Comparative study of molecular diversity in environmental and clinical isolates of *Klebsiella pneumoniae*: focus on resistance mechanism, virulence factors and clonal diversity

By

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NATIONAL INSTITUTE OF SCIENCE EDUCATION AND RESEARCH
Bhubaneswar

A thesis submitted to the
Board of Studies in Life Sciences
In partial fulfillment of the requirements
For the Degree of

DOCTOR OF PHILOSOPHY
Of
HOMI BHABHA NATIONAL INSTITUTE

May, 2017

Homi Bhabha National Institute

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Santosh Kumar Singh

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

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Publications in Refereed Journal:

a. Published:

1. ***Santosh Kumar Singh**[#], Mitali Mishra[#], MinuSahoo[#], Shashank Patole, Harapriya Mohapatra. Efflux mediated colistin resistance in diverse clones of *Klebsiella pneumoniae* from aquatic environment, Microbial pathogenesis, Volume 102, January 2017:109–112. PMID: 27914962.
2. ***Santosh Kumar Singh**, Roseleen Ekka, Mitali Mishra, Harapriya Mohapatra. 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. Environmental Monitoring and Assessment, 2017: http://dx.doi.org/10.1007/s10661-017-6005-4

b. Communicated:

1. ***Santosh Kumar Singh**, Mitali Mishra, MinuSahoo, Shashank Patole, SuneetaSahu, SudhiRanjan Mishra, Harapriya Mohapatra. Molecular basis of resistance and clonal relationship of multidrug resistant clinical isolates of *Klebsiella pneumoniae*. (**Under review**)
2. Mitali Mishra, Debi Prasad Mohapatra, **Santosh Kumar Singh**, Shashank Patole, Nagendra Debata and Harapriya Mohapatra, Differential expression of AcrAB-TolC in clinical and non-clinical isolates of *Enterobacter cloacae*: Implication in persistence of the opportunistic pathogen.(**Under review**)

c. Manuscript in preparation:

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* - pertaining to this thesis

CONFERENCES

1. Roseleen Ekka, Santosh Kumar Singh, Harapriya Mohapatra. "Prevalence and diversity of antibiotic resistant bacteria isolated from aqueous environment" 1st Global Forum on Bacterial Infections, New Delhi, October 3-5, 2011.
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WORKSHOPS

1. Attended workshop on Applications of Systems and Mathematical Biology in Public Health: An International Workshop held on February 23 & 24, 2015 at NISER, Jatni Campus, Odisha.
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3. Attended Science Communication Workshop organized by The Wellcome Trust/DBT India Alliance held on 11^{th} September 2014 at ILS, Bhubaneswar,

GENBANK SUBMISSION

1. Partial 16S rRNA gene sequences of 113 different bacterial isolated from aquatic environments. NCBI accession numbers: JQ912514 to JQ912626

DEDICATION

I dedicate this thesis to my parents and sisters without whom none of my success would be possible

ACKNOWLEDGEMENTS

I would like to express my special appreciation and thanks to my advisor **Dr. Harapriya Mohapatra**, you have been a tremendous mentor for me. Her broad knowledge in the field and enthusiasm for science has always been prompting me to learn and work in lab. I would like to thank you for encouraging my research and for allowing me to grow as a research scientist. Your advice on both research as well as on my career have been priceless.

I would like to thank my Doctoral Committee chairman **Dr. D. V. Singh** for his advice and time. It gives me great pleasure to thank my thesis committee members **Dr. Asima Bhattacharyya**, **Dr. Subhasis Chattopadhyay** and **Dr. Chandan Goswami** for their comments and suggestions during the course of my research work. I also gratefully acknowledge the help rendered by all the faculty members of School of Biological Sciences. I would also take the opportunity to extend a word of thanks to **Prof. V. Chandrasekhar,** Director, NISER and DAE for the wonderful infrastructural facilities and SBS (School of Biological Sciences) family for the cooperative work environment which helped me carry out research smoothly. I am thankful to the funding agencies; DAE, DBT and DST, Government of India for funding this project.

I would like to thanks my past and present lab members **Roseleen, Mitali, Shasank, Bipasha, Minu, Sunil** with whom working in the lab was a pleasure. I also express my heartfelt thanks to my friends **Ashutosh, Sanjima, Sushri, Lopa, Biswaranjan, Ankit, Uday, Anup and Depanjan** for their numerous help, support and enjoyable company for making my life more cheerful.

I would like to express my sincere gratitude to a number of people to whom I am really indebted to for their help, support and motivation in all my endeavors.

I owe my heartfelt gratitude to my parents who have always encouraged me throughout my life.

Santosh Kumar Singh

Contents

Homi Bhabha National Institute

Ph.D. PROGRAMME
Synopsis of Ph.D thesis

1. **Name of the candidate:** SANTOSH KUMAR SINGH
2. **Name of the Constituent Institution:** National Institute of Science Education and Research
3. **Enrolment No. :** LIFE11201004005
4. **Title of the Thesis:** Comparative study of molecular diversity in environmental and clinical isolates of *Klebsiella pneumoniae*: focus on resistance mechanism, virulence factors and clonal diversity
5. **Board of Studies:** Life Sciences

SYNOPSIS

This thesis work explores diversity and extent of multidrug resistant bacteria circulating in fresh water aquatic environments. As rise in infections caused due to opportunistic pathogens has been possibly been attributed to transfer of environmental antibiotic resistance bacteria to clinics, this work compares resistance mechanisms, virulence factors and clonal relationship between clinical and environmental isolates of *Klebsiella pneumoniae*.

Aquatic environment forms an interface, connecting the terrestrial environment and human population [1]. These systems also serve as sink receiving effluents and discharges from different sources often containing unused residual antimicrobials [2]. Since antibiotics are not completely metabolized, in the aquatic system their presence results in a selective pressure on the resident bacteria to develop resistance toward antibiotics [3,4]. Moreover, the aquatic environment also provides a platform for horizontal gene transfer resulting in acquisition of resistance genes. Thus, the aquatic ecosystem serves as an excellent niche for the bacteria to survive, share and shuffle their genes [5,6]. Owing to the above facts, spread of multidrug resistant bacteria in the aquatic environment is recognized as an important public health issue as resistance genes can transfer from environmental bacteria to human pathogens [7]. In developing countries, unsafe water supply, sanitation and hygiene, attributed an estimated 842000 deaths per year that includes 42.87% death to children under age of five (WHO 2014). Developing countries, including India, are challenged with complex socio-economic problems that escalate infectious disease burden [8]. Higher bacterial disease burden thus, increases the use of antibiotics to limit morbidity and mortality [9]. An integrated approach towards analyzing antibiotic resistance and pathogenic potential, especially from environmental isolates is vital as it provides information on extent of threat to public health due to circulation of multidrug resistant bacteria [7]; which however is less addressed [10].

Greater resistance problems have been associated with bacteria belonging to genera *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* [11,12,13]. *Klebsiella pneumoniae*, a member of Enterobacteriaceae are ubiquitous in the natural environment. Recent multicenter prospective study conducted in several Asian countries showed that *K. pneumoniae* is the second

most important pathogen accounting for 15.4% of community acquired pneumonia (CAP) which raises serious concerns as it initially was thought of an opportunistic pathogen [14]. The observed mortality rate for *K. pneumoniae* infection is higher (50%), while in case of hospitalized immunocompromised patient with underlying diseases such as diabetes mellitus it can approach to 100% [15].

With this background, my thesis work aimed at "Comparative study of molecular diversity in environmental and clinical isolates of *Klebsiella pneumoniae*: focus on resistance mechanism, virulence factors and clonal diversity". To achieve the above stated aim following objectives were laid down for the study:

1. Isolate and identify antibiotic resistant bacteria from aquatic environment and determine their phenotypic virulence factors.

2. Compare diversity in resistance mechanisms in *Klebsiella pneumoniae* isolates from aquatic environment and clinical settings.

3. Determine clonal relationship between *Klebsiella pneumoniae* isolates from aquatic environment and clinical settings

The thesis has been organized into four chapters and contents of which have been discussed as follows:

Chapter 1 (Introduction): This chapter provides an introduction to the thesis and review the literatures related to the prevalence and diversity of antibiotics resistant bacteria from aquatic environment. The chapter presents a detailed review of antibiotic resistance genes reported from aquatic environment, the antibiotic resistance patterns observed, clonal diversity of the circulating isolates, virulence factors associated with the organism, diseases caused and

treatment options for *Klebsiella pneumoniae*. The concluding part of this chapter provides recent update on bacterial persister cell.

Chapter 2 (Materials & Methods): This section describes the materials and methodology used to attain the objectives. It includes description of the sampling sites, methodology used for isolation, culturing and identification of pure colonies. Also it details the molecular biology protocols and microscopy techniques used in the study.

Chapter 3 (Results and Observations): This chapter reports the results and observation of the experiments carried out and discusses them in light of the current literature. Chapter 3 has been divided into sub-section described as follows:

Section 3.1 (**Isolation, identification of antibiotic resistant bacteria from aquatic environment and phenotypic characterization of virulence factors)**: This section reports predominance of isolates belonging to *Enterobacter* sp, *Pseudomonas* sp, *Proteus* sp, *Aeromonas* sp, *Acinetobacter* sp and *Klebsiella* sp, in the population under analysis. Resistance profiling of the 113 selected isolates showed 98% of the environmental isolates to be resistant to two or more than two different classes of antibiotic, which could categorize them as multidrug resistance. Seventeen isolates belong to *Aeromonas* sp (n=2), *Acinetobacter* sp (n=2), *Klebsiella* sp (n=5), *E. coli* (n=2), *Kluyevera* sp (n=1), *Enterobacter* sp (n=5) were resistant to last resort antibiotic colistin. Among the genera analyzed, isolates belonging to *Pseudomonas* followed by *Klebsiella* exhibited high multiple antibiotic resistance (MAR) indices indicating high risk associated with these genera of opportunistic pathogens. Association between MAR index and virulence index indicated isolates belonging to genus *Pseudomonas* as high threat group. Based on these findings we could conclude that association analysis between multiple antibiotic resistance (MAR) index and virulence index can aid in identification of potential high risk pathogenic

populations/isolates. This finding raises concern of these acting as potential pathogens, in the event of being transmitted to an appropriate host.

Section 3.2 (Comparative study on diversity in resistance mechanisms in *Klebsiella pneumoniae* isolates from aquatic environment and clinical settings): Findings of this work revealed clinical isolates exhibited greater MAR indices than environmental isolates. Results of phenotypic and genotypic screening for carbapenem resistance corroborated well with each other. Twenty two (9 environmental and all clinical) out of 27 isolates included in the study were positive for extended spectrum beta-lactamase genes. bla_{SHV} was major determinant of ESBL phenotype in the isolates under study. Three clinical isolates co-harbored bla_{SHV}, bla_{OXA} responsible for ESBL and carbapenem resistance respectively. Clinical isolates of *Klebsiella pneumoniae* exhibited wide spread resistance to quinolone mediated by mutations in the nucleotide sequences of Quinolone Resistance Determining Region (QRDR) of *gyrase A*. In contrast environmental isolates of *Klebsiella pneumoniae* did not have any mutation in the QRDR of *gyrase A*. Resistance towards quinolones in environmental isolates presumable could be attributed to efflux pumps. The present study is the first to report efflux mediated colistin resistance in environmental isolates of *Klebsiella pneumoniae*. The study also reports for the first time persister cell formation as a mechanism of antimicrobial tolerance in environmental *Klebsiella pneumoniae*.

Section 3.3 (Clonal relationship among *Klebsiella pneumoniae* isolates from aquatic sources and clinical settings): This section reports sequence types of *Klebsiella pneumoniae* isolates used in the preceding section. It was observed that clinical and environmental isolates belonged to diverse sequence types. Sequence types of many of the isolates could not be determined as these showed new allelic variations in the house keeping genes used for the study. Results of

phylogenetic analysis using concatenated sequences of seven housekeeping genes exhibited formation of two branches and two clades, with isolate KpAH7 belonging to ST231 forming an out group indicating divergent evolution of the isolate. Low bootstrap values at the nodes indicated less divergence and a possible common ancestral origin for most of the isolates.

Chapter 4 (Summary and Conclusions): This chapter provides concluding remarks about findings of this study, which emphasizes on the need to quantitatively evaluate the extent of threat posed by multidrug resistant bacteria from environment both at population and genus level, particularly from Indian sub-continent. Further it shows that association analysis of MAR index and virulence factors in multidrug resistant isolates can aid in identification of potential high risk pathogenic populations/isolates

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Publications in refereed journals:

(a) Published

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(c) Manuscript under preparation

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(d) Others:

A. Poster presented and conferences attended:

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Doctoral Committee:

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2.	DR. HARAPRIYA MOHAPATRA	Guide/ Convener		5.12.16
3.		Co-guide (if any)		
4.	DR. ACIMA BHATTACHARYYA	Member		5.12.16
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6.	DR D.V. SINGH	EXTERNAL Member		05/12/16

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ABC	ATP-binding components
ABM	Acute bacterial meningitis
AR	Antibiotic resistance
ARGs	Antibiotic resistance genes
AS	Ankylosing spondylitis
ATCC	American type culture collection
BATH	Bacterial adherence to hydrocarbon
BDNF	Brain-derived neurotrophic factor
BGA	Brilliant green agar
BHI	Brain Heart infusion
BLAST	Basic Local Alignment Search Tool
BRB	Blood-retinal barriers
CAUTIs	Catheter-associated urinary tract infections
CDC	Centers for Disease Control and Prevention
CFDA	Carboxyfluorescein diacetate
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CTns	Conjugative transposons
dfr	Dihydrofolate-reductase
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
eARB	Environmental antibiotic resistant bacteria
EDTA	Ethylene diamine tetraacetic acid
EE	Endogenous endophthalmitis
EMBL agar	Eosin methylene blue agar
ESBL	Extended spectrum β-lactamase
ETC	Extracellular toxic complex
FDA	Food and Drug Administration
GC	Guanine-cytosine

HEC	Hungarian epidemic clone
HGT	Horizontal gene transfer
HMV	Hypermucoviscocity
ICEs	Integrated Conjugative Elements
ICU	Intensive care unit
IL	Interleukin
KEE	*Klebsiella* endogenous endophthalmitis
KPC	*Klebsiella pneumoniae* carbapenemase
KPLA	*K. pneumoniae* liver abscess
LB	Luria-Bertani
LF	Lactose fermenter
LPS	Lipopolysaccharides
MAR index	Multiple antibiotic resistances index
MDR	Multi-drug-resistant
MGEs	Mobile genetic elements
MHA	Muller-Hinton agar
MHB	Muller-Hinton broth
MIC	Minimum inhibitory concentration
MLST	Multi locus sequence typing
MR-HA	Mannose-resistant Haemagglutination
MS-HA	Mannose-sensitive Haemagglutination
NDM	New Delhi metallo-β-lactamase
ng/L	Nano gram per liter
NHS	Normal human serum
NLF	Non lactose fermenter
nmol/L	Nano mole per liter
OD	Optical density
ORF	Open reading frames
OTC	Oxytetracycline
pARB	pathogenic antibiotic resistant bacteria
PCR	Polymerase chain reaction

PLA	Pyogenic liver abscess
PLC	Phospholipase C
PMQR	Plasmid-mediated quinolone resistance
QRDR	Quinolone-resistance determining region
SDS	Sodium dodecyl sulfate
ST	Sequence type
TCP	Toxin-coregulated pilus
TNF	Tumor necrosis factor
URTI	Upper respiratory tract infection
VC	Viable counts
WHO	World health organization
WWTP	Waste water treatment plant

Chapter-1
Introduction & Review of literature

1.1. Antibiotics and antibiotics resistance mechanisms

Antibiotics are low molecular weight (<3000 D) natural, semi-synthetic or synthetic organic compound that causes microbial growth inhibition (bacteriostatic) or cell death (bactericidal) due to specific interactions with bacterial targets. These are ubiquitously and perpetually present at a basal level in environments as it naturally produced by fungi, actinomycetes and bacteria to inhibit the surrounding organisms [16]. The first antibiotic to be discovered was penicillin in the 1920s by the Scottish scientist Alexander Fleming from a mould *Penicillium notatum* which inhibited growth of *Staphylococcus aureus* [17]. Thereafter, pioneering work of Domagk and Fleming inspired the subsequent discovery of a number of newer antimicrobial compounds that played a vital role in the treatment of infectious diseases in human and animals [16]. Efforts were continuous to discover more antibiotics which would treat bacterial infection but not harm the host. Most of the antibiotics in current use are compounds that were discovered during the 1940s to 1960s — "the golden era" of antibiotic discovery or their derivatives (**Fig.1.1**). The therapeutic use of antibiotic in the 1940s brought about a paradigm shift in the infectious diseases by treating deadly bacterial diseases successfully as well as opened the possibilities in the medical field for major surgical interventions and organ transplantation [18]. From several decades, the use of antibiotics in clinical setting saved many lives and thus it was called "magic bullet" or "miracle drug" [16]. The great success of antibiotics in human therapy prompted the compounds to be used out of clinical contexts. Thereafter, antibiotics had been used in large scale as growth promoters and for prophylactics in livestock, usually administered by addition to the feed [19,20]. Other non-clinical uses of antibiotics include their usage in fishery [21] and poultry farming [22].

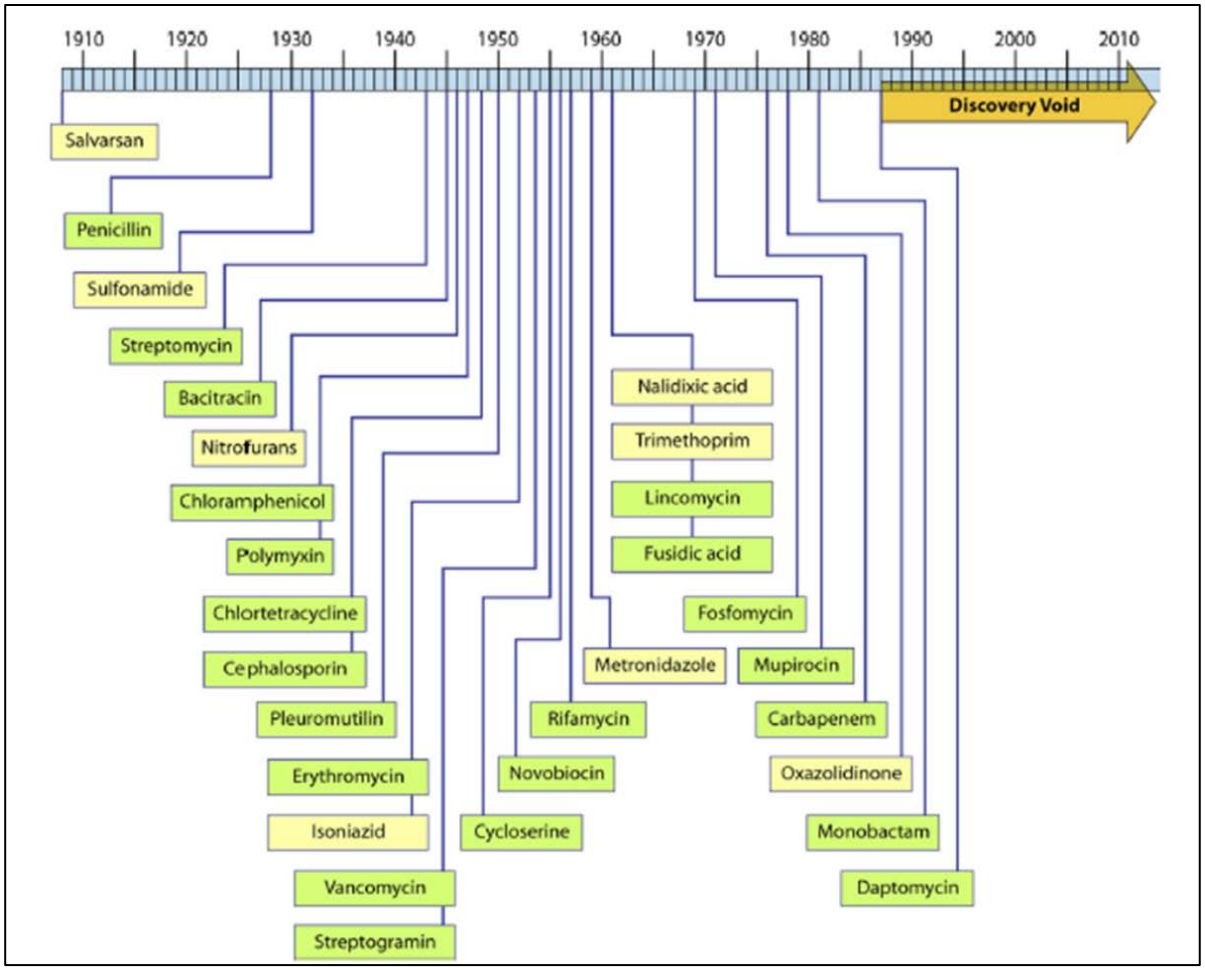


Figure 1.1 Timeline of the discovery of antibiotics.

Dates indicated are those of reported initial discovery or patent [23].

Antibiotics have been classified based on bacterial spectrum (broad versus narrow), type of activity (bactericidal versus bacteriostatic), origin (natural versus synthesized) and route of administration (injectable versus oral versus topical). However, antibiotics by their common chemical structure is the most useful way to classify because those with similar structure often have similar functionality, mode of action, substrate spectrum as well as toxicities. Different classes of antibiotic based on chemical structure, their mode of action and spectrum are listed in the **table 1.1**.

Table 1. 1 Different classes of antibiotic, mode of action and spectrum

Class	Examples	Mode of action	Effect on bacteria	Spectrum
β-Lactams	**Aminobenzylpenicillins** Penicillin G*, Ampicillin*, Amoxicillin*	Inhibit the synthesis of **cell walls** by binding to enzymes involved in peptidoglycan production.	Bactericidal	Active mainly against Gram-positive bacteria
	Ureidopenicillins Mezlocillin, Azlocillin, Piperacillin, Ticarcillin			
	Penicillinase resistant penicillins Oxacillin, Dicloxacillin, Flucloxacillin, cloxacillin*			Active against Gram-positive and Gram-negative bacteria.
	Cephalosporins 1st generation: Cefazolin, Cefalexin, Cefadroxil 2nd generation: Cefuroxime, Cefotiam, Cefuroxime axetil, Cefaclor, Loracarbef 3rd generation: Cefotaxime, Ceftriaxone, Ceftazidime, Cefixime, Cefpodoxime, proxetil, Ceftibuten 4th generation: Cefepime, Cefpirome			More effective against some Gram-negative bacteria, e.g. *Klebsiella, Proteus, Enterobacter, Hemophilus* spp. Has less effect upon Gram-positive bacteria
	Carbapenems Imipenem, Meropenem, Ertapenem, Doripenem			
	β-Lactam/ß-lactamase inhibitors Ampicillin/sulbactam, Amoxicillin/clavulanate, Piperacillin/tazobactam			Broad spectrum of activity against both Gram-positive and Gram-negative microorganisms
	Monobactam Aztreonam			
	β-Lactamase inhibitors Clavulanic acid, Sulbactam, Tazobactam			

Class	Examples	Mode of action	Effect on bacteria	Spectrum
Quinolones	**1st generation**: Nalidixic acid, Cinoxacin, Enoxacin **2nd generation**: Ciprofloxacin, Norfloxacin, ofloxacin **3rd generation**: Levofloxacin, Pefloxacin, Sparfloxacin **4th generation**: Moxifloxacin, gatifloxacin, Gamifloxacin	Interfere with bacteria DNA replication by binding with topoisomerases i.e. DNA gyrase and topoisomerase IV.	Bactericidal	
Aminoglycosides	Streptomycin*, Gentamicin*, Tobramycin, Netilmicin, Amikacin, Neomycin*	Inhibit protein synthesis by binding to 30S ribosomal subunit and inhibits the translocation of the peptidyl-tRNA from the A-site to the P-site.	Bactericidal	Gram-positive and Gram-negative bacteria but not acting upon anaerobic bacteria
Macrolides	Erythromycin*, Spiramycin, Roxithromycin, Clarithromycin, Azithromycin	Inhibit protein synthesis by binding to the large ribosomal subunit in the vicinity of the peptidyl transferase center	Bacteriostatic	

Class	Examples	Mode of action	Effect on bacteria	Spectrum
Tetracyclines	Tetracycline*, Doxycycline, Minocycline	Inhibit bacterial protein synthesis by preventing the association of aminoacyl tRNA with the bacterial ribosome	Bacteriostatic	Broad-spectrum. Exhibits activity against a wide range of Gram-positive, Gram-negative bacteria, atypical organisms such as Chlamydia, mycoplasmas
Sulphonamides	Sulphamethoxazole*,	Sulfonamides interfere with folic acid synthesis	Bacteriostatic	Broad-spectrum; affects Gram-positive and many Gram-negative bacteria
Polypeptides	Colistin*, Polymyxin-B	Disrupt the structure of cell membrane phospholipids and increase cell permeability	Bactericidal	Carbapenem resistant Gram's negative bacteria

Sources: http://www.springer.com/978-3-642-18401-7

*Antimicrobial drugs also approved for use in food-producing animals by FDA-2012 (http://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActADUFA/UCM416983.pdf).

To counter the effect, antibiotic producing bacteria themselves carry resistance genes, thus gaining advantage of niche and in turn exposing other bacteria to a pool of various antibiotic resistance genes. With developments of new antibiotics, bacteria have adapted defenses against these antibiotics and continue to develop resistances. Antibiotic resistance (AR) is the ability of bacteria to withstand the effects of antibiotic. Antibiotic resistance in bacteria are either intrinsic (naturally present in the genome of the bacteria) or acquired (from their natural habitat via various transfer mechanism like conjugation, transformation and transduction) [5]. Indiscriminate and inappropriate use of antibiotics in human as well as veterinary medicine has caused accumulation and bio-magnification of these chemicals and development of resistant bacteria in the environments [24,25]. The World Health Organization, the US Center for Disease Control, and numerous other global and national agencies recognize that the rate of antibiotic resistance among many disease-causing bacteria has been increasing every year. Thus, several antibiotics that were once successfully used against bacterial infections are now futile because of the development of antibiotic resistance through horizontal gene transfer and/or spontaneous mutations [26,27]. Some of the documented mechanisms of antibiotic resistance are as shown in **Fig.1.2**:

(i) Antibiotic inactivation or modification occurs mainly via hydrolysis, group transfer and redox mechanism [28]. Hydrolysis by β-lactamases, antibiotics inactivation by group transfer and modification via acyl transfer, glycosylation, phosphorylation, nucleotidylation, ribosylation and thiol transfer applies to β-Lactam and aminoglycoside resistance whereas redox reactions promote detoxification of antibiotics via oxidation and reduction for instance oxidation of tetracycline [28].

(ii) Specific interaction between an antibiotic and a target molecule is very important for effective antibiotic action, so even a small change in a target molecule can make antibiotic ineffective [29] and,

(iii) Most of the antibiotics are designed to target the intracellular processes hence their penetration through the bacterial cell wall is important for their function [30]. Both Gram-positive and negative bacteria have membrane proteins called efflux pumps that export antibiotics from the cell and maintain their low intracellular concentrations [29,31].

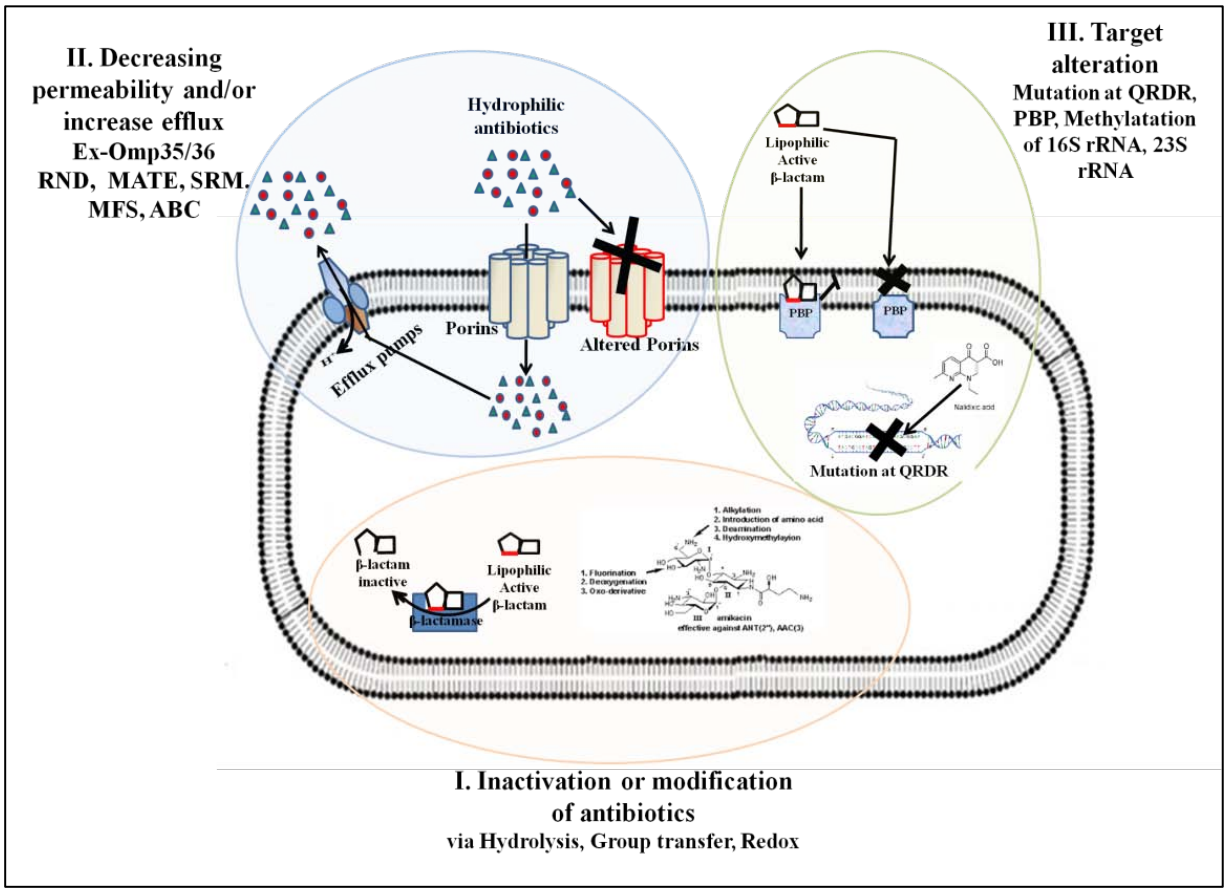


Figure 1.2 Mechanisms of antibiotics resistance.

(I) Antibiotic inactivation or modification, (II) permeability and/or increase efflux and (III) target alteration.

1.2. Gene transfer among bacteria

Antibiotic resistant genes are often encoded either on chromosome or on extra-chromosomal genetic elements (plasmids) or in segments that appear to have been recombined into the chromosome from other genomes (transposons, mobile genetic elements, integrons). Plasmid encoded mechanisms are capable of transfer to other bacterial cell. In two independent studies, 9.4% isolates collected from sediment of unpolluted river site in Wales Korea and 15% isolates collected from a polluted river site in Korea contained plasmids; of which 86% were large enough to be conjugal plasmids [32]. Kessie *et al*., (1998) found that different species of *Staphylococcal* isolates from polluted streams in Morocco contained plasmids of same size encoding tetracycline, erythromycin, neomycin, and streptomycin resistance genes [33].

The mobile genetic elements (MGEs) mediate the movement of DNA within genomes (intracellular mobility) or between bacterial cells (intercellular mobility). Transposons are segments in plasmids which often contain the ARGs. Genes embedded into plasmids and transposons are capable of transfer into a wide range of bacteria. Integrons are one of the major elements for antibiotic resistance globally because of their capability to capture and express diverse member of resistance genes cassettes. Integrons incorporate and rearrange open reading frames (ORF) embedded in them as gene cassettes, in a rec-independent site-specific recombination and thereby convert them into functional genes ensuring their correct expression [34]. Park *et al*., (2003) reported12.6% of coliform isolates in a Korean river contained integrons [35].

Conjugation process discovered in the 1950s has been regarded as the main facilitator of ARG transfer between bacteria [27]. Conjugation requires independently replicating genetic elements such as conjugative plasmids or chromosomally Integrated Conjugative Elements

(ICEs), including a conjugative transposons (CTns). The genetic materials encode proteins which facilitate their own transfer and also the transfer of other cellular DNA from the donor to a recipient cell, lacking the plasmids or ICEs (**Fig. 1.3A**). It is important to note that DNA transfer from the donor will be maintained and expressed in the recipient only if they integrate into the genome of the recipient by recombination. Resistance plasmids are common among multi-drug resistant clinical pathogens such as *Staphylococcus aureus*, *Enterococcus species*, *Clostridium difficile*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella* sp [36]. This mode of transfer includes the potential to transfer DNA among a broad host-range of species [37]. Transformation is ability to uptake free DNA from environment, was the first mechanism of prokaryotic horizontal gene transfer (HGT) to be discovered. (**Fig.1.3B**). Free DNA in the environments may be stabilized by sticking to particles from sediment as well as soil, with greater concentration in case of biofilm. In biofilms, it was observed that DNA of newly lysed bacteria was taken up by neighboring bacteria [38]. Natural transformation have been reported in more them 60 bacterial species [39]. Transduction is another form of gene transfer in environment mediated by independently replicating bacteriophage (Frost *et al*., 2005) as shown in **Fig. 1.3C**. DNA is protected inside phage particles to environmental degradation and their compact size simplifies their dissemination [38].

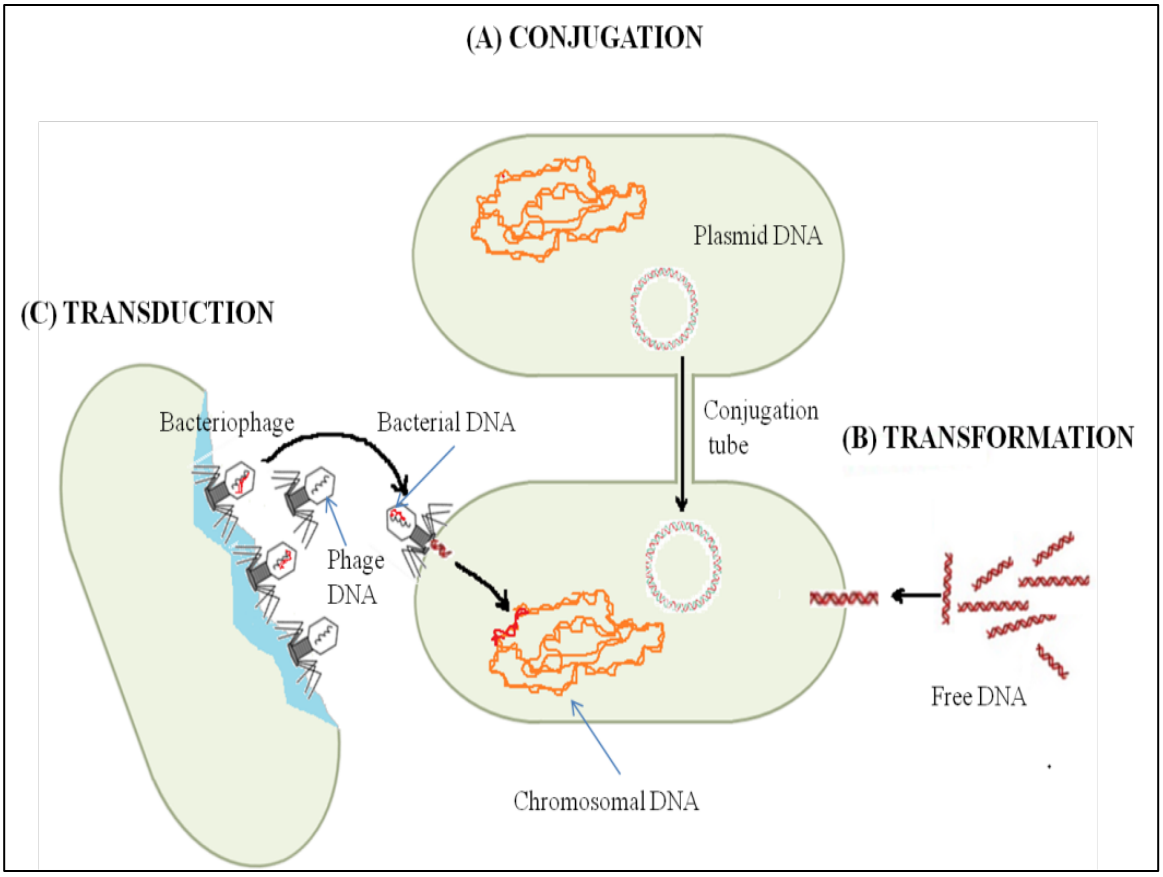


Figure 1.3 Gene transfer in bacterial cell.

(A) Conjugation: transfer of DNA *via* conjugation tube, (B) Transformation: direct uptake of free DNA from environments and (C) Transduction: transfer of DNA *via.* phage.

1.3. Antibiotics and antibiotic resistance genes in aquatic environments

Water is a natural resource essential for the survival of humankind and all species on earth. Fresh water comprises 3% of the total water on earth and of that less than 1% is available for human use in the form of lake, rivers, streams and aquifers [40]. Since the middle of the 20th century, pollution of fresh water resource by different types of contaminants is a major environmental problem in many parts of the world [41]. Industrials wastes, hospitals waste and anthropogenic activities constitute major sources of aquatic environment contamination by inorganic as well as organic micro-pollutants comprising toxic metals, hydrophobic organic

compounds and antibiotics [42,43]. Additionally, it also receives pathogenic bacteria and antibiotic resistance genes (ARGs) from hospital wastes and waste water treatment plants [2,5,44,45,46].

Antibiotic consumption

Over the last decade (2000 to 2010), global total antibiotic consumption increased by more than 30%, with the highest increase of 41% for penicillin and cephalosporins [25]. Top global consumers countries of the most antibiotics in 2010 were India (13 billion SU), China (10 billion SU) and the United States (7 billion SU) as shown in **fig. 1.4**. However, in terms of per capita antibiotic consumption among these countries in 2010, the United States led with 22 SU per person, compared with 11 SU in India and 7 SU in China [25].

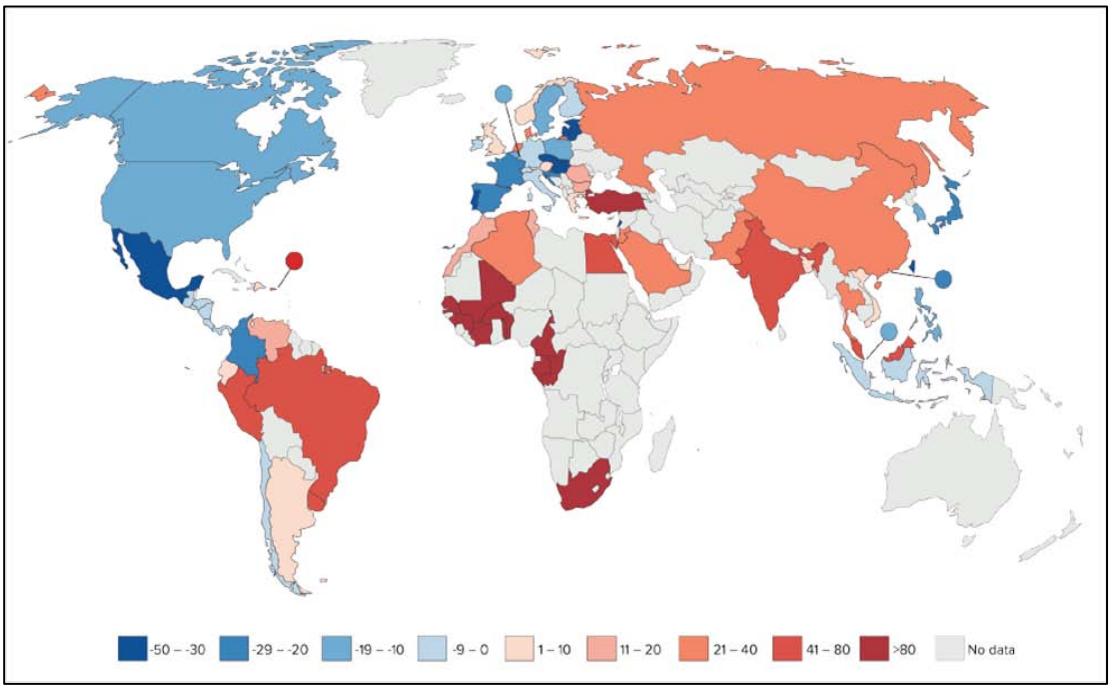


Figure 1.4 Antibiotic consumption per capita by country in the year 2000–2010 [25]

Misuse and overuse of antibiotics in therapies that include inappropriate choice of antibiotics, inappropriate dose regimen, inappropriate combination in case of combined therapy, failure to complete therapy, skipping of doses, use of antibiotics for non-bacterial infections and reuse of leftover antibiotics is still a widespread and common phenomenon across the world. Werner *et al*., (2011) showed that among hospitalized patients in the Metro Health Medical Center (Ohio, US), 39% of all requested therapeutic fluoroquinolones were used for non-bacterial or non-infectious diseases and inappropriate length of treatment [47]. Sun *et al*., (2015) from China, the most populated country in the world, showed that 55% (869/1590) prescriptions included an antibiotic with diagnosis of the common cold [48]. These antibiotic prescriptions were more from the village clinics (71%) as compare to other institutions (47%). In a study from India, out of 46% patients who had consumed antibiotics for a self-limiting upper respiratory tract infection (URTI) caused by bacteria or virus, only 7% had taken prescription from the physician, rest all were over the counter purchases [49]. The most commonly used antibiotics were amoxicillin and amoxiclav (36%), roxithromycin (21%), azithromycin (16%), ciprofloxacin (12%), cefixime (9%) and levofloxacin (6%). Only 21% patients completed the full course of antibiotic treatment whereas 18% patients stopped taking antibiotic after relief from the symptoms and 61% stopped after experiencing diarrhea as a side effect. Use of antibiotics does not leave the host unaffected, even though the individual might seem healthy. The overuse of antibiotics has most likely changes microbiomes in the human. Robinson and Young, (2010) observed an increase of *Proteobacteria* at the expense of *Firmicutes* and *Bacteroidetes* in the gut of human and mice during antibiotic treatment [50]. Apart from change of gut microbiomes and spreading resistance, overprescribing antibiotic is also associated with other problems ranged from allergic reactions, gastrointestinal to neurologic and psychiatric disorders [51]. After their

use in man and animals, a substantial part of all antibiotics consumed are not absorbed or metabolized by the body and excreted in their active or semi active form in the urine (90%) and feces (75%) and depending on their polarity, water solubility and persistence, antibiotics are released into rivers or accumulate with sewage sludge [52,53].

Antibiotics in environment

Of late, antibiotics are among the emerging contaminants in water because of concerns of their potential adverse effects on the ecosystem and possibly on human health. The increasing dependence on antibiotics for, treatment of bacterial infection in human, enhancing growth rate and treatment of veterinary and aquatic animals, over the years has resulted in dissemination of antibiotics in the aquatic environment [3,46,54]. It is important to realize that the many antibiotics can be used in both human and animal treatments that are dispersed either through treated urine and feces or direct disposal into the aquatic systems (FDA-2012). Hirsch *et al*., (1999) reported that up to 90% of the antibiotics administered to human and animals could be excreted unchanged and reach into the aquatic environment [24]. Once released into the environment, antibiotics can be transported either in a dissolved phase or adsorbed to colloids or soil particles into surface- and groundwater [55,56,57]. Such discharges elevate the concentration of antibiotic beyond normal threshold that cause a selective pressure on the resident bacteria leading to the development of resistance toward antibiotics in bacteria which may find its way back into the human population [3,4,7,58] as shown in the **Fig.1.5**.

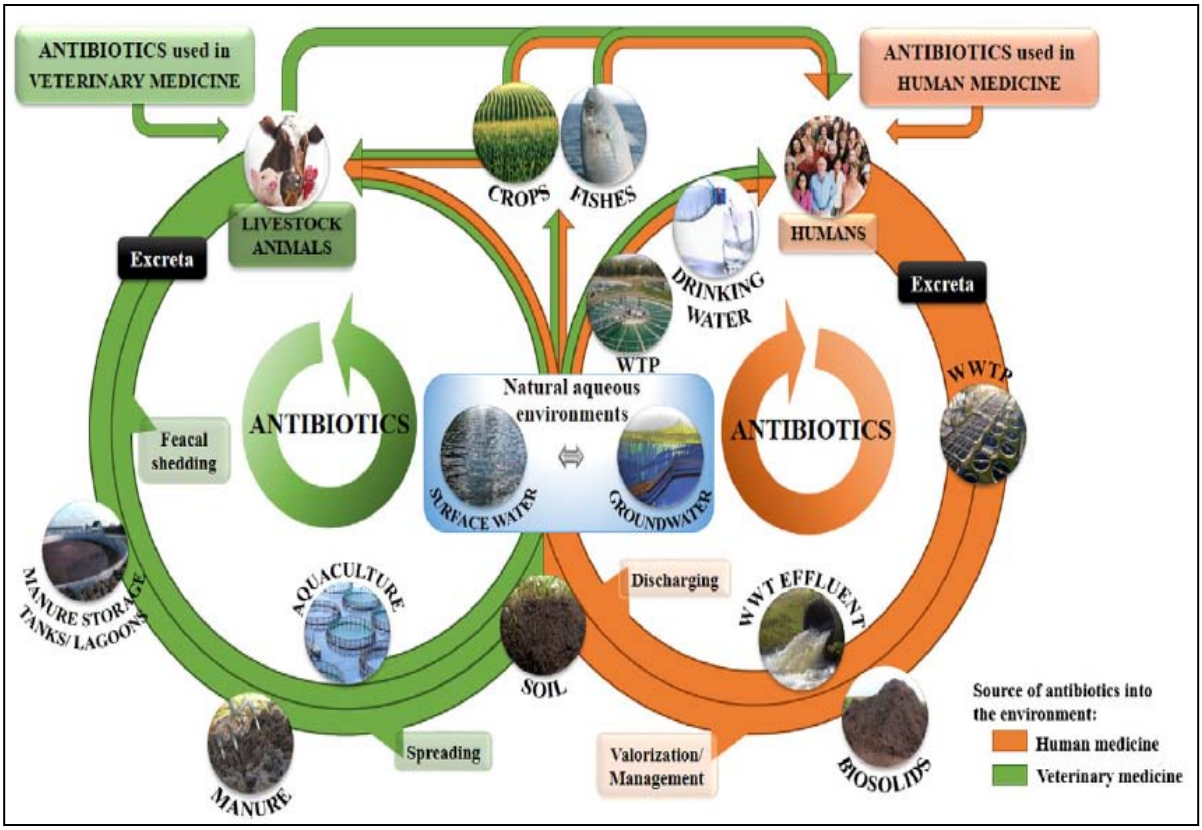


Figure 1.5 Routes of antibiotics in the aquatic environments.

Antibiotic from the veterinary sources (green) and human sources (orange) were comes in to natural aquatic environment via different routes [59].

In 1980, first report of surface water contamination by antibiotics was from England [60]. They detected at least one compound from the sulfonamide, macrolide and tetracycline group of antibiotics show the highest persistence and are frequently detected with concentrations of 1µg/L in river water [60]. Another most commonly used antibiotic in human medicine, veterinary and aquaculture is ciprofloxacin with substantial persistence in sewage treatment plants (removal efficiencies are around 60%) resulting in their emissions into the aquatic environment [61,62]. The ciprofloxacin concentrations detected in surface waters ranged from 0.0018 nmol/L in Brazil [63] to almost 20,000 nmol/L in India, with a worldwide median concentration of 500 nmol/L [62]. β-lactams are among the most prescribed antibiotics, but they readily undergo hydrolysis

and thus have been detected in very low concentrations in treated wastewater, or are not at all detected [64]. With the advancement in the detection technology, the occurrence of pharmaceuticals as contaminants in the aquatic systems has attracted increase attention. The occurrence of antibiotics in different aquatic environments of several countries is presented in **table 1.2**.

Table 1. 2 Occurrence of antibiotics in the aquatic environment

Country	Source	Antibiotics	Concentration	References
Germany	Surface water	Clarithromycin, Roxithromycin, Sulfamethoxazole, trimethoprim, Sulfamethazine	28-5ng/L	[24,65]
Europe	Surface waters span	Ciprofloxacin	0.04 nmol/L	[66]
Portugal	Mondego River water	Ciprofloxacin	119.2ng/L	[67]
France	Allier River water	Tetracycline	1.8ng/L	[68]
Portugal	Douro River water		53.3ng/L	[69]
North America	Surface waters	Ciprofloxacin	0.009 to 1.09 nmol/L	[70,71,72,73]
Italy	Po and Arno Rivers			[66]
Spain	Guadalquivir Rivers	Macrolides	AZT=0.09-153.72; CTR=0.09-65.63; ERT=nd-18.58ng/L	[74]
UK	Ely and Taff Rivers	Macrolides Erythromycin	144-10,025ng/L	[75]
	Trent River, Shardlow	Sulfachlorpyridazine	613,200ng/L	[76]

India	Hospital effluent, Ujjain	Ceftriaxone	59.5 μg/l	[77]
	Groundwater	ciprofloxacin	0.77 μg/l	
	River sediment	ciprofloxacin	914 mg/kg	
	Yamuna River water	ampicillin	13.75 μg/l	[78]
	Wastewater		0.172μg/l	[79]
		Amoxicillin	(Untreated)	
			and 0.062μg/l	
			(Treated)	

Resistant genes in environment

Apart from antibiotics, antibiotic resistance genes have been widely distributed in the environment even before the introduction of antibiotic chemotherapies. But human activities have probably increased the prevalence of resistant genes in the aquatic environment. Mobile genetic elements (MGEs) like conjugative plasmids, integrons and transposons associated with antibiotic resistance genes (ARGs) are considered to be high risk to public health as they are capable of self-transferring and dispersion *via* horizontal gene transfer and these resistant determinants are transmitted across taxonomic boundaries forming a bulk of resistant bacteria of diverse phylogenetic groups [37,80,81,82]. In Germany, β-lactam (bla_{oxa-2}) and aminoglycoside (*aac*A4, *aad*A1) resistance genes have been isolated from activated sludge [83] whereas TEM, IMP, and OXA-2 derivatives have been isolated in aquatic systems and sewage sludge from Portugal [84]. Dihydrofolate reductase (*dfr*) gene family are generally associated with integrons conferring resistance to trimethoprim and have been reported from different aquatic systems of several countries [85,86]. *dfr*A1, *dfr*A5, *dfr*A6, *dfr*A12, and *dfr*A17 from the Torsa river of India [87], *dfr*A1, *dfr*A7, *dfr*A12, and *dfr*A17 from polluted lagoon of Portugal [88] and *drf*A1 in surface waters from Germany and Australia [89] has been detected.

Similarly, quinolone resistance genes (*qnr*) have been reported from the environmental sources. *qnr*S has been isolated from several different sources like the activated sludge of a waste water treatment plants (WWTP), river, lake from Germany, France and Switzerland respectively [90,91,92]. Other variant like *qnr*B has been found in wastewater effluent and wastewater irrigated soils from Italy and Mexico respectively [93,94], *qnr*A, *qnr*B, and *qnr*S have been found in an urban coastal wetland close to the US–Mexico border [93].

