGTGTAC AAG–3'. Single colony of pure culture from LB agar plate

was mixed with 500µl of sterile Milli-Q water and cell lysate was prepared which was used directly as template DNA.

Amplified PCR product was run in 1.2% agarose gel in 1X TAE at 60V with appropriate DNA markers (NEB, USA) and bands visualized in Chemidoc (Bio-Rad, USA).

Table 3.2.1: Components used for PCR amplification of 16S rRNA gene

Components	Volume (µl)
5X buffer	5.0
dNTP (10mM@ 2.5mM each)	2.0
Primer F(10µMolar)	1.5
Primer R(10µMolar)	1.5
Template	1.5
Go Taq polymerase	0.5
H_2O	13.0
Total volume	25

Table 3.2.2: PCR reaction condition of 16S rRNA gene amplification

Steps	Temperature	Time	No. of cycles
Initial Denaturation	94°C	2 min	1
Denaturation	94°C	1 min	
Primer Annealing	54°C	1 min	35
Primer Extension	72°C	1 min	
Final Extension	72°C	10 min	1

PCR amplified products of 16S rRNA gene (1500bp) were purified using QIAquick@ Gel Extraction Kit following manufacturer's instructions and quantity and quality of eluted DNA was observed checked using Nanodrop (Thermofisher, USA) and agarose gel electrophoresis respectively. Nucleotide sequences were determined using the BigDye Terminator v3.1 Cycle Sequencing Kit as perfollowing the manufacturer's protocol, in an automated 3130XL Genetic Analyzer (Applied Biosystems, USA).

Bacterial isolates were assigned genera based upon the non-chimeric sequences that showed greater than 97% identity following BLAST alignment with the NCBI nucleotide database, which is used for identifying the bacterial isolates. The 16S rRNA gene sequences of all environmental *Enterobacter* isolates were submitted at NCBI GenBank bearing assigned accession numbers (JQ912514 to JQ912531).

3. 2. 3. Preservation and storage of bacterial culture

3. 2. 3. 1. Soft agar preparation

Bacterial isolates were inoculated onto LB agar plates and incubated overnight at 37ºC. Soft agar was prepared by LB broth with 0.8% bacteriological agar, was autoclaved at 15 lbs psi at 121°C for 15 min and 3-3.5ml of LB soft agar was poured into 5 ml cryovials (Tarsons, India) and allowed to solidify. Single colony was picked using inoculation rod and stabbed into soft agar tube. The inoculated tubes were labeled, incubated at 37ºC overnight, sealed with parafilm and subsequently stored at room temperature for further use.

3. 2. 3. 2. Glycerol stock preparation

Bacterial strains were inoculated onto LB agar plates and incubated overnight at 37ºC. A single colony was inoculated into 2ml sterile LB broth and incubated for 6-8 hours till log phase ($O.D._{600}$nm- 0.8). 800µl Eight hundred µl of this culture and 200µl of autoclaved glycerol (100%) were added into 1.8ml sterile cryovials (Tarsons, India). The cryovials were mixed by gentle vortex, sealed with parafilm, labeled and stored at -80ºC immediately.

3. 2. 4. Determination of antibiotic susceptibility by disc diffusion method

3. 2. 4. 1. Antibiogram of environmental and clinical *Enterobacter* isolates

Each of the bacterial strains was tested for susceptibility to different groups of antibiotics, using the disc diffusion method described by [166]. Bacterial colonies from Mueller-Hinton agar (MHA) plates were inoculated into a tube containing 2ml of Muller Hinton broth (MHB) (Himedia-India). The culture was incubated at 37ºC and 220 rpm for 6-8 hours till the $O.D._{600\ nm}$ reaches to 0.6 to 0.8 corresponding to approximately 2 x 10^8 CFU/ml. Bacterial lawn culture was made using a sterile cotton swab to spread the inoculum over the entire surface of MHA plates to make an uniform bacterial suspension. Antibiotic discs were then put on equidistant from each otherly using sterile forceps and held in place by pressing down the discs on the agar plates properly. The plates were then incubated overnight at 37°C in incubator (New Brunswick, USA). After incubation each plate was examined and zone of inhibition were measured using millimeter range scale (Hi-Media, India). The diameter of the inhibition zones was interpreted following CLSI standard guidelines and standards [167].

3.2. 4. 2. Antibiogram of ATCC control strains using Dodeca discs

Antibiotic susceptibility assay was also performed determined for ATCC strains under study i.e. *Enterobacter cloacae* ATCC 13047, *Enterobacter aerogenes* ATCC 13048, *Enterobacter cloacae* ATCC MDR BAA-1143, *Escherichia coli* ATCC 25922 and *Escherichia coli* ATCC MDR BAA-2469. However, we utilized Dodeca antibiotic disc (Himedia, India) and followed the same protocol mentioned earlier above.

3. 2. 5. Minimum inhibitory concentration (MIC) determination

3. 2. 5. 1. MIC of antibiotics in presence or absence of CCCP

Minimum inhibitory concentrations (MICs) are considered the `gold standard' for determining the antimicrobial susceptibility of organisms [168]. MIC of all *Enterobacter* isolates was tested by broth-double dilution method following the CLSI guidelines [167]. Minimum inhibitory concentrations (MICs) are considered the `gold standard' for determining the antimicrobials susceptibility of organisms [168]. MIC of all *Enterobacter* isolates were tested by broth-double dilution method following the CLSI guidelines [167]. The bacteria were grown in Muller-Hinton broth (MHB) to mid exponential phase to get turbidity 0.5 McFarland standards. Minimum inhibitory concentrations were performed by inoculating 10µl culture in 2ml of MHB containing the antimicrobial agents at different concentrations. The MIC was deemed to be the lowest antibiotic concentration that inhibits all visible growth.

To determine the possible effect of efflux pumps on the minimum inhibitory concentration (MIC) of antibiotics, MIC was determined by broth-double dilution method, in the presence or absence of efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at an optimized concentration of 50µMolar. Effect of CCCP (50 µMolar) on growth of *Enterobacter* isolates was determined. MIC results were interpreted following breakpoints as per CLSI 2014 guideline. Appropriate controls without CCCP and antibiotics were also included. Two or more fold decrease of MIC in presence CCCP was interpreted positive for efflux pump mediated antibiotic resistance [169].

3. 2. 6. Effect of pH dependent on MIC of antibiotic susceptibility

To check ascertain the role effect of pH on efflux activity, the same MIC was determined at different pH viz. 4.0, 6.0, 7.0 and 8.0 using protocol as mentioned previously, albeit in absence of without efflux pump inhibitor CCCP and at different pH viz. 4.0, 6.0, 7.0 and 8.0. Bacterial isolates grown in Mueller Hinton broth at pH 7.0±0.2 with OD_{600}nm of 0.6 to 0.8 was used for inoculation into 2ml of MHB of mentioned specified pH (i.e. 4.0, 6.0, 7.0 and 8.0) containing appropriate dilutions of antibiotics. Appropriate controls without antibiotic for every each pH were also included. Change in MIC values were recorded and confirmed by spread plating the cultures onto Mueller Hinton agar (MHA) plates and determining the colony forming units (CFU). Change in the MIC value with varying pH, was indicative of pH-dependent modulation of efflux activity.

3. 2. 7. Sodium (Na^+) dependent MIC of antibiotics

As few efflux pumps like MATE family proteins use sodium (Na^+ ions) gradient as their energy source, the effect of increasing Na^+ ions (in the form of NaCl) on the MIC was assessed. To find out the effect of Na^+ on efflux activity, bacterial cultures were grown till mid-log phase, incubated into MHB containing range of antibiotics with and without CCCP supplemented with varying concentrations of NaCl (0mM, 50mM, 100mM). The cultures were incubated at 37ºC overnight under standard incubation conditions. The experiment was conducted with two biological duplicates and three technical replicates each of the above-mentioned conditions. Bacterial growth was subsequently judged by visual inspection of turbidity in comparison to appropriate controls, followed by OD and CFU determination.

3. 2. 8. pH dependent Ethidium bromide exclusion assay

Further to confirm the pH dependent modulation of efflux, we determined ethidium bromide exclusion as a function of varying pH viz. 4.0, 6.0 and 8.0. Briefly, bacterial isolates were grown in 2 ml of Mueller Hinton broth till OD_{600nm} of 0.6 to 0.8. One ml of culture aliquot was centrifuged at 13,200 rpm for 5 minutes. The pellets were re-suspended in PBS buffer of specific pH mentioned above (i.e. 4.0, 6.0 and 8.0), followed by addition of ethidium bromide (EtBr) to a final concentration of 0.5µg/ml. The cultures were incubated at 37ºC, 220 rpm for 1 hour. Following incubation, 50µMolar CCCP was added and further incubated for 30 minutes under same conditions as mentioned above. Appropriate controls at pH without EtBr were included. The tubes were then centrifuged at 13,200 rpm for 2 minutes at 4ºC (Eppendorf, Germany).

The bacterial pellet was washed twice with PBS of respective pH to remove excess and adsorbed EtBr. Finally, the cell pellet was suspended in 1 ml of PBS of specific pH and incubated for 30 minutes at 37ºC and 220 rpm. The culture was finally centrifuged to collect supernatant that represented EtBr effluxed out of the system. Ethidium bromide effluxed at different pH was quantified by fluorimetry detection at 620 nm wavelength (Perkin-Elmer LS55 fluorescence spectrophotometer).

3. 2. 9. Virulence factor determination by microbiological assay

3. 2. 9. 1. Serum resistance assay

Normal Human serum (Millipore, India) was aliquoted into two sets of tubes; out of which one set was heat-inactivated at 56ºC for 30 minutes. Briefly, bacterial cultures were incubated overnight in LB at 37ºC, 220 rpm and then diluted to 1:100 using fresh LB and allowed to grow further till $O.D._{600}$ reaches 0.6-0.8. Bacterial cell suspension

was then prepared using sterile 1X PBS buffer pH 7.4. The plates were incubated at 37ºC for 24 hours. Serum resistance profile was categorized into grade-1 being non-resistant to grade-6 with highest level of resistance according to [170], as described in table no. 3.2.3.

Table 3.2.3.: Interpretation of serum resistance test

Grade	Viable counts	Viable counts	Interpretation
1	< 10% after 1 and 2 hours	<0-1% after 3 hours	Highly sensitive
2	10-100% after 1 hour	< 10% after 3 hours	
3	> 100 % after 1 hour	< 100% after 2 and 3 hour	Intermediatel y susceptible
4	> 100 % after 1 and 2 hours	< 100% after 3 hour	
5	>100% after 1, 2 and 3 hour	fell at some time during the 3 h period	Resistant
6	>100% after 1, 2 and 3 hour	Rise in cell count throughout 3 h	

3. 2. 9. 2. Biofilm assay

The biofilm formation ability of both clinical and non-clinical *Enterobacter* isolates was determined as described by [171] with some modifications. Briefly, overnight bacterial cultures grown in Brain Heart Infusion (BHI) broth (Himedia, India) were diluted to 1:100 with fresh BHI medium and 200ul of this was added to 96 well flat bottom tissue culture plate. Four technical replicates for each isolate were taken along with unincoulated media as control. The culture plates were incubated at 37ºC for 24 hours and 48 hours at 50 rpm to mimic water flow in aquatic environment. Following incubation, plates were washed thrice with 1X PBS to remove free-floating bacteria

and dried at room temperature. Again, cells were fixed with methanol for 15 min and stained with 0.1% Crystal Violet solution (Fisher Scientific, Mumbai, India) to stain the bound bacteria. The plates were subsequently incubated at room temperature for 30 min before excess dye was removed by washing with water. The bound dye was finally dissolved in 33% acetic acid and optical densities of isolates and control were measured at 595 nm in an Elisa Reader (iMark Microplate Reader, Biorad, USA). We defined the cutoff of OD for test samples, where ODc is the control with standard deviations from five technical replicates. Isolates were then classified as follows: OD < ODc – non-adherent, ODc < OD < 2X ODc –weakly adherent, 2 ODc < OD < 4X ODc – moderately adherent and 4X ODc < OD– strongly adherent [171].

3. 2. 9. 3. Haemagglutination assay

The presence of type 1 fimbriae (mannose-sensitive hemagglutination [MSHA]) at the bacterial cell surface was assessed using commercial baker's yeast (*Saccharomyces cerevisiae*) suspended in phosphate-buffered saline (5 mg dry weight per ml) as described by Claire [172]. Bacteria were grown under static conditions and after 48h, 50µl of bacterial suspension (approximately 10^{11} bacteria/ml) and 50 µl of *Saccharomyces cerevisiae* culture were mixed in sterile transparent glass slides, mixed well and observed for 3 mins at room temperature for visible clumping. Aggregation was monitored visually and interpreted with cells forming clumps to be positive for presence of type-1 fimbriae accordingly.

3. 2. 10. PCR screening of efflux pumps and outer membrane proteins

3. 2. 10. 1. Preparation of bacterial cell lysate for PCR

Overnight grown pure culture on nutrient agar plate was inoculated onto 2 ml of nutrient broth and incubated at 37ºC 220 rpm till late log phase (6-8 hours). Cultures

were centrifuged and bacterial pellet was washed once with 1X PBS pH 7.4. Finally cells were dissolved in 500µl of autoclaved Milli-Q water, lysed at 94ºC for 10 minutes, and then immediately chilled on ice for 15 minutes. Bacterial suspension was then centrifuged at 13,200 rpm for 5 minute at 4ºC. This cell lysate supernatant was stored at -20ºC until further use as template DNA for PCR.

Table no. 3.2.4: Oligonulceotides used for screening of efflux pump genes

Gene	Primer	Primer sequence (5'-->3')	Primer Length	Amplicon size (in bp)
AcrA	AcrA-Full-F	TACAGGATGTGACGACAAACAG	22	1220
	AcrA-Full-R	CCCGGCCAGCATGATAATAA	20	
AcrB	AcrB-Full-F	CCATGACGCAGGAGGATATTT	21	2704
	AcrB-Full-R	CAGTGAGGTTCTACCGAATGAC	22	
	TolC-Full-F	GGGACATGACATGGGAAAGA	20	1236
TolC	TolC-Full-R	CGTGAGCCAAGGTCAAGATA	20	
	Mate-Full-F	TTGTCGATACCGTGATGGCAGG	22	465
MATE	Mate-Full-R	AGTTAAGCGGGATGTTCAGCA	22	
-Full		G		
MATE	Mate-F	GCGTACGATGCGCGATATCC	20	105
	Mate-R	AACAGCGCCAGAGCAATGGG	20	
	Mfs-F	GCTGGGCGTGATTTCAATGG	20	107
Mfs	Mfs-R	CCTGCGGACGCATTAATACC	20	
Smr	Smr-F	ACAATTCCGATGGGGATTGC	20	106
	Smr-R	ATGCTGCCACGTCTAAATGC	20	

3. 2. 10. 2. PCR screening of efflux pumps

Presence of efflux genes belonging to different families e.g. RND superfamily (*acrA*, *acrB* and *tolC*, *Mate* (MATE superfamily), *Mfs* (MFS family) and *Smr* (SMR family) were determined sequentially by PCR with primers given in Table no. 3.2.4. 30 µl of PCR reaction mixture contained 3µl of (10X) PCR buffer (NEB, USA), 2 µl of

(2.5mM/dNTP) dNTP mix (Promega, USA), 1 µl of 25mM MgCl2, 1.5 µl each of (10µMolar) forward and (10µMolar) reverse primer, 2µl of template DNA and 0.125µl (2,000U) Taq DNA polymerase (NEB, USA) in a thermal cycler (Eppendorf, Germany).

3. 2. 10. 3. Multiplex PCR setup for detection of OMPs

To simultaneously detect the presence of genes belonging to different outer membrane proteins such as *OmpA*, *OmpF*, *OmpX*, *OmpC*, *LamB* & *FhuA*; a multiplex PCR was developed using primers listed below in table no. 3.2.5.

Hundred microlitre of reaction mixture contained 20 µl of (5X) Gotaq flexi buffer (Promega, USA), 2 µl of (2.5mM/dNTP) dNTP mix (Promega, USA), 2.5 µl of 25mM MgCl2, 1.5 µl each of (10µMolar) forward and (10µMolar) reverse primer belonging to six OMPs genes, 2.5 µl of template DNA and 0.2 µl (100U) of Gotaq flexi DNA polymerase (Promega, USA) in a thermal cycler (Eppendorf, Germany). PCR was programmed as follows: an initial denaturation at 94ºC for 2 min, followed by 35 cycles consisting of denaturation at 94ºC for 45 secs, annealing at 53ºC for 45 secs, and extension at 72ºC for 45 secs, with final extension at 72ºC for 10 mins.

3. 2. 10. 5. Agarose gel electrophoresis for PCR products

The PCR products were separated on 1-1.2% agarose gel (Hi-media, Mumbai) prepared in 1X TAE (Tris- Acetate-EDTA) buffer (pH 8.0-8.5) at 4 V/cm with ethidium bromide at a final concentration of 0.5µg/ml and visualized using a gel documentation system (Biorad, USA).

Table no. 3.2.5: **Oligonucleotides used in multiplex PCR of OMPs**

Gene	Primer	Primer sequence (5'-->3')	Primer Length	Amplicon size (in bp)
OmpF	OmpF-F2	GATCTGTACGGGAAAGCAGTT	21	1001
	OmpF-R2	GCCGAAGCCCTGTTCATTA	19	
OmpC	OmpC-F3	AGGGTTAATCAGTAAGCAGTGG	22	-
	OmpC-R3	AGGGTGAATTGTAAGAACCGAA	22	
OmpA	OmpA-F2	GGATGATAACGAGGCGCAAA	20	1196
	OmpA-R2	CAACCAGATGTCTACGCTGAAG	22	
OmpX	OmpX-F1	GGATTTACTTGAAGCACATTTGAGG	25	~500
	OmpX-R1	CCGAAGTGATTAGAAGCGGTAA	22	
FhuA	FhuA-F3	TCTTCCGTGACGCTTCATTC	20	-
	FhuA-R3	ACAACGTACCCTGGCAAATAA	21	
LamB	LamB-F3	CCCAGACGCTTTACCAGATT	20	419
	LamB-R3	GGCCTTCTGGCATCTCTTTAT	21	

3. 2. 11. Slot blot hybridization of efflux pumps and OMPs

3. 2. 11.1. Bacterial genomic DNA extraction using Gentra Puregene Bacteria/yeast DNA isolation kit

For genomic DNA purification isolation forom gram-negative bacteria, gentra Gentra puregene bacteria isolation kit (Qiagen, USA**)** was used. From an overnight grown culture on LB broth, 500µl (containing approximately 0.5-1.5x10^9 cells) was transferred to 1.5 ml microcentrifuge tube on ice and then centrifuged for 2 mins at 13,000 rpm to pellet cells. Pellet was mixed with 300 µl of cell lysis solution by pipetting up and down. Samples were incubated at 80ºC for 5 min to lyse the cells. To this, 1.5 µl of RNase A solution was added, and mixed by inverting 25 times and incubated for 60 mins at 37ºC. Samples were snap-chilled for 1 min on ice. To this,

100 µl of protein precipitation solution was added and vortexed vigorously for 20 secs at high speed. It was then centrifuged for 3 min at 13,000 rpm to form tight pellet of precipitated proteins. 300 Three hundred microlitreµl of isopropanol was taken in a clean 1.5 ml microcentrifuge tube and carefully add the supernatant from the previous step was added to it carefully and the tube was mixed properly by inverting gently at least 50 times. The solution was centrifuged for 1 min at 13,000 rpm, after which the supernatant was carefully, discarded leaving the DNA pellet. 300 µl of 70% ethanol was added and inverted several times to wash the DNA pellet. Finally, tube was centrifuged for 1 min at 13,000 rpm; supernatant was discarded and allowed to air dry for 5 mins at room temperature. To the DNA pellet, 50-100 µl of DNA Hydration solution was added and vortexed for 5 secs at medium speed, and incubated at 65ºC for 1 hour to dissolve the DNA. The genomic DNA obtained was further quantified using Nanodrop (Thermo Fisher, USA) and quality checked by subjecting to 260/280 ratio as well as visually on a 0.8% agarose gel electrophoresis running on a 0.8% agarose gel electrophoresis assessed quality.

3. 2. 11. 2. Probe Preparation, DNA hybridization for blotting

200 ng of genomic DNA were lysed with equal volume of denaturation buffer (0.5 M NaOH, 1.5 M NaCl). Slot blots were prepared with nylon filters (Hybond; Amersham International, London, UK) using PR 648 Slot Blot Manifold, 48-well (GE healthcare Life sciences, USA) and neutralized in neutralizing solution (0.5 M Tris-HCl, pH 8.0, 1.5 M NaCl). Finally, the liberated DNA was fixed to nylon membranes by exposure to UV light for 1 min (1800×100 uJ/cm^2) in a UV-crosslinker, in accordance with the manufacturer's instructions. All of the PCR-amplified products OMP genes were sequenced to confirm their identity and then they were used as probes by random labelling with [α-^{32}P] dCTP (3,000 Ci/mmol, BARC, Bombay, India) and hybridized

at 65ºC in phosphate buffer containing 500 mM Na_2HPO_4 (pH 7.2), 7% (wt/vol) sodium dodecyl sulfate, 1 mM EDTA, and 1% (wt/vol) bovine serum albumin.

3. 2. 11. 3. Washing and detection

Hybridized blots were washed once in 2× SSC buffer (1× SSC is 0.15 M NaCl with 0.015 M sodium citrate) for 5 min at room temperature, two times in 2× SSC-0.1% sodium dodecyl sulfate for 10 min at 65ºC, and once in 0.1× SSC-0.1% sodium dodecyl sulfate for 15 min at 65ºC. Autoradiographs were developed from the hybridized filters with the Bio-Rad Phosphor Imager screen (Bio-Rad, USA) and visualized in a Phosphor Imager (Bio-Rad, USA).

3. 2. 14. Bacterial growth curve determination

To assess the growth kinetics of the organisms under study, bacterial cultures were freshly revived on Tryptone soy agar (TSA, Himedia, India). Single, pure colony was then inoculated onto 5 ml of sterile Tryptone soy broth (TSB, Himedia, India) overnight at 37ºC and 220 rpm. O.D. at 600 nm was measured for this culture and it served as the primary inoculum to inoculate the fresh media at 1:100 dilutions (a 50 ml of TSB in a flask). The cultures were incubated at 37ºC and 220 rpm for 24 hours. O.D. at 600nm was determined at an interval of 1 hour by aliquoting 1.2 ml of culture. This was followed by dilution spread plating the culture onto TSA plates in duplicate. Plates were incubated at 37ºC overnight and the Colony forming unit (CFU) was calculated. Both spectrophotometric and CFU readings was used analyze bacterial growth.

3. 2. 12. RNA extraction, cDNA synthesis and real time PCR

3. 2. 12. 1. *Buffers needed:*

Table no. 3.2.6: 10X Formaldehyde agarose gel running buffer/10X MOPS buffer

Components	For 1 litre
200mM 3-N-morpholinopropanesulfonic acid (MOPS)	41.9 gms
Sodium acetate (50mM)	8.2 gms
EDTA (10mM)	3.72 gms

Dissolve in 800ml of double-distilled water & adjust the pH to 7.0 with conc. NaOH (10M). Make up the volume afterwards.

Table no. 3.2.7: 1X Formaldehyde agarose gel running buffer

Components	For 1 litre
10X MOPS buffer	100 ml
37% Formaldehyde (12.3M)	20 ml
Rnase- Free water	880 ml

Table no. 3.2.8: 5X RNA gel loading buffer

Components	For 5 ml
Saturated aqueous bromophenol blue solution	8 µl
500mM EDTA (pH-8)	40 µl
37% Formaldehyde (12.3M)	360 µl
100% glycerol	1 ml
Formamide	1542 µl
10X formaldehyde agarose gel buffer	2 ml
Rnase free water	Up to 5 ml

3. 2. 12. 2. RNA extraction using bacterial RNA-protect & RNeasy mini spin kit
(Utilizing Protocol-4: for gram-negative bacteria, cell lysis by proteinase-k and lysozyme; followed by protocol-7 for RNeasy minikit, Qiagen, USA)

Bacterial cultures were inoculated into 2 ml of Luria-bertani (LB) broth and grown at 37ºC and 220 rpm till mid log phase when the O.D. reaches 0.8 to 1.0 (~12×10^8 cells/ml). In a 2 ml RNase-free micro centrifuge tube, 500 µl of culture was added to 1 ml of RNA protect bacteria reagent and mixed by vortexing for 5 secs, incubated for 5-10 minutes at room tempertaure. The tube was centrifuged for 10 minutes at 5000g and supernatant was discarded. The bacterial pellet was resuspended with 100-200 µl of TE buffer containing Lysozyme and 10-20 µl of Qiagen Proteinase-K by pipetting up and down several times. Tubes were incubated at room temperature for 10 minutes with intermittent vortexing every 2 minutes. To this, 350 µl of buffer RLT (containing β-mercaptoethanol) was added, vortexed vigorously and centrifuged for 2 mins at max speed. Two hundred fifty microlitre of ethanol (96-100%) was added in a separate RNase-free 1.5 ml microcentrifuge tube to which the supernatant was added and mixed by pipetting. Now the lysate (~700µl) was transferred to a RNeasy mini spin column and spinned at >10,000 rpm for 15 secs. Flow through was discarded and add 700 µl of buffer RW1 was added to column and spinned at >10,000 rpm 15 secs. 40-80 µl of DnaseI enzyme incubation mix was added to the RNeasy silica gel membrane and allowed to stand for 15 minutes at the tabletop. 350 µl of buffer RW1 was added to column, kept for 30 secs and spinned down at >10,000 rpm 15 secs. The column was washed twice with 500 µl of buffer RPE and centrifuged at >10,000 rpm for 1 min. Then the column was placed in 1.5 ml collection tube and 30-50 µl of RNase free water was added to column and incubated for 1 min. Finally it was centrifuged at >10,000 rpm for 1min to elute the RNA, which was stored at -80ºC.

3. 2. 12. 2. Quantification of RNA

The concentration of RNA was determined by measuring 1µl of eluted RNA in the spectrophotometer with absorbance at 260 nm. Pure RNA has A260/A280 ratio in the range of 1.9–2.1. Denaturing agarose gel electrophoresis (1.2% formaldehyde agarose gel) was used to check the integrity and quality of total RNA. Formaldehyde in the gel disrupts secondary RNA structure so that RNA molecules can be separated by their charge migration. The concentration of RNA was determined by measuring 1 µl of eluted RNA in the spectrophotometer with absorbance at 260 nm (A260). Pure RNA has an A260/A280 ratio of 1.9–2.1. Denaturing agarose gel electrophoresis (1.2% formaldehyde agarose gel) was used to check the integrity and quality of total RNA. Formaldehyde in the gel disrupts secondary RNA structure so that RNA molecules can be separated by their charge migration.

3. 2. 12. 3. cDNA synthesis using superscript III reverse transcriptase

SuperScriptTM First-Strand Synthesis System for RT-PCR using random hexamer primers (Invitrogen, USA).

The RNA/primer mixture was prepared by adding the reagents as mentioned in table no. 3.2.9; then incubated at 65ºC for 5 minutes and placed on ice for at least 1 minute.

Table no. 3.2.9: Master mix preparation for RNA-primer mix

Components	Amount
RNA	n µl
10 mM dNTP mix	1 µl
Randon hexamers (50 ng/ μ l)	1–5 µl
DEPC-treated water up to	10 µl

In a separate tube, the following 2X reaction mix was prepared as mentioned below in table no.3.2.10. Nine microlitre of this 2X reaction mix was added to each tube containing RNA/primer mixture and incubated at room temperature for 2 minutes.

Table no. 3.2.10: Master mix preparation for cDNA synthesis

Components	For 1 Reaction (Rxn)
10X RT buffer	2 µl
25 mM MgCl2	4 µl
0.1 M DTT	2 µl
RNaseOUT ™ (40 U/µl)	1 µl

To this, 1 µl of SuperScriptTM II RT (50 U/µl) was added to each tube and further incubated at room temperature for 10 minutes. Tubes were then incubated at 42ºC for 50 minutes and the reaction was terminated by incubation at 70ºC for 15 minutes. Tubes were chilled on ice and the reaction was collected by brief centrifugation. To remove RNA complementary to the cDNA, 1 µl (2 units) of *E. coli* RNase H was added and incubated at 37ºC for 20 minutes. The cDNA was stored at -20ºC for further use or used for PCR immediately.

3. 2. 12. 4. Real time PCR using double delta Ct relative quantification method

The cDNA obtained were used as template for setting up real time PCR. The SYBR® Green PCR Master Mix was supplied as a 2X concentration and contains SYBR® Green I Dye, AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, Passive Reference, and optimized buffer components. The primers used for real time PCR are listed below in table no.3.2.11. Before setting up the real time PCR with the template, working solution of primers (250nM) was prepared afresh in nuclease free water. Prerequisite control module was run to validate the endogenous as well as target primer pair's efficiency, melting curve and Tm parameters. After this, standard curve

using various concentrations of cDNA (1ng, 10ng, 50ng and 100ng), was generated to determine dynamic range of Ct value in relation to template dilution.

Table no. 3.2.11: Primers used in Real time PCR study

Oligo Name	Sequence (5'-3')	Length	Amplicon Size (in bp)
AcrA-F	GCGGTCGTATTGGTAAATCC	20	112
AcrA-R	TGCGTCACGTCAACGTAG	18	
AcrB-F	TTCAGAAAGGCGGTCACG	18	107
AcrB-R	GCGCAGAATGTTCCCTAC	18	
TolC-F	GACCGATGCCCGTATCGAAG	20	115
TolC-R	CACCTCGTTGAGGGTGTTCC	20	
RpoB-F	AGAACAACCCGCTGTCTGAG	20	119
RpoB-R	ACCGTAGTGAGTCGGGTGTA	20	

After standardizing the necessary parameters, real time experiment was set up taking three biological replicate with three technical repeats each. The 20 µl of reaction mix was prepared as mentioned below:

Table no. 3.2.12: Reaction mixture for Real time PCR

Components	Amount
2X SYBR Green master Mix	10 µl
Forward primer (250 nanomolar)	1 µl
Reverse primer (250 nanomolar)	1 µl
Template cDNA (10 ng/µl)	3 µl
Nuclease Free water	5 µl

3. 2 .12. 5. Statistical analysis of real time data.

The comparative CT Method (ΔΔ CT Method) was utilized to achieve the result for relative quantitation in terms of fold change in expression. Firstly, the mean CT value and standard deviation values of the replicate sample were calculated. The ΔCT value was calculated (ΔCT = CT target – CT reference), followed by standard deviations of the target and reference values using the formula:

S = (s12+ s22)1/2; where X1/2 is the square root of X and S= standard deviation.

The ΔΔCT is then calculated by: ΔΔCT = ΔCT test sample – ΔCT calibrator sample. Fold-differences calculated using the ΔΔCT method is usually expressed as a range. The range for targetN, relative to a calibrator sample, is calculated by: 2–ΔΔCt with ΔΔCT +s and ΔΔCT – s, where s is the standard deviation of the ΔΔCT value.

3. 2. 13. High performance liquid chromatography for antibiotics

Stability of antibiotics, Cefotaxime and Cefuroxime, at different pH was determined by ZORBAX eclipse XDB HPLC (Agilent technologies, USA) using C18 column in a binary gradient pump of mobile phase- acetonitrile and 10milimolar phosphate buffer. Stock solutions of antibiotics (Cefuroxime sodium-CXM & Cefotaxime sodium salt-CTX) were prepared freshly using HPLC-grade water. The stock solutions were stored at -80ºC till further use. Standard points for antibiotics (512, 256, 128 μg/ml) were prepared in 2 ml HPLC-grade water by accordingly diluting the stock solution. All the standard antibiotic solutions were stored at -20ºC until further use.

HPLC run module:

Acetonitrile, Water and ortho-phosphoric acid of HPLC grade was filtered through 0.22μ membrane filter and transferred into 500 ml sterile bottles (inside fume hood).

Ten miliMolar phophoric acid solution (sodium) was prepared using HPLC grade water and pH was adjusted to 2.6 using 85% ortho-phosphoric acid and then filtered using 0.22µ membrane filter. Acetonitrile and 10 miliMolar phosphate buffer (pH 2.6) was degassed for 20 mins using ultrasonication. The mobile phase alone was pumped into HPLC column for approximately 30 minutes till a baseline was stabilized.

After observing a stable baseline, 20 µl of HPLC-grade water was injected using a clean Hamilton syringe as blank and run with the mobile phase for 20 minutes. Standard samples (25 µl) and experimental samples (25 µl) were then loaded, Chromatogram peaks were observed and the retention time were noted down. The retention time of each pH was compared with that of neutral pH 7.0 to determine the stability of drugs subjected to diverse pH. HPLC program parameters were set up as below.

Data acquisition- Photometric diode array (PDA) HPLC detectors (at 254 nm). Total flow- 1ml/min, Run time- 21 min. Pump mode- binary gradient Column oven- 25ºC.

Table no. 3.2.13: HPLC Gradient elution programme

Time	Acetonitrile conc.	Phosphate buffer conc.
0	7%	93%
6	19%	81%
16	49%	51%
20	7%	93%
21	---	Stop

3. 2. 15. Western blotting technique using bacterial cell lysate

3. 2. 15. 1. Starting material and buffers preparation

A] 1.5 M tris Tris HCL HCl (pH 8.8): 18.15 grams of Tris base was added to 50 ml of miliQ water. The pH was adjusted to 8.8 with 6N HCl and volume was made up to 100ml. Buffer was filter sterilized using vacuum pump before before usagefurther use.

B] 0.5 M Tris HCL HCl (pH 6.8): 3 grams of Tris base was added to 30 ml of miliQ water. The pH was adjusted to 6.8 with 6N HCl and volume was made up to 50ml. Buffer was filter sterilized before further usage.

C] 10X SDS PAGE (Tris glycine buffer): 250 mM Tris, 192 M glycine, 1% SDS:, pH 8.3 30.0 grams of Tris base, 144.0 grams of glycine, and 10.0 grams of SDS was dissolved in 1000 ml of H_2O. The pH of the buffer should be 8.3-8.5 and no pH adjustment was required. The running buffer was filtered and stored at room temperature and diluted to 1X before use.

D] Transfer buffer (Semi-dry): 25mM Tris, 192mM glycine, 20% (v/v) methanol pH 8.3: 5.8 grams of Tris base, 2.9 grams of glycine was added to 300 ml of distilled water. 200 ml of methanol was added and volume was made up to 1000 ml with H_2O.

E] 10X Tris buffered saline (TBS) pH 7.6: For 1000 ml of TBS buffer, 24 grams of Tris base, 88 grams of NaCl was dissolved in 900 ml of distilled water and pH was adjusted to 7.6 with 12 N HCl. Volume was made up to 1000 ml. For working 1x solution, mix 1 part of the 10x solution with 9 parts of distilled water. The final molar concentrations of the 1x TBS solution are 20 mM Tris and 150 mM NaCl.

F] Table no. 3.2.14: 5X Laemelli buffer/5X SDS-loading buffer composition:

Components	For 10 ml
0.5 M Tris-HCl, pH 6.8	1.75 ml
Glycerol (50%)	2.25 ml
SDS (10%)	1 ml
0.25% Bromophenol blue	0.5 ml
ß-Mercaptoethanol	1.25 ml

3.2. 15. 2. Bacterial whole cell lysate preparation

Following experimental set up as described earlier, bacterial isolates were grown upto 3-4 hours till $O.D._{600nm}$ reaches range of 0.8-1.0, Thereafter, bacterial cells were centrifuged at 10,000 rpm for 5 min at 4ºC and the supernatant was discarded. Pellets were washed in 1X sterile PBS buffer (pH 7.4) and centrifuged again at the same condition. Bacterial pellet was resuspended in 1X PBS buffer and required amount of protease inhibitor cocktail (Roche, Cat. No. 11836153001, USA) was added to each tube, vortexed and kept on ice. Approximate volume of 5x Laemmli sample buffer was added to the lysate to reach a final 1x dilution followed by vortexing to mix.

Finally, samples were denatured at 95ºC (in a dry bath) for 5 minutes and stored at -80ºC until further use.

3. 2. 15. 3. Quantification of protein by Bradford method

The Bradford assay, a colorimetric protein assay, is based on an absorbance shift of the dye Coomassie Brilliant Blue G-250. Under acidic conditions the red form of the dye is converted into its bluer form, binding to the protein being assayed.

The protein standard (Bovine serum albumin) was prepared as stock of 10mg/ml and further diluted as 250 mg, 500 mg, 750 mg, 1000 mg/ml. After standard protein curve was generated, the unknown protein samples were mixed with Bradford reagent, incubated for 5 minutes and spectroscopic readings were recorded at 595 nm.

3.2.15.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS PAGE is the method of separation of protein samples based on their molecular weight. Components of SDS-PAGE gel are listed below:

1. Acrylamide-bisacrylamide (29:1) solution was prepared by adding 29.2 grams of acrylamide (Sigma Aldrich, USA) and 1 gram of Bisacrylamide (Sigma Aldrich, USA) to 100 ml of autoclaved miliqQ water. The solution was mixed properly at 37ºC, filter-sterilized and stored in dark at 4ºC.
2. 10% (w/v) SDS (w/v)- 4.5 grams of sodium dodecyl sulphate (SDS) was added to 45 ml of miliQ water and the solution was dissolved at 37ºC with gentle stirring.
3. 10% (w/v) APS (w/v)- 0.6 gram of Ammonium persulphate (APS) was mixed well in 6 ml of miliQ water. This is catalysest in polymerization and it is made fresh prior to use.
4. TEMED: N, N, N', N'-Tetramethylethylenediamine (Sigma Aldrich, Cat No. 161-0800, USA) was used as a catalyst of polymerization.

Resolving gel solution: The stock solutions were mixed in the following order to obtain a resolving gel of suitable percentage. I have mostly used 12% and 10% resolving gel where components were added in the following order as mentioned in table no. 3.2.15.

Casting of the SDS polyacrylamide gel: This is the first step in which the gels were casted between the assembled glass plates. A gel is composed of resolving solution (three fourth of the total height) at the lower region of the glass plate assembly and stacking solution towards the upper region. A 10/15-well comb was inserted into the stacking gel solution.

Table no. 3.2.15: Resolving gel composition

Components	**For 10% gel**	**For 12% gel**
Autoclaved MiliQ water	1.9 ml	1.65 ml
Acrylamide-bisacrylamide (29:1) solution	1.7 ml	2.0 ml
1.5 M Tris HCL (pH 8.8)	1.3 ml	1.25 ml
10% SDS solution	0.05 ml	0.05 ml
10% APS solution	0.05 ml	0.05 ml
TEMED	0.002 ml	0.002ml

The gel solutions were allowed to polymerize at room temperature for 20-30 mins, after which the comb was gently removed. Electrophoresis buffer was added to the upper surface of the stacking gel and the whole glass plate assembly was kept inside the Mini-PROTEAN gel apparatus (Bio-Rad, Cat No. 165- 8003, USA) filled with 1X SDS-PAGE electrophoresis buffer.

Stacking gel solution: The stock solutions were mixed in the following order to obtain a 5% stacking gel as mentioned in table no. 3.2.16.

Table no. 3.2.16: Stacking gel composition

Components	3ml	2 ml
Autoclaved MiliQ water	2.1 ml	1.4 ml
Acrylamide-bisacrylamide (29:1) solution	0.5 ml	0.33 ml
0.5 M Tris HCL (pH 6.8)	0.38 ml	0.25 ml
10% SDS solution	0.03 ml	0.02 ml
10% APS solution	0.03 ml	0.02 ml
TEMED	0.003 ml	0.002 ml

Sample loading and electrophoresis: Samples were taken out at room temperature and properly thawed before loading. Denatured protein samples were loaded into individual wells in the stacking gel following the desired sequence. Standard protein molecular weight marker (Prestained protein ladder (10-250 kDa) Thermo-scientific, Cat No. BM008-500, USA) was loaded to obtain molecular masses of sample proteins. The electrophoresis chamber was connected to power pack and gel run was carried out at a constant current of 20miliAmpere 20 mAmp for 2-2.5 hours, till the bromophenol blue dye front reached 0.5 cm above the base of the gel.

Coomassie staining for visualization: To observe the expression pattern of proteins and proper cell lysis, a coomassie staining was performed. For this the staining and destaining solutions were prepared as mentioned below in table no. 3.2.17.

Table no. 3.2.17: ***Coomassie staining solution***

Components	**For 250 ml**
Coomassie brilliant blue (CBB) R-250	0.25 gm
Glacial acetic acid (GAA)	25 ml
Methanol	100 ml
Autoclaved distilled water	125 ml

Destaining solution **(40% methanol, 10% GAA)**

For 250 ml of destaining solution, 100ml of methanol was mixed with 25ml of Glacial acetic acid (GAA) in 125ml of autoclaved distilled water.

3. 2. 15. 6. Hybridization with primary antibodies and secondary antibodies

After the transfer of all proteins onto PVDF membrane, membrane was blocked with blocking buffer [5% skimmed milk prepared in 1X TBST i.e. 1X Tris buffered saline with 0.05% Tween-20 (Sigma Aldrich, USA)] for 1 hour in shaking at room temperature. After blocking, the membranes were washed thrice (10 mins each) with 1X TBST. Primary antibodies for AcrA, AcrB and TolC (Rabbit polyclonal) were prepared in blocking buffer at 1:1000 dilutions and added to the membrane uniformly. The membranes were incubated at 4ºC overnight. After incubation, membranes were washed thrice (10 mins each) with 1X TBST and secondary anti-rabbit IgG antibodies in blocking buffer was added at 1:10,000 dilutions and incubated for 1 hour at room temperature in shaking. Blots were then washed thrice (10 mins each) with 1X TBST and finally once with 1X TBS and proceeded for image development.

3. 2. 15. 7. Chemiluminescence detection

Super Signal West Femto Chemiluminescent Substrate kit (Thermo Fisher Scientific, Cat No. 34094, USA) was used for blot development and images were taken with Chemi Doc XRS (Bio-Rad Laboratories, Model No. #1708265, USA) equipped with Quantity One 1-D Analysis software version 4.6.9 (Bio-Rad Laboratories). For GroEL based blot images, Pierce ECL western blotting substrate (Thermo Fisher Scientific, USA) was used for image acquisition.

3. 2. 15. 8. Stripping and reprobing the membrane

Ready to use Restore Plus Western Blot Stripping Buffer (Thermo scientific, Cat No. 46430, USA) was used for stripping the western blotted membranes to probe with different sets of antibodies.

One time western-blotted PVDF membrane was washed twice with 1X TBS and was kept in the reprobing buffer for 30 min with shaking. Membrane was then washed three times (10 mins each) with TBST followed by 1 hour of blocking with 5% skimmed milk in 1X TBST. Primary antibody GroEL at 1:1000 dilutions was added to the membrane and incubated at room temperature for 3-4 hours. Membranes were washed with TBST thrice (10 minutes each) and secondary antibody (1:10,000) was added and kept in shaker for 1 hour. Finally, membranes were washed with 1X TBST thrice and developed as mentioned previously to get desired bands.

3. 2. 16. Blocking peptide validation

To ensure specificity of polyclonal antibodies of AcrA, AcrB and TolC, their specific blocking peptide has been utilized, which are listed below.

Details of blocking peptides used in this study:

- **AcrA**-CTSDGIKFPQDGTLE
- **AcrB**-CRRFSRKNEDIEHSH
- **TolC**-CGISDTSYSGSKTRG

All of these blocking peptides (Genscript, USA) were 15 aminoacids in length, supplied as lyophilized powder of 9 mg each. Each of these peptides were reconstituted as per directions and used as mentioned below.

Necessary amount of antibody was diluted in blocking buffer in two sets of tube: one 'Control' without blocking peptide and one 'Blocked' with blocking peptide. The ratio of the blocking peptide and antibody was tested for 1:1, 3:1, to 10:1. The tubes were spinned down and incubated at 37°C for 30 minutes. The membranes with transferred proteins were then incubated with the diluted primary antibody as mentioned above; without (control tube) and with varying concentrations of blocking peptides (blocked tube) from different tubes overnight at 4ºC. After primary antibody incubation, the membranes were probed with secondary antibody and signals were recorded as mentioned previously.

3. 2. 18. Bacterial membrane extraction using Proteoprep (Sigma) kit

ProteoPrep Membrane Extraction Kit (Sigma) is designed to prepare highly enriched soluble membrane protein fractions from many types of cells, including bacteria. The components of the kit include-

- Soluble cytoplasmic and loosely bound membrane protein extraction reagent- 125 ml of ultrapure water was added to the contents of the container and was mixed properly and stored at 4ºC.
- Protein extraction reagent type 4- 15 ml of ultrapure water was added to the contents of the container, and warmed to 20-25ºC to get properly mixed.

