POLYPHASIC APPROACH TO UNDERSTAND THE DISTRIBUTION AND DYNAMICS OF TEM β-LACATAMASE IN ENVIRONMENTAL BACTERIAL POPULATIONS

by

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INDIAN INSTITUTE OF TECHNOLOGY DELHI

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CERTIFICATE

This is to certify that the thesis **entitled "*Polyphasic approach to understand the distribution and dynamics of TEM β-Lactamase in environmental bacterial populations*"** being submitted by **Ms. Priyanka Gehlot** to the Indian Institute of Technology Delhi for the award of **"Doctor of Philosophy"** is a record of bonafide research work carried out by her. She has worked under my guidance and supervision and has fulfilled the requirements for submission of this thesis. To the best of my knowledge, the results contained in this thesis have not been submitted in part or full to any other university or institute for the award of any degree or diploma**.**

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"The roots of all goodness lie in the soil of appreciation for goodness." – Dalai Lama

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ABSTRACT

Antimicrobial resistance (AMR) is a global health threat, driven by multidrug-resistant (MDR) bacteria, including strains resistant to key drugs like carbapenems and colistin. Antibiotics have improved life expectancy and food security while their overuse in medicine, agriculture, and aquaculture practices has accelerated bacterial resistance in various environmental spheres across the globe. The emergences of β-lactam resistant bacteria (BLRB) especially carbapenem-resistant *Enterobacteriaceae* (CRE) designated as an urgent priority pathogen posing significant threats by infiltrating into water systems and food chains. Therefore, understanding and constantly monitoring BLRB and their genomic evolution in response to antibiotics and inhibitors, particularly to detect the emergence of novel mutants that may pose serious threats to both human and animal health. This study is crucial as India (largest antibiotic consumer) faces significant AMR risks, particularly in region with higher population density like Delhi. This research is the first comprehensive analysis of the environmental risks posed by BLRB along with their resistant genes in Delhi-National Capital Region (Delhi-NCR).

Among the various classes of β-lactamase enzymes, TEM-type β-lactamases are the most widely distributed and exhibit a high mutability rate, making them key contributors to the development of extended-spectrum and inhibitor-resistant β-lactamase variants that mediate bacterial resistance to β-lactam antibiotics. There are almost 474 variants of Temoneria (TEM) β-lactamase (BLs) identified in Lactamase Engineering Database (LacED). The first aim was to understand dynamics and distribution of TEM type β-Lactamase through Computational and Data Mining approach. The computational study results demonstrated that most TEM mutants recorded the least binding energy to penicillin and cephalosporin class of antibiotics and higher binding energy to carbapenem and monobactam class of antibiotics. Among the BLs inhibitors, tazobactam recorded the least binding energy against most of the TEM mutants, indicating that it can lower the catalytic activity of TEM BLs, thereby potentiating antibiotic action. Through the data mining approach TEM mutant database was generated, highlighting TEM mutations, bacterial diversity, Michaelis-Menten Constant's (Km), Minimum inhibitory concentration (MIC), and Inhibitor resistant TEM (IRT) types. It has been noted that earlier released antibiotics like amoxicillin and ampicillin had lower Km and higher MIC values, which indicates the prevalence of bacterial resistance. By analysing the differential binding energy (ΔBE) of the selected TEM mutants against β-lactam and BLs inhibitors, the most effective combination of β-lactam (carbapenem and monobactam class of antibiotics) and BLs inhibitors

(tazobactam) was identified, to cure bacterial diseases/infections and to prevent similar antibiotic resistance outbreaks in near future.

After understanding the dynamics and distributions of already reported TEM mutants, the second aim was to assess the prevalence and resistance characteristics of BLRB and β-lactamase resistant bacterial genes (BLRBG) under various environmental conditions within Delhi NCR, India. Using a culture-dependent method, isolated 130 BLRB from 75 different environmental samples, including lakes, ponds, the Yamuna River, agricultural soil, aquatic weeds, drains, dumping yards, sewage treatment plants (STPs) and gaushalas. Antibiotic susceptibility, phenotypic, and genotypic tests for β-lactamases and integron genes were conducted. Water and sediment samples of waterbodies was found to be substantial reservoir of BLRB and BLRBGs. The majority of the BLRB discovered are opportunistic pathogens from the *Bacillus, Aeromonas, Pseudomonas, Enterobacter, Escherichia* and *Klebsiella* genera, with Multiple Antibiotic Resistance (MAR) index ≥ 0.2 against a wide variety of β-lactams and β-lactamase inhibitor combinations. Interestingly, we discovered that 10 isolates of various origins produce both Extended Spectrum BLs and Metallo BLs, as well as found harbouring *blaTEM, blaCTX, blaOXA, blaSHV, int-1* and *int-3* genes.