Erythromycin is widely used in the veterinary and poultry and thus *erm* genes responsible for macrolides resistance are prevalent in the related environments [95]. *erm*A, *erm*B, *erm*C, *erm*F, *erm*T, and *erm*X have been found in bovine and swine manure as well as a swine waste lagoon [96]. Additionally, *erm*B have been found in wastewater in Portugal [97], and surface waters in Germany and Australia [89]. Owing to stability of tetracycline, *tet* gene family (*tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*G, *tet*M, *tet*O, *tet*S, and *tet*Q) which confer resistance to tetracycline was widely reported in the different environments from several countries [98,99,100,101].

Thus, together with antibiotics and antibiotic resistance genes, the aquatic systems provide an excellent sink for the emergence of resistance bacteria as it provided a selective pressure to share and shuffle these genes [5,6]. On the other hand, many of the environmental bacteria exhibit intrinsic resistance towards many antimicrobial agents [102].

1.4. Antibiotics resistant bacteria in the rivers of India and abroad

In developed country like U.S, 12% and 60% isolates from rivers the Ohio and Colorado respectively were resistance to ampicillin. A stunning 25% to 98% isolates were resistant to cephalothin and up to 68% isolates were resistant to amoxicillin/clavulanic acid [103]. A study carried out in Australia surface water suggested that more than 50% of *E. coli* have originated

from wastewater effluents [104], underlining the importance of municipal wastewater treatment plants as potential point sources of pathogens into surface water. Resistance is common in polluted rivers of other developed countries as well. For examples, *Aeromonas* sp resistant to quinolone (59% to nalidixic acid, 2% to ciprofloxacin) was reported to occur in rivers of Spain and France [105]. Blasco *et al*., (2008), isolated multidrug resistant *Pseudomonas* sp with multiple antibiotic resistant indexes greater than 0.5 from natural water reservoirs and industrial cooling towers in Spain [106]. Coagulase-negative *Staphylococci* resistant to combinations of antibiotics like ampicillin, chloramphenicol, tetracycline, and erythromycin were isolated from polluted rivers in Morocco [33]. Li *et al*., (2010) reported the presence of multidrug resistant *Gammaproteobacteria* from surface water receiving effluent from an oxytetracycline (OTC) production plant in China [107]. They showed that almost all bacteria showed multidrug-resistant (MDR) phenotypes isolated from the waste water and downstream river water samples whereas 28% bacteria showed multidrug-resistant (MDR) phenotypes isolated from upstream water samples.

In developing countries, surface water bodies are facing numerous threats originating from anthropogenic activities due to rapid population growth, poor sanitation facilities and industrialization [108,109]. Unregulated sale and dispensing of antibiotics is very common in these countries [110,111]. Thus, apart from hospital effluents, general communities are also the contributor for the input of antibiotic-resistant bacteria to the aquatic environment. Environmental water/sewage samples collected from the 7 regions (58 sites) in Dhaka, Bangladesh during 2012, showed multidrug resistant *K. pneumoniae, Pseudomonas sp, E. coli, Aeromonas sp, Acinetobacter sp* and *Citrobacter sp* in which 62% carried $bla_{\text{NDM-1}}$ gene [112]. From January to February 2014, 98% (n=163) *E. coli* isolated from the Apies River, Gauteng,

South Africa showed resistances to at least one of the nine antibiotics tested whereas 80% of isolates showed resistance to ≥3 antibiotics [113]. The concerns of pollution of aquatic environment in India are large. Religious beliefs, cultural traditions, poverty and large population interactive cause severe pollution. Central Pollution Control Board, Ministry of Environment & Forests, Government of India (2005), reported that 90% of the sewage generated by rural municipalities and 50% of sewage by urban municipal discharged to the fresh water ecosystem without any treatments that added various antibiotic resistant bacteria in the water ecosystem (http://cpcb.nic.in/newitems/12.pdf). **Table 1.3** showed the presence of antibiotic-resistant bacteria in India aquatic environments.

Table 1. 3 Antibiotics resistant bacteria in the Indian Rivers

River	City	Period	Pathogens	Resistance information	Resistance genes	Reference
Yamuna River	Delhi	2010	*E. coli*	Quinolone and β-lactam	$bla_{\text{CTX-M-15}}$, *CMY*-42, *qnr*-S $bla_{\text{NDM-1}}$	[114,115]
Ganges River	Rishikesh-Haridwar region	2010			$bla_{\text{NDM-1}}$	[115]
	West Bengal		*Escherichia coli* *Enterobacter sp*	Ampicillin, co-trimaxazole, chloramphenicol		[116]
			shiga toxin and enterotoxin producing *Escherichia coli*	multidrug-resistant		[109]
Cauvery River	Karnataka	2011-212	*E. coli, Enterobacter cloacae, Pseudomonas trivialis,* and *Shigella*	multidrug-resistant	bla_{TEM}, *dfr*	[117]

			sonnei		
Gomti river			*E. coli* and *Enterobacter*	ampicillin, chloramphenicol, sulfonamides, tetracycline, and streptomycin	[118]
			shiga toxin and enterotoxin producing *Escherichia coli*	multidrug-resistant	[109]
River Hooghly	Kolkata	2012-2013			[119]
River Kangsabati	Kharagpur	2012-2013			[119]
Saryu (Ghagra) River	Ayodhya	2009	*E. coli*	multidrug-resistant	[120]
River Mahananda	Siliguri, West Bengal	2007-2009	Oligotrophic bacteria	multidrug-resistant	[121]

1.5. Antibiotic resistant gene transfer from environmental to pathogenic bacteria

Environmental bacteria have been a reservoir of antibiotic resistance genes and a potential source of novel resistance genes in clinical pathogens [122,123]. Recently, metagenomic tools have identified resistance genes in environment isolates released from dead bacteria [123,124]. In a river basin in China, concentrations of extracellular DNA including ARGs were found more abundant than intracellular DNA, implying that environmental is an important reservoir for genes [125]. Antibiotic resistant bacteria developing in these environments adversely affect human health by direct exposure of patients to antibiotic resistant pathogen(s) (e.g. anthropogenic activities or drinking unsafe water) or exposure of patients to

resistance determinants and subsequent horizontal gene transfer (HGT) to bacterial pathogen(s) on or within a human host [7].

Dissemination of penicillin-resistance genes in *Streptococcus spp*. through natural transformation in many different environments is an excellent example [39]. The extended spectrum β-lactamase gene $bla_{\text{CTX-m}}$ has originated from a soil bacteria *Kluyevera* sp, is now very common in the clinical isolates [126]. New Delhi Metallo-β-lactamase-1 (NDM-1), a class B β-lactamase has been isolated from different bacteria recovered from many infection sites both in hospital- and community-acquired infections worldwide [127,128,129,130]. It hydrolyzes all β-lactam antimicrobials except for monobactam. Based on chaperonin homologue, phylogenetic analysis and guanine-cytosine content (GC) percentage, the putative original source of the $bla_{\text{NDM-1}}$ gene could be traced from a chromosome of plant pathogens, such as *Pseudoxanthomonas* and related bacteria that are widespread in the environment [131].

Aminoglycoside methylase is an enzyme that methylates 16S rRNA responsible for aminoglycosides resistance [132]. Recently, it was reported from many clinical isolates belonging to different genus like *Citrobacter freundii, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus mirabilis, Serratia marcescens*, *Salmonella* spp., *Enterobacter aerogenes, Escherichia coli, Shigella flexneri, Klebsiella oxytoca* and *Acinetobacter* spp. This enzyme has been associated with gene families including the *armA, rmtA, rmtB, rmtC* and *rmtD* genes. The nucleotide and amino acid alignment demonstrates that these genes have originated from the *Actinomicetales*, including *Streptomyces* and *Micromonospora* spp. [133].

Recently identified plasmid-mediated quinolone resistance (PMQR) genes *qnr*A and *qnr*S have been shown to originate from water-borne environmental bacteria, *Shewanella algae* and *Vibrio splendidus*, respectively [91,134]. Cattoir *et al*., (2008) identified qnrS gene from

Aeromonas punctata subsp. *punctata* and *A. media* isolated from water samples collected at diverse locations from the Seine River (Paris, France) [135]. The *qnr*S1 variant gene has subsequently been reported from several enterobacterial isolates from many developed and developing countries [135]. From an activated sludge bacteria isolated from a wastewater treatment plant in Germany, *qnr*S2 a variant of *qnr*S1, was identified on transferable IncQ-related plasmid (pGNB2) [92]. The same *qnr*S2 was reported from a single non-Typhi Salmonella clinical isolate from the United States [136]. Another plasmid-mediated quinolone resistance efflux pump gene *Qep*A, first characterized in multiresistant *E. coli* isolates recovered in Japan in 2002 and in Europe (Belgium) in 2005. Sequence analysis of the *qepA* gene has revealed a potential origin from environmental isolates, such as *Actinomycetales* (*Nocardia farcinica, Streptomyces globisporus*, and *Streptomyces clavuligerus*) [126].

First case of a plasmid-mediated colistin resistance mechanism was reported in 2016 [137]. In this *mcr*-1 gene that encode for the phosphoethanolamine transferase enzyme responsible for the addition of phosphoethanolamine to lipid A in *E. coli* and *Klebsiella pneumoniae* isolated from raw meat, animals and inpatient with infection were reported. The same *mcr-1* genes have also been found in *E. coli* isolates from marine birds' herring gulls in Europe and kelp gulls in South America [138]. Enzyme ethanolamine phosphotransferase (EptA, PmrC) that modify LPC was detected in aquatic bacterium *Shewanella algae* [139]. Similar enzyme was also reported from *Paenibacillus sophorae*, *Enhydrobacter aerosaccus*, *Moraxella catarrhalis*, *Vibrio halioticoli*, *Psychrobacter* sp and *Dichelobacter nodosus*. Amino acid sequence of the mcr-1 gene product, showed 63%, 63%, 59% and 54% identity with amino acid sequence of phosphoethanolamine transferase (EptA) enzyme of *Paenibacillus sophorae* (WP_036596266.1), *Enhydrobacter aerosaccus* (WP_007116571), *Moraxella catarrhalis*

(WP_003672704) and *Dichelobacter nodosus* (WP_012030864) respectively [137]. This suggested that aquaculture could be the origin of transferable colistin resistant *mcr* gene

1.6. Antibiotic resistance in *Klebsiella pneumoniae*

Klebsiella pneumoniae belonging to family Enterobacteriaceae was first described in 1885 as an opportunistic pathogen infecting only neonates and immunocompromised persons [14]. *K. pneumoniae* are ubiquitous in nature and commonly found in sewage, soils, vegetation, salt water, brackish water and fresh water [140,141] as well as a commensal resident of healthy human oropharynx, upper respiratory track and nasopharyngeal track [14,142,143]. It stays long in the hospital environment and can spread easily among humans through ventilators (breathing machines), catheters or wounds (caused by injury or surgery), intravenous (vein), from patient to patient via the contaminated hands of health care providers, from animal pet to human, causing rapid hospital and community acquired infections [14,144,145]. In recent few decades, it has successfully emerged as an opportunistic nosocomial pathogen frequently associated with both hospital and community acquired infections leading to increased morbidity and mortality [146]. In developing countries, incidence rate of respiratory tract infection and bacteremia in neonatal varies between 4.1 and 6.3 per 1000 live births with fatality rate of 18-68% [147]. Besides earlier reported diseases, liver and brain abscess has been reported a distinctive syndrome of community-acquired *K. pneumoniae* septicemia [148,149]. This syndrome is characterized by high mortality (10 to 40%) and in some cases, had been complicated by meningitis or endophthalmitis [150].

K. pneumoniae called “superbugs” had wide array of antimicrobial resistance mechanisms as it had both intrinsic resistance to some antibiotics as well as strong propensity to acquire resistance via acquisition of mobile genetic elements containing resistance determinants

[151]. In this fashion, *K. pneumoniae* expressing extensively drug-resistant (XDR) and pan drug resistant (PDR) phenotypes with limited or no therapeutic option have resulted in inclusion of *K. pneumoniae* within the ESKAPE list of pathogens by the Infectious Diseases Society of America [11].

The production of β-lactamase enzymes in *K. pneumoniae* have attracted much attention owing of their clinical relevance as these enzymes hydrolyzed the amide bond of the β-lactam ring resulting in the antimicrobial ineffective [152]. Extended spectrum β-lactamase (ESBL) belonging to the Ambler class A group having serine at active site was first identified in 1983 from a nosocomial *K. pneumoniae* strain in Germany [153]. Worldwide occurrence of ESBLs producer isolates among clinical setting are changing rapidly over time giving a variant with superior spectrum [154]. Global data from 266 center reported that the rate of *K. pneumoniae* ESBL producing bacteria was highest in America (44%) followed by Asia pacific (22%) and Europe (13%) [155]. The presence of both SHV (sulfhydryl variable) and TEM (first three latter of patient's name "Temoniera") enzymes is very common in *K. pneumoniae*, particularly in clinical isolates, and these enzymes represent a means by which strains can potentially become resistant to the cephalosporin antibiotics [156]. It has been reported that the SHV enzymes are 'universal' in the *K. pneumoniae* chromosome, although they can additionally be found on plasmids [157]. TEM and SHV hydrolyze penicillins and narrow spectrum cephalosporins, such as cephalothin or cefazolin. 190 bla_{TEM} and 161 bla_{SHV} variants having single or multiple amino acid substitutions from the parental strains has been reported in *K. pneumoniae* (*Klebsiella pneumoniae* MLST database). These variants showed variable levels of resistance to cefotaxime, ceftazidime and other broad-spectrum cephalosporins and monobactams such as aztreonam by lowering the Km values (increasing the affinity) for the β-lactams [158].

In 1987, other types of ESBLs, CTX-M (active on Cefotaxime) was identified in *K. pneumoniae* that had less than 40% homology with TEM and SHV types [159,160]. Some minor β-lactamase GES-1 (Guiana extended spectrum) was first detected in a *K. pneumoniae* isolate from France in 1998 [161]. A worrying aspect is that some of these enzymes may extend their spectrum of hydrolysis to include carbapenems through single point mutations, as illustrated by GES-2, with $^{Gly}170^{Asn}$ substitution [162] and GES-7, with $^{Ala}126^{Leu}$ substitution [163], with GES-2 being poorly inhibited by clavulanic acid. During 9-year period analyzed (1997-2005) an increase in frequency from 8% to 18% of ESBL-positive *K. pneumoniae* strains with an average frequency of 24% in hospitals in the Czech Republic is found with prevalence of 39% and 13% in the ICUs and standard wards, respectively [164,165]. Similarly, high resistance rate of up to 40% in *K. pneumoniae* to ceftazidime was found in France with prevalence of 40% in the ICU and 16% in standard hospital departments [166,167].

In addition to ESBLs production, Ambler Class C β-Lactamases (*Amp*C β-lactamases) which are plasmid mediated enzymes are being reported increasingly among *E. coli* and *K. pneumoniae* worldwide. *Amp*C enzymes of group 1 and 1e, in terms of functional classification [168] hydrolyze both oxyimino- and 7-α-methoxy-cephalosporins (cefoxitin and cefotetan), and are not inhibited by β-lactamase inhibitors, such as clavulanic acid [169]. *Amp*C β-lactamases are usually either not active or very poorly active on aztreonam and on the zwitterionic oxyimino-cephalosporins (such as cefepime and cefpirome). *Amp*C-producing isolates are typically susceptible to carbapenems. In Ireland *Amp*C mediated resistance in *Klebsiella* within a tertiary care hospital was not due to the spread of a single clone but found to be because of transfer of a mobile genetic element. This result suggested a need for continuous surveillance for the prevalence and evolution of *Amp*C enzymes among enteric bacteria [170].

Metallo β-lactamase (MBL) belongs to Amber class B having zinc at their active site. These β-lactamases were active against penicillins, different generation of cephalosporins and carbapenems but incapable of inactivating aztreonam [171]. Of eleven MBL groups described to date, only three have been identified in *K. pneumoniae,* including IMP (active on imipenem), VIM (Verona integron-encoded metallo β-lactamase) and NDM (New Delhi metallo β-lactamase). Different variants of afore mentioned MBL groups, with significant variations in hydrolytic efficiency, even between enzymes of the same type, has been reported. The amino acid similarity between different variants ranges from 36% to 99.7% for the IMPs, 74.3% to 99.6% for the VIMs, and 98.6% to 99.6% among NDM variants [172]. NDM-1 is a most recently discovered transferable subclass of the B1 group of MBLs having only 32.4% identity with VIM-1/VIM-2 and possesses novel amino acids near the active site along with an additional sequence at positions 226 to 228 that are not present in other MBLs [172,173]. NDM-1 was first identified in 2008 in Swedish resident of Indian origin patient, who had been previously hospitalized in New Delhi due to urinary tract infection caused by carbapenem-resistant *K. pneumoniae* during his visit to India [172]. NDM-1 is a carbapenemase that hydrolyzes all β-lactams except monobactams and is susceptible to EDTA but not to clavulanic acid [129,174]. Most of the NDM-1 infection cases reported does not have a history of hospital admission in India, but the presence of NDM-1 β-lactamase producing bacteria in environmental samples in New Delhi has important implications for people living in the city who are reliant on public water and sanitation facilities. Kumarasamy *et al.*, (2010) has reported that NDM-1 positive bacteria are known to be circulating in the Indian community through drinking water (tap water) and seepage samples (water pools in streets or rivulets) [175]. In an analysis from New Delhi,

NDM-1 gene was detected in 2 of 50 drinking water samples and 51 of 171 seepage samples [176].

Carbapenemase belong to Ambler class D have serine at active site. The first discovered OXA genes, such as OXA-1, were only found to confer resistance to the penicillins and had no effect on other β-lactam antibiotics. These were predominantly present in *Acinetobacter spp.* and *Pseudomonas aeruginosa* and to a lesser extent in members of the *Enterobacteriaceae* including *E. coli* and *K. pneumoniae* [177]. More than 400 bla_{OXA} derivatives had been assigned in which 229 bla_{OXA} variants were reported in *K. pneumoniae* that are capable of conferring resistance to the cephalosporins and carbapenems (http://www.lahey.org/studies/). The most dominant variant of bla_{OXA} is OXA-48 type and it is widely prevalent in North Africa, the Middle East, and the Indian subcontinent with various unrelated clonal types [178]. Plasmid-mediated KPC (*K. pneumoniae* carbapenemase) was first detected in a clinical isolate of *K. pneumoniae* from North Carolina in 1996 [179] and very soon it has become common in the east coast of the United States especially with emergence of new variants KPC-2 and KPC-3 in Baltimore, MD and New York respectively [180,181]. Among the KPC variants, KPC-2 and KPC-3 were also reported to be predominant worldwide [182,183]. European Centre for Disease Control and Prevention in 2010 had estimated that approximately 49% of all *K. pneumoniae* isolates from Greece were carbapenem-resistant in which approximately 53% were KPC-2-producers [184]. A report from Dhaka city, Bangladesh showed that 4.79% carbapenem-resistant *K. pneumoniae* isolates were KPC positive [185]. Even when monotherapy or combination therapy used, the overall mortality with KPC-associated infections has been estimated to be between 22% and 59% [184].

Successful treatment outcomes of bacterial infections with quinolones have led to increasing and the extensive use of fluoroquinolones. This in turn, has led to mounting resistance

to these antimicrobials [186]. High rates of resistance to quinolones have been reported from different parts of the world. Quinolone resistance generally results from stepwise chromosomal mutations [187]. Target alterations are predominantly in the quinolone-resistance determining region (QRDR), a portion of the DNA-binding surface of the topoisomerases at which amino acid substitutions can diminish quinolone binding and subsequently cause resistance to quinolones [187]. Recently, plasmid-mediated quinolone resistance (PMQR) has been discovered. This resistance comprises the production of Qnr proteins protecting the targets against the effects of quinolones [50]. In *K. pneumoniae*, the first plasmid-mediated quinolone resistance gene, named *qnr*A1, was detected from the USA in 1998 [188]. Later, the *qnr*B, *qnr*C, *qnr*D and *qnr*S genes were first reported from *K. pneumoniae, Proteus mirabilis, Salmonella enteric* and *Shigella flexneri* respectively [189,190,191].

Aminoglycosides are a complex family of compounds and the classification can be based on the chemical structure. There are different structural classes of aminoglycosides, characterized by having an aminocyclitol nucleus (streptamine, 2-deoxystreptamine (DOS) or streptidine) linked to amino sugars through glycosidic bonds [192].

1.7. Clonal relationship of *Klebsiella pneumoniae*

From the past three decades, numerous new molecular techniques have been developed and replaced old phenotypic systems in order to identifying the clonal nature of strains. Such approaches can help obtain information about the source of infection, trace their dissemination route of infection and understand their epidemiology, thus assisting in halting an outbreak and providing knowledge on the spread of an infection [193].

A highly discriminating nucleotides-sequence based molecular typing method called Multi locus sequence typing (MLST) is used for the clonal determination of most the bacteria.

Up to July 2016, there are 113 different MLST schemes for important bacterial genus including the *K. pneumoniae* have been reported (http://pubmlst.org/databases.shtml). Sequence type showed that specific clones are often found to be responsible for outbreaks of infections and dissemination of multidrug-resistant *K. pneumoniae* in the community [193]. Turton *et al.*, (2007) have reported *magA* gene, part of the serotype-specific region of the K1 capsule gene cluster responsible for the increasing prevalence of invasive liver abscesses in Korea, belonging to the main cluster ST23 [194]. This finding was supported by Chung *et al.*, (2007), where they demonstrated that increasing frequency of *K. pneumoniae* serotype K1 causing liver abscesses were attributable to the nationwide spreading of the ST23 strain throughout the country [195]. In a study conducted by Damjanova *et al.,* (2008) on Hungarian epidemic clone (HEC) prototype isolate and 10 non-epidemic isolates from an outbreaks occurred in 2003, three distinct allelic profiles were reported [196]. ST15 (allelic profile: 1-1-1-1-1-1-1) corresponding to Hungarian epidemic clone (HEC) prototype isolate, ST11 (allelic profile: 1-3-1-1-1-3-4) corresponding to epidemic clone III (EC III) and the novel ST147 (allelic profile: 4-3-6-1-7-4-38) corresponding to epidemic clone II (EC II). Later they also reported these three ECs accounting ~70% of the ESBL positive *K. pneumoniae* of which 36% belong to the nosocomial blood stream infection caused by *K. pneumoniae* in 2005. In 2008, first KPC-producing *K. pneumoniae* strain FIPP-1 belonging to ST258 was isolated from an inpatient with a complicated intra-abdominal infection at Florence University Hospital, Italy. The major concern of KPC-positive *K. pneumoniae* belonging to ST258 was their greater spreading propensity having remarkable consequences for the epidemiology of antibiotic resistance [197]. Samuelsen *et al.*, (2009) have shown the emergence of multi-resistant *K. pneumoniae* ST258 clone (closely related to the CTX-M-15-producing ST11 clone earlier described in Hungary) from Norway and Sweden [198]. These

KPC-producing *K. pneumoniae* isolates are disseminated internationally by multiple import events and local transmission of the biologically fit clones of *K. pneumoniae* with a high potential to gain resistance mechanisms. In another study, Kitchel *et al*., (2009) reported that in the CDC's KPC-producing *K. pneumoniae* PFGE database, a single lineage ST258 account for almost 70% of all isolates collected throughout the United States as well as isolates from India and Israel [199]. An outbreak in Israel due to KPC-3- producing *K. pneumoniae* ST 258 isolate was isolated from 10 different states and same ST was also isolated from the many regions of the United States [200]. This suggested the possible international dissemination of these strain. A single-locus *ton*B variant of ST258, a predominant clone of KPC-producing *K. pneumoniae* ST11 has been reported from seven hospitals in Zhejiang, Jiangsu and Anhui province, China [201]. Phylogenetic analysis of *ton*B ST258 variant and ST11 have grouped these clones into a single clonal complex CC258 that includes ST11, ST258 and another five sequence types *viz.* ST270, ST340, ST379, ST407 and ST418. The presence of ST11 in *K. pneumoniae* isolated from pets (dog and cat) in Spain, with the resistance genes $bla_{\text{DHA-1}}$, $bla_{\text{SHV-11}}$, and *qnr*B4 indicated a new reservoir responsible for the dissemination of ST11 epidemic clone and resistance genes [202]. Curiao *et al.*, (2010) reported two new clones ST384 and ST388 from Madrid (Spain) associated with KPC-3b *K. pneumoniae* and were resistant to all β-lactams including meropenem, imipenem and aztreonam [203]. Among them ST388, was a persistent clone found since 1998 at their institution and harbored the $bla_{\text{CTX-M-10}}$ gene whereas ST384 was represented an emerging highly infectious *K. pneumoniae* clone.

In a study, Giske *et al.*, (2012), showed that the dissemination of $bla_{\text{NDM-1}}$ in India, Sweden, and the United Kingdom were due to diverse sequence types (STs) [204]. The most common sequence type was ST14 (33.33%) belonging to serotype K2, found in all three

countries. Rest isolates belonged to ST11 (12.82%), ST149 (7.69%), ST231 (7.69%), ST625 (7.9%), ST147 (5.12%) and ST273 (2.56%) sequence type. Yong *et al.*, (2009) have reported a new sequence type ST14 from India having metallo-β-lactamase gene, carried on a unique genetic structure blaNDM-1 and a novel erythromycin esterase in *K. pneumoniae* [172]. Another *inf*B single-locus variant of ST14, ST15 has been reported from Midwestern USA [201].

1.8. Virulence and pathogenic factors of *Klebsiella pneumoniae*

Virulence factors are viewed as the properties (gene products) that alter host–microbe interaction and enable a microorganism to establish itself on or within a host of a particular species and enhance its potential to cause disease [205]. Identification of virulence factors is important in understanding bacterial pathogenesis and their interactions with the host. Several researchers have described different virulence factors in *K. pneumoniae*, that favor the pathogen's ability to cause infection, such as fimbrial & non-fimbrial adhesins, hypermucoviscocity, biofilm forming ability, iron-scavenging systems, capsular serotype, lipopolysaccharide and toxin production that are useful for adherence to host, pathogen's survival, self-defense and establishment of pathogenicity.

1.8.1. Adherence to host cell

1.8.1.1. Fimbriae mediated adhesin

First step in development of colonization and infection is adhesion of micro-organism to mucosal and epithelial cell surface. A filamentous organelle expressed on the bacterial cell surface called fimbriae contain adhesins (often called haemagglutinins) which bind with molecular receptors in the host to facilitate adherence to specific tissue surfaces [206]. These structures are typically as long as 10μm and diameter vary in between 1-11 nm and are extra stiff than flagella [14,207]. *Klebsiella* spp. produces two types of fimbriae based on whether adhesin

interaction can be inhibited by D-mannose. Based on this thick channelled type 1 fimbriae with dimension 6-8 nm width, 1-2μm length, having ability to agglutinate guinea pig erythrocytes in the absence of α-D-mannose and are commonly referred to as mannose-sensitive haemagglutination (MS) [14] and other are thin non-channelled type 3 fimbriae that only agglutinate tannic acid treated erythrocytes and are unaffected by the presence of α-D-mannose commonly referred as mannose resistant haemagglutination [208,209]. Mostly type 1 fimbriae were present on clinical isolates of *K. pneumoniae* (80%), but few *K. oxytoca* strains also express type-1 fimbriae [210]. Clinical and fecal carriage isolates of *K. pneumoniae* express type 1 fimbriae in higher proportion than environmental strains [210]. The genetic determinant*s of* type 1 fimbriae consisting of eight genes expressed from a chromosomal *fim* gene cluster. The *K. pneumoniae* type 1 fimbrial genes are like those of the *E. coli* type 1 fimbrial gene cluster and are capable of complementing type 1 fimbrial mutations in *E. coli* [211].

The genetic determinants of type 3 fimbriae in *Klebsiella*, carry a copy of the fimbrial gene cluster with five genes which encode for structural components of the fimbrial appendage [212]. These genes, termed *mrk* for the mannose resistant *Klebsiella*-like hemagglutination associated with the type 3 fimbriae, are *mrkABCDF*. The genomic sequencing of several strains of *K. pneumoniae* has shown that all possess a chromosomal copy of the *mrk* gene cluster. Indeed, the *mrk* gene cluster in *K. pneumoniae* isolates is constantly associated with the bacterial chromosome, whereas the initial identification of the plasmid encoded *mrk* in IA565, showed relatively few strains also possess an additional plasmid-borne copy of this gene cluster. Type 3 fimbriae have been consistently shown to play an important *in vitro* role in the *K. pneumoniae* to initially adhere to and from multicellular communities, called biofilms, on both abiotic and biotic substrates. Initially, the type 3 fimbriae were found to mediate adherence to respiratory tract

tissue at both the basolateral surface of tracheal cells and the basement membrane regions of bronchial epithelia [208]. Martino *et al*., (1996) found a novel fimbrial adhesin termed "KPF-28" [213]. It is a long, thin, and flexible fimbria having diameter 4–5nm and 0.5–2μm long. This adhesin was also encoded on a plasmid associated with strains producing SHV-4 extended-spectrum β-lactamase and involved in the adherence to the human carcinoma cell line Caco-2.

1.8.1.2. Non-Fimbriae mediated adhesin

A non-fimbrial adhesin called "CF29K." (29 kDa) was isolated by Darfeuille-Michaud *et al*., (1992) which is responsible for the adhesion of *K. pneumoniae* to intestinal cells. Gene of "CF29K." was encoded on a large conjugative plasmid, along with genes for broad-spectrum cephalosporin resistance and aerobactin [214].

1.8.1.3. Hypermucoviscocity

The formation of mucoviscous string greater than 5mm [215] or 10 mm [216] when a standard bacteriologic loop/needle is passed through a colony is characterized as the strain having the hypermucoviscocity (HMV) phenotype. In contrast to diseases caused by classical *K. pneumoniae* to immunocompromised, hospitalized or aged patients, the HMV strains caused infection to healthy individuals [216]. Fang *et al*., (2004) reported the liver abscess caused by hypermucoviscocity positive strains of *K. pneumoniae* was high (98%) as compared to other sites of *K. pneumoniae* infection (17%) [150]. Clinical *K. pneumoniae* isolates with the HMV phenotype are commonly associated with bacteremia and distinct invasive syndromes such as primary liver abscesses with destructive syndrome like metastatic meningitis and endophthalmitis, osteomyelitis and brain abscess [217]. These hypermucoid isolates show high resistance to phagocytosis, reduced serum sensitive and were more virulent in animal studies in comparison with non-mucoid isolates [218]. The genes associated with the HMV phenotype in

K. pneumoniae strains were *magA* (mucoviscosity-associated gene A) and *rmpA* (regulator of the mucoid phenotype). The *magA* gene has been found to be restricted to K1 isolates and more often found in liver invasive strains (98%) as compared to non-liver-invasive strains (29%) in Taiwan [215,216]. The *rmpA* gene is found in K1, K2, and other serotypes and associated with tissue abscess [215,217].

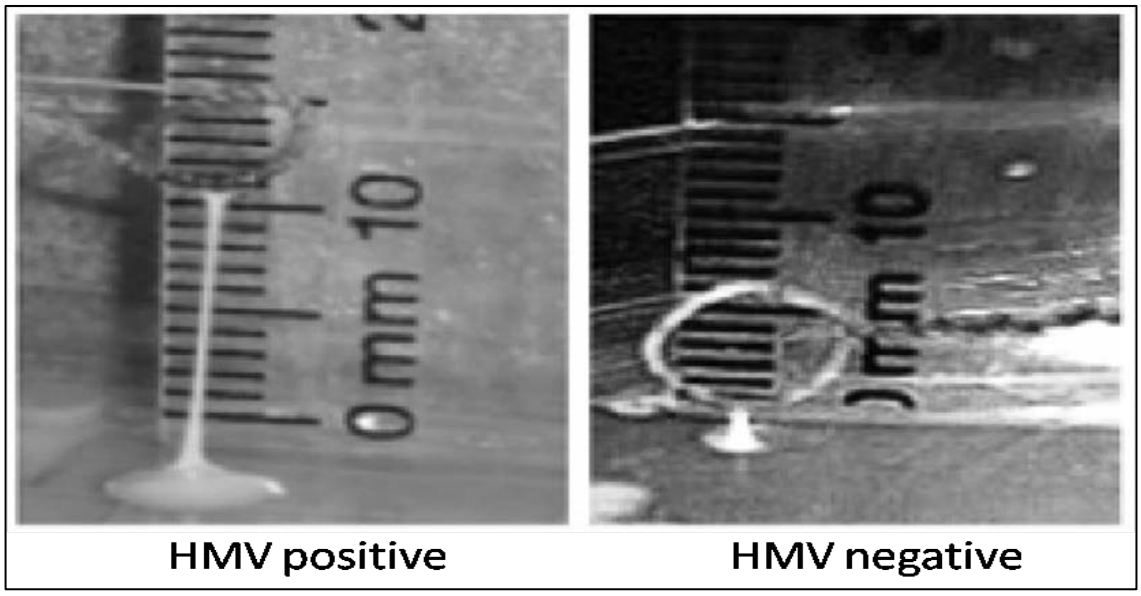


Figure 1.6 Modified string test for determination of the hypermucoviscocity phenotype of *K. pneumoniae* [215].

1.8.1.4. Hydrophobicity

The surface hydrophobicity of *Klebsiella* is a physio-chemical property highly influenced by the presence of capsular (K) and lipopolysaccharides (O) antigens [219,220]. Hydrophobicity is an indirect way to determine adhesion properties as micro-organism may adhere to a substratum via hydrophobic effect if the association sites possess sufficiently high densities of apolar areas. Williams *et al.*, (1988) had reported that not the loss of the capsular polysaccharide alone but loss of both K and O antigens (O^- : K^-) resulted in a substantial (all most 100%)

increase in surface hydrophobicity [219]. Benedi *et al.*, (1989) had shown that the capsular mutant strains (derive by UV irradiation) and the unencapsulated strain (KT717) was more hydrophobic (70%) as compared to (30%) by the encapsulated strains which is contradictory to result by Williams *et al.*, (1988) [220]. They also found that capsular mutants having similar susceptibility to antimicrobial agent as wild type but showed resistant toward serum bactericidal activity. In another study, Merino *et al*., (2000) showed that the O5-antigen LPS is an important factor for the adhesion to uroepithelial cells possibly because of its surface charge and hydrophobicity properties [221].

1.8.2. Survival means in host cell and self-defense mechanism

1.8.2.1. Biofilm production

Biofilm are complex three-dimensional structure formed by communities of inter or intra species microorganisms attached to a surface or interface enclosed in an exopolysaccharide matrix of microbial and host origin [222]. It provides protection to antibiotic treatment, attack by phagocytosis and harmful molecules and facilitates bacterial communication leading to expression of virulence determinants. In 1980, *in vitro* biofilm formation in *K. pneumoniae* was reported, but *in vivo* biofilm of *K. pneumoniae* in bladder epithelial cells with asymptomatic urinary tract infection was reported in 1992 [223,224]. Biofilm formation proceeds in two stages: a rapid attachment of the bacteria to the polymeric surface is followed by a more prolonged accumulation phase that involves cell proliferation and intercellular adhesion. The ability to adhere to materials and to form biofilm is an important feature in the pathogenesis of *Klebsiella* associated community acquired infections (CAIs) due to the colonization of the polymeric surface by forming multi-layered cell clusters, embebbed in extra cellular material [225]. Genes associated with biofilm formation in *K. pneumoniae* strain are *treC* [226] *mrk*A [227] and *celB*

[222]. *K. pneumoniae* is one of the most frequent causes of catheter-associated urinary tract infections (CAUTIs). The type 3 fimbriae of *Klebsiella pneumoniae* influence the development of biofilms in plastic, continuous flow-through chambers [228].

1.8.2.2. Siderophore production

Iron is one of the essential macro-nutrients for most bacterial species as it plays an important role in the electron transport chain and for various other enzymes as cofactor. To grow successfully in host tissues, where iron is present as complex to carrier molecules like transferrin, lactoferrin and heme [229], bacteria must be able to obtain iron from these host transport proteins. At this lower iron condition, bacteria produce siderophore, low molecular weight iron chelators to solubilize and utilize iron from host and environment. Different species have evolved a variety of secreted factors to obtain iron. Among the genus *Klebsiella*, three siderophore systems are present: enterobactin, aerobactin, and yersiniabactin [14,229]. Enterobactin (also known as enterochelin, a cyclic trimer of 2, 3-dihydroxybenzoylserine) is a catecholate siderophore that was produced by all the clinical isolates [230,231]. Aerobactin is a hydroxamate siderophore which is produced by a smaller fraction of *Klebsiella* strains and has a lower affinity for free Fe^{+3} than either enterobactin or yersiniabactin [231,232]. Yersiniabactin found among even fewer isolates is the phenolate siderophore [229].

1.8.2.3. Lipopolysaccharide

LPS is found in the outer membrane of all Gram-negative bacteria and comprises three components: the outer leaflet of the outer membrane forms of Lipid A attached via second component core oligosaccharide to the O-antigen side-chain polysaccharide which extends from the cell surface [233]. Nine O antigen types have been distinguished in *K. pneumoniae* O1 to O9, of which O1 is the most frequent antigen [234]. Due to long O-polysaccharide side chains of

LPS, it provide protection from complement mediated killing as after activation of C3b complement system, by formation of the lytic membrane attacking complex (C5b–C9) at a more distant from the bacterial outer membrane which results in a steric hindrance effect to O-Antigen [206].

The lipid A anchors the LPS molecule into the outer membrane and is also an endotoxin, stimulating the immune system through agonism of Toll-like receptor 4 (TLR4) which is present on macrophages, dendritic cells and other cell types inducing NF-kB mediated production of cytokines [235]. The genes cluster responsible for the synthesis of the core lipopolysaccharides of *K. pneumoniae* has been genetically characterized and contain six genes [236] as shown in **fig 1.7**. The first loci encode the transmembrane (*wzm*) and ATP-binding components of the ABC-2 transporter (*wzt*), which was formerly called as *rfb*AB, *glf* encodes a UDP-galactopyranose mutase, which generates uridine 59-diphospho-a-D-galactofuranose (UDP-Galf), the biosynthetic precursor of galactofuranosyl residues and the remaining three genes (*wbb*M, *wbb*N, and *wbb*O) encode three galactosyltransferases and form a membrane-localized glycosyltransferase complex [56,237,238]. *wbb*O is the last gene of the wb cluster; the WbbO gene product is the first dedicated enzyme in the assembly pathway for the O1 antigen. Disruption of one of the ORFs, orf10, resulted in a two-log-fold reduction of virulence in mice, as well as in a strong reduction in the capsule amount [239]. A monoclonal antibody that is specific for the genus *Klebsiella* was identified that binds to a conserved epitope of the core region [240].

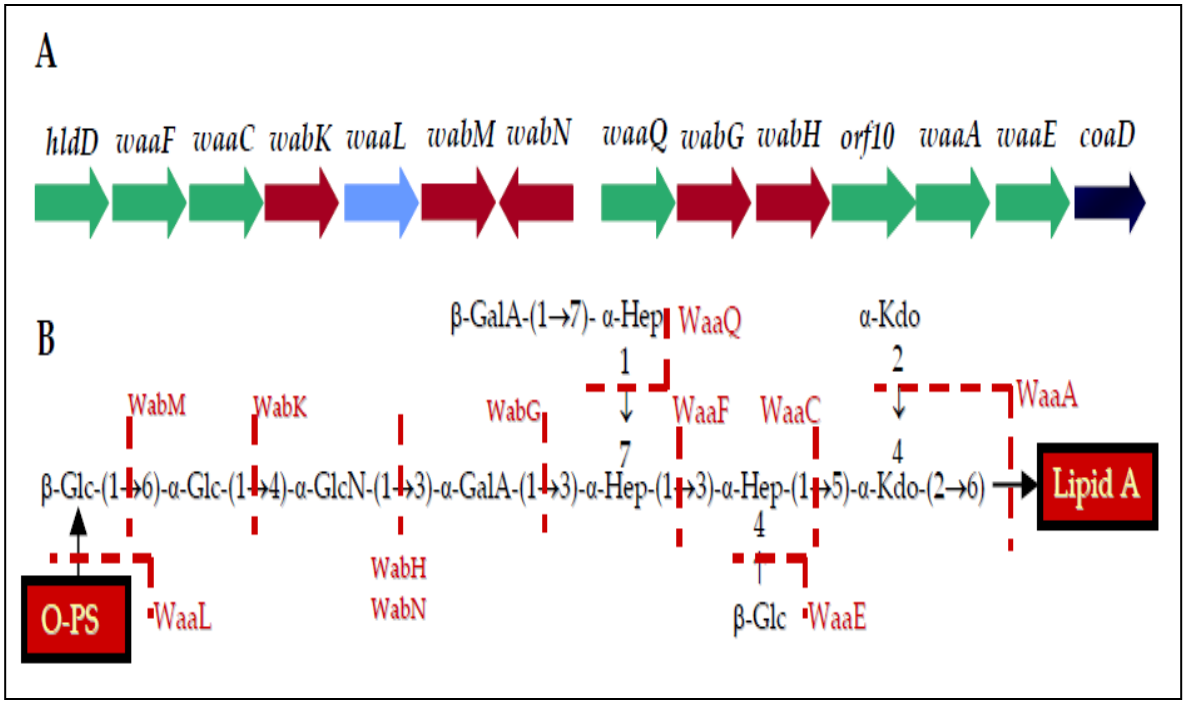


Figure 1.7 Genes cluster of the core lipopolysaccharide of *K. pneumoniae*

(A) Cluster *waa* of the genes involved in the core OS biosynthesis of *Klebsiella pneumoniae* 52145. (B) Structure of the core OS from *Klebsiella pneumoniae* 52145. Dotted lines identify the known or predicted genetic determinants involved in the indicated linkages [236].

1.8.3. Establishment of pathogenicity by Toxin production

The factor responsible for *K. pneumoniae* pathogenicity includes the production of heat-labile and heat-stable endotoxins [241]. The O1 lipopolysaccharide has been linked with the extensive tissue necrosis that complicates *Klebsiella* infections [242]. The production of an extracellular toxic complex (ETC) that has been shown to be responsible in mice for lethality and extensive lung necrosis is composed of 63% capsular polysaccharide, 30% lipopolysaccharide, and 7% protein [242]. Other factors KvgAS, a two-component system [243], capsular polysaccharide synthesis enzymes protein-tyrosine kinase & phosphotyrosine-protein

phosphatase [244] and HtrA (which may be involved in serum complement resistance [245] are responsible for the pathogenicity caused by *K. pneumoniae*.

1.9. Diseases caused by *Klebsiella pneumoniae*

Global studies indicated that in Europe and North America, rate of nosocomial infections of all hospitalizations were 5%-10% whereas in Asia, Latin America, and sub-Saharan Africa this rate was more than 40% [246]. *K. pneumoniae*, ranked second to *E. coli* as opportunistic nosocomial gram's negative pathogen is responsible for hospital-acquired infection and community-acquired infection depending on whether *Klebsiella* [14]. Since its first description in 1885, it has been recognized as most common etiological agent for urinary tract infections (UTI), nosocomial pneumonia, lower respiratory tract infection (LRTI), bacteraemia and septicaemia in neonates, immunocompromised, hospitalized or aged patients. *Klebsiella pneumoniae* hospital-acquired infections include severe symptoms like rapid onset of high fever, and haemoptysis (currant jelly sputum) whereas community-acquired infection have bulging interlobar fissure and cavitary abscesses with pragmatic mortality rates range from about 25 to 50% [247]. Mortality rates are as high as 50% and approach 100% in hospitalized, immunocompromised patient with primary diseases such as diabetes mellitus and acute alcoholism [15,248]. Most human and animal infections are caused by *K. pneumoniae* subspecies *pneumoniae* whereas subspecies *rhinoscleromatis* and *ozaenae* causing a minority of infections [14]. But from last 2-3 decade increased frequency of *K. pneumoniae* liver abscess (KPLA), *Klebsiella* endogenous endophthalmitis (KEE), meningitis, ankylosing spondylitis and acute arthritis have been reported globally that were very rare in the past.

1.9.1. Pyogenic liver abscess (PLA)

Since 1981, a distinctive syndrome of community-acquired *K. pneumoniae* septicemia with pyogenic liver abscess (PLA) with percentage rising from 30% in the 1980s to over 80% in the 1990s has been reported and contributed to the endemic feature of the disease in Taiwan with its annual incidence increasing steadily to 6.4/100000 population in 1996 to 2004 [249,250]. This syndrome is characterized by high mortality rate up to 10 to 40% even with the introduction of modern diagnostic imaging techniques that accurately locate the abscess along with the progress of image-guided percutaneous aspiration and drainage which abridged the risk of death. The symptoms of the disease characterized by fatigue, anorexia, nausea, diffuse abdominal discomfort, pleuritic chest pain, jaundice and fever [251,252]. About 11% to 12% reported patients with liver abscess reported are associated with septic metastatic endophthalmitis, septic pulmonary emboli, pyogenic meningitis, brain abscess, prostatic abscess, septic arthritis osteomyelitis, endocarditis or psoas abscess [253,254]. A retrospective study of 110 cases of primary liver abscess caused by *K. pneumoniae* during 2001-2002 showed presence of diabetes, metastatic infection, meningitis and endophthalmitis in 60.9%, 15.5%, 64.7% and 23.5% patients respectively with overall 10.0% mortality rate [255]. The K1 capsular serotype is the predominant serotype of *K. pneumoniae* strains causing liver abscess [256]. In both Taiwan and Korea, K1 capsular serotype is accounted for about 60% of *K. pneumoniae* strains causing liver abscess [195,257,258]. Rahimian *et al*., (2004) elucidated that the liver abscess usually occurs in patients of Eastern Asian countries, indicating a possible genetic linkage to disease susceptibility [259]. Lederman and Crum (2005) reported 67% cases of pyogenic liver abscess caused by *K. pneumoniae* occurred in the patients of Filipinos compared with only 29% non- Filipino patients have liver abscesses caused by *K. pneumoniae* along with different bacterial pathogens [251].

The presence of variable underlying diseases or conditions such as diabetes mellitus, heavy alcohol drinking, biliary tract diseases, malignancy, liver cirrhosis, end-stage renal disease, intra-abdominal infections, history of abdominopelvic surgery, history of steroid use and history of previous antibiotic use considered as risk factors for the K1 serotype *K. pneumoniae* liver abscess [260]. Recently, many cases of PLA associated with non-metastatic colorectal carcinoma (CRC) have been reported worldwide in which *K. pneumoniae* is predominant PLA pathogen with significantly higher rate of subsequent CRC [261]. The microbiological analysis showed that *K. pneumoniae* account for 50% colorectal cancer- related PLA in the East Asian than non-East Asian countries where as demographical analysis reported the frequency of colorectal cancer- related PLA was more common in age groups of 61-70 years with 1.5:1 male-to-female ratio without any geographical differences [252]. The children are not the exception as global incident of PLA is ranges from more than 79 per 100,000, 1 out of 140, 25 per 100,000 and 11 out of 100,00,00 pediatric admissions in India, Brazil, USA and Denmark respectively with significant morbidity and mortality [262].

1.9.2. Endogenous endophthalmitis

Endogenous endophthalmitis (EE) is a relatively rare but complicated infection caused by a haematogenous spread of bacteria from a distant anatomical site to the interior of the eye with characteristic symptoms of severe anterior chamber inflammation, ocular pain, blurred vision, swollen eyelids, vitreous haze, or choroidal abscess [263]. Before the mid-1980s, approximately 100 patients were reported with endogenous endophthalmitis caused by *K. pneumoniae* [264]. After that an increasing number of cases being reported in Asia and around the world that comprises 2% to 15% of all cases of endophthalmitis with very high prevalence 54% to 61% caused by *K. pneumoniae* in Asian countries [265,266]. Even after successful treatment with

both intravenous and local ocular antimicrobial therapy for *K. pneumoniae* endophthalmitis, 89% eyes have visual outcome worse than ability to count fingers [267]. The poor outcome of endogenous *K. pneumoniae* endophthalmitis therapies were due to the anatomical and physiological infirmity of the eye as well as eye's natural barriers, the blood-retinal barriers (BRB) and the blood-aqueous humor barrier (BAB) that complicate the delivery of antimicrobial and anti-inflammatory therapy that can effectively reduce the response of host immunity [268]. About 13% of *K. pneumoniae* endogenous endophthalmitis are complication of liver abscesses with the predisposing factors of diabetic and impaired immune function in diabetic's patients [269,270,271].

1.9.3. Community acquired bacterial meningitis

Bacterial meningitis is considered as one of the most perilous infectious diseases with several complications and high mortality. Among Gram negative bacteria as etiological agents, *K. pneumoniae* has one of the commonest causes of community acquired bacterial meningitis. It accounted for 20% of all bacterial meningitis in South-East Asia and North-East Asia with septic metastatic complications [272]. The proportion of cases of bacterial meningitis due to *K. pneumoniae* in one Taiwanese hospital increased from 8% during 1981 through 1986 to 18% during 1987 through 1995 [273]. Another ten years retrospective study in Iran, reported that 5.6% children suffering from acute bacterial meningitis had positive *K. pneumoniae* culture from cerebrospinal fluid [274]. Apart from Taiwan, cases of *K. pneumoniae* meningitis have remained common in other parts of Asia like South Africa, Australia and USA [275,276,277]. The rarity of these cases outside Asia raises the possibility of ethnicity or country of origin predisposing individuals to invasive disease [248]. The pathophysiology of the *K. pneumoniae* induced acute bacterial meningitis (ABM) in rat model, showed association of strong calcium signaling with

enhanced microglial activation in hippocampus prior to production of pro-inflammatory cytokines and oxidative damage [278]. One year later, Barichello *et al*., (2014) reported a significant increase of the pro-inflammatory mediator cytokines (TNF-α, IL-1β, IL-6), cytokine-induced neutrophil chemoattractant-1 and brain-derived neurotrophic factor (BDNF) levels in the central nervous system in adult wistar rats with *K. pneumoniae* meningitis [279]. Pneumocephalus a rare complication associated with *K. pneumoniae* meningitis had been reported from India in which air filled inside the cranial vault, lead to cranio-facial trauma [280].

1.9.4. Ankylosing spondylitis

Ankylosing spondylitis (AS) associated with Crohn's disease (CD) was mainly affecting males of young age groups with characteristic chronic inflammatory of spinal and large-joint arthritic and potentially disabling condition, included within the group spondyloarthropathic [281]. Throughout the world, various independent reports showed that *K. pneumoniae* could be the most likely etiopathogenic agent implicated in the development of ankylosing spondylitis in genetically prone individuals possessing HLA-B27 allelotype [282,283]. It has been reported that 96% patients having ankylosing spondylitis possess HLA-B27 as compared to 8% in general population [284]. Enzymes pullulanase PulA and PulD produced by *K. pneumoniae* share certain molecular structures with various self-antigens present in collagens and HLA-B27 molecules respectively [281]. High starch consumption enhanced *K. pneumoniae* growth in the bowel lead to elevated anti-*Klebsiella* antibodies as well as production of auto antibodies due to the cross-reactive self-antigens with the resultant inflammation at the pathological sites [285]. Further, Kirveskari *et al*., (1999), had reported that acute *K. pneumoniae* infection in patients possess HLA-B27 had significant decrease of monomorphic MHC class I as compared to patients negative for the HLA-B27 genotype, or healthy HLA-B27-positive individuals [286].