- Tributylphosphine (TBP) stock solution
- Alkylating reagent (Iodoacetamide)

3. 2. 18. 1. Bacterial cell pellet preparation & lyophilization

Bacterial isolate was grown as a pure culture on LBA agar and a single colony was inoculated onto 50 ml of sterile LB broth and incubated at 37ºC, 220 rpm for appropriate time point (3-6 hours).

Bacterial cultures were then centrifuged at 4500 rpm for 10 minutes at 4ºC to get pellet, which was washed twice with 15 ml of 1X PBS buffer (pH 7.4). Finally the bacterial pellet was stored at -80ºC before proceeding for lyophilization. Dry ice bench top freeze-dry system (Labconco, USA) was programmed and samples were lyophilized at -75ºC overnight.

3.2. 18. 2. Extraction of membrane protein and western blotting

The lyophilized product was weighed and suspended in ice-cold soluble cytoplasmic and loosely bound membrane protein extraction reagent. Samples were sonicated for one to two minutes to disrupt the cells and break down DNA. 50 ml of ice-cold soluble cytoplasmic and loosely bound membrane protein extraction reagent was added to this suspension and was incubated on ice for 1 hour.

The suspension was ultra-centrifuged (115,000g for 1 hour at 4ºC) to pellet membranes and membrane proteins. Pellet was washed twice with 2 ml of sterile miliQ water (with centrifugation at 20,000g for 20 minutes at 4ºC). The cell pellet was dissolved in 2 ml of protein extraction reagent type 4 (provided in the kit) and sonicated on ice (70% for 15 secs four times). The suspension was then centrifuged (14,000g for 45 minutes at 15ºC) to pellet cell debris.

The supernatant was transferred into a clean tube and 50 µl of TBP solution was added followed by incubation at room temperature for 1 hour that reduced this fraction. Sixty microlitre of alkylating reagent was added to this fraction and incubated at room temperature for 1.5 hours. Finally the sample was centrifuged at 20,000 g for five minutes at room temperature. The membrane fractions obtained were quantified and 3µg of protein was loaded into 12% SDS polyacrylamide resolving gels.

SDS-PAGE resolved proteins were transferred onto PVDF membranes with a Mini-Trans blot cell (Biorad, USA) at 17V for 1 hour. After blotting, membranes were blocked for one hour with PBS-0.05% Tween 20 (PBS-T) containing 5% non-fat dry milk. Membranes were incubated overnight at 4ºC with polyclonal anti-rabbit antibodies against AcrA and AcrB efflux proteins (Genscript, USA). Membranes were washed thrice with PBS-T and incubated with the appropriate HRP-conjugated secondary antibodies (Abcam, USA). After four washes, membranes were developed with SuperSignal West Femto Chemiluminescent Substrate (Pierce, USA) and images were acquired with Chemidoc Imaging System (Bio-Rad, USA).

3. 2. 19. Indirect Immunofluorescence assay and fluorescence microscopy

To ascertain that proteins being expressed under antibiotic treated and untreated sets of different pH are functionally active, we tried to examine visualize their localization expression on bacterial membrane in real time by following an indirect immunofluorescence assay as mentioned below. Bacterial pellet obtained from log phase cultures of $O.D._{600nm}$ 0.6-0.8 (~1×10^8 cells) were washed and dissolved with 1XPBS pH7.4. To this bacterial culture 10µg/ml of FM 464-FX (Thermo Fisher Scientific, USA), fixable analog of membrane dye was added and incubated at 37ºC for 20 minutes. Cultures were centrifuged at 4ºC, 8000 rpm for 5 minutes at 4C, fixed

with 4% PFA for 20 minutes followed which, cells were washed twice with 1XPBS (pH7.4). Anti-Rabbit polyclonal antibodies (for AcrA, AcrB & TolC) dissolved in 1X PBS with 3% BSA was then added to DL4.3 and EspIMS6 in the ratio of 1:10 and 1:30 respectively and incubated overnight at 4ºC. Further cells were washed twice with wash solution (1X PBS with containing 1% w/v BSA and 0.02% v/v Tween-20) and incubated with alexa Alexa fluor 488 tagged anti-rabbit IgG secondary antibodies (Life Technologies, USA) as instructed. Briefly cells were washed twice with 1X PBS with 1% BSA and 0.02% Tween-20 and once with 1X PBS pH7.4. Samples were imaged using 60X oil-immersion inverted fluorescence microscope (Olympus, Japan).

3. 2. 21. In-vitro cell culture assay

To confer the adhesion and/or invasive properties of selected clinical and non-clinical *Enterobacter* isolates gentamicin protection assay was performed.

3. 2. 21. 1. Cell lines used, growth conditions and their maintenance

RAW 264.7 murine macrophage cell line was used for gentamicin protection assay. The cell line was maintained in RPMI-1640 media (Himedia, India) supplemented with antibiotics cocktail containing 1x penicillin-streptomycin & 250 µg of Amphotericin-B (Himedia, India) and 10% Fetal Bovine Serum-US origin (PAN Biotech, India).

RAW 264.7 cell line was allowed to grow till 70-80% confluence at 37ºC at 5% CO_2 in NewBrunswick incubator (Eppendorf, India). After reaching confluency, cells were trypsinized using 1x Trypsin-EDTA (Himedia, India), and cells were given a passage and maintained with fresh complete media. For cryopreservation, cells were dissolved suspended inwith complete media containing 5% DMSO (MP Biomedicals, USA) and

stored at -80ºC for shorter time period and thereafter transferred to at liquid nitrogen for longer timeuntil further use.

3. 2. 21. 2. Multiplicity of infection (MOI) and Time of infection optimization

To initiate bacterial challenge to RAW 264.7 cell line, firstly the multiplicity of infection (MOI) was standardized. The MOI is the ratio of infectious agents (e.g. phage or bacteria) to infection targets (i.e. cells). Firstly, macrophage cells were seeded onto 12 well tissue culture plate at seeding density of 2×10^5 cells/ml in each well without antibiotics and incubated overnight in CO_2 incubator. Bacterial pure culture was inoculated into 3 ml of tryptic soy broth (Himedia, India) and incubated at 37ºC overnight. Culture was then diluted 1:1000 in fresh 5 ml of TSB and allowed to grow for 3-4 hours until $O.D_{600nm}$ reaches 0.6-0.8. Bacterial cultures were then centrifuged at 4500 rpm for 10 minutes at 4ºC. Pellet was then suspended in 1x PBS pH 7.4 and before infection was mixed with RPMI-1640+10% FBS media without antibiotics. Bacterial cells were then added to RAW cell line at MOI of 1:1, 10:1, 50:1 and 100:1.

After optimizing the MOI of 1:50, time of infection was standardized. For this, bacterial cultures at specific MOI of 50:1 was added to RAW 264.7 cell line grown on 35 mm tissue culture dishes and incubated for different time points i.e. 0, 15, 30, 45, 60, 90, 120 and 150 minutes. After each time points, the cells were washed twice with 1X PBS and lysed using 0.05% Ttriton-x X 100 and spread plated onto TSA plates and incubated overnight. Later CFU count was made and the optimal time point with maximum infectivity was found to be 1 hour.

3. 2. 21. 3. Gentamicin protection assay for *Enterobacter* isolates

With this set up, macrophage cell line was seeded at 2×10^5 cells/ml in each well without antibiotics in a 12 well tissue culture plate and incubated overnight. Bacterial cultures were prepared as mentioned previously. Cells were washed once with 1X PBS pH 7.4 and bacterial cultures at MOI 50:1 was added to RAW 264.7 cell line in two set of plates and incubated for 1 hour at 37ºC with 5% CO_2. After 1 hour, plates were washed twice with 1X PBS pH 7.4. One set of plate was lysed using 0.05% Triton-X which indicated in-vitro cell attachment of isolates. Another plate was incubated further for 1 hour with RPMI-1640 containing 200µg/ml of gentamicin to kill any extracellular bacteria. After incubation plate was washed twice with 1x 1X PBS (pH 7.4) and cells were lysed with 0.05% Triton-X 100, this indicated in-vitro cell invasive property of isolates. Samples were plated onto TSA in duplicates and incubated overnight at 37ºC. CFU count was done followed by statistical analysis taking three biological replicates with three technical repeats each. The same in-vitro cell adhesion and in-vitro cell invasion experiments were replicated in C43-DE3 knockout and C43-DE3 KO strains expressing AcrA/TolC to further compare with the observed phenotype of wild type *Enterobacter* isolates.

3. 2. 21. 4. In-vitro cell multiplication/ persistence assay

Survival to macrophage's killing activity for a prolonged period of time is an important virulent feature of a pathogen. To assess this, RAW 264.7 cell line were seeded onto 12 well tissue culture flask with 2×10^5 cells/ml in each well without antibiotics and incubated overnight at 37ºC with 5% CO2 in New Brunswick incubator (Eppendorf, India). Fresh bacterial cultures (EspIMS6 and *E.cloacae* ATCC 13047) of O.D. 0.8 were dissolved in RPMI-1640 media without antibiotics, and transfected to

the adhered macrophage cell line at an optimized MOI of 1:50, as mentioned previously. After 1 hour of incubation at 37ºC, media were removed and cells were washed twice in 1X PBS, and fresh media with 200µg/ml of gentamicin was added. After 1 hour of gentamicin treatment, cells were again washed with PBS and fresh RPMI-1640 media supplemented with 50µg/ml of gentamicin was added. Plates were incubated for 3, 6, 24, 48 and 72 hours with intermittent media change for all plates, except 3 hours. After incubation at the above mentioned time points, plates were washed twice with PBS and cells were lysed with 0.05% tritonTriton-X 100 and plated onto TSA. Plates were incubated overnight at 37ºC to determine the CFU/ml. Graphs and statistical analysis was made taking three biological replicates with three technical repeats each.

3. 2. 22. Effect of Silver Nanoparticles on MDR isolates

In this study, we have utilized two polysaccharide-capped nanoparticles; silver (AgNP) and silver-gold (AgAuNP) and tested their effect on multidrug efflux pump AcrAB-TolC protein expression in MDR isolates. To determine the antimicrobial efficacy of nanoparticles on multi-drug resistant MDR isolates MIC wasevaluated. The effect of silver nanoparticless on efflux pump protein expression was also then determined by western blotting.

3. 2. 22. 1. Physical Characterization of the Nanoparticles Used

The synthesis of these polysaccharide capped silver and bimetallic nanoparticles was performed at Luna Goswami's Lab (KIIT University) by green-synthesis approach, followed by its physical characterization. Particle size and zeta potential (ζ) of these silver nanoparticles were measured by Zetasizer Nano ZS instrument (Malvern Instruments, United Kingdom) at a constant temperature of 25 ± 1°C. The samples (0.1

mg/ml) were suspended in Milli-Q water and sonicated for 1 min. The mean hydrodynamic diameter and zeta potential for each sample was measured in triplicate and the results were measured as mean size ± SD. The surface plasmon resonance (SPR) was recorded at different time points (10–150 min) for polysaccharide-capped silver nanoparticles (AgNP) against the only carboxyl methyl tamarind (CMT) polysaccharide solution as blank.

3. 2. 22. 2. Determining hemocompatibility of silver nanoparticle

Hemocompatibility of polysaccharide capped silver nanoparticle was determined in terms of percent hemolysis using sheep blood [173]. The diluted suspension of extracted RBCs (0.2 ml) was mixed with varied concentrations (1, 3, 6, 9 and 12 µg/ml) of AgNPs in PBS (0.8 ml). Diluted suspension of RBCs mixed with 0.8 ml PBS and 0.8 ml double distilled water were used as negative and positive control, respectively. The mixture was gently vortexed and incubated at room temperature for 3 hours. After centrifugation (1600 rpm, 5 min) of the incubated mixture, absorbance of the supernatant was recorded at 541 nm by UV–Vis spectrophotometer (Agilent Cary 100 UV–Vis, Germany). Finally, hemocompatibility was evaluated in terms of percent hemolysis using the formula:

(AS − AN)/(AP − AN) × 100; Where "AS" is the sample absorbance, "AN" is the absorbance of negative control and "AP" is the absorbance of positive control [174].

3. 2. 22. 3. MIC of silver nanoparticle (AgNP) and silver-gold nanoparticle (AgAuNP) in MDR isolates

The antimicrobial efficacy of AgNPs and AgAuNPs were evaluated against multiple gram-negative (*E.coli*, *Enterobacter* spp. and *Klebsiella* sps.) and gram-positive bacteria (*Staphylococcus aureus, Staphylococcus epidermidis*) including control susceptible isolate ATCC 25922 *E.coli*. Bacterial cultures were sub-cultured in Muller

Hinton Broth till OD_{600nm} is 0.6-0.8 and used for MIC determination by standard double broth dilution tube method as mentioned previously. MIC break point values (in µg/ml) were noted after overnight incubation at the above-mentioned conditions. Further, we checked survival of the bacteria by dilution plating onto MHA plates to enumerate viable colonies.

3. 2. 22. 4. Western blotting form nanoparticle treated bacterial cells.

Two sets of flasks were maintained; one is control without nanoparticle and another is treated, with AgNP (6 µg/ml) and AgAuNP (0.75 µg/ml) at sub-MIC concentrations. All the flasks were incubated for 24 hours with 1 ml of samples removed intermittently after 0, 30, 60, 120, 180 and 1440 minutes of treatment of nanoparticles. 1 ml of bacterial sample was pelleted, washed once with 1x PBS pH7.4, dissolved in PBS. Afterwards, the sample was processed for protein extraction. The bacterial cells (treated and untreated) with nanoparticles were pelleted down, washed once with 1X PBS pH7.4, dissolved in PBS and kept on ice. The protein samples were preserved by adding proteinase-K, lysed by treating with 5X Lamelli buffer and denatured at 95ºC for 5 mins. Extracted protein samples were then loaded onto 10-12% polyacrylamide gels and western blotting for AcrA, AcrB and TolC was performed as described earlier. The images were acquired using ChemiDoc and analyzed using Quantity-one software (Biorad, USA) for densitometry.

3. 2. 23. Whole genome sequencing of *Enterobacter* isolates

From this study, we had selected environmental *Enterobacter cloacae* isolate DL4.3 (showing multi-drug resistance phenotype) and clinical *Enterobacter cloacae* isolate EspIMS6 (having extreme drug resistance phenotype) for whole genome sequencing. Genomic DNA from both isolates was extracted using the Gentra Puregene Yeast/Bact. Kit (Qiagen, GmbH) as described earlier. Whole genome sequencing was carried out at laboratory of ThermoFisher Scientific (India), Gurgaon, India.

Briefly, libraries for individual genomes were prepared using the workflow prescribed by the Ion Xpress™ Plus Fragment Library Kit (ThermoFisher Scientific, USA). Subsequently, the reads were amplified using the Ion OneTouch™ 2 System (ThermoFisher Scientific, USA) and sequenced using the Ion S5 system (ThermoFisher Scientific, USA). The sequences were then uploaded to the RAST server, and also in Prokaryotic Genome Annotation Pipeline (PGAP) to annotate genomes.

Results and Discussion

Results 4.1

Comparative analysis of multidrug resistance phenotype in environmental and clinical Enterobacter isolates: occurrence and diversity of bacterial MDR efflux pump

Section 4.1: Comparative analysis of multi-drug resistance phenotype in environmental and clinical *Enterobacter* isolates: contribution of efflux pumps

Background of the study

Antimicrobial resistance is emerging globally as a new threat to modern advances in medicine. Membranes embedded transporters elucidated by efflux pumps are known to mediate multi-drug resistance phenotype in opportunistic pathogens [1]. Moreover, tripartite constitution in RND type efflux pumps (exemplified by AcrAB-TolC) make it more effective in extruding the substrates from cytoplasm to the external environment utilizing proton motive force [50]. Hence these RND efflux pumps are predominantly associated with clinically significant antibiotic resistance in many gram-negative pathogens including *Enterobacter* Spp. [8].

This chapter presents results of antibiotic susceptibility profile of all the clinical and environmental *Enterobacter* isolates used in the study, and findings on the occurrence and diversity of bacterial MDR efflux pumps.

4.1.1: Identification and characterization of bacterial strains

Water samples were collected at periodic interval of two months except rainy season from four different water sources located in Jamshedpur city (as shown in figure no.4.1.1 below), India, following standard procedure for water collection. Two of these were natural fresh water rivers (Subarnarekha river, Kharkai river) and two were artificial reservoirs (Dimna lake, Hudco dam) (figure no.4.1.1).

Collected water samples were processed for bacteriological analysis. After routine microbiological identification; genus level identification of *Enterobacter* isolates were performed by 16S rRNA sequencing. The 16S rRNA gene sequences of environmental

Enterobacter isolates were submitted at NCBI Genbank, whose details and accession number has been listed in table no. 4.1.1 below.

Figure no. 4.1.1: Geographical distribution of sample collection point along Subarnarekha, Kharkai rivers and Dimna lake, Hudco dam in Jamshedpur, India. Dark circles represent the precise location of sampling sites.

Clinical *Enterobacter* isolates were collected from patients admitted to tertiary care hospitals in Bhubaneswar, Odisha, India. Primarily clinical specimens for isolation of *Enterobacter* isolates included urine, wound swab, pus, tracheal aspirate and blood. Preliminary identification up to genus level was done by colony characteristics on differential and enriched media, followed by more stringent identification by 16S rRNA sequencing method. The details of clinical *Enterobacter* strains are listed below in table no. 4.1.2. All the isolates were tested for their amino acid decarboxylation pattern towards lysine, arginine and ornithine that were concurrent with our species level identification.

Table no. 4.1.1: Details of environmental *Enterobacter* isolates used in this study

Strain ID	Source of isolation	Genbank accession no.	Lysine	Arginine	Ornithine	Identification
SR 1.1	Subarnarekha River	JQ912523	-Ve	+Ve	+Ve	*E. cloacae*
SR 1.5	Subarnarekha River	JQ912528	-Ve	+Ve	+Ve	*E. cloacae*
KR 1.8	Kharkhai River	JQ912527	+Ve	-Ve	+Ve	*E. aerogenes*
HD 1.9	Hudko Dam	JQ912525	-Ve	-Ve	+Ve	*Enterobacter* spp.
SR 2.2	Subarnarekha River	JQ912529	-Ve	+Ve	+Ve	*E. cloacae*
SR 2.3	Subarnarekha River	JQ912531	-Ve	+Ve	+Ve	*E. cloacae*
SR 2.4	Subarnarekha River	JQ912530	-Ve	+Ve	+Ve	*E. cloacae*
HD 2.1	Hudko Dam	Not deposited	-Ve	+Ve	+Ve	*E. cloacae*
SR 4.9	Subarnarekha River	Not deposited	-Ve	+Ve	+Ve	*E. cloacae*
KR 4.2	Kharkhai River	JQ912520	-Ve	-Ve	-Ve	*Enterobacter* spp.
DL 4.3	Dimna Lake	JQ912514	-Ve	+Ve	+Ve	*E. cloacae*
DL 4.6	Dimna Lake	JQ912515	-Ve	+Ve	+Ve	*E. cloacae*
DL 4.7	Dimna Lake	JQ912516	-Ve	+Ve	+Ve	*E. cloacae*
HD 4.3	Hudko Dam	JQ912519	-Ve	+Ve	+Ve	*E. cloacae*
SR 5.7	Subarnarekha River	JQ912524	-Ve	-Ve	+Ve	*Enterobacter* spp.
KR 5.2	Kharkhai River	JQ912526	-Ve	-Ve	+Ve	*Enterobacter* spp.
KR 5.3	Kharkhai River	JQ912521	-Ve	+Ve	+Ve	*E. cloacae*
KR 5.9	Kharkhai River	JQ912522	-Ve	+Ve	+Ve	*E. cloacae*
DL 5.1	Dimna Lake	JQ912517	-Ve	+Ve	+Ve	*E. cloacae*
DL 5.6	Dimna Lake	JQ912518	-Ve	-Ve	-Ve	*Enterobacter* spp.

Table no. 4.1.2: Details of clinical *Enterobacter* isolates used in this study

		Aminoacid decarboxylation			
Strain ID	**Specimen sample**	**Lysine**	**Arginine**	**Ornithine**	**Identification**
E Sp. IMS 1	Pus	+Ve	-Ve	p+Ve	*E. aerogenes*
E Sp. IMS 4	Tracheal aspirate	+Ve	-Ve	-Ve	*Enterobacter* spp.
E Sp. IMS 5	Wound Swab	+Ve	-Ve	-Ve	*Enterobacter* spp.
E Sp. IMS 6	Urine	-Ve	+Ve	+Ve	*E. cloacae*
E Sp. IMS 7	Urine	-Ve	+Ve	+Ve	*E. cloacae*
E Sp. IMS 8	Urine	+Ve	-Ve	-Ve	*Enterobacter* spp.
E Sp. IMS 9	Pus	+Ve	-Ve	-Ve	*Enterobacter* spp.
E Sp. IMS 10	Pus	+Ve	-Ve	-Ve	*Enterobacter* spp.
E Sp. IMS 11	Unknown	+Ve	-Ve	-Ve	*Enterobacter* spp.
E Sp. IMS 13	Pus	-Ve	+Ve	+Ve	*E. cloacae*
E Sp. IMS 16	Wound Swab	+Ve	p+Ve	-Ve	*Enterobacter* spp.
E Sp. IMS 17	Urine	+Ve	-Ve	-Ve	*Enterobacter* spp.
EC IMS 18	Blood	-Ve	+Ve	+Ve	*E. cloacae*
EC IMS 19	Pus	-Ve	+Ve	+Ve	*E. cloacae* sp. *cloacae*
EC IMS 20	Blood	-Ve	+Ve	+Ve	*E. cloacae* sp. *dissolvens*
EC IMS 21	Tracheal aspirate	+Ve	-Ve	-Ve	*E. cloacae*
Esp.TATAH 56	Urine	+Ve	-Ve	-Ve	*Enterobacter* spp.
EC TATAH 41	Urine	-Ve	+Ve	+Ve	*E. cloacae*
E Sp. AH1	Pus	-Ve	+Ve	+Ve	*E. cloacae*
E Sp. AH2	Unknown	+Ve	+Ve	+Ve	*Enterobacter* spp.
E Sp. AH3	Wound swab	+Ve	-Ve	-Ve	*Enterobacter* spp.
EC AH4	Wound swab	-Ve	+Ve	+Ve	*E. cloacae*

Note: '+ve'-Positive, '-ve'-Negative, 'p+'-partially positive or late positive. Lysine, Arginine and Ornithine represented aminoacid decarboxylation pattern.

4.1.2. Antibiotic susceptibility profile of *Enterobacter* isolates

The *Enterobacter* isolates were screened for their antibiotic susceptibility profiles by disk diffusion method [166]. Antibiotic sensitivity towards forty different antibiotics belonging to major groups like β-lactams, cephalosporins, quinolones, polypeptides, carbapenems, colistin was determined for environmental (n=20) and clinical isolates (n=22) as represented in figure no. 4.1.2A and figure no. 4.1.2B respectively. The antibiotic disks included in the study along with their potencies and zone diameter break points were listed in table no. 3.1.3. *Escherichia coli* strain ATCC 25922 was used as control for each assay. The diameter of the inhibition zones was recorded and interpreted following CLSI standards [167]. Based on their antibiotic susceptibility pattern, heat map was generated along with dendrogram using Bionumerics software version 7.0 (Applied Maths, Biomeriux company) as depicted in the figure no. 4.1.2A (for environmental, n=20) and figure no. 4.1.2B (for clinical, n=22) *Enterobacter* isolates.

All clinical isolates were showing 100% resistance pattern towards β-lactams whereas, >70% of environmental populations were resistant to β-lactams (figure no. 4.1.2A vs. 2B). Moreover, in case of cephalosporins, all clinical isolates were showing 100% resistance towards first and second generation of cephalosporins and around 75% of resistance was observed to third generation cephalosporins like ceftriaxone, cefotaxime, ceftazidime (figure no. 4.1.2B). Further, majority of clinical isolates were not susceptible towards fourth generation cephalosporin antibiotics like cefpirome and cefepime. Environmental isolates were mostly resistant towards first generation of cephalosporins like cefuroxime (figure no. 4.1.2A). While most of the environmental *Enterobacter* isolates were susceptible to quinolones, more than 50% of clinical isolates were found to be resistant towards quinolones (figure no. 4.1.2A vs. 2B).

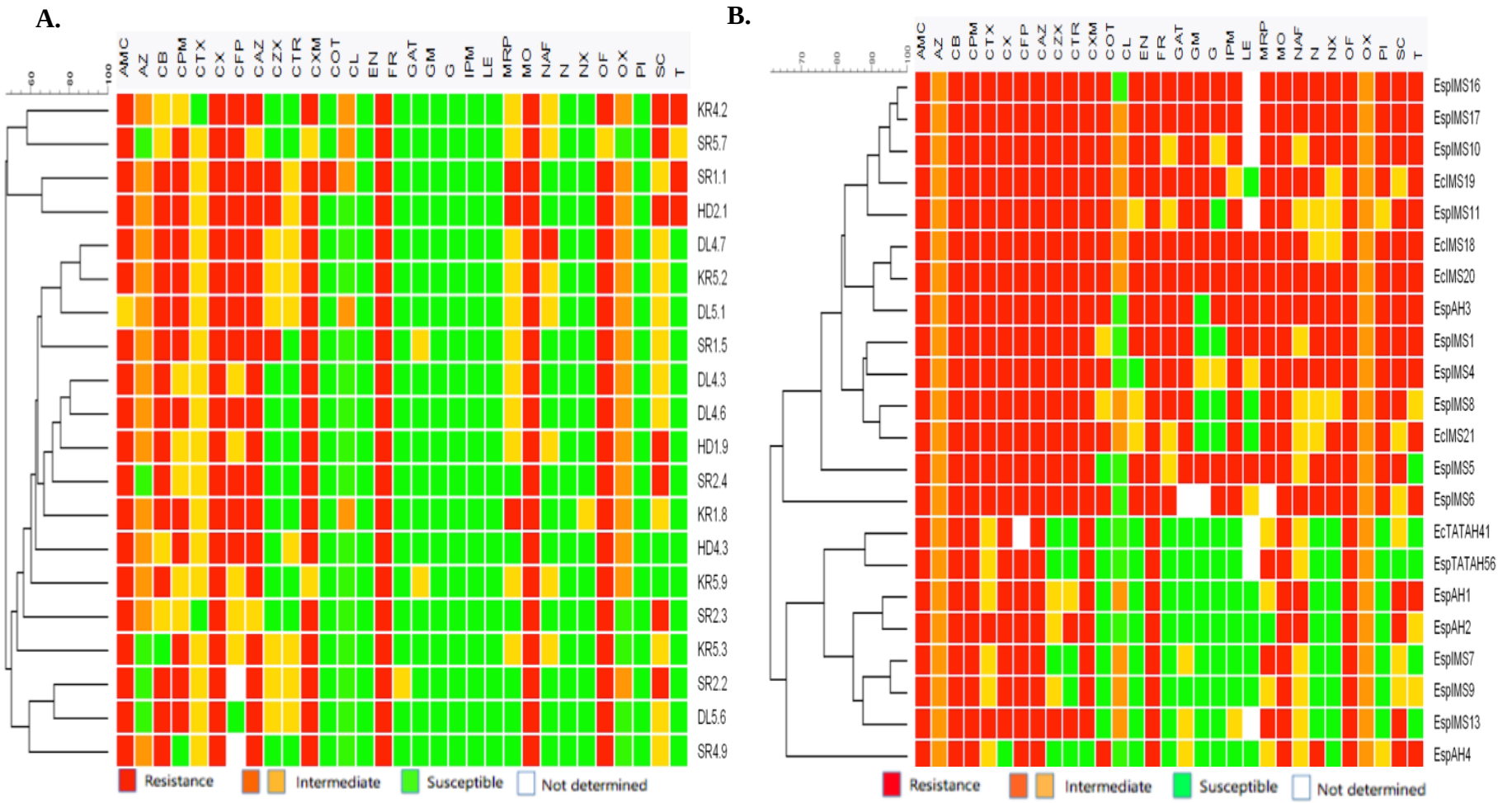


Figure no. 4.1.2: Representing heat map with dendrogram showing antibiotic susceptibility patterns of A] environmental (n=20) and B] clinical (n=22) *Enterobacter* isolates by disc diffusion assay. Abbreviations used for antibiotics: CX-Cloxacillin, NAF-Nafcillin, OX-Oxacillin, AZ-Azlocillin, PI-Piperacillin, AMC-Amoxyclav, nCXM-Cefuroxime, CAZ-Ceftazidime, CTX-Cefotaxime, CZX-Ceftizoxime, CTR-Ceftriaxone, CFP-Cefpirome, CPM-Cefepime, EN-Enoxacin, NX-Norfloxacin, OF-Ofloxacin,,LE-Levofloxacin, SC-Sparfloxacin, GAT-Gatifloxacin, N-Neomycin, COT-Cotrimaxazole, AZM/AT-Azithromycin, FR-Furazolidone, T-Tetracycline, IPM-Imipenem.

Similarly, for antibiotics belonging to aminoglycosides, carbapenems, macrolides majority of environmental isolates were found to be susceptible with a less intermediate populations; however, 50% of clinical isolates were observed to be resistant towards colistin, and around 30% of them were found to be resistant towards imipenem and meropenem (figure no. 4.1.2A vs. 4.1.2B). Alarmingly, 25% environmental isolates were resistant towards colistin; as against 50% of clinical *Enterobacter* isolates had developed resistance to colistin.

Overall, many of these environmental isolates were found to be showing resistance towards multiple antibiotics belonging to two or more group of antibiotics, indicating them to be multi-drug resistant (MDR) isolates as represented in figure no. 4.1.2A. However, most of the clinical *Enterobacter* isolates have developed an extensive MDR phenotype, where isolates EspIMS5, EspIMS6, EspIMS16, EspIMS17 were found to be pan-drug resistant and isolates EcIMS18, EcIMS19, EcIMS20, EspAH3 were found to be extreme drug resistant (XDR) in nature (figure no. 4.1.2B).

As noticed form the antibiogram profile above, most of the *Enterobacter* isolates exhibited resistance towards multiple groups of antibiotics. This prompted us to determine the multiple antibiotic resistance (MAR) index of these isolates as described by Krumperman [175] using the following equation:

$MAR_{index} = a/b$

Where “a” represents number of antibiotics to which isolate is resistant, “b” represents the number of antibiotics to which isolate was exposed. Individual MAR index of all the isolates were then plotted as presented in figure no. 4.1.3.

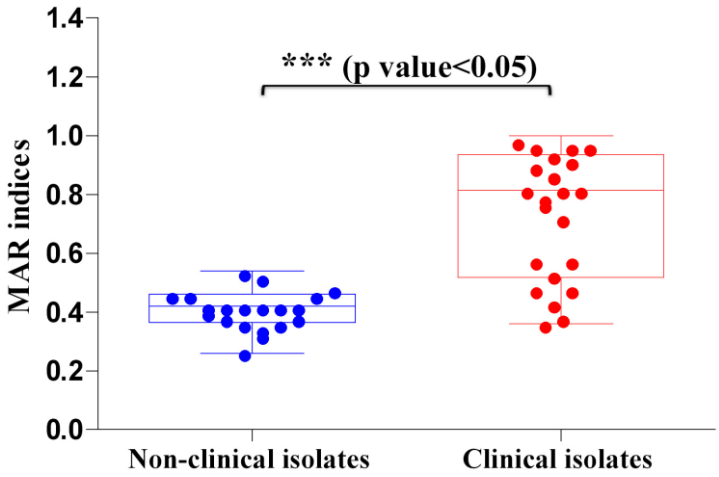


Figure no. 4.1.3: Multiple antibiotic resistance (MAR) indices of *Enterobacter* isolates (Environmental vs. Clinical).

As depicted in the above figure, the average MAR index in case of environmental isolates was 0.4, where as mean MAR index of clinical isolates was 0.8. This suggested that clinical isolates exhibited extreme multi-drug resistance phenotype. The difference between MAR indices of these two groups was found to be significant (p value<0.05).

4.1.3: Determination of minimum inhibitory concentration (MIC) of antibiotics for *Enterobacter* isolates and effect of CCCP in MIC breakpoint

With the data on resistance profile of the isolates, we proceeded to determine their minimum inhibitory concentration (MIC) values of antibiotics following macro broth dilution method as per CLSI guidelines. To observe the contribution of efflux pumps in mediating multiple drug resistance, ionophore CCCP was used at an optimized 50 µMolar concentration and the MIC values for each isolate was compared in presence and absence of CCCP, as presented in table below (table no. 4.1.3 and 4.1.4).

In addition to the antibiotics tested by disk diffusion, for clinical isolates MIC of trimethoprim, neomycin and levofloxacin in presence and absence of CCCP was also measured (table no. 4.1.4).

Table no. 4.1.3: MIC breakpoints towards antibiotics in environmental *Enterobacter* isolates

Antibiotics tested	Ampicillin (Amp)		Cefotaxime (CTX)		Cefuroxime (CXM)		Cefepime (CPM)		Tetracycline (T)		Erythromycin (Ery)	
CCCP (50µMolar)	-	+	-	+	-	+	-	+	-	+	-	+
SR 1.1	> 256	> 32	128	< 2	-	-	-	-	-	-	256	64
KR 1.8	256	16	256	4	-	-	-	-	-	-	128	64
HD 1.9	> 128	32	> 256	> 8	-	-	-	-	> 16	> 16	> 1024	> 16
SR 2.2	1024	512	-	-	-	-	-	-	-	-	128	64
SR 2.3	> 1024	1024	-	-	1024	512	-	-	-	-	256	128
SR 2.4	> 1024	> 1024	-	-	-	-	-	-	-	-	512	128
HD 2.1	256	128	64	64	512	512	-	-	16	16	64	64
SR 4.9	1024	256	512	256	1024	256	-	-	-	-	1024	128
KR 4.2	1024	512	-	-	512	256	> 512	> 512	512	512	1024	256
HD 4.3	> 1024	> 1024	> 512	> 512	> 512	512	64	16	-	-	> 512	256
DL 4.3	128	64	256	256	1024	512	-	-	128	32	1024	512
DL 4.6	128	64	512	256	1024	1024	64	32	64	16	256	256
SR 5.7	1024	256	512	256	512	256	1024	1024	1024	256	1024	1024
KR 5.2	1024	1024	512	128	256	256	-	-	-	-	256	128
KR 5.3	512	512	512	> 256	512	512	1024	1024	256	64	512	512
KR 5.9	1024	1024	128	512	1024	256	128	64	-	-	> 512	128
DL 5.1	> 1024	> 1024	512	128	>512	>1024	256	64	64	512	512	>1024
DL 5.6	1024	> 1024	256	256	256	256	512	128	512	64	> 1024	256

Note: The highlighted in yellow indicated two or more fold decrease in MIC in the presence of CCCP as compared to the MIC in absence of CCCP in environmental *Enterobacter* isolates. '-' indicated not determined.

Table no. 4.1.4: MIC breakpoints towards antibiotics in clinical *Enterobacter* isolates

Antibiotics tested	Cefotaxime (CTX)		Cefepime (CPM)		Tetracycline (Tet)		Erythromycin (Ery)		Trimethoprim (Tr)		Levofloxacin (Le)	
CCCP 50µMolar)	-	+	-	+	-	+	-	+	-	+	-	+
EcTATAH41	>512	256	>1024	512	256	256	>1024	1024	-	-	-	-
EspTATAH56	512	512	1024	1024	-	-	>1024	>1024	-	-	-	-
Esp IMS4	>1280	>1024	>1280	>1024	>1024	1024	>1024	>1024	-	-	-	-
Esp IMS5	>1024	>1024	>1024	1024	>1024	>1024	>1024	1024	-	-	>512	128
Esp IMS6	>1280	1024	>1280	>1280	-	-	-	-	1024	>512	256	512
Esp IMS9	512	<512	1024	1280	1024	1024	512	256	>1024	1024	-	-
Esp IMS11	>1280	>1280	>1280	1280	>1024	512	>1024	>1024	>1024	>1024	256	>256
Esp IMS13	>1280	1280	>1024	512	>1024	256	>1024	1024	-	-	512	512
Esp IMS 17	>1280	>1280	>1280	1024	>1024	>1024	>1024	>1024	>1024	1024	1024	512
Ec IMS18	>1280	>1024	>1280	>512	256	>256	>1024	>256	>1024	>512	128	>128
Ec IMS19	>1280	512	>1280	512	>128	>128	>512	>512	>1024	>512	>128	>128
Ec IMS20	>1280	>1280	>1280	>512	512	512	>1024	>256	>1024	>256	>128	128
Ec IMS21	>1280	>512	>1280	>512	256	>256	>1024	>1024	>1024	512	>64	>256
Esp AH1	256	256	<256	<256	256	>256	>1024	512	1024	<256	128	128
Esp AH2	>1024	>1024	>1024	512	>1024	1024	>512	512	>512	512	-	-
Esp AH3	>1024	>1024	>1024	512	>1024	512	>1024	1024	>1024	512	1024	512
Esp AH4	128	>128	256	>256	512	512	>512	256	>1024	512	-	-

Note: The highlighted in yellow indicated two or more fold decrease in MIC in the presence of CCCP as compared to the MIC in absence of CCCP in clinical *Enterobacter* isolates. '-' indicated not determined.

As observed in table no. 4.1.3, environmental *Enterobacter* isolates SR1.1, KR1.8, SR4.9, DL4.3, and DL4.6 showed two-fold decrease in MIC level of antibiotics belonging to varied groups like ampicillin, cefuroxime, cefotaxime, and erythromycin in presence of CCCP. Isolates HD2.1, SR2.4 and KR5.3 did not show much effect in MIC level in the presence of efflux pump inhibitor CCCP. Moreover, isolates like SR1.1, SR4.9, DL4.3, DL4.6, and SR5.7 displayed efflux pump mediated resistance to more than two different classes of antibiotics. Few isolates like DL5.1, DL5.6 and KR5.9 showed higher MIC in the presence of efflux inhibitor CCCP, which was unexplainable.

As presented in table no 4.1.4, most of the clinical isolates showed higher MIC values without CCCP for antibiotics tested in comparison to environmental isolates. Isolate EspTATAH56 did not show any change in its MIC value in the presence of CCCP. Isolates EcTATAH41, EspIMS6, EspIMS13, EcIMS18 showed a two-fold decrease in MIC value in presence of CCCP to cefotaxime and cefepime. Isolates EspIMS6, EspIMS13, EcIMS18, EcIMS19, and EspAH3 showed similar phenotype of reduced MIC level with CCCP towards two to three different group of antibiotics. Interestingly, like few environmental isolates, in clinical isolates EspIMS9, EspIMS11, EcIMS21 and EspAH1 reverse trend of increased MIC in the presence of CCCP was noticed. In EspIMS11 and EcIMS21, this phenotype was observed only in response to levofloxacin antibiotic, where there was also efflux-mediated resistance for other antibiotics (like cephalosporins) tested in both these isolates. This particular reverse MIC phenotype in presence of CCCP was therefore not restricted only to one particular antibiotic or one isolate, which needs further investigation.

Overall, from this MIC assay in presence of CCCP, we could notice involvement of bacterial multidrug efflux pumps in exhibiting multidrug resistance phenotype in these isolates.

4.1.4: PCR based screening of different efflux pumps genes

After observing multidrug resistance phenotype in the isolates tested, we investigated the molecular mechanisms of antibiotic resistance, and hence different efflux pump genes were screened in both clinical and environmental *Enterobacter* isolates. PCR assay for checking the presence of efflux genes belonging to RND superfamily (*acrA-acrB-tolC* genes), MATE superfamily, MFS superfamily, SMR superfamily was performed with bacterial cell lysate as the template DNA. PCR products were visualized on agarose gel and results were compiled.

4.1.4.1: PCR based detection of RND efflux genes

AcrAB-TolC tripartite efflux pumps belonging to RND superfamily are predominantly associated with multi-drug resistance phenotypes in many gram-negative pathogens [50]. Therefore, we screened for the presence of *acrA* (A1 & A2), *acrB* (B), *and tolC* (C1 & C2) efflux genes in our environmental and clinical *Enterobacter* isolates by PCR and images were recorded as shown in figure no. 4.1.4.

PCR screening revealed that AcrAB-TolC type of efflux pumps was more predominant in environmental *Enterobacter* isolates than clinical isolates (figure no.4.1.4: *acrA*-A1 Vs. A2, *tolC*-C1 Vs. C2). Moreover, in case of environmental isolates, 75% were positive for *acrA*, 20% were positive for *tolC* and 50% of isolates were positive for *acrB*; whereas in clinical isolates 37% were positive for *acrA*, 10% were positive for *acrB* and 50% of isolates were positive for *tolC*. The PCR-amplified products of *acrAB-tolC* efflux genes was further validated by sequencing and identity was confirmed by NCBI BLAST.

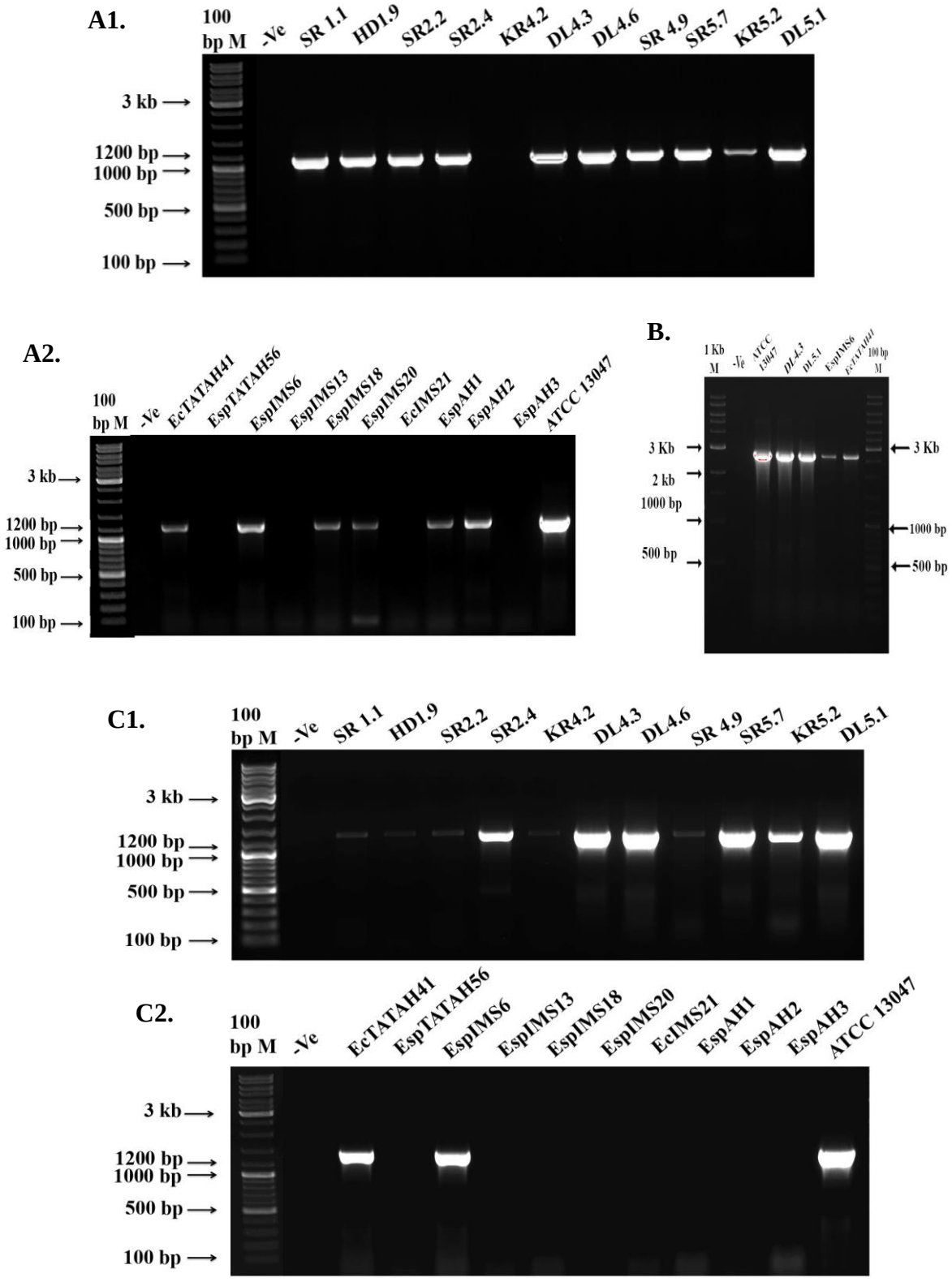


Figure no. 4.1.4: Gel electrophoresis of *acrA* (A1 & A2), *acrB* (B), *and tolC* (C1 & C2) efflux genes- PCR amplified products of A-*acrA* (1220 bp), B-*acrB* (2704 bp), C-*tolC* (1236 bp) were subjected to agarose gel (1% w/v) electrophoresis. A1 and C1 represented the PCR amplified products of environmental *Enterobacter* isolates, whereas A2 and C2 represented the PCR amplified products of clinical *Enterobacter* isolates.