Further, the third objective aimed to identify novel TEM mutant from isolated environmental BLRBs. For this 6 BLRBs were selected from the 10 BLRBs which has shown the presence of blaTEM gene in our second objective work. The minimum inhibitory concentration against the shortlisted 6 BLRBs showed that *Escherichia ruysiae, Pseudomonas aeruginosa* and *Bacillus* sp., have high MIC to the most of antibiotics and inhibitors combinations, indicating their highly resistance phenotype. To explore TEM mutant diversity, blunt end cloning, plasmid isolation and sequencing of the blaTEM gene was performed and found to harbour different TEM BLs mutants such TEM-142, 10, 22, 205 and novel TEM mutant, TEM(N) with multiple amino acid mutations at Met 55 Leu, Trp 151 Gly, Asn 255 Thr and Arg 256 Leu. Identified the TEM(N) mutant from *Escherichia ruysiae* isolated from the Sewage treatment plant of Okhla, New Delhi. The 3D structure of the novel TEM mutant was generated by Swiss protein modeller. Further, Molecular Dynamics (MD) Simulations were performed on the wildtype TEM-1 and TEM(N) to analysis the effect and interaction of mutating residues with the β-lactams and BLs inhibitors. *In- vitro* and MD analysis suggests that TEM(N) mutant resist to penicillin, cephalosporin and carbapenem classes, as well as to β-lactamase inhibitors like avibactam, tazobactam and relebactam, posing a significant public and environmental health threat. Our

study emphasizes the importance of surveillance which will monitor the emergence of novel BLRB and BLRG and will aid in predicting epidemics and devising appropriate preventive measures.

सार

रोगाणुरोधी प्रतिरोध (एएमआर) एक वैश्विक स्वास्थ्य खतरा है, जो बहुऔषधि प्रतिरोधी (एमडीआर) बैक्टीरिया द्वारा संचालित होता है, जिसमें कार्बापेनम और कोलिस्टिन जैसी प्रमुख दवाओं के प्रतिरोधी उपभेद शामिल हैं। एंटीबायोटिक्स ने जीवन प्रत्याशा और खाद्य सुरक्षा में सुधार किया है, जबकि चिकित्सा, कृषि और जलीय कृषि प्रथाओं में उनके अत्यधिक उपयोग ने दुनिया भर में विभिन्न पर्यावरणीय क्षेत्रों में जीवाणु प्रतिरोध को तेज कर दिया है। बीटा-लैक्टम प्रतिरोधी बैक्टीरिया (बीएलआरबी) विशेष रूप से कार्बापेनम प्रतिरोधी एंटरोबैक्टीरिया (सीआरई) का उद्भव एक तत्काल प्राथमिकता वाले रोगज़नक़ के रूप में नामित किया गया है जो जल प्रणालियों और खाद्य श्रृंखलाओं में घुसपैठ करके महत्वपूर्ण खतरे पैदा कर रहा है। इसलिए, एंटीबायोटिक्स और अवरोधकों के जवाब में बीएलआरबी और उनके जीनोमिक विकास को समझना और लगातार निगरानी करना, विशेष रूप से नए म्यूटेंट के उद्भव का पता लगाना जो मानव और पशु स्वास्थ्य दोनों के लिए गंभीर खतरे पैदा कर सकते हैं। यह अध्ययन महत्वपूर्ण है क्योंकि भारत (सबसे बड़ा एंटीबायोटिक उपभोक्ता) महत्वपूर्ण एएमआर जोखिमों का सामना करता है, खासकर दिल्ली जैसे उच्च जनसंख्या घनत्व वाले क्षेत्र में। यह शोध दिल्ली-राष्ट्रीय राजधानी क्षेत्र (दिल्ली-एनसीआर) में बीएलआरबी के साथ-साथ उनके प्रतिरोधी जीनों द्वारा उत्पन्न पर्यावरणीय जोखिमों का पहला व्यापक विश्लेषण है।