1.9.5. Septic arthritis

K. pneumoniae as an etiological agent for septic arthritis is very rare but required an early diagnostic arthrocentesis as severe infection may cause serious immobility by destroying the joints. Two cases of acute septic arthritis due to extended-spectrum beta-lactamase (ESBL) producing *K. pneumoniae* in renal transplant adult patients following a hospital acquired bacteraemia [287]. Suzuki *et al*., (2013) report the first case of *rmp*A-positive community-acquired *K. pneumoniae* showed phenotypic hypermucoviscocity causing septic arthritis in a 65-year-old Japanese woman [288]. Also, *K. pneumoniae* was associated with chronic diarrhea in HIV-infected persons [289]. *K. pneumoniae* as an etiological agent for necrotizing fasciitis have become more frequent in recent years due to emergence of the highly virulent K1 capsular serotype than previous with only 11 documented cases in the literature and all reported from Asia and the Middle East [290].

1.10. Treatment options of *Klebsiella pneumoniae*

Owing to the increasing antimicrobial resistance, therapeutic options for *K. pneumoniae* infections often represent a challenge worldwide [291]. Now-a-days, only a few of the major antimicrobial agents are effective for the treatment of severe nosocomial infections caused by *K. pneumoniae* due to their natural resistance towards ampicillin and amoxicillin with mean minimum inhibitory levels ranging from 200 to > 1000 mg/L [292]. Over the past two decades, *K. pneumoniae* presenting 50–88% pyogenic liver abscesses in Taiwan. After diagnosis of Klebsiella liver abscess (KLA), broad-spectrum antibiotics are started instantly to control ongoing bacteraemia and its associated complications. Due to natural resistance to ampicillin and penicillin, the third and fourth-generation cephalosporins, quinolones, aminoglycosides, and carbapenems have a drug of choice for KLA treatment [293,294]. KLA with septic

endophthalmitis or other distal metastases has been treated with systemic intravenous and intravitreous third-generation cephalosporin (ceftriaxone or ceftazidime) due to good penetration power of these antibiotics into the vitreous compartment [257]. The optimal duration for intravenous therapy and subsequent oral therapy remains unclear. In a study in Taiwan, therapy with cefazolin plus gentamicin in which treatment with gentamicin usually discontinued after 2 weeks to avoid nephrotoxicity but cefazolin treatment continued for at least 3 weeks or longer, depending on the clinical response and satisfactoriness of abscess drainage [295]. However, Rahimian *et al.*, (2004) reported shorter courses of antibiotic therapy with intravenous therapy for 17.5 days and oral therapy for 13.6 days with extremely low mortality [259]. *Klebsiella* meningitis may be treated with third-generation cephalosporins which are the drugs of choice because of their superior central nervous system penetration as well as their excellent activity against the organism [296]. Other measures include removal of infected shunts [297]. The suggested duration of treatment of KLA is 3 weeks because higher relapse rates have been noted in patients treated with shorter courses of therapy [157]. *K. pneumoniae* endophthalmitis report have been increased in the patient with diabetes mellitus and more than 50% patients with hepatic abscess [270,298]. Therapy for endophthalmitis may be intravitreal, intravenous, or both with ceftazidime and aminoglycosides particularly amikacin, as the recommended drugs [299]. Systemically given antibiotics into the eye have variable penetration power as over time antibiotic levels slowly decline in the vitreous cavity. Thus an appropriate dosage should be given to treat endophthalmitis as third generation cephalosporins (1-2g intramuscularly ceftriaxone) had vitreous levels ranging from 1.4-19.4µg/ml at the first 4.5hr and averaged to 5.9µg/ml [300], oral ciprofloxacin achieved a vitreous concentration of 0.2-0.5mg/L [301], where as a single dose of imipenem (1g) resulted in a mean vitreous level of 2mg/L after 2 to 4h

of infusion [302,303]. *Klebsiella* endocarditis is very rare with associate mortality rate 49% [304]. Baddour *et al.,* (2005) had reported a combination of intravenous aminoglycoside and beta-lactam antibiotic (most likely a third-generation cephalosporin) to treat *Klebsiella* endocarditis as gentamicin monotherapy was not effective for total sterilization [305]. Early consideration of cardiac surgery in combination with prolonged courses of combined antibiotic therapy is essential to cure ESBL-producing *Klebsiella* endocarditis [305]. Benenson *et al.*, (2009) had first shown that combination of colistin and gentamicin for at least six weeks was effective and resulted in the cure of patient's endocarditis, without need of surgical requirement [306]. Serious infection with ESBL-producing *K. pneumoniae* is difficult to treat because of β-lactamase produced by this organism that cleaved the β-lactam ring of antibiotic, making them ineffective [307]. However, *in vitro*, the combination therapy with β-lactams/β-lactamase inhibitors, third and fourth-generation cephalosporins, aminoglycosides, and quinolones may sometimes appear to be effective. Depending on the geographical location of the study site up to 0%–80% susceptibility rates was found for ESBL producing *K. pneumoniae* [308]. However, ESBL producing isolates are capable of hydrolyzing cephalosporins except carbapenems, therefore, carbapenems are the last resort to treat severe infections caused by ESBLs positive *K. pneumoniae* [309]. A new glycylcycline agent, tigecycline, has got US FDA approval for treating complicated intra-abdominal and complicated skin and skin-structure infections [310,311]. Tigecycline has now provided hope for the treatment of infections caused by resistant gram-negative organisms [312]. Furthermore, pharmacokinetic studies have demonstrated that tigecycline can be used for treating serious infections caused by KPC-producing *K. pneumoniae*, as 91.2 % of isolates remained susceptible [313].

1.11. Persister cell

Resistant bacteria developed various drug resistance mechanisms likes acquisition of resistance genes via horizontal genes transfer [314], enzymatic inactivation/modification of antibiotics via hydrolysis, group transfer and redox mechanism [315,316,317] and increased mutagenesis in hypermutator strains [318] which follow regular trend of growth in presence of antimicrobial agents. Unlike resistant bacteria, persister cells are dormant or extremely slow replicating subpopulation (approximately 1% at stationary phase and in biofilms) of bacteria that survive exposure to lethal concentrations of antibiotics without expressing a resistance mechanism. It was first described by Joseph W. Bigger in *Staphylococci* in 1944, ever since the first widespread use of antibiotics in the mid-1940s [319]. Persister cells show characteristic biphasic growth pattern in which a small number of non-growing or dormant cells survive for longer time after rapid killing of majority of bacteria in presence of the lethal doses of antimicrobials [320,321] as shown in **Fig.1.8**. The longer survival of the persister cells in the lethal dose of antibiotics was due to growth-arrested or dormant state in which most cellular processes such as DNA replication, cell wall synthesis, transcription and translation are inactive, that antibiotics targets [322]. Persister formation is not heritable as it reverts to normal growth once antibiotic is removed and their offspring are as susceptible as the original bacterial population and exhibit characteristic bi-phasic growth [320].

Since the first description of persister cell formation in *Staphylococci* by Joseph W. Bigger, persister formation has been reported in many bacteria belonging to different genus like *E.coli* [320,323], *Pseudomonas* sp [324,325,326], *Mycobacterium tuberculosis* [327], *Salmonella sp* [328], *Klebsiella pneumoniae* [329] and *Candida albicans* [330]. In these organisms, persister cell formation elucidated a protective mechanism responsible for survival of bacteria in the

different stressed conditions likes high level of signaling nucleotide (p)ppGpp in *E. coli* persister cells induces slow growth and reduced β-lactam susceptibility. In limited nutritional stress, reduced metabolic activity in *P. aeruginosa, C. albicans* persister cells inside biofilm contributed to the antibiotic tolerance under hostile condition [324,330]. By using various antibiotics, different model organisms like *E. coli*, *P. aeruginosa*, *S. aureus* and *S. epidermidis* showed few persisters cell formation in the lag and exponential phases of growth and the highest number of persisters in the stationary phase [320,331,332].

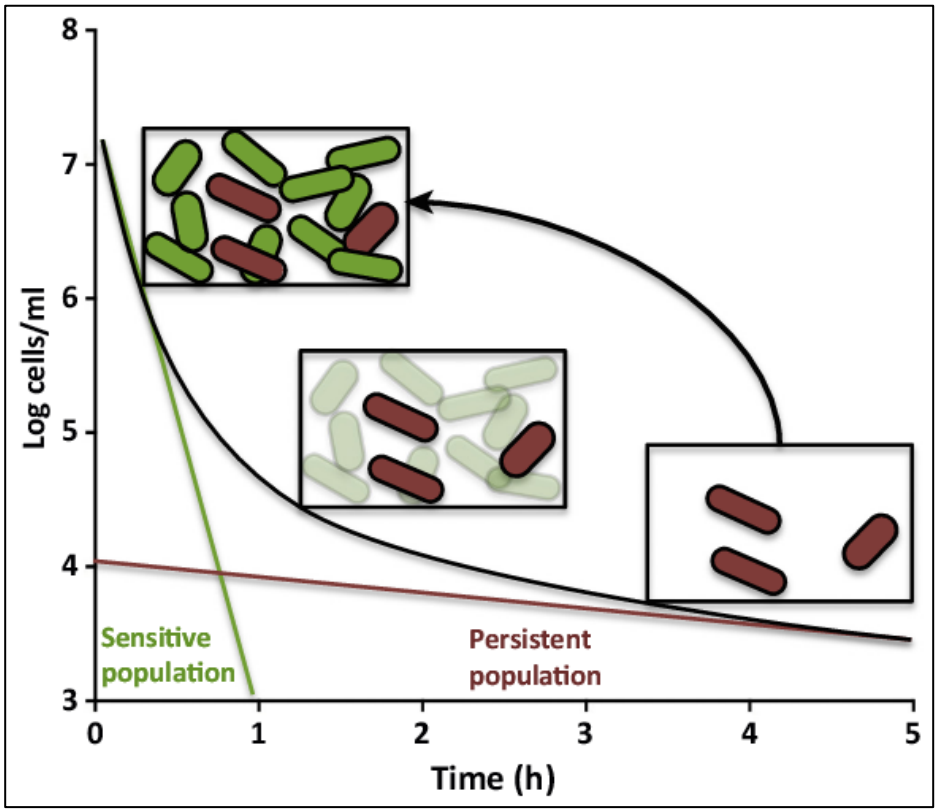


Figure 1. 8 Biphasic killing curve of persister cells

Persister cell showed biphasic killing curve in which the slope of the initial phase of killing (green line) represents the rapid death of the sensitive population and the slope of the second phase (red line) represents the much slower death of persisters. After removal of antibiotic (black arrow), persisters can regrow and give rise to antibiotic-sensitive cells that are genetically identical to the original population [333].

The molecular mechanism of persister formation is not well understood, although many transcriptome studies suggested that the dormant persisters phenotype were due to upregulation of several toxin-antitoxin modules [334] with diverse function like; the HipA toxin inhibitor of elongation factor EF-Tu [335,336]; the MqsR toxin cleaves most of the transcripts in the cell [337]; the RelE toxin cleaves mRNA [320,338] and the TisB toxin formed membrane pore thus decreasing proton motive force & ATP [339].

Apart from toxin-antitoxin related genes, *glp*D and *pls*B encode glycerol-3-phosphate (G3P) dehydrogenase, and G3P acetyltransferases respectively have been linked to persister formation in *E. coli* [340]. Individual knockouts of either of these genes, led to an increase in persister levels. In both *E. coli* and *Pseudomonas aeruginosa,* amino acid biosynthesis has also been reported to be linked to persister cell formation [341]. They showed leucine-starved biofilm along with carbon source starvation, induces persistence and tolerance towards fluoroquinolones.

Stress and signaling molecules also induced persister cell formation in the bacteria. SOS-induced expression of the TisB toxin of the type I *tisAB*/*istR* TA system is an example of stress-induced persister formation in *E. coli* [339]. (p)ppGpp, an alarmone has been identified in relation to persister formation [342]. Stringent response activation by (p)ppGpp switches bacterial metabolism towards a slower growth state through regulation of ~500 genes. The levels of (p)ppGpp typically fluctuates from cell to cell, but its synthesis increase under amino acid starvation that subsequently binds directly to RNA polymerase in conjunction with its partner transcriptional factor DksA to inhibit the transcription of stable rRNA and tRNA genes, leads to reduced growth and increased persistence [343]. Furthermore, (p)ppGpp mediated the activation of Lon protease through a regulatory cascade via PolyP (inorganic polyphosphate) to degrade antitoxins of several type II TA systems also leads to persister formation [342]. Indole is another

stress-induced intercellular signaling molecule that was found to increase persister levels and tolerance towards multiple antibiotics in *E. coli* [344]. Two quorum-sensing molecules namely pyocyanin and 3-OC12-HSL that expressed during logarithmic and stationary phases of growth in *Acinetobacter baumannii* and *P. aeruginosa* reported to increases persistence possibly by activating the expression of catalase and superoxide dismutase enzymes [345]. But as such, the actual mechanism of persister cell formation remains to be elucidated.

AIM AND OBJECTIVES OF THE RESEARCH

Review of the literature revealed availability of significant information on the abundance and diversity of resistance genes, imparting multidrug resistance phenotype in environmental bacterial isolates. However, there was lack of an integrated approach to address the extent of threat posed by such environmental isolates to human health. Secondly, there was lack of comparative study between multidrug resistant environmental isolates and their clinical counterparts, that would help understand the extent of dissemination and/or evolving nature of the isolates. With this background, my thesis work aimed at "Comparative study of molecular diversity in environmental and clinical isolates of *Klebsiella pneumoniae*: focus on resistance mechanism, virulence factors and clonal diversity". To achieve the above stated aim following objectives were laid down for the study:

1. Isolate and identify antibiotic resistant bacteria from aquatic environment and determine their phenotypic virulence factors.
2. Compare diversity in resistance mechanisms in *Klebsiella pneumoniae* isolates from aquatic environment and clinical settings.
3. Determine clonal relationship between *Klebsiella pneumoniae* isolates from aquatic environment and clinical settings.

Chapter-2
Materials & Methods

2.1 MATERIALS

2.1.1. Bacterial strains used in the study

One hundred and thirteen non-duplicate pure colonies of bacteria isolated from aquatic environment of Jamshedpur, Jharkhand and fourteen clinical isolates of *Klebsiella pneumoniae* obtained from tertiary care hospitals located in Jamshedpur and Bhubaneswar, Odisha, were used in the present study. Clinical isolates were from urine (n=12), sputum (n=1) and unknown (n=1) culture. Clinical isolates of *K. pneumoniae* were identified using automated identification system (Vitek 2.0) at the hospitals. *E. coli* ATCC 25922 was used as control.

2.1.2 Media and Reagents

A) Bacterial culture media

Table 2.1 Bacterial growth and differential media

Medium	Use	Compositions	gram/liter	Reference
Nutrient Agar (Difco, USA) pH-6.8 ± 0.2	Routine growth and maintenance of nonfastidious bacteria	Beef Extract	3.0	
		Peptone	5.0	
		Agar	15.0	
Luria-Bertani, Miller (Difco, USA) pH-7 ± 0.2	Growth and maintenance of pure cultures as well as recombinant strains	Tryptone	10.0	[346]
		Yeast Extract	5.0	
		Sodium Chloride	10.0	
		Agar	15.0	
Brain Heart infusion Agar (Difco, USA) pH-7.4 ± 0.2	Aerobic bacteriology and specific test	Beef heart infusion	10.0	[347]
		Tryptose	10.0	
		NaCl	5.0	
		Agar	13.5	

Muller-Hinton Agar (Difco, USA) pH-7.3 ± 0.2	Antimicrobial susceptibility testing	Beef extract	2.0	[348]
		Acid digest of casein	17.5	
		Starch	1.5	
		Agar	17.0	
Tryptic Soy Broth (Difco, USA) pH-7.3 ± 0.2	Enrichment medium used in qualitative procedures	Bacto Tryptone	17.0	[349]
		Bacto Soyatone	3.0	
		Glucose	2.5	
		NaCl	5.0	
		K_2HPO_4	2.5	
		Agar	17.0	
Mac-Conkey agars (Difco, USA) pH-7.1 ± 0.2	Differential media	Pancreatic Digest of Gelatin	17.0	[350]
		Peptones	3.0	
		Lactose	10.0	
		Bile salt	1.5	
		NaCl	5.0	
		Neutral red	0.03	
		Crystal violet	0.001	
		Agar	13.5	
Eosin methylene blue agar (Difco, USA) pH-7.2 ± 0.2	Differential media	Pancreatic Digest of Gelatin	10.0	[351]
		Lactose	5.0	
		Sucrose	5.0	
		K_2HPO_4	2.0	
		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	
		NaCl	5.0	
		Agar	13.5	
BIND (Brilliant green containing Inositol-Nitrate-Deoxycholate agar)	Selective media for *Klebsiella pneumoniae*	Myo-inositol	80.0	[352]
		Sodium nitrate	20.0	
		NaH_2PO_4	342.0	
		K_2HPO_4	60.0	
		NaCl	10.0	
		$MgSO_4$	2.4	
		sodium deoxycholate	10%	
		Brilliant green	1.0	

Broth consists of the same ingredients without agar

(B) Chemicals and Reagents

Table 2.2 Antibiotic used in study

Antibiotics	Solvent	Supplier
Amikacin	Water	Sigma, USA
Ampicillin	Water	Sigma, USA
Cefepime	Water	Cepime, Alembic
Cefuroxime	Water	Altacef, Glenmark
Cephotaxime	Water	Sigma, USA
Chloramphenicol	Water	Sigma, USA
Ciprofloxacin	0.01N HCl	Fluka, Germany
Erythromycin	50% methanol	Sigma, USA
Gentamycin	Water	USB, USA
Levofloxacin	Water	Sigma, USA
Norfloxacin	33% acetic acid	MP biomedical, USA
Tetracycline	Water	USB, USA
Trimethoprim	50% methanol	Fluka, Germany

The above antibiotics were supplied in powder form and kept at the optimum temperature (4˚C). Stock solutions (10mg/ml or 5mg/ml) were prepared by dissolving appropriate amounts of these antibiotics in respective solvent in 15 ml sterile container and mixed until the antibiotic had dissolved. Dissolved antibiotic solutions were filter sterilized using 0.22µm syringe filter (Millipore, USA) into a sterile 15 mL screw-cap tube and stored at 4˚C until further use.

Table 2.3 Antibiotic discs (Hi-Media, India) used in this study

Antibiotic class	Antibiotics	Symbol	Disc potency
β-lactams	Ampicillin-A	A	10
	Penicillin-G	P	10
	Cloxacillin	CX	10
	Naficillin	NAF	1
	Oxacillin	OX	10
	Azlocillin	AZ	75
	Mezlocillin	MZ	75
	Piperacillin	PI	100
	Ticarcillin	TI	75
	Carbenicillin	CB	100
	Amoxiclav	AMC	30
	Cephalothin	CEP	30
	Cefoxitin	CX	30
	Cefuroxime	CXM	30
	Ceftazidime	CAZ	30
	Cephotaxime	CTX	30
	Ceftizoxime	CZX	30
	Ceftriaxone	CTR	30
	Cefpirome	CFP	30
	Cefepime	CPM	30
Carbapenems	Meropenem	MER	10
	Imipenem	IMP	10
Quinolones	Nalidixic Acid	NA	30
	Cinoxacin	CIN	100
	Enoxacin	EN	10
	Ciprofloxacin	CIP	5
	Norfloxacin	NX	10
	Ofloxacin	OF	5
	Pefloxacin	PF	5
	Lemofloxacin	LO	10
	Levofloxacin	LE	5
	Sparfloxacin	SC	5
	Gemifloxacin	GM	5
	Gatifloxacin	GAT	5
	Moxifloxacin	MO	5
Polypeptides	Polymyxin-B	PB	300
	Colistin	CL	50
Aminoglycosides	Gentamycin	G	120
	Neomycin	N	30
	Co-Trimaxazole	CO	25
	Azithromycin	AZM	5

	Chloramphenicol	C	30
	Tetracycline	T	30
	Sulphafurazole	SF	300
	Trimethoprim	TR	5

Table 2.4 List of Chemicals used

S. No	Chemical name	Supplier
1	Acetic acid	Sigma-Aldrich, USA
2	Agar	Hi-Media, India
3	Agarose	Sigma-Aldrich, USA
4	Carbonyl cyanide 3-chlorophenylhydrazone	MP Biomedical, USA
5	Carboxyfluorescein diacetate	Sigma-Aldrich, USA
6	Crystal Violet	Fisher Scientific, India
7	Ethanol	Merck, Germany
8	Ethidium bromide	Sigma-Aldrich, USA
9	Ethylene diamine tetraacetic acid	Sigma-Aldrich, USA
10	Gelatin	Hi-Media, India
11	Glycerol	Sigma-Aldrich, USA
12	Hi Di formamide	Applied Biosystems, USA
13	Mannose	Hi-Media, India
15	Methanol	Hi-Media, India
16	Pop7 polymer	Applied Biosystems, USA
17	Propan-2-ol	Hi-Media, India
18	Sodium azide	Hi-Media, India
19	Sodium dodecyl sulphate	Sigma-Aldrich, USA
20	Tris HCl	Sigma-Aldrich, USA
21	Xylene	Hi-Media, India

Table 2.5 Reagents and buffers used

S. No	Reagent	Components	Supplier
1	PCR reagent	Buffer (5X) MgCl2 (25mM) Go Taq DNA polymerase (500 units/µl)	Promega, USA
2	DNA sequencing reagent	Sequencing buffer Ready reaction mix	Applied Biosystems, USA
3	DNA ladder	100 base pair 1 Kilo base	NEB, USA
4	DNA gel loading dyes (6X)		Promega, USA
5	DNA sequencer Anode/cathode buffer (10X)	Buffer with EDTA	Applied Biosystems, USA
6	Tris-acetate-EDTA buffer, pH-8.2 (50X)	Tris base Acetic acid EDTA (0.5M)	Promega, USA

Restriction Enzymes (NEB, USA)

i. EcoRV

ii. Bgl

iii. Not-1

2.1.3 Commercial Kits

i. QIAquick@ Gel Extraction Kit (QIAGEN GmbH, Germany)

ii. QIA mini plasmid isolation Kit (QIAGEN GmbH, Germany)

iii. BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, USA)

2.1.4 Instruments used in the study

i. Sterile water filtration unit (Millipore, USA)

ii. CELLGARD Class II Biological Safety cabinet (NUAIRE, USA)

iii. INNOVA 44R Incubator Shaker (Eppendorf, Germany)

iv. Automated 3130xl Genetic Analyzer System (Applied Biosystems, USA)

v. iMark Microplate Reader (Biorad, USA)

vi. FACS Calibur Flow Cytometer (BD Biosciences, USA)

vii. Laser-scanning confocal microscope LSM 780 (Carl Zeiss, Jena, Germany)

viii. Vapo protect Master Cycler (Biorad, USA)

ix. Centrifuge 5415R (Eppendorf, Germany)

x. AccuBlock™ Digital Dry Baths (Labnet International, USA)

2.1.5 Software used

i. SeqScape V2.6 software (Applied Biosystems).

ii. MEGA 4.0 for phylogenetic analysis.

iii. Cell Quest Pro software (BD Biosciences).

iv. ZEN 2010 software (Carl Zeiss).

v. LSM software (Carl Zeiss) for Images analysis.

vi. Adobe Photoshop CS4 (Adobe Systems Incorporated, San Jose, CA, USA).

vii. Online ClustalW/Muscle tool (www.genome.jp/tools/clustalw/) for sequence alignment.

viii. Online BLAST programs of the National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/blast/) for sequence similarity.

ix. Online Non-synonymous substitutions (dN) to synonymous substitutions (dS) ratio *(*www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html*).*

x. Online MLST database (http://*Klebsilla*.mlst.net) for determining ST's of the isolates.

2.1.6 Statistical soft wares used

i. SigmaStat version 3.5 (USA), was used to perform – (i) Kruskal-Wallis ANOVA on Ranks for statistical comparisons of the MAR indices between genera; (ii) Pairwise multiple comparisons by Dunn's method.

ii. SPSS version 17.0 was used to determine Pearson correlation coefficients (r) for correlation.

iii. Graphical software Origin 8.0 (USA) and GraphPad Prism were used for pictorial depiction of data.

iv. MS Excel was used for percentage and various other mathematical calculations.

2.2 Protocols followed commercial Kits

2.2.1 Isolation of plasmid DNA using QIAprep Spin Miniprep plasmid isolation Kit (QIAGEN, GmbH)

Plasmid DNA was isolated following the manufacturer's instructions as mentioned herewith. One milliliter of freshly grown culture was centrifuged into 1.5ml eppendorf tubes and pelleted bacterial cells were resuspended in 250 µl Buffer P1. Buffer P2 (240 µl) was added and tubes were inverted gently 4-6 times. Colour of the solution became blue due to presence of LyseBlue reagent. Next, 350 µl Buffer N3 was added. To avoid localized precipitation, immediately after addition of Buffer N3, solution in the tube was inverted gently 4-6 times that turned the blue colour into white and cloudy indicating proper lysis. The mixture was centrifuged at 13,000 rpm for 10min in a table-top microcentrifuge. A compact white pellet was formed. Supernatant were decanted into QIAprep Spin Columns carefully and centrifuged for 60S. After

discarding the flow-through, QIAprep Spin Columns was washed by adding 0.5 ml Buffer PB to remove high carbohydrate content and followed by centrifugation for 60S. Flow-through was discarded and Spin Column was washed by adding 0.75 ml Buffer PE and centrifuging for 60S. After discarding the flow-through QIAprep Spin Column was centrifuged for an additional 1 min to remove residual wash buffer. Plasmid DNA was eluted by placing the QIAprep column in a sterile 1.5 ml centrifuge tube and EB buffer was added to the center of each Spin Column, allowed to stand for 1 min and centrifuged for 1 min. Isolated plasmid DNA was separated in 1% agarose-TAE containing 0.5µg/ml ethidium bromide gel at 60V for 45min and visualized using the gel documentation system (Bio-Rad, USA).

2.2.2 Purification of PCR product using QIAquick@ Gel Extraction Kit (QIAGEN GmbH, Germany)

PCR amplified products from agarose gel were purified using QIAquick@ Gel Extraction Kit (QIAGEN, GmbH) according to the manufacturer's instructions. Briefly, gel slices were put in pre-weighed colourless eppendorf tubes and 3 volumes of QG buffer was added to 1 volume of gel weight and incubated at 50°C with intermittent vortexing until the gel slices completely dissolved . One gel volume of isopropanol was added to dissolve PCR products and mixed well. The sample mixture (isopropanol and dissolved PCR product) was passed through QIAquick columns, and centrifuged for 1 min and flow-through was discarded. QIAquick columns were placed back in the same collection tube and 0.5 ml of QG buffer was added and centrifuged for 1 min and flow-through discarded. The QIAquick column were then washed with 0.75 ml of PE Buffer and centrifuged for 1 min, the flow-through discarded and the QIAquick column centrifuged for an additional 1 min at 13,000 rpm (~17,900 x g). Finally, DNA was eluted in

sterile 1.5ml eppendorf by adding 25 µl of sterile Milli-Q water to the center of the QIAquick membrane by centrifuged the column for 1 min.

2.2.3 DNA sequencing using BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, USA)

PCR products/plasmid DNA was sequenced on 3130XL Genetic Analyzer using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) as per the manufacturer's protocol. PCR mixture contained 0.5µl of 2.5X Ready reaction mix, 1.75µl of 5X Dilution buffer, 2µl of 0.8 pM of primer, 1µl of 30ng/µl template and volume to 10µl made up with sterile Milli-Q water. The PCR reaction conditions included denaturation at 96°C for 0.10 min, annealing at 50°C for 0.05 min and extension at 60°C for 4 min. The sequencing PCR products were cleaned up to remove unused dNTP, ddNTP and other PCR components by Big Dye Terminator v3.1 Tube Clean up method. Briefly, PCR product were transferred into 0.6 ml tube and 12 µl master mix I (10 µl Milli-Q water and 2 µl of 125mM EDTA per reaction) and 52 µl master mix II (2 µl of 3M NaOAc at pH 4.6 and 50 µl of ethanol per reaction) were added, mixed well and incubated for 15 min at room temperature. The mixture was centrifuged at 12000 rpm for 20 min at room temperature, supernatant were decanted and 250 µl of 70% ethanol was added and further spun at 12000 rpm for 10 min at room temperature. Supernatant was decanted, 15 µl of Hi-Di formamide was added to dissolve the products. Dissolved products were transferred to 96 wells plate, denatured at 95°C for 5 min; snap chilled and proceeded for sequencing electrophoresis and data acquisition. The resultant sequences were aligned using Seq-Scape software (Applied Biosystems) to get full length sequence.

2.3 METHODOLOGY

The following section describes in detail the methodology followed to achieve the laid down objectives. Unless otherwise specified all incubations were done at 37°C at 220rpm and all experiments were carried out in triplicate and repeated at least thrice.

2.3.1 Description of water collection sites and sampling procedure

Jamshedpur city constitutes an economically significant part of state Jharkhand, India. It is located in the coordinate of 22°48'0" North, 86°11'0" East and is bordered by the states of Bihar, Odisha and West Bengal. The areas surrounding Jamshedpur are rich in minerals, including iron ore, coal, manganese bauxite and lime. It is the home of steel producing, truck manufacturing, tinplate production, pigment production, cement industries and many small and medium scale industries revolving around these products. Water samples were collected from four different water sources located in Jamshedpur, namely Subarnarekha River, Kharkai River, Dimna Lake and Hudco Dam. The Subarnarekha and Kharkai are natural fresh water rivers flowing on the north and west side of the city. Dimna lake (22°50’0” North, 86°10”East) and Hudco Dam (22°45'48"North, 86°15'5"East) are artificial reservoirs situated on the north and east side of the city as shown in **Fig 2.1**. These water sources serve as fresh water source for the city residents and are used for various purposes including domestic and industrial consumption as well as for recreational activities. The sampling sites were located upstream of the rivers before it traversed the city. These selected sites did not have any waste water discharge opening into the river.

Water samples were collected five times over a period of one year from March 2010 to March 2011. Sampling and isolation of bacteria were done using standard protocol (WHO, 1987). Briefly, approximately one liter water was sampled in an autoclaved container from one

to two feet below the surface from each of the above mentioned sites. The samples were stored immediately at 4°C, away from sunlight and transported to the lab within 12h for processing. Each water sample was filtered through Whatman no 1 filter paper to remove all suspended particles. The particle free water was passed through 0.22µm membrane with help of Millipore filtration unit (Millipore Corporation, USA). These membrane filters containing trapped bacteria were kept on the LB plates and incubated overnight at 37°C. All these procedures were performed inside BSL-2 cabinet (NuAire, USA). The overnight grown bacteria from membrane filters were transferred to 25ml of LB broth (Difco, USA), mixed vigorously, serially diluted. Ten microliters of each dilution *viz*. 10^{-6}, 10^{-7} and 10^{-8} were spread plated on LB agar plates. After the growth of different microorganisms on the plates, each bacterial colony on the basis of their morphology was picked up and streaked on LB media. Isolated strains were further characterized on differential media for their tentative identification.

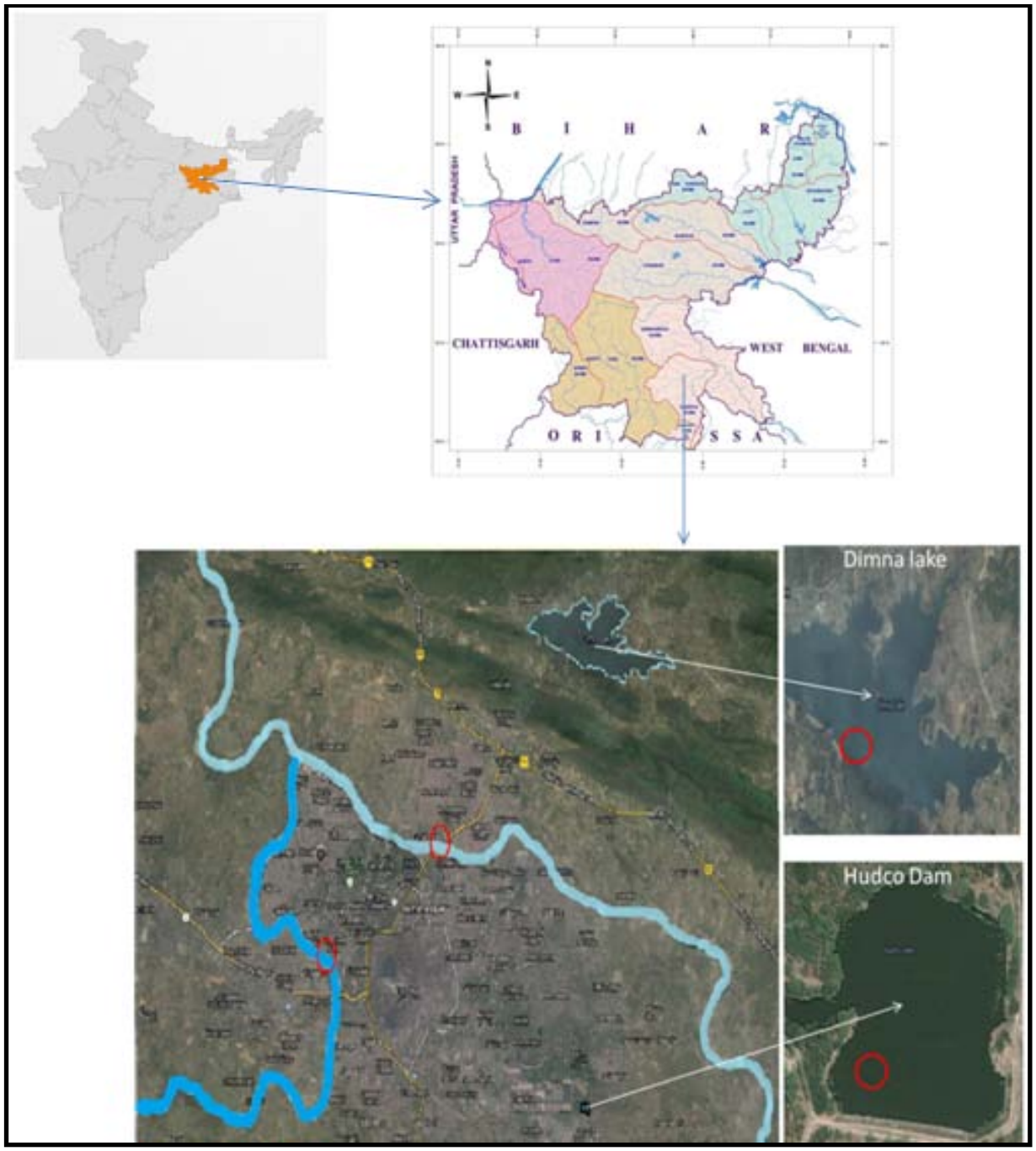


Figure 2.1: Water sampling sites.

Subarnarekha river denoted by light blue line, Kharkai river denoted by deep blue line, Dimna lake denoted by light blue outline and Hudco dam denoted by black outline. Outside figures are large view of Dimna Lake and Hudco dam. Red circle denote point of water collection site.

2.3.2 Culture preservation and storage

2.3.2.1 Soft agar preparation

Bacterial isolates were inoculated onto LB agar plates and incubated overnight at 37ºC. LB broth with 0.8% bacteriological agar was autoclaved at 15 psi at 121°C for 15 min and 3.5ml LB of soft agar was poured into 5 ml cryovials and allowed to solidify. Single colony was picked using straight wire inoculation rod and stabbed into soft agar tube. The inoculated tubes were incubated at 37˚C overnight and subsequently stored at room temperature for further use.

2.3.2.2 Glycerol stock preparation

Bacterial strains were inoculated onto Luria-Bertani agar plates and incubated overnight at 37ºC. A single colony was inoculated into 2ml LB broth and incubated approximately till 4-5h when it reached exponential phase. 800µl of culture and 200µl glycerol (100%) were added into 1.8ml cryovials. The cryovials were mixed by vortex and stored at -80ºC immediately until further use.

2.3.3 Identification of bacterial isolates

2.3.3.1 Culture based identification using differential media

Differential media are widely used for isolation and tentative identification of closely related organisms or groups of organisms. Differentiation of microorganisms was done on the basis of characteristic changes or growth patterns in the presence of certain dyes or chemicals in the media. Five differential media were used and tentative bacterial identification was done as described in Difco manual.

I. Brilliant green agar –is a suitable medium for isolation of Salmonella other than *S. typhi*. It is used for examination of water and wastewater. The brilliant green dye inhibits most of the gram positive bacteria and a majority of gram negative bacilli while the phenol red

serves as a pH indicator that gives yellow colour due to acid production during utilization of lactose and /or sucrose. Colony morphology on Brilliant Green Agar was determined as shown in **Table 2.6**.

Table 2.6 Colony morphology on Brilliant Green Agar plate

Bacterial strains	Morphology
Salmonella (other than S. typhi and S. paratyphi)	White to red, opaque colonies surrounded by red zones in the medium
S. typhi and S. paratyphi	No growth to trace growth
Shigella	No growth to trace growth
Escherichia coli and Enterobacter/Klebsiella	Yellow to greenish-yellow colonies surrounded by intense yellow-green zones in medium
Proteus	No growth to trace growth
Pseudomonas	Pink to red colonies
Gram-positive bacteria	No growth to trace growth

II. Eosin methylene blue agar –is a selective and differential medium for the isolation and differentiation of gram-negative enteric bacilli. It contains eosin Y and methylene blue dyes that inhibit gram-positive bacteria to a limited degree. The dyes also serve as differential indicators in response to the fermentation of lactose and/or sucrose by microorganisms (**Table 2.7**).

Table 2.7 Colonial morphology on Eosin Methylene Blue Agar plate

Bacterial strains	Morphology
Escherichia coli	Large, blue-black, green metallic sheen
Enterobacter/Klebsiella	Large, mucoid, blue-black
Proteus/ Shigella	Large, colorless
Salmonella	Large, colorless
Pseudomonas	Irregular, colorless
Gram-positive bacteria	No growth to slight growth

III. Columbia blood agar – 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 (**Table 2.8**).

Table 2.8 Culture/medium appearance on Columbia blood agar plate

Bacterial strains	Result
α-hemolytic	Agar under the colony is dark and greenish due to partial hemolysis
β-hemolytic	Complete hemolytic, area appear lightened and transparent
γ-hemolytic	No hemolytic

IV. Mac-Conkey agar- Mac-Conkey agar is selective and differential plating media mainly used for the detection and isolation of lactose fermenting and non-lactose fermenting gram-negative organisms (**Table 2.9**).

Table 2.9 Typical media appearance on Mac-Conkey Agar plate

Bacterial strains	Result
Lactose fermenter	Light pink to dark pink
Non-Lactose fermenter	Colorless

2.3.3.2 16S rRNA gene sequencing

16S rRNA gene was PCR amplified using the universal primer Bact_63f_62C 5'–CAGGCCTAACACATGCAAGTC-3' and Bact_1389r_63C 5'–ACGGGCGGTGTGTAC AAG–3' [122]. Single colony of overnight grown pure culture from LB plate was taken into 500µl of Milli-Q sterile water and cell lysate prepared by incubating at 94°C for 10 min followed by snap chilling on ice for 10 min. Suspension was centrifuged at 13,000 rpm for 1 min at 4°C and 1.5 µl of this was used directly as template DNA.

Table 2.10: 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(10µM)	1.5
Primer R(10µM)	1.5
Template	1.5
Go Taq polymerase	0.5
H_2O	13.0
Total volume	25

Table 2.11: 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

Amplified PCR product was run in 1.2% agarose gel in 1X TAE at 60V for 45 minute with appropriate DNA markers (NEB, USA) and bands visualized in Chemidoc (Bio-Rad, USA). PCR amplified products of 16S rRNA gene (1500bp) were purified using QIAquick$^{@}$ Gel Extraction Kit following protocol mentioned previously and quantified using Nanodrop (Thermofisher, USA) by taking 1µl sterile water as blank. Quality of eluted PCR products were checked by loading 2µl of purified product onto 1.2% agarose-TAE gel containing ethidium bromide (0.5µg/ml final concentration) at 60V for 45 minute and visualized in Chemidoc (Bio-Red, USA). The resulting eluted PCR products were sequenced, aligned using SeqScape software (Applied Biosystems) to get full length sequence and analyzed using BLAST programs

of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nhi.gov) for extent of sequence identity and genus identification. 16S rRNA gene of all isolates were submitted to NCBI to get accession number.

2.3.4 Determining resistance profiles of bacterial isolates

2.3.4.1 Antibiotic susceptibility test by disc diffusion method

One hundred and thirteen non-duplicate bacterial pure colonies isolated from aquatic environment and fourteen bacteria isolates from clinical samples were tested for their antibiotic susceptibility to forty five antibiotics belonging to different classes *viz.* β-lactam including cephalosporin, aminoglycoside, quinolones, macrolide, nitrofuran, polypeptide and sulphonamides (**Table 2.3**) using disc diffusion method as describe by Bauer *et al*., (1966) [353]. Briefly, single colony of bacteria was inoculated into 2ml Muller-Hinton broth (Difco, USA) and incubated at 37°C with 220 rpm until it achieved the turbidity of the 0.5 McFarland standards. The resulting suspension approximately contained $2x10^8$ CFU/ml. A sterile cotton swab was dipped into the culture, rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove the excess inoculum. The dried surface of the Muller-Hinton agar plate was then inoculated by swabbing the entire surface. The procedure was repeated by streaking two or more times, rotating the plate 60 degree each time to ensure uniform distribution of the inoculum. Proper application of disks was ensured by pressing down the disk on to the medium, so that there is complete contact between media and disk. *E. coli* ATCC 25922 was used as control for each assay. The diameter of the inhibition zones was recorded and interpreted following CLSI standard proposed for the different bacteria (CLSI 2007) [354].

2.3.4.2 Determination of Minimum inhibitory concentrations (MIC) for antibiotics

Minimum inhibitory concentrations (MICs) are considered the `gold standard' for determining the antimicrobials susceptibility of organisms [355]. MIC of all *K. pneumoniae* isolates were tested by broth-double dilution method following the CLSI guidelines [354]. 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 MHB containing the antimicrobial agents (**Table 2.2)** at different concentrations. The MIC was deemed to be the lowest antibiotic concentration that inhibits all visible growth.

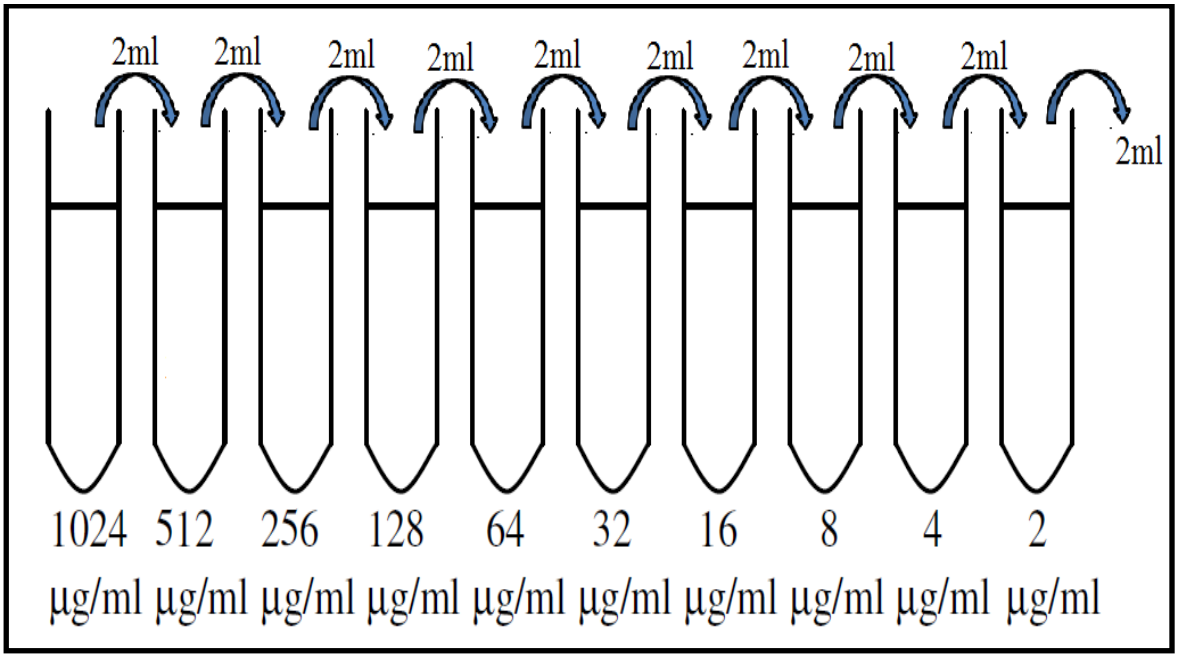


Figure 2.2: Schematic of double dilution method for minimum inhibitory concentration determination.

2.3.5 Mechanism of antibiotic resistance

2.3.5.1 Phenotypic screening for extended spectrum β-lactamase, carbapenemase and metallo- β-lactamase resistance

A) Screening for detection of ESBL by Double Disc synergy Test

Double disc synergy test was carried as described by (CLSI-2010). The isolated colony was inoculated in MH broth for 5-6 h to reach the turbidity equal to 0.5 McFarland standard and lawn culture was made on Mueller-Hinton agar using sterile cotton swab. Third generation cephalosporins *viz.* ceftazidime (30 µg) disc and ceftazidime + clavulinic acid (30 µg + 10µg) disc was placed 25mm apart. An increase of ≥5mm in zone of inhibition for ceftazidime + clavulinic acid compared to ceftazidime confirmed ESBL production by the organism.

B) Screening for Carbapenemase detection by Modified Hodge test

Phenotypic detection of carbapenemase by Modified Hodge test was done following the method outlined by CLSI (2010). A lawn of 0.5 McFarland suspension of *E. coli* ATCC 25922 was made on Mueller-Hinton agar plate using sterile cotton swab. A 10-µg imipenem disk was placed on the center and each test isolates were streaked from the disk to the edge of the plate. After overnight incubation positive isolate would show a clover leaf-like indentation of the *Escherichia coli* 25922 growing along with the test organism within the zone of inhibition.

C) Screening for metallo- β-lactamase by EDTA-Double disc diffusion method

EDTA-Double disc diffusion test was carried as described by (CLSI-2010). The isolated colonies were inoculated in MH broth for 5-6h to reach the turbidity equal to 0.5McFarland standard and lawn culture was made on Mueller-Hinton agar using sterile cotton swab. Carbapenem antibiotic *viz.* imipenem (10 µg) disc and imipenem (10 µg) + EDTA (750 µg) disc was placed 25mm apart from each other. An increase of ≥5mm in zone of inhibition for imipenem + EDTA compared to imipenem alone was confirmed the organism as MBL producer.

2.3.5.2. Genotypic screening for β-lactamase genes

Single colony of pure bacterial culture from an overnight grown LB plate was inoculated into 500µl of sterile Milli-Q water for cell lysate preparation as mentioned previously. One microliter of this was used directly as template DNA. Primers used for screening of Class A, Class B, Class C and Class D β-lactamase genes and annealing temperature for each of the primer pairs is listed in **Table 2.12**. PCR products were confirmed by restriction digestion and/or sequencing of amplified product.

Table 2.12: Oligonucleotides for resistance genes

Gene	Primer	Primer sequence (5'------>3')	Amplicon size (bp)	Annealing temperature	Reference
bla$_{SHV}$	shv-34-F	GCGTTATTTTCGCCTGTGTA	200	51°C	[356]
	shv-34-R	AGGTGCTCATCATGGGAAAG			
bla$_{TEM}$	tem-1-F	CATTTTCGTGTCGCCCTTAT	169	51°C	
	tem-1-R	GGGCGAAAACTCTCAAGGAT			
bla$_{CTX}$	ctx-4-F	CGTCACGCTGTTGTTAGGAA	155	53°C	
	ctx-4-R	CGCTCATCAGCACGATAAAG			
	ctx-27-F	CTGGAGAAAAGCAGCGGAG	158	52°C	
	ctx-27-R	TGCTTTTGCGTTTCACTCTG			
	ctx-32-F	CGTCACGCTGTTGTTAGGAA	156	51°C	
	ctx-32-R	CGCTCATCAGCACGATAAAG			
bla$_{VIM}$	vim-4-F	TCCGACTTTACCAGATTGCC	171	51°C	
	vim-4-R	TTTCAATCTCCGCGAGAAGT			
	vim-7-F	CGCAGCTTTCTGGTTGGTAT	180	52°C	
	vim-7-R	CGTGTCACCGAGTTTCTGAG			
bla$_{IMP}$	imp-2-F	CGGTTTGGTGGTTCTTGTAAA	200	51°C	

	imp-2-R	ATTCAGATGCATACGTGGGA			
	imp-5-F	GTGGAACGCGGCTATAAAAT	186	52°C	
	imp-5-R	TAGCCAATAGCTAGCTCCGC			
	imp-13-F	AGGAGCGGCTTTACCTGATT	198	52°C	
	imp-13-R	CGCTCCACAAACCAATTGAC			
bla$_{NDM}$	NDM-F	GGTTTGGCGATCTGGTTTTC	621	52°C	[129]
	NDM-R	CGGAATGGCTCATCACGATC			
*amp*C	AmpC-F	CCTCTTGCTCCACATTTGCT	189	53°C	
	AmpC-R	ACAACGTTTGCTGTGTGACG			
bla$_{OXA}$	oxa-1-F	TATCTACAGCAGCGCCAGTG	199	52°C	
	oxa-1-R	CGCATCAAATGCCATAAGTG			
	oxa-5-F	GCACGTGCATCTACAGCCTA	175	54°C	
	oxa-5-R	AAACCTGTATAGCGCCCCTT			
	oxa-9-F	GCTGCATATGTTGGTGTTCG	162	53°C	
	oxa-9-R	AAAGACGAGCACGGAGACAC			
	oxa-10-F	AGAGGCTTTGGTAACGGAGG	191	52°C	
	oxa-10-R	TGGATTTTCTTAGCGGCAAC			
bla$_{KPC}$	KPC-3-F	CAGCTCATTCAAGGGCTTTC	196	51°C	
	KPC-3-R	GGCGGCGTTATCACTGTATT			
*qnr*A	qnrA-F	AGAGGATTTCTCACGCCAGG	580		This study
	qnrA-R	TGCCAGGCACAGATCTTGAC			
*qnr*B	qnrB-F	GGMATHGAAATTCGCCACTG	264		This study
	qnrB-R	TTTGCYGYYCGCCAGTCGAA			

2.3.5.3 Screening for plasmid mediated antibiotic resistance

Plasmid DNA of all the *K. pneumoniae* isolates were extracted from overnight grown cultures, based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt using QIAGEN plasmid mini kit following protocol mentioned

previously. Isolated plasmid DNA was separated in 1% agarose-TAE containing 0.5µg/ml ethidium bromide gel at 60V for 45min and visualized using the gel documentation system.