4.1.4.2: Slot blot hybridization of *acrAB-tolC* efflux genes

Since acrAB-tolC type of RND efflux family was abundant in most of the *Enterobacter* isolates screened, we further validated their presence by slot blot hybridization. Extracted genomic DNA from each of the bacterial isolate was probed with purified PCR products and detection of hybridized blots was performed. Genomic DNA Samples loading order for slot blot arrangement was mentioned below in table no. 4.1.5. The slot blot images for *acrAb-tolC* efflux genes were depicted below in figure no. 4.1.5 (A-AcrA, B-AcrB, and C-TolC). The results obtained in slot blot hybridization corroborated well with PCR results; *acrA* was equally predominant in both clinical as well as environmental *Enterobacter* isolates and *tolC* was more abundant in clinical strains than environmental isolates. Moreover, the RND transporter *acrB* was again highly abundant in clinical isolates followed by environmental isolates (figure no. 4.1.5).

4.1.4.3: PCR based detection of other efflux genes

After exploring the presence of RND superfamily efflux genes, we also investigated the presence of other families of efflux pumps. We had designed degenerated short length PCR primers for MATE (105 bp), MFS (107 bp) and SMR (106 bp) superfamily of efflux pumps. The PCR amplified products were then subjected to agarose gel (1.2% w/v) electrophoresis and images were recorded. Presented below are few representative images of the PCR products (figure no. 4.1.6.). The PCR amplified products of *Mate*, *Mfs* and *Smr* efflux genes were gel-purified and purified product was sequenced. Sequences obtained were validated by BLAST for their identity.

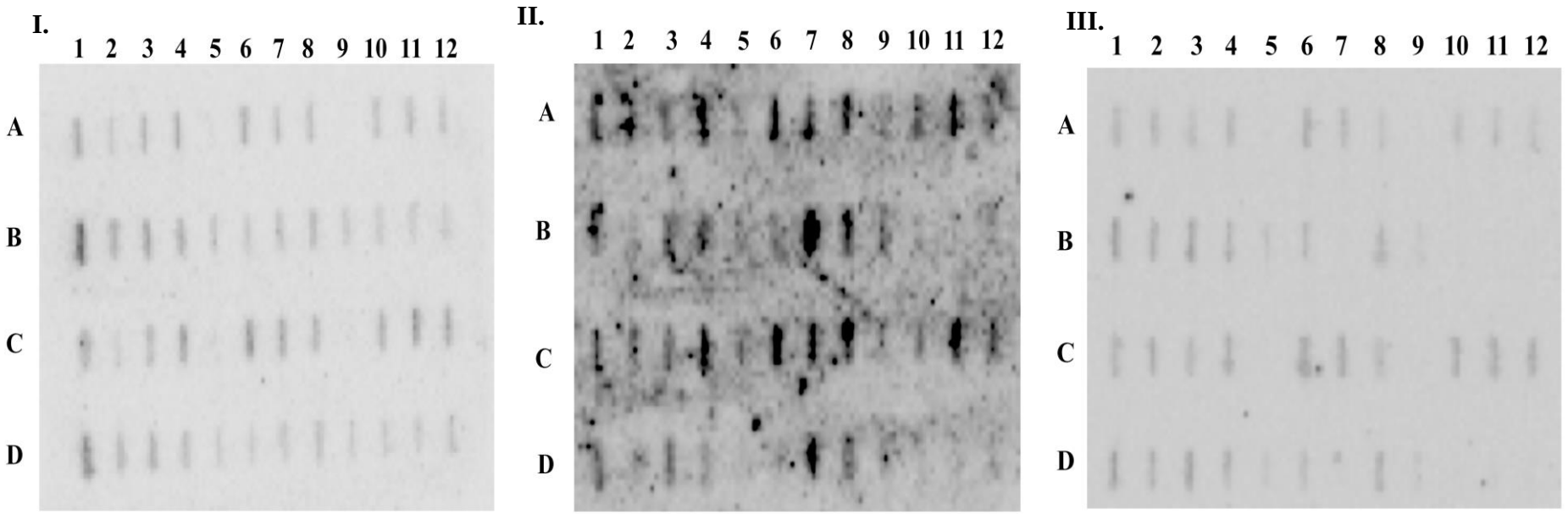


Figure no. 4.1.5: Slot blot images of *acrA* (panel I) *acrB* (panel II) and *tolC* (panel III) efflux genes- Row A and B contained clinical *Enterobacter* isolates whereas row C and D contained environmental *Enterobacter* isolates (Except for positions-D9, D10 and D11 which contained ATCC type strain and D12 remained blank). Bacterial genomic DNA samples were loaded in columns (1-12) order as mentioned below in table no. 4.1.5 for all the blots.

Table no. 4.1.5: Genomic DNA samples loading order for slot-blot arrangement

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	EcTATAH41	EspTATAH56	EspIMS1	EspIMS4	EspIMS5	EspIMS6	EspIMS7	EspIMS8	EspIMS9	EspIMS10	EspIMS11	EspIMS13
B	EspIMS16	EspIMS17	EcIMS18	EspIMS19	EspIMS20	EcIMS21	EspAH1	EspAH2	EspAH3	EspAH4	*E.cloacae* 13047	*E.aerogenes* 13048
C	SR1.1	SR1.5	HD1.9	KR1.8	SR2.2	SR2.3	SR2.4	HD2.1	SR4.9	KR4.2	DL4.3	DL4.6
D	DL4.7	HD4.3	SR5.7	KR5.2	KR5.3	KR5.9	DL5.1	DL5.6	*Klebsiella* 13883	*E.coli* 25922	*Pseudomonas* 27853	-

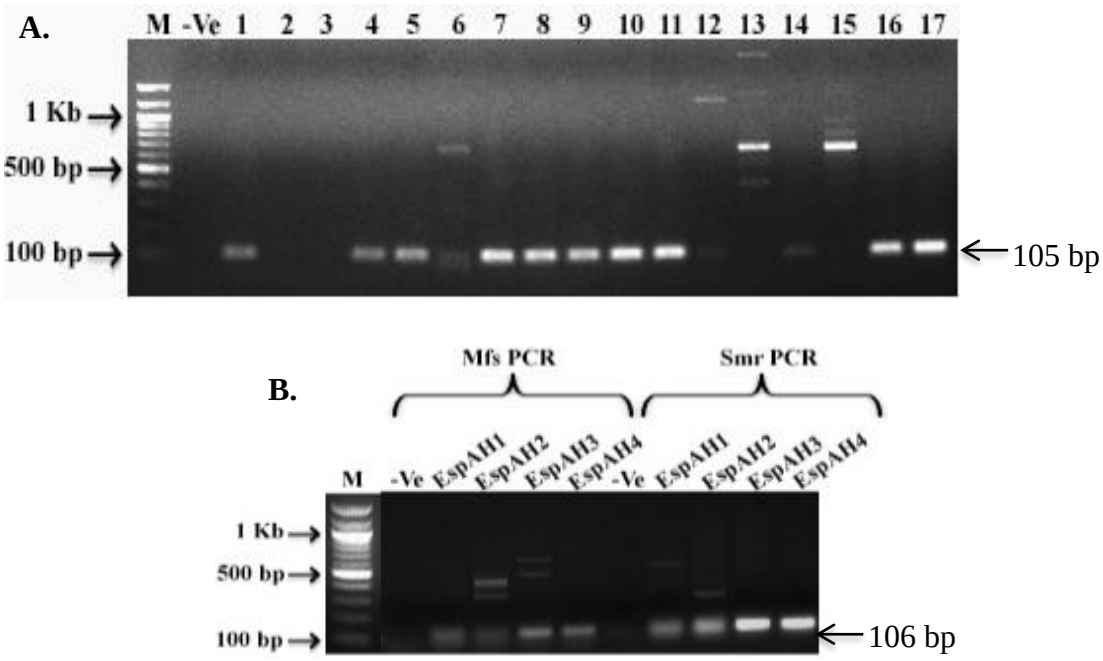


Figure no. 4.1.6: Gel electrophoresis (1.2% w/v) of PCR screening for other efflux pump genes - *Mate* (A) *Mfs* and *Smr* (B) Samples order for *Mate* PCR: M- 100bp ladder, -Ve- Negative control, 1-EspIMS1, 2-EspIMS2, 3-EspIMS3, 4-EspIMS4, 5-EspIMS5, 6-EspIMS6, 7-EspIMS7, 8-EspIMS8, 9-EspIMS9, 10-EspIMS10, 11-EspIMS11, 12-EspIMS12, 13-EspIMS13, 14-EspIMS14, 15-EspIMS15, 16-EspIMS16, 17-EspIMS17.

It was noteworthy that MFS and SMR type of efflux pumps were exclusively present in clinical isolate EspAH3 and EspAH4. On the contrary, all the environmental isolates tested here were devoid of these types of efflux pumps. Positive PCR amplicons for MATE type of efflux pumps was obtained more in clinical isolates as compared to environmental *Enterobacter* isolates. Overall the PCR results were compiled and distribution of efflux genes in the *Enterobacter* populations as a pie chart is shown below in figure no. 4.1.7. PCR based screening and slot blot suggested wide abundance of AcrAB-TolC type of efflux pumps in both clinical (figure no. 4.1.7A) and environmental *Enterobacter* isolates (figure no. 4.1.7B).

In case of environmental isolates SR1.5, SR2.2, SR2.3, SR2.4, HD2.1, DL4.3, DL4.6 and DL5.1 all three parts of a functional tripartite efflux unit i.e. *acrA, acrB* & *tolC*

were present. Interestingly, MATE efflux pumps were the second most abundant efflux pumps in both clinical (29%) and environmental (19%) isolates (figure no. 4.1.6A vs. 4.1.6B). This gave us an idea about the distribution of several efflux genes in *Enterobacter* isolates in the context of their source of isolation.

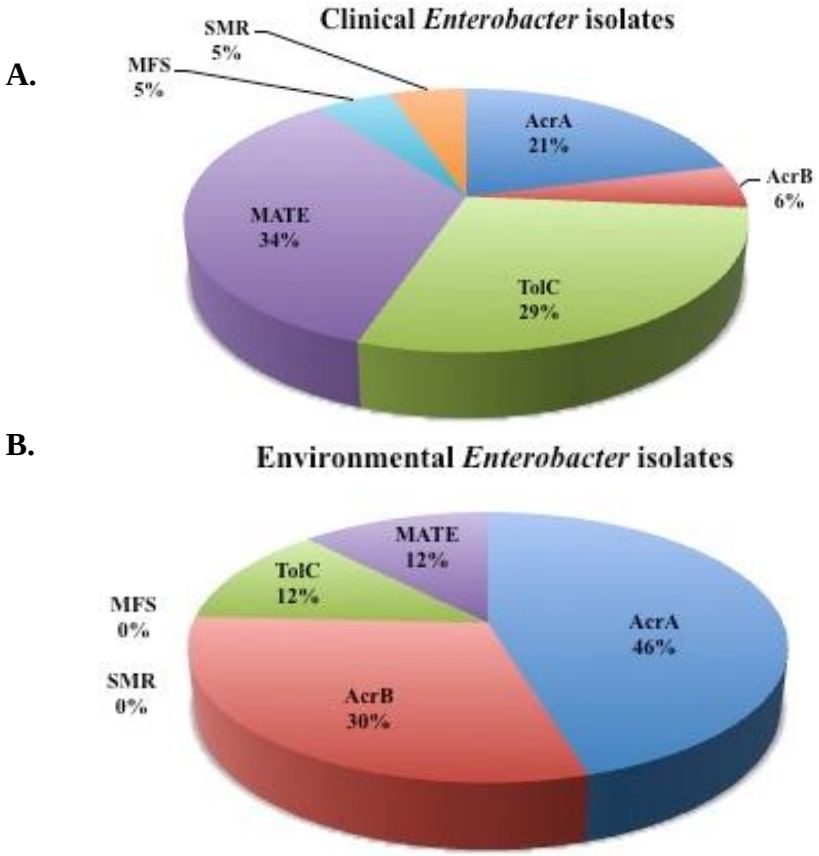


Figure no. 4.1.7: Distribution of efflux genes in *Enterobacter* isolates: Pie chart represented the occurrence of different efflux genes belonging to RND, MFS, SMR and MATE super families of efflux pumps in clinical (A) and environmental (B) *Enterobacter* isolates studied.

4.1.5. Effect of sodium (Na^+) on MATE efflux activity

MATE (Multiple antibiotic and toxic extrusion) superfamily of efflux pumps is known to utilize H^+/Na^+ as its energy source for effluxing substrates. Therefore, we investigated the effect of varying concentrations of sodium (0, 50, 100 miliMolar of NaCl) in the presence/absence of ionophore CCCP (that blocks the proton motive force gradient) to efflux cephalexin (1^{st} generation cephalosporin drug). Normal saline solution (0.89% w/v NaCl) used in clinics for injecting antibiotics is equivalent to

152mM of NaCl, this guided us to use 100 mM of NaCl here. We tested this phenomenon in an environmental *Enterobacter* isolate KR4.2, which contained MATE efflux pump.

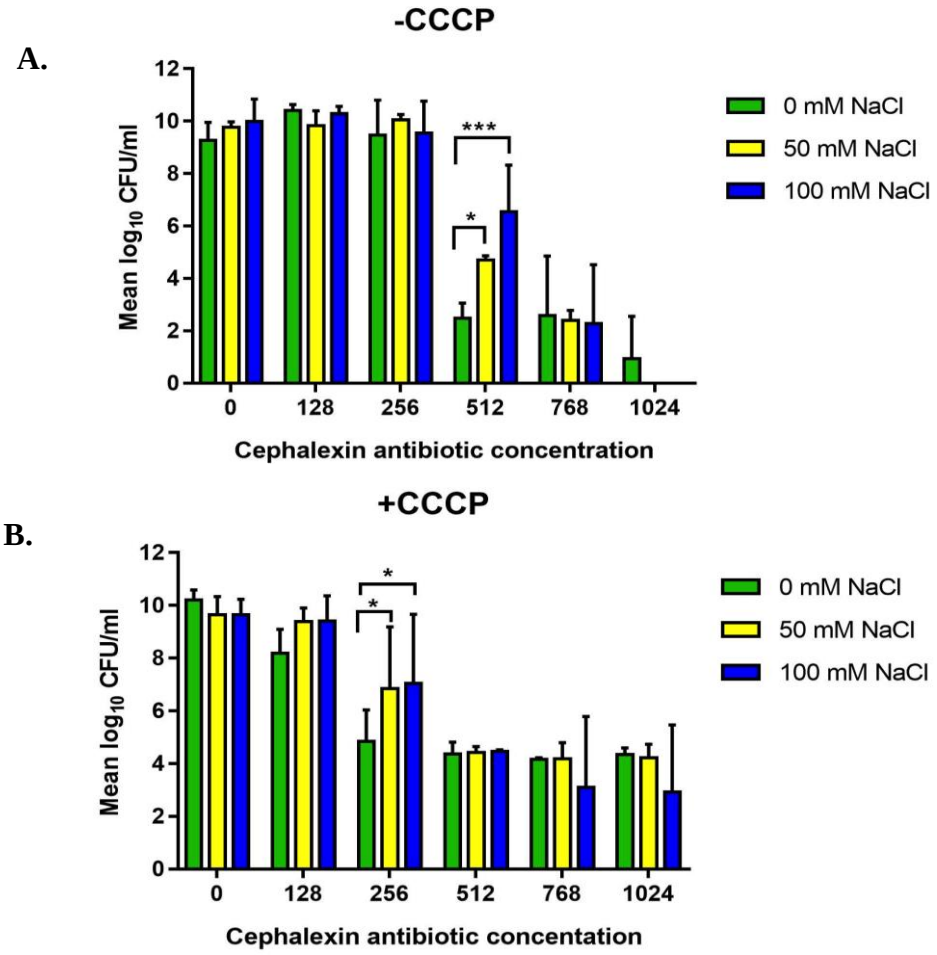


Figure no. 4.1.8: Effect of sodium on bacterial growth in presence of varying concentrations of cephalexin antibiotic without (A) or with CCCP (B). Bacterial growth was recorded from three biological replicates with three technical replicates each subjected to different treatment combinations of NaCl and CCCP, as mentioned in the text in detail. Two-way ANOVA statistical test was applied where * represented $p<0.05$ and *** represented $p<0.001$.

The MIC value for cephalexin in isolate KR4.2 was 512 µg/ml. Without antibiotic, addition of NaCl at 50 mM and 100mM concentrations did not show any significant differences in bacterial growth (figure no. 4.1.8A and 4.1.8B). However, without the efflux pump inhibitor CCCP, at 512 µg/ml of cephalexin concentration (figure no. 4.1.8A), addition of sodium chloride at 100mM and 50 concentration, showed three and

two fold increase in CFU counts respectively in comparison to cells without sodium treatment. There was certainly no increase in MIC without CCCP, even after addition of 50 or 100 mM NaCl. On the contrary, in presence of CCCP (figure no. 4.1.8B), irrespective of NaCl concentrations, bacterial growth was found to be indifferent at 512 µg/ml of cephalexin concentration. Compared to without CCCP, the bacterial CFU count at higher antibiotic concentration (i.e. 1024 µg/ml) was greater in the presence of CCCP (figure no. 4.1.8A vs. 4.1.8B). In presence of CCCP, when the primary energy source (i.e. H^+/PMF) of the efflux system is blocked, the organism could utilize 50/100 mM of NaCl as an alternative energy source for effluxing the drugs, as in here MATE efflux pumps. This resulted in the organism's tolerance towards higher concentration of cephalexin (1024 µg/ml) (figure no. 4.1.8B).

Discussion

Efflux pumps are prominent drivers of multidrug resistance in several bacterial isolates [1]. These membrane embedded transporters are highly efficient in excluding multiple drugs, noxious dyes and toxic compounds owing to their poly-substrate specificity, helps the pathogen in attaining multidrug resistance phenotype [57]; [176]. Based upon substrate specificity, energy requirements, membrane architecture and distribution; bacterial multidrug efflux pumps have been categorized into five different super-families: (i) ATP-binding cassette (ABC) superfamily, (ii) The major facilitator superfamily (MFS), (iii) The drug/metabolite transporter (DMT) superfamily (including the small multidrug resistance [SMR] family), (iv) The multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily (including the multidrug and toxic compound extrusion [MATE] family) and (v) The resistance-nodulation-cell division (RND) superfamily [32].

Of the above-mentioned efflux pumps, the tripartite constitution in RND efflux pumps exemplified by AcrAB-TolC, are predominant in many gram-negative pathogens including *Escherichia coli*, *Salmonella* spp., *Klebsiella* spp., & *Enterobacter* spp. [48], [177], [50]. *Enterobacter* species; majorly *E. cloacae* & *E. aerogenes* are important causative agents for nosocomial infections, belong to group of 'ESKAPE' pathogens, owing to their multiple drug resistance (MDR) phenotypes, alarming a threat to current medicine [178], [179]. Both these species are significantly associated with nosocomial outbreaks, clinical infections like catheter related UTI's, abdominal cavity/intestinal infections, wound infections, pneumonia, and septicemia [7]; [8]. Reports also suggest the importance of MDR efflux pumps in maintaining cellular homeostasis and virulence in *E. cloacae,* besides its primary role of antibiotic resistance. With this background, we investigated the distribution of different MDR efflux pump families in environmental and clinical *Enterobacter* isolates used in this study.

First, total 137 strains from environmental sources (Water samples) and total 130 strains isolated from clinical sources (mainly Urinary tract infections, and few from sputum, tracheal swabs, pus, and wound swabs) were collected under aseptic conditions. Samples were processed through routine microbiological identification procedures involving enriched and differential media (Eosin methylene blue agar-EMBA and MacConkey agar-MA) to identify gram-negative *Enterobacter* isolates from other members of family Enterobacteriaceae. Aminoacid decarboxylation based biochemical test was also conducted for presence of three decarboxylase enzymes each utilizing lysine, arginine or ornithine in *Enterobacter* spp. Based on 16S rRNA gene sequencing and microbiological assays, *Enterobacter* isolates were identified up to genus level; 20 from environmental (as presented in table no. 4.1.1) and 22 from clinical samples (as

presented in table no. 4.1.2) respectively were then selected for further study. Moreover, in environmental group of isolates, 8% belonged to *E.cloacae* and 5% were *E. aerogenes*; and in clinical isolates, 5% were *E.cloacae* and 9% belonged to *E. aerogenes. E. cloacae* and *E. aerogenes* were prevalent in the populations under study, which is coinciding with earlier reports [8], suggesting these two to be predominant species of *Enterobacter* in the environment and clinics.

Disc diffusion assay for forty different antibiotics belonging to major groups like β-lactams, cephalosporins, quinolones, polypeptides, carbapenems, colistin was determined for environmental (n=20) and clinical *Enterobacter* isolates (n=22). Many of these environmental and clinical isolates were found to be showing resistance towards multiple antibiotics belonging to two or more group of antibiotics, indicating them to be multi-drug resistant (MDR) isolates (figure no. 4.1.1). It was noteworthy that most of the environmental *Enterobacter* isolates have developed resistance to more than one antimicrobial agent and thus displayed MDR phenotype. On contrast, most of the clinical isolates were resistant to more than two key classes of antibiotics thus extreme drug resistant (XDR) in nature; and few isolates showed resistance to all commercially available antibiotics tested, i.e. pan-drug resistant (PDR) phenotype [180]. The extent of drug resistance was also validated by calculating multiple antibiotic resistance (MAR) index; which suggested that the clinical isolates have significantly greater MAR indices (=0.8) as compared to environmental isolates (MAR index=0.4).

The minimum inhibitory concentration (MIC) for antibiotics was determined for all the *Enterobacter* isolates by macro broth dilution method as per the CLSI guidelines. CCCP an ionophore, acts as an energy decoupler, dissipating membrane potential by blocking protein motive force [181]. CCCP, at an optimized 50µMolar concentration, was used in MIC of screening of efflux pump mediated resistance for all the isolates.

Two or more fold decrease in MIC values of several antibiotics tested in presence of CCCP indicated towards possible inhibition of efflux pump, leading to inefficient exclusion of antibiotics as observed by less antibiotic concentration becoming fatal for bacterial growth [182]. From the noticed reduction in MIC in presence of CCCP towards multiple antibiotics, it was evident that efflux pumps contributed towards observed MDR phenotype in both clinical and environmental *Enterobacter* isolates.

Next, *Enterobacter* isolates were screened for occurrence of efflux pumps genes belonging to different super families such as RND (*AcrAB-TolC*), *MATE, MFS* and *SMR*. PCR screening followed by slot blot and sequence confirmation helped us in determining the distribution of MDR efflux pumps. Results suggested that AcrAB-TolC type of efflux pumps was predominately present in the isolates under study followed by MATE efflux pump, which was more prevalent in clinical isolates. This approved earlier reports as AcrAB-TolC efflux pumps to be key MDR efflux pump in *Enterobacter* spp. [72]. MFS and SMR type of efflux pumps were only found in clinical isolates. Again, greater efflux activity was evident in presence of NaCl, as observed from inhibition of bacterial growth even at >MIC concentrations of cephalexin antibiotic, in an *Enterobacter* isolate possessing MATE efflux pump. This emphasized on the importance of Na^+ ions, apart from PMF on MATE efflux pump activity [50].

Overall, such screening of efflux pump genes provided a comparative overview of extent of drug resistance amongst environmental and clinical *Enterobacter* isolates and the contribution of efflux pumps in mediating such MDR phenotypes.

Results 4.2

Outer membrane proteins (OMPs) in Enterobacter spp: Association with antibiotic resistance and virulence

Section 4.2: Association studies of Outer membrane proteins with antibiotic resistance and virulence in clinical and environmental *Enterobacter* spp.

Background of the study

Outer membrane proteins (OMPs) are water-filled open channels, that allow passive penetration of molecules. Besides their solute-carrier properties, OMPs are also known to function in various other cellular processes. In addition, OMPs act as receptors for bacteriocins, hemolysin, other toxins and antibodies [127]. A well-coordinated interplay between outer membrane permeability through the regulation of the porin channels and increased efflux activity; facilitated the emergence of multi-drug resistant bacteria [146]. Development of multidrug resistance phenotype in gram-negative pathogens has recently been associated with porin modification in three ways: alterations in porin expression, decreased porin expression and mutation in porins. All of these factors individually or together affect the susceptibility towards antibiotics, β-lactams in particular [14]. However, the association pattern of OMPs with antibiotic resistance and virulence is a less explored aspect, which we investigated and presented the results of the same in this section.

4.2.1. Screening for presence of OMPs in *Enterobacter* isolates

Both clinical and environmental *Enterobacter* isolates were screened for the presence of OMPs genes by multiplex PCR, followed by slot blot hybridization and sequencing of the PCR products for confirmation. After optimizing the PCR cycling parameters with *Enterobacter cloacae* ATCC 13047 isolate, multiplex PCR was performed for *OmpA*, *OmpX*, *OmpF*, *OmpC*, *FhuA* and *LamB* for 20 environmental and 22 clinical

Enterobacter isolates along with ATCC type strains (figure no.4.2.1). PCR products were subjected to agarose gel (1.5% w/v) electrophoresis and images were recorded.

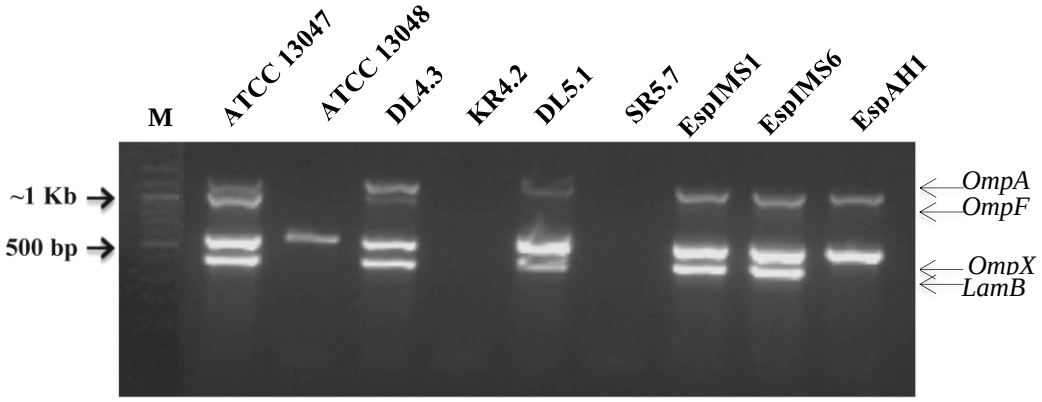


Figure no. 4.2.1: Agarose gel (1.5%) images of multiplex PCR to detect OMPs in Clinical and Environmental isolates. Multiplex PCR products resulted in four distinct bands of OMPs in *E. cloacae* ATCC 13047, *OmpA* (1196 bp), *OmpF* (1001 bp), *OmpX* (~500 bp) and *LamB* (419 bp). Taking ATCC 13047 as control, multiplex PCR based screening was performed taking other clinical and environmental *Enterobacter* isolates.

As observed form the multiplex PCR for OMPs, *OmpA* and *OmpX* were present in both clinical (30% and 24%) and environmental isolates (36% and 35%) respectively; with higher occurrence in environmental *Enterobacter* isolates (figure no. 4.2.1). Similarly, *LamB* and *OmpF* were found in 24% and 22% of clinical isolates respectively, whereas only 14-15% of environmental isolates were positive for them. However, none of these isolates tested, were positive for presence of *OmpC* and *FhuA* (figure no. 4.2.1).

Sequencing further validated the PCR amplified products and the identity of different OMPs was confirmed by NCBI BLAST algorithm. Results obtained from multiplex PCR were validated by slot blot hybridization for each *Enterobacter* isolates tested, as shown in the figure no. 4.2.2. The results obtained in slot blot hybridization corroborated well our multiplex PCR results; indicating *OmpA* and *OmpX* to be

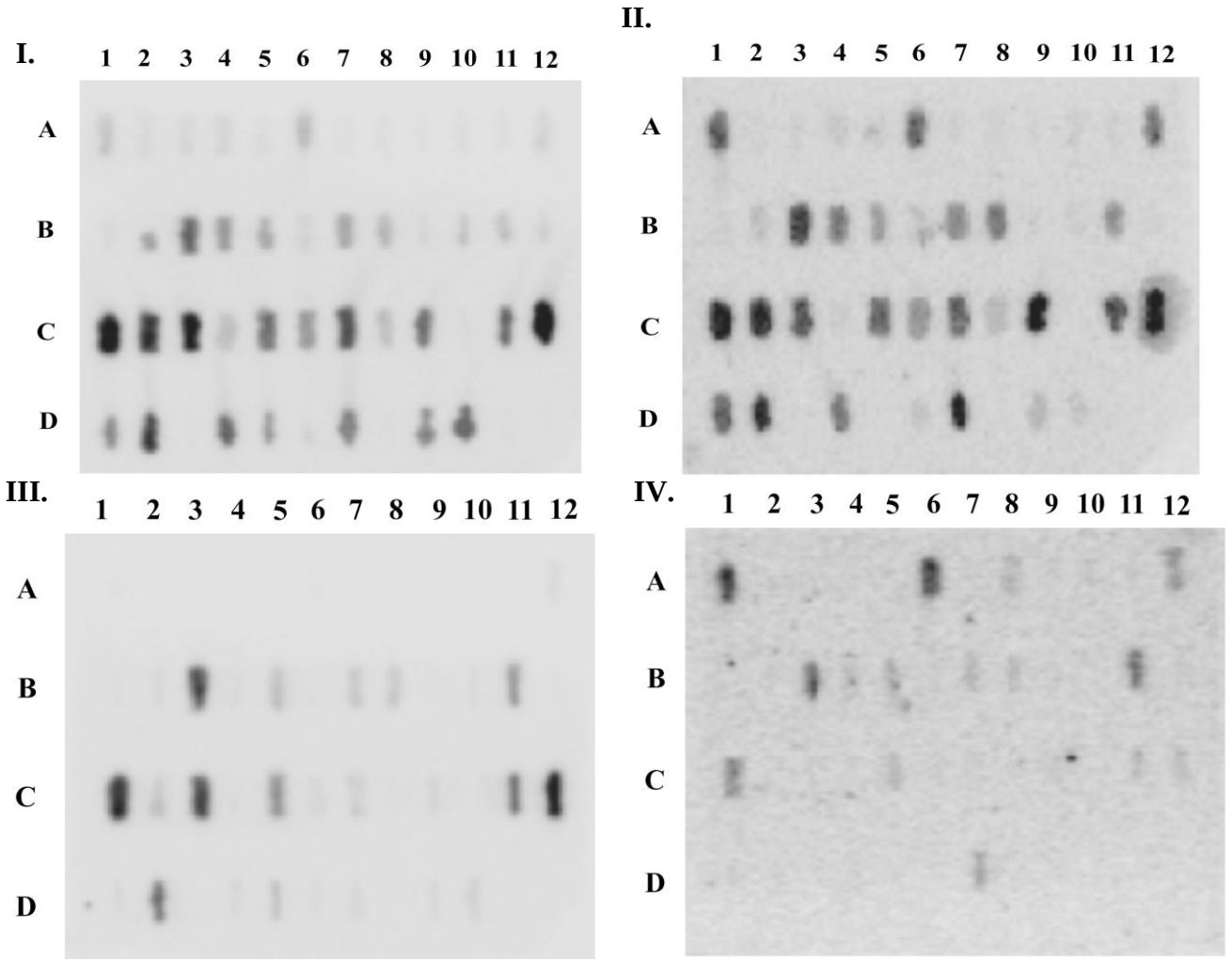
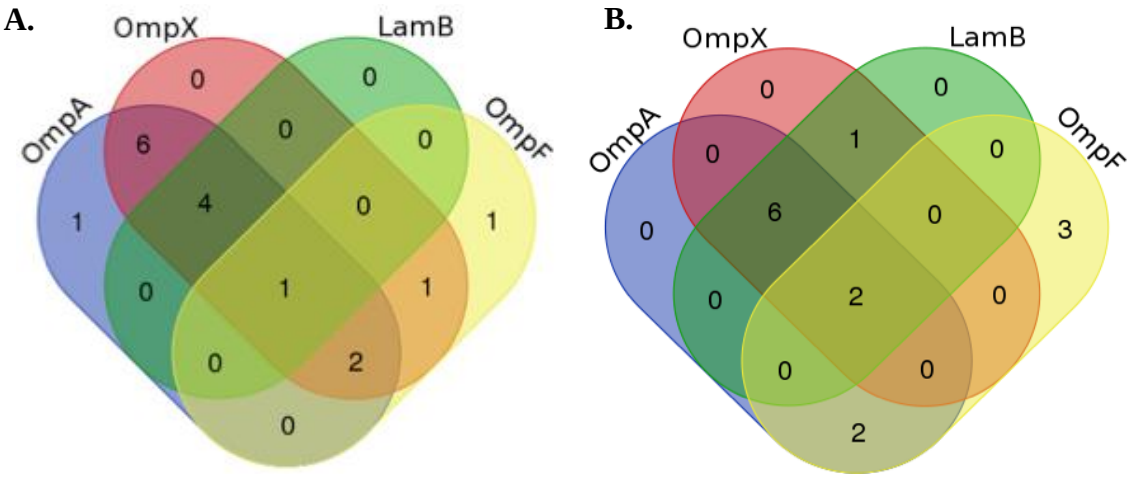


Figure no. 4.2.2: Slot Blot images of OmpA (I), OmpX (II), OmpF (III) and LamB (IV)- Panel I and II contained clinical *Enterobacter* isolates whereas panel III and IV contained environmental *Enterobacter* isolates (Except for positions-B11, B12, D9, D10 and D11 which contained ATCC type strain and D12 remained blank). The detailed sample loading order is same as mentioned earlier in table no 4.1.5.

predominant OMPs in both environmental and clinical *Enterobacter* isolates (figure no. 4.2.2). Overall, the multiplex PCR and slot blot results were compiled and distribution of OMPs in *Enterobacter* isolates as a venn diagram, is presented in figure no. 4.2.3.



A. In Environmental *Enterobacter* Isolates

LamB OmpA OmpF OmpX **(1):** SR2.2

LamB OmpA OmpX **(4):** DL4.3, DL4.6, DL5.1, SR1.1

OmpA OmpF OmpX **(2):** SR1.5, SR2.3

OmpA OmpX **(6):** DL4.7, KR5.2, SR4.9, SR2.4, HD4.3, HD1.9

OmpF OmpX **(1):** HD2.1

OmpA **(1):** KR5.3

B. In clinical *Enterobacter* Isolates

LamB OmpA OmpF OmpX **(2):** *E.cloacae* ATCC 13047, EspAH2

LamB OmpA OmpX **(6):** EspIMS6, EcIMS18, EspAH1, EcTATAH 41, EcIMS19, EcIMS20

OmpA OmpF **(2):** EspIMS17, EspAH4

LamB OmpX **(1):** EspIMS13

OmpF **(3):** EspIMS16, EspIMS8, EspIMS11

Figure no. 4.2.3. Overview on occurrence of OMPs in *Enterobacter* isolates- Venn diagram representing distribution pattern of OMPs in (A) Environmental and (B) Clinical *Enterobacter* isolates.

Nevertheless, *LamB-OmpA-OmpX* and *OmpA-OmpX* combination of OMPs were more prevalent in clinical (n=6) and environmental (n=6) isolates respectively. There were also environmental (n=1), and clinical (n=3) *Enterobacter* isolates, where only *OmpF* was present (Figure no. 4.2.3). Two clinical isolates (EspAH2 and *E.cloacae* ATCC-

13047) and one environmental isolate (SR2.2) contained four OMPs (*OmpA*, *OmpX*, *OmpF* and *LamB*) (Figure no.4.2.3).

4.2.2. Pheotypic detection of presence of different virulence factors

Bacterial virulence factors enable the pathogen to replicate and disseminate within host cells in part by evading the host-defense system. Evaluation of such virulence factors is important to determine their pathogenic potential. We selected few MDR environmental as well as clinical *Enterobacter* isolates and screened their virulence potential by Haemagglutination assay, biofilm assay and serum resistance assay; results of the same has been summarized and represented below in table no. 4.2.1.

Detection of type-I fimbriae by Haemagglutination test revealed that all of the environmental and clinical *Enterobacter* isolates tested to be positive for fimbriae; including DL4.6 (environmental) and EspAH3 (clinical) isolates being late positive for agglutination (table no. 4.2.1).

Serum resistance assay also stated the pathogenic potential of these isolates, which ranged from grade-1 (being non-resistant) to grade-6 (highly serum resistant), as mentioned previously in table no. 3.2.3. This was determined as per their ability to maintain viable count post-treatment with human serum from 1 hour to 3 hours [170]. As evident from table no. 4.2.2, environmental isolates DL4.3, DL4.6, SR5.7 of displayed low serum resistant activity and belonged to grade-2, where as DL5.1 was of grade-3 with moderate serum resistance. SR4.9, however, being an environmental isolate, showed grade-5 of highly serum resistant phenotype. Out of the clinical isolates, EspAH2 and EspAH3 were of grade-2 and EcIMS21 and EspAH4 were of

grade-3 serum resistant type. Moreover, isolates EcTATAH41, EspIMS6 and EcIMS18 were of grade-5 serum resistant phenotype indicating towards their strong pathogenicity (table no. 4.2.1).

Table no. 4.2.1: Screening of virulence factors in *Enterobacter* isolates

Isolates	Strains tested	Haemagglutination assay (Fimbriae)	Serum resistance assay	Biofilm assay
Environmental	SR4.9	Positive	Grade 5	Weakly adherent
	DL4.3	Positive	Grade-2	Weakly adherent
	DL4.6	Late Positive	Grade-2	Weakly adherent
	SR5.7	Positive	Grade-2	Strongly adherent
	DL5.1	Positive	Grade-3	Moderately adherent
Clinical	EcTATAH41	Positive	Grade 5	Strongly adherent
	EspIMS6	Positive	Grade 5	Strongly adherent
	EcIMS18	Positive	Grade 5	Weakly adherent
	EcIMS21	Positive	Grade-3	Strongly adherent
	EspAH2	Positive	Grade-2	Moderately adherent
	EspAH3	Late Positive	Grade-2	Strongly adherent
	EspAH4	Positive	Grade-3	Strongly adherent
	E.cloacae 13047	Positive	Grade-5	Strongly adherent

Microtitre plate based crystal violet staining method was performed for evaluating biofilm formation ability of the isolates at 24 and 48 hours as described in [171]. Biofilm assay suggested that most of the clinical isolates i.e. EcTATAH41, EspIMS6, EcIMS21, EspAH3 and EspAH4 were strongly adherent in nature even upto 48 hours (figure no. 4.2.4B). Where as isolate EspAH2 was moderately adherent and isolate EcIMS18 was weakly adherent in nature. On the contrary, environmental isolates SR4.9, DL4.3 and DL4.6 were weakly adherent and DL5.1 was moderate adherent in nature (figure no. 4.2.4A and B). Interestingly, environmental isolate SR5.7 was found

to be strong biofilm producer (figure no.4.2.4A and B). *Enterobacter cloacae* type strain ATCC 13047 was also found to be strongly adherent in the biofilm assay.

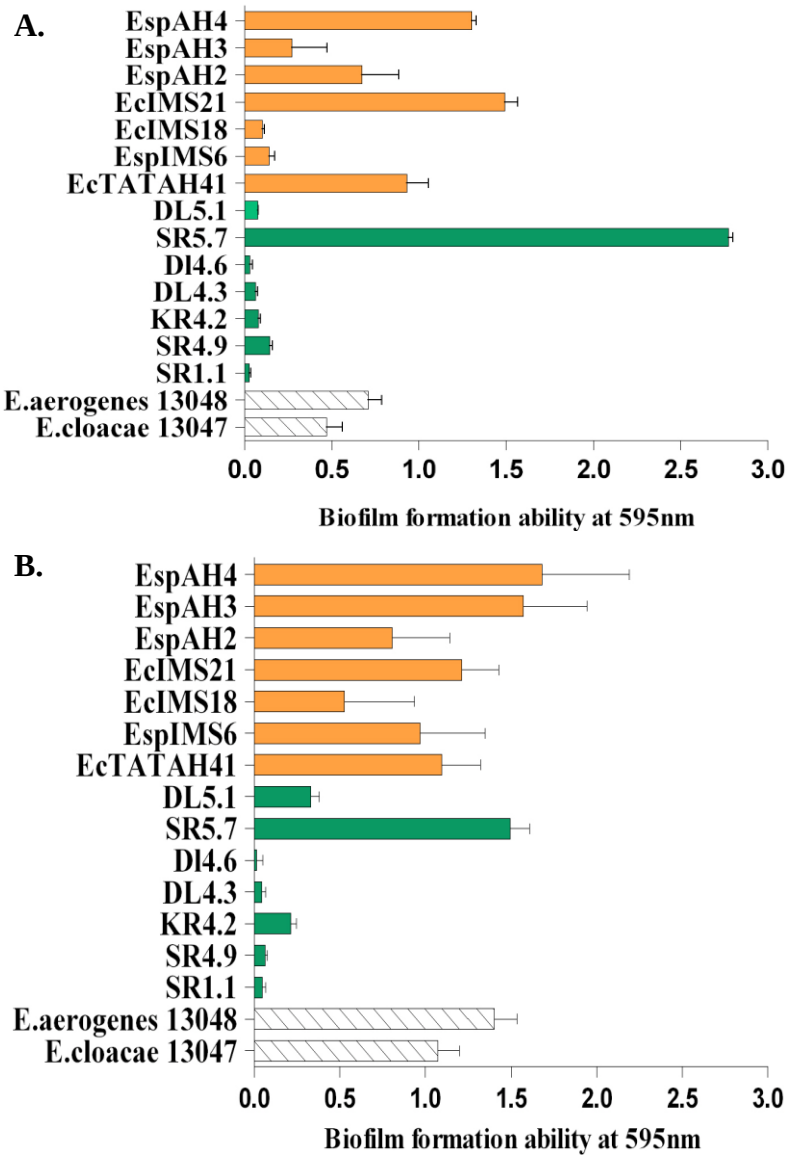


Figure no. 4.2.4: Biofilm formation of *Enterobacter* isolates by crystal violet staining method at 24 hours (A) and 48 hours (B). The X-axis represented the O.D. value of bound crystal violet at 595 nm. The Y-axis represented the *Enterobacter* isolates being tested. The orange bar and green bar in the graph indicated clinical and environmental *Enterobacter* isolates respectively, whereas white bar represented the ATCC *Enterobacter* type strains: ATCC 13047 and ATCC 13048.

4.2.3. Bacterial infection to murine macrophage RAW 264.7 cell line

For a pathogen, to be successful it is important to adhere to the host cell and infiltrate to enable colonization, which boost bacterial pathogenicity. To determine in-vitro

pathogenic potential of the MDR *Enterobacter* isolates, we used murine macrophage cell line RAW 264.7. Macrophage cell lines are widely used for testing bacterial infection process, as they are the flag-bearer of host innate immune response.

First, we standardized the multiplicity of infection (MOI) and time of infection (TOI) for *Enterobacter* isolates as shown in figure no. 4.2.5.