β -लैक्टम एंटीबायोटिक दवाओं के खिलाफ बैक्टीरिया का प्रतिरोध मुख्य रूप से टेमोनेरिया (टीईएम) β-लैक्टामेज (बीएल) के उत्पादन के माध्यम से होता है, जिसमें लैक्टामेज इंजीनियरिंग डेटाबेस (एलएसीईडी) में लगभग 474 वेरिएंट की पहचान की गई है। पहला उद्देश्य कम्प्यूटेशनल और डेटा माइनिंग दृष्टिकोण के माध्यम से टीईएम प्रकार β-लैक्टामेज की गतिशीलता और वितरण को समझना था। कम्प्यूटेशनल अध्ययन के परिणामों ने प्रदर्शित किया कि अधिकांश टीईएम म्यूटेंट ने पेनिसिलिन और सेफलोस्पोरिन वर्ग के एंटीबायोटिक दवाओं के लिए सबसे कम बंधन ऊर्जा और कार्बापेनम और मोनोबैक्टम वर्ग के एंटीबायोटिक दवाओं के लिए उच्च बंधन ऊर्जा दर्ज की डेटा माइनिंग दृष्टिकोण के माध्यम से TEM म्यूटेंट डेटाबेस तैयार किया गया, जिसमें TEM म्यूटेशन, बैक्टीरियल विविधता, माइकेलिस-मेन्टेन कॉन्स्टेंट (Km), न्यूनतम अवरोधक सांद्रता (MIC), और अवरोधक प्रतिरोधी TEM (IRT) प्रकारों पर प्रकाश डाला गया। यह देखा गया है कि पहले जारी किए गए एंटीबायोटिक्स जैसे एमोक्सिसिलिन और एम्पीसिलीन में Km कम और MIC मान अधिक थे, जो बैक्टीरिया प्रतिरोध की व्यापकता को दर्शाता है। β-लैक्टम और BLs अवरोधकों के खिलाफ चयनित TEM म्यूटेंट की अंतर बंधन ऊर्जा (ΔBE) का विश्लेषण करके, बैक्टीरियल रोगों/संक्रमणों को ठीक करने और निकट भविष्य में इसी तरह के एंटीबायोटिक प्रतिरोध प्रकोपों को रोकने के लिए β-लैक्टम (कार्बापेनम और