To determine the role of plasmids in mediating resistance, elimination of plasmid DNA from *K. pneumoniae* isolates was done as described by El-Mansi *et al*., (2000) using 5 to 10% SDS in LB broth [357]. *K. pneumoniae* isolates that contained plasmid were inoculated onto LB agar plates and incubated at 37°C for overnight. Single colony from the plate was picked up and inoculated into LB broth and incubated till the cells reached log phase. Twenty microliters of the grown culture was inoculated in LB broth having 10% SDS and incubated for 24h at 37°C and 220 rpm. After 24h, culture was serial diluted and 10µl was spread plated on LB agar plate for determination of colony-formation. For screening the plasmid cured colony, each single colony was spotted onto MHA plate as well as MHA with 64µg/ml antibiotics (cephotaxime, cefepime, cefuroxime, ceftriaxone) and 16µg/ml of levofloxacin and incubated at 37°C for 16-18h. The colonies which did not show visible growth in sub lethal antibiotic concentrations were taken for plasmid isolation. Confirmation of plasmid loss was done by isolating the plasmid and visualization by agarose gel. To attribute the role of plasmids in resistance, antibiotics susceptibility and fold change of MIC of the cured strains were compared with those of the wild type isolates.

2.3.5.4 Screening for presence of integrons mediating resistance

The presence of Class 1, Class 2 and Class 4 integron and their resistance cassettes in all environmental and clinical isolates of *Klebsiella pneumoniae* were determined sequentially by PCR using primers listed in **table 2.13**.

Table 2.13: Oligonucleotides for Class 1, Class 2 integron integrase and their variable regions

Gene	Primer	Primer sequence (5'-->3')	Amplicon size	Reference
intI1	int-1U	GTTCGGTCAAGGTTCTG	923bp	[358]
	int-1D	GCCAACTTTCAGCACATG		
	inF	GGCATCCAAGCAGCAAGC	variable	[359]
	inB	AAGCAGACTTGACCTGAT		
intI2	int-2U	ATGTCTAACAGTCCATTTT	450bp	[360]
	int-2D	AAATCTTTAACCCGCAAAC		
	hep74	CGGGATCCCGGACGGCATGCACG ATTTGTA	variable	[361]
	hep51	GATGCCATCGCAAGTACGAG		

Table 2.14: Components used for PCR amplification for Class 1, Class 2 integron integrase and their variable regions (for 25 µl)

Components	Volume (µl)
5X buffer	5.0
$MgCl_2$	2.0
dNTP	2.0
Primer F (10µM)	1.5
Primer R (10µM)	1.5
Template	1.5
Go Taq polymerase	0.5
H_2O	11.0

Table 2.15: PCR reaction condition Class 1, Class 2 integron integrase and their variable regions

Class 1 &2 integron integrase

Steps	Class 1		Class 2		No. of cycles
	Temperature	Time	Temperature	Time	
Initial Denaturation	94°C	2 min	94°C	2 min	1
Denaturation	94°C	30 S	94°C	30 S	35
Primer Annealing	50°C	30 S	50°C	30 S	
Primer Extension	72°C	1 min	72°C	1 min	
Final Extension	72°C	10 min	72°C	10 min	1

Class 1 &2 integron variable regions

Steps	Class 1 variable region		Class 2 variable region		No. of cycles
	Temperature	Time	Temperature	Time	
Initial Denaturation	94°C	2 min	94°C	5 min	1
Denaturation	94°C	1 min	94°C	1 min	35
Primer Annealing	54°C	1 min	55°C	1 min	
Primer Extension	72°C	2 min	72°C	5 min	
Final Extension	72°C	10 min	72°C	8 min	1

PCR band of Class1 and Class 2 variable region were sequenced and analyzed using BLAST programs of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nhi.gov).

2.3.5.5 Screening for mutation in the Quinolone Resistance Determining Region of *gyr*A and *gyr*B genes mediating quinolone resistance

Quinolone resistance is mainly due to mutation in quinolone targets (QRDR) of DNA gyrase genes *gyr*A and *gyr*B [362]. To check for such mutations, QRDR of *gyr*A and *gyr*B gene was amplified and sequenced using primer listed in **table 2.16**. The resulting eluted PCR products were sequenced, analyzed and aligned with *Klebsiella pneumoniae* strain NTUH-K2044 (AP006725.1) as reference.

Table 2.16: Oligonucleotides for *gyr*A and *gyr*B genes

Gene	Primer	Primer sequence (5'-->3')	Amplicon size	Reference
*gyr*A	gyrA-F1	ATGAGCGACCTTGCGAGAGA	2634 bp	This study
	gyrA-R3	TTATTCTTCGTCTTCGGCG		
*gyr*B	gryB-F2	GAGATGACTCGTCGTAAAGGC	184 bp	This study
	gyrB-R2	GGATTTTACCCTTCAGCGG		

Reaction Mix (for 25 µl)

Table 2.17: Components used for PCR amplification for *gyr*A and *gyr*B gene

Components	Volume (µl)
5X buffer	5.0
$MgCl_2$	2.5
dNTP	2.0
Primer F (10µM)	2.0
Primer R (10µM)	2.0
Template	1.5
Go Taq polymerase	0.5
H_2O	9.5
Total volume	25

Table 2.18 PCR condition for *gyr*A and *gyr*B gene amplification

Steps	***gyr*A**		***gyr*B**		**No. of cycles**
	Temperature	Time	Temperature	Time	
Initial Denaturation	94°C	2 min	94°C	2 min	1
Denaturation	94°C	1 min	94°C	1 min	35
Primer Annealing	60°C	1 min	58°C	1 min	
Primer Extension	72°C	2 min	72°C	2.5 min	
Final Extension	72°C	10 min	72°C	10 min	1

2.3.5.6 Role of efflux pump in antibiotics resistance

Efflux pump play important role in tolerance towards antimicrobial agents. There are five types of efflux pump present in bacteria but RND type efflux pump was more responsible for antibiotics resistance. To check the effect of RND type efflux pump, carbonyl cyanide m-chlorophenyl hydrazone (CCCP) inhibitor of RND type efflux pump was used with 50µM concentration that doesn't inhibit the growth of any isolates. MIC for each antibiotic using double dilution method was determined in the presence of CCCP and compare with MIC without CCCP.

2.3.5.7 Mechanism of colistin resistance in environmental isolates of *Klebsiella pneumoniae*

Molecular basis of colistin resistance has been attributed either to the presence of deletions/insertions/amino acid substitutions in *mgrB* – encoding a regulatory peptide of PhoP/PhoQ regulon, that in turn regulates PmrAB system, responsible for lipopolysaccharide modifications [363]. PCR amplification using primers targeted at *mgrB* (**Table 2.19**) followed by

sequencing of the amplified products was carried out to determine the deletions/insertions/amino acid substitutions in *mgrB* gene. Further, as these were environmental isolates, we also checked for the presence and possible role of efflux pump genes in mediating colistin resistance. To ascertain the contribution of efflux pumps towards colistin resistance, we determined MIC of colistin in presence of efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at an optimized final concentration of 50µM followed by PCR screening and sequencing of MATE efflux pump genes.

Table 2.19: Oligonucleotides for *mgrB* gene and MATE efflux pump gene

Gene	Primer	Primer sequence (5'-->3')	Amplicon size (bp)	Reference
mgrB	mgrB-Kp-F	TTAAGAAGGCCGTGCTATCC	253	[364]
	mgrB-Kp-R	AAGGCGTTCATTCTACCACC		
MATE	MATE-F	TTGTCGATACCGTGATGGCAGG	465	This study
	MATE-R	AGTTAAGCGGGATGTTCAGCAG		

2.3.5.8 Non-genetic basis of antibiotic resistance in Klebsiella pneumoniae: Persister cell formation

While performing MIC, the environmental isolate DL5.4 exhibited a distinct behavior. The isolate did not show any visible growth but when plated onto solid media showed growth, which is not likely to happen if the organism is killed by the drug. This serendipitous observation leads us to characterize a non-genetic phenomenon of drug tolerance – persister cell formation in *Klebsiella pneumoniae*.

2.3.5.8.1 Determination of DL5.4 survival in response to antibiotic treatment

The time dependent killing of the *Klebsiella pneumoniae* isolate DL5.4 was determined as described by Keren *et al.*, (2004) [320]. Briefly, 1:1000 diluted overnight grown culture of DL5.4 was inoculated in 20ml MHB in a 125 ml baffled flask and incubated at 250 rpm, 37°C for 4 h, thereby reaching early exponential phase. One milliliter of cell suspension was re-inoculated in flasks containing 25ml MHB supplemented with antibiotics. Individual experiments were performed with antibiotics *viz.* ciprofloxacin (Fluka, Germany), levofloxacin (Sigma-Aldrich, <country?>), and norfloxacin (MP biomedical, <country?>) at final concentration of 2µg/ml; while erythromycin (Sigma-Aldrich, <country?>) and tetracycline (USB, USA) were at final concentration of 256 and 128µg/ml respectively. The flasks were incubated on a shaker with 220 rpm at 37°C. Ten microliters were drawn at 0, 1, 2, 4, 6 and 8h post antibiotic exposure, appropriately diluted and 10 µl spread plated on LB agar plate for determination of colony-forming units.

2.3.5.8.2 Effect of growth phase of the inoculum on persister cell formation

An overnight culture of DL5.4 was diluted 1:1000 into 20 ml fresh media contained in 125 ml flask and cultured on a shaker with 220 rpm at 37°C. Aliquot of 1 ml were drawn at 3, 6 and 9h representing early exponential, exponential and stationary growth phase respectively, and inoculated in 25ml MHB containing ciprofloxacin, levofloxacin, and norfloxacin at final concentration of 2 µg/ml, while erythromycin and tetracycline were used at final concentration of 256 µg/ml 128 µg/ml respectively. The flasks were incubated in a shaker at 37°C with 220 rpm. Ten microliters were drawn at 0, 1, 2, 4, 6 and 8h post antibiotic exposure, appropriately diluted and 10 µl was spread plated on LB agar plate for determination of colony-forming units.

2.3.5.8.3 Effect of varying antibiotic concentration on persister cell formation

Overnight grown culture of isolate DL5.4 was diluted 1:1000 into 20 ml fresh media in 100 ml conical flasks and cultured in a shaker with 220 rpm at 37°C. After 4h of incubation, 1ml culture aliquots were inoculated in five flasks containing 25ml MHB supplemented with antibiotics ciprofloxacin, levofloxacin and norfloxacin at final concentration of 2µg/ml, while erythromycin and tetracycline were at final concentrations of 256µg/ml and 128 µg/ml respectively (referred to as 1X). The cultures were grown on a shaker with 220 rpm at 37°C for 3h. After 3h of incubation, 10µl of sample were removed, diluted to 10^{-4} and 10^{-6} and 10 µl was spread plated on an LB agar plate for determination of colony-forming units. The remaining culture was centrifuged (10,000xg, 5 min, 10°C) and cell pellet resuspended in 2 ml fresh MHB with 2X concentration of respective antibiotics and further incubated for 4h. After 4h of incubation, 10µl of sample were removed, diluted to 10^{-4} and 10^{-6} and 10 µl was spread plated on an LB agar plate for determination of colony-forming units. The above steps were repeated with increasing antibiotic concentrations of 3X and 4X.

2.3.5.8.4 Determining the non-heritable nature of persister cells formed by DL5.4

As mentioned previously, 1:1000 dilution of overnight grown culture was inoculated in 25ml MH broth in 100ml flask and incubated at 37°C for 4h with 220 rpm. Ciprofloxacin, levofloxacin, norfloxacin were added to a final concentration of 2µg/ml and erythromycin and tetracycline to a final concentration of 256 and 128µg/ml respectively at 37°C for 4h with 220rpm. Surviving cells were pelleted (10000xg, 5 min, 4°C), and resuspended in 25ml fresh MHB with same concentration of respective antibiotics and further incubated for 4h. The process

was repeated for four generations and at each generation cells 10µl samples were aliquoted, diluted 10^{-4} and 10^{-6} and 10µl was spread plated for determination of colony-forming units.

2.3.5.8.5 Determining change in morphology of persister cells formed by DL5.4 by laser scanning microscope

The change in morphology, if any, in presence of antibiotics was examined by confocal microscope. An overnight culture was diluted 1:1000 in 25ml MH broth in culture flask and cultured on a shaker with 220 rpm at 37°C for 6h. After addition of antibiotics ciprofloxacin, levofloxacin, norfloxacin, erythromycin and ampicillin at 1X final concentrations, culture was allowed to grow for 6hour. Two microliters of 5-(and-6)-Carboxyfluorescein Diacetate (CFDA) of final concentration of 100µM was added and incubated for 15 min in dark. The stained cells (5 µl) were added onto glass slide and assessed by a laser-scanning confocal microscope (LSM 780; Carl Zeiss, Jena, Germany) with Argon laser 480 excitation and a 633X/1.4 NA oil immersion objective (Carl Zeiss). The microscope was equipped with an LSM-TPMT camera system and ZEN 2010 software (Carl Zeiss). Images were analyzed using LSM software (Carl Zeiss). All images were acquired at room temperature and digitally processed for presentation using Adobe Photoshop CS4 (Adobe Systems Incorporated, San Jose, CA, USA).

2.3.6. Clonal relationship by Multi locus sequence typing (MLST)

Sequence types of the isolates were determined by Multi locus sequence typing (MLST). Seven housekeeping genes, namely, beta-subunit of RNA polymerase (*rpoB),* glyceraldehyde-3-phosphate dehydrogenase *(gapA),* malate dehydrogenase (*mdh),* phosphoglucose isomerase (*pgi),* phosphorine E (*phoE),* translation initiation factor 2 (*infB)* and periplasmic energy

transducer *(tonB)* as described in the MLST database (http://*Klebsiella*.mlst.net). The primers used for the amplification of the housekeeping gene (**Table 2.20**) were according to Diancourt *et al*., 2005 [365].

Table 2.20: Oligonucleotides for multi locus sequence typing of *Klebsiella pneumoniae*

Gene	Primer	Primer sequence (5'-->3')	Amplicon size (bp)	Annealing temperature
pgi	pgi1F	GAGAAAAACCTGCCTGTACTGCTGGC	690	69°C
	pgi1R	CGCGCCACGCTTTATAGCGGTTAAT		
mdh	mdh130F	CCCAACTCGCTTCAGGTTCAG	740	66°C
	mdh867R	CCGTTTTTCCCCAGCAGCAG		
rpoB	Vic3	GGCGAAATGGCWGAGAACCA	501	60°C
	Vic2	GAGTCTTCGAAGTTGTAACC		
gapA	173F	TGAAATATGACTCCACTCACGG	450	64°C
	181R	CTTCAGAAGCGGCTTTGATGGCTT		
phoE	604.1	ACCTACCGCAACACCGACTTCTTCGG	420	64°C
	604.2	TGATCAGAACTGGTAGGTGAT		
infB	1F	CTCGCTGCTGGACTATATTCG	318	60°C
	1R	CGCTTTCAGCTCAAGAACTTC		
tonB	1F	CTTTATACCTCGGTACATCAGGTT	414	65°C
	1R	ATTCGCCGGCTGRGCRGAGAG		

Sequencing of all seven housekeeping genes was carried out on both the strands. Same primers were used for both amplification and sequencing. Sequence analysis was done using Seq-Scape v2.6 software (Applied Biosystems). Alleles and Sequence Types (STs) were assigned using the Institute Pasteur MLST databases (www.pasteur.fr/mlst/Kpneumoniae.html).Clonal relationship among *Klebsiella pneumoniae* isolates was determined by phylogenetic analysis. All housekeeping genes sequences obtained in

this study were concatenated in the order of *gap*A, *inf*B, *mdh*, *pgi*, *pho*E, *rpo*B and *ton*B and analyzed for their clonal relationships using the MEGA 6.0 (Molecular Evolutionary Genetics Analysis) software. Evolutionary history was inferred using the Neighbor-Joining method and evolutionary distances were computed using the Nei-Gojobori (assumed transition/transversion bias = 2) method and are in the units of the number of non-synonymous substitutions per synonymous site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. dN/dS ratio of the average of non-synonymous substitutions (dN) to the average of synonymous substitutions (dS) were calculates using Synonymous Non-synonymous Analysis Program (www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html*).*

2.3.7. Phenotypic screening for virulence factors

In addition to resistance mechanism, possession of virulence factors is equally important for bacteria to be successful as an opportunistic pathogen.

2.3.7.1 Adherence factors

2.3.7.1 1 Hemagglutination assays

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 Hennequin *et al*., (2009) [366] and type 3 pili (mannose-resistant *Klebsiella*-like hemagglutination [MR/KHA]) was examined as described by Podschun *et al*, 2001 [141]. For MSHA, 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 min at room temperature for visible

clumping. Type 3 pili (MR-HA) were assayed using tanned ox red blood cells. For the test with tanned Ox red cells, Ox blood was allowed to stand for some time for red blood cells to settle down. Serum was drawn carefully using pasture pipette. Red blood cells was washed twice in normal saline and treated with 0.003 % (w/v) tannic acid for 10 min at 37°C by the method of Boyden (1951) and then washed twice in normal saline before making up to a 3% (v/v) suspension in saline [367]. Bacteria were grown under static conditions for 48h and 50µl of bacterial suspension (approximately 10^{11} bacteria/ml) and 50 µl of erythrocytes were mixed in sterile glass slides, mixed well and observed for 3 min at room temperature. Agglutination was finally read after further incubation for 10 min at 4°C.

2.3.7.1.2 Hypermucoviscocity test

The string test for hypermucoviscocity was determined according to Lin *et al*., 2011 [368]. All the strains were inoculated on 5% sheep blood agar plates and incubated at 37°C over night. A standard bacteriological loop was used to stretch a mucoviscous string. The formation of a viscous string ≥5 mm long, representing the characteristic of hypermucoviscocity and regarded as a positive string test.

2.3.7.1.3 Hydrophobicity test

Bacterial hydrophobicity is assessed by hydrocarbon-xylene (BATH) method according to Wojnicz *et al.*, (2007) [369]. All isolates were grown in LB broth and harvested by centrifugation at 5000 rpm for 5 min. The harvested cells were washed in sterile phosphate buffer saline (pH 7.4) and suspended in the same buffer. Three milliliter of bacterial culture was vortexed with 1ml of xylene for 60S and left for 30 min. After the sample had separated in to two phase, optical density of the aqueous phase was measured at 470 nm. The degree of

hydrophobicity was expressed as the percentage decrease in optical density of lower aqueous phase compared with that of cell suspension without xylene.

2.3.7.2 Cell damage factors

2.3.7.2.1 Gelatinase production

Single colony from overnight grown pure culture was stabbed into tubes containing 12% gelatin in 0.8% Nutrient Broth (Difco Labs, Detroit, MI). Tubes were incubated at 37°C for 48 and 60h and then placed into the refrigerator for approximately 30 min. Extent of liquefaction of gelatin indicated extent of gelatinase production by the organism.

2.3.7.2.2 Phospholipase C production

Phospholipase C (PLC) has been suggested to be a key virulence factor in several infectious diseases and has been recovered from infected tissue and from bacterial isolates [370]. Apart from primary function in membrane maintenance, it damage fibroblast and muscle cell membrane, causes contraction of the isolated rat aorta, and stimulates the release of arachidonic acid [371]. To check presence of extracellular phospholipase activity in the aquatic environmental isolates, we used egg yolk agar plate method as egg yolk contain phospholipids and if bacteria produced phospholipase it depredate phospholipase around the bacteria. Egg yolk emulsion was prepared according to Billing *et al.,* (1957) by mixing four parts of sterile distilled water to one part of egg yolk (V/V) and heating at 45^{o}C in water bath for 2h [372]. The precipitate obtained was centrifuged at 7000xg for 10min. The supernatant was decanted and filter sterilized using a 0.22 µm membrane filter. One milliliter of this emulsion was added to 10ml of pre-sterilized LB nutrient agar to prepare egg yolk agar. The egg yolk agar had a translucent appearance initially. One hundred microliter of each culture supernatant was added into each well. The plates were incubated overnight at 37^{o}C and then observed for zone of

clearance around the well. The degree of enzymatic activity was measured at 24h and 48h by the diameter of the clear zone (mm) around the colony.

2.3.7.3 Survival in host

2.3.7.3.1 Serum resistance

The host's first line of defense after inflammation against invading microorganisms includes the bactericidal effect of serum, which is mediated primarily by complement proteins. The susceptibility of bacteria to human serum was determined [373]. Bacteria were diluted to $2x10^6$cells/ml in physiological saline. Twenty five microliters of bacterial suspensions and 75 µl of normal human serum (NHS) were put into micro titer trays, mixed, and incubated at 37°C. Viability was determined immediately and after 1, 2 and 3h of incubation. After mixing, samples were taken and serial dilutions were spread plated on brain heart infusion agar for colony counts. Responses were graded from 1 to 6 according to Hughes *et al.,* (1982) as shown in **table 2.21** [374]:

Table 2.21 Interpretation of serum resistance test

Grade	Viable counts	Viable counts	Interpretation
1	< 10% after 1 and 2h	<0-1% after 3h	highly
2	10-100% after 1h	< 10% after 3h	sensitive
3	> 100 % after 1h	< 100% after 2 and 3h	intermediately
4	> 100 % after 1 and 2h	< 100% after 3h	susceptible
5	>100% after 1, 2 and 3h	fell at some time during the 3h period	Resistant
6	>100% after 1, 2 and 3h	rise in cell count throughout 3h	

2.3.7.3.2 Biofilm formation ability

The biofilm forming ability of all isolates was determined as described by Stepanovic *et al*., (2000) with some modification [375]. Briefly, 2ml of BHI broth was inoculated with a loopful of test organism and incubated at 37^{o}C for 24 h. The culture was further diluted 1:100 with fresh medium and flat bottom tissue culture plates (96 wells) were filled with 200µl of diluted cultures individually. Uninoculated sterile broth served as control. The culture plates were incubated at 37^{o}C for 48h at 50 rpm rotation to mimic water flow in aquatic environment. Following incubation, the plates were washed with water to remove free-floating bacteria and dried at room temperature and fixed with methanol for 15 min. 0.1% Crystal Violet solution (fisher scientific) used for stained 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 dissolved in 200 mL of 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 (OD_c) for the micro titter plate test as three standard deviations. Isolated were classified as follows: OD < OD_c – non adherent, OD_c< OD < 2X OD_c –weakly adherent, 2 OD_c< OD < 4X ODc – moderately adherent and 4X OD_c< OD– strong adherent.

Chapter-3
Results & Discussion

The present study was undertaken with the aim to compare antibiotic resistance profiles, diversity of antibiotic resistance mechanisms and virulence factors in environmental and clinical isolates of *Klebsiella pneumoniae*. Results of this work are presented in this chapter along with major findings being discussed in light of the available literature. Based on the objectives laid down for the study, this chapter is been divided into three sub-sections.

Section 3.1: Isolation, identification of antibiotic resistant bacteria from aquatic environment and phenotypic characterization of virulence factors

In the first objective towards attaining our aim, we isolated bacteria from water samples and screened them for their antibiotic resistant profile and for presence of virulence factors.

3.1.1. Isolation and identification of bacteria from aquatic environment

Culture-based approaches were used for the determination of bacterial abundance and diversity in these aquatic environments. The total bacterial count in the four water sources varied considerably. Preliminary identification of bacterial isolates was done on the basis of the colony characteristic on the differential media as described in **Table 3.1.1**).

Table 3.1.1 Preliminary identification of bacterial isolates on the basis of growth on differential media

Organism	LB agar	Mac-Conkey	EMB
Escherichia coli	Low convex, round	LF/NLF	Green Metallic sheen
Klebsiella sp	Convex, round, mucoid	LF	Brown dark at center
Enterobacter sp	Convex, round	LF	
Pseudomonas sp	Swarming, pigmented	NLF	
Acinetobacter sp	Convex, round, mucoid	NLF	
Aeromonas sp			
Proteus sp	Concentric ring, Swarming	NLF	Colorless
Bacillus sp			

LF- Lactose fermenter, NLF- Non lactose fermenter, EMB agar-Eosin methylene blue agar, LB agar-Luria-Bertani, BGA-Brilliant green agar

Based on colony morphology in LB agar and differential media, 113 non-duplicate bacterial isolates were selected for further studies. Identification of these non-duplicate isolates up to genus level was done using 16S rRNA gene sequencing. The 1500bp PCR product of 16S rRNA amplified gene (**Fig 3.1.1**) was sequenced and analyzed. Sequences having ≥98% sequence similarity using online Basic Local Alignment Search Tool (BLAST) of the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) were assigned to the respective bacterial phylotypes.

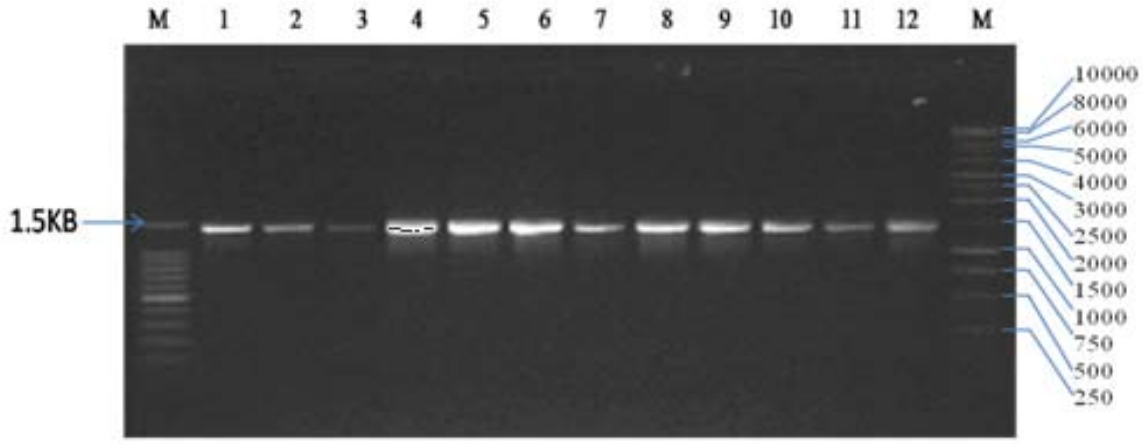


Figure 3.1.1: Agarose gel of PCR-amplified 16S rRNA gene.

Lanes 1-12: PCR positive products of 16S rRNA gene (~1400 bp) for isolates SR1.1, SR1.2, SR1.3, SR1.4, SR1.5, SR1.6, DL1.11, HD1.9, HD1.12, KR1.8, KR1.10 and KR1.13 respectively. M: DNA ladder (NEB, USA).

The 16S rRNA gene sequence analysis revealed the presence of 16 different genera of gram negative bacteria. These included pure colony isolates belonging to *Enterobacter* sp (n=18), *Pseudomonas* sp (n=16), *Aeromonas* sp (n=15), *Proteus* sp (n=15), *Acinetobacter* sp (n=14), *Klebsiella* sp (n=13), *E. coli* (n=3), *Citrobacter* sp (n=3), *Comamonas* sp (n=3), *Vibrio* sp (n=1), *Acidovorax* sp (n=1), *Providencia* sp (n=1), *Kluyevera* sp (n=1), *Burkholderia* sp

(n=1), *Cronobacter* sp (n=1) and *Ochrobacter* sp (n=1) (**Fig 3.1.2**). Beside these, gram positive bacteria belonging to genus *Bacillus* sp (n=4) and *Kurthia* sp (n=2) were also isolated.

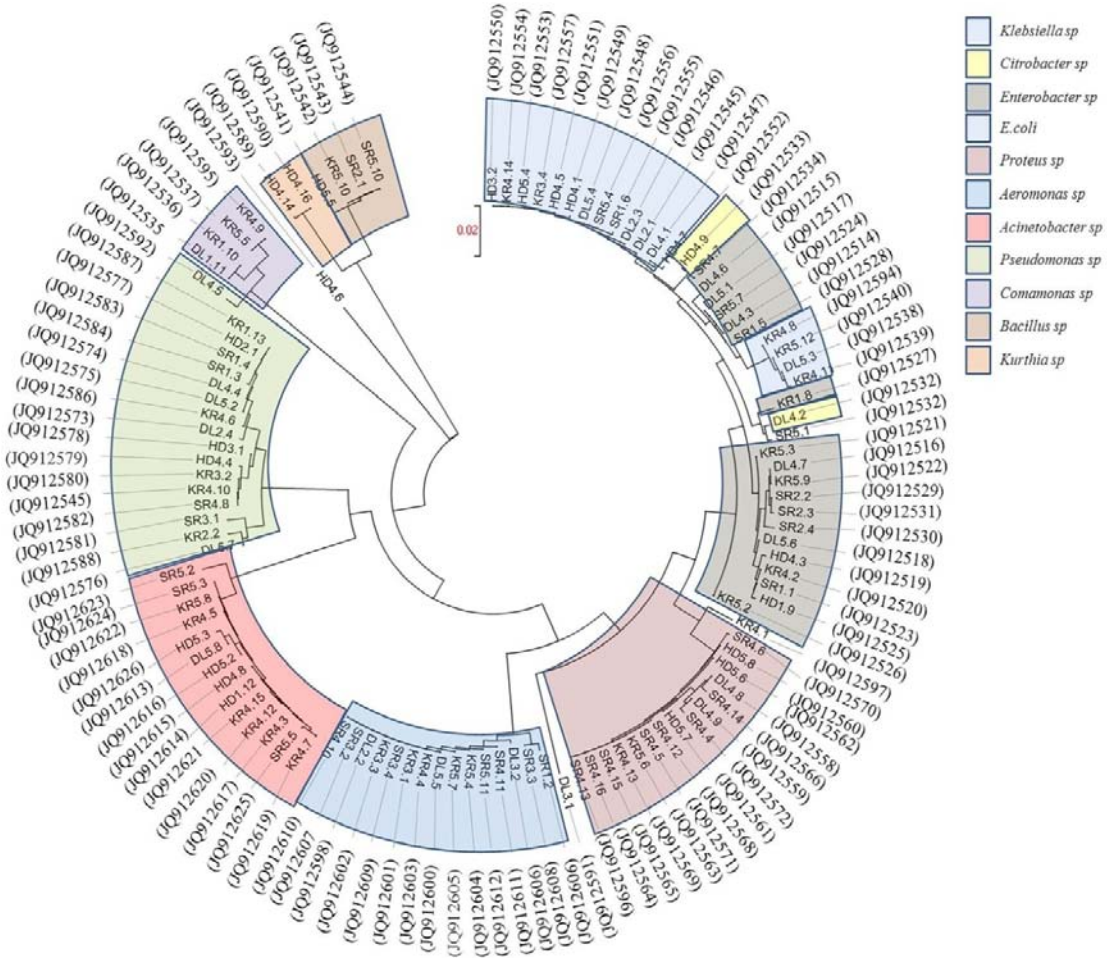


Figure 3.1.2: Phylogenetic analysis using 16S rRNA nucleotide sequences of 113 environmental bacterial isolates.

The evolutionary distances were computed using the modified Nei-Gojobori (assumed transition/transversion bias = 2) method and are in the units of the number of synonymous differences per synonymous site. There were a total of 410 positions in the final dataset. One thousand bootstrap replicates were performed for each analysis. Branches corresponding to partitions reproduced in less than 75% bootstrap replicates are collapsed (The final publication is available at Springer via http://dx.doi.org/10.1007/s10661-017-6005-4).

Identification of bacteria showed the presence of six major genera *Enterobacter, Pseudomonas, Aeromonas, Proteus, Acinetobacter* and *Klebsiella* covered approximately 81%

(n=90) of total bacterial isolates. These isolates accounted 26.5% (n=30), 22% (n=25), 17% (n=19) and 15% (n=17) from Subarnarekha River (SR), Kharkai River (KR), Dimna Lake (DL) and Hudco Dam (HD) respectively (**Table 3.1.2)**.

Table 3.1.2 Distribution of six major genera among different water sampling sites

Source	*Acinetobacter* sp (%)	*Aeromonas* sp (%)	*Enterobacter* sp (%)	*Klebsiella* sp (%)	*Proteus* sp (%)	*Pseudomonas* sp (%)
Subarnarekha river	3 (9)	7 (21)	6 (18)	2 (6)	8 (24)	4 (12)
Kharkai river	6 (18)	5 (15)	5 (15)	2 (6)	2 (6)	5 (15)
Dimna lake	1 (4)	3 (13)	5 (21)	4 (17)	2 (8)	4 (17)
Hudco dam	4 (18)	0 (0.00)	2 (9)	5 (23)	3 (14)	3 (14)
Total	14 (12%)	15 (13%)	18 (16%)	13 (12%)	15 (13%)	16 (14%)

Values in brackets indicate percentage of total bacterial isolates

3.1.2. Antimicrobial resistance profiling of bacterial isolates from aquatic environment

Resistance profiles of the 113 bacterial isolates towards 44 antimicrobial agents representing different groups, viz., β-lactams including cephalosporin, aminoglycoside, quinolones, macrolide, nitrofuran, polypeptide and sulfonamides revealed wide spread resistance albeit with varying degrees of resistance to different antimicrobials tested (**Fig 3.1.3)**. Most of the isolates were resistant to β-lactam group. Among the antibiotics belonging to penicillin group, about 90-100% isolates showed resistance towards ampicillin, cloxacillin, mezlocillin, naficillin, oxacillin and penicillin-G while least resistance was observed towards azlocillin (32%), piperacillin (35%) and carbenicillin (35%).

Among cephalosporin group, greater resistance was seen towards 1st and 2nd generation of the drugs represented by cephalothin (83%), cefoxitin (73%), and cefuroxime (49%). Least resistance was exhibited towards 3rd generation cephalosporin represented by as ceftazidime (22%), cefotaxime (5%), ceftriaxone (6%) and ceftizoxime (10%). It was interesting that isolates

exhibited greater resistance towards the 4th generation cephalosporins represented by cefepime (30%) and cefpirome (39%) as compare to 3rd generation cephalosporin.

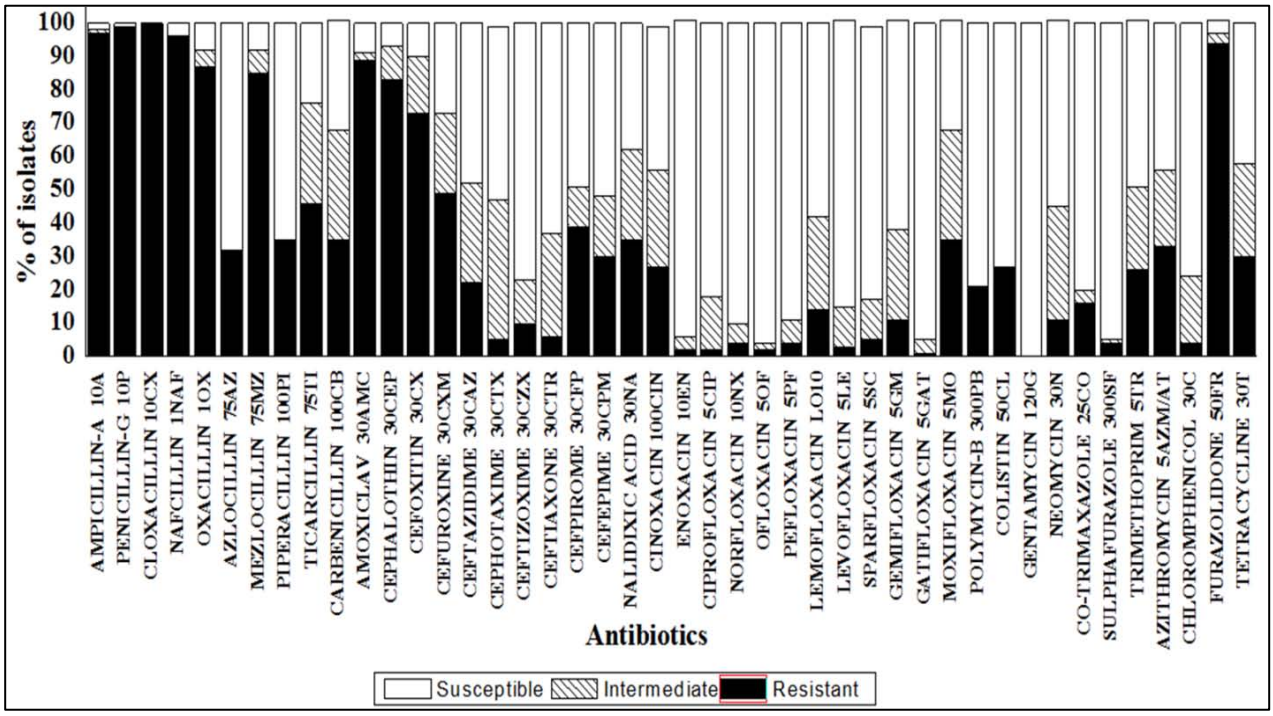


Figure 3.1.3: Antibiotic resistance profile of 113 environmental bacterial isolates.

Graph depicting percentage of isolates showing Resistance-Intermediate-Sensitivity towards different classes of antibiotics tested by disk diffusion method. X-axis: antibiotics with their potency and abbreviation; Y-axis: Percentage of isolates showing different phenotypes (The final publication is available at Springer via http://dx.doi.org/10.1007/s10661-017-6005-4).

Resistance towards quinolones was relatively less compared to β-lactams and cephalosporins. Only 34% isolates showed resistance towards at least one commonly used quinolone. The resistance pattern towards individual quinolones was as follows: nalidixic acid (35%), cinoxacin (27%), ciprofloxacin (2%), norfloxacin (4%), gatifloxacin (1%), ofloxacin (2%), levofloxacin (3%), pefloxacin (4%), sparfloxacin (5%) and moxifloxacin (35%). Overall resistances towards aminoglycosides tested were less as compared to other antimicrobial

categories. Eleven percent isolates (n=12) were resistant against neomycin while no resistance was observed against gentamicin. It was observed that 24% and 28% isolates were resistant towards polypeptides polymyxin-B and colistin respectively. Apart from the above-mentioned antibiotics, 33%, 30% and 3% isolates were resistant to azithromycin, tetracycline and chloramphenicol respectively, representing antimicrobials that are protein synthesis inhibitors. Similarly 26% and 16% isolates were resistant to trimethoprim and co-trimaxazole respectively that are folic acid synthesis inhibitors. Box plot showing distribution of resistance patterns of individual isolates towards antimicrobial agents used for therapeutic purposes against respective genera **Fig. 3.1.4A-F**.

The minimum inhibitory concentration (MIC) for seven antibiotics was tested against all the isolates using broth double dilution method and breakpoint values were interpreted as per CLSI-(2007) guideline. As shown in the **Table 3.1.3,** MIC breakpoint values corroborated well with the antibiotic susceptibility results. Isolates categorized as resistant based on disc diffusion results, also exhibited very high MIC values. Out of 91 isolates resistant to ampicillin, 75 isolates exhibited MIC break point ≥128µg/ml. Similarly, out of 27 isolates resistant to cefotaxime, 23 isolates exhibited MIC break point ≥256µg/ml. Four isolates were resistant to ciprofloxacin with breakpoint of 8µg/ml while two isolates were resistant to levofloxacin (16µg/ml). 76% (n=39), 84% (n=76) and 63% (n=25) isolates resistant to tetracycline, erythromycin and trimethoprim had MIC value ≥64µg/ml.

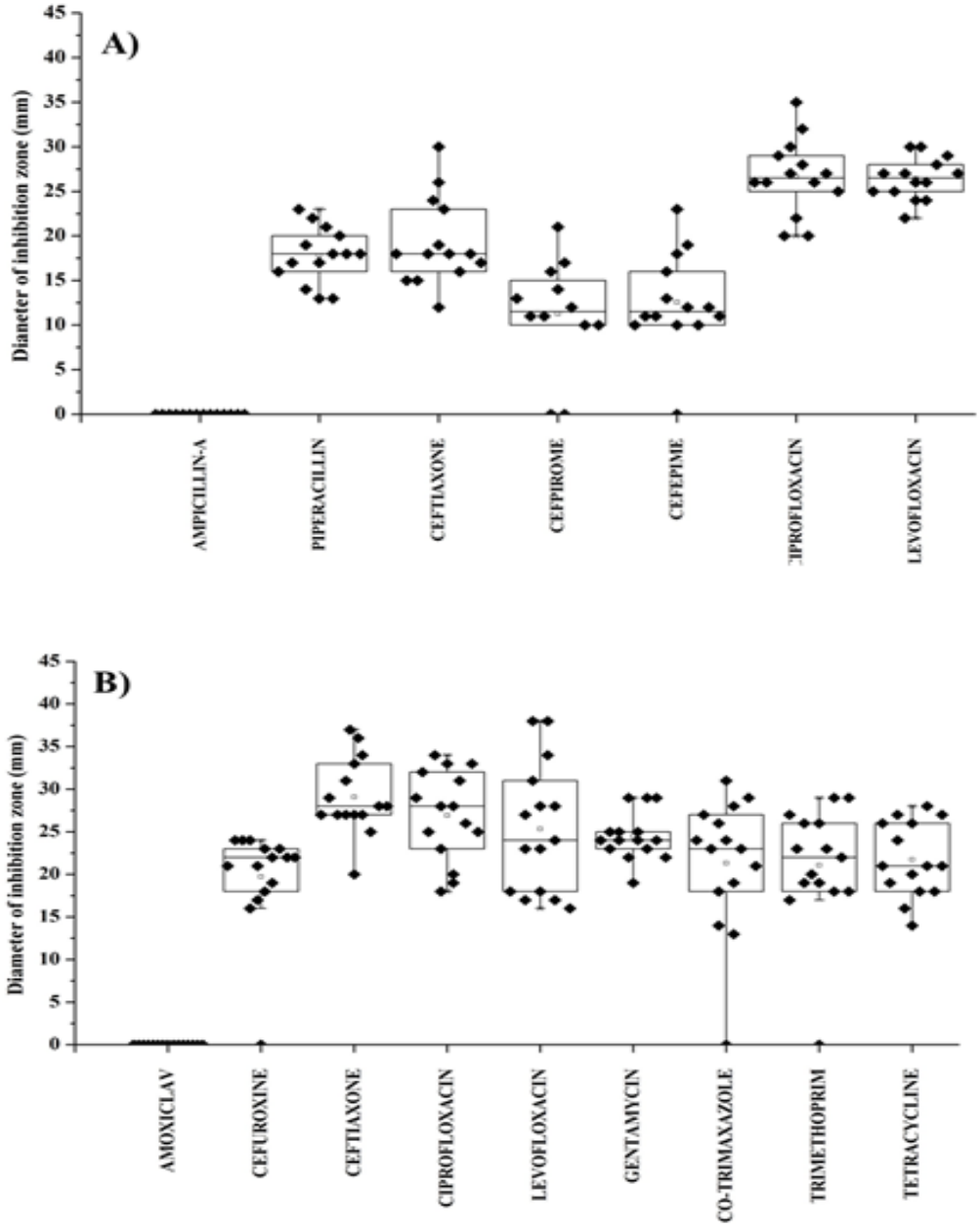


Figure 3.1.4: Box plot showing distribution of resistance patterns of individual isolates towards antimicrobial agents used for therapeutic purposes against respective genera.

A – *Acinetobacter* isolates and B - *Aeromonas* isolates. The box extends from the 25th to the 75th percentile, with a line at the median (50th percentile). The whiskers are extended to show the highest and lowest values. The crosses represent outliers and empty squares represent mean values.

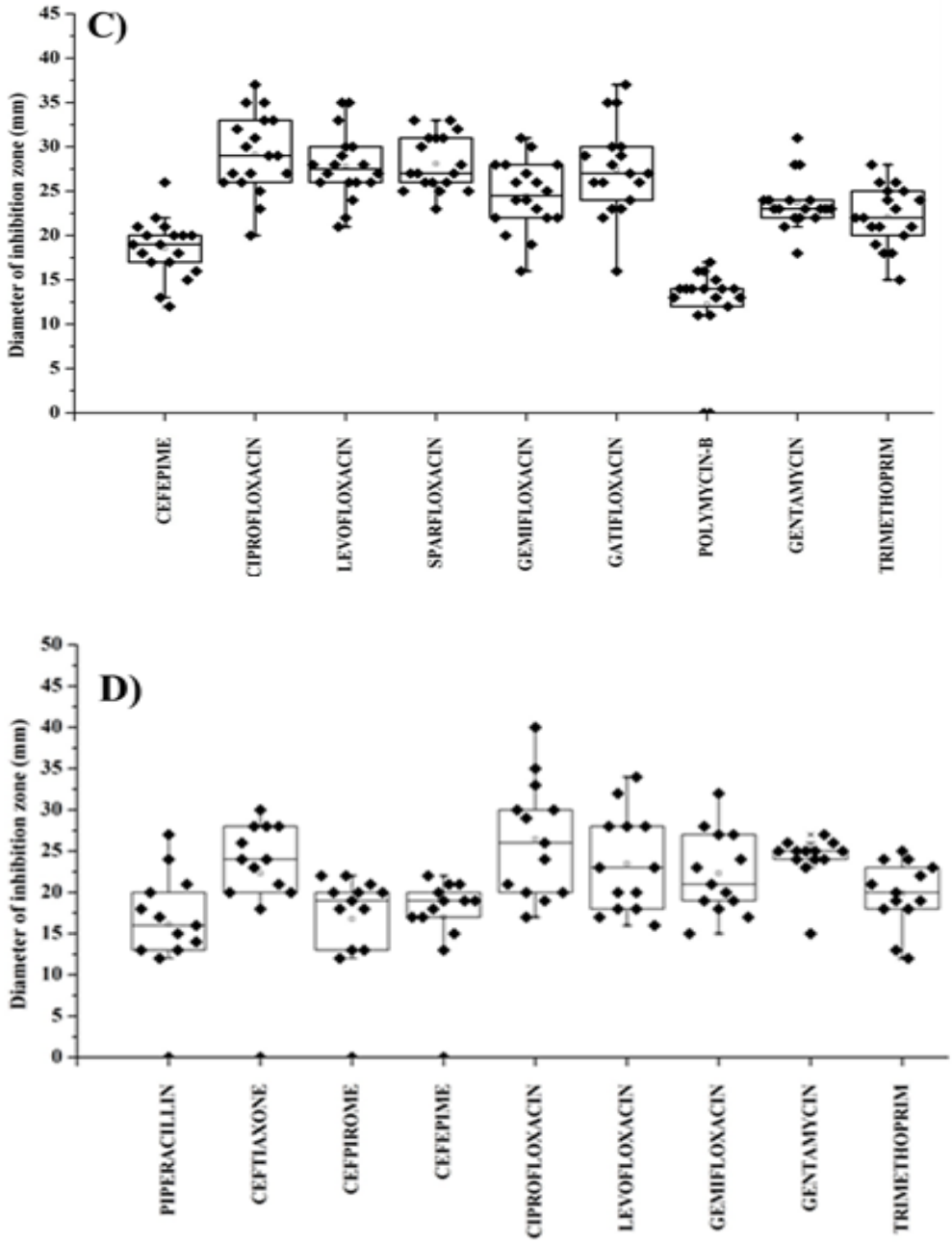


Figure 3.1.4: Box plot showing distribution of resistance patterns of individual isolates towards antimicrobial agents used for therapeutic purposes against respective genera.

C - *Enterobacter* isolates and D - *Klebsiella* isolates. The box extends from the 25th to the 75th percentile, with a line at the median (50th percentile). The whiskers are extended to show the highest and lowest values. The crosses represent outliers and empty squares represent mean values.

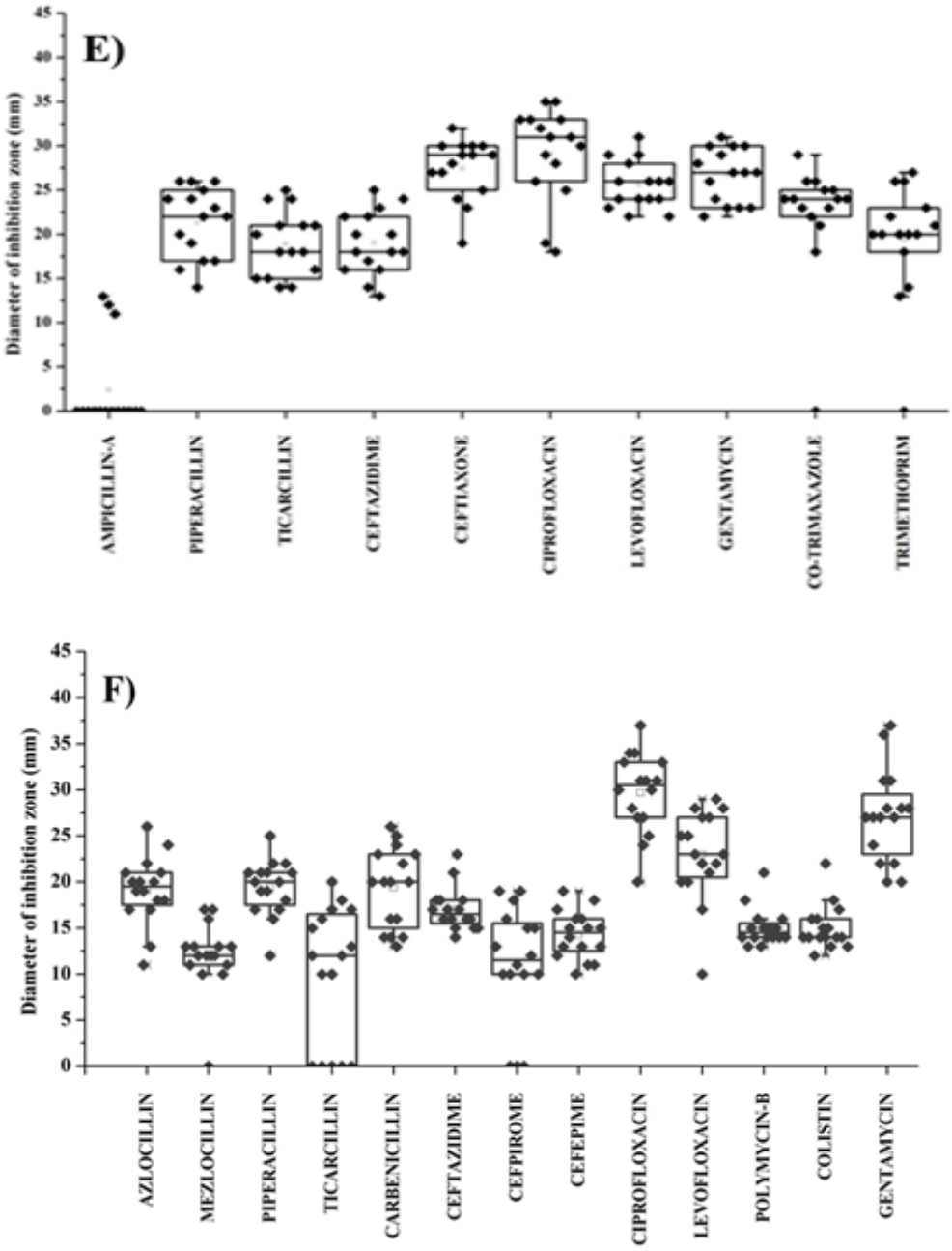


Figure 3.1.4: Box plot showing distribution of resistance patterns of individual isolates towards antimicrobial agents used for therapeutic purposes against respective genera.

E – *Proteus* isolates and, F – *Pseudomonas* isolates. The box extends from the 25th to the 75th percentile, with a line at the median (50th percentile). The whiskers are extended to show the highest and lowest values. The crosses represent outliers and empty squares represent mean values.