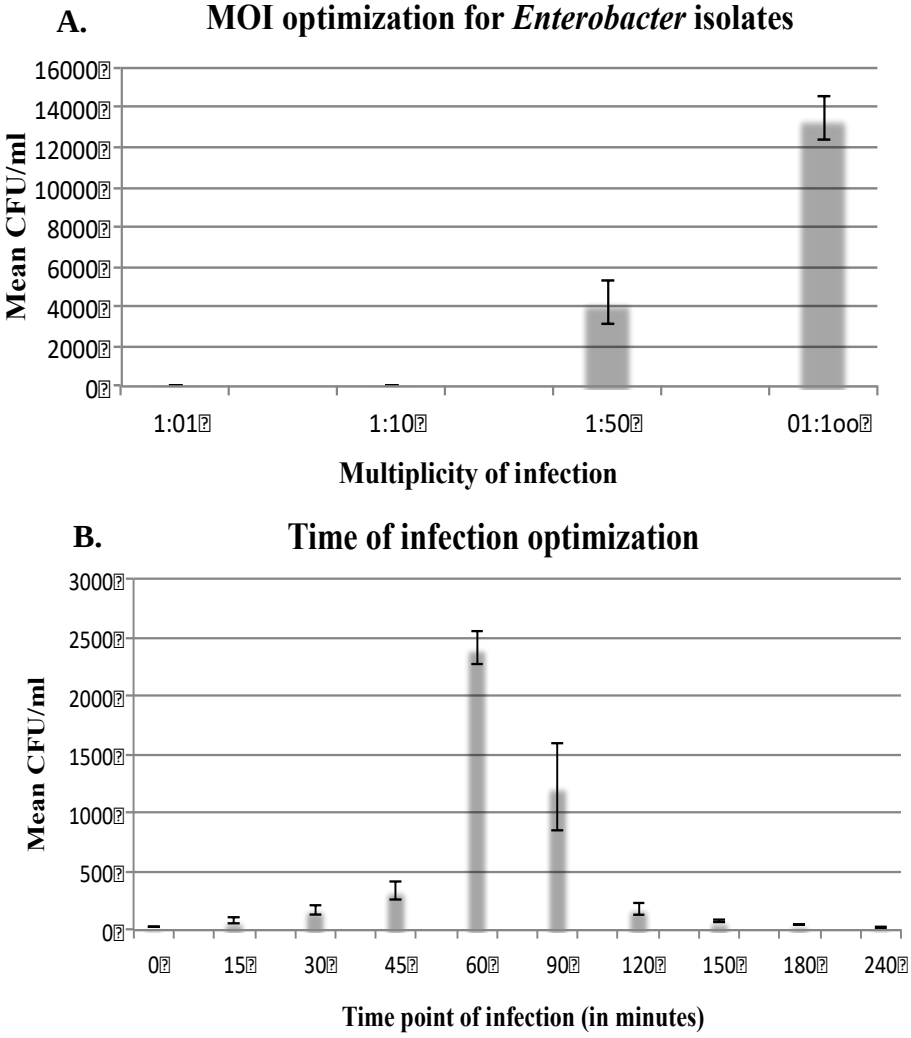


Figure no. 4.2.5: Optimization of MOI and TOI in cell culture based assays
A) Represent the standardization of multiplicity of infection (MOI)
B) Represent the standardization of time point of infection (TOI)

As represented in figure no. 4.2.5A, at multiplicity of infection (MOI) (ratio of host cells to bacterial cells) of 1:1, 1:10, there was not readable signs of infection, which indicated that the number of bacterial cells used for infection was not sufficient to

establish infections. However at MOI of 1:50 and 1:100, *Enterobacter* isolates were capable of successful infection as observed from their viable count (figure no. 4.2.5A). Though, MOI of 1:100, yielded maximum number of viable bacterial cells, three times CFU than that observed at MOI 1:50 (figure no. 4.2.5A), this possibly could be due to saturation of pathogens in the medium to such an extent that overpowered the killing activity of macrophages. Hence, we selected MOI of 1:50 as our standardized MOI for rest of the infection processes. The rationale behind this is to enhance the chance of infection by optimal but not saturated bacterial cells that can adhere and invade the mammalian cells subsequently.

With a MOI of 1:50, we tried to optimize the suitable time of exposure of pathogen to macrophage RAW 264.7 cell line. For this, we had incubated the mammalian cells with bacterial cells at different time points from 0, 15, 30, 45, 60, 90, 120, 150, 180 to 240 minutes (figure no. 4.2.5B). After each TOI, bacterial cells were enumerated, which revealed that after 60 minutes of exposure maximum number of *Enterobacter* cells were viable, suggesting that the organism is able to survive the counter-defense mechanism of macrophages. Hence pathogen exposure at MOI of 1:50 for 60 minutes of TOI is best suited for establishing a successful *Enterobacter* infection in macrophage cell line.

With the optimized parameters, we infected RAW 264.7 cell line with clinical and environmental *Enterobacter* isolates following gentamicin protection assay to observe in-vitro cell attachment and invasion features. On comparison with *in-vitro* cell-attachment and invasion potential of standard pathogenic *Salmonellae typhii* isolate ATCC 13324 (in terms of viable bacterial cells), tested *Enterobacter* isolates were categorized into three major groups:

I) Highest pathogenic potential ($P.P(_{Entero.}) \geq P.P(_{S.typhii})$)

II) Moderate pathogenic potential ($P.P(_{Entero.}) \leq P.P(_{S.typhii})$)

III) Minimal pathogenic potential ($P.P(_{Entero.}) << P.P(_{S.typhii})$)

Where P.P is pathogenic potential of the tested organism that was determined based on the CFU of bacterial cells post infection into RAW 264.7 macrophage cell line. Results obtained from three independent biological replicates for each isolate was calculated and represented as bar graphs below in figure no. 4.2.6. (*in-vitro* Adhesion) and figure no. 4.2.7. (*in-vitro* Invasion).

Cell attachment assay revealed overall higher ability of environmental isolates to adhere to RAW 264.7 cell line as compared to clinical isolates (figure no. 4.2.6A and 6B). Environmental isolate SR4.9 showed highest *in-vitro* cell attachment ability with phenotype similar to that of ATCC *Salmonellae typhii* 13324 (figure no. 4.2.6A). Isolates DL5.1 and SR5.7 showed moderate attachment. Whereas clinical isolates EcTATAH41, EcIMS21 displayed moderate attachment and isolates EspIMS6, EspAH4 showed minimal *in vitro* attachment (figure no. 4.2.6B).

On the contrary, out of the clinical isolates only EspIMS6 and EcTATAH41 showed moderate to strong invasiveness in RAW 264.7 cell line (figure no. 4.2.7B), where as amongst environmental isolates specifically SR4.9 and DL5.1 showed weaker invasive properties (figure no. 4.2.7A). This indicated the emergence of virulent multi-drug resistant clones in environmental samples.

Looking into this, we have calculated the % ratio of invasion frequency to adhesion frequency for % fraction of populations that are invading, which is tabulated in table no. 4.2.2. for the *Enterobacter* isolates tested.

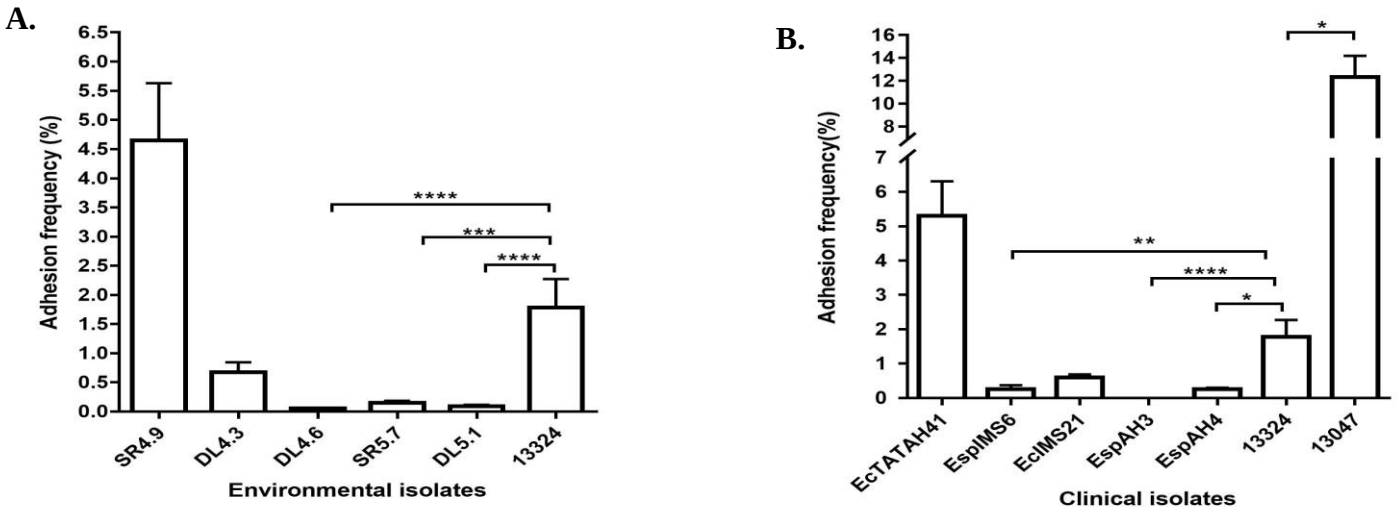


Figure no. 4.2.6: *In-vitro* cell-attachment ability of *Enterobacter* isolates in RAW 264.7 cell line. A) Environmental and B) Clinical isolates

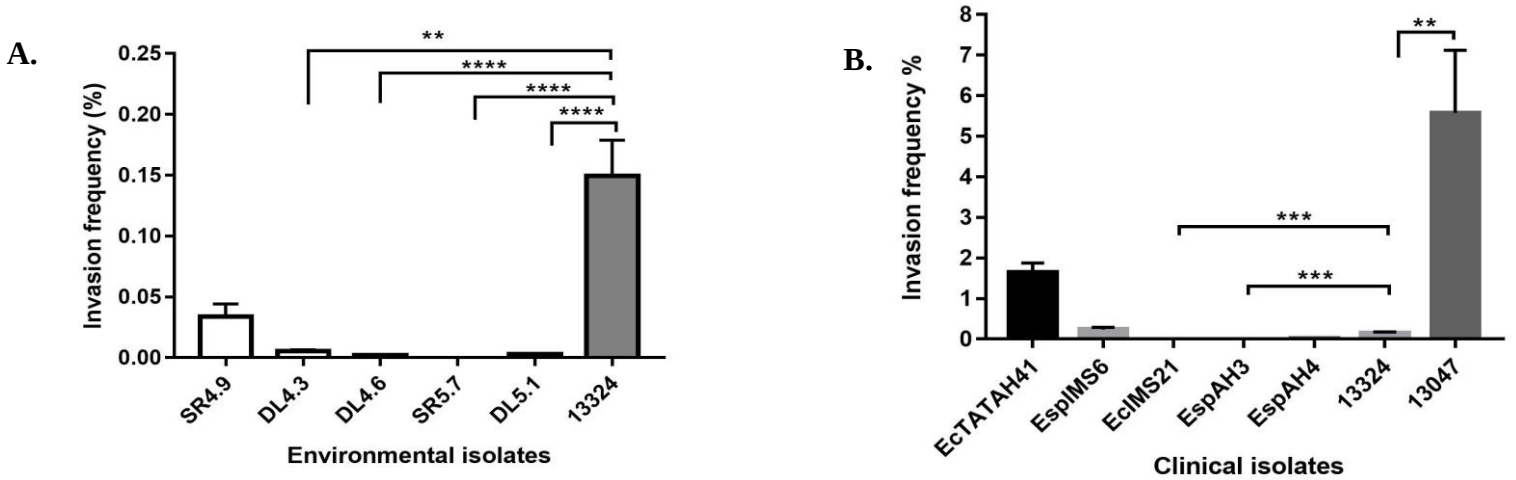


Figure no. 4.2.7: *In-vitro* cell-invasion ability of *Enterobacter* isolates in RAW 264.7 cell line. A) Environmental and B) Clinical isolates.

Table no. 4.2.2: Fraction of populations (%) invading the RAW 264.7 cell line

Isolates	% Ratio (Invasion frequency/Adhesion frequency)
SR4.9	0.730
DL4.3	0.827
DL4.6	3.962
SR5.7	0.312
DL5.1	3.100
EcTATAH41	**30.957**
EspIMS6	**91.957**
EcIMS21	0.261
EspAH3	**29.692**
EspAH4	2.885
E.cloacae 13047	**45.164**

It showed clinical isolates EcTATAH41, EspAH3 and Ec13047 showed 30-45% of invading populations and EspIMS6 displayed >90% of invading populations. This data implied that the %populations adhered in EspIMS6 are actually invading the cells (Table no. 4.2.2).

Bacterial persistence inside macrophage cells after internalization is an important aspect for establishing successful infection. This feature aids in pathogen's survival against the killing activity of macrophages. Hence, we examined the duration of intracellular survival of two clinical *Enterobacter cloacae* isolates; EspIMS6 and ATCC 13047 inside murine macrophage RAW 264.7 cells. At 3 hours and 6 hours post infection, there was no significant change in bacterial growth (figure no. 4.2.8), indicating survival of the internalized bacterial cells. Further, both *Enterobacter cloacae* isolates survived inside macrophage up to 72 hours, though there was significant decrease in viable count after 24 hours post infection inside macrophage cells (figure no. 4.2.8). This suggested that the clinical *E.cloacae* isolates (EspIMS6 and ATCC 13047) could persist in macrophage cells for a longer period of time.

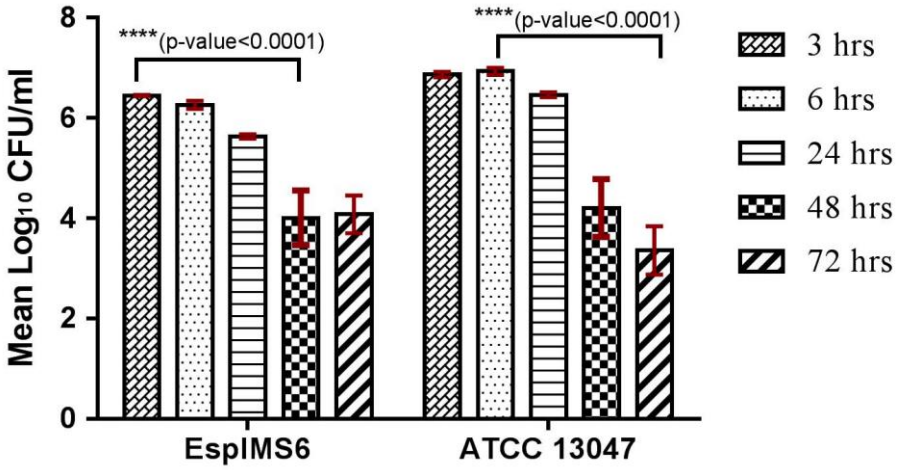


Figure no. 4.2.8: Intracellular survival assay in macrophages: This bar graph represented bacterial growth of *Enterobacter cloacae* isolates EspIMS6 and ATCC 13047 up to 72 hours inside macrophage cells. The results shown are highly significant, **** p-value <0.0001.

4.2.4. Association of OMPs with antibiotic resistance

With the afore-mentioned results of OMPs screening and antibiotic resistance profile of the isolates, we further analyzed association between OMPs and antibiotic resistance in the sample population under study. To investigate the association of drug resistance with OMPs, we separated the clinical and environmental *Enterobacter* isolates into two major groups: OMP positive isolates and OMP negative isolates. Between both these groups we measured their antibiotic resistance pattern and determined the contribution of that particular OMP with drug resistance in the context of clinical and environmental origin.

Greater percent of environmental *Enterobacter* isolates positive for *OmpA* and *OmpX* displayed resistance to β-lactams and cephalosporins in comparison to those negative for *OmpA* and *OmpX* (figure no. 4.2.9A and 9B). On the contrary, we observed that greater percent of *Enterobacter* isolates negative for *LamB* and *OmpF* showed antibiotic resistance, suggesting low association of *LamB* and *OmpF* in β-lactams and cephalosporins resistance amongst environmental isolates (figure no. 4.2.9C and 9D).

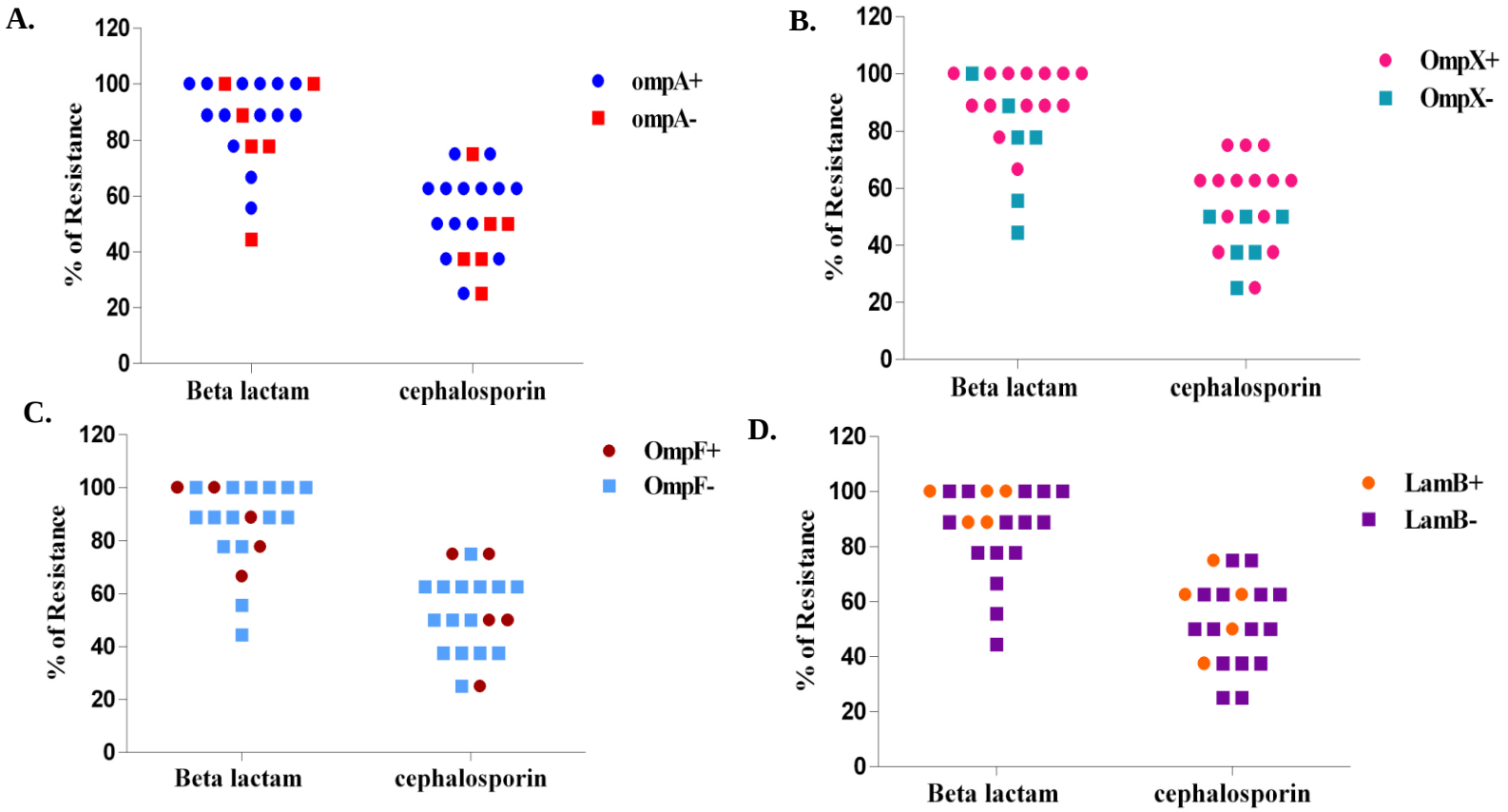


Figure no. 4.2.9: Association of OMPs with antibiotic resistance in environmental *Enterobacter* isolates. The scattered dot plot represents the %of resistance towards β-lactams and cephalosporins observed in different *Omp* positive isolates and *Omp* negative isolates. A) *OmpA* positive and *OmpA* negative, B) *OmpX* positive and *OmpX* negative, C) *OmpF* positive and *OmpF* negative and D) *LamB* positive and *LamB* negative isolates.

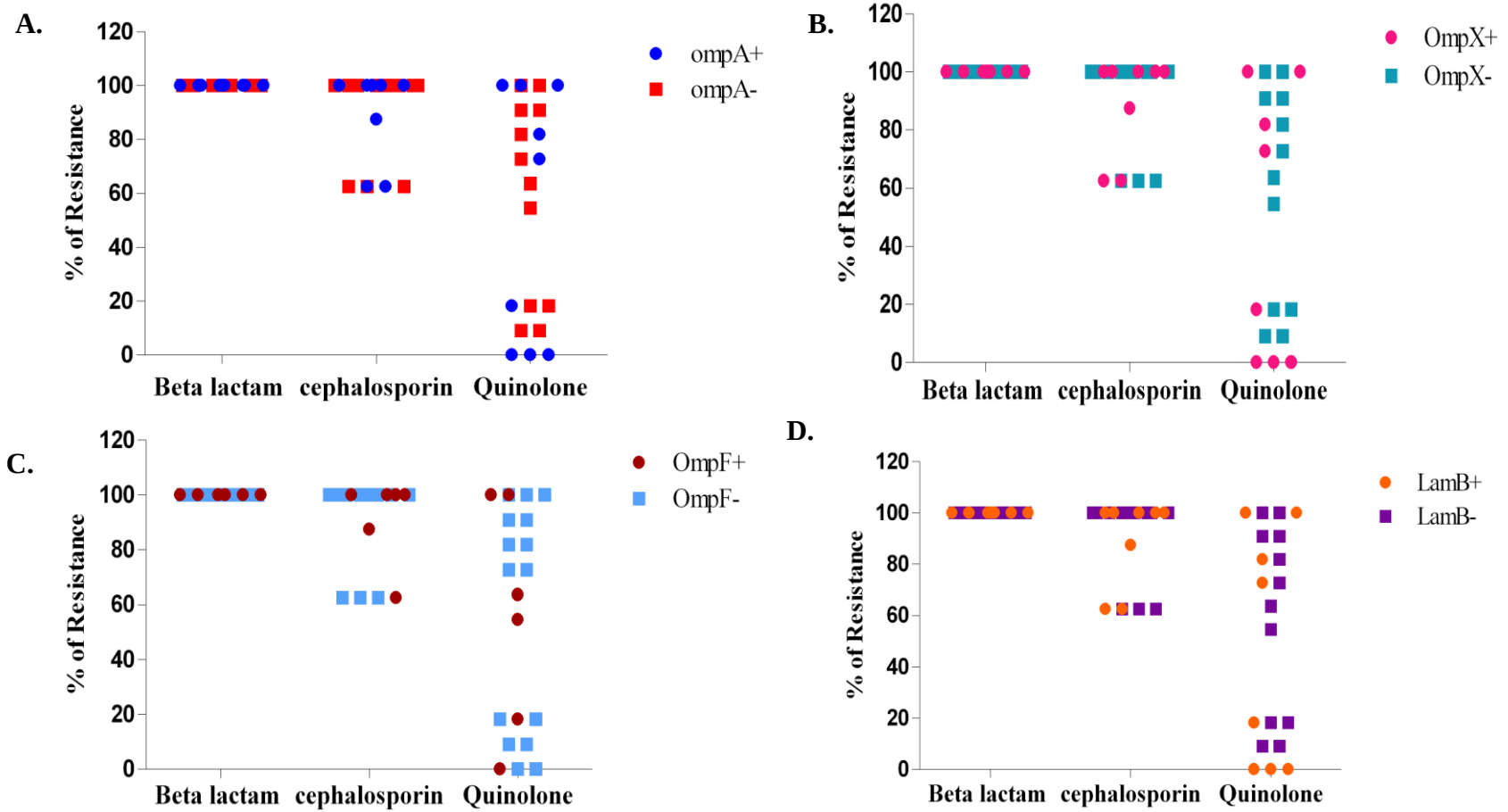


Figure no. 4.2.10: Association of OMPs with antibiotic resistance in clinical *Enterobacter* isolates. The scattered dot plot represents the %of resistance towards β-lactams, cephalosporins and quinolones observed in different *Omp* positive isolates and *Omp* negative isolates. A) *OmpA* positive and *OmpA* negative, B) *OmpX* positive and *OmpX* negative, C) *OmpF* positive and *OmpF* negative and D) *LamB* positive and *LamB* negative isolates.

On the contrary, among the clinical *Enterobacter* isolates, there was no significant difference in resistance pattern observed between those isolates being positive and negative for OMPs tested (figure no. 4.2.10A-D). All clinical *Enterobacter* isolates exhibited complete resistance towards β-lactams, 60-100% isolates were resistant towards cephalosporins. From figure no. 4.2.10 A-D, it was well understood that OMPs in clinical *Enterobacter* isolates had significantly less or no association with antibiotic resistance. This association data was further analyzed using linear regression curve, which showed significant association of Omp profile in environmental isolates with resistance to β- lactams and cephalosporins (figure no. 4.2.11-A), which corroborated well with our previous observation. Nonethess, the association of OMPs with antibiotic resistance was insignificant in clinical isolates (figure no. 4.2.11-B).

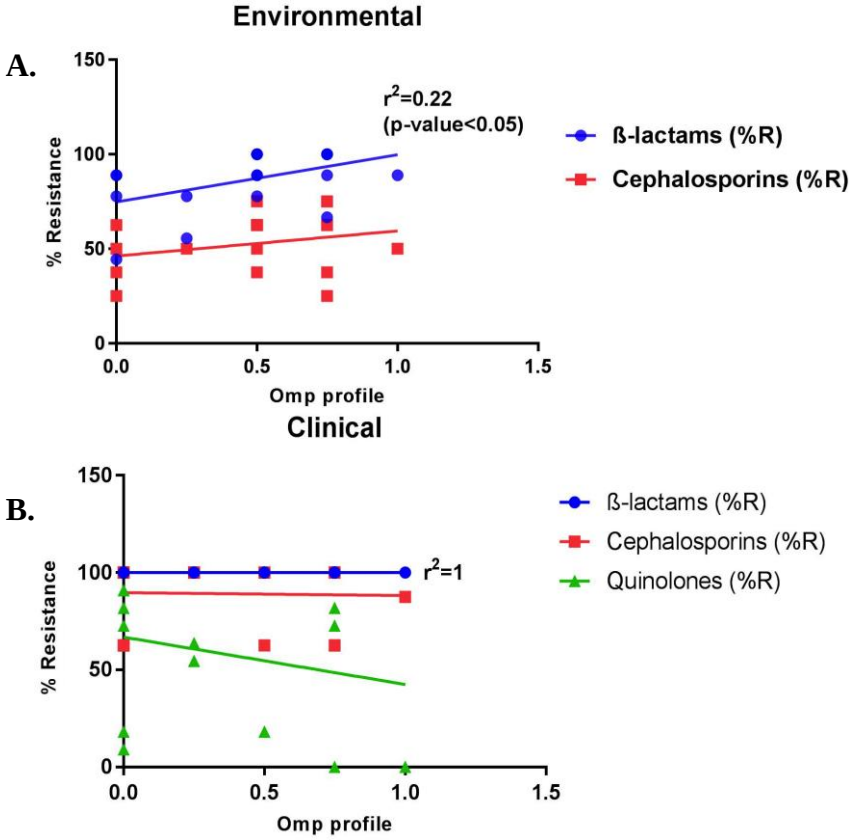


Figure no. 4.2.11: Linear regression analysis showing association of Omps with antibiotic resistance in environmental (A) and clinical (B) *Enterobacter* isolates.

Moreover, environmental isolates positive for *OmpA* and *OmpX* were found to be multi-drug resistant (MDR) (n=11) in comparison to those isolates being negative for *OmpA* and *OmpX* (n=4) (figure no. 4.2.12A). In clinical isolates, most of the MDR isolates were devoid of OMPs (figure no. 4.2.12B). It was evident that association of *OmpA* and *OmpX* with MDR phenotype is prevalent primarily in environmental *Enterobacter* isolates.

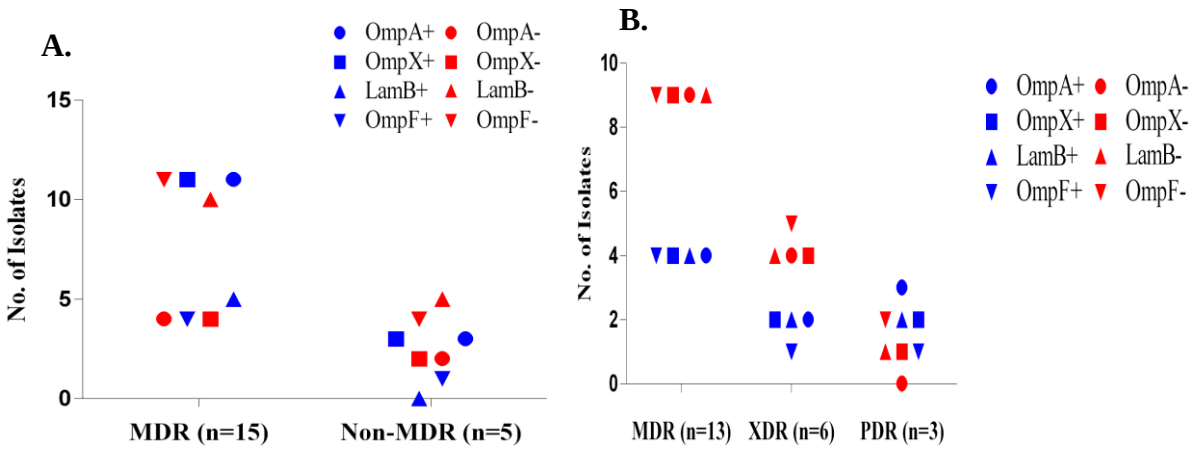


Figure no. 4.2.12: Association of OMPs with Multidrug resistance (MDR) phenotype in environmental (A) and clinical (B) *Enterobacter* isolates.

4.2.5. Association of OMPs with virulence factors

For any opportunistic pathogen like *Enterobacter* spp., cell adherence and invasion are essential steps for successful colonization and subsequent infection. This prompted us to dissect the role of OMPs with virulence properties. Pearson-correlation matrix with *Enterobacter* isolates positive for OMP and their respective in-vitro cell adhesion and cell invasion frequency was generated using XLSTAT software (www.xlstat.com/en/). Analyzed pearson-correlation matrix (as shown in table no. 4.2.3) was then plotted in correlation circle (figure no. 4.2.13) and biplot (figure no. 4.2.14) as shown below, to demonstrate the association between variables.

Table no. 4.2.3: Correlation matrix (Pearson (n)) between variables

Variables	*OmpA*	*OmpX*	*OmpF*	*LamB*	*Adhesion frequency*	*Invasion frequency*
OmpA	1					
OmpX	**0.810**	1				
OmpF	0.289	-0.134	1			
LamB	**0.671**	**0.828**	-0.043	1		
Adhesion frequency	0.327	0.404	**0.523**	0.269	1	
Invasion frequency	0.259	0.318	**0.615**	0.381	**0.934**	1
Values in bold are different from 0 with a significance level alpha=0.05						

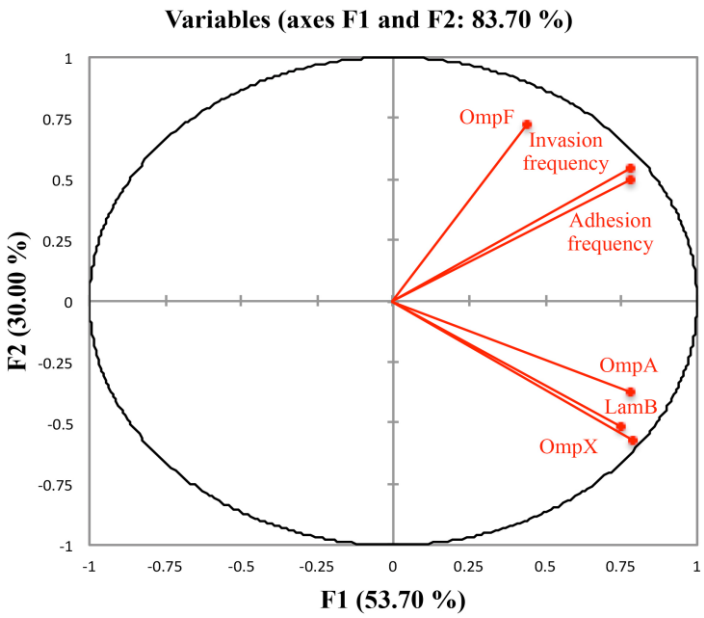


Figure no. 4.2.13: Correlation circle showing association of OMPs with virulence

It is indicative from the matrix (table no. 4.2.3) and figure no. 4.2.13, that *OmpA* and *OmpX* are strongly correlated (r=0.810), which is reflected in their similar pattern for association with antibiotic resistance. Moreover, *LamB* showed a positive correlation with other OMPs; more significantly with *OmpX* (r=0.828) than with *OmpA* (r=0.671). *OmpF* displayed a negative correlation with *OmpX*

and *LamB;* while *OmpF* was found to be positively associated with adhesion (r=0.523) and invasion frequency (r=0.615). Further, adhesion and invasion frequency were strongly correlated, as observed from the correlation circle (figure no. 4.2.13), suggesting that isolates with higher invasive properties also had greater adhesive features, which indicates it to be perhaps prerequisite for a successful infection progression (table no. 4.2.3).

Similar association between variables (i.e. OMPs and adhesion/invasion frequency), in the context of *Enterobacter* isolates is presented in figure no. 4.2.14. In this biplot, the isolates were segregated into three major categories: (i) *Omp* positive isolates with higher adhesion/invasion frequency, (ii) *Omp* positive isolates with moderate adhesion/invasion frequency and (ii) isolates lacking *Omp* and displaying low adhesion/invasion frequency.

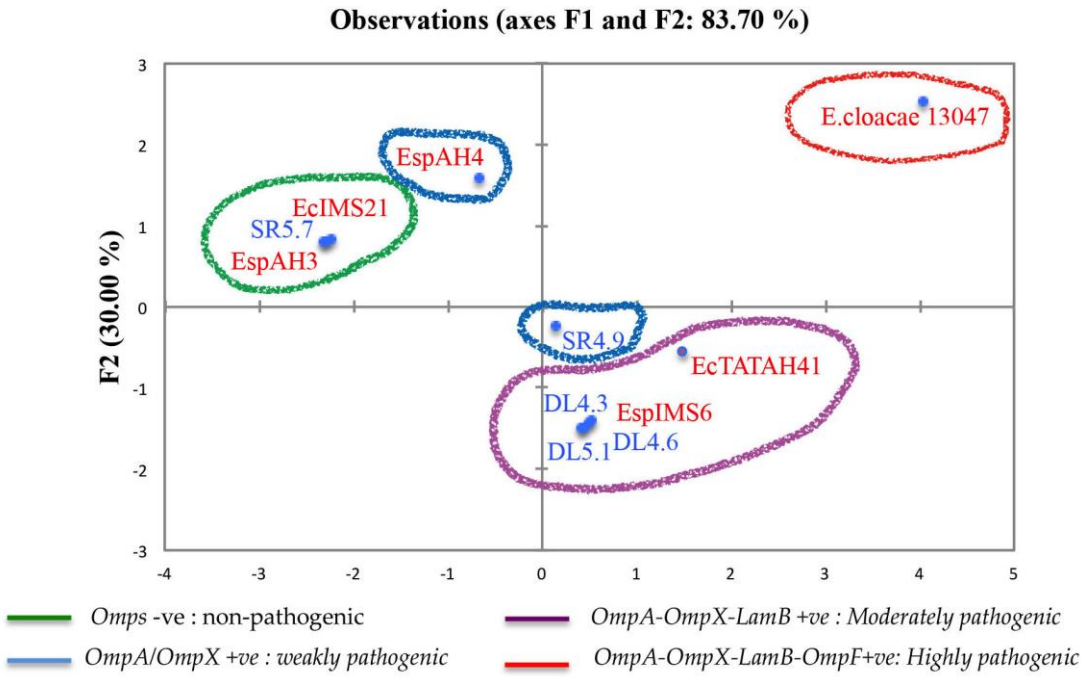


Figure no. 4.2.14: Biplot showing association of OMPs with virulence in *Enterobacter* isolates (two component analysis)- Organisms in red indicated clinical *Enterobacter* isolates and in blue indicated environmental *Enterobacter* isolates.

Clinical isolate *Enterobacter cloacae* ATCC 13047, was positive for all *OmpA-OmpX-LamB-OmpF* and exhibited highest adhesion-invasion frequency, hence belonged to the first category. Second group of *Omp* positive isolates was again divided into isolates bearing different combinations of OMPs, with three/two/one out of four OMPs tested. For instance, isolates positive for *OmpA-OmpX-LamB* were EcTATAH41, EspIMS6 (clinical) and DL4.3, DL4.6 and DL5.1 (environmental) (figure no. 4.2.13). While the clinical isolates bearing three *Omps* showed moderate adhesion-invasion frequency, environmental isolates displayed weaker pathogenicity. Environmental isolate SR4.9 that was positive for *OmpA-OmpX* could show only strong cell-adhesive property. Interestingly, EspAH4 that was only positive for *OmpA* could only display weaker cell adhesive quality (Figure no. 4.2.13). Isolates belonging to third category, which were devoid of any OMPs, namely SR5.7 (environmental), and EcIMS21, EspAH3 (clinical) did not exhibit in-vitro adhesion or invasion features, and hence were grouped together (figure no. 4.2.13).

Discussion

Outer membrane proteins (OMPs) form major source of bacterial membrane transport system. These proteins are involved in important physiological functions: as porins aid in penetration of small solutes, act as receptors for bacteriophages and help maintain cellular integrity. Classically, OMP family consists of small β-barrel membrane anchors (OmpA, OmpX), general non-specific porins (OmpF/OmpC), substrate-specific porins (LamB) and TonB-dependent receptors (FhuA) [129].

Regulation of outer membrane protein expression is known to contribute significantly towards antibiotic resistance in gram negative bacteria including *Klebsiella pneumoniae* [183], *E. coli* [184] and *Enterobacter* spp. [9]. Despite the advances in understanding the role of OMPs in resistance and virulence, the association between them is less addressed. In this section, the association of OMPs with antibiotic resistance and virulence factors in *Enterobacter* isolates (both from environmental and clinical settings) is elucidated.

Antibiotic Resistance in *Enterobacter* spp., in particular towards β-lactams, and carbapenems, is well-coordinated mechanisms involving overproduction of chromosomally encoded β-lactamase (AmpC), altered membrane permeability and overexpression of efflux pumps [185]. It has been studied that expression of OMPs are significantly affected in carbepenem-resistant *Enterobacter* isolates particularly by two ways; downregulation of *OmpF* and *OmpC* gene and/or *OmpC*-directed polarization of the outer membrane [185]. This altered outer membrane protein balance in the context of OmpF/OmpC greatly regulates the β-lactams resistance by selecting porins with preferable transmembrane channel diameter [186].

This prompted us to investigate the distribution of OMPs (both non-specific and substrate-specific) in clinical and environmental *Enterobacter* isolates by developing a multiplex PCR. We designed a hexaplex PCR for rapid and easy detection of different OMPs present in *Enterobacter* isolates. Multiplex PCR based screening followed by slot blot hybridization and sequencing of the purified PCR products confirmed presence of *OmpA*, *OmpX*, *LamB* and *OmpF* in the

isolates. Results indicated that majority of environmental isolates (n=13) were positive for *OmpA* and *OmpX*, out of which n=5 were also positive for *LamB*, n=2 co-harboured *OmpF*. In contrast, greater number of clinical isolates (n=8) co-harboured *OmpA*, *OmpX* and *LamB*; out of which n=2 isolates were positive for *OmpF* as well. Predominance of OmpA and OmpX in these isolates coincided with earlier reports, suggesting these two OMPs to be integral part of bacterial membrane [187]. Overexpression of OmpX in *E. coli* and *E. aerogenes* strains was found to reduce expression of non-specific porins (e.g. OmpC and OmpF), leading to restricted permeability of β-lactams [146], [187]. It was interesting to note that none of these isolates tested were positive for presence of *OmpC*.

Reduced/no expression of two major non-specific porins, OmpC and OmpF in *E. cloacae* isolates, could be due to point mutations affecting their transcription/translation/insertion into outer membrane [9]. It has been shown that resistant isolates expressed only OmpF-type porins in low ionic strength conditions, and complete impermeability to β-lactams is achieved through total loss of OmpC-type porins in resistant isolates [14]. Noteworthy is the absence of OmpC in the *Enterobacter* isolates tested in this study that echoed the previous findings. We had also investigated the presence of substrate-specific porins such as LamB and FhuA in these *Enterobacter* isolates but couldnot find any FhuA positive isolates. However, LamB was present in 25% of environmental and 37% of clinical isolates, making LamB as the third most abundant OMPs, next to OmpA and OmpX. To the best of our knowledge, the present study elucidated multiplex PCR based OMPs screening in *Enterobacter* isolates for the first time. This study also highlighted the importance of different OMPs in clinical and

environmental settings for better survival of the multi drug resistant opportunistic pathogen.

To be able to survive effectively in host environment, the organism must be able to adapt itself to different physiological conditions. Thus, we investigated important virulence factors in *Enterobacter* isolates such as biofilm forming ability, presence of adhesive structure (such as type 1 fimbriae) and serum resistance (that is important for avoidance of complementation). Since all of the isolates tested positive for fimbriae in Haemagglutination test (table no. 4.2.1), we further investigated their *in-vitro* cell adhesion and invasion ability in RAW 264.7 macrophage cell line. Although environmental isolates lacked the cell invasion property, but isolates SR4.9 and DL5.1, SR5.7 showed strong and moderate cell attachment phenotype respectively (figure no. 4.2.6). Isolate SR4.9 was highly serum resistant and had weaker biofilm producer, whereas isolates DL5.1 and SR5.7 were moderate serum resistant profile but were stronger biofilm producer (table no. 4.2.1). Apart from maintaining cellular integrity, OmpA plays a vital role in biofilm formation and adherence to biotic and abiotic surfaces [188]. We too observed that the clinical isolates possessing *OmpA,* were strong biofilm producers.

On the contrary, clinical isolates EspIMS6, EspAH4 and EcTATAH41, EcIMS21 displayed moderate to strong *in-vitro* cell attachment abilities respectively (Figure no.4.2.6). Moreover, isolates EspIMS6 and EcTATAH41, possessing *OmpA-OmpX-LamB,* could show moderate cell invasion properties, both being strong biofilm producer, highly serum resistant (figure no. 4.2.7, table no. 4.2.1).

Enterobacter cloacae ATCC 13047 contained all the four OMPs (*OmpA-OmpX-LamB-OmpF*) and displayed strongest adhesive and invasive properties (figure no.4.2.6, figure no. 4.2.7). *E.cloacae* isolates ATCC 13047 and EspIMS6 could even persist inside macrophage cell compartment for 72 hours, though the viable counts decreased after 24 hours (figure no. 4.2.8), a characteristic feature of chronic infection caused by opportunistic pathogens.

Association studies between OMPs and antibiotic resistance revealed greater association of *OmpA* and *OmpX* with resistance towards β-lactams and cephalosporins (figure no. 4.2.9 and figure no. 4.2.11); indicating role of these two porins in drug resistance in environmental *Enterobacter* isolates. On the contrary, significantly low association of *LamB* and *OmpF* in antibiotic resistance in environmental isolates was noticed (figure No. 4.2.9C and 9D).

Moreover, we dissected the role of OMPs with virulence factors in *Enterobacter* isolates by generating a pearson-correlation matrix with OMPs and *in-vitro* cell adhesion and cell invasion frequency (table no. 4.2.3). The backdrop of this analysis was represented in figure no. 4.2.13 that categorized these isolates into different groups, depending on their OMPs profile and respective adhesion-invasion frequency. *Enterobacter* isolate that harbored all OMPs such as *E. cloacae* ATCC 13047, being positive for all *OmpA-OmpX-LamB-OmpF*, was found to have higher pathogenic index. Whereas, *Enterobacter* isolates like SR5.7, EciMS21 and EspAH3, which were devoid of OMPs, observed to lack *in-vitro* cell adhesion and invasion features, and represented minimal pathogenic potential. Isolates possessing two or three OMPs, most preferably, *OmpA-OmpX-LamB* or *OmpA-OmpX*, combinations conveyed moderate pathogenic potential.

Put together, it was confirmatory that presence of OMPs help *Enterobacter* spp. in establishing infection in host cells. Summarizing all the findings, we presented here that clinical *Enterobacter* isolates harbored multiple OMPs as compared to their environmental counterparts. Further, we have established an association of OmpA and OmpX with antibiotic resistance and OmpF with *in-vitro* pathogenic potential in *Enterobacter* spp.

Results 4.3

AcrAB-TolC Efflux pump in Enterobacter isolates: Modulation in expression under physiological conditions and its significance

Section 4.3.1: Effect of pH and antibiotics on AcrAB-TolC expression in clinical and environmental *Enterobacter* isolates

Background of the study

AcrAB-TolC efflux pumps are primarily associated with multiple antibiotic resistance in clinically significant many gram-negative pathogens including *Enterobacter* spp., *Escherichia coli*, *Salmonella*, *Pseudomonas aeruginosa* and *Klebsiella* spp. [50], [48], [189]. Compared to other bacteria such as *E. coli*, the *Enterobacter* spp. are versatile in their adaptability to varied pH conditions as observed with their ubiquitous presence [7]. This enhances better survival ability of the organisms under extreme pH conditions in external environments [190]. AcrAB-TolC efflux pumps belong to tripartite RND transporters, and hence efficiently extrude drug molecules, noxious dyes and toxic compounds from cytoplasm and periplasmic space across the outer membrane [176],[46]. AcrAB-TolC efflux pumps are drug/H^+ antiporter that utilize proton gradient as energy source. Previous reports indicated pH to affect activities of RND efflux pump proteins and different types of efflux proteins are affected differentially by pH. Most of these studies done on *acrAB-tolC* expression in multidrug resistant clinical isolates are based upon transcript analysis. However, from such studies it is difficult to decipher whether the clinical isolates constitutively express high levels of protein, or, is there an induced up-regulation of these proteins, in presence of the drugs.

With this background, we investigated the impact of physiological stimuli, such as pH and cephalosporin antibiotics, as observed in many pathological conditions such as urinary tract infections, on AcrAB-TolC efflux protein expression and survival fitness in environmental and clinical isolates of *E. cloacae*.

