Characterizing the Co-Existence of Metallo-β-lactamase and Extended Spectrum Beta-Lactamase genes in *Klebsiella pneumoniae* isolates in community wastewater samples of Dhaka, Bangladesh.

Submitted By

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A thesis submitted to the Department of Mathematics and Natural Science in partial fulfillment of the requirements for the degree of B.Sc in Microbiology

Department of Mathematics and Natural Science

September, 2022

Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.

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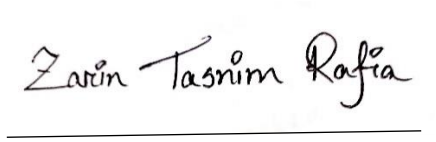

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Approval

The thesis/project titled "Characterizing the Co-Existence of Metallo-β-lactamase and Extended Spectrum Beta-Lactamase genes in *Klebsiella pneumoniae* isolates in community wastewater samples of Dhaka, Bangladesh" submitted by Zarin Tasnim Rafia, ID-18126002 of summer 2021 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of B.sc in Microbiology on 29th September, 2022.

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ABSTRACT

Background: The co-existence of MBL and ESBL in Gram-negative bacteria poses greater risk in public health and limit the range of treatment choices, even with β-lactams which is the most widely used class of antibiotics. Untreated wastewater from hospitals increasing the opportunities to transfer the antibiotics resistance gene between pathogens which ultimately reach communities and poses health risks. Metallo-β-lactamase (MBL), that is one type of carbapenems enzyme, exhibit the ability to inactivate all classes of β-lactams antibiotics. With discovering of bla_{NDM-1} gene, a novel β-lactamase, produced the New Delhi Metallo- β-lactamase, poses a larger threat as this gene usually found along the side of other genes that provide resistance to almost all antibiotics. This bla_{NDM-1} gene is easily transferable between Enterobacteriaceae. ESBL is another commonly found enzyme of Enterobacteriaceae that inactivate cephalosporins and monobactams. Both these genes are important to be identified and prevention of their spread.

Materials and Methods: Wastewater samples were collected from 18 sites in Dhaka and transferred to the laboratory. Bacterial cultures were grown on selective agar media for isolation of *Klebsiella pneumoniae*. Antibiotic resistance profiles of colonies were determined with Kirby-Bauer disc diffusion testing with PCR identification of MBL encoding genes, bla_{NDM-1}, and bla_{IMP}, as well as ESBL encoding genes, bla_{SHV}, bla_{TEM}, and bla_{CTX-M}.

Results: From this study it can be perceived that, 29.88% of *Klebsiella pneumoniae* which were either imipenem or meropenem resistant or both of antibiotic resistant, most of them carried the bla_{NDM-1} or bla_{SHV} gene which is the most common gene responsible for coding the MBL & ESBL enzyme known as Metallo-beta-lactamase that makes bacteria resistant to a broad range of beta-lactam antibiotics.

Keywords: Metallo-beta-lactamase; Extended spectrum-beta-lactamase; Carbapenem, blaNDM-1; Wastewater; Antibiotics

Acknowledgement

First of all, I would like to express my gratitude to the Almighty for giving me the strength to complete the thesis.

I would like to express my sincere gratitude to **Professor A F M Yusuf Haider** (Chairperson, Department of Mathematics and Natural Sciences) and **Dr. Fahim Kabir Monjurul Haque** (Assistant Professor, Department of Mathematics and Natural Sciences) for giving me the opportunity to pursue my thesis in our BRAC University Microbiology Lab. I extend my deepest gratitude and special thanks to **Adeeba Raydah** (Biotechnologist) and **Tahsin Shahrin Khan** (Former Teaching Assistant) to direct me, assist me and encourage me to conduct my thesis. I am obliged to use all the necessary machinery and vital materials to complete the thesis with the of the Microbiology laboratory of BRAC University who opened heartily. In addition, my sincere appreciation goes to my supervisors for sharing their wealth of wisdom with us over the course of this thesis.

The oversight and encouragement of these esteemed individuals influenced this report to be completed flawlessly in every step of the project. I regard this opportunity as a significant achievement in the growth of my career. I will certainly aspire to use the skills and experience learned in my near future in the best possible way.