Table3.1.3 Minimal inhibitory concentration of different antibiotics for multidrug resistant bacteria isolated from water sources

Antibiotic	CLSI (2007) breakpoint for resistant (µg/ml)	Number of resistant isolates as per CLSI breakpoint
AMP	≥32	101
CTX	≥32	40
CIP	>4	4
LE	>8	2
T	>16	51
E	≥8	113
TRI	>8	40

AMP- Ampicillin, CTX- Cephotaxime, CIP- Ciprofloxacin, LE- Levofloxacin, T- Tetracycline, E- Erythromycin, Tri- Trimethoprim; Shaded portion showed resistant categories.

Determining multiple antibiotic resistance (MAR) index

Bacterial isolates exhibited varied resistance patterns towards multiple antimicrobial agents. These observations lead us to analyze the multiple antibiotic resistances (MAR) index of the isolates belonging to the different genera. MAR index is useful in analyzing potential high-risk populations and high-risk environment. MAR index of individual isolates showed that 73 isolates (65%) had MAR index >0.3 with the highest being 0.68 in the population under study.

Comparative analysis of MAR indices of the representative populations indicated that *Pseudomonas* isolates exhibited highest multiple drug resistant phenotypes with an average MAR index of 0.41. Isolates belonging to *Acinetobacter*, *Klebsiella* were next with MAR indices 0.36 while *Proteus* and *Aeromonas* had MAR indices of 0.33 and 0.32 respectively. However, as evident from the box-plots, MAR indices of *Enterobacter* isolates exhibited relatively lower values with average of 0.29. Of the three *E. coli* isolates, one isolate KR5.12 exhibited highest MAR index of 0.68. The isolate was resistant towards 30 different antibiotics. Since average MAR indices of the genera differed from each other, we performed Kruskal-Wallis ANOVA.

Kruskal-Wallis ANOVA on ranks test statistic gave H value of 23.995 with $p < 0.001$ indicating that differences in the median values among the groups are statistically significant. Pair-wise multiple comparisons by Dunn's method were carried out to isolate the group or groups that differ from the others. The results of the pair-wise multiple comparison revealed significant difference in MAR indices ($p < 0.05$) between *Pseudomonas* and *Enterobacter* with Q value of 4.539, *Pseudomonas* and *Aeromonas* with Q value of 3.206, *Pseudomonas* and *Proteus* with Q value of 3.153 (**Fig 3.1.5**). These observations lead us to conclude that propensity of multiple antibiotic resistance phenotype amongst the genera could be ranked in the order, *Pseudomonas* >*Klebsiella = Acinetobacter* >*Proteus* >*Aeromonas* >*Enterobacter*.

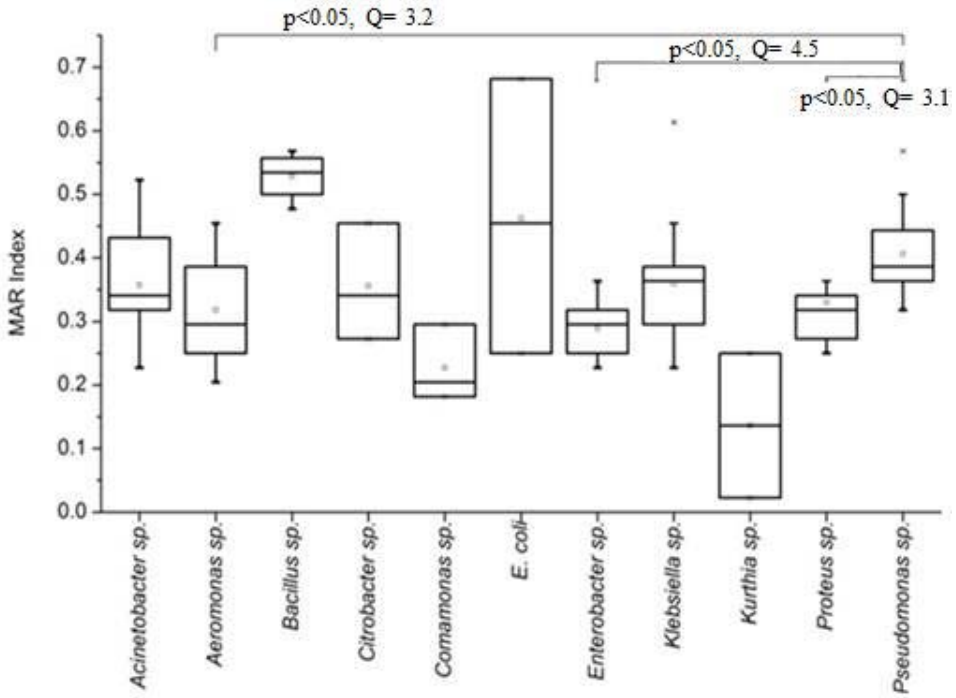


Figure 3.1.5: Box plot of Multiple Antibiotic Resistance Index of different groups of bacteria derived from disk diffusion experiment.

The box extends from the 25th to the 75th percentile, with a line at the median (50th percentile). The whiskers are extended to show the highest and lowest values. The crosses represent outliers and empty squares represent mean values.

For four water sources, average MAR indices were 0.352, 0.368, 0.303 and 0.353 for Subarnarekha River, Kharkai River, Dimna Lake and Hudco Dam respectively that indicated high antibiotic usage at these areas (**Fig 3.1.6)**.

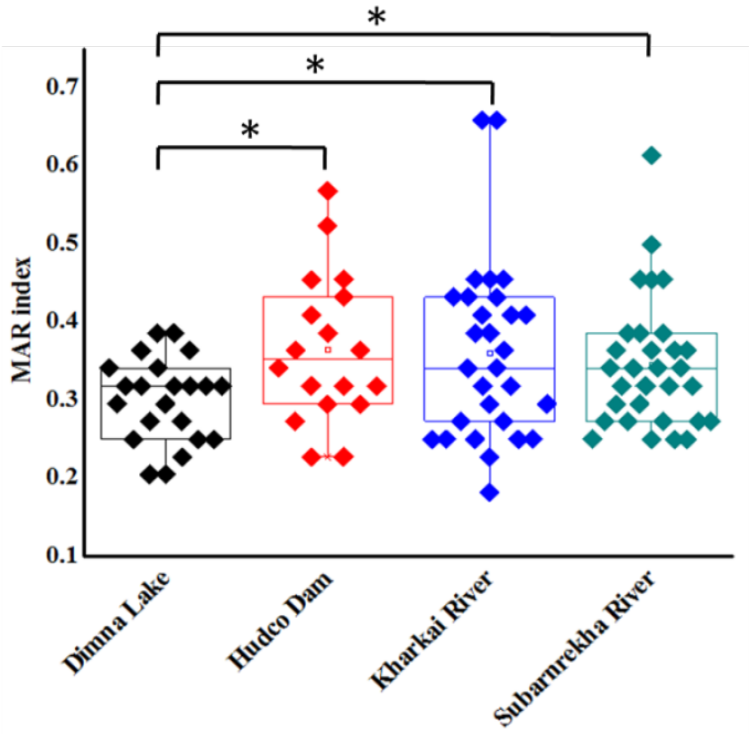


Figure 3.1.6: Box plot of Multiple Antibiotic Resistance Index.

MAR index of different water sources (P value ≤ 0.005 was taken as significant). The box extends from the 25th to the 75th percentile, with a line at the median (50th percentile). The whiskers are extended to show the highest and lowest values. The crosses represent outliers and empty squares represent mean values.

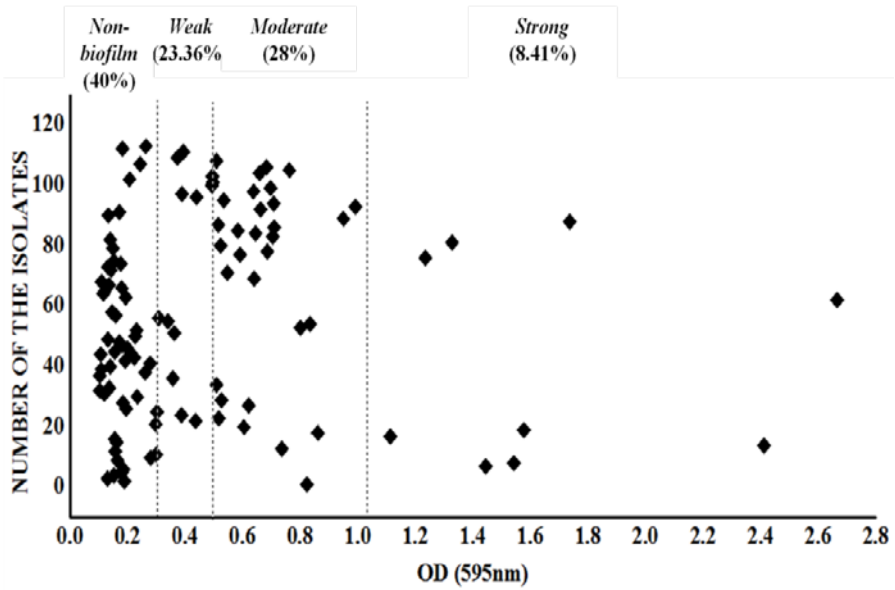
3.1.3. Phenotypic screening for virulence factors of bacteria isolated from aquatic environment

Virulence factors are viewed as the properties (gene products) that alter host–microbe interactions and enable a microorganism to establish itself on or within a host of a particular species and enhance its potential to cause disease. Therefore, in addition to resistance mechanism, possession of virulence factors is equally important for a bacterium to be successful

as an opportunistic pathogen. Hence we analyzed the possession of virulence factors that help in adherence and colonization. Phenotypic assays for biofilm forming ability type 1 fimbriae, hydrophobicity, phospholipase-C, gelatinase production, haemolytic activity and were carried out to determine the virulence factors responsible for adherence, invasion, and survival in the host cells.

The biofilm formation by bacteria has been suggested to be an important stage in the pathogenesis of numerous bacterial species as biofilm is resistance to antimicrobial agents and are the major cause of recalcitrant infections. It provides protection from environmental stress and protection against host defense mechanisms, thus responsible for 65% of all bacterial infections. Biofilm forming ability of all the isolates were shown in the **figure 3.1.7**. Only 8% (n=9) isolates were strong biofilm former whereas 28% (n=30), 23% (n=25) and 40% (n=43) isolates were moderate, weak and non-biofilm former (**fig. 3.1.7A)**. Genus wise comparative analysis of biofilm forming ability revealed that majority of *Klebsiella* (n=11) isolates were strong to moderate biofilm formers while majority of *Enterobacter* (n=10) isolates were non-biofilm formers, except isolate DL5.1 which was a strong biofilm former having fimbriae (**Figure 3.1.7B**). Similar trend was observed in case of genera *Pseudomonas* and *Aeromonas.* Among *Acinetobacter* isolates, exceptions were isolates DL5.8 and HD4.8, rest of the isolates could be ranked as none or moderate biofilm producers. In case of *Proteus* the biofilm forming ability was weak to moderate (**Figure 3.1.7B**).

A)



B)

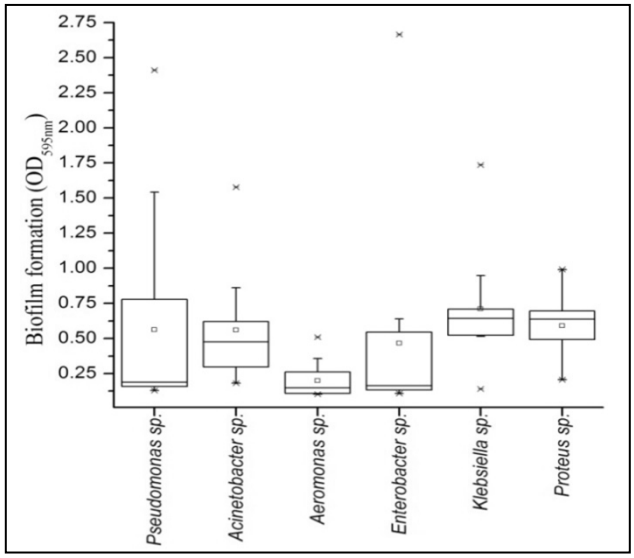


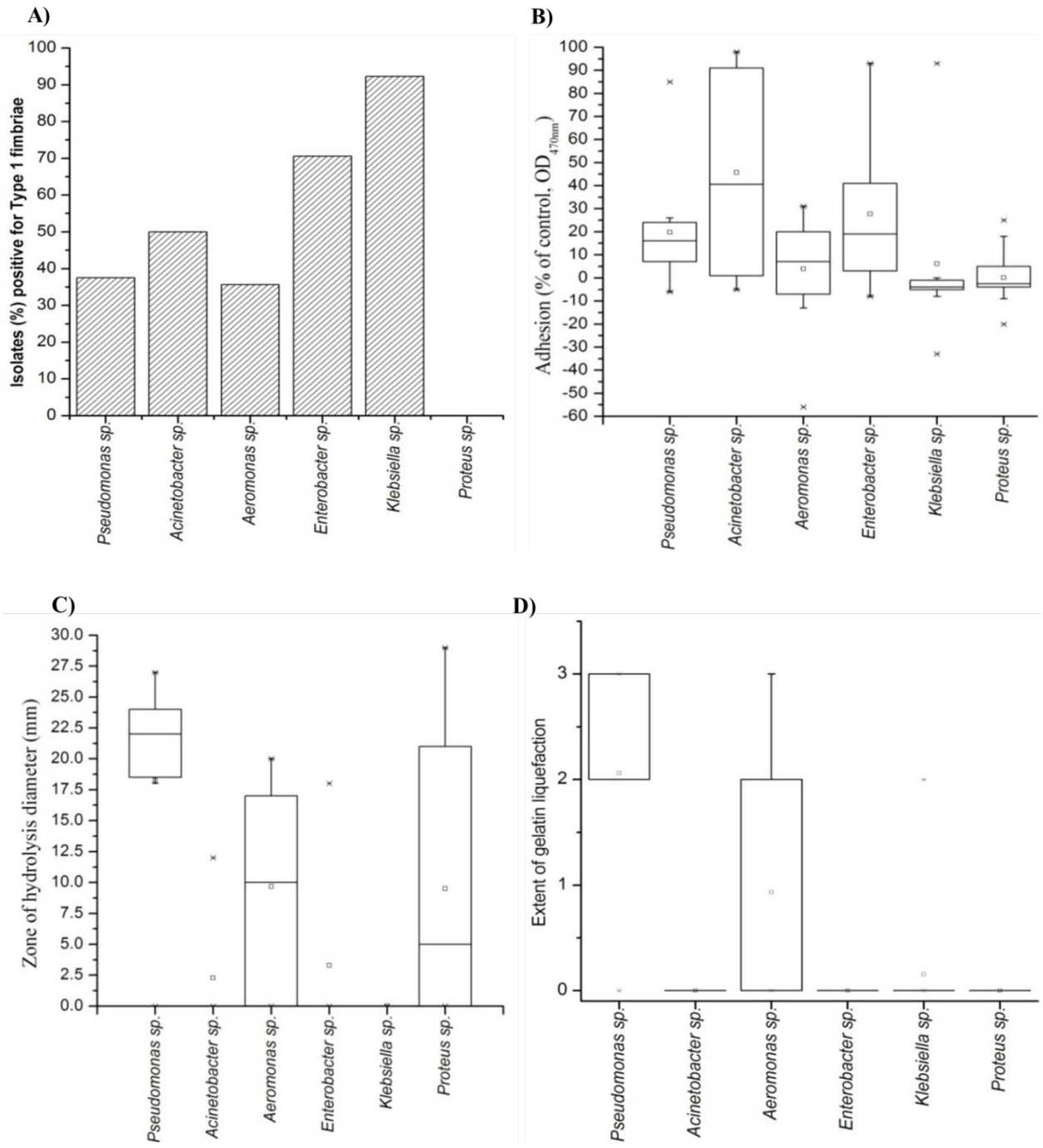
Figure 3.1.7: Biofilm formation by aquatic environmental isolates.

(A) Biofilm formation ability of all bacteria isolate and (B) Genus wise comparative analysis of biofilm forming ability. $OD_{595nm} \leq 0.253$; $0.253 < OD_{595nm} \geq 0.506$; $0.506 < OD_{595nm} \geq 1.012$ and $OD5_{595nm} > 1.012$ were called none, weak, moderate and strong biofilm former respectively. (B) Box plot of biofilm forming ability of different genera of bacteria.

Type 1 fimbriae are one of the factors for adherence to host. We screened all the isolates for presence of type 1 fimbriae using Hemagglutination assay. Hemagglutination assay revealed 45.13% isolates have type 1 fimbriae. Results showed that 92% *Klebsiella pneumoniae* isolates possessed type 1 fimbriae. Except for DL4.1, all the isolates of *Klebsiella* sp. were positive for

type 1 fimbriae whereas all the *Proteus* sp. isolates were negative for type 1 fimbriae. 77% of *Enterobacte*r, 50% of *Acinetobacter*, 38% of *Pseudomonas* and 33% of *Aeromonas* isolates were positive for type 1 fimbriae (**Figure 3.1.8A**). Hydrophobicity is an indirect way of determining adhesion properties; as microorganism may adhere to a substratum via hydrophobic effect if the association sites possess sufficiently high densities of apolar areas. Overall across the six predominant gram-negative genera tested, greater hydrophobicity was observed in the genera *Klebsiella* followed by *Proteus* while *Pseudomonas* and *Enterobacter* were least hydrophobic. Interestingly all the three *E. coli* and *Bacillus* isolates were highly hydrophobic (**Figure 3.1.8B**).

Phospholipase C (PLC) and gelatinase have been suggested to be a key virulence factor in several infectious diseases and has been recovered from infected tissue and from bacterial isolates. Apart from primary function in membrane maintenance, it damages fibroblast and muscle cell membrane, causing contraction of the isolated rat aorta, and stimulates the release of arachidonic acid. Extracellular PLC (**Figure 3.1.8C**) and gelatinase (**Figure 3.1.8D**) was produced at varied amount by the six prominent gram-negative genera in the present study. Significant amount of extracellular PLC and gelatinase production was seen in case of *Pseudomonas* spp. followed by *Aeromonas* and *Proteus*. Majority of *Acinetobacter*, *Klebsiella* and *Enterobacter* isolates were either weak or none producers. Isolates belonging to *Acinetobacter, Citrobacter, Comamonas* and *E. coli* were non-haemolytic in nature (**Figure 3.1.8E**). A completely clear zone of hydrolysis – beta hemolysis, was observed in following isolates; six belonging to *Pseudomonas* sp., two belonging to *Aeromonas* sp, two belonging to *Enterobacter* sp, two belonging to *Proteus* sp and one each belonging to *Klebsiella* and *Providencia* sp. An incomplete hydrolysis – alpha hemolysis, was seen in one *Aeromonas* isolate.


A)
Isolates (%) positive for Type 1 fimbriae
100
90
80
70
60
50
40
30
20
10
0
Pseudomonas sp.
Acinetobacter sp.
Aeromonas sp.
Enterobacter sp.
Klebsiella sp.
Proteus sp.
B)
Adhesion (% of control, OD470nm)
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
Pseudomonas sp.
Acinetobacter sp.
Aeromonas sp.
Enterobacter sp.
Klebsiella sp.
Proteus sp.
C)
Zone of hydrolysis diameter (mm)
30.0
27.5
25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Pseudomonas sp.
Acinetobacter sp.
Aeromonas sp.
Enterobacter sp.
Klebsiella sp.
Proteus sp.
D)
Extent of gelatin liquefaction
3
2
1
0
Pseudomonas sp.
Acinetobacter sp.
Aeromonas sp.
Enterobacter sp.
Klebsiella sp.
Proteus sp.

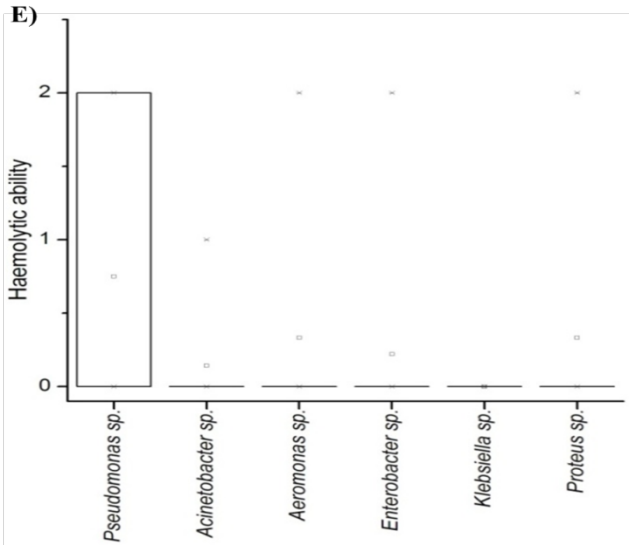


Figure 3.1.8: Phenotypic screening for virulence factors.

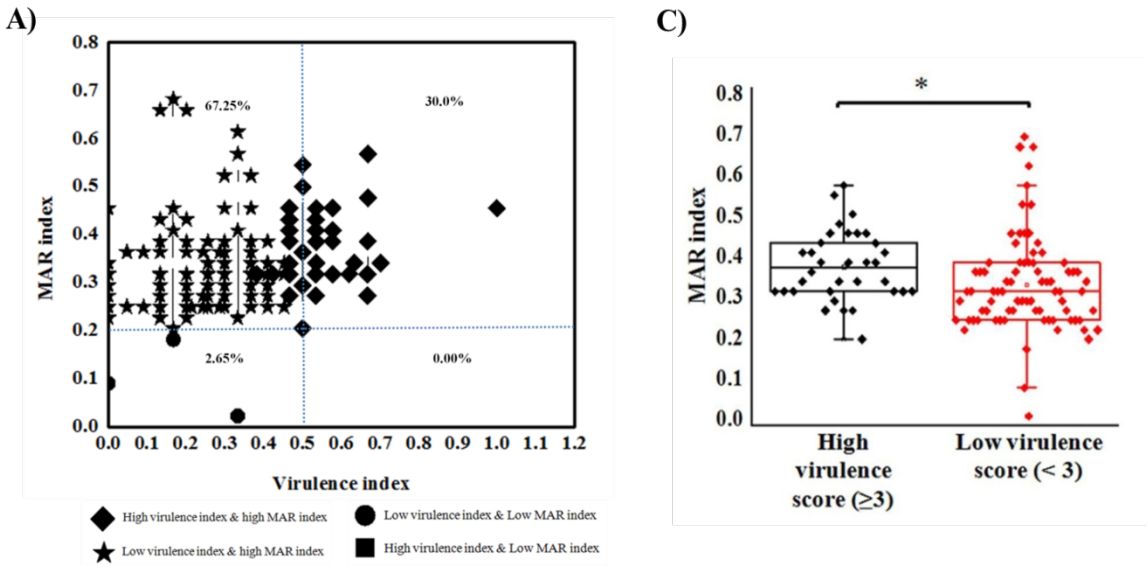
A - Percent of isolates positive for type 1 fimbriae. B- Box plot depicting hydrophobicity of isolates belonging to six gram negative genera assayed by bacterial adherence to hydrocarbon-xylene. C - Box plot of phospholipase C production by isolates belonging to six gram negative genera measured as zone of hydrolysis on egg yolk emulsion plates. D - Box plot of gelatinase production as determined by the extent of hydrolysis of gelatin. Values on the Y-axis represent extent of gelatin liquefaction ranging from no hydrolysis (numeric value 0) to complete liquefaction (numeric value 3). E - Box plot depicting haemolytic ability of different bacterial isolates as determined by the extent of hydrolysis of sheep blood RBC's. Values on Y-axis correspond to complete, partial or no hemolysis in descending numerical values respectively (The final publication is available at Springer via http://dx.doi.org/10.1007/s10661-017-6005-4).

3.1.4 Association between MAR index and virulence index

From our previous results we observed differences in MAR indices and expression of virulence factors across the genera. So we tried to find out if there exists any association between MAR index and virulence index in environmental multidrug resistant isolates used the present study. In literature MAR index of 0.2 (arbitrary) has been used as cut off [376]. The mean value of MAR indices of the population under study was 0.34. Hence, MAR index $\geq$ 0.3 and virulence index $\geq$ 0.5 was taken as cut-off. Isolates of each genus was categorized into four groups: (i)

High threat –wherein isolates have virulence and MAR indices greater than or equal to cut-off values, (ii) Moderate threat –comprising of isolates having virulence index≥ 0.5 value but MAR index <0.3, (iii) Low threat – comprising of isolates having virulence index <0.5 with MAR index ≥0.3, and (iv) No threat –comprising of isolates having low virulence and MAR index below cut-off values

As is depicted in **Fig 3.1.9A**, 30% of isolates in the population under study were under high threat category. Analysis at the genus level indicated that 75% of isolates belonging to *Pseudomonas* sp could be categorized under high threat category as they have high MAR index and high virulence index. Whereas isolates belonging to genera *Aeromonas*, *Acinetobacter*, *Klebsiella* and *Proteus* ranged in the category of moderate to low threat (**Fig 3.1.9B**). With these observations we were tempted to determine whether there existed any significant association between extent of multidrug resistance and virulence properties exhibited by the entire population under study. Unpaired t-test performed to compare variance between MAR indices of isolates having high and low virulence score indicated that there existed significance difference between the two groups (p<0.02) (**Fig 3.1.9C**).



B)

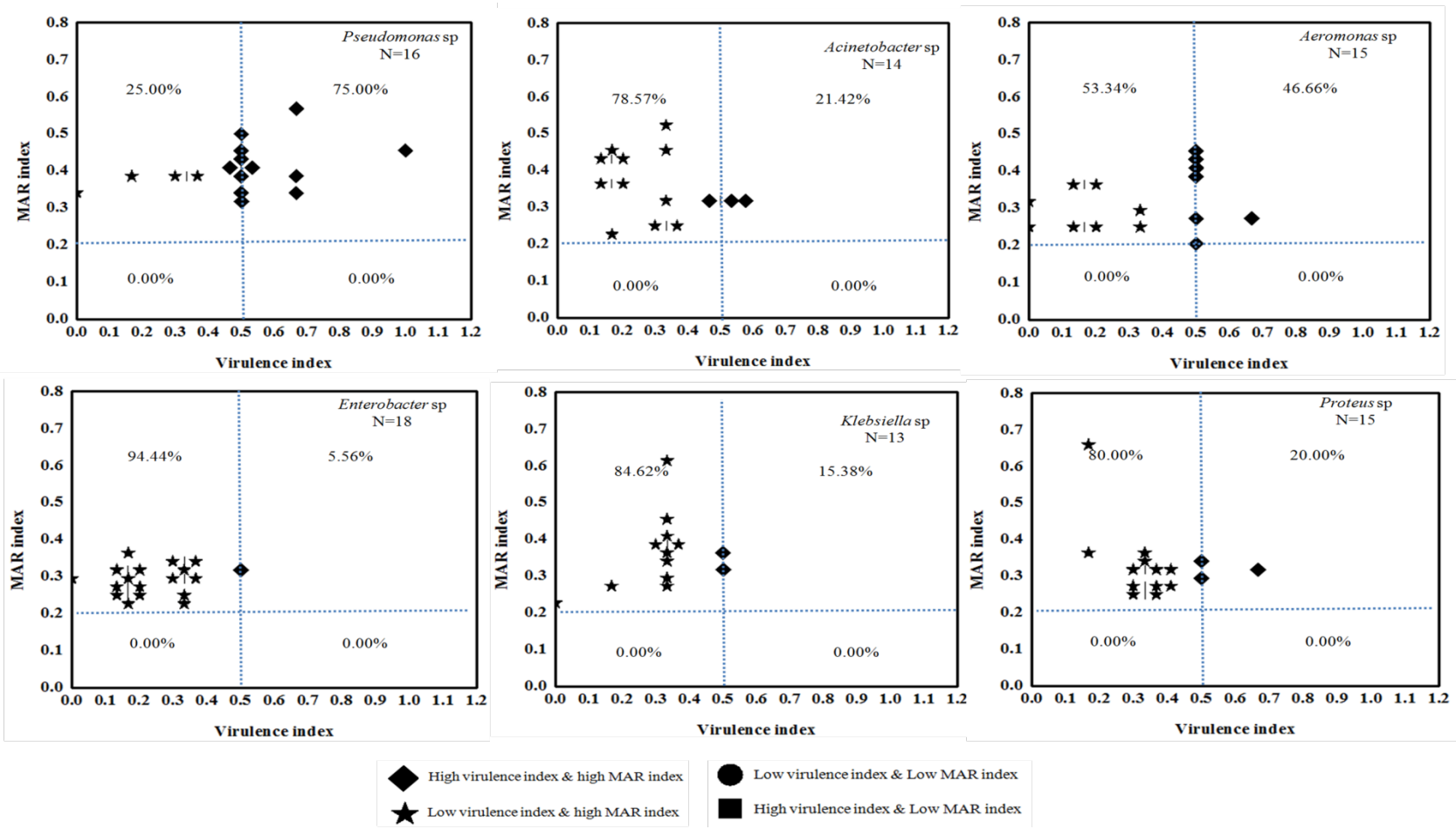


Figure 3.1.9: Association of MAR index and virulence index.

(A) All isolates in the population under study representing (B) genus wise and (C) sub-sets of the population with high and low virulence score. Dotted lines represent cut off values of 0.3 that divides the plane to four quadrants where individual isolates are placed as per the legend (The final publication is available at Springer via http://dx.doi.org/10.1007/s10661-017-6005-4).

3.1.5 Discussion

Anthropogenic activities exert significant influence and affect the physio-chemical and biological parameters of the surrounding environment. Aquatic environment is first and most to be affected by such interferences, as it serves as a sink receiving treated and untreated municipal waste discharge from different sources. In addition it also receives various contaminants such as residual pesticides, herbicides, fertilizers, growth promoters etc. from fields as run-off water. Amongst various contaminants, antibiotics are regarded as emerging contaminant that has an unintended negative consequence as it lead to an increase in antibiotic resistant bacteria (ARBs) in the environment [377,378,379,380,381]. Bacterial resistance to different classes of antimicrobial agents in a natural water environment such as a river represents a source of threat to human health and with a presumption of leading the world towards the return of the pre-antimicrobial era [123]. These antibiotic resistant bacteria can infect people by consumption of water or through direct contact with the water during bathing or through food weds (consumption of fish containing AR bacteria). An integrated approach towards analyzing antibiotic resistance and pathogenic potential, especially from environmental isolates is vital as it provides information on extent of threat to public health due to circulation of multidrug resistant bacteria in the environment and helps to confine or at least delay this development. The number of studies investigating Indian rivers as reservoirs of multidrug resistant potential pathogenic bacteria is still very limited. Data mostly for MDR *E. coli* as an indicator of faecal coliforms in the rivers are available [109,114]. In the present study, we isolated and identified multidrug resistant bacteria and did association analysis of MAR index and virulence factors for the detection of potential multidrug resistant pathogenic bacteria in the aquatic environment.

Water samples were taken from river, dam and Lake of Jamshedpur city. This aquatic system receives biologically-treated effluent from the nearby sewage works. 16S rRNA gene sequencing of bacteria isolated from these aquatic system revealed majority of isolates were represented by the six gram negative genera *viz. Pseudomonas, Aeromonas, Acinetobacter*, *Klebsiella*, *Proteus* and *Enterobacter*. It is worth mentioning that except *Proteus*, these bacteria are known opportunistic pathogens and have been implicated as etiological agents of hospital acquired infections both in developed and developing countries [382]. Rest of the isolates were identified as *Bacillus, Kluyevera, Acidovorax, Ochrobacter, Vibrio, Cronobacter, Providencia, Burkholderia* and *Kurthia*.

Percentage antibiotic susceptibility in terms of resistant, intermediate and susceptible (RIS) analysis indicated that the majority of the isolates were resistant towards penicillin and commonly used cephalosporins belonging to first, second and fourth generations. Consistent with our results Papandreou *et al*., (2000) have reported higher resistance towards cefoxitin and cefuroxime and lower resistance towards cefotaxime and ceftriaxone in isolates from drinking water samples in Greece [383]. Similarly, Skariyachan *et al.,* (2015) have also demonstrated that most of the isolates from Cauvery River, India were also found to be resistant to third and fourth generation cephalosporins [117]. High resistance toward cephalosporins have been due to extensive use of the drug as an alternative to penicillin G in respiratory and soft tissue infection caused by gram negative organisms especially *Klebsiella* sp, *Proteus* sp, *Enterobacter* sp. [384]. Second and third generation cephalosporins are also frequently used in surgical prophylaxis including treatment of mixed-aerobic and anaerobic infections in cancer patients. Ceftizoxime has been reported to have good cerebrospinal fluid penetration in patients with bacterial meningitis [385,386,387] and is not metabolized in body and eliminated through kidney [384].

For the above-mentioned reasons this is the drug of choice in wide range of serious infections including bacterial meningitis especially in children [388]. A disturbing observation was of nearly 89% isolates showing resistance towards Amoxiclav, which is the drug of choice for treating β-lactamase resistant *E. coli, Proteus* sp *and Klebsiella* [389]. Low resistance was seen towards piperacillin and carbenicillin. Carbenicillin still remains the preferred drug of choice for treating *Pseudomonas* and indole positive *Proteus* which is not inhibited by penicillin G or aminopenicillins [384]. In the present study, resistance among isolates towards quinolones was relatively less compared to β-lactams and cephalosporins. Resistance towards fluoroquinolones has been attributed to the occurrence of mutations in the target enzymes and/or due to reduced permeability or efflux of these drugs. Low resistance towards commonly used fluoroquinolones obtained in the present study provides a circumstantial evidence for the fact that accumulation of mutations resulting in target modifications is a less prevalent mechanism of resistance amongst the environmental isolates. Results of our study differ from Alouache *et al*., (2014), where the authors have observed similarity between antibiotic resistance mechanisms in the clinical setting and the environmental isolates of *E. coli* and *Klebsiella pneumoniae* from a wastewater treatment plant [390].

MAR indices are useful in analyzing potential high risk populations and high risk environment [376]. Site wise analysis of MAR index revealed that the highest risk environments were Hudco Dam and Kharkai River and lowest risk environment was that of the Subarnarekha River and Dimna Lake. These observations are in agreement with the water quality and anthropogenic activities witnessed by these two fresh water systems [391]. As in the present study, prior works have indicated greater percent of resistant isolates to be associated with stagnant water [392,393]. Often rapid urbanization results in obstruction of free flowing rivers

for catering to the needs of growing population. Further studies need to be conducted to conclusively ascertain whether such anthropogenic interventions, resulting in development of stagnant/closed water systems, unintentionally lead to creation of conductive environment for evolution, maintenance and spread of the resistance mechanism thereby increasing the risk of community acquired multi drug resistant infections.

Based on comparative MAR indices, the propensity of exhibiting multiple drug resistant phenotype among the genera ranked in the order *Pseudomonas >Klebsiella = Acinetobacter >Proteus >Aeromonas >Enterobacter*. It is worth mentioning that genus *Klebsiella*, *Acinetobacter*, *Pseudomonas* and *Enterobacter* have been implicated as etiological agents of hospital acquired infections and show resistance to majority of the antibacterial therapeutic agents [11]. It is worth mentioning that the above genus, have also been implicated as significant contributors to nosocomial and community associated infections in India as well [9,394]. Drug resistant *Pseudomonas aeruginosa* have been reported from aquatic environments in water parks and lakes of Brazil [392] and Detroit river in USA [395]. Analysis of gram negative bacteria from surface and underground water sources in Nigeria have revealed occurrence of high resistance in the members of genera *Enterobacter*, *Pseudomonas* and *Proteus* [396]. Similarly, Papandreou *et al*., (2000) have reported multi antibiotic resistant gram negative bacteria belonging to *Pseudomonas*, Vibrionaceae, *Acinetobacter* and Enterobacteriaceae from drinking water samples in Greece [383]. In contrast to the present study, Papandreou *et al*., (2000) did not find any strain resistant towards quinolones, aminoglycosides and ceftazidime.

Members of gram negative bacteria belonging to Enterobacteriaceae are etiological agents implicated in nosocomial infections and are also ubiquitously distributed as non-pathogenic forms in natural environments [378]. Often opportunistic bacterial pathogens can

acquire resistance mechanisms from environmental donors and are able to transmit these acquired resistance determinants in clinical settings [10,378]. Rise in the antibiotic resistance phenotypes of environmental isolates thus confers upon them survival fitness, as compared to susceptible phenotypes, wherein the former upon exposure to antibiotic pressure are able to adapt better and survive. Nevertheless, conversion of a non-pathogenic isolate into a pathogen is possible only if such isolates are able to attach, colonize, invade and establish themselves in the host. With the objective to assess the pathogenic potential of the isolates, we carried out phenotypic assay for detection of five virulence factors *viz*. biofilm forming ability, presence of type 1 fimbriae, production of phospholipase C and gelatinase, extent of hydrophobicity and haemolytic ability.

Environmental isolates, which are resistant to multiple antimicrobial agents and also contains virulence factors are critical in increasing the infection burden. This aspect prompted us to find out what fraction of environmental antibiotic resistant bacteria (eARB) isolates in the present study can be considered as potential high risk isolates capable of transforming into pathogenic antibiotic resistant bacteria (pARB) given the chance passage to susceptible hosts. Hence we investigated association between MAR index and virulence factors that can complement eARB to transform into pARB. We observed that environmental multidrug resistant isolates of genus *Pseudomonas* co-exhibit high MAR and virulence indices. This observation is significant as previous studies have indicated high degree of genetic relatedness [397] coupled with phenotypic diversity [398] between clinical and environmental isolates of *Pseudomonas aeruginosa*. Nevertheless, association studies at genetic level with virulence and antibiotic resistance are poorly understood with correlations often being due to clonal nature [399]. It is worth mentioning that recently Davis and Brown (2016) have reported association study based

on MAR index and phenotypic assay in clinical isolates of *Pseudomonas aeruginosa* [400]. Studies addressing antibiotic resistance and virulence factors in bacteria from non-clinical sources have mostly elucidated only the genetic diversity/clonal relationship aspect [401].

To summarize, results of the study indicated that significant differences in the propensity to acquire multiple antibiotic resistance phenotype exists among the environmental bacteria. Further, association analysis of MAR index and virulence index revealed 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. We strongly believe our study to be the first of its type quantitatively evaluating the extent of threat posed by multidrug resistant bacteria from environment both at population and genus level, particularly from Indian sub-continent. Findings of this study have resulted in isolation of clones that are being characterized for their similarity/dissimilarities with clinical counterparts; the aspect is under investigation at the time of writing this thesis. Such organisms pose greater threat especially under man made and/or natural disasters. Further, the present study utilized low cost, established protocols which can be implemented by laboratories in developing nations to monitor environmental health risks associated with multidrug resistant isolates. This is reiterated by a recent published study [400] wherein the methodology has been used in analyzing clinical isolates. Association analysis of MAR index and virulence factors in multidrug resistant isolates can aid in identification of potential high risk pathogenic populations/isolates as shown in **Fig 3.1.10**.

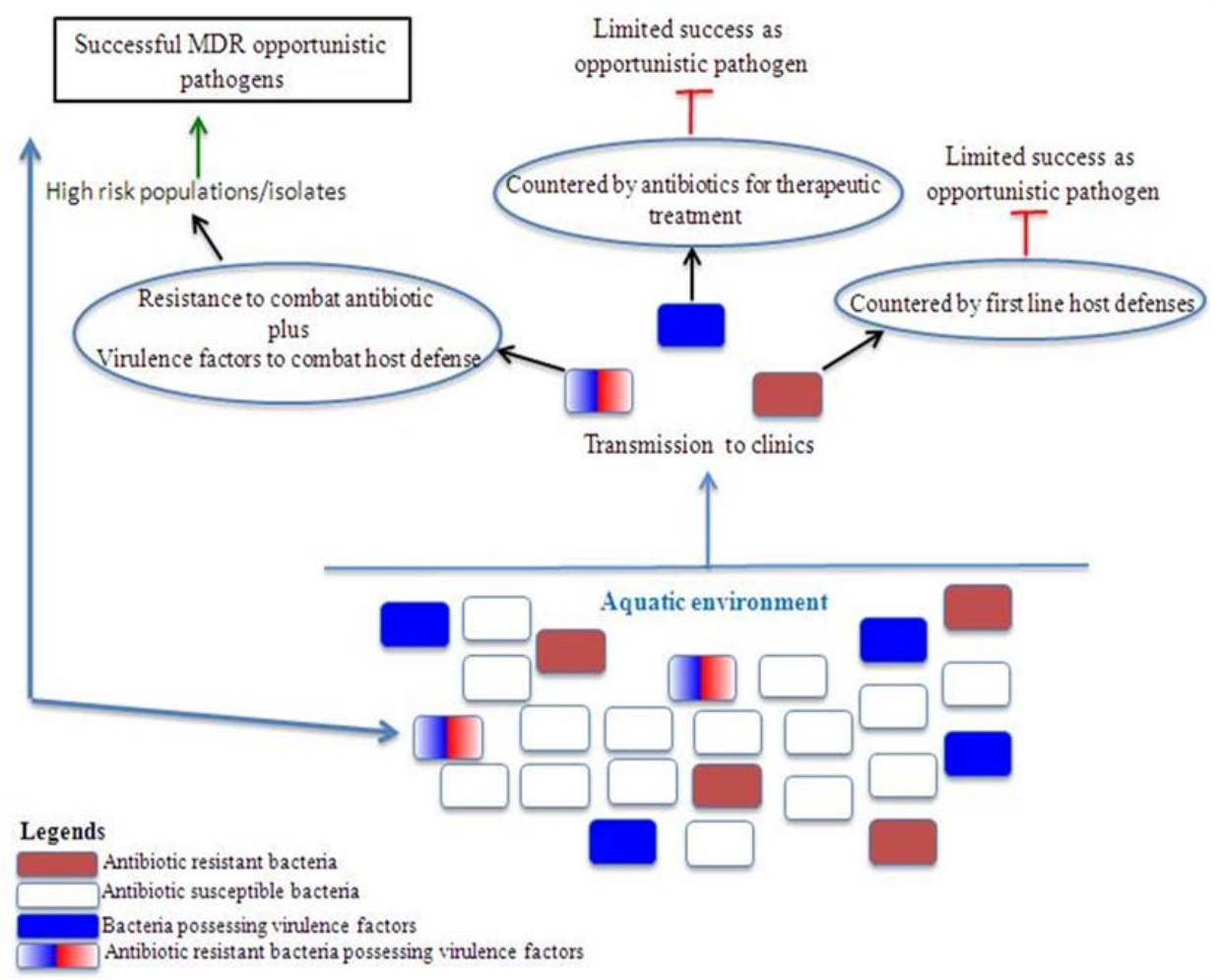


Figure 3.1.10: Schematic depiction of transit of environmental Antibiotic Resistant Bacteria (eARB) to pathogenic Antibiotic Resistant Bacteria facilitated by presence of virulence factors.

Association analysis of MAR index and virulence factors in environmental multidrug resistant isolates can aid in identification of potential high risk pathogenic populations/isolates that can be transmitted and establish as pathogenic antibiotic resistant bacteria (The final publication is available at Springer via http://dx.doi.org/10.1007/s10661-017-6005-4).

Section 3.2: Comparative study of diversity in resistance mechanisms in *Klebsiella pneumoniae* isolates from aquatic environment and clinical settings

Results of the previous section clearly indicated that environmental *Klebsiella pneumoniae* isolates had second highest MAR index within the genera identified in this study. Further, these categorized in moderate to low threat category based on association analysis of MAR and virulence index. Moreover, resistance mechanisms and genetic analysis of multi drug resistant environmental *Klebsiella pneumoniae* has been less addressed. The above mentioned aspects enthused us to study diversity in resistance profile and resistance mechanisms in environmental and clinical isolates of *Klebsiella pneumoniae*. Results of the above mentioned study are presented in this section.

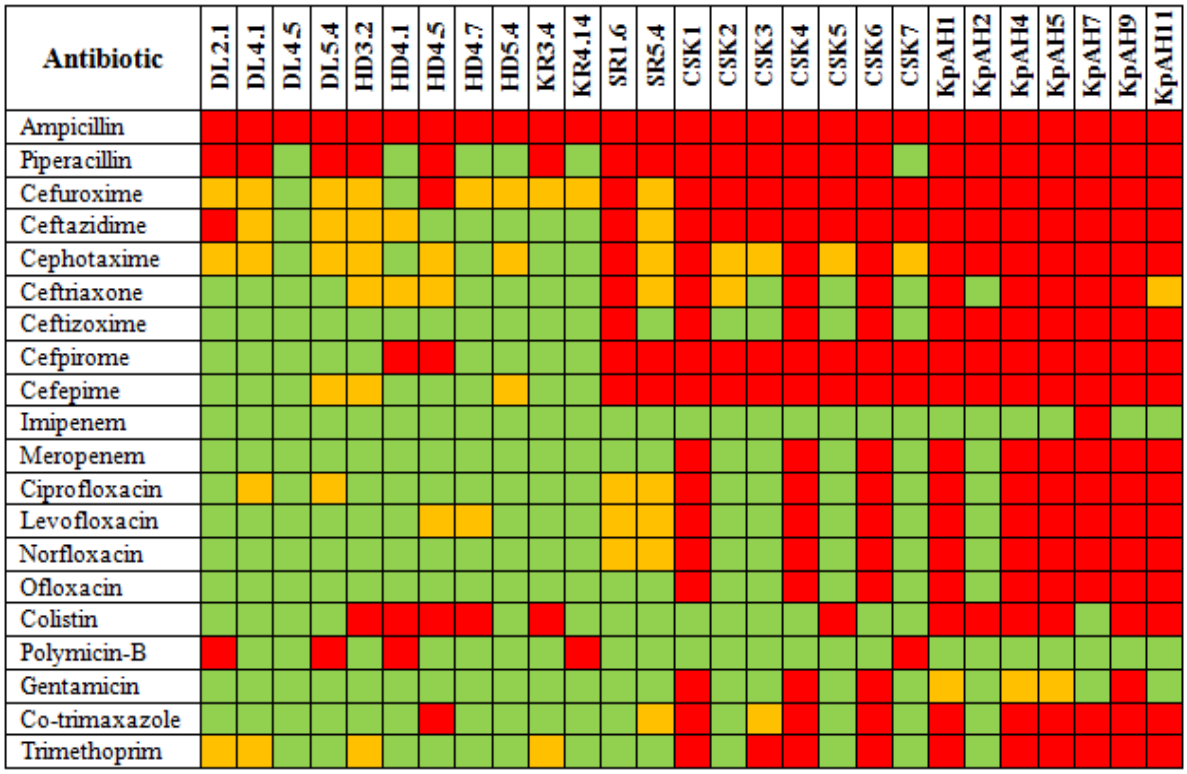
3.2.1 *Klebsiella pneumoniae* isolates used

Thirteen *K. pneumoniae* (JQ912545 to JQ912557) isolated from aquatic environment mentioned in previous section - 3.1 and fourteen clinical isolates of *K. pneumoniae* obtained from tertiary care hospitals in Jamshedpur and Bhubaneswar were studied for resistance profiles, molecular mechanisms of resistance and phenotypically screened for presence of virulence factors.

3.2.1. Antimicrobial resistance profiling

All the environmental and clinical isolates of *K. pneumoniae* were multidrug resistant as they were resistant to two or more than two classes of antibiotics. A significant difference in resistant and susceptibility pattern was observed in environmental and clinical isolates of *K. pneumoniae* to different class of antibiotics tested (**Fig. 3.2.1A)**.

A)



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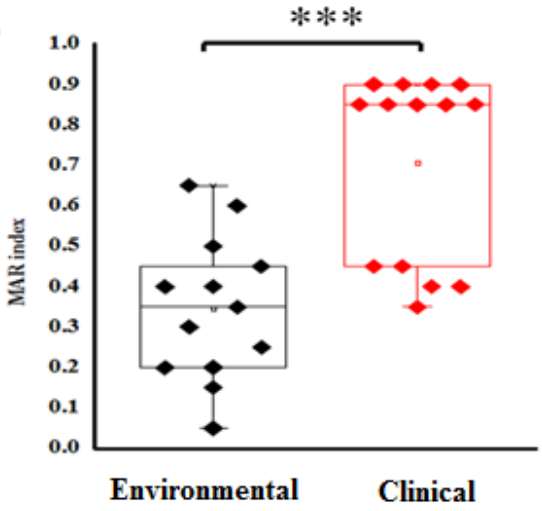


Figure 3.2.1: (A) Antibiotic-susceptibility profiles of 27 *K. pneumoniae* (13 environmental + 14 clinical) isolates determined by disc diffusion method and (B) Multiple antibiotics resistance (MAR) index of environmental and clinical isolates of *K. pneumoniae*.

Red squares indicate resistance; green squares indicate susceptible and orange squares indicate intermediate. *** showed p=0.001

The antibiotic disk diffusion results indicated that environmental isolates of *K. pneumoniae* were non-susceptible toward β-lactams antibiotics such as ampicillin (100%), cefuroxime (84.6%), piperacillin (61.5%) ceftazidime (53.8%), cefotaxime (46%), ceftriaxone (46%), ceftizoxime (23%), cefepime (30.7%) and cefpirome (30.7%). They showed comparatively less resistance toward polypeptide such as colistin (23%), polymyxin-B (23%). Further, the isolates exhibited intermediate resistance toward quinolones such as ciprofloxacin (15.4%), levofloxacin (7.7%) and norfloxacin (23%). Nearly, 15.3% isolates were also resistant towards co-trimaxazole (15.4%) and trimethoprim (15.4%). The isolates were susceptible toward carbapenem antimicrobials such as imipenem and meropenem. Isolates were also susceptible to aminoglycoside like gentamicin (**Fig 3.2.1A**).

In contrast to environmental isolates, clinical isolates of *K. pneumoniae* showed resistance toward different classes of antibiotics. Antibiotic disk diffusion result indicated that clinical isolates of *K. pneumoniae* were highly resistant towards β-lactams, cephalosporin and quinolone group of antibiotics like ampicillin (100%), piperacillin (93%), cefuroxime (100%), ceftazidime (100%), cephotaxime (71%), ceftriaxone (57%), ceftizoxime (71%), cefepime (100%) and cefpirome (100%), ciprofloxacin (64%), levofloxacin (64%), norfloxacin (64%) ofloxacin (64%), colistin (50%) and co-trimaxazole (41.17%) but less resistant towards polymyxin-B (7%), carbapenem such as imipenem (7%) and gentamycin (26%) **(Fig.3.2.1A**). This difference in antibiotic resistance was portrayed as significant difference in the MAR index between environmental and clinical isolates of *K. pneumoniae* (**Fig. 3.2.1B**).

Determining minimum inhibitory concentration (MIC)

With the above results, we went on further to check the minimum inhibitory concentration for selected antibiotics. The MIC values for the antibiotics tested co-related well with disc diffusion data as shown in **table 3.2.1**.

MIC results of environmental isolates of *K. pneumoniae* showed higher breakpoint value for β-lactam antibiotics such as ampicillin (ranging from 256-1024µg/ml), cephalosporins such as cefuroxime (ranging from 256-1024µg/ml), cephotaxime (ranging from 128-512µg/ml) but low MIC breakpoint value toward fourth generation cephalosporin such as cefepime (ranging from 4-256µg/ml) and macrolides like erythromycin (ranging from 64-512µg/ml). Exception was isolate KR4.14 having comparatively low MIC breakpoint value for all β-lactam antibiotics. As compared to β-lactams and macrolides, all environmental isolates of *K. pneumoniae* showed low MIC breakpoint value for quinolone group of antibiotics such as ciprofloxacin (ranging from 0.5-4µg/ml except KR3.4 and SR5.4), levofloxacin (ranging from 0.5-4µg/ml except DL2.1, DL2.3, HD4.1, KR3.4 and SR5.4) and norfloxacin (range from 1-8µg/ml except DL5.4 and SR5.4). In contrast to environmental isolates of *K. pneumoniae*, clinical isolates of *K. pneumoniae* showed very high MIC breakpoint value quinolone group of antibiotics such as ciprofloxacin (range from 8-512µg/ml except CSK2, CSK5 and CSK7), levofloxacin (range from >8-128µg/ml except CSK2, CSK5 and CSK7) and norfloxacin (range from >8-512µg/ml except CSK2, CSK5 and CSK7). Clinical *K. pneumoniae* isolates from Bhubaneswar showed resistance toward gentamicin with MIC values ranging from 128 - 512µg/ml except KpAH2 that was susceptible to gentamicin.