4.3.1.1: Bacterial strains used

In the initial screening of the isolates, we selected few environmental (DL4.3, SR2.3) and clinical (EspIMS6, EcTATAH41) *Enterobacter* isolates along with *Enterobacter cloacae* type strain ATCC 13047, which were screened positive for *acrAB-tolC* genes. In addition, we also investigated other clinical isolates namely *E.coli* AH, ATCC MDR *E.coli* BAA-2469) and *Klebsiella* spp. (Ksp AH). The selection of these specific isolates was done taking into account MAR indices and source of isolation, whereas ATCC type strains were included for comparison purpose.

4.3.1.2: Resistance pattern, MIC profile and efflux genes screening

4.3.1.2.1. Resistance profile of the isolates under study

Resistance profiling of environmental *E. cloacae* isolate DL4.3 and clinical *E. cloacae* isolate EspIMS6, indicated DL4.3 to be multi drug resistant (MDR) and EspIMS6 to be extensive drug resistant (XDR) (table no. 4.3.1.1).

Table no. 4.3.1.1: Antibiotic susceptibility profile of the isolates under study

Strains	Resistance profile to antibiotics*
DL4.3*	CX, NAF, OX, AZ, MZ, PI, TI, CB, AMC, CX, CXM, CAZ, AZM/AT, FR
EspIMS6*	CX, NAF, OX, AZ, MZ, PI, TI, CB, AMC, CX, CXM, CAZ, CTX, CZX, CTR, CFP, CPM, EN, NX, OF, PF, LO, LE, SC, GAT, N, COT, SF, TR, AZM/AT, FR, IPM
SR2.3*	AZ, AMC, CXM, CX, CEP/CH, CX, FR, M, MZ, NAF, OX, R, T
MDR 2469**	AMC, AK, AMP, CXM, CZX, CX, CPZ, CTR, TCC, MRP, PIT, AT, GAT, A/S, CTX, IPM, CPM, CAC, CAZ, OF, GEN, CIP
ATCC 13047**	AMC, CXM, AMP, CAZ, CX, CAC, A/S

***** Abbreviations used for antibiotics: CX-Cloxacillin, NAF-Nafcillin, OX-Oxacillin, AZ-Azlocillin, MZ-Mezlocillin, PI-Piperacillin, TI-Ticarcillin, CB-Carbenicillin, AMC-Amoxyclav, CX-Cefoxitin, CXM-Cefuroxime, CAZ-Ceftazidime, CTX-Cefotaxime, CZX-Ceftizoxime, CTR-Ceftriaxone, CFP-Cefpirome, CPM-Cefepime, EN-Enoxacin, NX-Norfloxacin, OF-Ofloxacin, PF-Pefloxacin, LO-Lemofloxacin, LE-Levofloxacin, SC-Sparfloxacin, GAT-Gatifloxacin, N-Neomycin, COT-Cotrimaxazole, SF-Sulphafurazole, TR-Trimethoprim, AZM/AT-Azithromycin, FR-Furazolidone, T-Tetracycline, IPM-Imipenem, CEP/CH-Cephalothin, R-Rifampicin**. ****Antibiotic resistance profile were done using DoDeca Enterobacteriaceae discs 1 & 2 (Himedia, India). Disc 1 contained antibiotics Ampicillin AMP (10 mcg), Gentamicin GEN (10 mcg), Amikacin AK (30 mcg), Ciprofloxacin CIP (5 mcg), Ofloxacin OF (5 mcg), Co-Trimoxazole COT (25 mcg), Amoxyclav AMC (30 mcg), Cefuroxime CXM (30 mcg), Ceftazidime CAZ (30 mcg), Ceftazidime/ClavulanicacidCAC (30/10mcg), Cefepime CPM (30 mcg), Imipenem IPM (10 mcg). Disc 2 contained antibiotics Cefotaxime CTX (30 mcg), Cetriaxone CTR (30 mcg), Cefoxitin CX (30 mcg), Meropenem MRP (10 mcg), Piperacillin/Tazobactam PIT (100/10 mcg), Aztreonam AT (30 mcg), Gatifloxacin GAT (5 mcg), Ampicillin/Sulbactam A/S (10/10 mcg), Cefoperazone (CPZ 75 mcg), Levofloxacin LE (5 mcg), Ceftizoxime CZX (30 mcg), Ticarcillin/Clavulanic acid TCC (75/10mcg).

EspIMS6 was resistant to cefepime, ceftazidime, imipenem, piperacillin/tazobactam, meropenem, ciprofloxacin and levofloxacin, and was susceptible to colistin, and amikacin. DL4.3 was resistant to all tested β-lactams, few cephalosporin antibiotics, macrolides and nitrofurans, as shown in table no. 4.3.1.1.

4.3.1.2.2: Determination of Minimum inhibitory concentration of antibiotics

As mentioned in section 3.2.5, minimum inhibitory concentration (MIC) of antibiotics were determined by broth double dilution method in presence or absence of ionophore carbonyl cyanide m- chlorophenylhydrazone (CCCP) at an optimized concentration of 50 µMolar. Effect of CCCP (50 µMolar) on growth of isolates DL4.3, EspIMS6 and *E.cloacae strain* ATCC 13047 was determined, to nullify the individual inhibitory role of CCCP on bacterial growth at the concentration used in this study (figure no. 4.3.1.1).

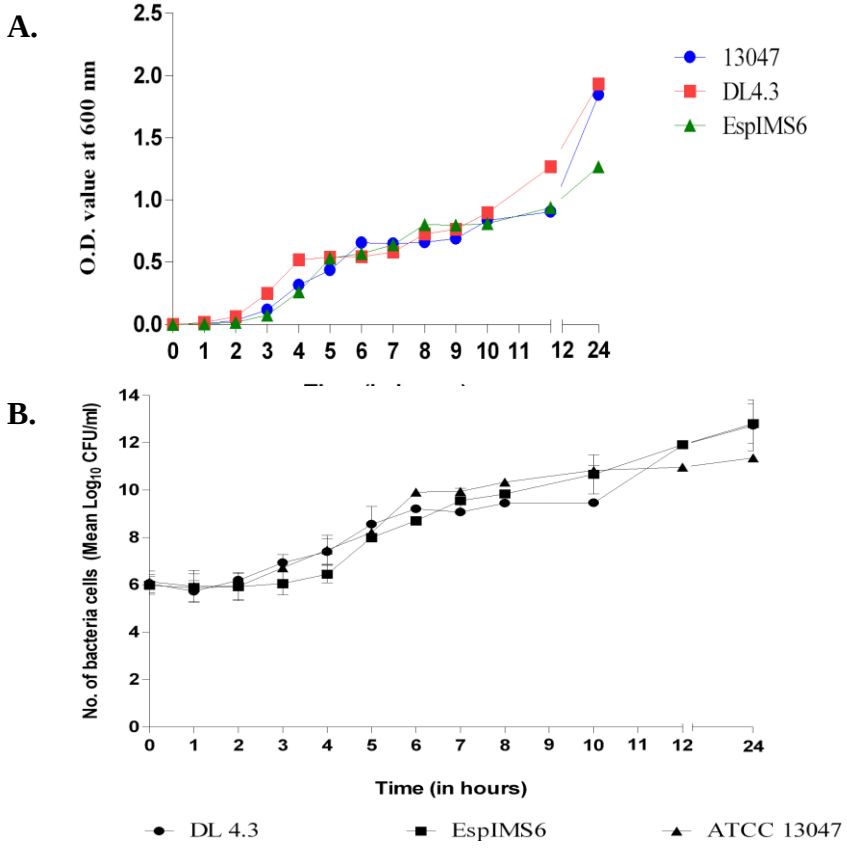


Figure no. 4.3.1.1: Effect of CCCP (50µMolar concentration) on bacterial growth: (A)-O.D. vs Time (in hours) and (B) Mean CFU/ml Vs. Time (in hours).

As observed from both the graphs of $O.D._{600}$nm (figure no. 4.3.1.1A) and CFU/ml (figure no. 4.3.1.1B), it was evident that CCCP at 50µMolar concentration was not detrimental to bacterial growth in the isolates studied. A decrease in MIC in presence of CCCP indicated the contribution of efflux pump towards multi-drug resistance (table no. 4.3.1.2). In presence of CCCP, *Enterobacter* isolate DL4.3, exhibited two-fold decrease in MIC value of ampicillin, cefuroxime, erythromycin, trimethoprim and four-fold decrease in MIC of tetracycline. Isolate EspIMS6 exhibited two-fold decrease in MIC value in presence of CCCP of cefotaxime, trimethoprim and neomycin.

Table no. 4.3.1.2: MIC breakpoint values (in µg/ml) of selected antibiotics in presence (+) or absence (-) of ionophore (CCCP) at 50µMolar concentration.

	Amp		CTX		CXM		Ery		TR	
CCCP	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**	**+**
DL4.3	128	64	256	256	1024	512	1024	512	1024	256
EspIMS6	ND	ND	>2048	1024	>1024	>1024	S#		1024	512
SR2.3	>1024	1024	S#		1024	512	256	128	S#	
ATCC 13047	ND		I*		>256	32	ND		ND	
MDR 2469	ND		>1024	512	ND		ND		ND	

S#-Susceptible; I*-Intermediate susceptible; ND–Not determined
Amp-Ampicillin, CTX-Cefotaxime, CXM-Cefuroxime, Ery-Erythromycin, TR-Trimethoprim

We again performed PCR based screening for the efflux pump genes in the isolates under study. The results confirmed presence of *acrAB-tolC* genes belonging to RND superfamily in isolates DL4.3 and EspIMS6 (figure no..4.3.1.2A). PCR screening results were confirmatory indicating these isolates to be negative for MATE, MFS and

SMR efflux pump genes, as presented in figure no. 4.3.1.2B. Thus, we went ahead with further investigations using DL4.3 and EspIMS6.

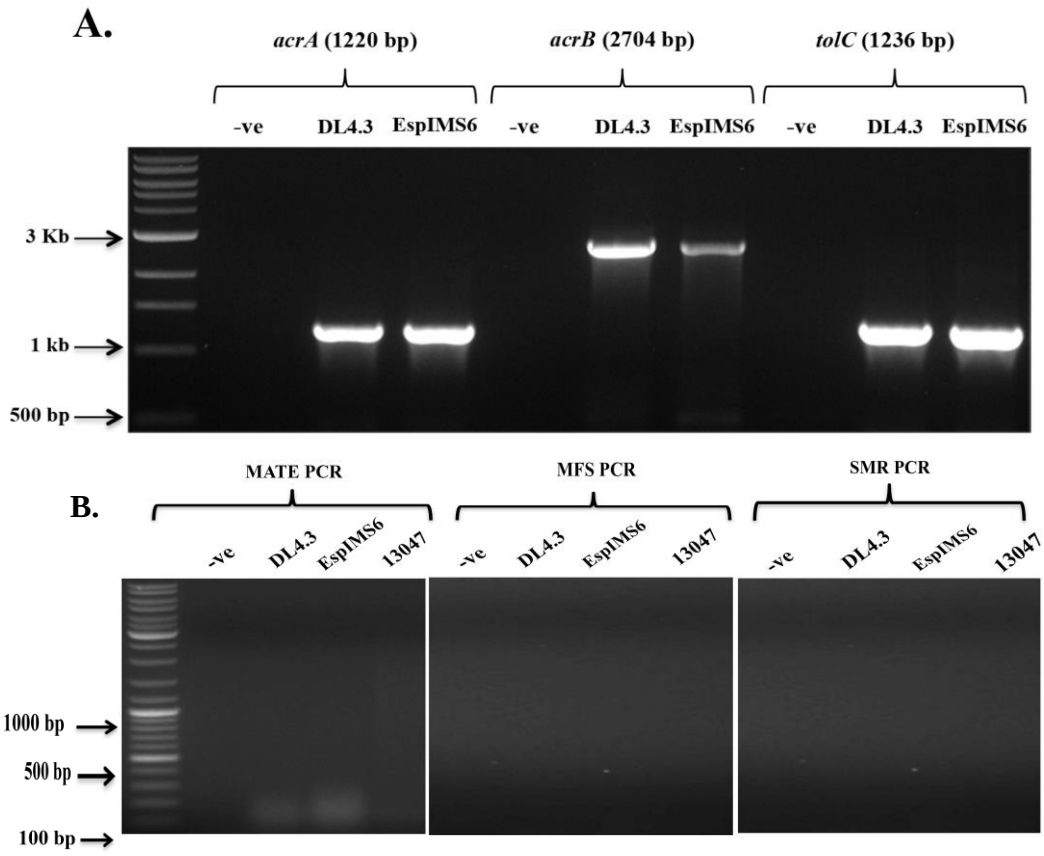


Figure no. 4.3.1.2: Screening of efflux genes in isolates under study- A) Amplicons of *acrA* (1220 bp), *acrB* (2704 bp) and *tolC* (1236 bp). B) PCR results for *Mate*, *Mfs* and *Smr* efflux genes

4.3.1.3: Effect of pH on bacterial growth

We investigated the effect of different pH (i.e. pH 4.0, pH 6.0 and pH 8.0) on bacterial growth. Overnight growth of the bacterial isolates at varying pH as mean Log_{10}CFU count/mL is presented in figure no. 4.3.1.3. As observed no significant differences in CFU count ($p>0.05$) was observed for *E. cloacae* isolates grown at varying pH *viz*. 4.0, 6.0 and 8.0. However, the environmental isolate DL4.3 grew faster than the clinical isolate EspIMS6 across all pH studied. However, in other isolates growth decreased

significantly at pH 4.0. Nevertheless, at pH 6.0 and 8.0 bacterial growth was healthy and difference was not significant (figure no. 4.3.1.3).

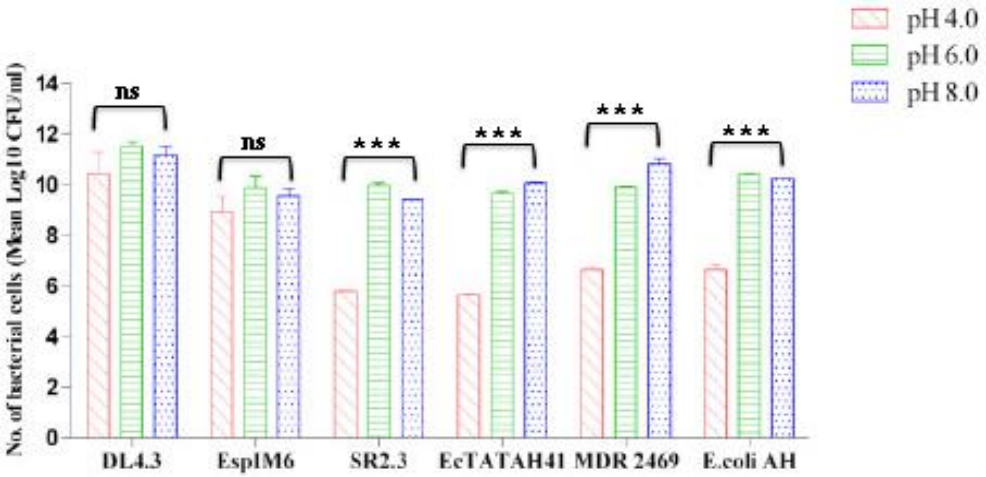


Figure no. 4.3.1.3: Effect of different pH on bacterial growth. Two-way ANOVA analysis between different pH treatment was performed where p-value <0.001 was found significant (***) and p-value>0.05 was not significant.

4.3.1.4: Effect of varied pH on antibiotic susceptibility

AcrAB-TolC functions by utilizing the proton motive force. So, we investigated whether variations in pH would alter the MIC of antibiotics. We determined MIC of DL4.3 (figure no. 4.3.1.4A) and EspIMS6 (figure no. 4.3.1.4B) towards cefuroxime and cefotaxime respectively. In addition we also documented survival of the isolates in the above stated experimental conditions by determining average $\log_{10}$CFU/ml.

Compared to control pH 7.0±0.2, where MIC of cefuroxime for isolate DL4.3 was 1024µg/ml (table no. 4.3.1.2), at pH 8.0 it decreased to 512µg/ml (figure no. 4.3.1.4A). However, at pH 4.0 and pH 6.0, DL4.3 was able to tolerate up to 1280µg/ml of cefuroxime, with two fold higher CFU count (2.87 $\log_{10}$ CFU/ml) at pH 4.0 than that at pH 6.0 (1.23 $\log_{10}$ CFU/ml). Nevertheless, overall a gradual decrease in CFU count was evident with increase in antibiotic concentration both at pH 4.0 and 6.0 (figure no. 4.3.1.4A). Similarly, at pH 7.0±0.2, the MIC of cefotaxime for EspIMS6 was >1024µg/ml (Table no. 4.3.1.2).

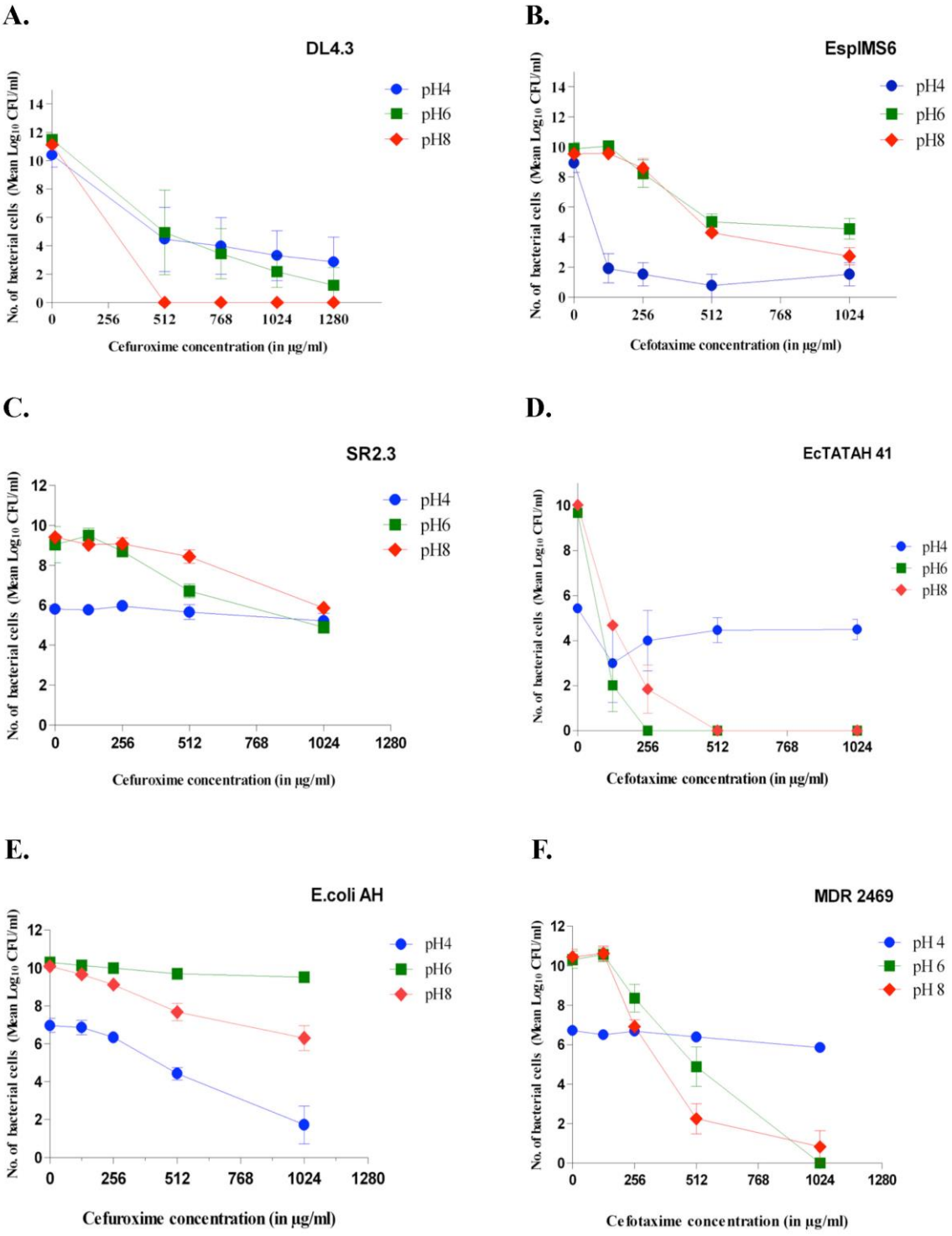


Figure no. 4.3.1.4: Effect of pH on cephalosporin antibiotic susceptibility-
E. cloacae isolates used: environmental isolates DL4.3 (A) and SR2.3 (C) with cefuroxime, and clinical isolates EspIMS6 (B), EcTATAH41 (D) with cefotaxime. Prototype *E. coli* ATCC MDR 2469 (F) with cefotaxime and clinical *E. coli* isolate *Ecoli*AH (E) with cefuroxime drug.

Unlike DL4.3, in presence of cefotaxime, EspIMS6 showed reduced growth at pH 4.0 compared to pH 6.0 and 8.0, with greater CFU being observed at pH 6.0. With cefotaxime concentration of 1024µg/ml, at pH 6.0, CFU count was 3 fold higher in comparison to pH 4.0 (4.5 $\log_{10}$ CFU/ml vs 1.5 $\log_{10}$ CFU/ml respectively) and 1.8 fold more (2.7 $\log_{10}$ CFU/ml) when compared to pH 8.0 (figure no. 4.3.1.4B).

Similar observations were also made in *E. cloacae* isolates, the susceptible SR2.3 (figure no. 4.3.1.4C) and clinical EcTATAH41 (figure no. 4.3.1.4D). In clinical *E.coli* isolate *E coli* AH, unlike pH 6.0 and 8.0, at pH 4.0, with the increase in antibiotic concentrations, there was a gradual decrease in bacterial growth (figure no. 4.3.1.4E). On the contrary, in prototype MDR *E.coli* isolate BAA-2469 (figure no. 4.3.1.4F), we observed similar growth pattern as of EcTATAH41 (figure no. 4.3.1.4D); where the organism tolerated higher concentration of antibiotics at pH 4.0, but succumbed to death at pH 6.0 and pH 8.0. Overall, these results indicated a change in antibiotic susceptibility with varying pH, suggesting possible modulation of efflux activity in bacterial isolates. All the isolates survived higher dosage of cephalosporin antibiotics at low pH 4.0.

4.3.1.5 Effect of pH on Ethidium bromide efflux in *Enterobacter* isolates

To demonstrate the effect of pH on efflux activity, Ethidium bromide (EtBr) was used, which is known as a fluorogenic substrate for AcrAB-TolC efflux pump. First, the concentration of EtBr was optimized at 0.5µg/ml (figure no. 4.3.1.5) without (A) and with (B) CCCP (50µMolar), to use in this fluorometric assay. Later, bacterial cells exposed to pH 4.0 (figure no. 4.3.1.5C) and pH 8.0 (figure no. 4.3.1.5D), with/without EtBr and with/without CCCP were also checked for standardizing the control, which

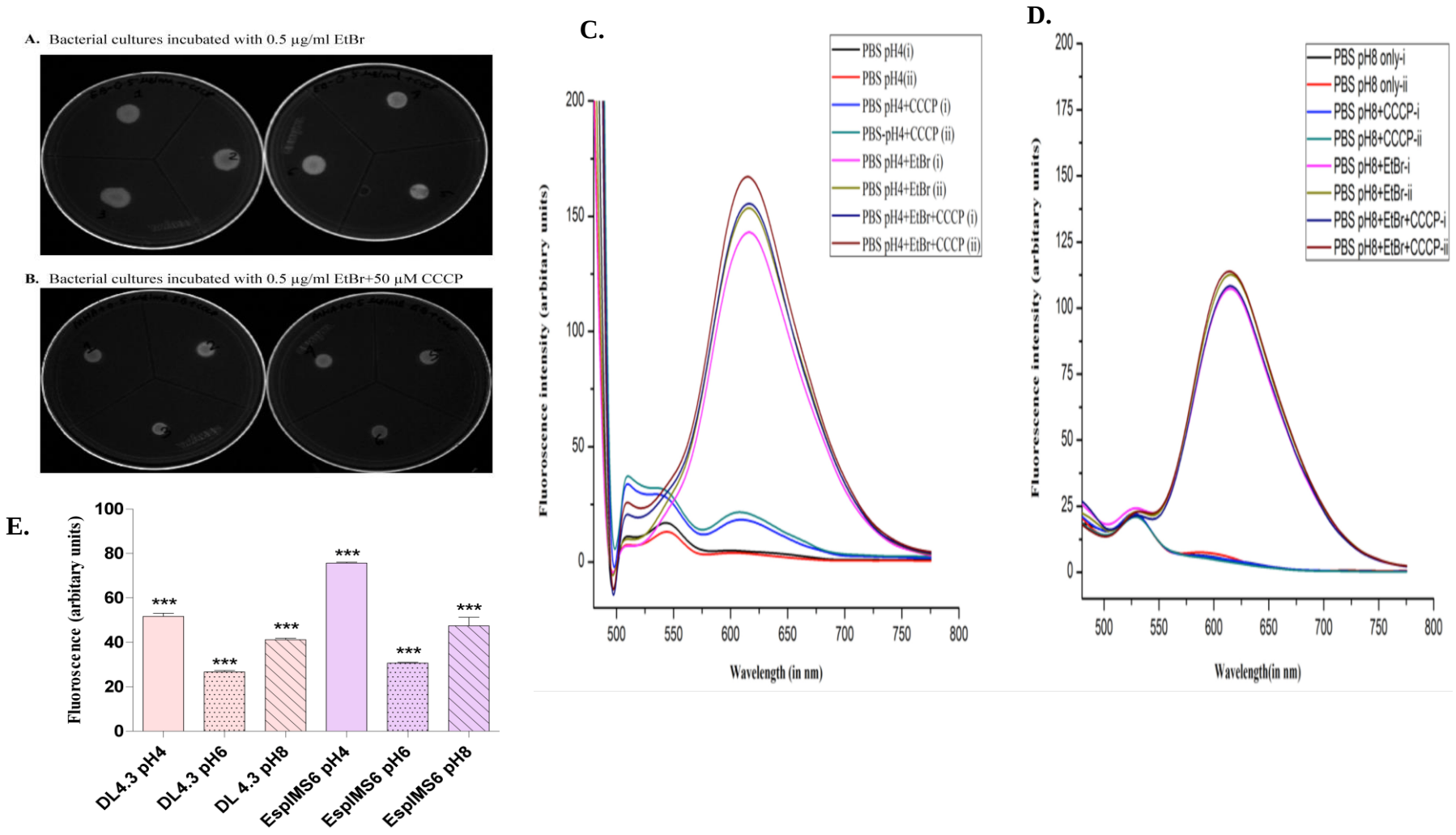


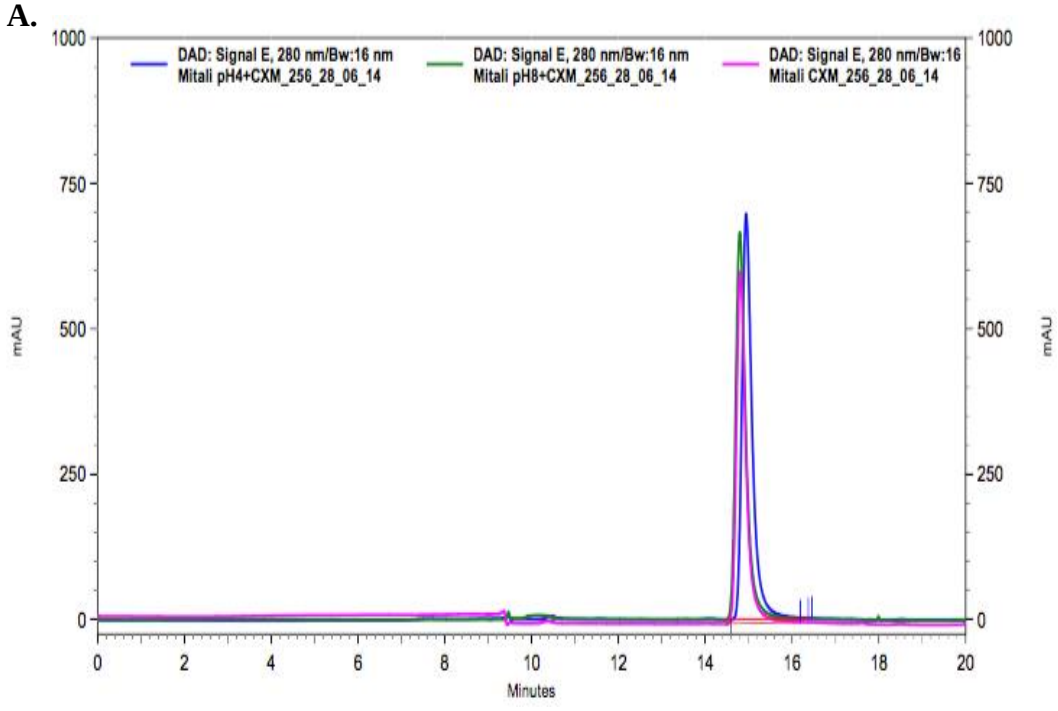
Figure no. 4.3.1.5: Ethidium bromide efflux at varied pH by fluorometric assay- A and B represent the optimization of EtBr concentrations to be used by agar method. C and D represent the standard run of EtBr at varied pH and CCCP. E represents the fluorescence intensity observed in experimental set of varied pH.

suggested that the fluorescence observed is higher at pH 4.0 than at pH 8.0, but addition of CCCP had minimal effect on EtBr fluorescence. Similar trend of higher fluorescence at pH 4.0 than at pH 8.0, was also observed in both DL4.3 and EspIMS6 (figure no. 4.3.1.5E). Greater fluorescence was observed for EspIMS6 as compared to DL4.3 at pH 4.0. At pH 6.0, the fluorescence obtained was minimum in both the isolates. This quantitative analysis also suggested that pH modulates the efflux activity in *Enterobacter* isolates.

4.3.1.6 Effect of pH on stability of cephalosporin drugs

After observing a possible pH-dependent modulation of antibiotic susceptibility, we checked the stability of the cephalosporin antibiotics used under different pH conditions by HPLC (figure no.4.3.1.6). Cefuroxime (A) and Cefotaxime (B) drug at 256 µg/ml, exposed to pH 4.0, 6.0 and 8.0 were run in triplicates in the standardized protocol to determine the retention time/elution time. From the results (figure no. 4.3.1.6A and figure no. 4.3.1.6B) it was affirmative that the antibiotics were stable and there was no degradation across the pH range of 4.0 to 8.0.

A.



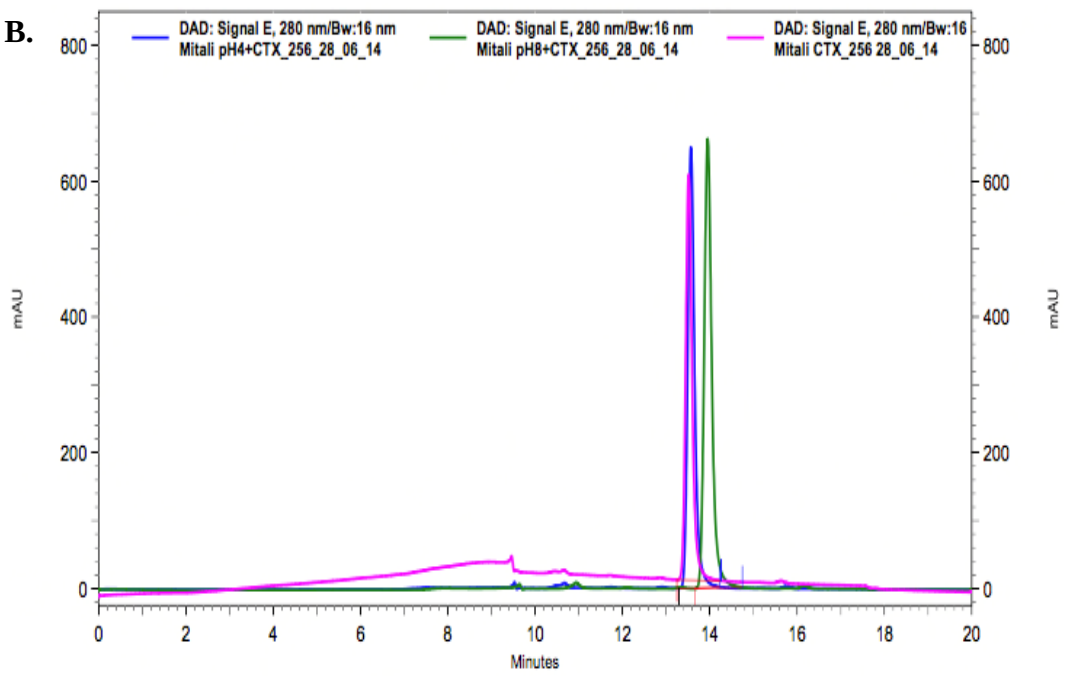


Figure no. 4.3.1.6: HPLC peaks of cephalosporin drugs exposed at varied pH
A) Cefuroxime and B) Cefotaxime drug at 256 µg/ml exposed to pH 4.0, 6.0 and 8.0.

4.3.1.7 Effect of pH and cephalosporin antibiotics on *acrAB-tolC* gene expression

To determine expression pattern of *acrAb-tolC* efflux genes in *Enterobacter* isolates exposed to varied pH and cephalosporin drugs, we have used quantitative Real time PCR techniques.

4.3.1.7.1 Bacterial RNA extraction and cDNA synthesis

Total RNA was isolated and purified from mid log phase cultures of *Enterobacter* isolates DL4.3 and EspIMS6, grown under different pH in presence or absence of 256 µg/ml of cefuroxime and cefotaxime antibiotic respectively. RNA samples were checked for its quality and quantity estimation (figure no. 4.3.1.7.1A). Reverse transcription was then performed and the cDNA samples were checked for its quality by 16s PCR (figure no. 4.3.1.7.1B).

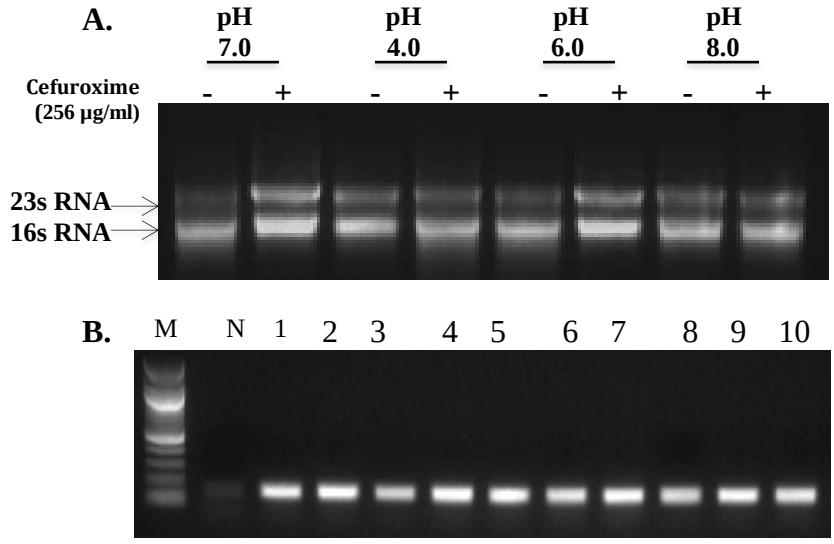


Figure no. 4.3.1.7.1: Agarose gel (1% w/v) images of extracted RNA and cDNA. Panel A is a representative image showing the quality of RNA extracted from environmental *Enterobacter* isolate DL4.3 subjected to varied pH (pH 7.0, pH 4.0, pH 6.0 and pH 8.0) in the presence ('+') and absence ('-') of cefuroxime (at 256 µg/ml concentration).
Panel B represented agarose gel (1.2% w/v) run of 16s PCR using cDNA samples from DL4.3 without antibiotic 1-pH 7.0, 2-pH 4.0, 3-pH 6.0, 4-pH 8.0, DL4.3 with antibiotic 5-pH 7.0, 6-pH 4.0, 7-pH 6.0, 8-pH 8.0, EspIMS6 9-pH 7.0 without and 10-pH7.0 with antibiotic.

4.3.1.7.2. Real time experiment validation and melt-curve analysis

Next, we determined the relative expression levels of *acrAB-tolC* mRNA transcripts by real time PCR using the double delta Ct quantification method, against expression levels of the housekeeping gene *rpoB*. For this first, we analyzed the expression patterns at pH 7.0 and normalized the expression pattern at pH 7.0 with other pH tested to obtain a relative quantification of fold change values. The results of the experiments are presented in figure no. 4.3.1.7.2.1 and figure no. 4.3.1.7.2.2 (A to F).

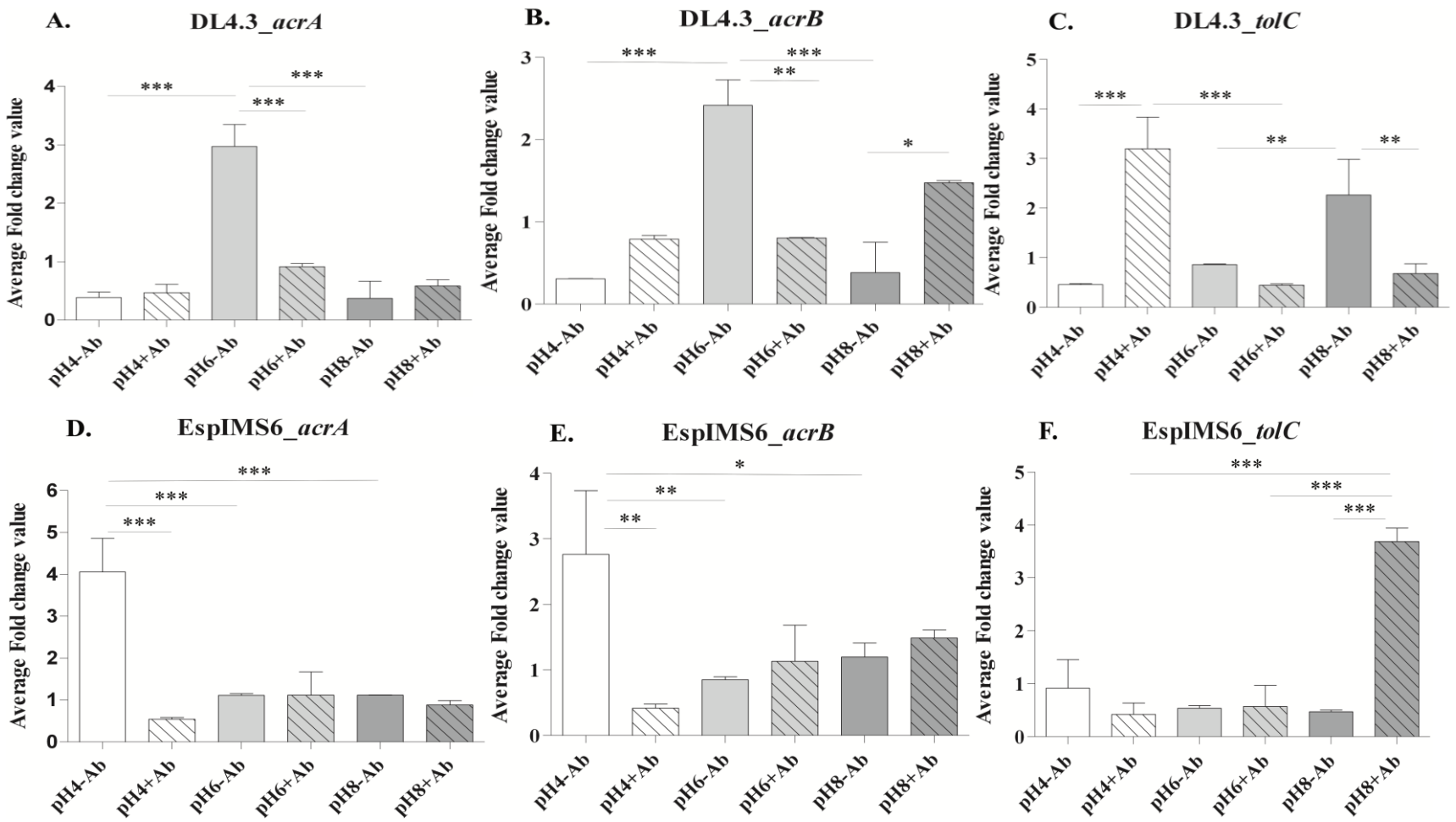


Figure no. 4.3.1.7.2.2: Relative Expression levels of *acrAB-tolC* transcripts. Mean Fold change values Data obtained from ΔΔCt relative quantification of three biological replicates with three technical replicates each are plotted and mean and SEM were calculated and plotted. Statistical significance was determined using two-way ANOVA with data being significant as ***- p-value<0.001,**-p-value <0.01,*- p-value <0.05. A], B] and C] represents effect of pH and cefuroxime (256ug/ml) in DL4.3 at transcript levels of *acrA*, *acrB* and *tolC*. D], E] and F] represents effect of pH and cefotaxime (256ug/ml) in EspIMS6 at transcript levels of *acrA*, *acrB* and *tolC*.

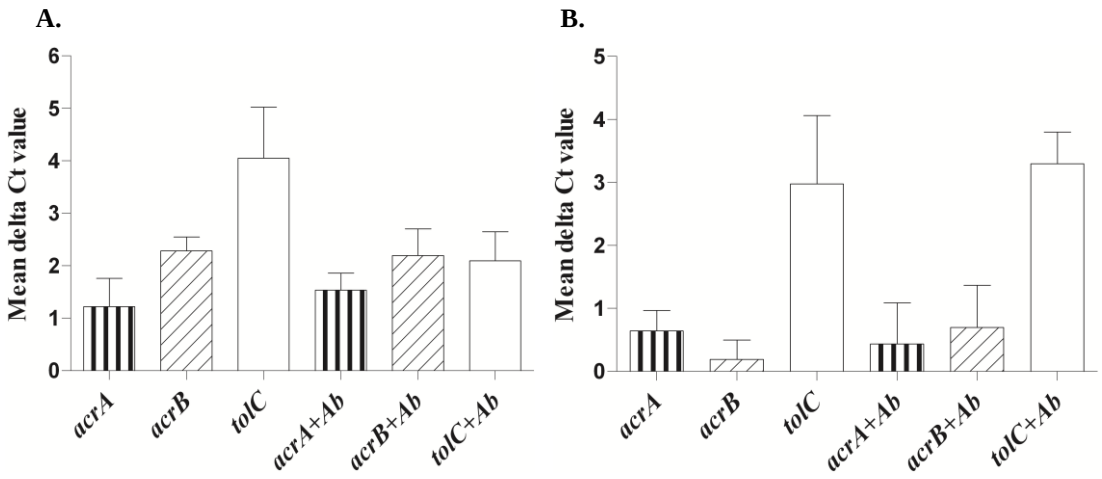


Figure no. 4.3.1.7.2.1: Expression levels of *acrAB-tolC* transcripts at pH 7.0: Panel A) represented the expression levels of EspIMS6 and Panel B) represented the expression levels of DL4.3 in the presence or absence of cephalosporin antibiotics.

At pH 4.0 in EspIMS6, *acrA* and *acrB* were up-regulated by 4 and 2.7 fold respectively (figure no. 4.3.1.7.2.2 D-E). Whereas in DL4.3, pH 6.0 induced 3 and 2.5 fold up-regulation of *acrA*, *acrB* transcript levels respectively (figure no. 4.3.1.7.2.2 A-B). In presence of antibiotic cefuroxime, at pH 4.0, in DL4.3, *tolC* was 3 fold up regulated. Whereas similar pattern of 3.5 fold higher expression of *tolC* was observed in EspIMS6 at pH 8.0, in presence of antibiotic cefotaxime (figure no. 4.3.1.7.2.2 C-F). To summarize, it was observed that in DL4.3, presence of antibiotic cefuroxime up regulated expression of *tolC* at pH 4.0 but put down its expression at pH 8.0 (figure no. 4.3.1.7.2.2C). In absence of cefuroxime drug, pH 6.0 induced up-regulation of *acrA* and *acrB* transcripts levels in DL4.3. On the contrary, in EspIMS6, at pH 4.0, absence of antibiotic increased expression of *acrA* and *acrB* genes, while at pH 8.0, addition of antibiotic cefotaxime up-regulated expression of *tolC* (figure no. 4.3.1.7.2.2 D-E-F).

4.3.1.8 Effect of pH and cephalosporin antibiotics on AcrAB-TolC protein expression in *Enterobacter* isolates

To validate the above reported transcript-level observations, we performed western blotting using customized polyclonal rabbit anti-AcrA, anti-AcrB and anti-TolC antibodies at 1:1000 dilutions (recommended for Immunoblot). To start with, we used customized antibodies that were generated against AcrA, AcrB and TolC using in-silico epitope based antigenic peptide. These antigenic peptides were derived from *E. coli* strain K12, whose protein structure has been deposited in the database (Courtesy: Genscript, USA & Biotech desk, Hyderabad). Proceeding further, we also checked the specificity of customized antibodies towards antigen in *Enterobacter* isolates, using supplied blocking peptides (Genscript, USA). Bacterial total cellular protein was extracted and subjecting it to 12% SDS-PAGE analysis, followed by Coomassie staining, helped to assess quality and quantity of the protein, as shown below (figure no. 4.3.1.8.1).