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LIST OF ACRONYMS

PBPs	Penicillin Binding Proteins
K. pneumoniae /*k. p.*	*Klebsiella pneumoniae*
E. coli	*Escherichia coli*
VIM	Verona integron-encoded metallo-β-lactamase
IMP	Imipenemase
NDM	New Delhi metallo-β-lactamase
EMB	Eosin Methylene Blue
MHA	Mueller Hinton Agar
PCR	Polymerase Chain Reaction

Chapter 1

Introduction

One of the most often used antimicrobial agents is Beta-lactam antibiotics. Penicillin binding proteins (PBPs), also known as transpeptidase enzymes, which are necessary for the formation of the peptidoglycan layer of the bacterial cell wall, are inhibited by these antibiotics (Sauvage E et. Al, 2008). Gram-negative bacteria that produce Metallo-β-lactamase and Extended spectrum-β-lactamases (MBL and ESBL) can cause infections that are particularly severe due to the antibiotic resistance they confer (Qamar M. U.e et al, 2012). Even with β-lactams, the most widely used antibiotics, the co-existence of MBL and ESBL genes into these pathogens might further restrict treatment choices (Ahmed O. B. et al, 2021). Inadequately treated wastewater, specifically from hospital sources, increases the chance that these microbes may exchange genes that make them resistant to antibiotics, as well as the danger that they will spread throughout the environment and eventually reach populations (Vaz-Moreira I. et al, 2014).

One type of Carbapenemase enzyme known as MBL may convert all classes of β-lactam antibiotics inactive, including last-resort carbapenems, except for monobactams (Chaudhary U. et al, 2004). The identification of the bla_{IMP} gene in clinically isolated samples led to the discovery of MBLs (Rupp M. E. et al, 2012). A greater risk poses with the discovery of the bla_{NDM-1} gene that generated the New Delhi Metallo-β-lactamase-1 enzyme, a novel β-lactamase that often detected with other genes that made bacteria resistant to almost all antibiotics. The bla_{NDM-1} gene is widely transferrable between *Enterobacteriaceae* and is most frequently detected in *Klebsiella pnuemoniae* (Brolund A. et al, 2014).

Cephalosporins and monobactams like aztreonam can be rendered inactive by ESBL enzymes, which are most frequently detected in *Enterobacteriaceae* (Plazkill T. et al, 2012). Although ESBL organisms were initially been of either of the TEM or SHV types, CTX-M type enzymes are currently the most frequently seen in clinical situations (Laraki N. et al,2010).

The public's health is at risk from MBLs and ESBLs, thus it's significant to identify and restrict their environmental spread. India-originated NDM-1 has already spread over the world and poses a serious risk of spreading to nearby nations like Bangladesh (Kumarasamy K. K. et al, 2010). Both MBL and ESBL-producing bacteria are present throughout the country, according to earlier research on wastewater, with increased prevalence found adjacent to hospitals and towns with access to healthcare facilities (Adnan N. et al, 2013).

The current investigation seeks to identify the current prevalence and describe the antibiotic resistances of ESBL and Carbapenemase-producing *K. pneumonia*e in wastewater from several community areas of Dhaka, Bangladesh. Additionally, it intends to confirm the molecular presence of MBL determinants, bla_{NDM-1}, bla_{IMP}, as well as ESBL genes, bla_{SHV}, bla_{TEM}, and bla_{CTX-M}, and confirm their co-existence patterns within the resistant isolates.

Chapter 2

2.Materials and Methods

2.1. Sample Collection and Processing

A total of 36 samples of wastewater were collected from 4 cluster areas, consisting of 18 sampling sites, within the Dhaka Metropolitan Area, Bangladesh. The samples were taken from open surface sewer drains and natural drainage canals linked to the city's rivers and lakes (Cahill N. et al, 2019). A minimum of 200ml of each sample was collected in sterile 500ml containers by lowering into the wastewater collection points, a process as described by Cahill et al (Tille P. M. et al,2022). These were immediately transported to the laboratory, where it was centrifuged to remove debris and kept at 4℃ prior to microbiological and molecular analysis.

2.2. Bacterial Isolation

Samples were serially diluted and plated onto HiCrome™ ESBL Agar using spread plate technique, kept for incubation at 37℃ for 24 hours. Isolates were preliminarily identified based on colony morphology. HiChrome ESBL (ESBL product sheet) was used to differentiate *Klebsiella pneumoniae* as bluish-green colonies (Devi et al, 2021). A total of 87 isolates were selected for *Klebsiella pnuemoniae* (n=87), with the species' identity further confirmed by standard biochemical testing (Hudzicki J. 2009).