Table 3.2.1 Minimum inhibitory concentration (µg/ml) of different antibiotics of *K. pneumoniae* isolated from environment and clinical samples

	Isolates	A	AK	CXM	CPM	CTX	CIP	LE	NX	T	E
	CLSI breakpoint for resistant	**≥32**	**16**	**≥32**	**≥32**	**≥64**	**>4**	**>8**	**≥16**	**>16**	**≥8**
Environmental isolates of *K. pneumoniae*	DL2.1	512	0.5	128	NG	128	4	>8	8	16	256
	DL2.3	1024	NG	256	128	256	2	>8	8	64	256
	DL4.1	>1024	2	512	16	512	2	4	4	128	>512
	DL5.4	512	16	256	128	256	4	4	16	128	256
	HD3.2	>1024	8	128	32	128	1	2	4	32	64
	HD4.1	>1024	8	128	4	256	2	8	2	256	512
	HD4.5	>1024	NG	128	32	128	0.5	1	2	16	128
	HD4.7	>1024	NG	128	NG	128	1	2	4	NG	256
	HD5.4	1024	NG	128	NG	NG	2	4	8	NG	256
	KR3.4	1024	8	128	8	256	>8	8	>8	32	64
	KR4.14	64	NG	NG	NG	256	0.5	0.5	16	32	128
	SR1.6	>1024	NG	256	128	128	NG	1	>32	128	>512
	SR5.4	256	NG	>256	>256	>512	8	>8	1	>256	256
Clinical isolates of *K. pneumoniae*	CSK1	>512	16	>512	>512	>512	16	16	16	128	>256
	CSK2	128	NG	64	32	128	NG	NG	NG	>128	128
	CSK3	>512	NG	256	32	128	8	>8	>8	>128	>128
	CSK4	>512	16	>512	>512	>512	256	128	256	256	>256
	CSK5	256	NG	128	64	128	NG	1	2	128	128
	CSK6	>512	32	>512	>512	>512	>256	128	256	>128	>256
	CSK7	256	NG	128	64	128	1	1	4	32	64
	KpAH1	>512	>512	>512	512	>512	512	64	-	>256	>512
	KpAH2	>512	256	>512	512	256	16	16	64	>256	256
	KpAH4	>512	64	>512	512	>512	512	128	512	>256	256
	KpAH5	>512	-	>512	512	>512	512	128	256	>256	>512
	KpAH7	>512	>512	>512	512	>512	8	64	-	>256	>512
	KpAH9	>512	-	>512	512	>512	512	256	-	64	>512
	KpAH11	>512	-	128	128	128	>512	64	256	NG	>512

A-Ampicillin, AK- Amikacin, CIP- Ciprofloxacin, LE- Levofloxacin, CXM- Cefuroxime, CPM-cefepime, CTX-cephotaxime, E- Erythromycin; NG-no growth at lowest concentration

.

As could be observed from the results of antibiotic susceptibility, many isolates showed resistance towards multiple antibiotics which are often used in treatment regimen. Hence we thought it pertinent to analyze if there existed any significant correlation between resistances towards a combination of antibiotics of different classes. We performed Pearson correlation between pair of antibiotics using SPSS software. Results of this analysis are tabulated in **Table 3.2.2.** The 'r' value indicated strength of the correlation. As was observed, except for ceftazidime –ciprofloxacin (r=0.81) and cefepime-ciprofloxacin (r=0.76) combination, environmental *K. pneumoniae* isolates had low value for correlation co-efficient toward other pairs of antibiotics, indicating that these antibiotics could be therapeutically effective. In case of clinical *K. pneumoniae* isolates, high values of correlation co-efficient were observed for 3rd generation cephalosporin ceftazidime and 4th generation cephalosporin cefepime (r = 1.0); ciprofloxacin with meropenem & Co-trimaxazole (r = 1.0), and meropenem with Co-trimaxazole (r = 1.0). This indicated that combinatorial therapies of these drugs would be ineffective towards treating *Klebsiella pneumoniae*. Lowest value for correlation co-efficient were observed between gentamicin and ceftazidime, cefepime and colistin (r <0.40), indicating that these could be therapeutically effective.

Table 3.2.2 Pearson correlation coefficient (r) matrix of resistance between pairs of antibiotics for *Klebsiella pneumoniae* isolates

		CAZ	CTR	CPM	MER	CIP	CL	COT	G
Environmental isolates of *K. pneumoniae*	**CAZ**	1							-
	CTR	.525	1						-
	CPM	**.766***	.338	1					-
	MER	-	-	-	-				-
	CIP	**.810***	.070	**.790***	-	1			-
	CL	-.162	.478	-.419	-	-.685	1		-
	COT	-.438	.221	-.403	-	-.395	0.000	1	-
	G	-	-	-	-	-	-	-	-
		CAZ	**CTR**	**CPM**	**MER**	**CIP**	**CL**	**COT**	**G**
Clinical isolates of *K. pneumoniae*	**CAZ**	1							
	CTR	.566	1						
	CPM	**1.0****	.566	1					
	MER	.666	**.967****	.666	1				
	CIP	.666	**.967****	.666	**1.0****	1			
	CL	.757*	.190	.757*	.382	.382	1		
	COT	.666	**.967****	.666	**1.0****	**1.0****	.382	1	
	G	.232	.766*	.232	.634	.634	-.391	.634	1

CAZ-ceftazidime (30µg/ml), CTR-ceftriaxone (30µg/ml), CPM-cefepime (30µg/ml), MER-meropenem (10µg/ml), CIP-ciprofloxacin (5µg/ml), CL-colistin (50µg/ml), COT-Co-trimaxazole (25µg/ml) and G-gentamicin (120µg/ml).

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

3.2.2. Determining genetic basis of multidrug resistance

3.2.2.1 Phenotypic and genotypic screening for β-lactam resistance mechanisms

The presence of resistant genes either on chromosome or plasmid responsible for hydrolysis or modifying the antibiotics is one of the important underlying genetic reasons for bacterial resistance to antimicrobials. The best example is a β-lactamase gene renders the antibiotics ineffective by hydrolysing β-lactam ring. β-lactamases are divided into four major classes namely extended spectrum β-lactamase (ESBL), metallo-β-lactamases (MBL), AmpC and carbapenemase which are frequently reported in the *Klebsiella pneumoniae*. According to CLSI (2010) guidelines, strains showing zone of inhibition of ≤22mm for ceftazidime, ≤27mm for cephotaxime and ≤25mm for ceftriaxone can be assumed to be potential producers of ESBL and isolates resistant to carbapenem could potentially encode carbapenemase.

Double disk-diffusion test for ESBL result showed 53.8% (n=7) and 64% (n=9) environmental and clinical *Klebsiella pneumoniae* isolates respectively, to be ESBL positive as the zone of inhibition for ceftazidime + clavulanic acid compared to ceftazidime alone was more than ≥5mm (**Fig.3.2.2A**). Some isolates like HD5.4, CSK6, KpAH5 and KpAH7 showed no zone of inhibition for both ceftazidime + clavulinic acid compared to ceftazidime alone suggesting that these isolates were not affected by inhibitor clavulanic acid. Two clinical isolates of *Klebsiella pneumoniae* (KpAH1, KpAH7) were MBL positive as the zone of inhibition for imipenem + EDTA compared to imipenem alone was more than ≥5mm (**Fig.3.2.2B**). Modified Hodge test for carbapenemase have showed only one clinical isolate CSK7 was positive for MHT (**Fig3.2.2C**).

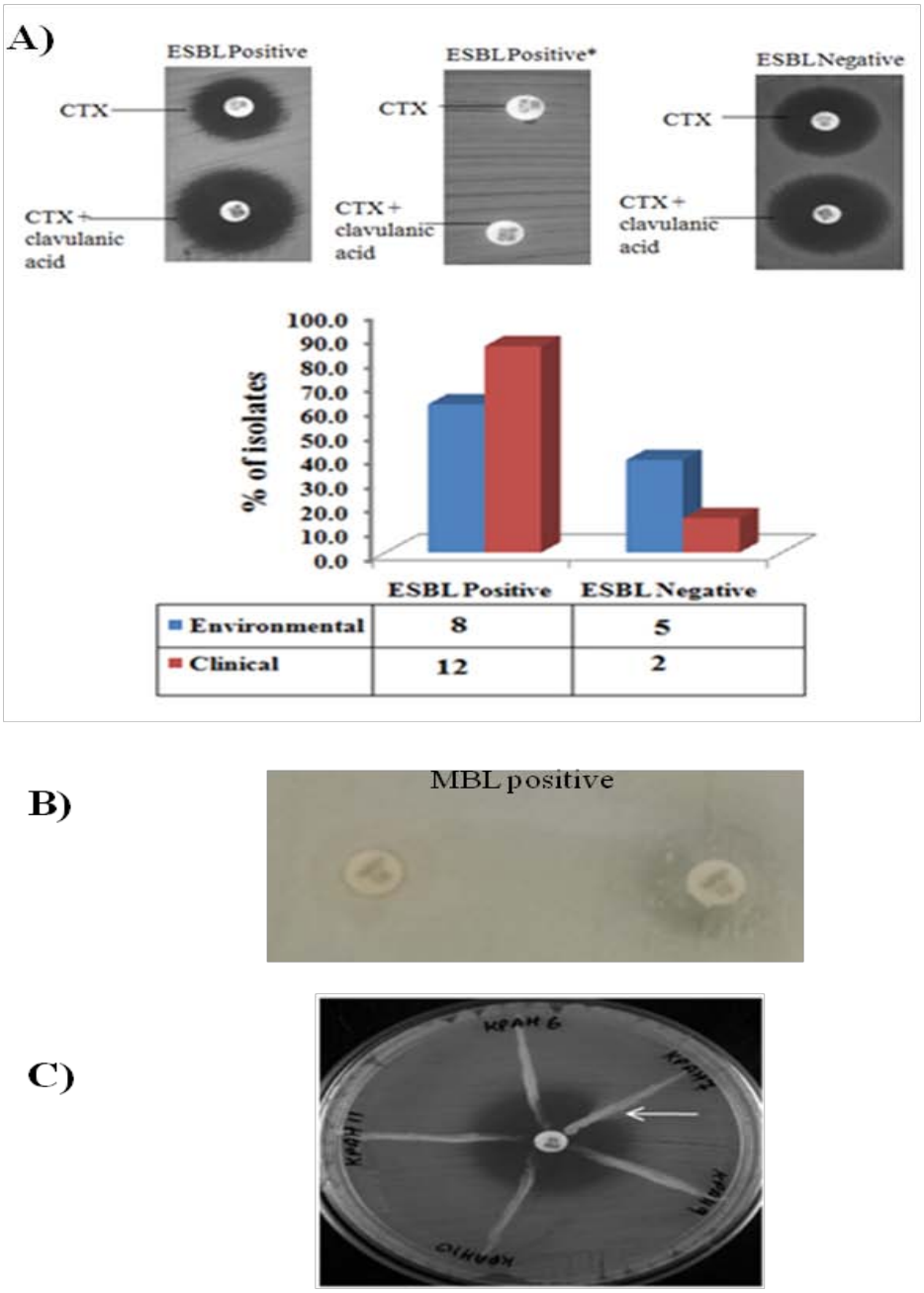


Figure 3.2.2: Phenotypic detection of β-lactamase

A) Double disc synergy test for ESBL. An increase in the inhibition zone diameter of >5 mm for a combination disc versus ceftazidime disc alone confirmed ESBL production, (B) EDTA double disc test and (C) Modified Hodge Test for carbapenemase

PCR amplification showed that the most abundant ESBL gene was *bla*$_{SHV-32}$ identified in 61.5% (n=8) environmental and 85.7% (n=12) clinical *K. pneumoniae* isolates (**Fig.3.2.3**). Only one environmental *K. pneumoniae* isolate SR5.4 contained *bla*$_{CTX-15}$.

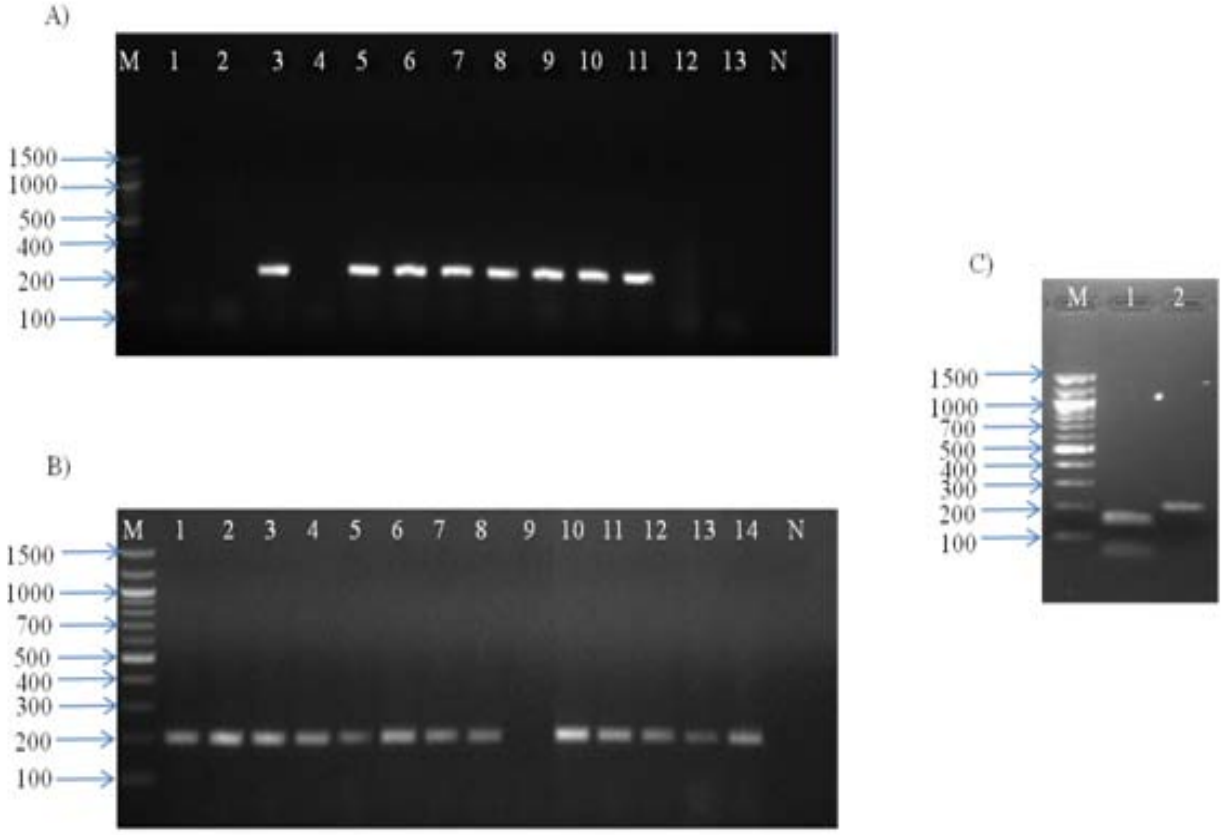


Figure 3.2.3: PCR amplification of *bla*$_{SHV-34}$.

(A) Environmental *K. pneumoniae* isolates. Lane 3, 5, 6, 7, 8, 9, 10, 11, 12 and 13 positive amplification of *bla*$_{SHV-34}$ of isolates DL4.1, HD3.2, HD4.1, HD4.5, HD4.7, HD5.4, KR3.4 AND KR4.1 respectively (B) Clinical *K. pneumoniae* isolates. Lane 1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13 & 14 positive amplification of *bla*$_{SHV-34}$ of isolates CSK1, CSK2, CSK3, CSK4, CSK5, CSK6, CSK7, KpAH1, KpAH4, KpAH5, KpAH7, KpAH9 & KpAH11 respectively, (C) Restriction digestion of *bla*$_{SHV-34}$ PCR product using Not1 restriction enzyme. Lane – 100bp DNA ladder, Lane 1- Not1 restriction digested PCR product, Lane 2 – undigested PCR product. Lane M – 100bp DNA ladder (NEB, USA), Lane N – Negative control (without template).

*Amp*C gene which confers resistance to cephalosporin and cephamycins without being affected by inhibitor clavulanic acid, tazobactam and sulbactam was found to be present in one environmental *K. pneumoniae* isolate (**Fig 3.2.4A)** and two clinical *K. pneumoniae* isolates **(Fig.3.2.4B**). This genotypic detection correlated well their phenotypic double disk diffusion results (**Fig. 3.2.2A**). Further, NDM-1 gene was present in two clinical *K. pneumoniae* isolates namely KpAH1and KpAH7.

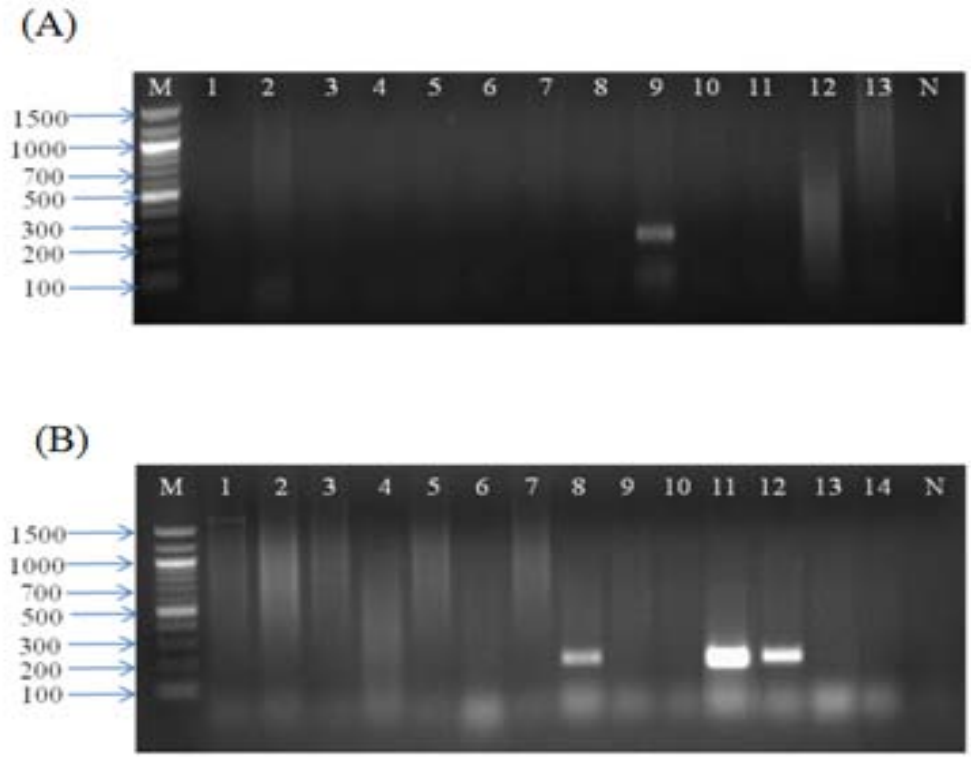


Figure 3.2.4: PCR amplification of *AmpC*.

(A) Environmental *K. pneumoniae* isolates. Lane 9 positive amplification *Amp*C of isolates HD5.4 (B) Clinical *K. pneumoniae* isolates. Lane 8, 11 & 12 positive amplification of *Amp*C of isolates KpAH1, KpAH5 & KpAH7 respectively, Lane M – 100bp DNA ladder (NEB, USA), Lane N – Negative control (without template).

PCR screening for presence of carbapenemase encoding gene was negative for all the environmental *K. pneumoniae* isolates. This genotypic result correlated with antibiotics susceptibility and Modified Hodge test. However, among the clinical *K. pneumoniae* isolates, three isolates were positive for bla_{OXA-1} (**Fig. 3.2.5A)** and two were positive for bla_{OXA-9} (**Fig. 3.2.5B).** Two isolates CSK6 & KpAH9 were positive for both bla_{OXA-1} and bla_{OXA-9}.

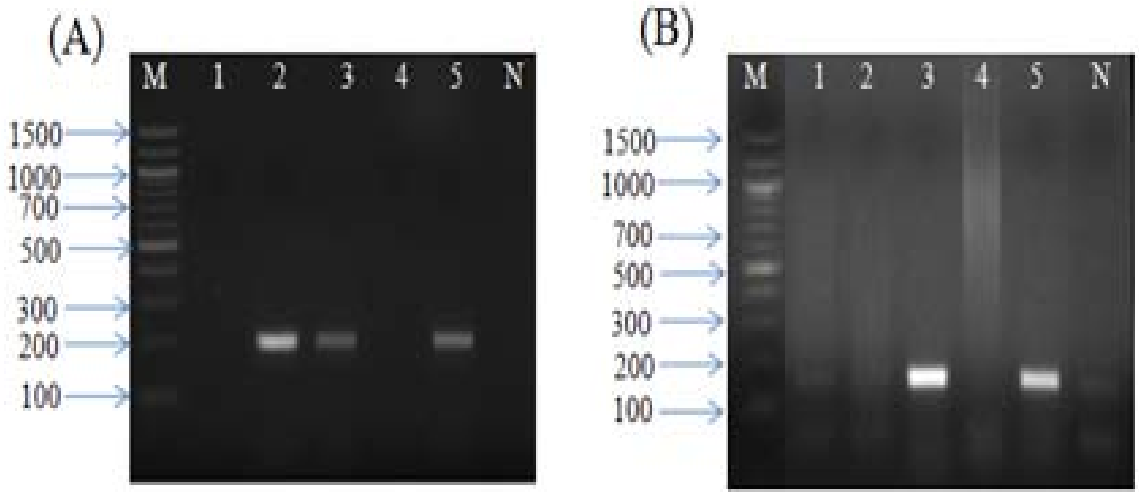


Figure 3.2.5: PCR amplification of *bla$_{OXA}$*.

(A) PCR amplification of bla_{OXA-1}, Lane 2, 3 and 5 were positive amplification of bla_{OXA-1} of isolates CSK4, CSK6 and KPAH9 respectively and (B) PCR amplification of bla_{OXA-9}. Lane 3, 5 and 6 were positive amplification of bla_{OXA9} of isolates CSK6 and KpAH9 respectively. Lane M- 100bp DNA ladder (NEB, USA) and Lane N- negative control (without template).

All the *Klebsiella pneumoniae* isolates included in this study were negative for other ESBLs and MBL genes like bla_{PER}, bla_{VIM}, bla_{IMP}, bla_{GES}, bla_{VEB} and bla_{CYM}.

3.2.2.2. Screening for mutations in Quinolone Resistant Determining Regions (QRDR) of gyrase A and gyrase B genes as quinolone resistance mechanism

Fluoroquinolones are broad-spectrum synthetic antimicrobial agents that target bacterial type II topoisomerases and act against both DNA gyrase and topoisomerases IV. Mutations in the quinolone binding active sites of the A and B subunit of DNA gyrase result in quinolone resistance. Common site of resistance-conferring mutations are Ser^{83} to Leu/Ile/Phe and Asp^{87} to Tyr/Asn in gyrase A and mutations to Asp^{426} to Asn and Lys^{437} to Glu in gyrase B. To check for possible presence of any such mutations in QRDR of the isolates under study, we sequenced the purified PCR amplified *gyr*A and *gyr*B genes. Sequences obtained were aligned with *Klebsiella pneumoniae* strain NTUH-K2044. The alignment revealed that none of the environmental isolates showed any mutation in the QRDRs region of *gyr*A (**Fig 3.2.6A**) and *gyr*B (**Fig 3.2.7A**). However, in the clinical isolates, three types of amino acid substitutions, Ser-Ile transition (in CSK4, KpAH1 and KpAH7), Ser-Tyr transition (in CSK6) and Ser-Phe transition (in KpAH5 and KpAH9) at 83 amino acid position was observed (**Fig3.2.6B**). In addition, Asp-Gly transition in CSK6 and KpAH9 and Asp-Ala transition in KpAH5 at 87 amino acid positions was observed (**Fig. 3.2.6B)**. There were no mutations observed in QRDRs of *gyr*B in these *K. pneumoniae* isolates (**Fig. 3.2.7B)**.

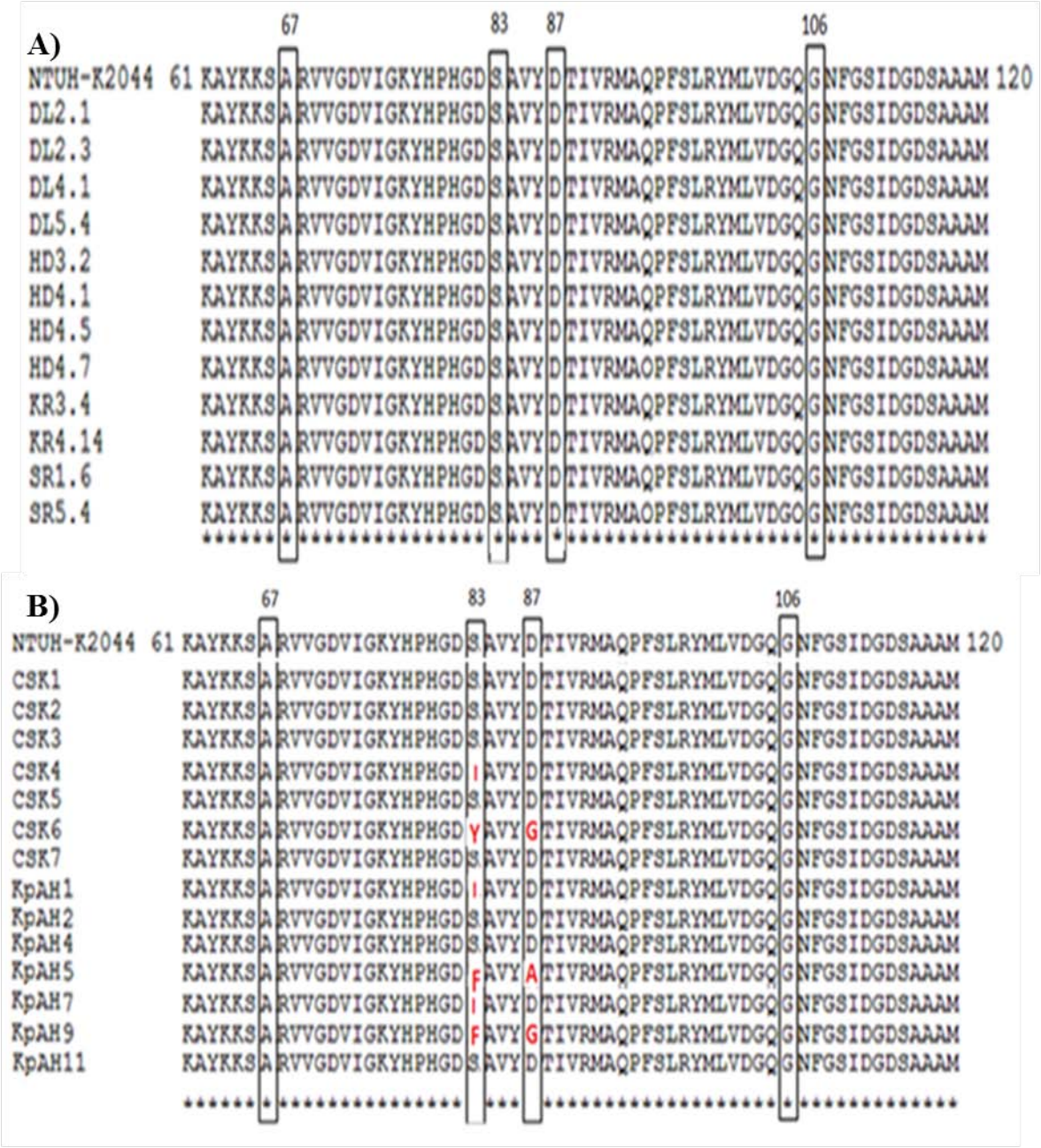


Figure 3.2.6: Alignment of Quinolone Resistance Determining Regions *of gyr*A.

(A) Environmental isolates of *K. pneumoniae* and (B) clinical isolates of *K. pneumoniae*. ClustalW alignment of coding regions of *gyr*A derived from test environmental and clinical *Klebsiella pneumoniae* strains compared to NTUH-K2044 strain.

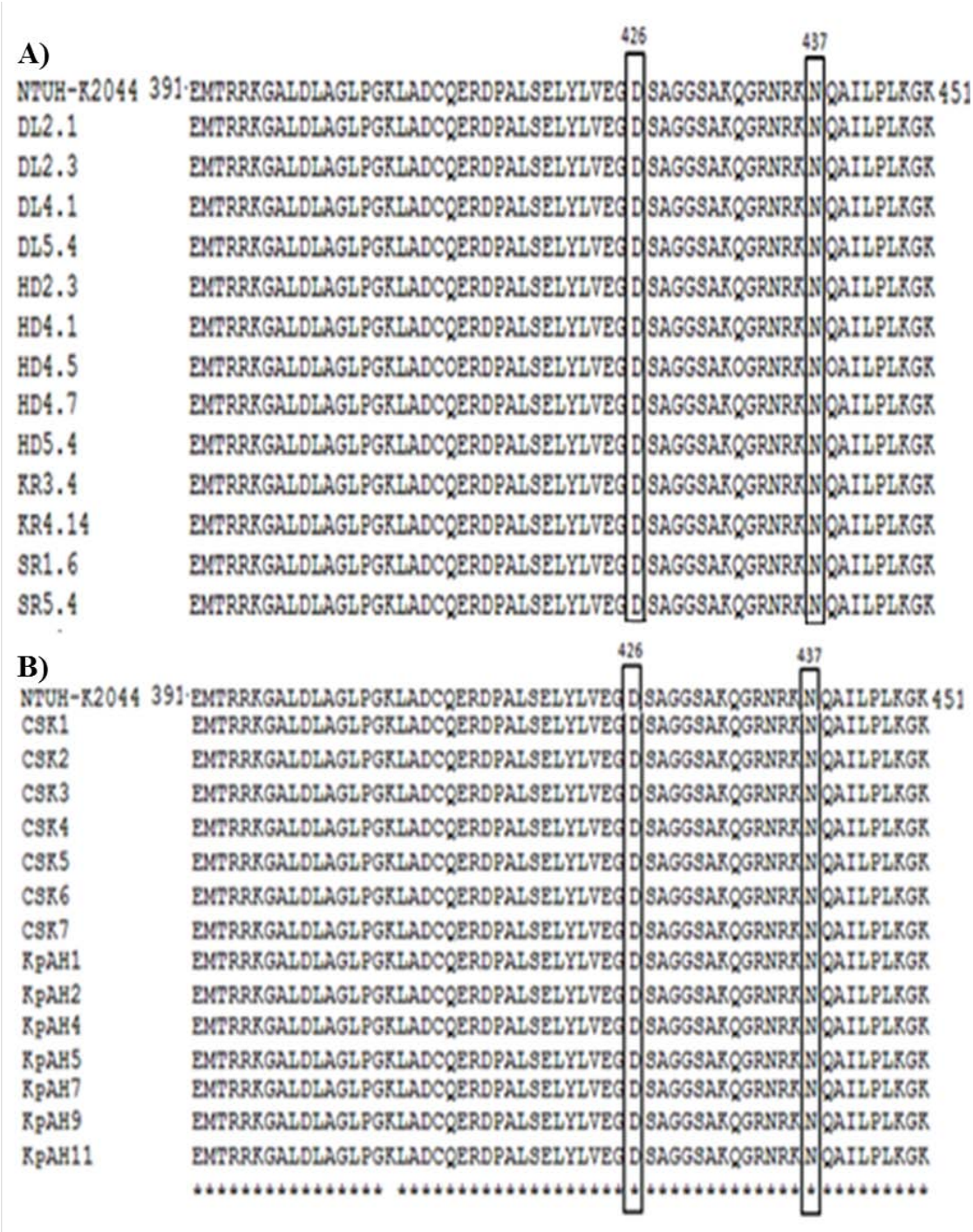


Figure 3.2.7: Alignment of Quinolone Resistance Determining Regions of *gyr*B.

(A) Environmental isolates of *K. pneumoniae* and (B) clinical isolates of *K. pneumoniae*. ClustalW alignment of coding regions of gyrB derived from test environmental and clinical *Klebsiella pneumoniae* strains compared to NTUH-K2044 strain.

3.2.2.3. Efflux as mechanism of antibiotic resistance

Efflux pump have been implicated in inducing antibiotics resistance among gram negative pathogens. Majority of the efflux pump utilize the proton motive force and efflux the drug out of the cell. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is an ionophore that disrupts the proton motive gradient and thus impairs activity of the efflux pump proteins. Thus, there occurs a decrease in MIC reading in presence of CCCP compared to that in its absence for organisms which exhibit efflux mediated resistance. This technique was used to screen isolates that possibly encode efflux mediated resistance mechanism. At least two fold decrease of MIC in presence of CCCP was taken as the cut off for possible involvement of efflux pump.

The MIC data with and without carbonyl cyanide m-chlorophenyl hydrazone (CCCP) for *K. pneumoniae* isolates are shown in **table 3.2.3**. Seven environmental isolates DL2.1, DL2.3, HD4.1, HD4.5, HD4.7, KR3.4 and SR5.4 showed efflux pump mediated resistance for many antibiotics. In presence of CCCP, DL2.1 showed 8-, 2-, 8-, 2- and 8- fold decreases in MIC for ciprofloxacin, cefuroxime, cephotaxime, tetracycline and erythromycin respectively The decrease in MIC of antibiotics for DL2.3 in presence of CCCP were 2-, >16-, 128-, 256-, 64-, 8-fold for ciprofloxacin, levofloxacin, cefepime, cephotaxime, tetracycline and erythromycin respectively (**Table3.2.3**). HD4.1 decreases in MIC for levofloxacin, cefuroxime and cefepime whereas HD4.7 decreases in MIC for cefuroxime and cephotaxime. HD4.5 showed 16- and 2-fold decrease in MIC for tetracycline and erythromycin respectively. The decrease in MIC of KR3.4 in presence of CCCP were >8-, 8-, 2-, 256-, 32-, 2-fold for ciprofloxacin, levofloxacin, cefuroxime, cephotaxime, tetracycline and erythromycin respectively (**Table3.2.3**). The decrease in MIC of antibiotics for SR5.4 in presence of CCCP was 4-fold for ciprofloxacin and levofloxacin.

Seven clinical isolates namely CSK2, CSK3, CSK5, CSK6, CSK7, KpAH4 and KpAH11 showed efflux pump mediated drug resistant phenotype. In presence of CCCP, the decrease in MIC of CSK2 was 8- and 4- fold for cefuroxime and cefepime respectively whereas CSK3 and CSK6 showed 4- and 2- fold decreases in MIC for ciprofloxacin and levofloxacin respectively. CSK5 showed 8-fold decrease in MIC were for tetracycline. Decrease in MIC of CSK7 was 32-fold for cefepime and 2- fold for erythromycin. KpAH4 and KpAH11 also showed decrease in MIC for ciprofloxacin and levofloxacin respectively (**Table3.2.3**).

3.2.2.4 Screening for plasmid mediated antibiotic resistance

Plasmids are extra chromosomal DNA occurring ubiquitously in bacteria and are known to encode resistance genes towards many antibiotics. Hence it was necessary to determine the presence of plasmids in the strains exhibiting drug resistance. From agarose gel analysis, it was found that two *K. pneumoniae* environmental isolates namely, KR3.4 and DL4.1 contained plasmid corresponding to 1.5 and 3kb respectively (**Fig. 3.2.8A**). In contrast, seven clinical isolates of *K. pneumoniae* namely KpAH1, KpAH2, KpAH4, KpAH5, KpAH7, KpAH9 and harbored plasmids (**Fig. 3.2.8B**). To determine possible role of plasmid in conferring antibiotic resistance, all plasmid positive *K. pneumoniae* isolates were cured of plasmid by using 10mM SDS. The SDS treated colonies were checked for curing of plasmids. This was confirmed by plasmid extraction and visualization on an ethidium bromide stained agarose gel. Absence of the band in the cured colonies confirmed expulsion of plasmids (**Fig. 3.2.9A**). Following confirmation of plasmid curing, possible role plasmid in mediating antibiotic resistance was determined by comparing the zone of inhibitions of the wild type (WT) to the cured colony (C) in an antibiotic disk diffusion test followed by determination of MIC values.

Table 3.2.3 MIC (µg/ml) of antibiotics with and without carbonyl cyanide m-chlorophenyl hydrazone of environmental *K. pneumoniae* isolates

	Isolates	CIP		LE		CXM		CPM		CTX		T		E	
		-	+	-	+	-	+	-	+	-	+	-	+	-	+
Environmental isolates of *K. pneumoniae*	DL2.1	**4**	**0.5**	>8	8	**128**	**64**	NG	NG	**128**	**16**	**16**	**8**	**256**	**32**
	DL2.3	**2**	**1**	**>8**	**0.5**	256	256	**128**	**NG**	**256**	**NG**	**64**	**NG**	**256**	**32**
	DL4.1	2	2	4	4	512	512	16	16	512	512	128	128	>512	512
	DL5.4	4	4	4	4	256	256	128	128	256	256	128	128	256	256
	HD3.2	1	1	2	2	128	128	32	32	128	128	32	32	64	256
	HD4.1	2	2	**8**	**4**	**128**	**64**	**4**	**2**	256	256	256	256	**512**	**256**
	HD4.5	0.5	0.5	1	1	128	128	32	32	128	128	**16**	**NG**	**128**	**64**
	HD4.7	1	1	2	1	**128**	**64**	NG	NG	**128**	**64**	NG	NG	256	256
	HD5.4	2	2	4	4	128	128	NG	NG	NG	NG	NG	NG	256	256
	KR3.4	**>8**	**NG**	**8**	**NG**	**128**	**64**	8	8	**256**	**NG**	**32**	**NG**	**64**	**32**
	KR4.14	0.5	0.5	0.5	0.5	NG	128	NG	64	256	256	32	NG	128	256
	SR1.6	NG	NG	1	1	256	256	128	128	128	128	128	128	>512	512
	SR5.4	**8**	**2**	**>8**	**2**	>256	>256	**>256**	**128**	>512	>512	>256	>256	**256**	**128**

	Isolates	CIP		LE		CXM		CPM		CTX		T		E	
		-	+	-	+	-	+	-	+	-	+	-	+	-	+
Clinical isolates of *K. pneumoniae*	CSK1	16	16	16	16	>512	>512	>512	>512	>512	>512	128	128	>256	>256
	CSK2	NG	NG	NG	NG	**64**	**8**	**32**	**8**	128	128	>128	>128	128	128
	CSK3	**8**	**2**	**>8**	**4**	**256**	**128**	32	32	128	128	>128	>128	>128	>128
	CSK4	256	256	128	128	>512	>512	>512	>512	>512	>512	256	256	>256	>256
	CSK5	NG	NG	1	1	128	128	64	64	128	128	**128**	**16**	128	128
	CSK6	**>256**	**64**	**128**	**64**	>512	>512	>512	>512	>512	>512	>128	>128	>256	>256
	CSK7	1	NG	1	NG	128	128	**64**	**NG**	128	128	32	32	64	32
	KpAH1	512	512	64	64	>512	>512	512	512	>512	>512	>256	>256	>512	>512
	KpAH2	16	16	16	16	>512	>512	512	512	256	256	>256	>256	256	128
	KPAH4	512	512	**128**	**64**	>512	>512	512	512	>512	>512	>256	>256	256	256
	KpAH5	512	512	128	128	>512	>512	512	512	>512	>512	>256	>256	>512	>512
	KpAH7	8	16	64	64	>512	>512	512	512	>512	>512	>256	>256	>512	>512
	KpAH9	512	512	256	256	>512	>512	512	512	>512	>512	64	64	>512	>512
	KpAH11	**>512**	**256**	64	64	128	128	128	128	128	128	NG	NG	>512	>512

CIP- ciprofloxacin, LE- levofloxacin, CXM- cefuroxime, CPM- cefepime, CTX- cephotaxime, T- tetracycline, E- erythromycin, "-" without CCCP, "+" with CCCP.

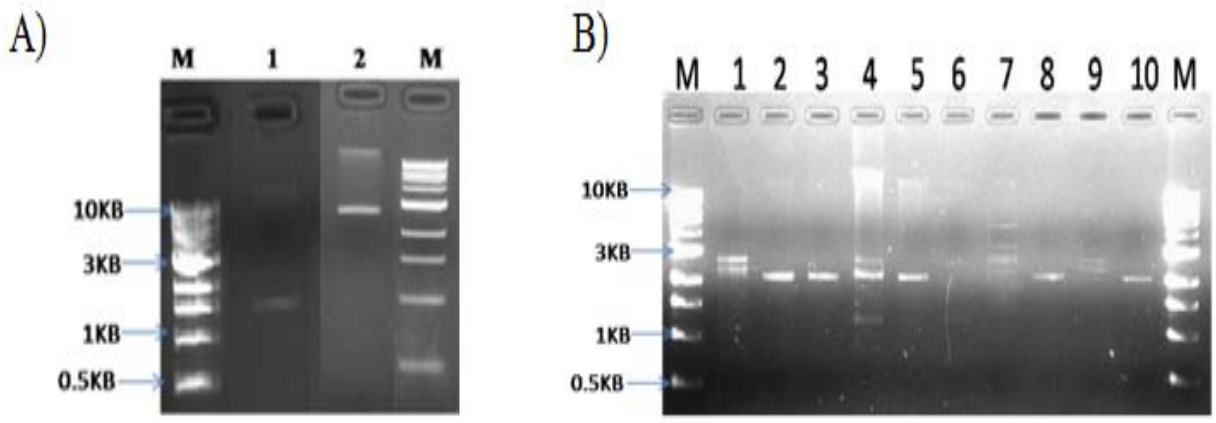


Figure 3.2.8: Plasmid profile of *K. pneumoniae*

(A) Environmental *K. pneumoniae* isolates. Lane-1, and 2 were KR3.4 and DL4.1 respectively and (B) Clinical *K. pneumoniae* isolates. Lane- 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 represent isolates KpAH1, KpAH2, KpAH4, KpAH5, KpAH7, KpAH9 and KpAH11 respectively. Lane M - 1KB DNA ladder (NEB, USA).

Cured and wild type environmental isolates of *K. pneumoniae* did not show any change in the inhibition zone diameter, indicative of no plasmid mediated resistance mechanism in them. Out of seven plasmid positive clinical *K. pneumoniae* isolates, loss of plasmid in five isolates was concomitant with loss of resistance (**Fig.3.2.9B**). Isolates KpAH1 lost resistance to quinolone (ciprofloxacin MIC 32 µg/ml to <2 µg/ml) and KpAH4 became susceptible to cephalosporin (cephotaxime MIC 512 µg/ml to <16 µg/ml). KpAH5 and KpAH9 became susceptible to cephotaxime (MIC 512 µg/ml to <8 µg/ml) and ciprofloxacin (MIC 512 µg/ml to <2 µg/ml). The isolate KpAH11 completely lost its resistant phenotype (ciprofloxacin MIC 512 µg/ml to <2 µg/ml; cephotaxime MIC 512 µg/ml to <32 µg/ml and azithromycin MIC MIC 512 µg/ml to <128). Two isolates were not cured as they were unable to grow in the lowest concentration of SDS.

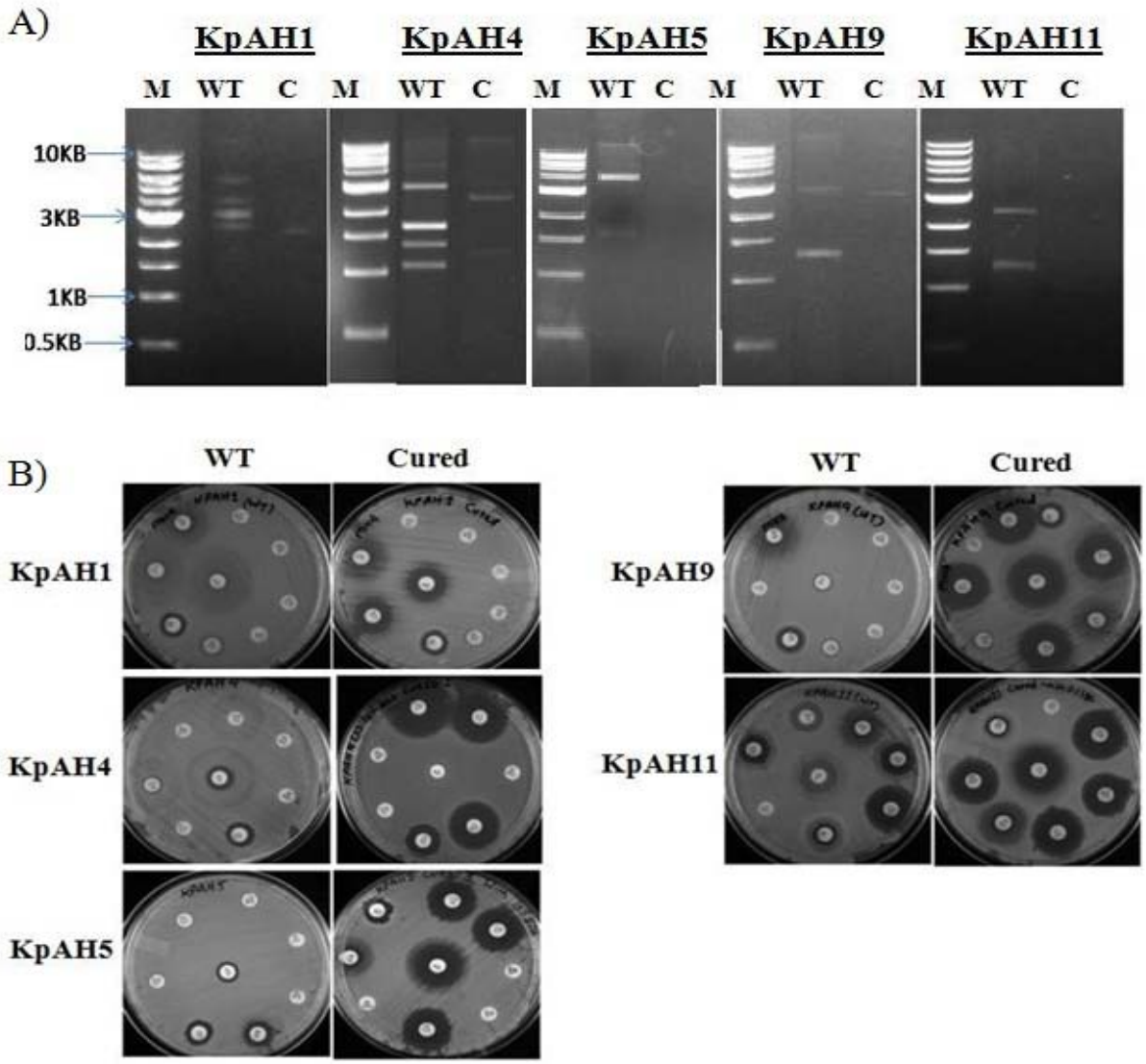


Figure 3.2.9: (A) Plasmid profile and (B) comparison of antibiotic susceptibility by disc diffusion of *K. pneumoniae* isolates before and after curing.

As the cured strains became susceptible for quinolone group of antibiotics with >32-fold decrease in MIC value indicative of presence of plasmid encoded quinolone resistance determinants in these isolates. To check the plasmid encoded quinolone resistance, PCR amplification of *qnr*A and *qnr*B were done and found that none of the isolates have *qnr*A but four isolates KAH1, KAH5, KpAH9 and KpAH11 were positive for *qnr*B (**Fig. 3.2.10**).

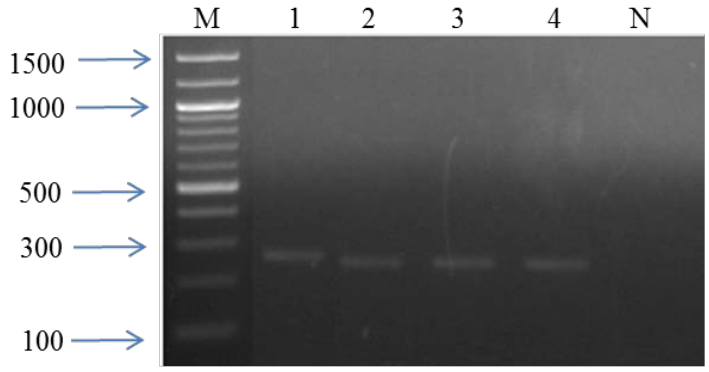


Figure 3.2.10: PCR amplification of *qnr*B

Lane 1, 2, 3 and 4 were positive amplification of *qnr*B of isolates KpAH1, KpAH5, KpAH9 and KpAH11 respectively. Lane M- 100bp DNA ladder (NEB, USA) and Lane N- negative control (without template).

3.2.2.5 Screening for presence of Class 1 and Class 2 integrons

Besides plasmids, integrons are also well known for spreading of resistant genes. Out of the 27 *K. pneumoniae* isolates under study, one environmental and nine clinical isolates carried Class 1 integron (**Fig. 3.2.11A**).

PCR with in-F and in-B primers targeting the variable region yielded an amplicon of 2.1KB in DL2.1. Upon sequencing it was found that, *arr*-3 gene that encodes rifampicin ADP-ribosylating transferase, *aac*A4 gene encoding aminoglycoside and *dfr*A1 gene encoding dihydrofolate reductase were present. The isolate CSK3 contained only dihydrofolate reductase gene (*dfr*A12). The isolate CSK4 contained dihydrofolate reductase (*dfr*2d) and *aacA*2 for aminoglycoside. The isolate CSK 6 had *arr*-2 gene that encodes rifampicin ADP-ribosylating transferase and *cml*A5 gene responsible for chloramphenicol resistance, while isolate CSK7 contained *aad*A2 for aminoglycoside and dihydrofolate reductase (*dfr*A12). KpAH7 contained dihydrofolate reductase (*dfr*A12), hypothetical protein (*orf*F), and aminoglycoside-3"-

adenylyltransferase (*aad*A2) genes whereas two isolates, KpAH5 and KpAH1, did not have any gene cassette in the variable region (**Fig. 3.2.11B)**.