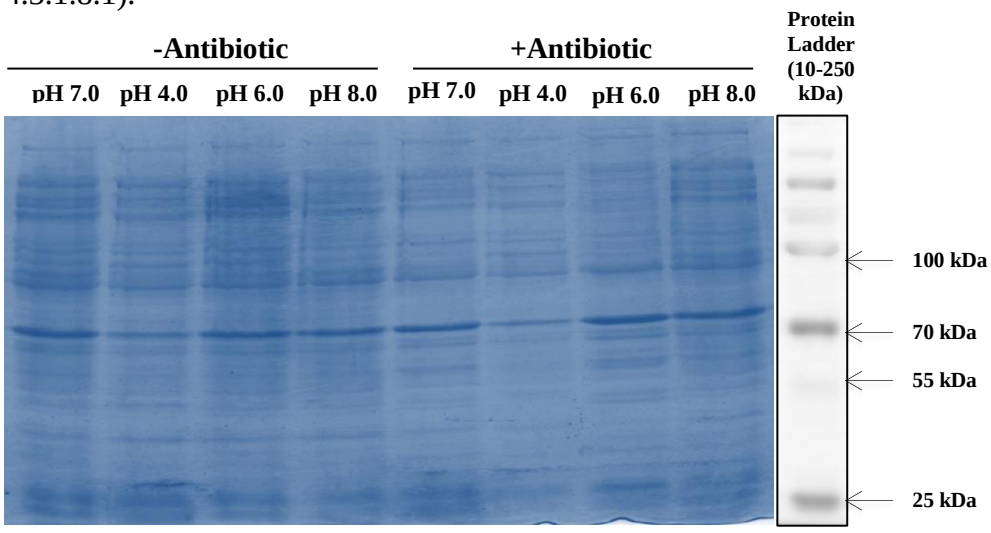


Figure no. 4.3.1.8.1: Coomassie brilliant blue stained gel showing extracted bacterial proteins. 12% SDS PAGE gels with 1µg of protein samples loaded extracted from *Enterobacter* isolates in the presence or absence of antibiotics.

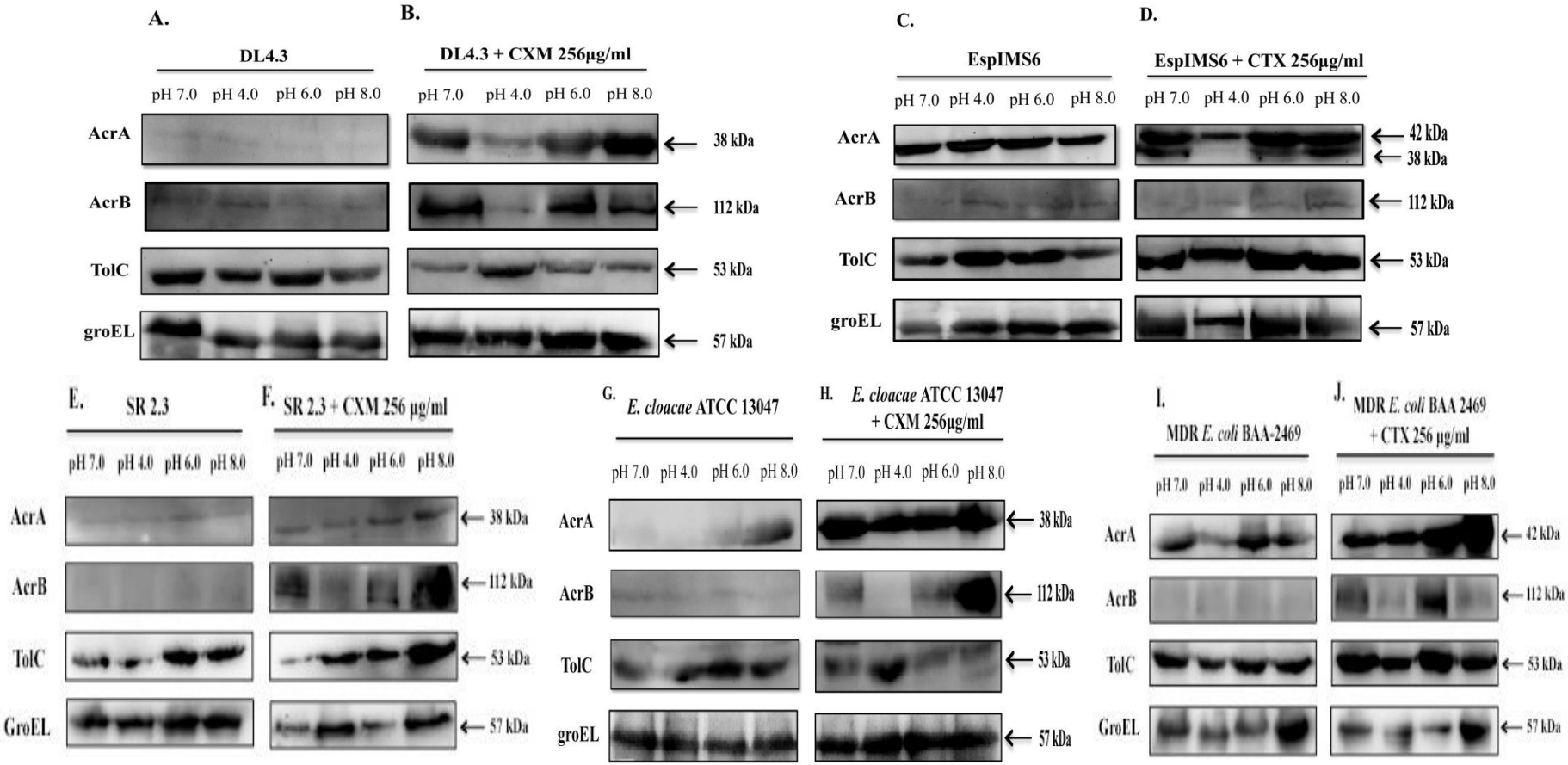


Figure no. 4.3.1.8.2: Immunoblot images showing expression of efflux proteins. 1µg of total cellular protein from each sample were loaded onto 12% SDS-PAGE, bands transferred to PVDF membranes and probed with 1:1000 dilutions of anti-AcrA, anti-AcrB and anti-TolC antibodies. Band intensities were normalized with expression of housekeeping chaperon anti-GroEL (1:3000) for all experiments. Environmental *E. cloacae* isolate DL4.3 and SR2.3 at different pH without (A), (E) and with 256 mg/L cefuroxime (B), (F). Clinical isolates *E. cloacae* EspIMS6 and *E. coli* ATCC MDR BAA-2469 at different pH without (C), (I) and with 256 mg/L cefotaxime (D), (J). *E. cloacae* type strain ATCC 13047 at different pH without (G) and with 256 mg/L cefuroxime (H).

Western blotting was then performed using the total cellular protein (whole cell lysate) of *E.cloacae* isolates and was probed with primary polyclonal rabbit anti-AcrA, anti-AcrB and anti-TolC antibodies. The blot images at different pH with and without antibiotic (figure no. 4.3.1.8.2 A-J) along with the mean ratio of normalized density of proteins obtained from three biological replicates (table no. 4.3.1.3) are presented below.

In the whole cell lysate obtained for non-clinical isolate DL4.3, AcrA was not detected across the pH studied *viz.* 7.0, 4.0, 6.0, and 8.0 (figure no. 4.3.1.8.2A). Similar observation was recorded with the multi susceptible non-clinical isolate SR2.3 (figure no. 4.3.1.8.2E). However, in presence of 256μg/ml (sub-MIC concentration) of cefuroxime (in DL4.3), AcrA was expressed as a 38kDa protein, in the whole cell lysate, with lowest expression at pH 4.0 against rest of the pH studied (figure no. 4.3.1.8.2B). In addition, cefuroxime induced expression of AcrB in DL4.3 and SR2.3 (figure no. 4.3.1.8.2B and 2F), with nearly 14 fold higher expression at pH 6.0, 7.0 and 8.0 in DL4.3, but much less (~9 fold) at pH 4.0 (figure no. 4.3.1.8.2B, table no. 4.3.1.3). Three fold up regulation of TolC in DL4.3 in presence of cefuroxime at pH 4.0 also echoed the real time data (figure no. 4.3.1.8.2B). In contrast, TolC in SR2.3 was 2.5 fold up-regulated in presence of cefuroxime (figure no. 4.3.1.8.2F).

Immunoblotting of whole cell lysate of clinical isolate EspIMS6, showed constitutive expression of AcrAB-TolC across all the pH studied (figure no. 4.3.1.8.2C). These results were concurrent with those obtained at transcript level analysis. In presence of 256μg/ml concentration cefotaxime, AcrA was up regulated by 6 to 8 fold higher in the whole cell lysate (figure no. 4.3.1.8.2D) in EspIMS6. Similarly, 12 to 16 fold up regulation of AcrA was observed in presence of cefuroxime in *E. cloacae* ATCC 13047 (figure no. 4.3.1.8.2H).

Table no. 4.3.1.3: Average normalized density (int/mm^2) of efflux proteins (in comparison to housekeeping GroEL)

Average band intensity (INT/mm2) for AcrA, AcrB and TolC proteins normalized against average band intensity of GroEL from three independent biological replicates.

Proteins	AcrA					AcrB					TolC				
Isolates used	**DL4.3**	**EspIMS6**	**ATCC 13047**	**SR2.3**	**MDR 2469**	**DL4.3**	**EspIMS6**	**ATCC 13047**	**SR2.3**	**MDR 2469**	**DL4.3**	**EspIMS6**	**ATCC 13047**	**SR2.3**	**MDR 2469**
pH 7.0-Ab	0.72	4.00	5.58	1.42	3.90	6.61	4.28	7.36	1.73	1.53	0.78	1.11	10.09	1.23	3.02
pH7.0+Ab	2.81	7.71	15.18	3.22	2.31	14.44	9.98	8.04	5.26	2.65	2.51	5.69	4.24	1.24	6.83
pH 4.0-Ab	0.82	3.01	8.10	1.45	1.13	5.99	4.04	9.61	1.75	2.02	0.87	1.08	14.43	1.31	3.53
pH 4.0+Ab	3.82	6.84	12.50	1.71	2.60	9.76	8.02	7.91	2.50	2.36	2.45	4.14	6.66	1.61	7.18
pH 6.0-Ab	1.00	2.82	5.22	1.56	1.94	4.35	4.75	7.17	1.58	2.21	0.64	1.28	12.23	2.04	2.95
pH 6.0+Ab	5.02	8.24	16.25	3.85	5.49	14.68	8.88	8.35	7.82	18.24	3.17	5.74	6.02	4.40	11.29
pH 8.0-Ab	1.25	2.44	12.49	1.13	2.01	4.12	4.78	6.63	1.85	1.18	0.65	1.25	7.09	1.30	3.07
pH 8.0+Ab	3.9	8.80	16.40	1.64	4.24	14.50	8.95	11.96	6.94	4.98	2.77	7.09	6.15	2.50	8.36

It was noteworthy that except pH 4.0, at all other pH tested *viz*. 6.0, 7.0 and 8.0, a 38 kDa form of AcrA was observed in lesser quantities along with a 42 kDa band in both EspIMS6 and ATCC 13047 (figure no. 4.3.1.8.2D and 2H).

Immunoblotting results indicated that in all the three *E. cloacae* isolates DL4.3, EspIMS6 and ATCC 13047, alkaline pH 8.0 reduced TolC expression (figure no. 4.3.1.8.2A, 2C, 2G); whereas in susceptible environmental *E. cloacae* isolate SR2.3, TolC was 1.3 fold up regulated at pH 8.0 (figure no. 4.3.1.8.2E). In contrast, TolC was expressed in significant quantities at acidic pH 4.0 in all the four isolates (figure no. 4.3.1.8.2A, 2C, 2G, 2I). TolC expression in *E. coli* ATCC BAA-2469 was constant across all the pH tested (figure no. 4.3.1.8.2I). It was also noted that, TolC expression was promiscus between pH 4.0 to pH 7.0. This effect of pH on TolC was more pronounced in the environmental isolate DL4.3 than in the clinical isolates EspIMS6 and *E. cloacae* type strain ATCC 13047 (figure no. 4.3.1.8.2A vs. 2C & 2G). It was noted that TolC is expressed constitutively across all the pH studied.

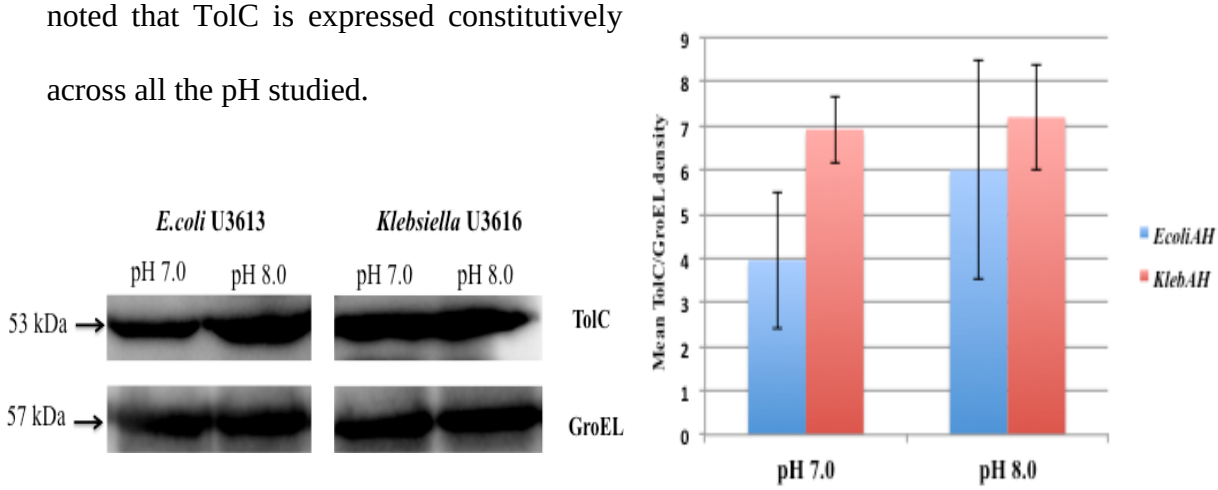


Figure no. 4.3.1.8.3: TolC expression at alkaline pH 8.0 in clinical UTI isolates, showing western blot and relative intensity of the band as compared to GroEL.

To reassure the induction of TolC expression at varied pH, we investigated this aspect in two other clinical isolates (Urinary tract infection isolate), *E.coli* (U3613) and *Klebsiella pneumoniae* (U3616) at pH 7.0 and 8.0 only. As observed form figure no.

4.3.1.8.3, it was evident that in both the isolates, alkali pH 8.0 induced TolC expression, more prominently in *E.coli* isolates. The protein expression was greater in *Klebsiella* spp. compared to *E.coli.*

From immunoblot results we could conclusively summarize the following:

I. Constitutive expression of AcrAB-TolC efflux proteins across pH 4.0 to 8.0, vary significantly between clinical and non-clinical isolates with former being greater than later.

II. Cephalosporin antibiotics induced significant up-regulation of the AcrAB-TolC efflux pump proteins in all isolates studied.

III. Increase in TolC expression at alkaline pH showed positive association with alkaline survival of isolates.

IV. We observed a similar pattern of protein expression in prototype *E. coli* ATCC MDR BAA-2469 and *E. cloacae* ATCC 13047 (Fig. no. 4.3.1.8.2I-J and 2G-H respectively), which further confirmed our conclusions.

4.3.1.9. Membrane protein extraction and immunoblotting of AcrA and AcrB in membrane fractions of *Enterobacter* isolates

It was interesting to note that upon probing of the cellular protein fraction of cefotaxime treated EspIMS6 with anti-AcrB antibody, a distinct and intense 112 kDa monomeric band of AcrB was induced, across all the pH studied (figure no.4.3.1.8.2D). Our hypothesis was supported by the results obtained with membrane fraction. Indeed, the membrane fraction of untreated cells of both the *Enterobacter* isolates, showed minimal expression of 112 kDa band of monomeric AcrB (figure no. 4.3.1.9C), whose expression was enhanced when cells were treated with cephalosporin antibiotics (figure no. 4.3.1.9D).

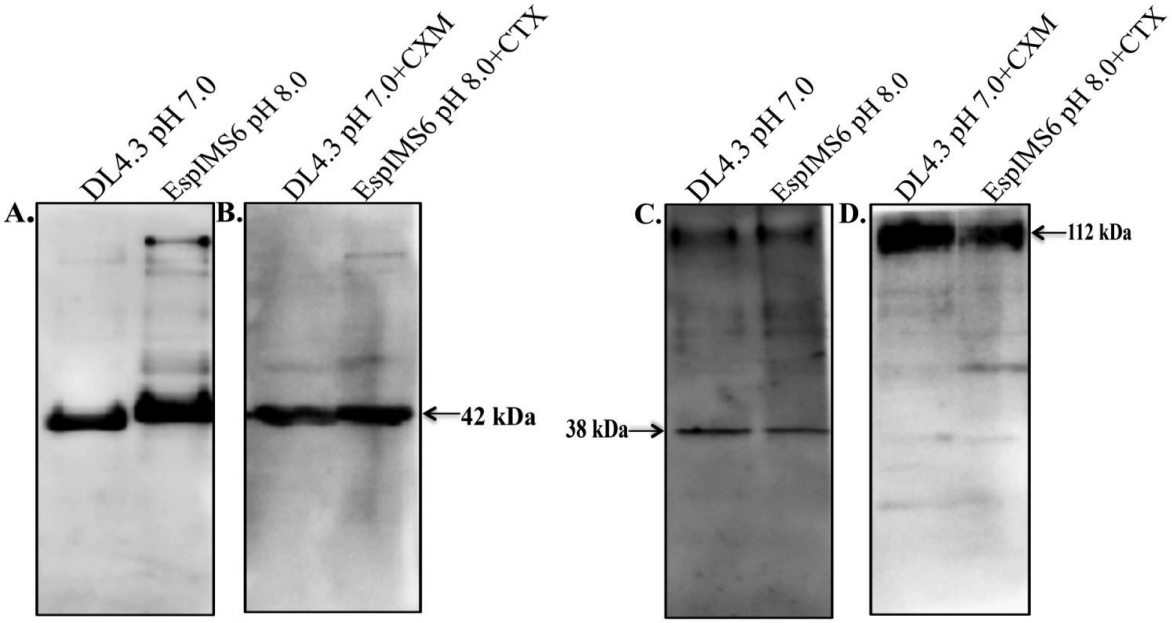


Figure no. 4.3.1.9: Immunoblot of membrane fractions. Environmental *E. cloacae* isolate DL4.3 and clinical *E. cloacae* isolate EspIMS6 were grown at pH 7.0 and pH 8.0 respectively (with and without 256 mg/L of respective cephalosporin antibiotics). Membrane fractions were extracted and 3 μg of protein were run in 12% SDS-PAGE, and probed with anti-AcrA and anti-AcrB antibodies. Membrane localized AcrA and AcrB protein in the absence (A, C) and presence of antibiotic (B, D) respectively.

It was worth noting that, immunoblotting with anti-AcrA antibody, of the membrane protein fraction, of isolate DL4.3 at pH 7.0 revealed the presence distinct 38 kDa band (figure no. 4.3.1.9A). Nevertheless, 42kDa band of AcrA was observed in the membrane fraction subjected to antibiotic treatment (figure no. 4.3.1.9B).

4.3.1.10: Immunofluorescence study for expression of AcrAB-TolC efflux proteins in response to pH and cephalosporin drugs

After looking into their expression pattern in total cellular and membrane fractions, we investigated AcrAB-TolC expression in bacterial cells treated with and without antibiotic by indirect immunofluorescence as shown below (figure no. 4.3.1.10.1-EspIMS6 and figure no. 4.3.1.10.2-DL4.3).

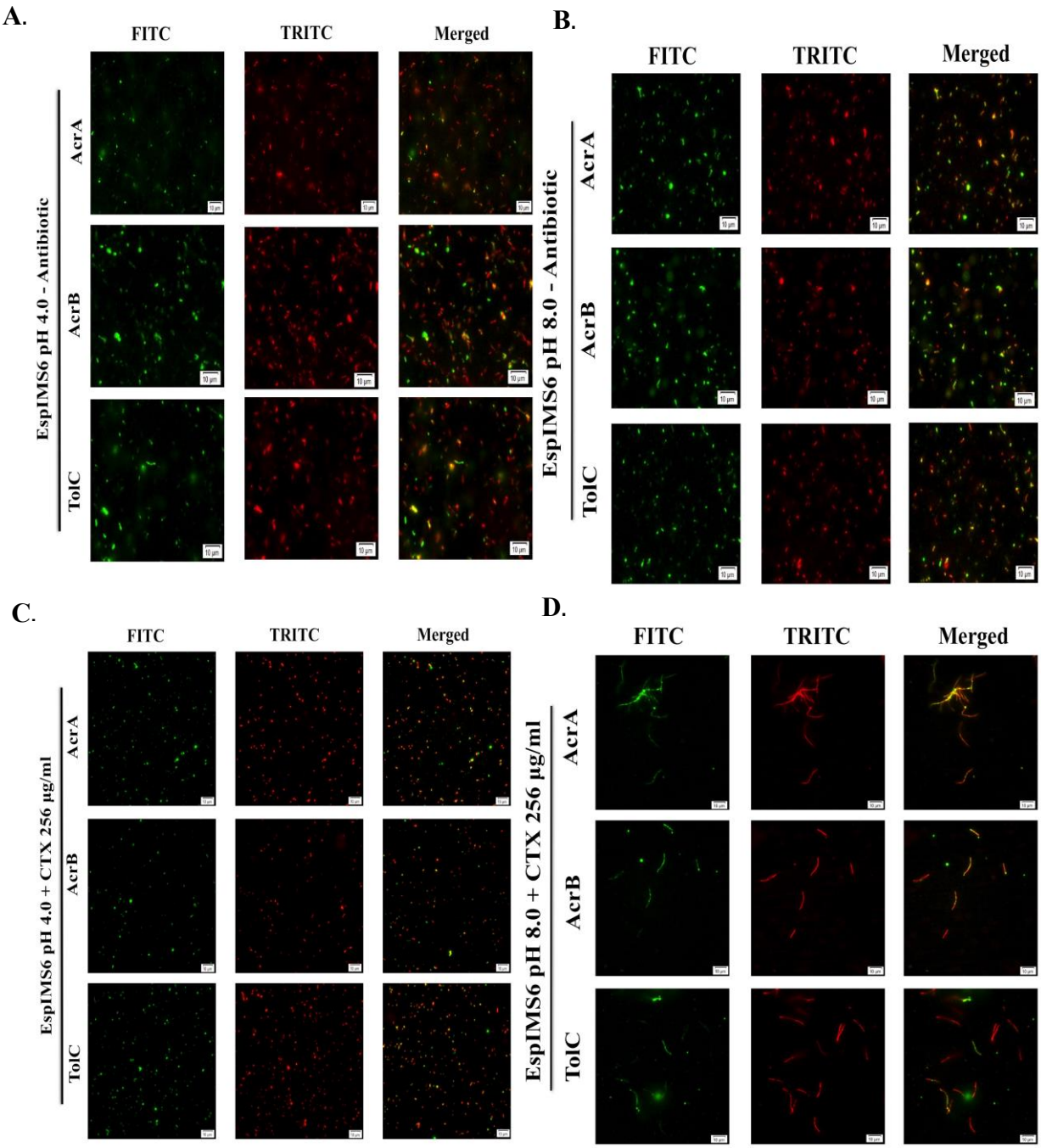


Figure no. 4.3.1.10.1: Expression of efflux proteins in EspIMS6 at pH 4.0 and pH 8.0. A and C- Without Cefuroxime, B and D- With cefuroxime at 256µg/ml.concentration.

Red channel (TRITC) indicated FM-464 dye labeling bacterial membrane and green channel (FITC) represented the Alexa-488 conjugated secondary antibody towards primary antibodies of interest. The merged panel represented co-expression pattern of the proteins.

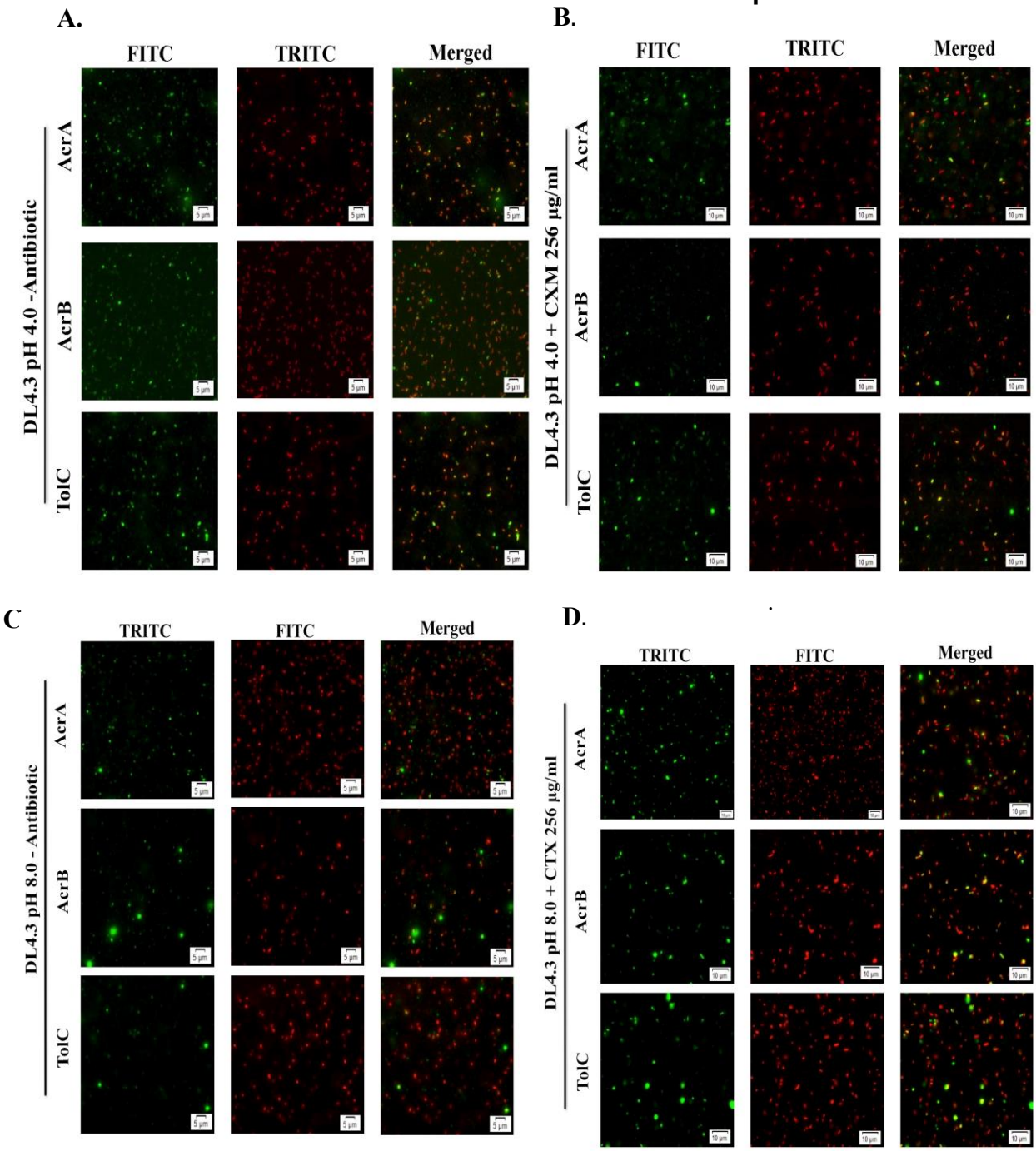


Figure no. 4.3.1.10.2: Expression of efflux proteins in DL4.3 at pH 4.0 and pH 8.0. A and C-Without Cefotaxime, B and D- With cefotaxime at 256µg/ml.concentration.

Red channel (TRITC) indicated FM-464 dye labeling bacterial membrane and green channel (FITC) represented the Alexa-488 conjugated secondary antibody towards primary antibodies of interest. The merged panel represented co-expression pattern of the proteins.

It was quite evident from co-expression pattern that in clinical isolate EspIMS6 at acidic pH 4.0 and alkaline pH 8.0, AcrAB-TolC efflux proteins were constitutively expressed (figure no. 4.3.1.10.2A-B). On the other hand, overlap of images in environmental isolate DL4.3 revealed AcrAB-TolC efflux proteins to be expressed at acidic pH 4.0 (figure no. 4.3.1.10.1A), but not at alkaline pH 8.0 (figure no. 4.3.1.10.1C). Again, we observed reduced expression of efflux proteins at alkaline pH 8.0 in DL4.3, which echoed our previous observations.

Moreover, expression of efflux proteins was enhanced after addition of cephalosporin antibiotics, which again concurred with our immunoblot results. In DL4.3 isolate, at pH 8.0 in presence of cefuroxime at 256µg/ml, expression of TolC was greater followed by AcrB and AcrA (figure no. 4.3.1.10.1D), against that observed at pH 4.0 (figure no. 4.3.1.10.1B). In clinical isolate EspIMS6, cefotaxime at 256µg/ml concentrations induced expression of AcrAB-TolC proteins both at pH 4.0 (figure no. 4.3.1.10.2C) and at pH 8.0 (figure no. 4.3.1.10.2D).

Particularly in clinical isolate EspIMS6, in imaging experiments with the treatment of antibiotic, dormant cells (relatively slow growth as compared to without antibiotics) were observed, which were of smaller in size at pH 4.0 (figure no. 4.3.1.10.2C) and were of extended thread-like structures at pH 8.0 (figure no. 4.3.1.10.2D). Being an urinary tract isolate, EspIMS6 was prone to such exposure of adverse pH and antibiotic concentrations, and may be the size difference in terms of dormancy is an example of greater adaptability feature of Enterobacterial pathogens.

Discussion

Over the years we have gained significant insight into the structure function aspect of AcrAB-TolC efflux pump proteins [58],[176]. Circumstantial evidences from the literature, indicates possible involvement of efflux systems in cellular pH homeostasis, which however has been less explored [32]. We carried out comparative investigation on the effect of pH and cephalosporin drugs on AcrAB-TolC expression in clinical and environmental isolates of *E. cloacae*. Our results also showed constitutive low expression of AcrAB-TolC in environmental isolates as compared to clinical isolates. Nevertheless, cephalosporin drugs-cefuroxime and cefotaxime; up regulated AcrAB-TolC expression in all isolates including *E. coli*.

We observed that while the clinical isolate EspIMS6 survived increasing antibiotic dosage at all pH, the environmental isolate tolerated acidic pH 4.0-6.0 but succumbed at alkaline pH 8.0. It was also observed that alkaline tolerance of environmental *E. cloacae* isolate SR2.3 was better than that of DL4.3. Experiments done to study the effect of pH on drug susceptibility revealed that at acidic pH 4.0, *E. cloacae* isolates DL4.3, EspIMS6, SR2.3 and *E. coli* BAA-2469 were more tolerant towards higher concentrations of the antibiotics. There may be two possible reasoning; either the drugs are pH labile or there exists other mechanisms of drug resistance.

It is well established that *E. cloacae* exhibit intrinsic resistance towards beta-lactams including first-generation cephalosporins, which is attributed to chromosomally encoded AmpC beta-lactamases while they possess inducible beta-lactamases imparting resistance towards rest of the cephalosporins [8]. Noteworthy is that beta-lactamases activity (including those of AmpC) is significantly lowered at acidic pH 5.8

[191]. These facts indicate possible existence of other mechanism that aid in decreased drug susceptibility of the isolates under study.

Results obtained from HPLC analysis negated any possibility of degradation of antibiotics tested. Except for cefuroxime and cefotaxime, pH stability of rest of the antibiotics *in vitro* was extremely poor, that rendered them unfit for inclusion in the present study. It is significant to mention here, that, cephalosporin structure allows relatively greater extent of side chain modifications including those at C-3 and C-7 positions. In cephalosporins, nature of substituents at C-7 positions influences stability against chemical/enzymatic hydrolysis of the drug [192].

As evident from real time PCR without antibiotics, at pH 4.0 in EspIMS6 and at pH 6.0 in DL4.3, transcript levels of *acrA* and *acrB* were up regulated by four and three folds respectively. Again presence of antibiotic cefotaxime upregulated expression of *tolC* in EspIMS6 at pH 8.0 where as similar phenomenon was observed at pH 4.0 in DL4.3 with cefuroxime, which echoed the noted pH modulatory effect on efflux in MIC assay. Association of *acrA* and *acrB* together in a single operon and involvement of common regulatory mechanisms might explain this observed harmony of *acrA* and *acrB* in expression studies [193]; [194].

As mentioned earlier pH is known to regulate a wide array of membrane proteins in *E. coli* K12 cells [89]. In *E. coli,* TolC associated proton driven efflux pumps were found to be significantly important in survivability under extreme acidic (pH 2.0) environment. We observed that, in clinical and environmental isolates TolC expression was promiscus at acidic pH 4.0. Our results are consistent with Deininger *et al* [195],

who have reported TolC to be required for maximal growth rates below pH 6.5, but not for aerobic survival in extreme alkaline condition (pH 10.0). However, we observed lowered expression of TolC at pH 8.0 than at pH 4.0, in environmental isolate DL4.3 and in *E. cloacae* ATCC 13047 coupled with decreased growth of these isolates at alkaline pH in presence of cefuroxime. In contrast, we observed positive association between up regulation of TolC and aerobic survival under alkaline condition (pH 8.0) for clinical isolates EspIMS6, *E. coli* BAA-2469, and environmental isolate SR2.3. These isolates survived at alkaline pH 8.0 in presence of antibiotics.

Whether TolC plays an important role in alkaline aerobic survival, or, survival of these isolates at alkaline pH is due to increased beta-lactamase activity remains to be further explored. *In vivo* conditions expose the opportunistic *E. cloacae* to face dual challenge of varying physical environment, such as pH and antibiotic concentrations. Under such conditions up regulation of TolC facilitates the organism in combating resistance and establishing infection. This is very conspicuous from protein expression data obtained in the present study. As mentioned previously, at acidic pH 4.0, beta-lactamase activity is highly compromised. Up regulation of AcrAB-TolC under acidic pH thus possibly facilitates extrusion of the antibiotics from the periplasmic space, reducing effective intracellular drug concentration. This implies probable role of AcrAB-TolC in survival of the organisms under acidic pH in presence of cephalosporin antibiotics.

In vitro and *in vivo* proteolytic profiling of AcrA, have shown that a ~28kDa of AcrA constitute a proteolytically stable core domain whereas, a ~38kDa of AcrA protein essentially plays a role in drug efflux [62, 196]. We hypothesize that the 38kDa band of AcrA, observed in membrane fraction of the non-clinical isolate DL4.3, to be the above mentioned functional version of the protein, retaining efflux activity. Also, in

presence of cefotaxime in EspIMS6, the 42kDa AcrA band observed could be post-translational modified product of the 38kDa band. We also observed enhanced expression of AcrB in the presence of antibiotics cefuroxime and cefotaxime in DL4.3 and EspIMS6 respectively. These results strongly indicated greater translational turnover of monomeric form of the AcrB protein (112 kDa) in the cellular fraction, which might trimerize and get functionally localized in the membrane, in response to antibiotic.

Our results thus conclusively indicated AcrAB to be up regulated in presence of cephalosporin antibiotics in both the wild type isolates EspIMS6 and DL4.3. Similar results in *E. cloacae* type strain ATCC 13047 and *E. coli* ATCC MDR BAA-2469 also substantiated this. Our results have shown antibiotics (cefuroxime and cefotaxime) to play a greater role than pH, in enhancing AcrAB expression in *E. cloacae* isolates. This is concurrent to the reported 8 fold increase in NorB efflux pump upon shift to pH 4.5 in *Staphylococcus aureus* associated with moxifloxacin resistance [197].

Put together, this study provides an insight into strain/isolate specific differential regulation of AcrAB-TolC. These efflux pump proteins have constitutively higher expression in clinical isolates compared to environmental isolates. However, in both types of isolates, cephalosporin drugs further enhance their expression. Based on these observations, we hypothesized a model (figure no. 4.3.1.11), which suggested greater role of TolC in either compromising or facilitating survival of MDR environmental/clinical isolates respectively.

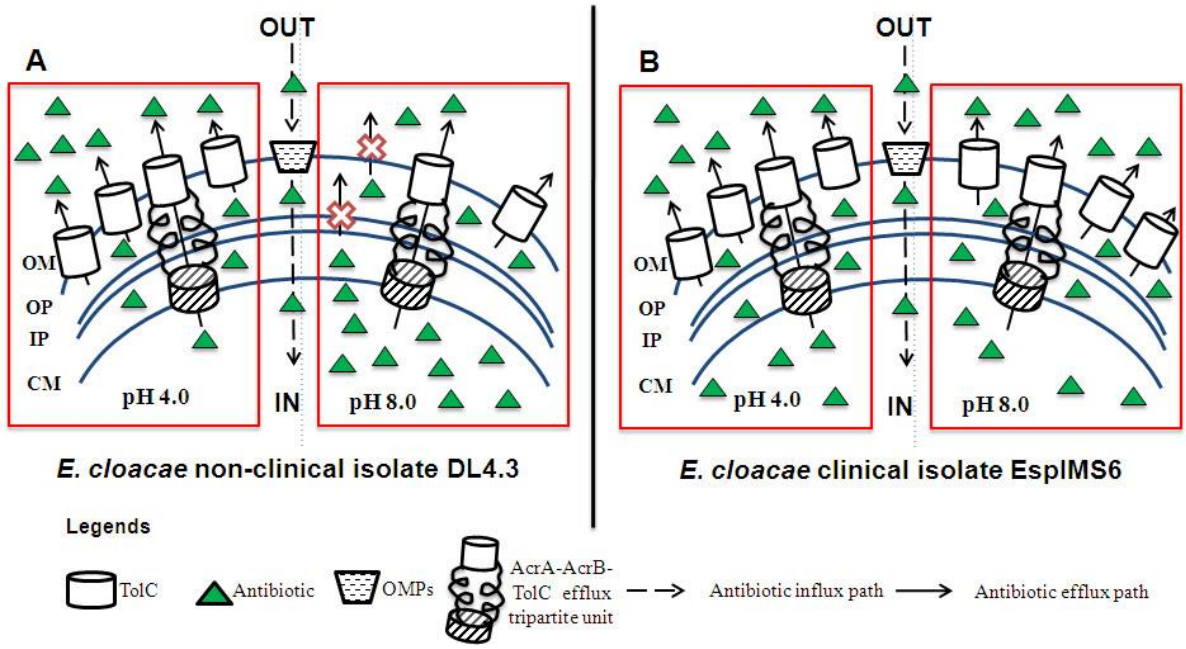


Figure no. 4.3.1.11: Graphical model hypothesizing role of TolC in bacterial survival in response to pH and antibiotics

This is an illustrative model proposing significance of AcrAB-TolC expression in survival and persistence of *E. cloacae* isolates. (A) In non-clinical isolate DL4.3, at pH 4.0 enhanced expression of TolC enable efflux of drugs, lowering their intracellular and periplasmic concentration, which is reverted at pH 8.0 due to reduced TolC expression. (B) In clinical isolate EspIMS6, similar levels of TolC expression across pH 4.0 to pH 8.0, lower intracellular and periplasmic drug concentration and facilitate survival of the bacteria.

Section 4.3.2: Effect of silver (AgNP) and silver-gold (AgAuNP) nanoparticles on AcrAB-TolC expression

Background of the study

The rise in antimicrobial resistance coupled with diminishing available options has necessitated search for alternative therapeutic options against MDR pathogens [198]. Over the years, nanoparticle-based approaches have gathered much attention due to their fast and effective antibacterial properties for various pathogens [199], [200]. Nanoparticles endowed with wide bactericidal spectrum, ability to inhibit biofilm formation, increased permeability and low dosage efficacy, form a powerful weapon to combat MDR bacteria [201], [202], [203], [204]. However, these therapeutic advantages posed by the nanoparticles could potentially be hindered with their extrusion by efflux pumps; particularly those belonging to RND super family, which exhibit substrate non-specificity. In this section we present results of polysaccharide capped silver nanoparticle (AgNP) and silver-gold nanoparticle (AgAuNP) as antibacterial agents and their effect on AcrAB-TolC protein expression.

4.3.2.1: Bacterial strains used, their antibiotic susceptibility profile

To evaluate the antimicrobial efficacy of AgNPs, we had used bacterium C19 (identified as a multi-drug resistant *E. coli* strain), *Klebsiella pneumoniae* Kp52, *Enterobacter cloacae* EcIMS18, *Staphylococcus epidermidis* and *staphylococcus haemolyticus. E. coli* strain MTCC 443 (i.e. ATCC 25922) was used as control in the antibiotic susceptibility test. Antibiotic disc diffusion test revealed the resistance pattern observed towards various groups of antibiotics (table no. 4.3.2.1). All of the isolates tested displayed MDR phenotype.

Table no. 4.3.2.1: Resistance profile of isolates used in this study for AgNPs and AgAuNP antibacterial efficacy

Sl. No.	Strains used	Resistance profile
1	*Escherichia coli (*C19)	AMP, AMC, PI, CXM CTR, NX, S, IPM, TE, RIF, SC
2	*Enterobacter cloacae* EcIMS18	CZX, CX, LE, CTR, TCC, MRP, PIT, GAT, A/S, CTX, AMC, GEN, CIP, AMP, CXM, IPM, CPM, COT, CAZ, AK
3	*Klebsiella pneumoniae* KpIMS52 (830)	AK, CZ, CTR, CIP, G, MRP, MO, TOB
4	*Staphlococcus epidermidis*	C, CZ, OF, CIP, GAT
5	*Staphlococcus haemolyticus*	OX, AMP, OF, CIP, GAT, E, TE, TR
6	*Escherichia coli (*MTCC 443)/ (ATCC 25922)	
7	*Enterobacter cloacae* EspIMS6	CZX, CX, LE, CTR, TCC, PIT, GAT, A/S, CTX, AMC, OF, CIP, AMP, CXM, IPM, CPM, COT, CAZ
8	ATCC 13047 (*E. cloacae* type strain)	AMC, CXM, AMP, CAZ, CX, CAC, A/S
9	BAA-1143 (ATCC MDR *E. cloacae*)	CZX, CX, CPZ, CTR, TCC, PIT, AT, A/S, CTX, AMC, AMP, CXM, CAC, CAZ
10	BAA-2469 (ATCC MDR *E. coli*)	CZX, CX, CPZ, CTR, TCC, PIT, AT, GAT, A/S, CTX, AMC, OF, GEN, CIP, AMP, CXM, IPM, CPM, CAC, CAZ, AK

Antibiotics used were: Ampicillin AMP (10 mcg), Gentamicin GEN (10 mcg), Amikacin AK (30 mcg), Ciprofloxacin CIP (5 mcg), Ofloxacin OF (5 mcg), Co-Trimoxazole COT (25 mcg), Amoxyclav AMC (30 mcg), Cefuroxime CXM (30 mcg), Ceftazidime CAZ (30 mcg), Ceftazidime/Clavulanic acidCAC (30/10mcg), Cefepime CPM (30 mcg), Imipenem IPM (10 mcg), Cefotaxime CTX (30 mcg), Cetriaxone CTR (30 mcg), Cefoxitin CX (30 mcg), Meropenem MRP (10 mcg), Piperacillin/Tazobactam PIT (100/10 mcg), Aztreonam AT (30 mcg), Gatifloxacin GAT (5 mcg), Ampicillin/Sulbactam A/S (10/10 mcg), Cefoperazone (CPZ 75 mcg), Levofloxacin LE (5 mcg), Ceftizoxime CZX (30 mcg), Ticarcillin/Clavulanic acid TCC (75/10mcg).

The results of disc diffusion test (table no. 4.3.2.1) revealed that the ATCC strains displayed MDR phenotype, wild type clinical *E.cloacae* isolates exhibited extensive drug resistance (XDR) phenotype, with resistance towards almost all of the antibiotics belonging to multiple classes. Out of the strains listed above, isolates C19, EcIMS18, KpIMS52, *S. epidermidis* and *S. haemolyticus* and *E. coli* MTCC 443 were used to study antibacterial efficacy of AgNP while those listed from sl nos. 6 to 10 were used in the study on Ag-AuNp. This selection was random with availability of the strains at the time of experimentation.

4.3.2.2: Determination of antibacterial efficacy of nanoparticles

We determined the minimum inhibitory concentration breakpoints of silver nanoparticle (AgNP) and silver-gold bimetallic nanoparticle (Ag-AuNP). We observed that both the nanoparticles tested significantly inhibited bacterial growth in a dose dependent manner as shown below (table no. 4.3.2.2).