मोनोबैक्टम एंटीबायोटिक्स का वर्ग) और BLs अवरोधकों (टैज़ोबैक्टम) का सबसे प्रभावी संयोजन पहचाना गया। पहले से रिपोर्ट किए गए TEM म्यूटेंट की गतिशीलता और वितरण को समझने के बाद, दूसरा उद्देश्य भारत के दिल्ली एनसीआर में विभिन्न पर्यावरणीय परिस्थितियों में BLRB और β-लैक्टामेस प्रतिरोधी जीवाणु जीन (BLRBG) की व्यापकता और प्रतिरोध विशेषताओं का आकलन करना था। संस्कृति-निर्भर विधि का उपयोग करते हुए, झीलों, तालाबों, यमुना नदी, कृषि मिट्टी, जलीय खरपतवार, नालों, डंपिंग यार्ड, सीवेज ट्रीटमेंट प्लांट (STP) और गौशालाओं सहित 75 विभिन्न पर्यावरणीय नमूनों से 130 BLRB को अलग किया गया। β-लैक्टामेस और इंटेग्रोन जीन के लिए एंटीबायोटिक संवेदनशीलता, फेनोटाइपिक और जीनोटाइपिक परीक्षण किए गए। जल निकायों के पानी और तलछट के नमूनों में BLRB और BLRBGs का पर्याप्त भंडार पाया गया। खोजे गए BLRB में से अधिकांश बैसिलस, एरोमोनस, स्यूडोमोनस, एंटरोबैक्टर, एस्चेरिचिया और क्लेबसिएला जेनेरा से अवसरवादी रोगजनक हैं, जिनमें कई प्रकार के β-लैक्टम और β-लैक्टामेज अवरोधक संयोजनों के खिलाफ मल्टीपल एंटीबायोटिक प्रतिरोध (MAR) सूचकांक ≥ 0.2 है। दिलचस्प बात यह है कि हमने पाया कि विभिन्न मूल के 10 आइसोलेट्स विस्तारित स्पेक्ट्रम BLs और मेटालो BLs दोनों का उत्पादन करते हैं, साथ ही blaTEM, blaCTX, blaOXA, blaSHV, int-1 और int-3 जीन को आश्रय देते हैं। इसके अलावा, तीसरे उद्देश्य का उद्देश्य पृथक पर्यावरणीय BLRBs से नए TEM उत्परिवर्ती की पहचान करना था। इसके लिए 10 BLRBs में से 6 BLRBs का चयन किया गया, जिसने हमारे दूसरे उद्देश्य कार्य में blaTEM जीन की उपस्थिति को दर्शाया है। शॉर्टलिस्ट किए गए 6 BLRBs के खिलाफ न्यूनतम निरोधात्मक सांद्रता ने दिखाया कि एस्चेरिचिया रुइसिया, स्यूडोमोनस एरुगिनोसा और बैसिलस एसपी. में अधिकांश एंटीबायोटिक्स और अवरोधक संयोजनों के लिए उच्च MIC है, जो उनके अत्यधिक प्रतिरोधी फेनोटाइप को दर्शाता है। TEM उत्परिवर्ती विविधता का पता लगाने के लिए, ब्लंट एंड क्लोनिंग, प्लास्मिड अलगाव और blaTEM जीन की अनुक्रमण किया गया और पाया गया कि इसमें विभिन्न TEM BLs उत्परिवर्ती जैसे TEM-142, 10, 22, 205 और नए TEM उत्परिवर्ती, TEM(N) में कई अमीनो एसिड उत्परिवर्तन हैं जो Met 55 Leu, Trp 151 Gly, Asn 255 Thr और Arg 256 Leu पर हैं। नई दिल्ली के ओखला के सीवेज उपचार संयंत्र से अलग किए गए Escherichia ruysiae से TEM(N) उत्परिवर्ती की पहचान की गई। स्विस प्रोटीन मॉडेलर द्वारा नए TEM उत्परिवर्ती की 3D संरचना तैयार की गई। इसके अलावा, β-लैक्टम और BLs अवरोधकों के साथ उत्परिवर्तित अवशेषों के प्रभाव और अंतःक्रिया का विश्लेषण करने के लिए वाइल्डटाइप TEM-1 और TEM(N) पर आणविक गतिशीलता (MD) सिमुलेशन किए गए। इन-विट्रो और एमडी विश्लेषण से पता चलता है कि टीईएम (एन) म्यूटेंट पेनिसिलिन, सेफलोस्पोरिन और कार्बापेनम वर्गों के साथ-साथ एविबैक्टम,

टैज़ोबैक्टम और रिलेबैक्टम जैसे बीटा-लैक्टामेज अवरोधकों का प्रतिरोध करता है, जो एक महत्वपूर्ण सार्वजनिक और पर्यावरणीय स्वास्थ्य खतरा पैदा करता है। हमारा अध्ययन निगरानी के महत्व पर जोर देता है जो नए बीएलआरबी और बीएलआरजी के उद्भव की निगरानी करेगा और महामारी की भविष्यवाणी करने और उचित निवारक उपाय तैयार करने में सहायता करेगा।

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Abbreviations	Description
AMR	Antimicrobial resistance
ARB	Antibiotic Resistant Bacteria
BLRB	β-Lactam Resistant Bacteria
BLRG	β-lactam resistant gene
MDR	Multidrug Resistant
PDB	Protein Data Bank
MAR Index	Multiple Antibiotic-Resistant Index
MIC	Minimum inhibitory concentration
BLs	β-lactamase
TEM BLs	Temoneria β-lactamase
Km	Michaelis-Menten Constant's
BE	Binding Energy
AAR	Amino Acid Residue
ESBL	Extended Spectrum β-lactamase
SBL	Serine β-lactamase
MBL	Metallo- β-lactamase
NCBI	National Center for Biotechnology
NCMR	National Center for Microbial Resources
BLAST	Basic Local Alignment Tool
nBLAST	Nucleotide Basic Local Alignment Tool
pBLAST	Protein Basic Local Alignment Tool
LACED	Lactamase Engineered Database
MD	Molecular dynamics
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation

SASA	Solvent Accessible Surface Area
Rg	Radius of Gyration
HBO	Hydrogen Bond Occupancy
HBN	Hydrogen Bond Number
MMPBSA	Molecular Mechanics Poisson-Boltzmann Surface Area
WHO	World Health Organisation
ECDC	European Center for Disease Control and Prevention
CLSI	The Clinical & Laboratory Standards Institute
EUCAST	The European Committee on Antimicrobial Susceptibility Testing