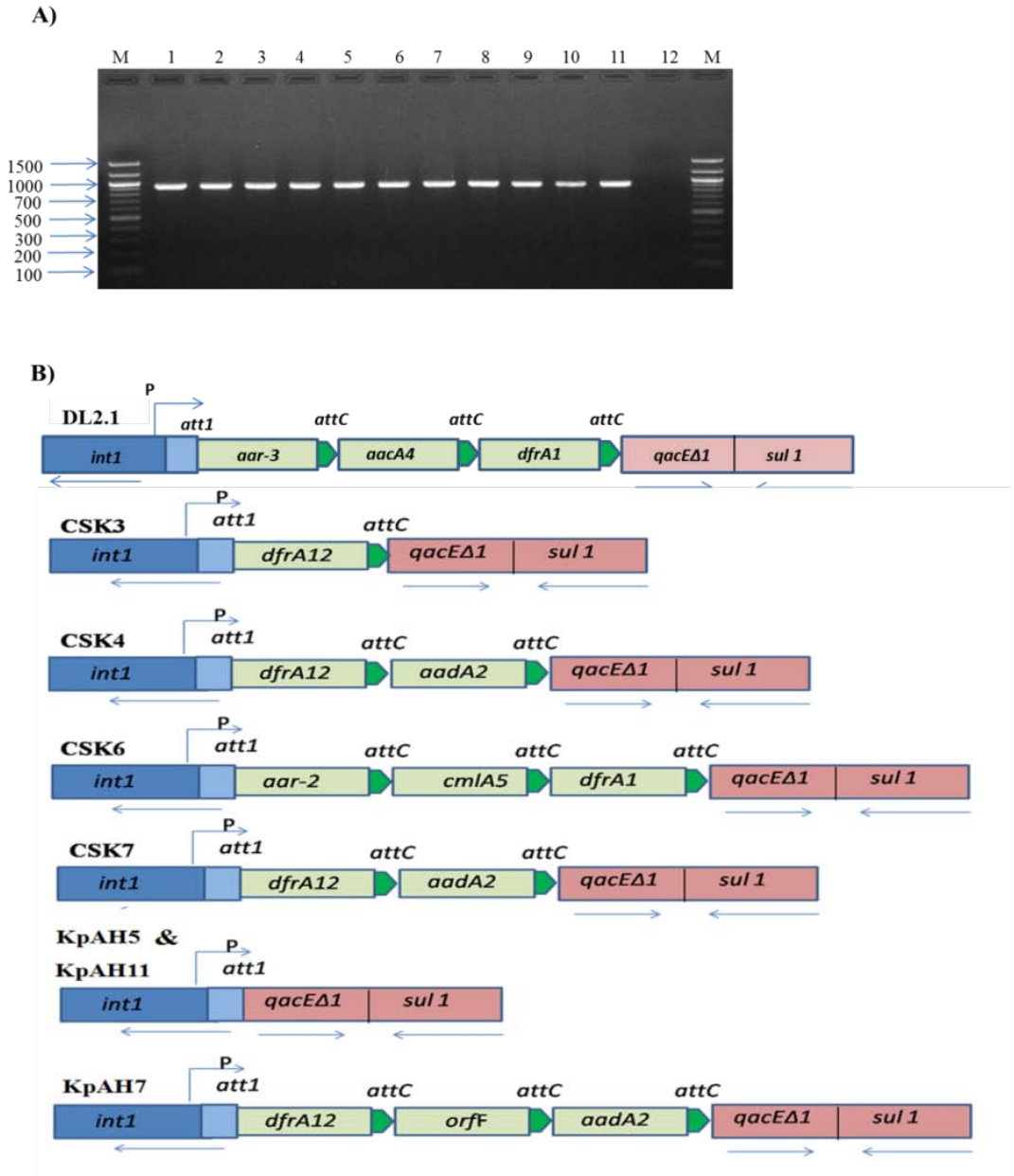


Figure 3.2.11: Detection of Class 1 integron.

(A) PCR amplification of Class 1 integrase and (B) variable regions of environmental and clinical *K. pneumoniae* isolates. Class 1 integron were found in one environmental isolates DL2.1 (lane 1) and nine clinical isolates CSK3, CSK4, CSK6, CSK7, KpAH4, KpAH5, KpAH7, KpAH9 and KpAH11 (lane 2-10). Lane 11 is positive control (VC20) and lane 12 is no template control. Lane M is 100 bp NEB ladder.

Class 2 integron was not found in any environmental *K. pneumoniae* isolate, but two clinical *K. pneumoniae* isolates namely CSK3 & CSK6 had Class 2 integron and insertion sequence IS5 TnpA (tnpA) gene respectively (F**ig. 3.2.12A**). The variable region of CSK3 contains an amplicon of ~7KB that was not able to sequence completely whereas CSK6 contain *dfr*1 gene for dihydrofolate reductase and *sat*-1 gene for streptothricin acetyltransferases (**Figure 3.2.12B/C**).

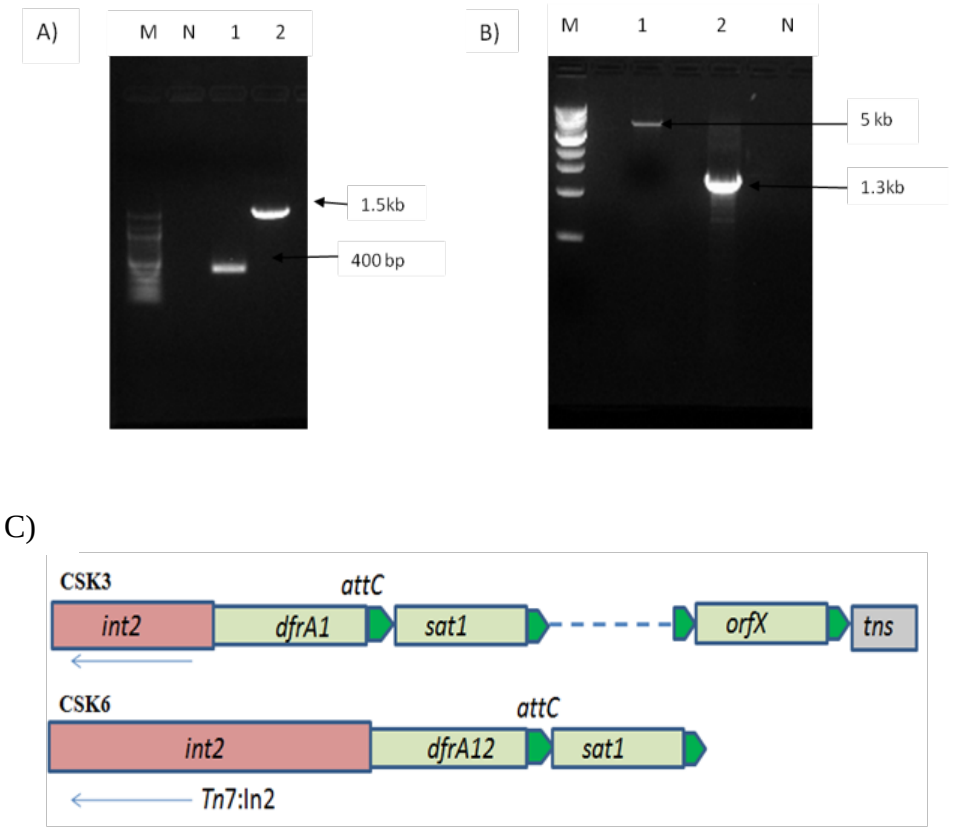


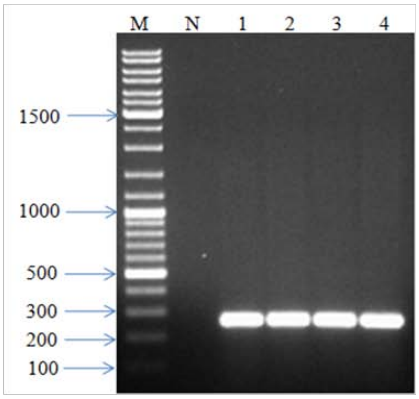
Figure 3.2.12: Detection of Class 2 integron.

(A) PCR amplification of Class 2 integrase, (B) PCR amplification of Class 2 integron gene cassette and C) The variable region of CSK3 (lane1) & CSK 6 (Lane 2). Two clinical *Klebsiella pneumoniae* isolates CSK3 (Lane1) & CSK6 (Lane2) having Class 2 integron & insertion sequence IS5 TnpA (tnpA) gene respectively.

Colistin resistance mechanisms in environmental *Klebsiella pneumoniae* isolates

Four environmental *Klebsiella pneumoniae* isolates namely HD3.2, HD4.1, HD4.5 and HD4.7 were resistant to peptide antibiotic colistin. Molecular basis of colistin resistance has been due to deletions/insertions/amino acid substitutions in *mgrB* – encoding a regulatory peptide of PhoP/PhoQ regulon that in turn regulates PmrAB system, responsible for lipopolysaccharide modifications. PCR amplification of colistin resistant isolates for *mgrB* yielded an amplicon of approximately 250bp and subsequent sequencing of the purified PCR product confirmed the absence of insertion/deletion, but showed two silent mutations (**Fig.3.2.13**). So, we checked for the presence and possible role of efflux pump genes in mediating colistin resistance. The MIC in presence of efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at an optimized final concentration of 50µM showed a drastic 3 to 4 fold reduction (**Table 3.2.4**). PCR screening followed by sequencing confirmed all the four isolates to be positive for presence of MATE efflux pump genes (**Fig.3.2.14**). These results indicated possible involvement of MATE efflux pump in mediating colistin resistance.

(A)



(B)

```
Ref       GTGAAAAAATTACGGTGGGTTTTACTGATAGTCATCATAGCAGGCTGCCTGTTGCTGTGG 60
HD3.2     GTGAAAAAATTACGGTGGGTTTTACTGATAGTCATCATAGCAGGCTGCCTGTTGCTGTGG 60
HD4.1     GTGAAAAAATTACGGTGGGTTTTACTGATAGTCATCATAGCAGGCTGCCTGTTGCTGTGG 60
HD4.5     GTGAAAAAATTACGGTGGGTTTTACTGATAGTCATCATAGCAGGCTGCCTGTTGCTGTGG 60
HD4.7     GTGAAAAAATTACGGTGGGTTTTACTGATAGTCATCATAGCAGGCTGCCTGTTGCTGTGG 60
          ************************************************************

Ref       ACTCAGATGCTTAACGTAATGTGCGACCAGGATGTTCAGTTTTTCAGCGGCATTTGCACT 120
HD3.2     ACTCAGATGCTTAACGTAATGTGCGACCAGGATGTTCAGTTTTTCAGCGGCATTTGCACT 120
HD4.1     ACTCAGATGCTTAACGTAATGTGCGACCAGGATGTTCAGTTTTTCAGCGGCATTTGCACT 120
HD4.5     ACTCAGATGCTTAACGTAATGTGCGACCAGGATGTTCAGTTTTTCAGCGGCATTTGCACT 120
HD4.7     ACTCAGATGCTTAACGTAATGTGCGACCAGGATGTTCAGTTTTTCAGCGGCATTTGCACT 120
          ************************************************************

Ref       ATCAATAAATTTATCCCGTGGTAA     144
HD3.2     ATTAATAAATTTATTCCGTGGTAA     144
HD4.1     ATTAATAAATTTATTCCGTGGTAA     144
HD4.5     ATTAATAAATTTATTCCGTGGTAA     144
HD4.7     ATTAATAAATTTATTCCGTGGTAA     144
          ** ********** *********
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(C)