Table no. 4.3.2.2: Antibacterial properties of nanoparticles as observed from their Minimum inhibitory concentration (MIC) values

Strains used	**AgNP (in µg/ml)**	**Ag-AuNP (in µg/ml)**
*Escherichia coli (*C19)	1.5	ND
E. cloacae EcIMS18	3	ND
K. pneumoniae KpIMS52	6	ND
Staphlococcus epidermidis	6	ND
Staphlococcus haemolyticus	>6	ND
E. cloacae EspIMS6	12	1.5
E. cloacae ATCC 13047	12	3
E. cloacae ATCC MDR BAA-1143	12	3
E. coli ATCC MDR BAA-2469	12	3

Note: ND-Not determined

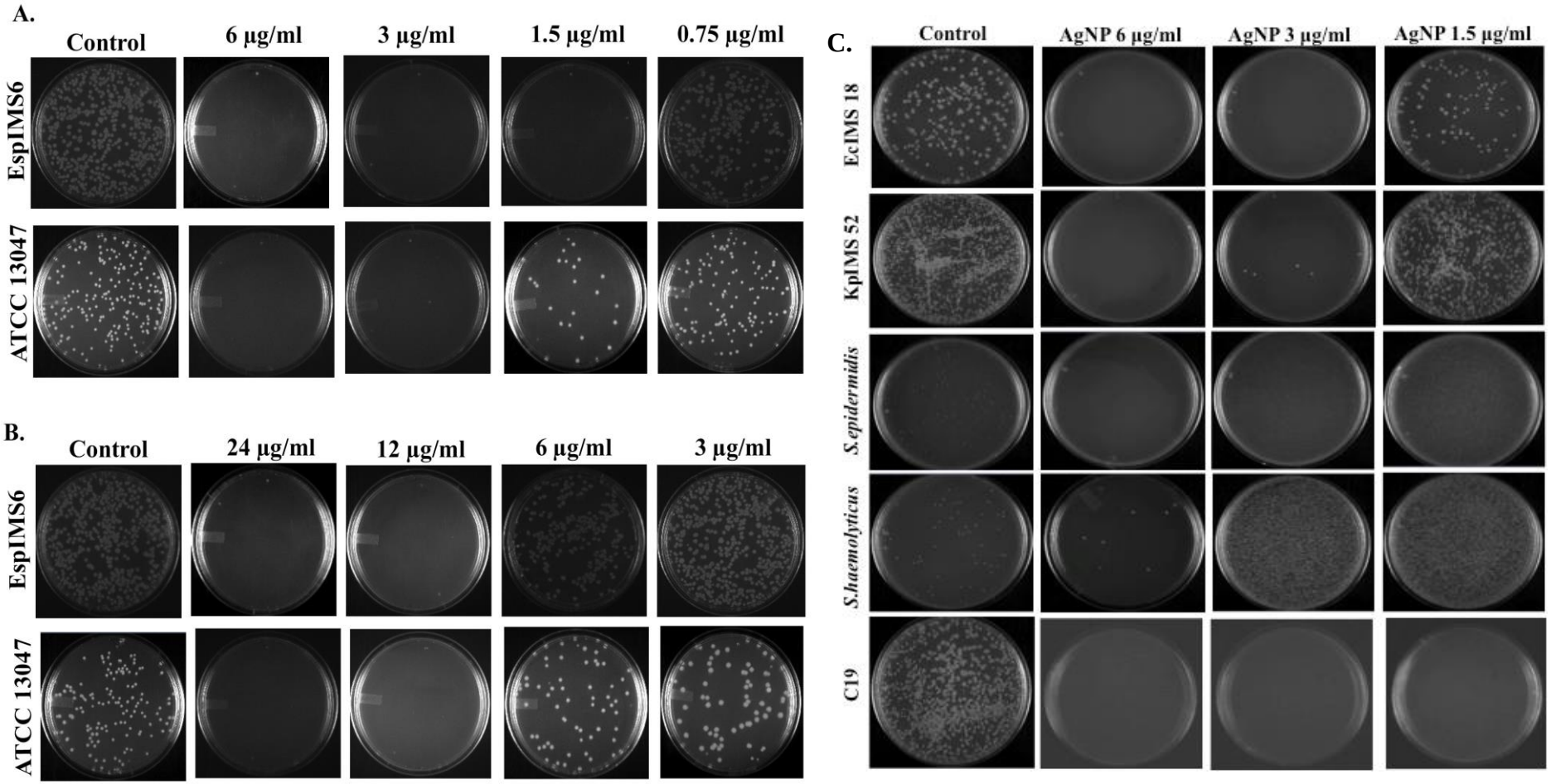


Figure. no. 4.3.2.1: Antibacterial efficacy of nanoparticles-Antibacterial properties of AgAuNPs (Panel A) and AgNPs (Panel B and C) nanoparticles on bacterial growth was determined by spread plating onto MHA plates after overnight exposure to nanoparticles at varying concentrations. Images were recorded using gel documentation system (Biorad, USA).

As compared to the commonly used antibiotics, the CMT-capped AgNPs strongly inhibited the growth of these MDR organisms at concentrations of 1.5µg/ml to 6µg/ml, indicating its efficacy to be used at such low concentration in vitro. However, MIC breakpoint for AgNPs was 12 µg/ml for EspIMS6, ATCC 13047, BAA 1143 and BAA 2469 (table no. 4.3.2.2). Interestingly, AgAuNPs had a much lower MIC breakpoint value of 1.5 and 3 µg/ml for isolates EspIMS6 and other MDR ATCC isolates respectively. The results clearly indicated that bimetallic nanoparticle Ag-AuNPs was more efficient in killing the MDR bacteria compared to AgNPs alone. From the MIC tubes after overnight incubation, the bacterial colonies were enumerated, viable growth was recorded which is represented in figure no. 4.3.2.1, which suggested antimicrobial properties of silver nanoparticles.

4.3.2.3. Immunoblotting for AcrAB-TolC efflux pump proteins in AgNp and AgAuNP nanoparticle-treated cells

Applications of silver nanoparticles depended on their efficacy to be retained in the cell vis-à-vis being effluxed out. We investigated this by studying the effect of silver nanoparticles on expression of multi-drug efflux pump proteins AcrAB-TolC in two *E.cloacae* isolates, clinical EspIMS6 and ATCC 13047 type strain. Overall immunoblotting results revealed that presence of silver nanoparticles did not significantly affect AcrAB-TolC efflux pump protein expression (figure no. 4.3.2.2).

Nevertheless, in the type strain ATCC 13047; AcrB and TolC did exhibit slight but insignificant changes in expression particularly in presence of AgAuNPs (figure no. 4.3.2.2B and 2C). Expression of outer membrane protein TolC decreased post 120 minutes in ATCC 13047; in presence both the silver nanoparticles tested *viz.* AgNPs and AgAuNPs (figure no. 4.3.2.2C- panel 3 and 4).

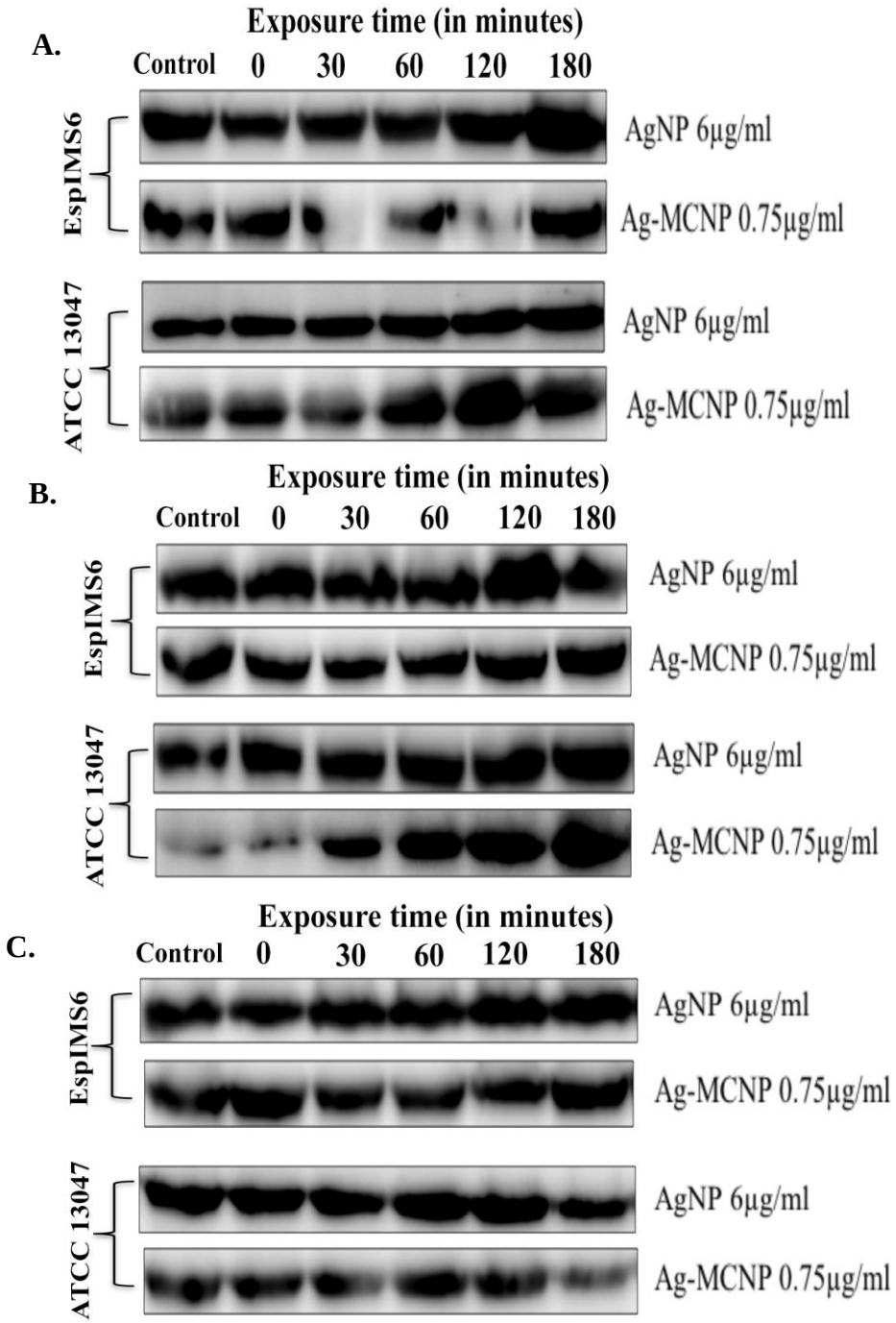


Figure no. 4.3.2.2: Western blot images of nanoparticle treated cells Note: Effect of silver and silver-metal composite nanoparticles on AcrAB-TolC expression. **(A)** AcrA (42 kDa) protein expression in EspIMS6 (panel 1.2) and *Enterobacter cloacae* ATCC 13047 (panel 3,4); **(B)** AcrB (~38 kDa) protein expression in EspIMS6 (panel 1,2) and *E. cloacae* ATCC 13047 (panel 3,4); **(C)** TolC (55 kDa) protein expression in EspIMS6 (panel 1,2) and *E. cloacae* ATCC 13047 (panel 3,4).

Here too we did not observe any significant changes in TolC expression for the wild type isolate EspIMS6 in presence of either of the nanoparticles.

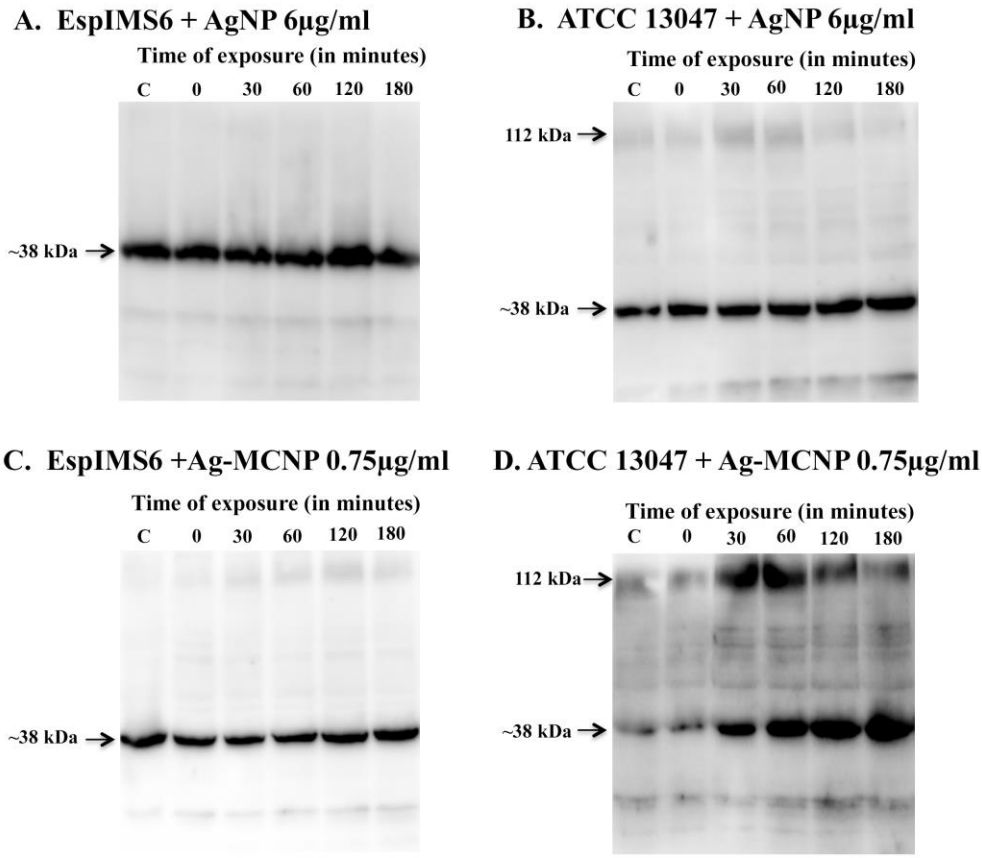


Figure no. 4.3.2.3: Whole blot showing expression of AcrB protein in response to silver nanoparticles. The whole immunoblot showed absence of 112 kDa of monomeric AcrB protein, instead ~38 kDa protein was expressed in response to sub lethal concentrations of AgNP (6µg/ml) and AgAuNP (0.75µg/ml) in EspIMS6 (A,C) and ATCC 13047 (B, D).

A 38 kDa band of AcrB was observed post 30minutes of treatment with Ag-MCNP at a concentration of 0.75 µg/ml in ATCC 13047 (figure no. 4.3.2.2B-Panel 3 and 4. figure no. 4.3.2.2D). No such changes were observed for wild type isolate EspIMS6, where only 38 kDa AcrB protein was consistently expressed at all the time points of exposure to AgNP as well as Ag-MCNP (figure no. 4.3.2.3A,3C). However, both the isolates EspIMS6 and ATCC 13047, when treated with the AgNP nanoparticles showed bands corresponding to ~38 kDa (figure no. 4.3.2.3A,3B).

4.3.2.4. Hemocompatibility of Silver Nanoparticles

Extent of hemolysis of red blood cells was determined to check hemocompatibility of silver nanoparticles. It was observed that AgNPs at concentrations of 1, 3 and 6 µg/ml, exhibited only 0.063, 0.31, and 0.63% of hemolysis, respectively (figure no. 4.3.2.3.). At higher concentrations of AgNP (i.e., 9 and 12 µg/ml) hemolysis was approximately 2%. Nanoparticles exhibiting hemolysis below 5% are considered hemocompatible [212].

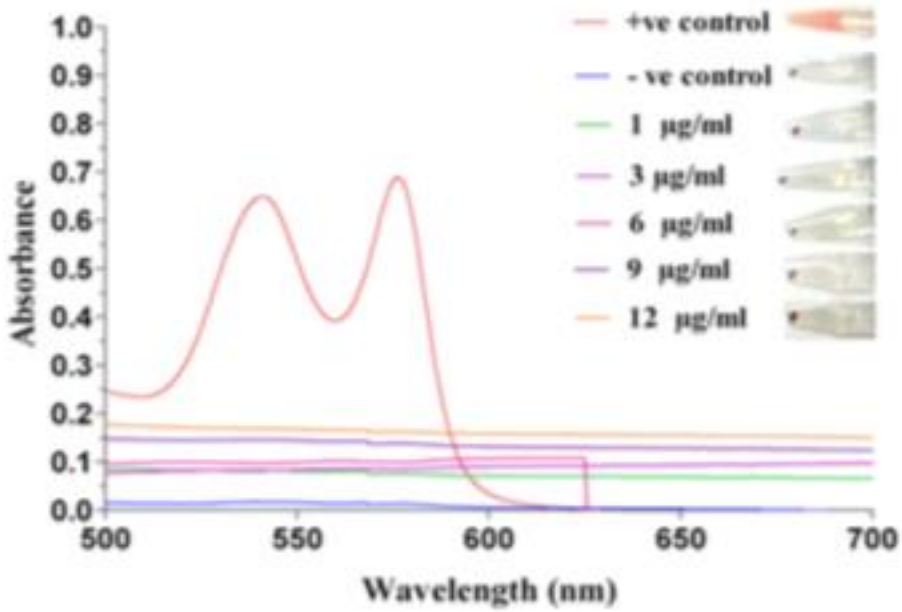


Figure no. 4.3.2.4: Hemocompatibility of silver nanoparticles. Determination of hemolytic activity at different concentrations (1, 3, 6, 9, and 12 µg/ml) of AgNPs, along with positive and negative control, recorded at 541 nm by UV-Vis spectrophotometer.

Discussion

The present study reports no effect of silver nanoparticles used on AcrAB-TolC efflux protein expression. This finding is significant as it confirms the viability of using these AgNPs and Ag-AuNPs as antibacterial agents.

AcrAB-TolC efflux pumps have been found to mediate multidrug resistant phenotype in many clinically significant gram-negative pathogens including *E. cloacae* [176],[50], [72]. Such efflux systems efficiently extrude drugs out of the bacterial cell irrespective of structural divergence, often resulting in multiple antibiotic resistances ([57], [46]). As these systems play crucial role in mediating MDR, inhibiting efflux-mediated antibiotic resistance by nanoparticles thus forms an attractive approach [198].

Past decade has seen substantial progress in the field of nanotechnology and application of nanoparticle. Nanoparticles have been used in several medicinal applications including antimicrobial therapy, drug delivery systems and for treatment of various diseases [205]. Amongst all these nanoparticles, silver nanoparticles (AgNPs) have gathered much attention and became widely accepted because of its medicinal implications [206]. Besides their well-known antibacterial properties, AgNPs had low toxicity and greater wound healing properties [207]. We had previously reported AgNPs to be less cytotoxic towards mammalian cells even at higher concentrations [204]). Biocompatibility is extremely important feature for nanoparticles to be used as therapeutic agents. For this, we had performed hemolysis assay that described AgNPs to be non-hemolytic.

There exist very few reports on effect of AgNPs on bacterial efflux systems. Kovács and groups [208] reported AgNPs to inhibit activity of p-glycoproteins efflux pump (belonging to ABC transporter family) in multidrug resistant cancer cells, and thereby enhancing efficacy of chemotherapy. Recently study by Christena and groups [209] has established the efflux inhibitory properties of copper nanoparticles (CuNPs) in tackling multidrug resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolates. However, effect of AgNPs on expression of bacterial multidrug resistant

efflux pumps is not elucidated. With this background, we checked the effect of AgNPs and Ag-AuNPs on AcrAB-TolC efflux pump protein expression. Our results indicated the AgNPs affected the expression of AcrB protein to a greater extent when compared to AcrA and TolC. In presence of AgNPs, though TolC and AcrA protein expression remains unaffected, the absence of a functional monomeric AcrB renders the bacterial cells impaired for efflux activity.

This is indicative of the application of AgNPs as efflux inhibitor, since AcrB protein is responsible for substrate recognition and binding of diverse structures [57]. Previous studies have shown that expression of AcrB transporter is affected by cellular metabolites [194] and hence forms an ideal target for efflux and virulence inhibitor design [210]. Also, AcrB is a proton motive force (PMF) dependent RND transporter. Interestingly, Dibrov [211] in a study on mechanism of action of metal nanoparticles observed that metal nanoparticles such as AgNPs, dissipate PMF by interfering with bacterial respiration. Hence, energy required for this tripartite efflux system may be hindered by the addition of AgNPs.

Although change in efflux activity in *Enterobacter* spp. by AgNPs have been reported in context of EmmDR efflux pump (belonging to Multiple antibiotic and toxic extrusion-MATE superfamily) (212), but they haven't looked into expression changes. To the best of our knowledge, this is the first report on effect of silver nanoparticles on AcrAB-TolC efflux protein expression. This study indicates potential efflux inhibitor property of the AgNPs. Along with their anti-biofilm activity as reported earlier [204], the nanoparticles used in this study have strong potential to be used as effective and alternate therapeutic candidates to combat multidrug resistant gram-negative Enterobacterial pathogens.

Section 4.3.3: Whole genome sequencing analysis of MDR *Enterobacter cloacae* DL4.3 (environmental) and EspIMS6 (clinical) isolates

Background of the study

This section of chapter dealt with the next-generation sequencing of the genome of two *Enterobacter cloacae* isolates; environmental multidrug resistant DL4.3 and clinical extensive drug resistant EspIMS6, to know the similarities and differences between both isolates in their genetic makeup that is reflected in their phenotype. This is a prerequisite for understanding the molecular basis of antibiotic resistance, the repertoire of efflux genes harbored by the organisms, the efflux pump regulatory proteins encoded by the organisms, and different virulence factors, particularly in the context of the source of isolation.

4.3.3.1: Next generation sequencing analysis

A total of 822,417,719 and 859,537,668 bases were obtained in the form of 2,444,256 and 2,549,556 reads, with average read lengths of 336 and 337 bp for EspIMS6 and DL4.3, respectively. The reads were assembled using the SPAdes algorithm version 3.1.0 into 203 and 145 contigs, with average sizes of 148,694 and 122,602 bases for EspIMS6 and DL4.3, respectively. The genomes were uploaded to the Rapid Annotations using Subsystems Technology (RAST) server that was used to annotate the genomes, which were 5,296,869 and 4,820,048 bp in size, with 54.7% and 54.9% G+C content for EspIMS6 and DL4.3, respectively. EspIMS6 and DL4.3 were found to have 5,013 and 4,545 protein-coding genes, of which 4,006 and 3,731 were assigned functions, respectively. Further, 102 and 104 genes were found to code for RNA in

EspIMS6 and DL4.3, respectively. Additionally, the sequences have been submitted to the Prokaryotic Genome Annotation Pipeline (PGAP).

4.3.3.2: Subsystems and categories distribution (RAST analysis)

Rapid Annotations using Subsystems Technology (RAST) server was utilized to annotate the bacterial draft genome sequences into different categories, sub-categories and sub-systems depending on their functional role. Few of the genes were also categorized as no-subcategory reflecting not-assigned function.

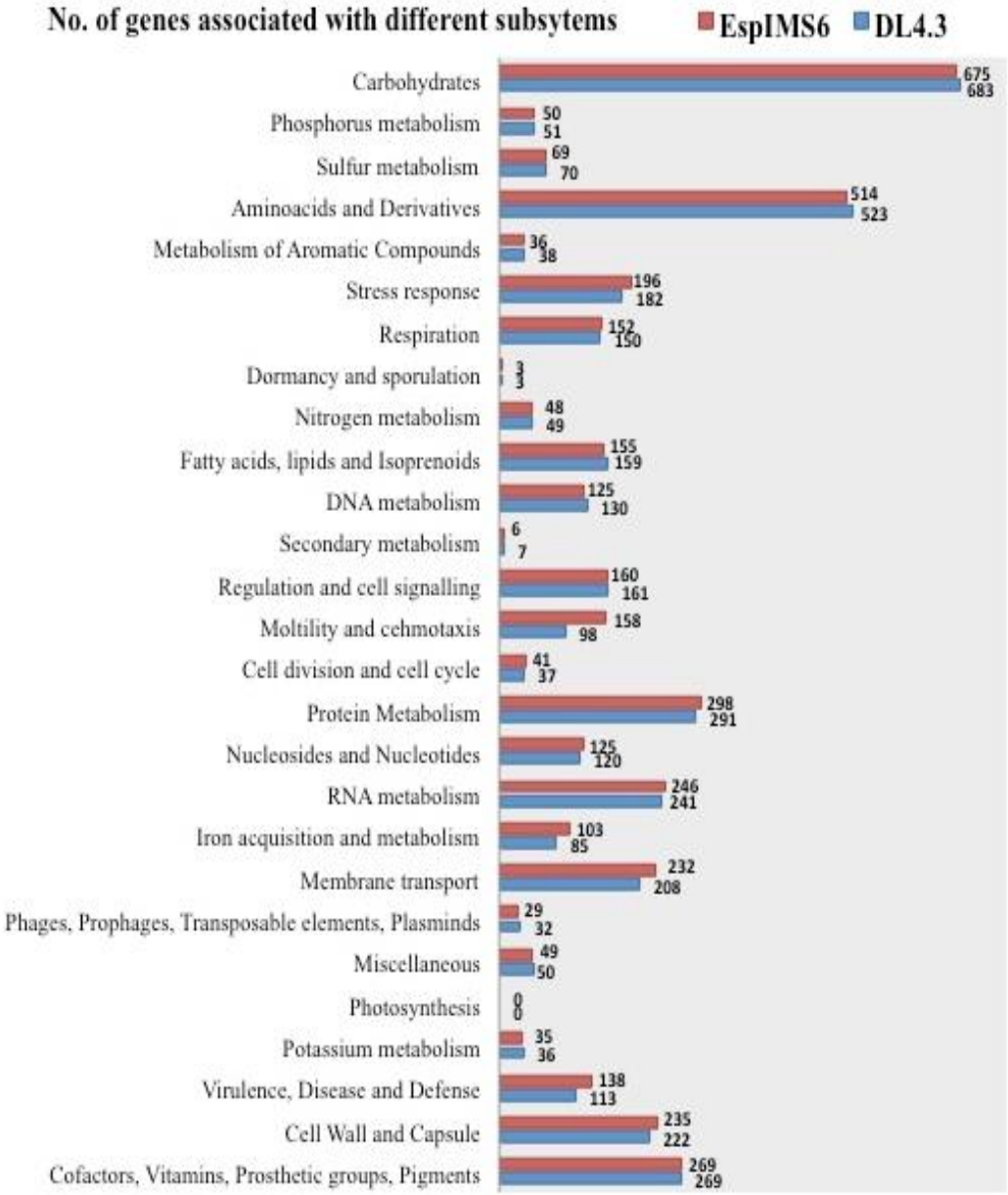


Figure no. 4.3.3.1: Comparative analysis of number of categories and subsystems

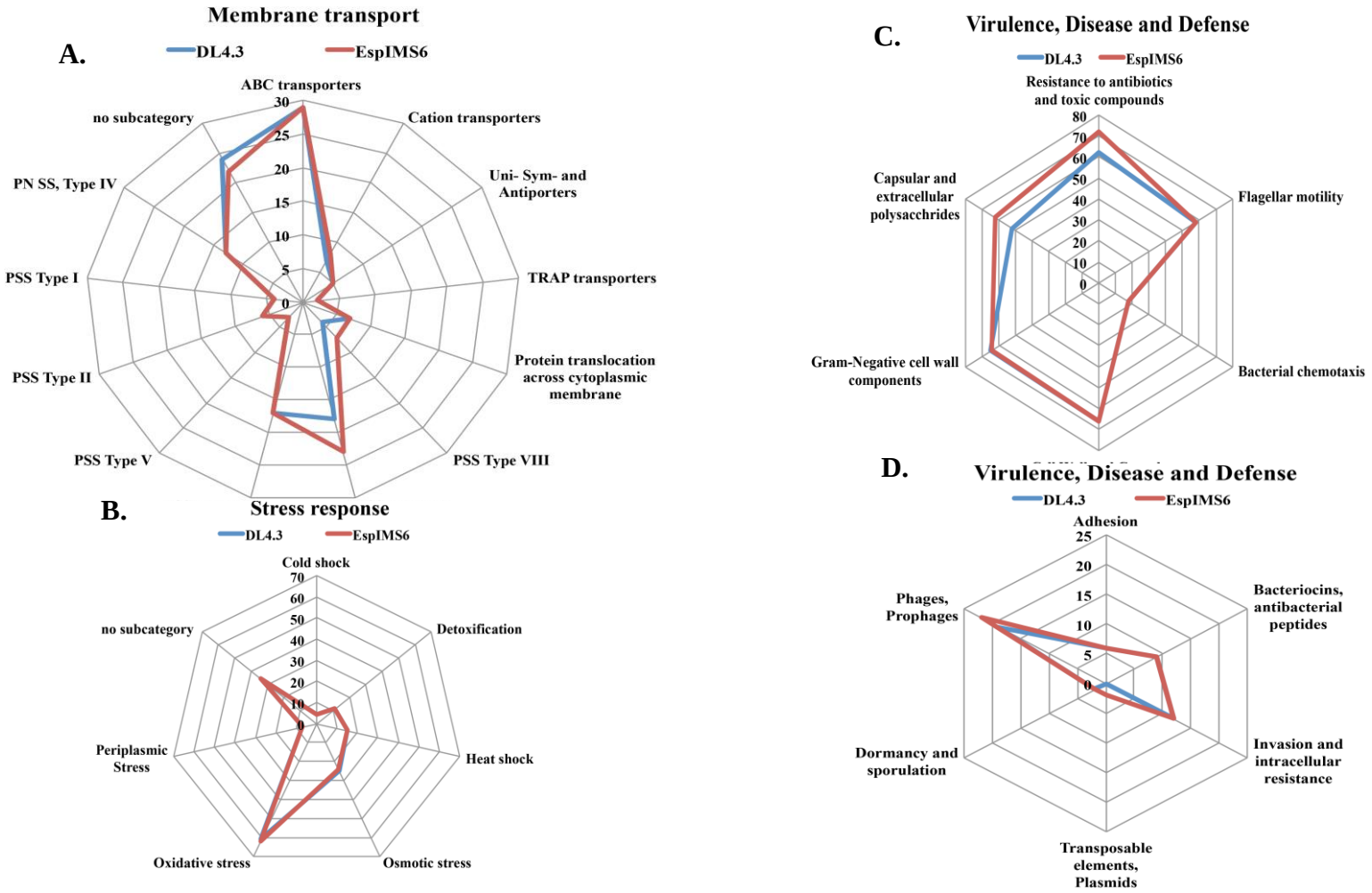


Figure no. 4.3.3.2: Spider-plots describing individual categories and subsystems: Panels represents different subsystems (A) Membrane-transport, (B) Stress response, (C) and (D) Virulence, Disease and Defense.

As evident from the comparative analysis of genes assigned under different categories (figure no. 4.3.3.1) in both *E.cloacae* isolates, larger amount of the genes were associated with, in order of carbohydrates> aminoacids and derivatives> protein metabolism> cofactors, vitamins, prosthetic groups, pigments> RNA metabolism> cell wall and capsule> membrane transport. We had also segregated individual category and its sub-systems involved to get a blueprint of the similarities and dissimilarities amongst the isolates as presented in figure no. 4.3.3.2.

As observed from figure no. 4.3.3.2A, that represented the genes involved in membrane transport machinery, both the isolates harbored ABC transporters, cation transporters along with protein secretion systems. However, protein secretion system type VII and type VIII were more prevalent in clinical isolate EspIMS6. Both the organisms were similar in terms of their stress response ability, including oxidative stress osmotic stress, heat shock, cold shock etc. (figure no. 4.3.3.2B). Moreover, in case of virulence, defense and disease (figure no. 4.3.3.2C, 2D), both DL4.3 and EspIMS6 contained flagellar motility, chemotaxis, capsules, antibacterial peptides, invasion and intracellular resistance. But clinical isolate EspIMS6 outnumbered DL4.3 (figure no. 4.3.3.2C), in terms of capsular and extracellular polysaccharides, resistance to antibiotics and toxic compounds and phages and prophages. Interestingly, EspIMS6 contained transposable elements and plasmids, which DL4.3 was devoid of (figure no. 4.3.3.2D).

The subsystem dealing with resistance to antibiotics and toxic compounds indicated presence of large number of efflux pumps involved in the two isolates. As observed form figure no. 4.3.3.3, both the isolates harbored mdtABCD multidrug resistance cluster, 12 multidrug resistance efflux pumps and more prominently AcrAB-TolC type

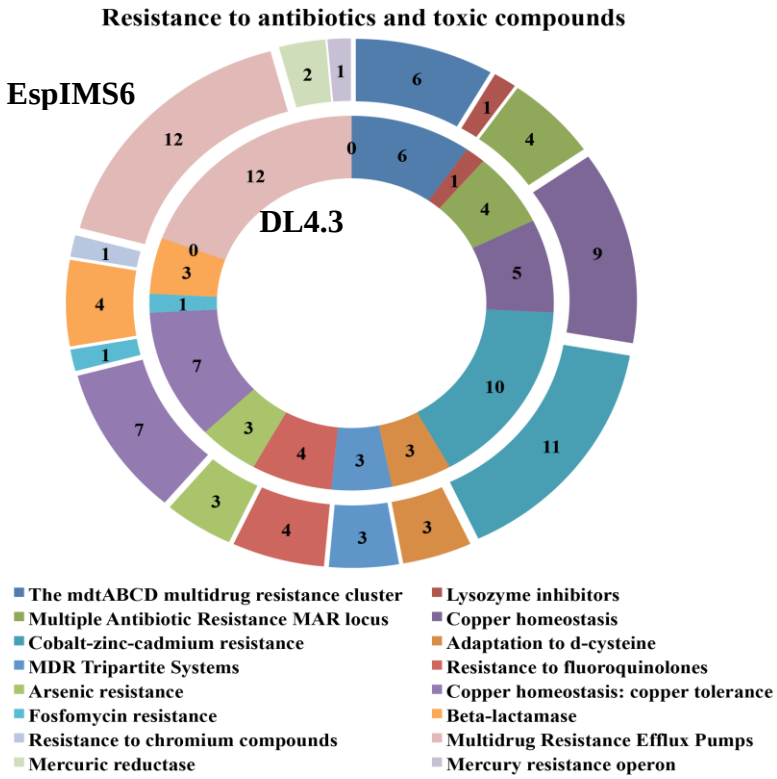


Figure no. 4.3.3.3: Comparison of efflux pumps involved in resistance to antibiotics, heavy metals and toxic compounds

MDR tripartite RND efflux system, along with multiple antibiotic resistance (MAR) locus. Both the organisms possessed beta-lactamase, resistance towards fluoroquinolones, fosfomycin, heavy metals such as copper, cobalt-zinc-cadmium. Interestingly, unlike DL4.3; the clinical isolate EspIMS6 exhibited resistance to mercury, chromium and copper to a greater extent.

4.3.3.3: Determination of resistance genes, virulence genes in *E. cloacae* isolate

To further investigate the presence of different antibiotic resistance determinants and acquired virulence genes, we had analyzed our draft genomic sequences in an internet-based platform (Center for genomic epidemiology http://www.genomicepidemiology.org). This enabled us to gather all the informations related to phenotyping and genotyping.

Table no. 4.3.3.1: Presence of different chromosomally encoded resistance determinants in *E. cloacae* isolates

	Resistance factors/genes	
Antibiotics class	**DL4.3**	**EspIMS6**
Aminoglycoside	No	***aac(6')Ib-cr*** (Fluoroquinolone and aminoglycoside resistance) ***aph(3')-Ia*** (Aminoglycoside resistance)
Beta-Lactam	***blaCMH-3*** (Beta-lactam resistance)	***blaDHA-1*** (Beta- lactam resistance AmpC- type) ***blaCMH-3, blaNDM-1, blaOXA-1*** (Beta-lactam resistance)
Colistin	No	No
Fluoroquinolone	No	***aac(6')Ib-cr*** (Fluoroquinolone and aminoglycoside resistance) ***QnrB4*** (Quinolone resistance)
Fosfomycin	***fosA*** (Fosfomycin resistance)	***fosA*** (Fosfomycin resistance)
Fusidic Acid	No	No
Glycopeptide	No	No
Macrolide, Lincosamide and Streptogramin B	No	***mph(A)*** (Macrolide resistance)
Nitroimidazole	No	No
Oxazolidinone	No	No
Phenicol	No	***catB3*** (Phenicol resistance)
Rifampicin	No	***ARR-3*** (Rifampicin resistance)
Sulphonamide	No	***sul1*** (Sulphonamide resistance)
Tetracycline	No	No
Trimethoprim	No	***dfrA1*** (Trimethoprim resistance)

However, multi-locus sequence typing (MLST) analysis at the Center for Genomic Epidemiology, suggested both to be *E.cloacae* isolates; where environmental DL4.3 was of Unknown sequence type (*dnaA*, *fusA*, *gyrB*, *leuS*, *pyrG*, *rplB*, and *rpoB*; 1-1-61- 214-1-22-1) and clinical EspIMS6 was of ST-456 (*dnaA*, *fusA*, *gyrB*, *leuS*, *pyrG*, *rplB*, and *rpoB*; 149-44-61- 180-152-1-1).

Antimicrobial resistance genes were predicted using ResFinder from the Center for Genomic Epidemiology, and only two antimicrobial resistance genes, *blaCMH-3* (responsible for resistance to ß-lactams) and *fosA* (conferring fosfomycin resistance) were identified in DL4.3. In clinical isolate EspIMS6, multiple genes were reported encoding resistance to β-lactams, cephalosporins, fluoroquinolones, aminoglycoside, groups of antibiotics (table no. 4.3.3.1). Moreover, presence of genes conferring resistance towards fosfomycin (*fosA*), Macrolide (*mph(A)),* Phenicol *(catB3),* Rifampicin (*ARR-3),* Sulphonamide (*sul1)* Trimethoprim *(dfrA1)* were also revealed.

Discussion:

The availability of genome sequences would enable us to undertake genomic comparisons of *Enterobacter cloacae* isolates from environmental and clinical sources. Previously, we studied the association between multiple-antibiotic resistance and virulence in environmental gram-negative bacterial isolates, including that belonging to *Enterobacter* species [212]. The draft genome sequences obtained from DL4.3 and EspIMS6 validated our earlier observations, as EspIMS6 harbored multiple antibiotic resistance determinants that were reflected in their XDR phenotype. Also EspIMS6 contained integrons and type VII and type VIII protein secretion systems, making the clinical strain more robust virulent pathogen. This kind of sequence analysis will enable us to understand the internal genetic background that is reflected in their phenotype.

CHAPTER 5

Summary & Conclusions

The last chapter of this thesis summarizes the salient findings from the present study with concluding remarks and future prospects.

Section 4.1: Occurrence and diversity of efflux pumps in MDR clinical and environmental Enterobacter isolates

- *Enterobacter cloacae* and *Enterobacter aerogenes* are ubiquitously distributed in the environment and clinical settings.
- Non-clinical *Enterobacter* isolates were multidrug resistant whereas few clinical *Enterobacter* isolates exhibited pandrug resistance.
- Efflux pumps presumably played a role in mediating MDR phenotypes.
- AcrAB-TolC efflux genes were wide spread in clinical and non-clinical *Enterobacter* isolates followed by MATE efflux pump genes. SMR and MFS family of efflux genes were present only in clinical isolates.

Section 4.2: Comparative study on association of outer membrane proteins in multidrug resistant environmental and clinical Enterobacter isolates

- Compared to environmental isolates greater number of clinical isolates possessed multiple OMPs i.e *OmpA, OmpX, LamB, OmpF*.
- Greater number of β-lactam and cephalosporin resistant isolates possessed *OmpA* and *OmpX*–presumably indicating their roles in the phenotype.
- Correlation was observed between presence of all the four OMPs and invasiveness of the isolate(s). Results suggested role of *OmpF* and *LamB* in bacterial adhesion and invasion *in vitro*.

Section 4.3: Effect of physico-chemical environment on expression of AcrAB-TolC multidrug efflux pump in Enterobacter isolates.

- Distinct differences in constitutive expression of AcrAB-TolC were observed between clinical and environmental *Enterobacter* isolates, with the former being relatively higher. Cephalosporin drugs up regulated AcrAB-TolC expression.
- Comparatively, expression of TolC was higher at acidic pH than at alkaline pH. Expression of TolC at alkaline pH showed association with survival of isolates at alkaline pH.
- The absence of a functional monomeric AcrB efflux transporter upon treatment to silver nanoparticles indicated the potential efflux inhibitory properties of AgNPs.
- Whole genome sequencing analysis helped us to explain better pathogenic adaptability of clinical isolate EspIMS6 over environmental isolate DL4.3.

Put together, all these key points have been highlighted as a model below in figure no. 5.1, to present the concluding remarks obtained from this study.

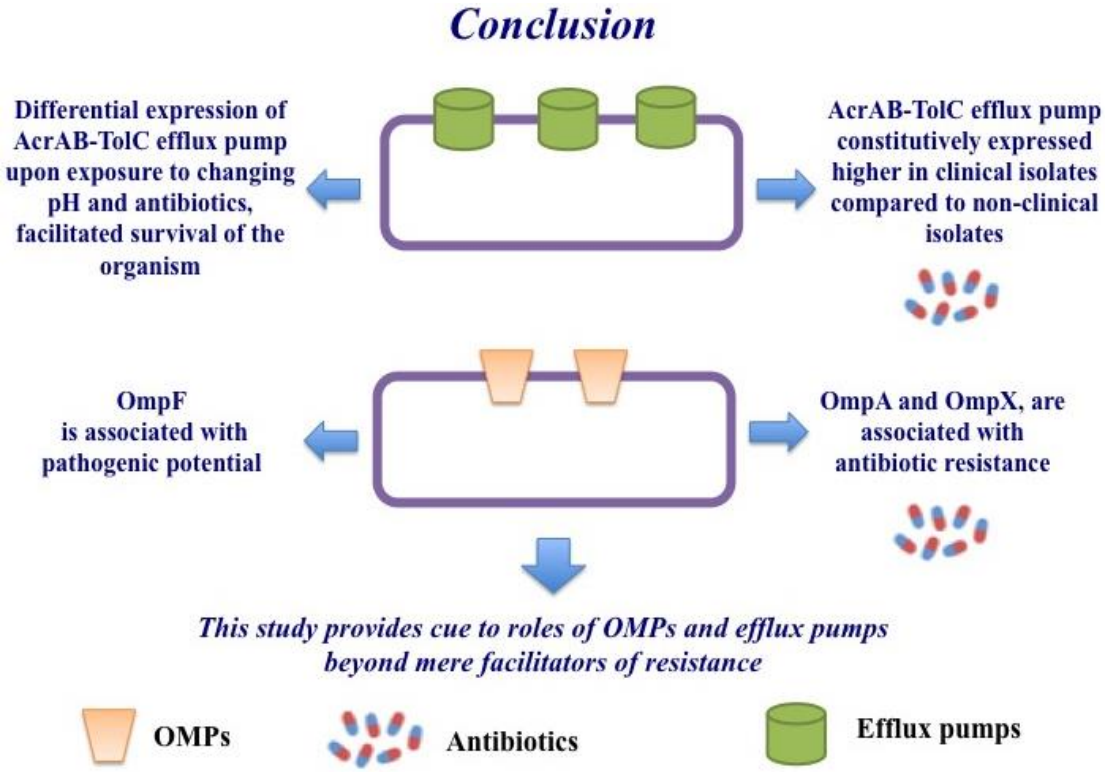


Figure no. 5.1: Graphical model summarizing the findings of the study

Significance of the thesis:

Earlier report from our lab explored the association between multiple antibiotic resistance (MAR) index and virulence index in environmental populations, to determine what percent of environmental antibiotic-resistant bacteria (eARB) could pose threat as potential pathogen. [213].

Detailed investigations done in the present work on environmental *Enterobacter* isolates further strengthens our earlier observation that eARB isolates may not pose high risk threat to human health owing to their limited pathogenic potential. However, clinical *Enterobacter* isolates, given an opportunity, may transform themselves into pathogens with antibiotic resistant bacteria (pARB). In addition, significant differences observed at the genomic level in two *Enterobacter* isolates of environmental and clinical origin further hints and necessitates exploring their evolutionary linkages.

Future directions:

The data presented in this pilot-scale study compared and contrasted the resistance phenotypes exhibited by MDR efflux pumps between environmental and clinical *Enterobacter* isolates. Although the physiological roles of AcrAB-TolC efflux pumps and OMPs, besides antibiotic resistance was significantly elucidated here in multiple *Enterobacter* isolates; but this observation needs to be scientifically validated in wild type overexpression/deletion mutants. It would be interesting to explore the observed association between antibiotic resistance determinants and virulence, in other closed species of *Enterobacter* and other opportunistic ESKAPE pathogens of clinical significance, which may reveal many unexplored areas in bacterial pathogenesis.

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Appendix

A1. Cloning and expression of AcrAB-TolC

To validate the previous expression studies, we proceeded to clone the AcrAB-TolC genes into expression vector pet24b via the following methodology.

A.1.1. Reagents required and buffer preparation

I. Inoue Buffer preparation

Inoue buffer was prepared fresh prior to use and filtered through 0.22µ membrane filter. Buffer was stored at 4ºC as mentioned in table below table no. A1.