```
Ref       MKKLRWVLLIVIIAGCLLLWTQMLNVMCDQDVQFFSGICTINKFIPW 47
HD3.2     MKKLRWVLLIVIIAGCLLLWTQMLNVMCDQDVQFFSGICTINKFIPW 47
HD4.1     MKKLRWVLLIVIIAGCLLLWTQMLNVMCDQDVQFFSGICTINKFIPW 47
HD4.5     MKKLRWVLLIVIIAGCLLLWTQMLNVMCDQDVQFFSGICTINKFIPW 47
HD4.7     MKKLRWVLLIVIIAGCLLLWTQMLNVMCDQDVQFFSGICTINKFIPW 47
          ***********************************************
```

Figure 3.2.13: **PCR amplification and sequence of *mgr*B.**

(A) PCR amplification *mgr*B gene, (B) Alignment of a fragment of the *mgrB* gene nucleotide sequences and (C) the *mgr*B translated amino acid sequences of the colistin-resistant isolate HD3.2, HD4.1, HD4.5 and HD4.7 on the basis of that of the wild-type reference strain *Klebsiella pneumoniae* ATCC 700603 (NCBI Reference Sequence: NZ_AOGO01000009.1). In panel B, gray shad indicate changed nucleotides in HD3.2, HD4.1, HD4.5 and HD4.7. In panel B and C, star indicates conserved nucleotide and protein respectively (The final publication is available at Elsevier via http://dx.doi.org/10.1016/j.micpath.2016.11.024).

Table 3.2.4 Minimum inhibitory concentration of colistin with and without CCCP

Isolate ID	Colistin MIC (mg/L)	
	-CCCP	+CCCP
HD3.2	8	2
HD4.1	16	2
HD4.5	>16	2
HD4.7	8	2

CCCP - Carbonyl cyanide 3-chlorophenylhydrazone

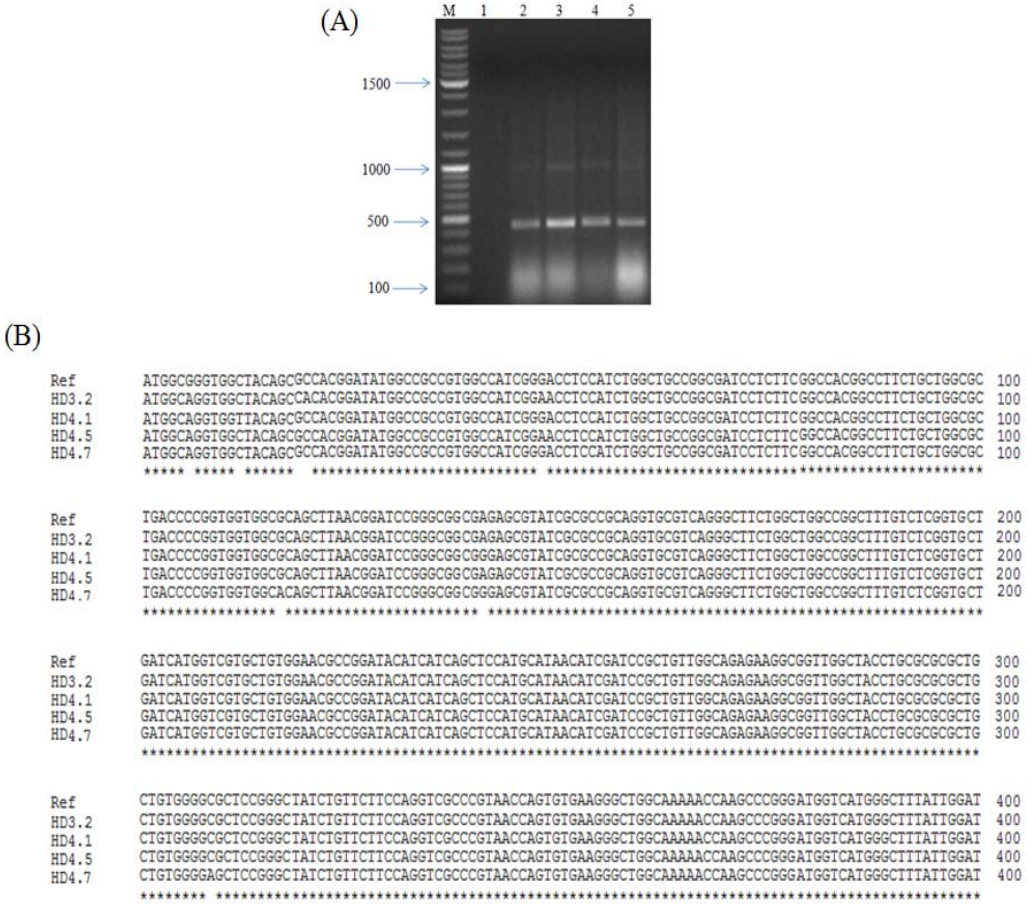


Figure 3.2.14: **PCR amplification and sequence of MATE efflux pump.**

(A) PCR amplification MATE efflux pump gene and (B) Alignment of a fragment of the MATE efflux pump nucleotide sequences with reference sequence (The final publication is available at Elsevier via http://dx.doi.org/10.1016/j.micpath.2016.11.024).

3.2.3. Non genetic basis of antibiotic tolerance in environmental *Klebsiella pneumoniae* isolate

During screening for the known resistance mechanisms, *Klebsiella pneumoniae* isolate DL5.4 from Dimna Lake was found to be devoid of β-lactamase genes like bla_{SHV}, bla_{TEM}, bla_{OXA}, bla_{NDM-1} and *Amp*C; did not contain plasmid/integron and mutations in QRDR of *gyr*A and *gyr*B. Also MIC in presence and absence of CCCP and varapamil was unchanged. Despite this, *K. pneumoniae viz.* DL5.4 was tolerant to a number of antibiotics that was intriguing.

Time dependent killing exhibits biphasic killing curve

From the last few decades, persister cell formation as a means of drug tolerance has been described where a small subpopulation of genetically identical susceptible bacterial inoculum survives exposure to lethal concentrations of antibiotics. A typical feature of persister cell formation is the biphasic killing curve generated upon treatment with antibiotics [320]. In order to test whether *K. pneumoniae* isolate DL5.4 was able to develop persisters, DL5.4 was treated with quinolone antibiotics (ciprofloxacin, levofloxacin and norfloxacin), tetracycline and erythromycin and monitored for biphasic killing curve. As shown in **Fig 3.2.15 and Table 3.2.5**, treatments by all antibiotics displayed a typical biphasic killing pattern, where the colony forming unit (cfu) was reduced due to rapid killing during the first 2h of antibiotic treatment followed by a plateau of surviving subpopulation, confirming the presence of multidrug tolerant persisters in *K. pneumoniae* isolate DL5.4.

Persister cells formed can tolerate much higher concentration of antibiotics

In order to determine the extent of antibiotic concentration tolerated by persister cell, concentration dependent persister formation was performed taking 4X MIC concentrations of respective antibiotics. Results showed that the persister cells formed in response to 1X concentration of respective antibiotics survived higher concentration (4X i.e. 8µg/ml of

ciprofloxacin and levofloxacin, 1024 µg/ml of erythromycin and 512 µg/ml of tetracycline) of the drugs tested (**Fig 3.2.16 and Table 3.2.6)**. These results highlighted the fact that once persister cells are induced, it becomes extremely difficult to eradicate the surviving population with any other treatment.

Table 3.2.5 Time dependent persister assay of DL5.4 at different time points

Time	Control[#] (CFU±SE)	Tetracycline (CFU±SE)	Erythromycin (CFU±SE)	Ciprofloxacin (CFU±SE)	Levofloxacin (CFU±SE)
2h	12.6 ± 0.68	3.7 ± 1.20	5.2 ± 0.11	4.3 ± 0.08	3.8 ± 0.24
4h	13.1 ± 0.21	4.0 ± 0.30	5.0 ± 0.30	4.4 ± 0.44	3.1 ± 0.55
6h	13.2 ± 0.13	3.4 ± 0.60	4.9 ± 0.33	4.0 ± 0.93	2.3 ± 0.00
8h	13.3 ± 0.04	3.2 ± 0.14	4.4 ± 0.45	3.5 ± 1.18	2.3 ± 0.00

#-without antibiotic; initial inoculums 10.7 ± 0.13 Log_{10} CFU/ml

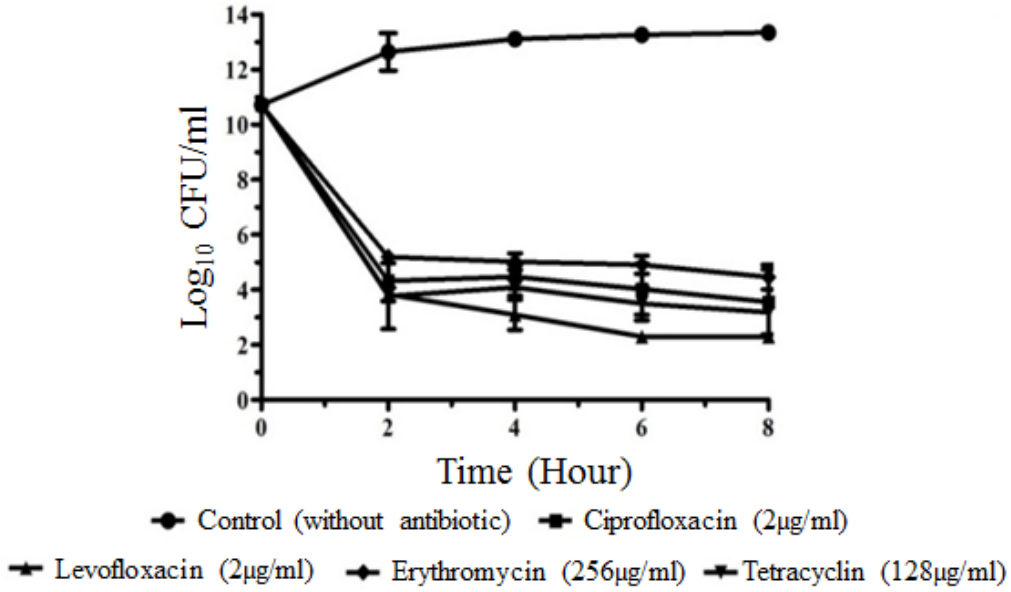


Figure 3.2.15: Time dependent persister assay

Log_{10} CFU/ml of control and antibiotic treated exponential phase culture of *K. pneumoniae* DL5.4 was plotted against different incubation periods. The values are averages of three replicates and error bar indicate standard error.

Table 3.2.6 Effect of increasing concentration of antibiotics on persister cell formation

MIC (µg/ml)	Control[#] (CFU±SE)	Tetracycline (CFU±SE)	Erythromycin (CFU±SE)	Ciprofloxacin (CFU±SE)	Levofloxacin (CFU±SE)
1X	12.8 ± 0.31	5.2 ± 0.11	5.2 ± 0.11	3.9 ± 0.28	4.0 ± 0.70
2X	11.9 ± 1.43	4.1 ± 0.35	4.0 ± 1.62	2.9 ± 0.31	3.0 ± 0.20
3X	11.9 ± 1.21	3.1 ± 0.60	3.9 ± 1.29	2.8 ± 0.14	2.5 ± 0.34
4X	11.8 ± 1.42	2.9 ± 0.93	3.8 ± 0.19	2.4 ± 0.18	2.9 ± 0.69

#-without antibiotic; Initial inoculums 10.71 ± 0.13Log_{10} CFU/ml

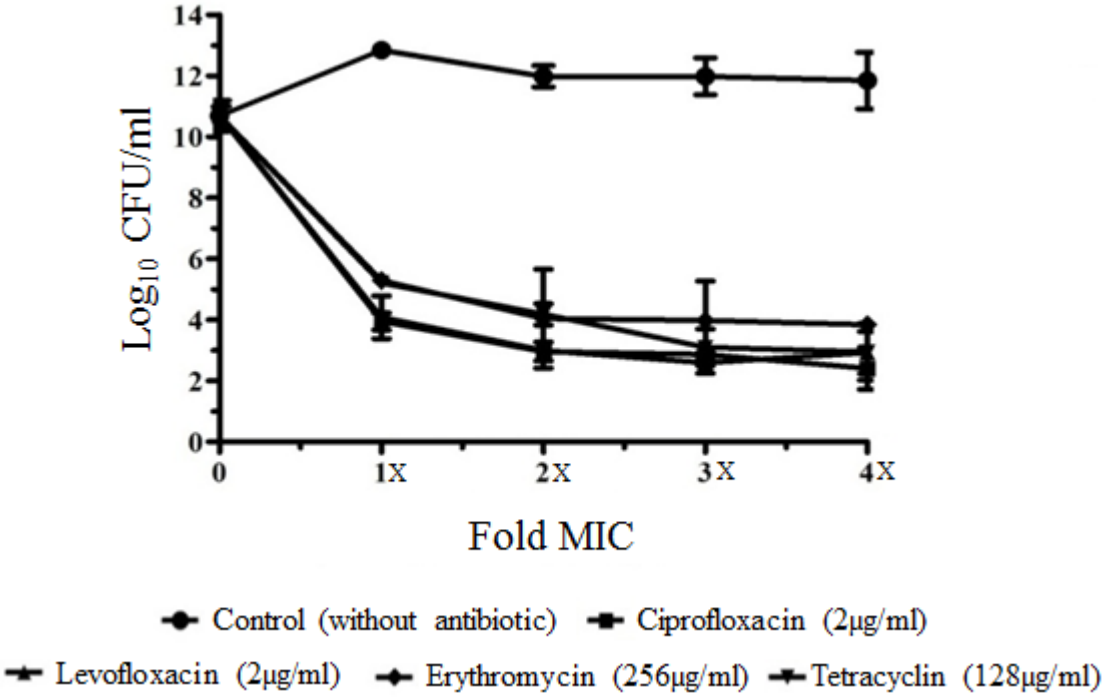


Figure 3.2.16: Concentration dependent persister assay

Log_{10} CFU/ml of control and antibiotics treated exponential phase culture of *K. pneumoniae viz.* DL5.4 was plotted against different incubation periods. The values are averages of three replicates and error bar indicate standard error.

Effect of growth phase of initial inoculum on persister formation

We next sought to determine the effect of growth phase of initial inoculum on persister formation. For this, 3h, 6h and 9h grown culture representing early exponential, late exponential and stationary phase inoculum were treated with 1X concentration of antibiotics *viz*. 2µg/ml ciprofloxacin, 2µg/ml levofloxacin, 2µg/ml norfloxacin, 256µg/ml erythromycin and 128µg/ml tetracycline. Results of this experiment are presented in **table3.2.7** and **fig. 3.2.17**. The results revealed that when cells in early exponential phase were used as inoculum, less number of persister cells was formed. A similar biphasic killing dynamics were observed in different growth phase (**Fig. 3.2.17**).

Table 3.2.7 Effect of growth phase of inoculum on persister cell formation

	Early exponential phase Log_{10} CFU/ml			Late exponential phase Log_{10} CFU/ml			Stationary phase Log_{10} CFU/ml		
	3h	6h	9h	3h	6h	9h	3h	6h	9h
Control[#]	12.5 ± 0.05	12.6 ± 0.07	12.2 ± 0.06	12.7 ±0.14	12.3 ±0.06	12.9 ±0.05	12.9 ±0.06	12.9 ±0.06	12.6 ±0.04
Tetracycline	5.2 ± 0.32	5.1 ± 0.36	4.8 ± 0.00	6.1 ±0.04	6.2 ±0.02	6.1 ±0.01	6.2 ±0.04	6.2 ±0.03	6.1 ±0.02
Erythromycin	5.1 ± 0.12	4.6 ± 0.26	4.8 ± 0.21	6.0 ±0.08	6.3 ±0.02	6.2 ±0.03	6.2 ±0.01	6.1 ±0.03	6.0 ±0.03
Ciprofloxacin	1.5 ± 0.10	1.3 ± 0.28	1.3 ± 0.21	4.1 ±0.08	4.0 ±0.07	3.7 ±0.19	4.1 ±0.10	3.8 ±0.09	3.4 ±0.02
Levofloxacin	1.4 ± 0.21	1.3 ± 0.00	1.3 ± 0.00	3.9 ±0.07	3.8 ±0.06	3.4 ±0.06	3.7 ±0.08	3.7 ±0.02	2.9 ±0.06

#-without antibiotic

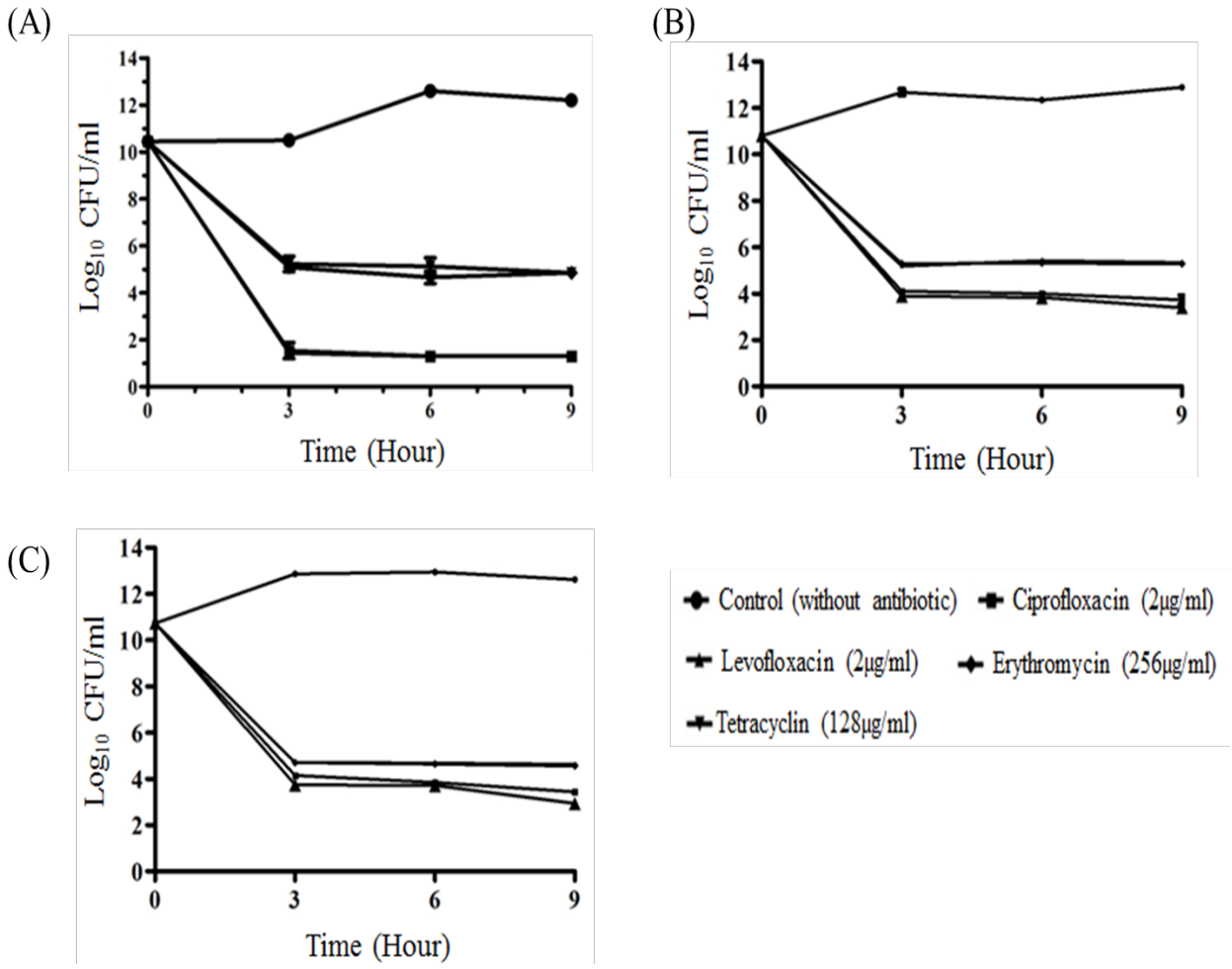


Figure 3.2.17: Effect of growth phase of inoculum on persister cell formation

A) Early exponential phase (B) Late exponential phase and (C) Stationary phase. Log_{10} CFU of control and antibiotics treated exponential phase culture of *K. pneumoniae viz.* DL5.4 was plotted against time points. The values are averages of three replicates and error bar indicate standard error.

Persistence is a non-heritable phenomenon

Non-heritable nature of persister cells was determined using same set of antibiotics for four generations. Results (**Table 3.2.8 and Fig**. **3.2.18**) indicated that one generation of persister cells formed upon exposure to an antibiotic, when re-inoculated into fresh medium with same concentration of antibiotic, did not exhibit any increase in cell numbers. Such a growth was also seen in subsequent generations. This observation was also confirmatory for the fact that the

subpopulation entering in to persister stage are not resistant mutants. The confocal image of persister cells showed thread like structure which indicated it non dividing nature (**Fig 3.2.19**).

Table 3.2.8 Log10 CFU of control and antibiotics treated *K. pneumoniae* viz. DL5.4 in different generation

Generation	Control	Tetracycline	Erythromycin	Ciprofloxacin	Levofloxacin
1st	13.09 ± 0.16	5.30 ± 0.02	5.38 ± 0.01	4.14 ± 0.09	3.54 ± 0.55
2nd	13.24 ± 0.10	5.01 ± 0.08	4.77 ± 0.18	3.88 ± 0.18	3.17 ± 0.38
3rd	13.17 ± 0.06	4.58 ± 0.12	4.98 ± 0.04	3.01 ± 0.33	2.63 ± 0.21
4th	13.28 ± 0.12	4.02 ± 0.01	4.61 ± 0.02	2.09 ± 0.01	3.39 ± 0.01

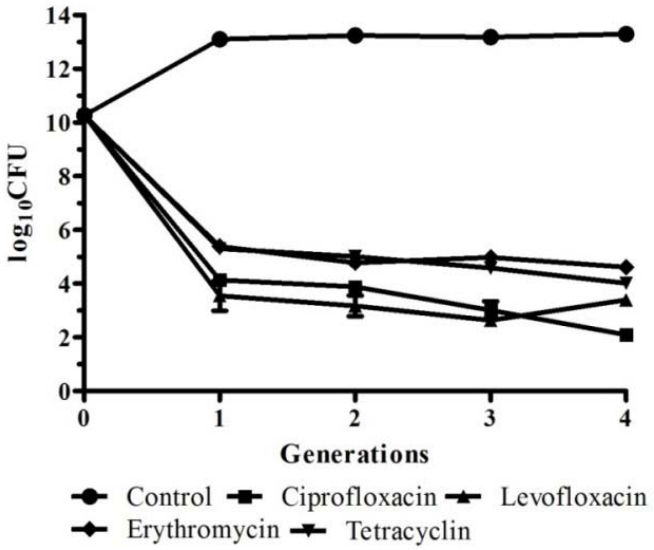


Figure 3.2.18: Non-Heritable nature of persister.

The values are averages of three replicates and error bar indicate standard error. Initial inoculums 10.26 ± 0.10 Log_{10} CFU/ml. Persister cell showed not further growth when re-inoculated in to fresh medium with same sets of antibiotics over multiple generations.

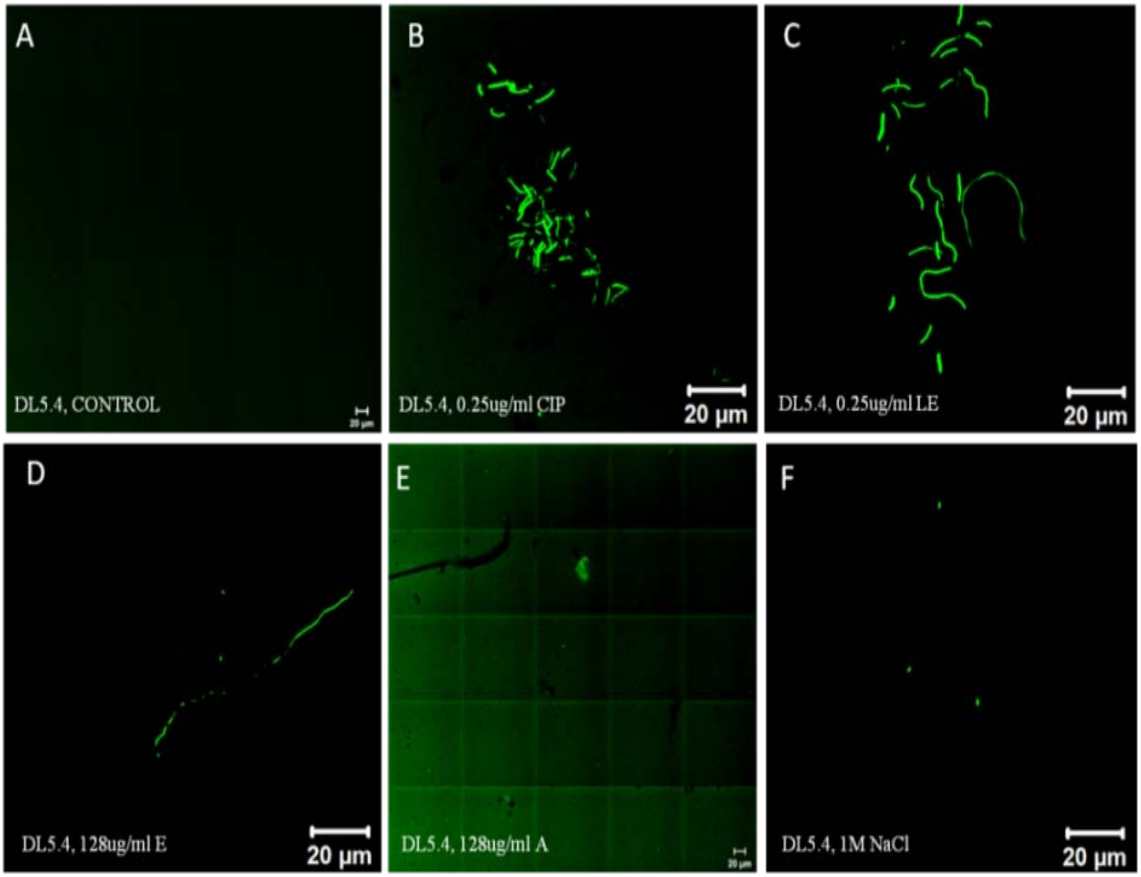


Figure 3.2.19: Morphological changes of persister cells.

Confocal microscopy image of (i) untreated (ii) Ciprofloxacin, (iii) Levofloxacin, (iv) Erythromycin and (v) 128µg/ml Ampicilin (vi) 1mM NaCl. Images A, E and F were taken in 5X5 tiles manner with 20µm magnification of each tile.

3.2.4 Detection of virulence factors

In addition to multiple antibiotic resistance, possession of virulence factors is advantageous for bacteria to be a successful pathogen. Hence we checked for the presence of fimbriae and hypermucoviscocity, which are known to aid pathogenicity in *Klebsiella pneumoniae*. In addition, we carried detailed analysis for genus *Klebsiella pneumoniae* of the phenotypic virulence assay results reported in chapter 1. Results and detail of the analysis done are presented here.

Adhesion to mucosal and epithelial cell surfaces is often the first step in the development of colonization and infection. Adhesins are often also Haemagglutinins located on fimbriae (pili) that protrude on the surface of the bacterial cells. Type 1 fimbriae were present in all, except three isolates of *Klebsiella pneumoniae*, the environmental isolate DL4.1 and clinical isolates CSK1 and CSK6. Type 3 fimbriae were not detected in any of the *K. pneumoniae* isolates (**table 3.2.9**). Another factor responsible for adhesion was hypermucoviscocity that was determined by string test. Five environmental isolates namely DL2.1, DL4.1, HD4.1, KR3.4 and KR4.14 showed hypermucoviscous phenotype (**Fig. 3.2.20A and Table 3.2.9**). Hydrophobicity is an indirect way to determine adhesion property, as micro-organism may adhere to a substratum via hydrophobic effect, provided association sites possess sufficiently high densities of apolar areas like mucosal surface. We checked for hydrophobicity by bacterial adherence to hydrocarbons (BATH) (**Fig. 3.2.20B**). Results indicated that only two environmental isolates DL2.3 and HD4.7 were hydrophobic (**Table 3.2.9**), while rests were highly hydrophilic.

Biofilm protect bacteria from environmental stress and against host defense. Also biofilms are an important stage in the pathogenesis of numerous bacterial species and are the major cause of recalcitrant infections. The strains were assayed for production of biofilm using crystal violet stain. The results indicate that each strain shows a different potential to form biofilm under the same conditions of experimentation. Two environmental isolates (HD3.2 & SR5.4) were strong biofilm former & two isolates were non-biofilm former (**Fig. 3.2.20C and Table 3.2.9**). Remaining environmental isolates were moderate biofilm former. Strong biofilm formation ability in clinical isolates was shown by CSK2 followed by CSK3, while the clinical isolate CSK1 showed moderate biofilm formation. Remaining clinical isolates were non-biofilm former (**Fig. 3.2.20C and Table 3.2.9**).

After the onset of invasion, bacteria meet the cellular and humoral bactericidal components of the innate immune system. Lipopolysaccharides (LPS) have been implicated as a major factor in the ability of bacteria to resist serum bactericidal activity by the host. To check the serum resistance properties, all isolates were incubated with human serum for 0h, 1h, 2h and 3h and found that all environmental isolates were intermediately susceptible by categorizing in to grade 3 and 4 while clinical isolates were resistant by categorizing in to with grade five toward human serum (**Table 3.2.9**).

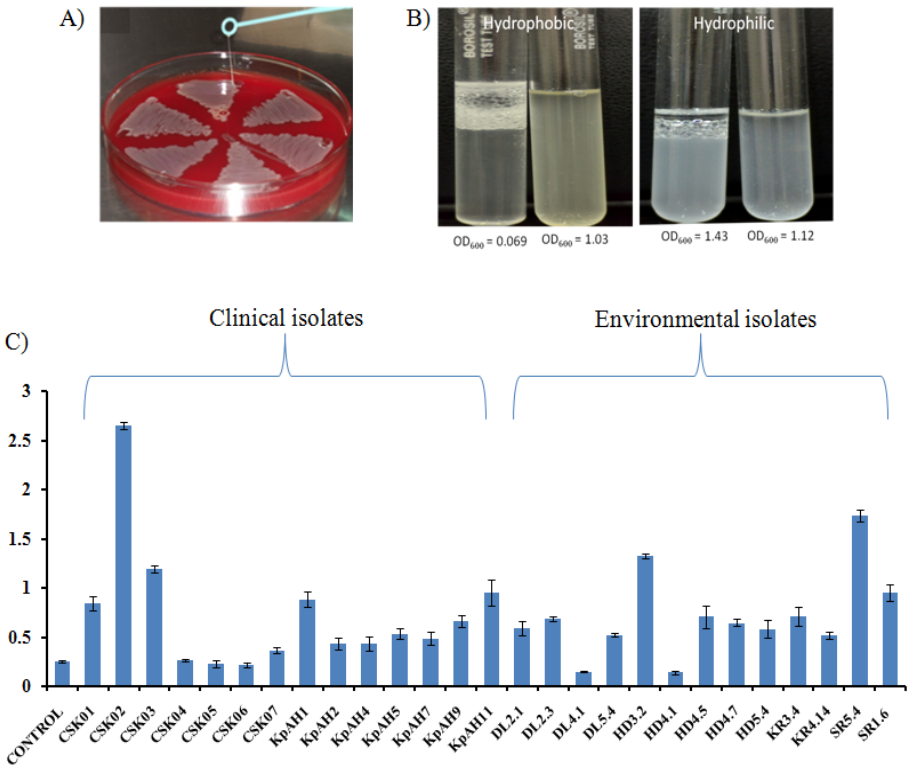


Figure 3.2.20: Phenotypic detection of virulence factors of *K. pneumoniae*

(A) String test for hypermucoviscocity, (B) BATH assay for Hydrophobicity and (C) Biofilm forming ability. The formation of a viscous string≥5 mm long, representing the characteristic of hypermucoviscous strain. Isolate with 35% decrease in optical density of lower aqueous phase compared with the OD of cell suspension without xylene was regarded as hydrophobic. Biofilm forming isolates were classified as follows: OD < OD_c – non adherent, OD_c < OD < 2X OD_c – weakly adherent, 2 OD_c < OD < 4X ODc – moderately adherent and 4X OD_c < OD– strong adherent.

Table 3.2.9 Summery of virulence factors present in environmental and clinical isolates of *K. pneumoniae*

Isolate	Fimbriae	HMV	% Hydrophobicity	Serum resistance	Biofilm forming ability
DL2.1	Type1	+	-26.5 ±9	Intermediate	Moderate
DL2.3	Type1	-	89.5 ±6	Intermediate	Moderate
DL4.1	-	+	-5.5 ±8	Intermediate	Non biofilm
DL5.4	Type1	-	-8 ±7	Intermediate	Moderate
HD3.2	Type1	-	-11.5 ±5	Intermediate	Strong
HD4.1	Type1	+	-10 ±8	Intermediate	Non biofilm
HD4.5	Type1	-	-4 ±4	Intermediate	Moderate
HD4.7	Type1	-	93 ±0	Intermediate	Moderate
HD5.4	Type1	-	-4.5 ±4	Intermediate	Moderate
KR3.4	Type1	-	-0.5 ±1	Intermediate	Moderate
KR4.14	Type1	+	-4.5 ±6	Intermediate	Moderate
SR5.4	Type1	-	-4.5 ±1	Intermediate	Strong
SR1.6	Type1	-	-2.5 ±4	Intermediate	Moderate
CSK1	-	-	-6.5 ±4	Resistant	Moderate
CSK2	Type1	-	-7.5 ±1	Resistant	Strong
CSK3	Type1	-	-3 ±3	Resistant	Strong
CSK4	Type1	-	-8.5 ±2	Resistant	Weak
CSK5	Type1	-	-8.5 ±5	Resistant	Non biofilm
CSK6	-	-	-10 ±3	Resistant	Non biofilm
CSK7	Type1	-	-2.5 ±4	Resistant	Weak
KpAH1	Type1	-	-6 ±5	Resistant	Strong
KpAH2	Type1	-	-3 ±2	Resistant	Weak
KpAH4	Type1	-	-5 ±3	Resistant	Weak
KpAH5	Type1	-	0 ±10	Resistant	Moderate
KpAH7	Type1	-	-24 ±3	Resistant	Moderate
KpAH9	Type1	-	-18 ±2	Resistant	Moderate
KpAH11	Type1	-	-17 ±2	Resistant	Strong

3.2.5 Discussion

Results of our study on resistance profiles and diversity of drug resistance mechanisms between environmental and clinical isolates of *Klebsiella pneumoniae* revealed the following

(1) All environmental and clinical isolates exhibited multidrug resistant phenotype, however the average MAR index of clinical isolates was much higher (0.7) compare to environmental isolates (0.3).

(2) Disc diffusion and MIC result corroborated well with each other and indicated that resistance to quinolone was much wide spread among clinical isolates than environmental isolates.

(3) Clinical isolates exhibited high co-relation co-efficient (at p=0.01) for cefepime and ceftazidime (1.0), meropenem and ceftriaxone (1.0), ciprofloxacin and meropenem (1.0), ciprofloxacin and ceftriaxone (0.9). Also the isolates exhibited significantly high correlation (at p=0.05) for colistin and cefepime/ceftazidime. These later results indicate strong diffusion barrier which perhaps couple with β-lactamase production renders these antibiotics ineffective. Further, the results of the analysis also indicate limited treatment options available with diminished effectivity for drug of choice.

(4) Screening for resistance genes and mechanisms indicated wide spread diversity. bla_{SHV} was detected in majority of clinical (n=12) and environmental (n=8) isolates. NDM-1 gene was present in only clinical isolates (n=2). Mutation in QRDR of gyrase A that confers high degree of quinolone resistance was present in only clinical isolates. Reduced MIC in presence of CCCP indicated possible involvement of efflux pumps in mediating quinolone resistance particularly in environmental isolates.

(5) Environmental isolates DL5.4 exhibited persister cell formation as a mechanism to overcome drug toxicity.

(6) Hypermucoviscous phenotype was associated with three environmental isolates but none of clinical isolates. Biofilm formation was significantly more pronounced in environmental than in clinical isolates.

(7) Four environmental isolates exhibited efflux pump mediated colistin resistance.

Klebsiella pneumoniae contend with great varieties of environmental stresses due to their ubiquitous lifestyle [206]. *K. pneumoniae* is capable of employing a multitude of mechanisms to confer their existence in such hostile conditions. Recent multicenter prospective study conducted in several Asian countries showed, *K. pneumoniae* ranked second accounting for 15.4% of the pathogens responsible for hospitalized community aquired pneumonia (CAP) [402]. This raises serious concerns as *K. pneumoniae* was primarily known to act as an opportunistic pathogen. The problem of infection caused by *K. pneumoniae* started intensifying as environmental isolates too showed multidrug resistant phenotype [141]. Apart from intrinsic resistance to aminopenicillin and carboxypenicillin, Struve *et al*., (2004) reported that none of the *K. pneumoniae* isolated from environment were resistant to antibiotics like chloromphenicol, tetracycline, nalidixic acid, sulphonamides, polymyxin, kanamycin, gentamicin, tobramycin, netilmicin and amikacin [403]. In contrast, in the present study, resistance rates in environmental isolates were high, varied considerably from nil for imipenem, meropenem, ofloxacin and gentamycin to 100% for ampicillin, 77% for cefuroxime, 62% for piperacillin, 38% for cefepime and 31% for cefpirome. Also notable was the fact that ≥30% were resistant to ciprofloxacin, levof loxacin, polymyxin, colistin and trimethoprime. Further, greater number of the clinical isolates were resistant toward

all the β-lactam, quinolone, trimethoprim, gentamycin and tetracycline as was also reported by Paterson *et al*, 2004. Greater percent resistance towards 3rd and 4th generation cephalosporin in the present study could be attributed towards their extensive use as an alternative to Penicillin G in treatment of respiratory and soft tissue infections caused by *Klebsiella* sp as well as in surgical prophylaxis [404,405].

In 1980-90s, clinical application of the third & fourth-generation cephalosporins caused a major breakthrough in treating resistant infections caused by β-lactamase producing bacteria. Production of extended-spectrum β-lactamases (ESBLs), particularly by *E. coli* and *K. pneumoniae*, is the most well known mechanism of resistance to third and fourth generation cephalosporins such as ceftazidime and cefepime and has also been associated with wide spread occurrence of antimicrobial resistance in the hospital setting [406]. The first ESBL was identified in *K. pneumoniae* strain isolated in Germany in 1983 [407]. Since then more than 200 ESBL variants have been identified, some of which have spread rapidly worldwide [408].

In 2009, CLSI introduced two-step procedure for the detection of ESBL producers. In the first step, isolates of *E. coli* and *K. pneumoniae* were to be screened for resistance to one or more of third-generation indicator cephalosporin (ceftriaxone, cefotaxime, ceftazidime, cefpodoxime or aztreonam). Since ESBLs vary in their hydrolysis of these cephalosporins as substrates, resistance to at least one of them was considered as positive in the screening test. In the second step, isolates that were positive in the screening test were confirmed for ESBL production by clavulanic acid based test. CLSI recommends that both cefotaxime or ceftazidime, alone and in combination with clavulanic acid must be used for detection of ESBLs. Of the twenty two isolates (8 environmental and 14 clinical) that were positive in the screening test in this study, twenty isolates (9 environmental isolates and 11 clinical *Klebsiella pneumoniae*

isolates) were phenotypically confirmed as ESBL producers by the CLSI-double disk synergy method. From the literature in pubmed excluding clonal outbreak and small sample data (<15) during 2000-2011 across India, the detection rates of ESBL-*Klebsiella pneumoniae* is shown to vary from 6 to 87% (**Table 3.2.10**).

Table 3.2.101 Prevalence of ESBL-KP data across India during 2000-2011

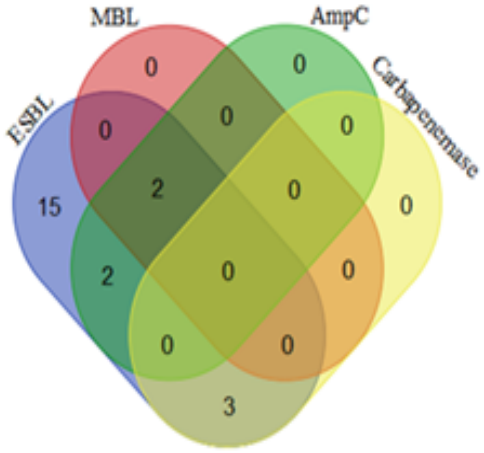
Region	Center	% ESBL	Reference
Northern	New Delhi	1.6-87%	[409,410,411,412]
	Banaras	46%	[413]
	Lucknow	66.7%	[414]
	Aligarh	30.18%	[415]
	Uttar Pradesh	24.6%	[416]
Eastern	Sikkim	57.14%	[417]
Western	Mumbai	20%	[418]
	Pune	16%	[419]
Southern	Chennai	6.66%-65.51%	[420,421]
	Puducherry	32%	[422]
	Coimbatore	40%	[423]

The most frequently encountered ESBLs belong to the TEM and SHV (sulfhydryl variable) families [424,425]. Additionally, other types of ESBLs, CTX-M-type and PER (*Pseudomonas* extended resistance)-type were also reported but were less prevelent. Genotypic detection in the present study showed that ESBL gene $bla_{\text{SHV-32}}$ was identified in 61.5% (n=8) environmental & 88% (n=12) clinical *K. pneumoniae* isolates. In one environmental isolate (SR5.4) $bla_{\text{CTX-m}}$ gene found.

Emergence of other mechanisms, like production of *Amp*C and carbapenemase in *K. pneumoniae* may have contributed to resistance towards β-lactam/β-lactamase inhibitor combinations and carbapenems respectively [426]. We reported *Amp*C production in 14% (n=4)

of *K. pneumoniae* isolates, which echoed to previous reports from eastern part (Kolkata) of the country with 13% (n=3) *Amp*C β-lactamase producers [427]. However, reports from southern and northern parts of India indicated 29% *K. pneumoniae* isolates to produce *Amp*C [428]. This reflects a possible regional localization of *Amp*C gene. It also put up an intriguing question on clonal relationship of the isolates studied, which is less addressed.

MBL genes like bla_{IMP}, bla_{VIM} were not detected in this study, similar to reports from China and Taiwan that suggests relatively low or null levels of MBL in clinical isolates *K. pneumoniae* [429,430]. ESBLs producing isolates capable of hydrolyzing cephalosporins except carbapenems, therefore, carbapenems are the last resort antibiotics to treat severe infections caused by ESBL positive *K. pneumoniae*. Unfortunately, over use of this drug creates a selective pressure to *K. pneumoniae* strains which resulted in the acquisition of resistance by the production of the enzyme carbapenemase. The most dominant carbapenemase in clinical *K. pneumoniae* strains were *bla*OXA-48 and plasmid-mediated KPC, that are reported to be widely prevalent in North Africa, the Middle East, and the Indian subcontinent in isolates that were clonally unrelated [178,182,183]. In the present study only three isolates (CSK4, CSK6 and KpAH9) carried *bla*OXA gene. Co-production of more than one type of β-lactamase confers resistance to wide range of β-lactam antibiotics including first, second and third generations of cephalosporins and carbapenems. During the past decennium, co-production of multiple β-lactamases especially ESBLs (e.g. bla_{SHV}, bla_{TEM} and/or bla_{CTX}) and carbapenemase (bla_{KPC} and/or bla_{OXA}) have reported from many countries [431,432]. In present study, we also found some isolates produced multiple β-lactamases as shown in **fig. 3.2.21**.



Name	Total	Isolates
AmpC, ESBL, MBL	2	KpAH1, KpAH7
AmpC, ESBL	2	HD5.4, KpAH5
ESBL, Carbapenemase	3	CSK4, CSK6, KpAH9

Figure 3.2.21: Co-occurrence of multiple β-lactamases in clinical isolates of *K. pneumoniae*

Quinolone resistance in *Klebsiella pneumoniae*

Fluoroquinolones are broad-spectrum, synthetic, antimicrobial agents that bind to a short DNA sequence known as quinolone resistance-determining region (QRDR) of the target DNA gyrase and topoisomerase IV [433]. Alterations due to mutation in amino acids of QRDR to which fluoroquinolones exactly bind and presence of plasmid mediated quinolone resistance gene (*qnr*) results in resistance to fluoroquinolones [362]. Common sites of resistance-conferring mutations and alterations are Ser^{83} to Leu/Ile/Phe and Asp^{87} to Tyr/Asn in gyrase A and mutations to Asp^{426} to Asn and Lys^{437} to Glu in gyrase B [362]. In this study, none of the *K. pneumoniae* isolated from aquatic environment have mutation in the QRDR region of gyrase A and gyrase B. In contrast, clinical isolates showed three types of amino acid substitutions, Ser-Ile transition, Ser-Tyr transition and Ser-Phe transition at 83 amino acid position and Asp-Gly transition and Asp-Ala transition at 87 amino acid positions. In present study, high prevalence (68.75%) of resistance towards commonly used fluoroquinolones provides circumstantial evidence for the fact that accumulation of mutations resulting in target modifications and

plasmid mediated quinolone resistance gene amongst clinical isolates of *K. pneumoniae*. Beside resistant genes and target alteration, energy-dependent efflux have also been shown to contribute to the antibiotics resistance phenotype in *K. pneumoniae* [434]. Efflux pumps reduced the antibiotic concentration inside the bacterial cell. Mazzariol *et al*., (2002) reported contribution of a multidrug efflux system *Acr*AB responsible for fluroquinolone resistant phenotype in clinical strain of *K. pneumoniae* [435]. In another study, Aathithan *et al*., (2011) have reported very high (92%) efflux pump mediated multidrug resistance in clinical *Klebsiella spp* [436]. But in present study we found low abundance (n=7) of efflux pump mediated multidrug resistance in environmental and clinical isolates of *K. pneumoniae*.

Colistin resistance in *Klebsiella pneumoniae*

Colistin, the last resort antibiotic has been used as a therapeutic option for treatment of carbapenem resistant *Klebsiella pneumoniae* [437]. Many studies have established emergence of colistin resistant *K. pneumoniae* (Col-RKP) in clinical settings associated with its increased clinical usage [437,438]. During 2013 to 2014, reports of colistin resistance in tested *Klebsiella pneumoniae* increased to 2% in India (www.resistancemap.org). Apart from clinical usage, colistin sulphate is also used as supplement in animal feed in poultries and fish farms as a growth promoter [438,439]. Colistin being very stable in water, microorganisms present in aquatic environment is exposed to the residual drug and such persistent exposure leads to mutations and development of new strains [440,441]. Here we reported the occurrence of colistin resistance *Klebsiella pneumoniae* namely HD4.5, HD4.7, HD3.2 and HD4.1 isolated from artificial stagnant water source. Colistin resistance in the clinical *K. pneumoniae* has been attributed either due to deletions/insertions/amino acid substitutions in *mgrB* [442,443,444]. Recently, plasmid mediated *mcr-1* gene responsible for colistin was reported in *K. pneumoniae* isolated from

animals [137]. In the present study, all the four isolates have two silence mutations without any insertion/deletion in the regulatory *mgr*B gene. Three to four fold reduction of MIC for colistin in presence of CCCP and presence of MATE efflux pump indicates probable role of efflux pump in mediating colistin resistance phenotype in these isolates. This finding is definitely alarming as colistin-resistant *K. pneumoniae* increase the difficulty level as treatment of infections become extremely difficult.

Plasmid and integron as resistance gene carriers

Plasmids and integrons play an important role in the antibiotic resistance in most bacterial isolates and *K. pneumoniae* is no exception. Many observations conclusively infer plasmids playing an important role in antibiotic resistance, carry a tremendous potential to disseminate resistance markers to distant recipient cells [445]. In the present study, two *K. pneumoniae* isolated from aquatic environment and seven *K. pneumoniae* isolated from clinical samples harbour plasmids. Plasmid curing using SDS confirmed the the presence of quinolone resistance determinants in wild type *K. pneumoniae* clinical isolates; as cured strain became susceptible for quinolone antibiotics such as levofloxacin, ciprofloxacin and norfloxacin. This observation was supported by presence of *qnr*B gene in wild type plasmid positive isolates.

Integrons are the major players in antimicrobial resistance globally, because they are able to capture, integrate and express diverse gene cassettes encoding proteins associated with antimicrobial resistance [446]. Presence of integrons thus boosts multidrug resistant phenotype in isolates bearing it, which severely limits treatment options. Occurrence of integrons in 90%, 73% and 70% of *K. pneumoniae* isolates was reported from Asia, Australia and US respectively [447,448,449]. Stockes and Gillings (2011), had reported that integrons are not only present in pathogenic organisms, but also present in bacteria isolated from environment like river, waste

water and soil [450]. We observed less number of isolates containing integrons among environmental (n=1) and clinical (n=9) isolates of *K. pneumoniae* under study. Sequence analysis of integron's variable region identified the gene *aad*A2, that confer resistance to aminoglycosides, which was also reported recently in *K. pneumoniae* sequence type 274 strains from companion animals [451]. Presence of three variants of *dfr* gene (*dfr*A2d, *dfr*A12, *dfr*A1) was identifid in the present study. *dfr* gene that sustain trimethoprim resistance, and are commonly reported associated with Class1 integron in many bacterial isolates [452]. We have reported the presence of class 2 integrons in two clinical isolates, which is less explored in *K pneumoniae* from India. The isolates CSK3 and CSK6, harbored class 2 integrons and showed different resistant pattern. Isolate CSK6 that carried both Class1 and Class 2 integron was resistant to most of the antibiotics except colistin, polymyxin-B and imipenem similar to Bhattacharjee *et al*., (2010) [446], whereas isolate CSK3 with both Class1 and Class 2 integron was resistant to only cephalosporin group of antibiotic.

Persister cell formation in *Klebsiella pneumoniae*

In contrast to antibiotics resistance resulting from mutations in existing genes or the acquisition of external resistance-encoding genes, persister cell formation in bacteria is non-inherited and is purely phenotypic which showed high tolerance toward antimicrobial agents [332]. Since the first description of persister cell formation in staphylococci by Joseph W. Bigger in 1944 [319], persister formation has been reported in *E. coli* [320,323,335], *Pseudomonas sp* [324,325,326], *Mycobacterium tuberculosis* [327], *Salmonella* sp [328] and *Candida albicans* [330]. In these organisms, persister cell formation elucidated a protective mechanism responsible for survival of bacteria in the different stressed conditions [324,330]. We characterized persister cell formation in *K. pneumoniae* isolate DL5.4. Isolate DL5.4 showed biphasic killing pattern

and was able to tolerate up to 4X concentration of MIC. The characteristic tolerant sub-population formed was non heritable in nature *i.e.* when tolerant bacteria were re-inoculated into fresh medium with antibiotic added, it showed the biphasic killing curve. In order to observe persisters, we used carboxyfluorescein diacetate, which specifically stains membrane of live cells. After treatment with antibiotics, most of the cells changed morphologically. More filamentous structure were formed which was also shown by Kim *et al.,* (2011) [453]. Numerous bacterial species used quorum-sensing mechanisms to regulate virulence factor expression or phenotypic changes. These controls of gene expressions were done by extracellular signaling molecules called auto inducers on a critical threshold concentration within a population, which triggered signal transduction cascade. Upon addition of *K. pneumoniae* DL5.4 filterable extracellular substance, no increases in persister number was observed. Similar results were shown in *E. coli* [331], in *P. aeruginosa* [326] and in *S. epidermidis* [332]. In all case no increase in persister numbers was shown in early exponential phase cultures that received growth medium from stationary phase cultures to induce persister production.

In spite of the extensive capabilities of host immune system to eliminate pathogens, a wide range of bacteria effectively and frequently infect humans and animal hosts by inactivating, defeating and/or by weaken immune defense systems [454] and causing tissue damage. These pathogenic bacteria have been associated with the various virulence traits that lead to pathogenicity. These virulence factors viewed as the properties (i.e., gene products) that alter host–microbe interaction and enable a microorganism to establish itself on or within a host of a particular species and enhance its potential to cause disease [205].

Our results on association analysis of MAR index and virulence index (chapter 1) indicated *Klebsiella pneumoniae* to hold second position, preceded by *Pseudomonas aeruginosa*.

Klebsiella pneumoniae cause infection in humans of all ages, however infants, the elderly and the immunocompromised group were at highest risk [455]. In general there was a wide variety of virulence factors that may contribute to pathogenicity in humans. The present study showed that the environmental and clinical isolates of *Klebsiella pneumoniae* carried different virulence factor, as has also been reported earlier [141,403].

Section 3.3: Determine clonal relationship between *Klebsiella pneumoniae* isolates from aquatic environment and clinical settings

Specific clones are often found to be responsible for outbreaks of infections and clonal dissemination of multidrug-resistant *K. pneumoniae* in the community [193]. Based on results obtained so far, it was intriguing to know if there existed any clonal relationship amongst the environmental and clinical isolates of *K. pneumoniae* under study.

3.3.1 Phylogenetic analysis based on 16S rRNA gene

All of the twenty seven isolates of *Klebsiella pneumoniae* (13 environmental and 14 clinical isolates) were subjected to multi-locus sequence typing (MLST) analysis and 16S rRNA sequences were used for phylogenetic analysis.

1167 nucleotides of 16S rRNA gene sequence contain an average 19.7% T, 22.9% C, 25.4% A and 32.0% G. Analysis of 16S rRNA sequence grouped these isolates in to four clusters with bootstrap value ≥50% as shown in **Fig 3.3.1**. Cluster 1- the largest group contained fourteen isolates, seven each clinical and environmental. The clinical isolates included both *K. pneumoniae* isolated from Jamshedpur (CSK1, CSK3, CSK5 and CSK7) as well as Bhubaneswar (KpAH1, KpAH2 and KpAH5). Within cluster-I, majority of *K. pneumoniae* isolates from Hudco dam were grouped. Additionally, one isolate (DL4.1) from Dimna lake and two isolates KR3.4 and KR3.14 also grouped. Cluster II contained six isolates, of which five were clinical (CSK2, CSK6, KpAH4, KpAH9 and KpAH11) and one (HD4.1) was an environmental isolate. Cluster III contained two clinical isolates (CSK4 and KpAH7) whereas cluster IV contained five environmental isolates from Subarnarekha River (SR1.6 and SR5.4) and Dimna Lake (DL2.1, DL2.3and DL5.4).

Thus, based on 16S rRNA sequencing it could be observed that there existed significant amount of phylogenetic similarity between environmental and clinical isolates of *K. pneumoniae*. It is also worth mentioning that clinical isolates from Bhubaneswar shared clonal relationship with those from Jamshedpur. This provides circumstantial evidence for spread of clones in this geographic region.

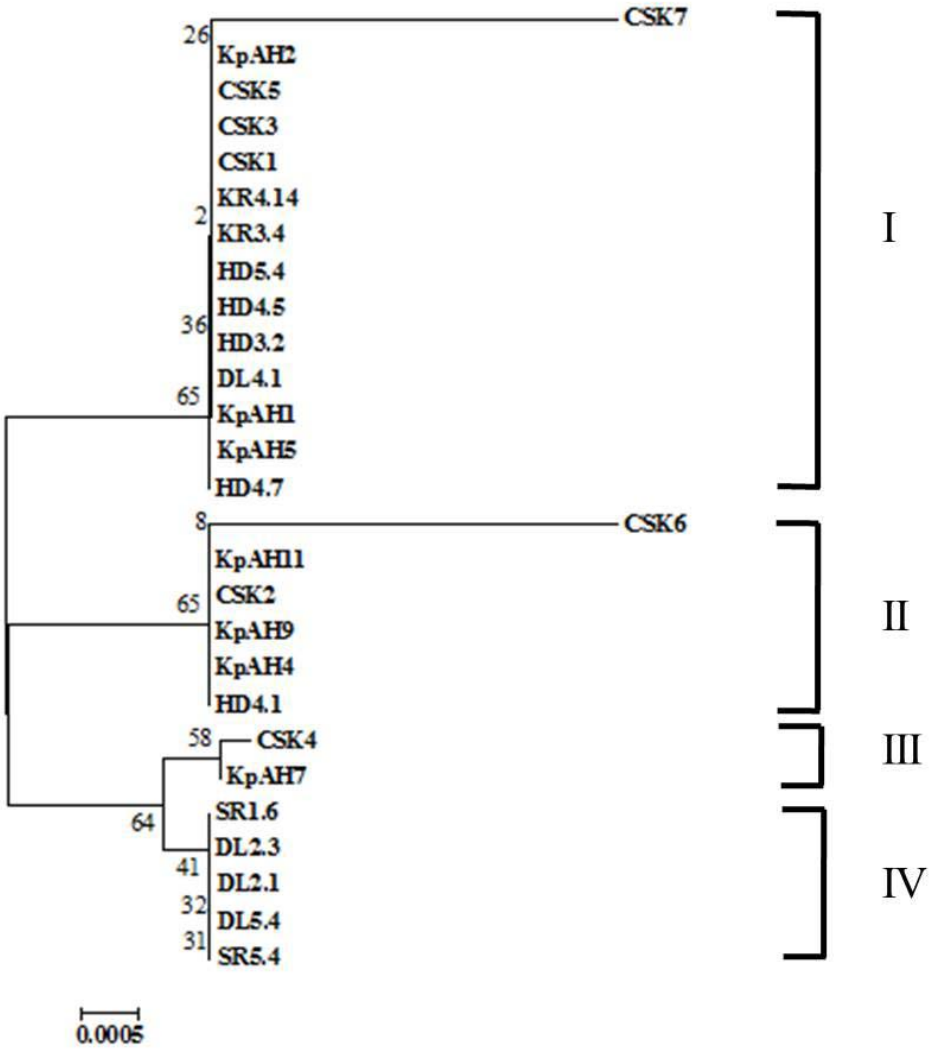


Figure 3.3.1: Phylogenetic tree of *K. pneumoniae* using 16S rRNA gene sequences

Unrooted tree constructed by using 16S rRNA gene sequences by neighbour-joining method, showing the phylogenetic relationships of *K. pneumoniae.* The numbers indicate the percentage occurrence in bootstrapped trees. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets.

3.3.2 Determination of Multi locus Sequence Type (MLST) of isolates under study

The results of 16S rRNA phylogenetic analyses further enthused us to determine the sequence type of these isolates. So we performed Multi locus sequence type analysis. PCR amplified products of seven housekeeping genes were obtained for all strains using respective primers (**Table 2.20 and Fig. 3.3.2**). Sequences of housekeeping genes of each isolate were analyzed for allelic variation using multi locus query of *Klebsiella pneumoniae* –MLST Pasteur Web Site. Multi locus sequence typing revealed diverse sequence types among the isolates (**Table 3.3.1**). *Klebsiella pneumoniae* DL4.1 belonged to ST337, HD4.5 belonged to ST200, HD4.7 belonged to ST 1296, CSK1 belonged to ST1310, CSK2 belonged toST14, CSK3 belonged toST721, CSK4 belonged to ST307, KpAH1 belonged to ST 327 and KpAH7 belonged to ST 231. Three isolates HD3.2, HD4.1 and HD5.4 have new allelic variations which have not been reported for any sequence type whereas sequence type for other isolates could not be determined as they contained variants for loci that have not been reported so far (**table 3.3.1**). The allelic variation showed that HD3.2 was a single locus variant (SLV) of ST200 on *pgi*, HD4.1 was a single locus variant of ST105, ST152 and ST1110 on *ton*B whereas HD5.4 was single locus variant of ST1471 on *ton*B.

3.3.3 Phylogenetic analysis using individual and concatenated housekeeping gene sequences

Since most of the isolates showed allelic variations, so we analyzed phylogenetic relatedness between the isolates utilizing individual gene and concatenated sequences of housekeeping genes. The sequence similarity for each gene showed that *pho*E gene had the highest level of sequence polymorphism (79% similarity), followed by *mdh* gene (81%). The GC content for individual genes exhibited very little variation between the genes (**Table 3.3.2**). Estimation of synonymous and non-synonymous substitution rates is important in understanding

the dynamics of molecular sequence evolution as it provides a powerful tool for understanding the mechanisms and driving forces of molecular evolution.

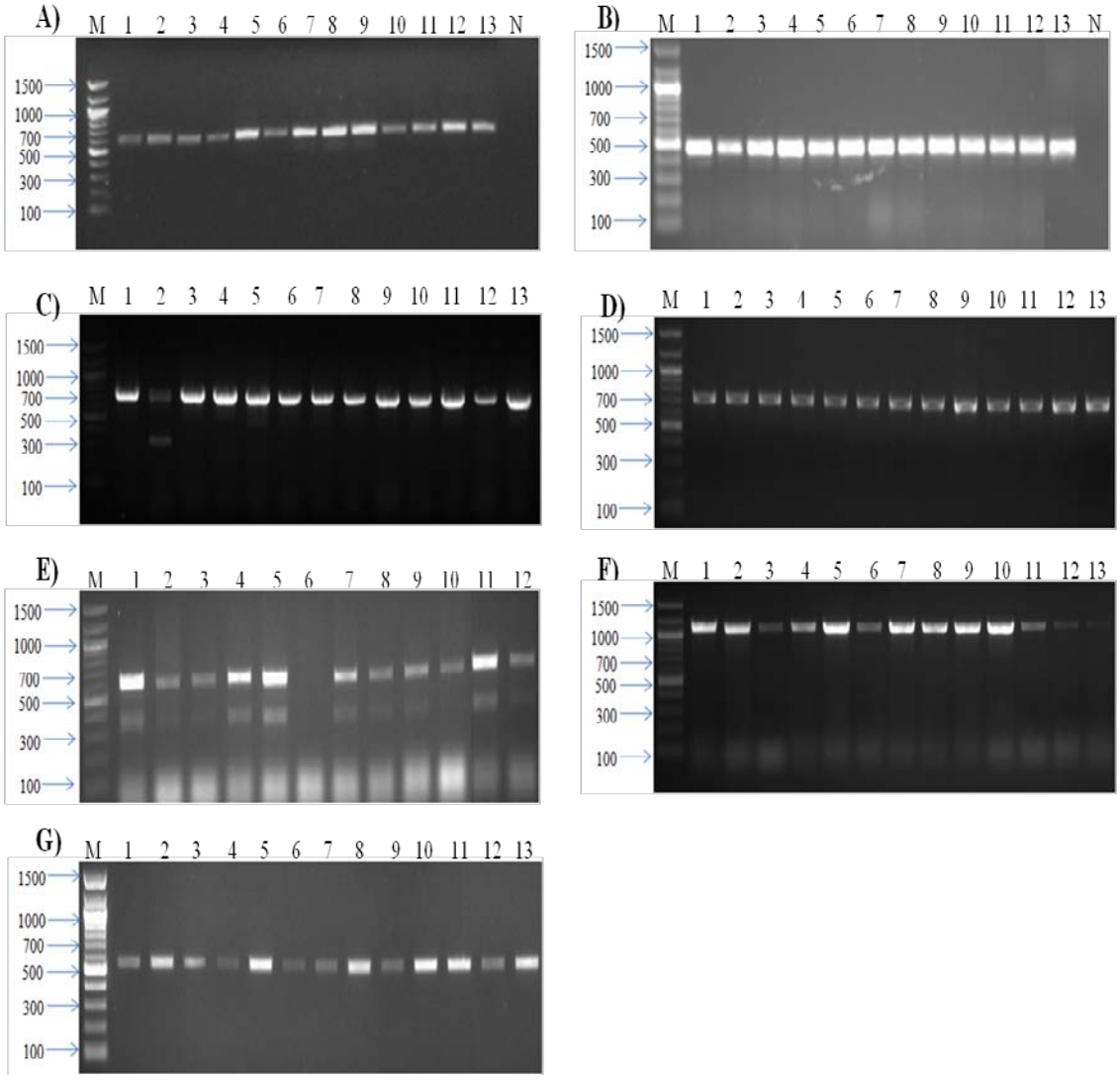


Figure 3.3.2: PCR amplification of seven housekeeping genes of environmental *K. pneumoniae* isolates.

Lane 1 to 13 show positive products of the (A) *gap*A (663bp), (B) *inf*B (450bp), (C) *mdh* (740bp), (D) *pgi* (690bp), (E) *pho*E (600bp), (F) *rpo*B (1100bp) and (G) *ton*B (540bp) gene for isolates DL2.1, DL2.3, DL4.1, DL5.4, HD3.2, HD4.5, HD4.7, HD5.4, KR3.4, KR3.14, SR1.6 and SR5.4. M: DNA ladder (NEB, size as indicated) and N: negative control (without template). Similar results were obtained from clinical *K. pneumoniae* isolates.

Table 3.3.1 MLST and allelic profile of housekeeping genes from environmental and clinical isolates of *Klebsiella pneumoniae*

Isolates ID	*gap*A	*inf*B	*mdh*	*pgi*	*pho*E	*rpo*B	*ton*B	Sequence Type
DL2.1	18	22	26	63* (98.84%)	107	20	87* (99.76%)	Un-ST
DL2.3	2	63* (91.19%)	115* (84.28%)	8* (97.45%)	39* (82.38%)	81* (97.01%)	51	Un-ST
DL4.1	2	1	11	1	1	1	13	ST-337
DL5.4	88	22	63	22	107	38	1* (99.76%)	Un-ST
HD3.2	2	1	2	22	12	1	68	Unreported
HD4.1	2	3	2	1	1	4	1	Unreported
HD4.5	2	1	2	1	12	1	68	ST-200
HD4.7	4	1	2	1	7	70	12	ST-1296
HD5.4	10	1	1	1	9	4	24	Unreported
KR3.4	2	1	99	1* (99.77%)	9	1	129	Un-ST
KR4.14	2	1	65	1	10* (99.76%)	4	9	Un-ST
SR1.6	18	22	18	63* (99.31%)	11	92* (99.80%)	1* (99.76%)	Un-ST
SR5.4	48	6	18	59* (99.77%)	92	13	51	Un-ST

CSK1	14	1	2	1	21	1	1	ST-1310
CSK2	1	6	1	1	1	1	1	ST-14
CSK3	2	1	11	1	9	8	43	ST-721
CSK4	4	1	2	52	1	1	7	ST-307
CSK5	1	1	1	85* (99.54%)	10	1	15	Un-ST
CSK6	10	6	1	1* (99.31%)	1	1	1	Un-ST
CSK7								Un-ST
KpAH1	10	6	1	1	1	1	1	ST-327
KpAH2	18	15	56	22	171* (99.76%)	94	51	Un-ST
KpAH4	1	1	56* (98.32%)	22	1	1	1	Un-ST
KpAH5	18	15	1* (99.79%)	8* (91.44%)	171* (99.76%)	38* (99.20%)	51	Un-ST
KpAH7	2	6	1	3	26	1	77	ST-231
KpAH9	18* (99.56%)	6	1	22* (99.54%)	171* (99.76%)	94	6	Un-ST
KpAH11	2	1	1* (98.74%)	3	10	1	19	Un-ST

Un-ST- unknown sequence type, “*”-Nearest allele match with percentage in bracket

The ratio dN/dS expected to exceed unity only if natural selection promotes changes in the protein sequence. Low dN/dS ratios (<1) in genes are considered to be evolving under strong functional constraints.

Table 3.3.2 Phylogenetic analysis of 27 (environmental and clinical) *Klebsiella pneumoniae* isolates

Locus	Strains analyzed (n)	Nucleotides (%)				dN/dS	Frequency T/C/A/G (%)
		Size of analyzed fragment (bp)	CN	VN	PIN		
*rpo*B	27	501	463 (92%)	38 (8%)	26 (5%)	0.03	22.5/27.9/22.6/27.0
mdh	27	477	386 (81%)	91 (19%)	22 (5%)	0.02	20.7/27.0/22.6/29.7
pgi	27	432	374 (87%)	58 (13%)	27 (6%)	0.01	19.2/31.8/22.4/26.6
phoE	27	420	330 (79%)	90 (21%)	22 (5%)	0.04	19.5/28.3/25.0/27.2
*gap*A	27	450	438 (97%)	12 (3%)	8 (2%)	0.07	19.6/31.2/24.2/25.0
infB	27	318	274 (86%)	44 (14%)	24 (78%)	0.03	17.0/28.6/20.8/33.6
*ton*B	26	414	367 (89%)	47 (11%)	41 (10%)	0.06	12.4/33.1/22.8/31.8
Concatenated genes	27	3012	2557 (85%)	455 (15%)	175 (6%)		18.9/29.7/23.0/28.4

CN- Conserved nucleotide, VN- Variable nucleotide, PIN- Parsimony-informative nucleotide

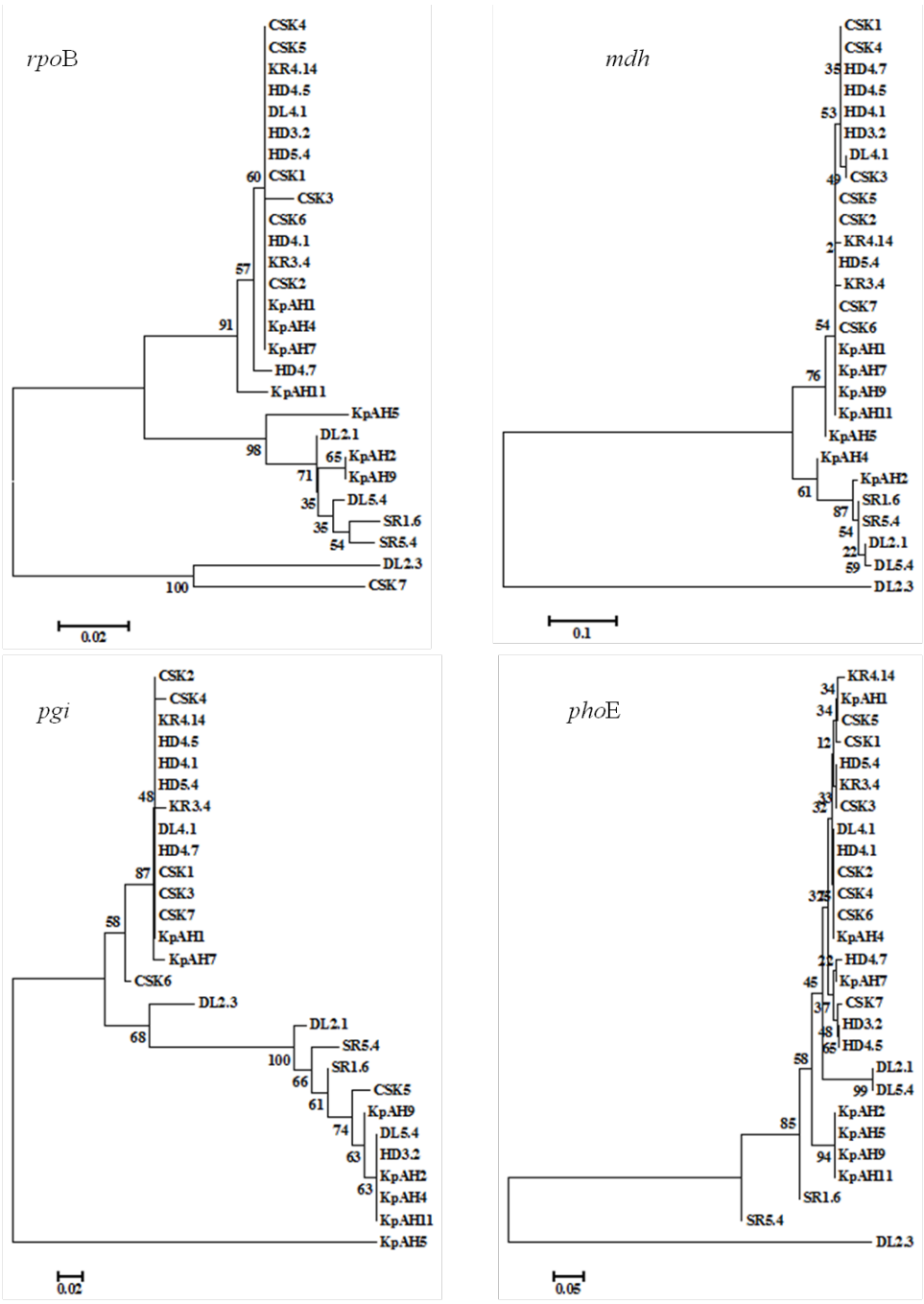
dN/dS, ratio of the average of non-synonymous substitutions (dN) to the average of synonymous substitutions (dS) calculated

*ton*B gene of CSK7 was excluded due to deletion present in the gene

A single-gene tree was built from the sequences for each of the seven genes (**Fig. 3.3.3**). CSK7 and DL2.3 grouped in one distinct branch in *inf*B, and *rpo*B trees. The trees built using *gap*A, *inf*B, *mdh*, *rpo*B and *ton*B sequences grouped DL2.1, DL4.1, SR1.6, SR5.4, KpAH2, KpAH5 and KpAH9 in one branch.

The concatenated sequence with 3,012bp had 2,731 (90.65%) conserved, 281 (9.32%) variable and 135 (4.48%) parsimony informative sites in environmental *K. pneumoniae* isolates whereas 2,731 (90.65%) conserved, 281 (9.32%) variable and 135 (4.48%) parsimony informative sites in clinical *K. pneumoniae* isolates (**Table 3.3.2**). Phylogenetic analysis using concatenated sequences of seven housekeeping genes exhibited formation of two branches (I and II) as shown in **Fig 3.3.4**. In branch one, it form two clades with bootstrap value 99%. Isolate KpAH7 belonging to ST231 formed a distinct out-group, indicating its diversification from rest of the clade members whereas low bootstrap values at the other nodes indicated less divergence and a possible common ancestral origin for most of the isolates. In branch-II, CSK7 and DL2.3 grouped similar to *inf*B, and *rpo*B individual trees.

Thus based on phylogenetic analysis, it could be observed that there existed significant amount of similarity between environmental and clinical isolates of *K. pneumoniae*. It is also worth mentioning that clinical isolates from Bhubaneswar shared clonal relationship with those from Jamshedpur. This provides circumstantial evidence for spread of clones in the geographic region.


rpoB
CSK4
CSK5
KR4.14
HD4.5
DL4.1
HD3.2
HD5.4
CSK1
CSK3
CSK6
HD4.1
KR3.4
CSK2
KpAH1
KpAH4
KpAH7
HD4.7
KpAH11
KpAH5
DL2.1
KpAH2
KpAH9
DL5.4
SR1.6
SR5.4
DL2.3
CSK7
0.02
mdh
CSK1
CSK4
HD4.7
HD4.5
HD4.1
HD3.2
DL4.1
CSK3
CSK5
CSK2
KR4.14
HD5.4
KR3.4
CSK7
CSK6
KpAH1
KpAH7
KpAH9
KpAH11
KpAH5
KpAH4
KpAH2
SR1.6
SR5.4
DL2.1
DL5.4
DL2.3
0.1
pgi
CSK2
CSK4
KR4.14
HD4.5
HD4.1
HD5.4
KR3.4
DL4.1
HD4.7
CSK1
CSK3
CSK7
KpAH1
KpAH7
CSK6
DL2.3
DL2.1
SR5.4
SR1.6
CSK5
KpAH9
DL5.4
HD3.2
KpAH2
KpAH4
KpAH11
KpAH5
0.02
phoE
KR4.14
KpAH1
CSK5
CSK1
HD5.4
KR3.4
CSK3
DL4.1
HD4.1
CSK2
CSK4
CSK6
KpAH4
HD4.7
KpAH7
CSK7
HD3.2
HD4.5
DL2.1
DL5.4
KpAH2
KpAH5
KpAH9
KpAH11
SR1.6
SR5.4
DL2.3
0.05

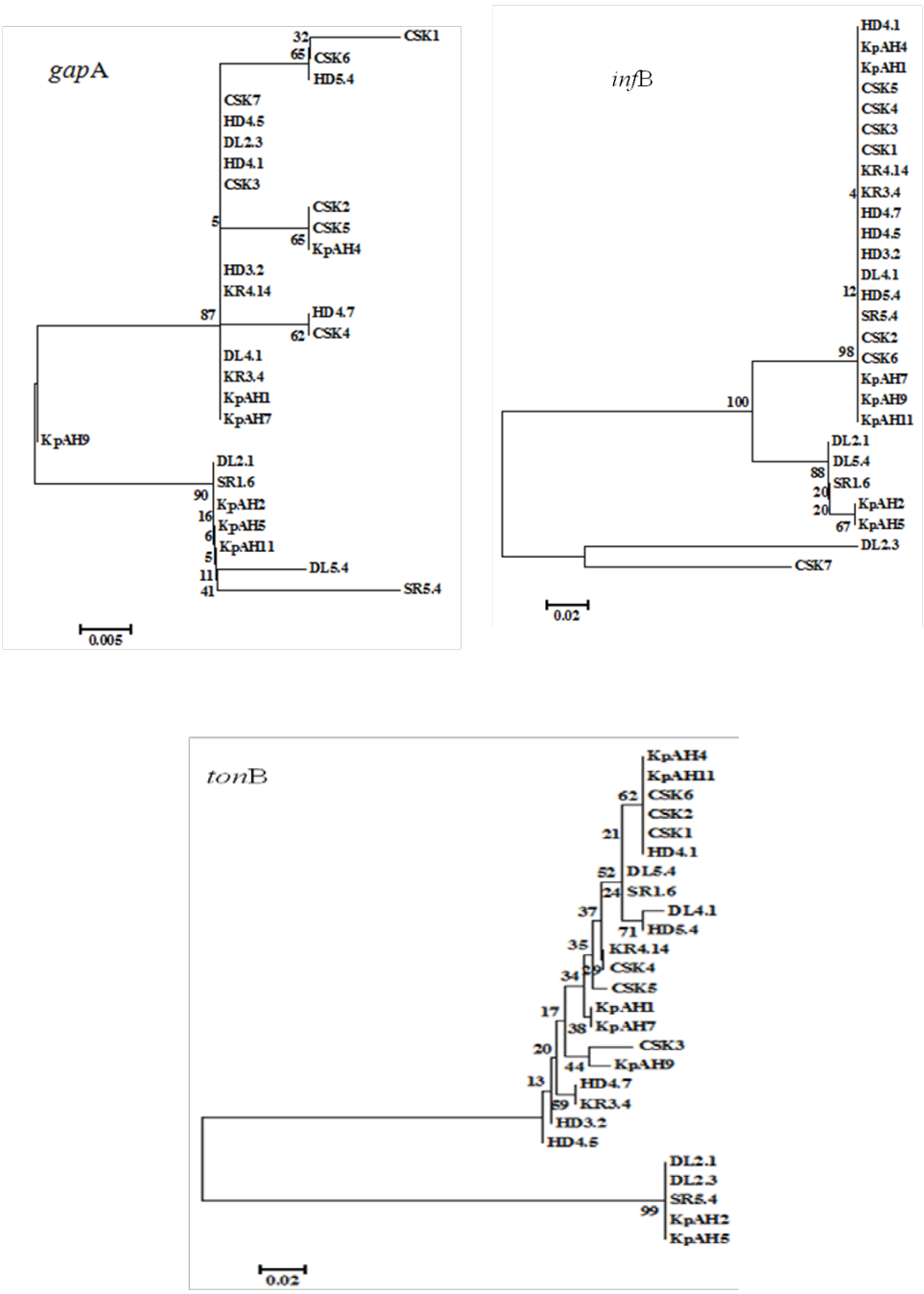


Figure 3.3.3: Phylogenetic tree of twenty seven *Klebsiella pneumoniae* based on individual house-keeping genes.

The trees for all studied strains (n = 27) were generated using the neighbor-joining method. Bootstrap support values (%) at each of the nodes are indicated at each node.

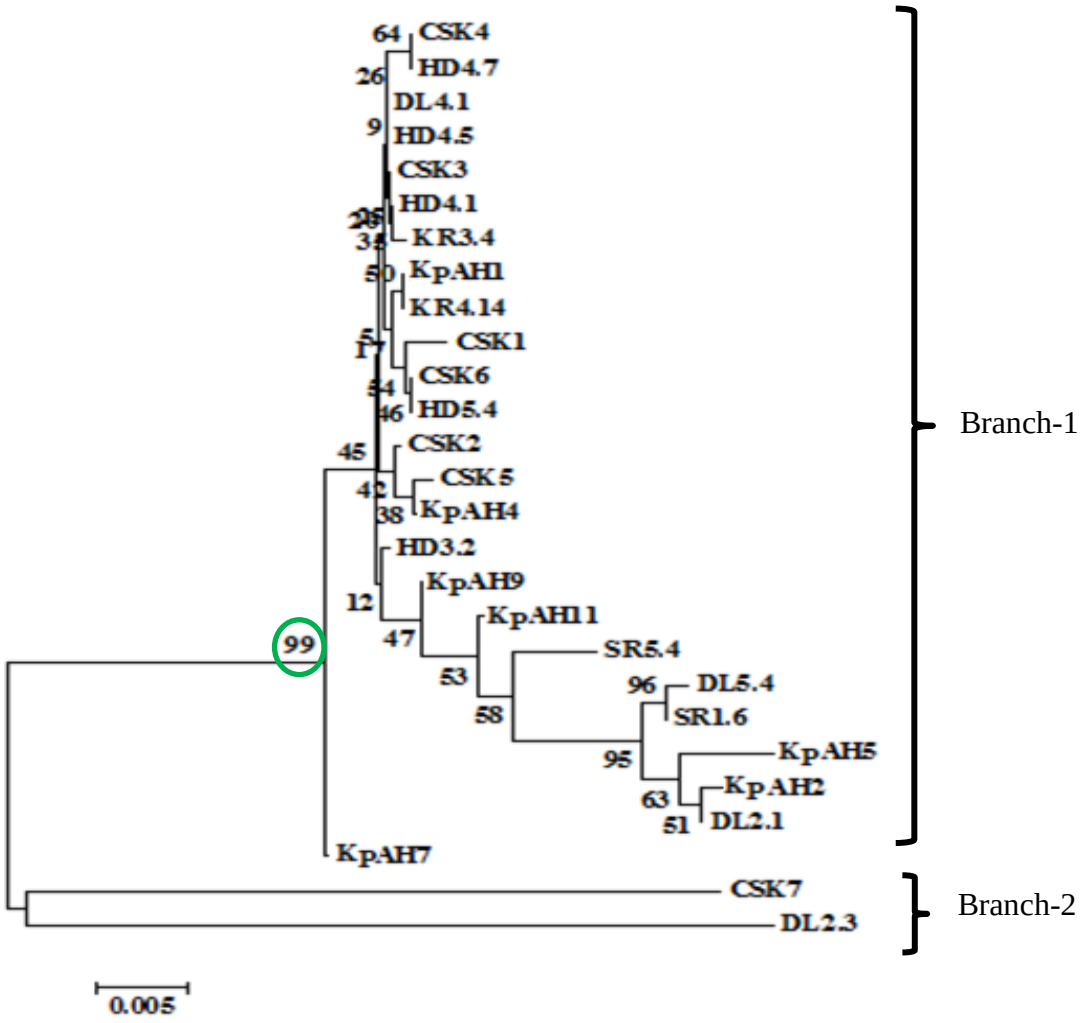


Figure 3.3.4: Phylogenetic tree of twenty seven *Klebsiella pneumoniae* computed from the concatenation sequences of seven house-keeping genes.

Concatenation sequences of seven house-keeping genes in the order of *gap*A, *inf*B, *mdh*, *pgi*, *pho*E, *rpo*B and *ton*B. Phylogenetic tree was prepared using the neighbour-joining (Nei-Gojobori method) method with 1000 boot strap. Construction and bootstrapping was carried out using MEGA 6 suite programs.

3.3.4 Discussion

Klebsiella pneumoniae has emerged as an important nosocomial pathogen responsible for hospital-acquired and long-term care–associated infections with high morbidity and mortality [456,457]. Identifying the clonal nature of strains can helps us to obtain information about the source of infection, trace their dissemination route of infection and to understand their epidemiology thereby assisting in halting a current outbreak or providing knowledge on the spread of an infection [168].

As a consequence of its low evolutionary rate, 16S rRNA sequencing has failed to distinguish many closely related but ecologically distinct groups of bacteria [458]. Due to their higher rate of neutral mutation, protein-coding genes are often more suitable for relatedness determinations at or below the species level. Multi locus sequence typing (MLST) is most useful method developed for typing of many bacterial species. There are 2269 sequence types of *K. pneumoniae* reported worldwide till June 2016 (http://pubmlst.org/databases.shtml). Among them, 39.70% (901 STs) different sequence types have been reported from Asian countries (http://pubmlst.org/databases.shtml). Among them, most frequently encountered sequence types (STs) were ST11 (10.32%), ST23 (6.77%), ST15 (1.55%), ST147 (1.44%), ST17 (1.44%), ST592 (1.22%) and ST45 (1.10%). From India, so far only 1.45% (33 STs) different sequence types had been reported (http://pubmlst.org/databases.shtml).

Multi locus sequence typing in the present study revealed nine different known sequence types belonging to ST337, ST200, ST1296, ST14, ST231, ST307, ST327, ST721 and ST1310 indicating spread of these clones in the geographic region of this study. *K. pneumoniae* ST337 was reported from Colombia carried $bla_{\text{KPC-2}}$ [182]. *K. pneumoniae* ST337 was also reported from skin of cat carrying ESBL genes like CTX-M-14, *SHV*-11 as well as plasmid mediated quinolone resistance genes *qnr*B, *qnr*S, aac(6) -Ib-cr, *oqx*AB from Japan [459]. In contrast

environmental isolates of *K. pneumoniae* ST337 in the present study, was resistance to ampicillin, piperacillin whereas intermediated resistance toward cefuroxime, ceftazidime and carried only SHV-34 gene. It showed hypermucoviscous phenotype, exhibited intermediate serum resistance and was positive for type1 fimbriae.

K. pneumoniae ST200 was reported from human infected with urinary tract infection from Brazil (http://pubmlst.org/databases.shtml). *K. pneumoniae* ST200 also reported from Taiwanese University Hospital too had typeA *omp*36 and ESBL gene [460]. In the present study, *K. pneumoniae* isolate HD4.5 with ST 200 exhibited intermediate serum resistance and was positive for type1 fimbriae. Apart from being resistant to cephalosporin, this isolates showed resistance toward last resort antibiotic colistin. In addition, two more isolates HD4.7 with ST1296 and HD3.2 (a single locus variant (SLV) of ST200 on *pgi*) had similar resistance and virulence profile, indicating emergence of clone in the aquatic environment. *K. pneumoniae* ST14 was earlier reported to be a frequent host of NDM-1, KPC and CTX-M enzymes from India and the United States [199,204]. Isolate CSK2 with ST14 in our study carried only $bla_{\text{SHV-1}}$ gene. Isolate KpAH7 with ST231 co-harbored multiple β-lactamase genes ($bla_{\text{SHV-34}}$, $bla_{\text{TEM-1}}$, $bla_{\text{NDM-1,}}$ *Amp*C) along with *aacA2*, *qnrB* and *dfrA12* genes. *K. pneumoniae* ST231 was first reported in 2011 from India and subsequently have been reported from Spain, Canada, Mauritius and Singapore with similar resistance genes. This indicates the global dissemination of *K. pneumoniae* ST231 [461,462,463,464]. *K. pneumoniae* isolate belonging to ST307 from Chennai, India contained *bla*NDM-1 and that reported from Brazil contained KPC-enzyme [204,465], but in the present study isolate CSK4 with ST307 carried $bla_{\text{SHV-34,}}$ $bla_{\text{TEM-1,}}$ bla_{OXA} genes. *K. pneumoniae* ST307 had been reported to be KPC-producing clones in USA [466] and Europe [465,467] but in the present study, isolate CSK4 with ST307 carried $bla_{\text{OXA-1}}$ instead of

KPC gene. ST 327 was reported from Spain and Israel [468,469] as hosts of only ESBLs and carbapenemase. Isolate KpAH1 with ST327 carried $bla_{\text{SHV-34}}$, $bla_{\text{TEM-1}}$ and $bla_{\text{NDM-1}}$ genes and showed resistance towards cephalosporin but susceptible to carbapenems. *K. pneumoniae* ST1310 was earlier reported from Vietnam and France whereas ST721 was reported from Vietnam and China (http://pubmlst.org/databases.shtml). In the present study, isolates CSK1 with ST1310 resistance to cephalosporins, quinolones and aminoglycosides and isolate CSK3 with ST721 resistance to cephalosporins and aminoglycosides carried $bla_{\text{SHV-34}}$ gene. Isolate CSK3 carried both Class 1 and Class 2 integron. This study is the first to report the resistance profile and resistance determinants of *K. pneumoniae* ST1310 and ST721.

Despite the isolates belonging to different sequence types, phylogenetic analysis of concatenated sequences showed that beside KpAH7, CSK7 and DL2.3, all other environmental and clinical isolates of *K. pneumoniae* were grouped into single cluster with low bootstrap values at the nodes indicating less divergence and a possible common ancestral origin for most of the isolates.

Chapter-4
Summary & Conclusions

The present investigation addresses two issues that were relevant in context of studies done on multidrug resistant environmental isolates. First, by utilizing an integrated approach of association analysis between multidrug resistance and virulence potential identified the extent of threat posed by the population as well as individual genera. Secondly, the comparative analysis of resistance mechanism between environmental and clinical isolates of *Klebsiella pneumoniae* showed that the later harbored multiple mechanisms as compared to environmental isolates. Subsequent phylogenetic analysis of these isolates exhibited less divergence signifying possible common ancestral origin. Thus, the salient features of the investigation can be listed as follows:

1. In the present study on basis of MAR indices, the propensity of exhibiting multiple drug resistant phenotype among the genera ranked in the order *Pseudomonas* >*Klebsiella* = *Acinetobacter* >*Proteus* >*Aeromonas* >*Enterobacter*.
2. It could be concluded that *Pseudomonas* isolates from the present environment posed highest threat as potential pathogens followed by *Klebsiella pneumoniae*.
3. Association analysis of MAR index and virulence factors in multidrug resistant isolates can aid in identification of potential high risk pathogenic populations/isolates.
4. All environmental and clinical isolates of *Klebsiella pneumoniae* exhibited multidrug resistant phenotype, however the average MAR index of clinical isolates was much higher (0.7) compared to environmental isolates (0.3).
5. Screening for resistance genes and mechanisms in *Klebsiella pneumoniae* isolates indicated wide spread diversity. bla_{SHV} was detected in majority of clinical (n=12) and environmental (n=8) isolates.
6. Environmental isolate of *Klebsiella pneumoniae* DL5.4 exhibited persister cell formation as a mechanism to overcome drug toxicity.

7. Hypermucoviscous phenotype was associated with three environmental *Klebsiella pneumoniae* isolates but none of clinical isolates.
8. Four environmental isolates exhibited efflux pump mediated colistin resistance.
9. Multi locus sequence typing in the present study revealed nine different known sequence types belonging to ST337, ST200, ST1296, ST14, ST231, ST307, ST327, ST721 and ST1310 indicating spread of these clones in the geographic region of this study.
10. Phylogenetic analysis of concatenated sequences showed that except three isolates namely, KpAH7, CSK7 and DL2.3, all other environmental and clinical isolates of *K. pneumoniae* grouped into single cluster with low bootstrap values; indicating less divergence and a possible common ancestral origin for most of the isolates.

The present study utilized low cost, established protocols which can easily be adapted by laboratories in developing nations. Such a study helps monitor environmental health risks associated with multidrug resistant isolates circulating in the environment. My study was on a pilot scale using less number of isolates for analysis. However, it provides a roadmap for easy scale up with a broader and deeper level of analysis. In other words, subsequent studies can be formulated either focusing on specific bacterial genus or increasing the number of isolates, which will provide a more robust association between the traits. Also, in such studies, investigations can be extended beyond phenotypic virulence to genetic basis. The above mentioned approach would help address the clonal nature of clinical and the environmental isolates, facilitating track source of origin.

Chapter-5
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