Table no. A1. **Inuoe buffer composition**

Composition	For 100 ml
$MnCl_2.4H_2O$	1.08 gms
$CaCl_2.2H_2O$	0.22 gms
Kcl	1.865 gms
PIPES (0.5M, pH 6.7)	2 ml
MiliQ water	Up to 100 ml

II. PIPES (piperazine-N,N'-bis (2-ethanesulfonic acid) 0.5M

15.1 grams of PIPES was dissolved in 80 ml of distilled water. The pH was adjusted to 6.7 with 5M KOH, and volume was made up to 100 ml.

III. Crack lysis buffer (50mM NaOH, 0.5% SDS and 5mM EDTA)

Composition	Amount (for 10 ml)
SDS (20%)	0.25 ml
EDTA (0.5M), pH 8.0	0.1 ml
NaOH (1 M)	0.5 ml

Volume was made up to 10 ml with miliQ water.

A.1.2. Bacterial strains & plasmid vector storage and maintenance

DH5α *E. coli* strain (for competent cell preparation) was received as a kind gift from Dr. T. K. Chowdhury's lab, NISER. C43-DE3 (Δ*AcrAB-tolC*) strain was received as a kind gift from Dr. Du D., Oxford University, UK. These strains were revived freshly on sterile LBA agar plates and cryopreserved at -80ºC in 30% glycerol. pET-24b expression vector used in this study is a transcription vector designed for expressing genes from bacterial cells under the control of the T7/Lac promoter, with a His-tag at the end of the translated product, and contains Kan^R (Kanamycin resistance) antibiotic selection marker.

A.1.3. DH5α competent cell preparation (By Inoue method)

DH5α *E.coli* strain was used as the host, and competent cells were prepared following protocol mentioned by Inoue method (Sambrook et al 2014). A single colony of DH5α from LBA plate was inoculated to 5ml of LB broth and incubated at 37ºC for 6-8 hours at 220rpm. The culture was then transferred to 250ml LB broth and incubated at 18ºC, 150-180 rpm, till O.D. at 600nm reached 0.55. Thereafter the culture was transferred to ice for 10minutes. Cells were harvested by centrifugation at 2500g for 10 minutes at 4ºC. Cells were resuspended gently in 80ml of ice- cold Inoue buffer. Cells were again harvested by centrifugation at 2500g for 10 minutes at 4ºC. The supernatant was removed and cells were resuspended gently with 20ml of ice-cold Inoue buffer. 1.5ml of DMSO was added and mixed by swirling the tube and incubated on ice for 10minutes. 100µl of aliquots were made from the bacterial suspension to chilled 1.5 ml microcentrifuge tubes and snap frozen in liquid nitrogen and stored at -80ºC. After preparation of competent cells, their transformation efficiency was checked.

A.1.4. PCR amplification of AcrAB-TolC, PCR product purification and quantification

The primers were used to amplify the AcrA (1104 bases), AcrB (2708 bases) and TolC (1254 bases) genes along with NdeI and XhoI restriction sites in forward and reverse primers respectively. Total 30 µl of PCR reaction mixture contained 6µl of (5X) go taq flexi buffer (Promega, USA), 2 µl of (2.5mM/dNTP) dNTP mix (Promega, USA), 2 µl of 25mM MgCl2, 1.5 µl each of (10µMolar) forward and (10µMolar) reverse primer, 2µl of template genomic DNA and 0.2µl (500 U) Taq DNA polymerase (NEB, USA) in a thermal cycler (Eppendorf, Germany). The PCR products were run in a 1% agarose gel electrophoresis, images were acquired using Geldoc imaging system (Biorad, USA).

Table no.A2. Oligonucleotides used for cloning of *AcrAB-TolC* genes

Primer name	Sequence (5'-3')	Length
AcrA_NdeI_FP	GCGCATATGAACAAAAACAGAGGGTTAACG	30
AcrA_XhoI_RP	GCGCTCGAGAGACTTGGTTTGTTCTGACTG	31
AcrB_NdeI_FP	GCGCATATGCCTAATTTCTTTATCGATCGC	30
AcrB_XhoI_RP	GCGCTCGAGGTGAGGTTCTACCGAATG	27
TolC_NdeI_FP	GCGCATATGGGAAAGATGATGCCTTACTGG	27
TolC_XhoI_RP	GCGCTCGAGTGGCTGGATCTCCACG	25

PCR product purification using QIAquick Gel Extraction Kit (QIAGEN, Germany)

PCR amplified products from agarose gel were purified using QIAquick Gel Extraction Kit (QIAGEN, Germany) according to manufacturer's instructions. Briefly, gel slices were put in pre-weighed eppendorf tubes. 3 volumes of buffer QG was added to 1 volume of gel weight and incubated at 50ºC with intermittent vortexing until the

gel slices completely dissolved. One gel volume of isopropanol was added to dissolved PCR products and mixed well and was transferred to the QIAquick columns. QIAquick columns were centrifuged for 1 min and flow-through was discarded, 0.5 ml of buffer QG was added and centrifuged at 13,000 rpm for 1 min. The QIAquick column were then washed with 0.75 ml of Buffer PE; discarded the flow-through and centrifuged again for 1 min at 13,000 rpm. Finally, DNA was eluted with 25-30 µl of milli-Q water by incubating for 1 min at RT and centrifuging the column for 1 min. The eluted DNA was quantified by nanodrop and quality was determined by agarose gel electrophoresis as mentioned earlier.

A.1.5. Plasmid DNA extraction using Mini and Midi Kit

Plasmid DNA extraction using QIAGEN Spin Miniprep Kit (Qiagen,USA)

The vector pET-24b and/or pET-21a was isolated by using QIAGEN spin Miniprep kit (Qiagen, USA) following manufacturer's protocol. Briefly, 3 ml of bacterial culture was grown overnight in LB broth with 50 µg/ml kanamycin. Culture was centrifuged for 5 mins at 13,000 rpm to pellet down cells. 250 µl of buffer-P1 (containing RNaseA) was added to the pellet and was resuspended with proper mixing. 300µl of Lysis buffer P2 was added followed by 350 µl of buffer N3 was added and mixed by slowly inverting the tube 4-5 times to precipitate proteins and leaving plasmid DNA in supernatant due to its small size. The white precipitate formed was pelleted by centrifugation at 13000 rpm for 10 minutes. The supernatant was carefully transferred to a spin column and centrifuged for 1 minute at 13000 rpm. The column was then washed with 0.75 ml of PE buffer, by centrifuging at 13000 rpm for 1 minute. To remove the residual PE buffer, the column was again centrifuged. The column was transferred to a sterile 1.5ml microcentrifuge tube and elution buffer (30-50 µl) was

added to the center of the column and incubated at RT for 2-3 minutes. Finally the tube was centrifuged at 13000 rpm for 1minute to elute the plasmid DNA from the column. Quantification of the plasmid was done by a Nanodrop 2000C system (Thermo, USA) and quality was determined by running the product on a 0.8% agarose gel.

Plasmid DNA extraction using QIAGEN plasmid midi Kit (Qiagen,USA)

For large-scale extraction of plasmid DNA, Qiagen plasmid midi kit was utilized following manufacturer's instructions. A single colony from freshly streaked plate was inoculated in 5 ml of LB broth containing appropriate selective antibiotic. It was incubated for 6-8 hours at 37ºC at 220 rpm. This starter culture was diluted 1:500 into 25 ml of selective LB medium and grown for 12-16 hours. Bacterial cells were harvested by centrifugation at 6000g for 15 mins at 4ºC. Bacterial pellet was resuspended in 4 ml of buffer P1 followed by 4 ml of buffer P2. It was mixed thoroughly and incubated at RT for 5 mins. To this, 4 ml of chilled buffer P3 was added, mixed thoroughly and incubated on ice for 15 mins. Sample was then centrifuged at 20,000g for 30 mins at 4ºC to remove the supernatant containing plasmid DNA. The supernatant was recentrifuged at 20,000g for 15 mins at 4ºC. Qiagen-tip 100 column was equilibrated with 4 ml of buffer QBT, to which the supernatant was allowed to enter the resin by gravity flow. Qiagen-tip was then washed twice with 10 ml of buffer QC, and finally DNA was eluted with 2-5 ml of buffer QF. DNA was precipitated by adding 3.5 ml of isopropanol and centrifugation at 15,000g for 30 mins at 4ºC. DNA pellet was washed with 2 ml of 70% ethanol and centrifuged at 15,000g for 10 mins. DNA pellet was then air-dried for 5-10 mins and redissolved in suitable amount of TE buffer for further use.

A.1.6. Restriction digestion and ligation set up

Gel eluted PCR amplified products and plasmids were double digested with NdeI and XhoI restriction enzymes by incubating at 37ºC for 3 hours. This digestion reaction contained 1- 2µg of DNA, 5µl of 10X NEB cutsmart buffer, restriction enzymes (5-10 Units). The digested products were gel purified using QIAquick gel extraction kit (Qiagen, USA) and quantified. Each ligation reaction mixture (20µl) contained approximately 50ng of vector and 33ng of the insert DNA (in 1:3 ratio), 10µl of 2X quick ligation buffer (NEB, USA), 1µl of quick ligase enzyme and nuclease free water. The ligation reaction mix was incubated at 25ºC for 15 minutes in a water bath.

A.1.7. Transformation into DH5α cells

Stored competent cells from -80ºC was removed and kept on ice to thaw for 15-20 minutes. Three to five µl of ligation mixture was added to the thawed competent cell tubes and mixed by gentle tapping and immediately kept on ice for 15-20 minutes.

Heat shock for 45seconds at 42ºC was given to the transformation tube and immediately transferred to ice for 2 minutes. 300-600 µl of LB broth was added to the transformation tube and incubated at 37ºC, 220 rpm for 1 hour. 200 µl of the mixture was spread plated onto LBA agar plate containing 50 µg/ml kanamycin, and incubated at 37ºC for 12-18 hours.

A.1.8. Screening for positive transformants

Grown colonies on antibiotic-selection plates were screened for presence of insert (*AcrA* and TolC) by multiple methods, firstly by colony PCR and secondly by crack lysis buffer to denature the cells. The lysed products were run in agarose gel to observe a shift in bands indicating insert DNA in comparison to vector control.

After confirmation of positive transformants, colony was re-streaked onto fresh plate and stored. Plasmid DNA was extracted from the positive colony and restriction digestion of the same was done to check the appropriate release of insert from the vector. Then the released insert DNA was gel purified and sequenced for validation

A.1.9. Expression of AcrA and TolC into C43-DE3 knockout strains

The C43-DE3 knockout (Δ*AcrAB-tolC*) strain was prepared as competent cells as mentioned earlier (Sambrook et al. 2014). The AcrA & TolC positive clone's plasmid DNA was then transformed onto these competent cells as mentioned previously. Colonies obtained were screened for positive transformants, plasmid DNA was extracted and digested for release of insert as described previously. The C43-DE3 knockout strain bearing AcrA/TolC only was then cryopreserved at -80ºC. This C43-DE3 expressing AcrA/TolC only was then utilized in cell culture and western blotting experiments to validate the expression pattern of AcrA and TolC observed in wild type isolates.

A.1.11. Cloning of *acrA* and *tolC* efflux genes into pET24b vector

To investigate the role of AcrAB-TolC efflux pump, we proceeded to clone the *acrA* and *tolC* genes into pet24b expression vector containing kanamycin cassette using NdeI and XhoI restriction enzymes. The recombinant products were then transformed into DH5α *E. coli* strain (ultra-competent cells). The transformed colonies were subsequently validated by colony PCR and positive transformants were selected further for plasmid extraction and sequencing of the plasmids to check for presence of insert *acrA* and *tolC (*as represented below in figure no. A1).

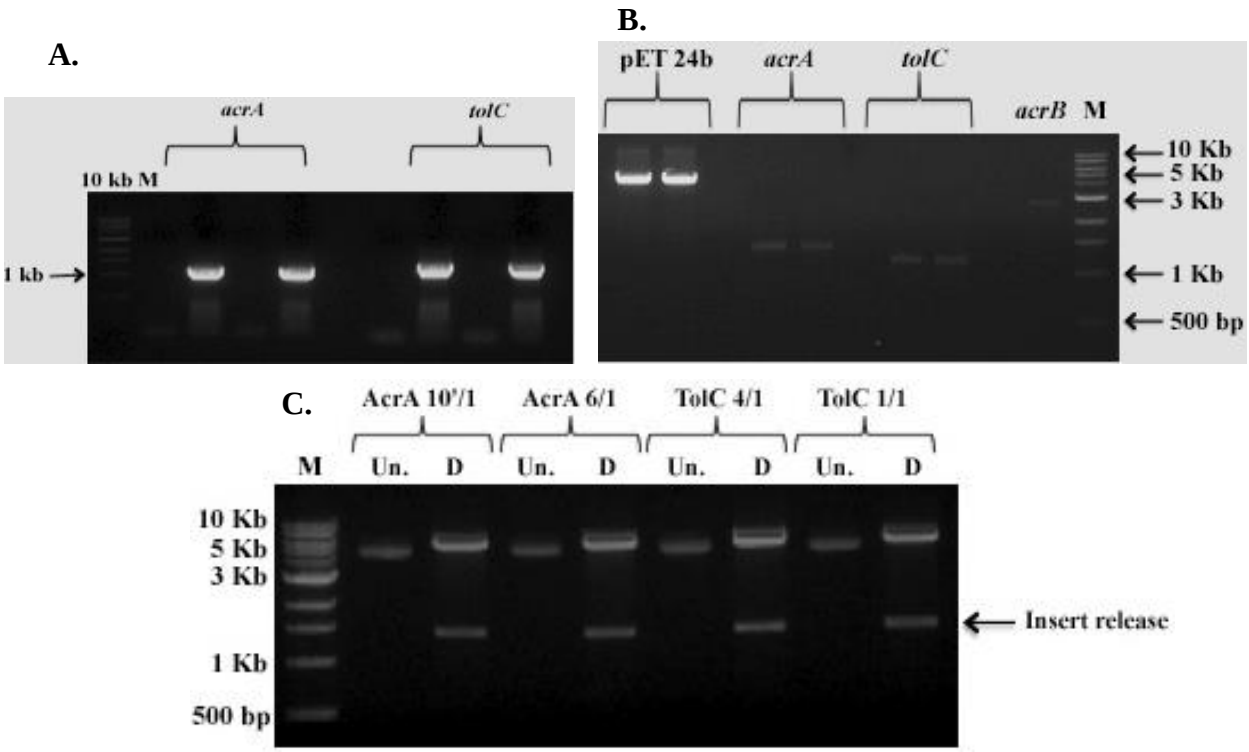

Figure no. A1: Cloning of AcrA and TolC into pET24b vector

A] PCR amplification for *acrA* and *tolC* using cloning primers with restriction enzyme NdeI and XhoI. **B]** Restriction digestion of the PCR amplified *acrA* and *tolC* (insert DNA) and plasmid vector (pET24b), with restriction enzymes NdeI and XhoI. **C]** The plasmids extracted from positive clones were digested and run with the undigested ones to check the release of desired insert DNA. Un. Represents Undigested product where D. represents digested product.

A.1.12. Generation of AcrA and TolC expression constructs

For generating AcrA and TolC protein expression construct, we used C43-DE3 KO (ΔacrAB), which was a generous gift from Dr. Dijun Du (Camridge University, England). Firstly the C43-DE3 KO strain was made competent using CaCl2 method (Sambrook J et al., 2000). The positive AcrA and TolC DH5α clones obtained previously, were then transformed into C43-DE3 KO (ΔacrAB) and positive transformants were validated by restriction digestion and PCR, as mentioned previously. All the positive clones were cryopreserved until further use.

A.1.13. In-vitro cell adhesion and cell invasion of expression constructs

The AcrA and TolC expression constructs generated using C43-DE3 KO was further investigated for their pathogenic potential, to address individual role of AcrA and TolC on in-vitro cell adhesion and cell invasion ability. For this, we performed gentamicin protection assay using appropriate controls and wild type MDR *Enterobacter* isolates.

As observed from figure no.A.3 below, in comparison to the wild type isolates DL4.3 and EspIMS6, C43-DE3 KO (ΔacrAB) could show an optimum level of attachment to RAW264.7 cell line. However, C43-DE3 KO with TolC expression construct showed one fold higher attachment ability to mammalian cells. On the contrary, in cell invasion assay except clinical wild type isolate EspIMS6, none of the C43 constructs containing acrA/tolC, C43 KO, even wild type non-clinical isolate DL4.3 could show invasive properties. This suggested possible role of outer membrane protein TolC in cell adhesion ability in pathogens.

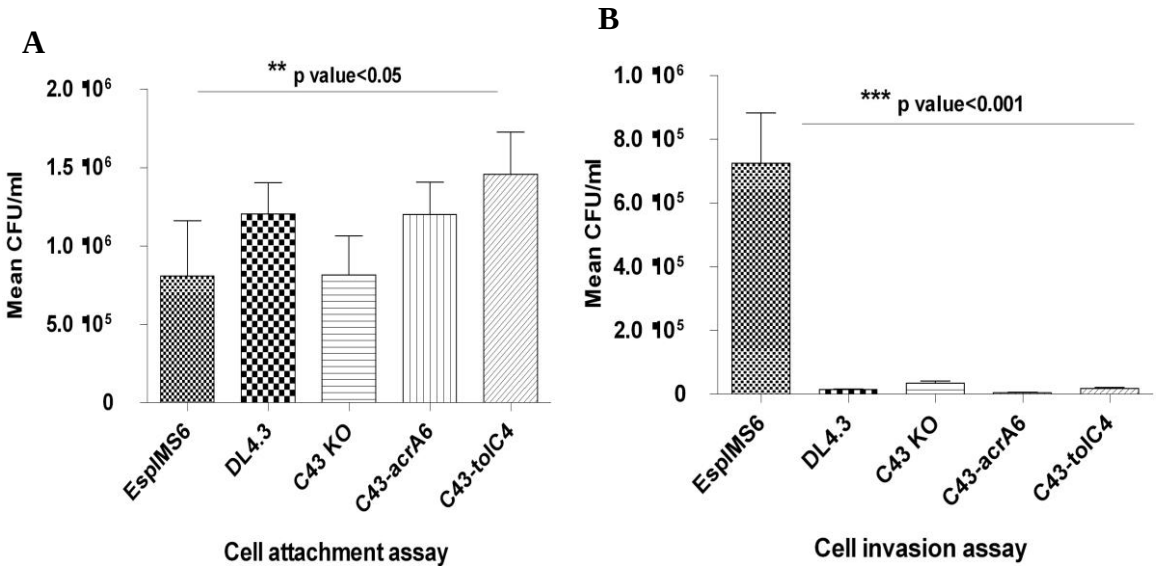

Figure no. A2: Determination of pathogenic potential of expression constructs.

A) In-vitro cell attachment assay and B) In-vitro cell invasion assay for *acrA* and *tolC* expression constructs in RAW 264.7 cell line.

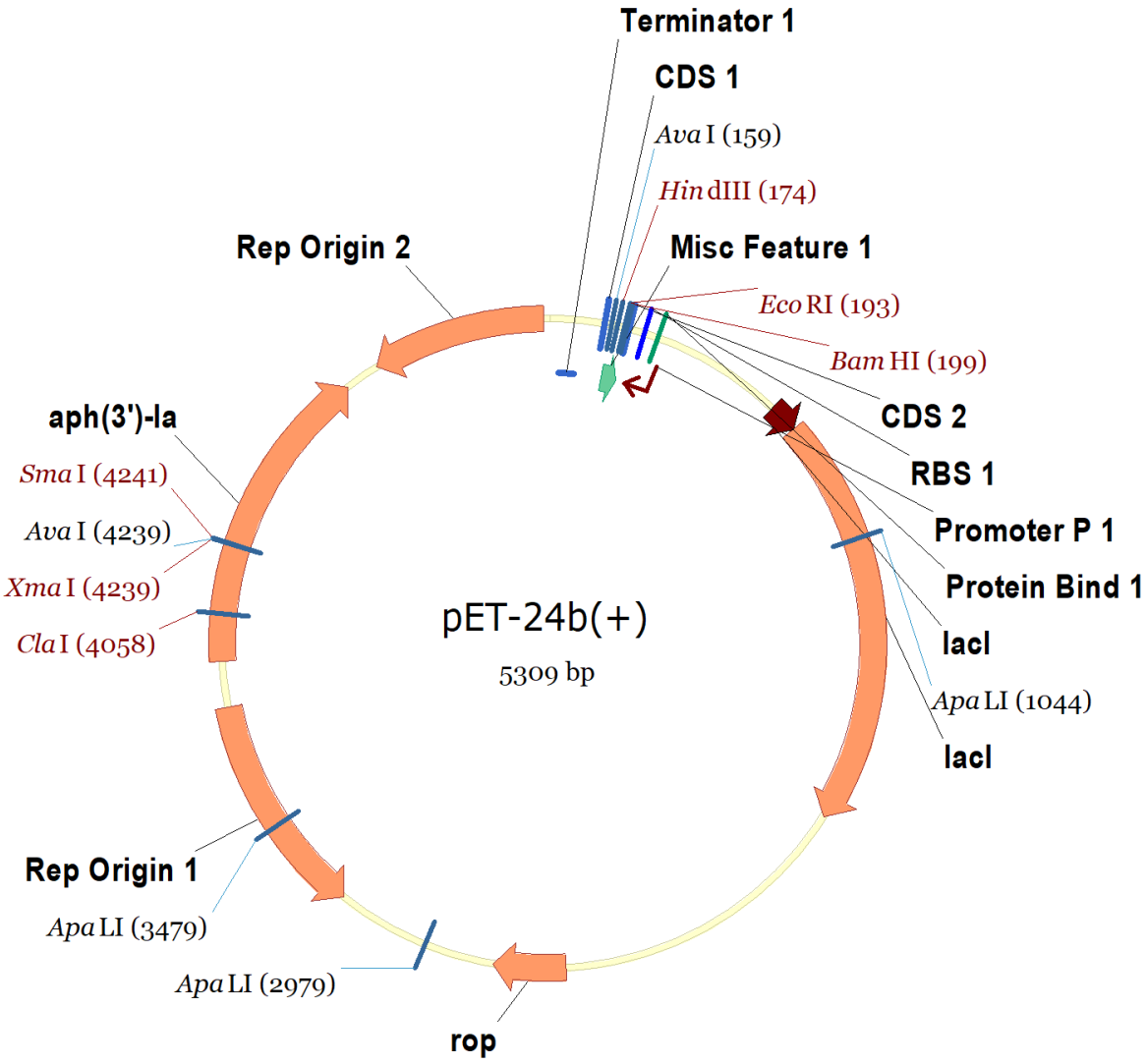

Figure No. A3: Vector map of pet24b (+) showing the size of the vector and position of restriction sites.

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Antibacterial Efficacy of Polysaccharide Capped Silver Nanoparticles Is Not Compromised by AcrAB-TolC Efflux Pump

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Antibacterial therapy is of paramount importance in treatment of several acute and chronic infectious diseases caused by pathogens. Over the years extensive use and misuse of antimicrobial agents has led to emergence of multidrug resistant (MDR) and extensive drug resistant (XDR) pathogens. This drastic escalation in resistant phenotype has limited the efficacy of available therapeutic options. Thus, the need of the hour is to look for alternative therapeutic approaches to mitigate healthcare concerns caused due to MDR bacterial infections. Nanoparticles have gathered much attention as potential candidates for antibacterial therapy. Equipped with advantages of, wide spectrum bactericidal activity at very low dosage, inhibitor of biofilm formation and ease of permeability, nanoparticles have been considered as leading therapeutic candidates to curtail infections resulting from MDR bacteria. However, substrate non-specificity of efflux pumps, particularly those belonging to resistance nodulation division super family, have been reported to reduce efficacy of many potent antibacterial therapeutic drugs. Previously, we had reported antibacterial activity of polysaccharide-capped silver nanoparticles (AgNPs) toward MDR bacteria. We showed that AgNPs inhibits biofilm formation and alters expression of cytoskeletal proteins FtsZ and FtsA, with minimal cytotoxicity toward mammalian cells. In the present study, we report no reduction in antibacterial efficacy of silver nanoparticles in presence of AcrAB-TolC efflux pump proteins. Antibacterial tests were performed according to CLSI macrobroth dilution method, which revealed that both silver nanoparticles exhibited bactericidal activity at very low concentrations. Further, immunoblotting results indicated that both the nanoparticles modulate the transporter AcrB protein expression. However, expression of the membrane fusion protein AcrA did show a significant increase after exposure to AgNPs. Our results indicate that both silver nanoparticles are effective in eliminating MDR *Enterobacter cloacae* isolates and their action was not inhibited by AcrAB-TolC efflux protein expression. As such, the above nanoparticles have strong potential to be used as effective and alternate therapeutic candidates to combat MDR gram-negative Enterobacterial pathogens.

Keywords: nanoparticles, efflux pump, AcrAB-TolC, *Enterobacter cloacae*, antibiotic resistance

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Polysaccharide-capped silver Nanoparticles inhibit biofilm formation and eliminate multi-drug-resistant bacteria by disrupting bacterial cytoskeleton with reduced cytotoxicity towards mammalian cells

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Development of effective anti-microbial therapeutics has been hindered by the emergence of bacterial strains with multi-drug resistance and biofilm formation capabilities. In this article, we report an efficient green synthesis of silver nanoparticle (AgNP) by *in situ* reduction and capping with a semi-synthetic polysaccharide-based biopolymer (carboxymethyl tamarind polysaccharide). The CMT-capped AgNPs were characterized by UV, DLS, FE-SEM, EDX and HR-TEM. These AgNPs have average particle size of ~20–40 nm, and show long time stability, indicated by their unchanged SPR and Zeta-potential values. These AgNPs inhibit growth and biofilm formation of both Gram positive (*B. subtilis*) and Gram negative (*E. coli* and *Salmonella typhimurium*) bacterial strains even at concentrations much lower than the minimum inhibitory concentration (MIC) breakpoints of antibiotics, but show reduced or no cytotoxicity against mammalian cells. These AgNPs alter expression and positioning of bacterial cytoskeletal proteins FtsZ and FtsA. CMT-capped AgNPs can effectively block growth of several clinical isolates and MDR strains representing different genera and resistant towards multiple antibiotics belonging to different classes. We propose that the CMT-capped AgNPs can have potential bio-medical application against multi-drug-resistant microbes with minimal cytotoxicity towards mammalian cells.

Infections caused by pathogenic bacteria have become a serious health and economic problem[1]. There has been constant decrease in effectiveness of antibiotics mainly due to unregulated use of antibiotics, leading to the development of multi-drug-resistant (MDR) bacterial strains[2,3]. Therefore, it has become necessary to search for alternative healthcare approaches to mitigate the problem of bacterial infections and contaminations. Typically, the bacterial infections can be categorised into two types, namely acute infection and chronic infections. The former, however are treated effectively by the development of modern vaccines, antibiotics and infection control measures[4]. However, the other type of infections has accentuated the infection related complications and therefore has poised a major challenge in controlling infection related issues[4]. Moreover, the treatment of acute infections have become difficult because the infection related diseases have been supplemented by chronic infections caused by

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Antibiotic resistance determinants and clonal relationships among multidrug-resistant isolates of *Klebsiella pneumoniae*

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ABSTRACT

In the present study, we performed PCR based screening to determine the presence of antibiotic resistance genes and sequencing to find mutation in QRDR region among fourteen isolates of *K. pneumoniae*. Association analysis was conducted to detect the co-resistance among the isolates. Multi-locus sequence analysis was carried out to determine the clonal relationship among them. All the *K. pneumoniae* isolates showed resistance to multiple antibiotics and exhibited cross-resistance to antibiotics. Although few isolates co-harbored variants of β-lactamase genes, others carried *qnrB* on plasmid and mutations in Quinolone-Resistant Determining Region (QRDR). This study thus indicates that clonally unrelated *K. pneumoniae* isolates exhibited co-resistance, harboured multiple antibiotic resistance genes present on the chromosome, plasmids and/or integron Therefore, the data from this study can provide guidelines for the prudent use of antibiotics to avert the impending danger of losing out on the available antibiotics for therapeutic use.

1. Introduction

Klebsiella pneumoniae has emerged as successful opportunistic nosocomial pathogen. It is an etiological agent of urinary tract infections (UTI), septicemia, respiratory tract infections (RTI), liver abscess, endogenous endophthalmitis and meningitis. Often such infections exhibit high mortality, especially in immunocompromised patients and neonates [1,2]. This bacterium has strong propensity to acquire antibiotic resistance and ability to persist longer in the hospital environment which makes it an extremely successful nosocomial pathogen [3].

Third and fourth generation cephalosporin antibiotics revolutionized treatment of infections caused by *K. pneumoniae*. However, efficacy of these therapeutic drugs was soon diminished by discovery of β-lactamases [4]. Emergence and rapid spread of *K. pneumoniae* isolates producing metallo-beta-lactamases (MBL) and *K. pneumoniae* carbapenemase (KPC) continues to pose serious threat for treatment of healthcare-associated infections [5]. High mortality upto 50% has been reported due to KPC-producing *K. pneumoniae* infections [5].

With occurrence of resistance to β-lactam antibiotics, therapeutic usage resorted to was combination of β-lactam-β-lactamase inhibitor [6]. However, this combinatorial therapy was soon challenged with discovery of enzyme AmpC β-lactamase that is resilient to inhibition by β-lactamase inhibitors [4]. To further complicate the situation, extended-spectrum β-lactamase (ESBL)-producing isolates, exhibited very high resistance to quinolones (up to 56%) that could be attributed to occurrence of amino acid substitution in QRDR region of either *gyr*A/*gyr*B or due to acquisition of plasmid-mediated quinolone resistance genes [7,8]. Such quick and wide spread acquisition of resistance to multiple therapeutic drugs has resulted in *K. pneumoniae* to be included in the list of ESKAPE pathogen by Infectious Diseases Society of America (IDSA) [9]. In developing countries, easy availability of antibiotics coupled with its higher consumption has resulted in disproportionately greater levels of resistance leading to therapeutic failure.

Resistance spectrum of a pathogen varies in different regions and thus knowledge about the local resistance pattern helps to guide appropriate usage of antibiotics. Therefore, the aim of this study was to investigate the molecular basis of resistance and sequence type of multidrug resistant (MDR) *K. pneumoniae* isolates from a tertiary care hospital in eastern India. We believe such

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Short communication

Efflux mediated colistin resistance in diverse clones of *Klebsiella pneumoniae* from aquatic environment

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ABSTRACT

The peptide drug colistin is commonly used to treat carbapenem resistant gram negative bacterial infections. In the present study, we report efflux mediated colistin resistance in multidrug resistant *Klebsiella pneumoniae* isolates belonging to ST200 and ST1296, isolated from a fresh water environment. The isolates exhibited intermediate resistance to human serum, possessed Type 1 fimbriae and harbored bla_{SHV-34} and bla_{TEM-1} genes. Our results highlight the evolving nature of these clones in the country. These observations emphasize the need for judicious usage of antibiotics to prevent the imminent danger of losing out on currently available therapeutic options.

Colistin, a last resort peptide antibiotic has been used as a therapeutic option for the treatment of carbapenem resistant *Klebsiella pneumoniae* [1]. Studies have established emergence of colistin resistant *K. pneumoniae* (Col-RKP) associated with increased clinical usage of the drug [1]. During 2013–2014 in India, reports of colistin resistance in *Klebsiella pneumoniae* isolates increased to 2% as compared to the preceeding year (www.resistancemap.org). Apart from its use in clinics, colistin sulphate is also used as a growth promoter supplement in animal feed in poultries and fish farms [2,3]. Colistin is very stable in water [4]. Microorganisms present in aquatic environment are exposed to residual colistin and such persistent exposure leads to mutations and development of new strains [5]. Molecular basis of colistin resistance has been attributed either to deletions, insertions or amino acid substitutions in *mgrB* gene encoding a regulatory peptide of the PhoP/PhoQ regulon, which in turn regulates PmrAB system, responsible for lipopolysaccharide (LPS) modifications [6–8]. Also, the presence of plasmid encoded *mcr-1* gene – a LPS modifying enzyme, has been identified to confer transferable resistance towards colistin [9–11]. However, there exist very few reports on efflux mediated colistin resistance [12]. In the present study, we report occurrence of colistin resistance in four isolates identified as *Klebsiella pneumoniae*, namely HD4.5, HD4.7, HD3.2 and HD4.1 (Accession no. JQ912549 to JQ912552) obtained from an artificial stagnant fresh water reservoir (Hudco dam) located in the state of Jharkhand, India. All four isolates were ESBL positive as determined by double disc diffusion test and harbored bla_{SHV-34} and bla_{TEM-1}. Furthermore, all four isolates exhibited intermediate resistance to human serum and were positive for Type1 fimbriae. In addition, isolate HD4.1 also exhibited hypermucoviscous phenotype.

Antibiotic susceptibility test by Kirby-Bauer disc diffusion method, showed all the four *Klebsiella pneumoniae* isolates to have a zone of inhibition diameter $\leq$11 mm (50 μg/ml disc potency, Hi-Media, India). Further, all the above mentioned isolates exhibited minimum inhibitory concentration (MIC) >4 mg/L for colistin. Both of these breakpoint values categorized them as colistin resistant according to the EUCAST (2015) guidelines [13]. We believe novelty of this study lies in detailing the occurrence of colistin resistant in fresh water environmental isolates of *Klebsiella pneumoniae*.

To determine the mechanism of colistin resistance, PCR amplification and sequencing was carried out using primers targeting *mgrB* gene [8]. Sequencing of the 250bp *mgrB* amplicon revealed presence of two silent mutations and absence of any insertion/deletion in the gene sequence (Fig. 1). Isolates in the present study

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Retrospective study on clonal relationship of multidrug-resistant *Klebsiella* spp. indicates closed circulation and initiation of clonal divergence

Debi Prasad Mohapatra,[1]† Santosh Kumar Singh,[2]† Minu Sahoo,[2]† Shashank Patole,[2] Mitali Mishra,[2] Nagen Kumar Debata[1] and Harapriya Mohapatra[2,*]

Abstract

Purpose. Antibiotic resistance patterns often exhibit geographical variations. Periodic analyses of resistance spectra and phylogenetic trends are important guides for facilitating judicious use of therapeutic interventions. The present study retrospectively analysed the infection trends and resistance patterns for, and clonal relationships between, isolates of *Klebsiella* spp. from a tertiary care hospital.

Methodology. Bacterial isolates were collected from January 2013 to June 2014 and their resistance profiles were identified using an automated bacterial identification system. A phylogenetic tree was constructed using housekeeping genes and molecular evolutionary genetic analysis software. The d_N/d_S ratio was determined by the Synonymous Non-synonymous Analysis Program and polymorphic sites, while the difference per site was calculated using DNA Sequence Polymorphism software. Statistical Package for Social Science software was used to perform all statistical analyses.

Key Findings. The results of this study indicated the prevalence of community-acquired urinary tract and lower respiratory tract infections caused by *Klebsiella* spp. among geriatric patients. The occurrence of new allelic profiles, a low d_N/d_S ratio and the lack of strong evolutionary descent between isolates indicated that mutations play a major role in the evolution of the organism.

Conclusion. The findings of this study highlight the consequences of antimicrobial agents exerting a silent and strong selective force on the evolution of *Klebsiella* spp. The expansion of such analyses is of great importance for addressing rapidly emerging antibiotic-resistant opportunistic pathogens.

INTRODUCTION

Antimicrobial resistance has been building into a crisis over decades and has become a global health threat in the 21st century [1, 2]. Many common and life-threatening infections are becoming difficult or even impossible to treat, resulting in an increased health and economic burden. *Klebsiella* spp. is identified as one of the rapidly emerging pathogens responsible for both hospital- and community-acquired infections [3, 4]. The spectrum of infections caused by*Klebsiella* spp. includes but is not limited to bacteraemia, pneumonia, urinary tract infection (UTI), lower respiratory tract infection (LRTI) and surgical site infections [4]. The frequency and rate of such infections is on the rise, with escalating mortality rates, especially in immunocompromised patients and neonates [5, 6]. Furthermore, the recent emergence of *K. pneumoniae* as an aetiological agent for a distinctive syndrome of community-acquired septicaemia with liver and brain abscesses has been reported [7, 8]. This syndrome is characterized by high mortality (10 to 40 %), and in some cases it has been complicated by meningitis or endophthalmitis, which was very rare in the past [9, 10]. The control and treatment of infections by *K. pneumoniae* has become more difficult because of intrinsic as well as acquired resistance to a variety of antimicrobial agents [11]. β-lactams and quinolones alone or in combination are the current drugs of choice for the treatment of *Klebsiella* infections [11]. However, the occurrence of extended-spectrum beta-lactamase (ESBL)

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Keywords: *Klebsiella*; multi drug resistance; phylogenetic analysis; clonal evolution.

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Three supplementary tables and three supplementary figures are available with the online version of this article.

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Association study of multiple antibiotic resistance and virulence: a strategy to assess the extent of risk posed by bacterial population in aquatic environment

Santosh Kumar Singh · Roseleen Ekka · Mitali Mishra · Harapriya Mohapatra

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Abstract The present study explored the association between multiple antibiotic resistance (MAR) index and virulence index to determine what percent of environmental antibiotic-resistant (eARB) bacteria could pose threat as potential pathogen. *16srRNA*-based sequencing of 113 non-duplicate isolates identified majority of them to be gram negative belonging to *Enterobacter, Pseudomonas*, *Aeromonas*, *Proteus*, *Acinetobacter*, and *Klebsiella*. Statistical comparison of MAR indices of the abovementioned genera indicated differences in the median values among the groups ($p < 0.001$). Pair-wise multiple comparison by Dunn's method indicated significant difference in MAR indices ($p < 0.05$), based on which multiple antibiotic resistance phenotype could be ranked in the order *Pseudomonas* > *Klebsiella* = *Acinetobacter* > *Proteus* > *Aeromonas* > *Enterobacter*. Association between MAR index and virulence index revealed that 25% of isolates in the population under study posed high threat to human/animal or both; out of which 75% isolates belonged to genus *Pseudomonas*. Based on observations of comparative analysis of the six gram-negative genera, it could be concluded that *Pseudomonas* isolates from environment pose significantly high threat as potential pathogens while *Enterobacter* isolates posed no threat.

Keywords *Pseudomonas* · *Klebsiella* · Antibiotic resistance · Virulence · Opportunistic pathogen

Introduction

Emergence and dissemination of drug-resistant bacteria has manifested itself as a public health problem globally (WHO 2014). Investigation of sources and reservoirs of antimicrobial resistance, beyond clinics, has shed new light on the role of environmental bacteria harboring diverse form of resistance mechanisms, even from antibiotic-free environments (Aminov 2009; Finley et al. 2013; Coutinho et al. 2014; Czekalski et al. 2014).

Aquatic environment forms an interface, connecting the terrestrial environment and human population. Rivers and fresh water sources, such as lakes and dams, often form an integral part of community life (Abraham 2011). In addition, such aquatic systems serve as sink receiving waste and bacteria from different sources (Taylor et al. 2011; Rizzo et al. 2013; Zurfluh et al. 2013; Marti et al. 2014, Czekalski et al. 2015). Over the years, increasing dependence on antibiotics for

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PROKARYOTES

Draft Genome Sequences of Nonclinical and Clinical *Enterobacter cloacae* Isolates Exhibiting Multiple Antibiotic Resistance and Virulence Factors

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ABSTRACT *Enterobacter* spp. have been implicated as opportunistic pathogens which over the years have gained resistance toward most of the available therapeutic drugs. We sequenced two multidrug-resistant *Enterobacter cloacae* isolates harboring multiple efflux pump genes. These isolates exhibited strain-specific modulation of efflux pump protein expression.

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Enterobacter spp. are ubiquitously present in the environment and are important nosocomial pathogens primarily affecting immunocompromised patients (1). Their ability to evade the biocidal action of several groups of antibiotics has resulted in their inclusion in the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) group of pathogens (2). *Enterobacter* spp. are implicated as etiological agents in a plethora of infections, such as urinary tract infections, meningitis, septicemia, bacteremia, wound infection, lower gastrointestinal infections, and nosocomial infections (3). Previously, we studied the association between multiple-antibiotic resistance and virulence in environmental Gram-negative bacterial isolates, including those belonging to *Enterobacter* species (4). From the aforementioned study, we selected the environmental *Enterobacter cloacae* isolate DL4.3 (GenBank accession number JQ912514) that showed a multidrug resistance phenotype. *Enterobacter cloacae* EsplMS6 was a urinary tract infection isolate received from a tertiary-care hospital in Bhubaneswar, Odisha, India, that exhibited extreme drug resistance. A decrease in MIC values for antibiotics in the presence of the proton motive gradient disrupter carbonyl cyanide *m*-chlorophenylhydrazone indicated a possible involvement of efflux pumps in mediating resistance in both the isolates. Immunoblotting has shown strain-specific modulation of AcrAB-TolC efflux proteins in response to pH and cephalosporin antibiotics. This study reports the whole-genome sequences of the above-mentioned two isolates, which is a prerequisite for understanding the molecular basis of antibiotic resistance, the repertoire of efflux genes harbored by the organisms, the efflux pump regulatory proteins encoded by the organisms, and the contribution of efflux proteins in physiological functions in the cell, particularly in the context of the source of isolation.

Genomic DNA from both isolates was extracted using the Gentra Puregene Yeast/Bact. kit (Qiagen GmbH), according to the manufacturer's instructions. Whole-genome sequencing was carried out at the laboratory of Thermo Fisher Scientific India, Gurgaon, India. Briefly, libraries for individual genomes were prepared using the workflow prescribed by the Ion Xpress Plus fragment library kit (Thermo Fisher Scientific, USA). Subsequently, the reads were amplified using the Ion OneTouch 2 system (Thermo Fisher Scientific) and sequenced using the Ion S5 system (Thermo Fisher Scientific). A

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PROKARYOTES

Draft Genome Sequences of Clinical and Nonclinical Isolates of *Klebsiella* spp. Exhibiting Nonheritable Tolerance toward Antimicrobial Compounds

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ABSTRACT A clinical isolate and a nonclinical isolate of *Klebsiella pneumoniae* were found to exhibit nonheritable tolerance in response to antimicrobial compounds. The draft genome sequences of both isolates are presented here.

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Klebsiella pneumoniae is found in natural habitats, such as soil and water and on vegetation, and is known to cause a variety of nosocomial infections in immunocompromised individuals, such as wound infections, urinary tract infections, and respiratory tract infections (1, 2). Further, the past decade has seen a drastic rise in community-acquired *Klebsiella pneumoniae* infections (3–6). Previously, we studied the association between multiple-antibiotic resistance and virulence in environmental bacterial isolates, including those belonging to *Klebsiella* (7). We observed the environmental *K. quasipneumoniae* isolate DL5.4 (GenBank accession number JQ912548) to exhibit nonheritable tolerance toward antimicrobial compounds. Simultaneously, we observed a persistence phenomenon in the wound infection isolate KpIMS38, which was obtained from a tertiary-care hospital in Bhubaneswar, India. Further, we noticed that the isolate KpIMS38 harbored a plasmid. This study reports whole-genome sequencing of these two isolates that will enable genomic comparisons specifically with respect to their sources of isolation and persister-forming ability.

Genomic DNA from both the isolates and plasmid DNA from KpIMS38 were extracted using the Gentra Puregene Yeast/Bact. kit and the QIAprep spin miniprep kit (Qiagen GmbH), respectively, according to the manufacturer's instructions. Whole-genome sequencing was carried out at a laboratory of Thermo Fisher Scientific, Gurgaon, India. Briefly, libraries were prepared for individual genomes and the plasmid using the workflow delineated by the Ion Xpress Plus fragment library kit (Thermo Fisher Scientific, USA), amplified using the Ion OneTouch 2 system (Thermo Fisher Scientific), and sequenced using the Ion S5 system (Thermo Fisher Scientific). A total of 597,006,766, 400,449,596, and 18,357,749 bases in the form of 1,700,049, 1,151,033, and 60,120 reads were obtained, with average read lengths of 351, 348, and 305 bp for KpIMS38, DL5.4, and the plasmid, respectively. These were assembled using the SPAdes algorithm version 3.1.0 (8) into 169, 170, and 71 contigs, with average sizes of 72,636, 84,693, and 398 bases for KpIMS38, DL5.4, and the plasmid DNA, respectively.

The Rapid Annotations using Subsystems Technology (RAST) server (9, 10) was used to annotate the genomes of KpIMS38 and DL5.4, which were 5,255,239 (inclusive of the 13,170-bp plasmid) and 5,134,131 bp in size, with G+C contents of 57.2% and 58.2%, respectively. Isolates KpIMS38 and DL5.4 contained 4,320 and 4,295 protein-coding genes with assigned functions, while 1,131 and 910 genes were annotated as coding for hypothetical proteins. Furthermore, 92 and 97 genes were found to code for RNA in KpIMS38 and DL5.4, respectively. The sequences have also been submitted to the