2.3. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the 87 isolates was tested using the Kirby-Bauer Disk Diffusion method (Lewis II J. S., 2022). Antibiotic discs were used for Gentamicin (10 μg), Amoxycillin (30 μg), Piperacillin/Tazobactam (100/10 μg), Imipenem (10 μg), Meropenem (10 μg), Ciprofloxacin (5 μg), Norfloxacin (10 μg), Amikacin (30 μg), Cefepime (30 μg), Azithromycin (15 μg), and Colistin (10 μg), (ref to the company). The tested isolates were classified as sensitive (S), intermediate (I), and resistant (R) based on inhibition zone measurements in accordance with Clinical Laboratory Standards Institute (CLSI) 2020 as shown in Table 1 (Gales A C. et al, 2001). Colistin resistance was determined at zone diameter ≤11mm and sensitive at ≥14mm (a limitation is that these parameters may be subject to error) (Galani I. et al, 2008)

Antibiotic	Disc Potency (µg)	Diameter Zone (mm)		
		R	I	S
Gentamicin	10	≤12	13-14	≥15
Amoxycillin-clavulanate	20/10	≤13	14-17	≥18
Piperacillin/Tazobactam	100/10	≤17	18-20	≥21
Imipenem	10	≤19	20-22	≥23
Meropenem	10	≤19	20-22	≥23
Ciprofloxacin	5	≤21	22-25	≥26
Amikacin	30	≤14	15-16	≥17
Cefepime	30	≤18	19-24	≥25
Azithromycin	15	≤12	-	≥13

Table 01: Zone diameters for antibiotics used for *Klebsiella pneumoniae* isolates according to CLSI

2.4. Phenotypic Selection of Carbapenemase and ESBL producers

Carbapenemase production was detected by a resistance to imipenem and meropenem antibiotics according to CLSI standards as shown in Table 1. The resistant isolates were selected for further analysis. Isolates were cultured onto HiChrome ESBL agar, containing supplements to differentiate ESBL-producing organisms. Isolates showing growth were suspected to produce ESBL enzymes.

2.5. Extraction of DNA from the Resistant spp.

For the extraction of the resistant bacterial DNA, 'Boiling method' was performed due to its simplicity, cost effectiveness and short handling time. The resistant bacterial species were grown in LB broth overnight which later went through a series of centrifugation and washing steps before finally the cells were incubated at 95°C for 15 minutes, and immediately cooled on ice for 10 minutes. The DNA rich supernatant was collected and stored at -20°C.

2.6. PCR assays

DNA extraction was carried out for all suspected carbapenemase and ESBL-producing isolates. These were further examined through gene amplification by conventional PCR method and identification through gel electrophoresis for MBL encoding genes, bla_{NDM-1}, and bla_{IMP}, as well as ESBL encoding genes, bla_{SHV}, bla_{TEM}, and bla_{CTX-M}. Primers used for the study have been detailed in Table 02.

Primer target type	Target gene	sequence	size	reference
ESBL	blaTEM	TEM-F 5’ AAAATTCTTGAAGACG-3’ TEM-R 5’ TTACCAATGCTTAATCA-3’	1073	Hayat S. et al, 2019
	blaSHV	SHV-F 5'-TACCATGAGCGATAACAGCG-3' SHV-R 5'-GATTTGCTGATTTCGCTCGG-3'	450	Doosti A. et al. 2015
	blaCTX-M	CTX-M F 5’-ACGCTGTTGTTAGGAAGTG-3’ CTX-M R 5’-TTGAGGCTGGGTGAAGT-3’	857	Seyedjavadi S. S., et al 2016
MBL	blaNDM-1	(F) 5'-ACCGCCTGGACCGATGACCA-3' (R) 5'-GCCAAAGTTGGGCGCGGTTG-3'	264	Solanki R., et al, 2014
	blaIMP	F 5′-GAAGGCGTTTATGTTCATAC-3′ R 5′-GTATGTTTCAAGAGTGATGC-3′	587	Solanki R. et al, 2014

Table 02: primers used for gene amplification in the study

Chapter 3

Results

For the study, a total of 87 *Klebsiella pneumoniae* (n=87) from 4 different cluster regions with differing prevalence as shown in Table 03.

Region	Number of samples	*Klebsiella pneumoniae* isolates (n)	*Klebsiella pneumoniae* isolates (%)
A	9	17	35.42
B	10	41	56.94
C	11	18	33.96
D	6	11	45.83

Table 03: Distribution of *Klebsiella pneumoniae* isolates across different sampling regions.

3.1. Antibiotic Susceptibility Profiling

A total 87 isolates of *klebsiella pneumoniae* were subjected to 11 antibiotics impregnated in disks and their resistance profiles were recorded. It was observed that Amoxicillin had the highest incidence among the strains (89.86%), followed by Cefepime (60.49%), Azithromycin (57.89%), Ciprofloxacin (37.25%). The strains recorded least resistance to Gentamycin (8.97%). Significant resistance could be observed in case of Piperacillin/Tazobactam (33.72%), Amikacin (20.99%), Imipenem (26.19%) Meropenem (13.51%), Colistin (19%) and Norfloxacin (13.33%).

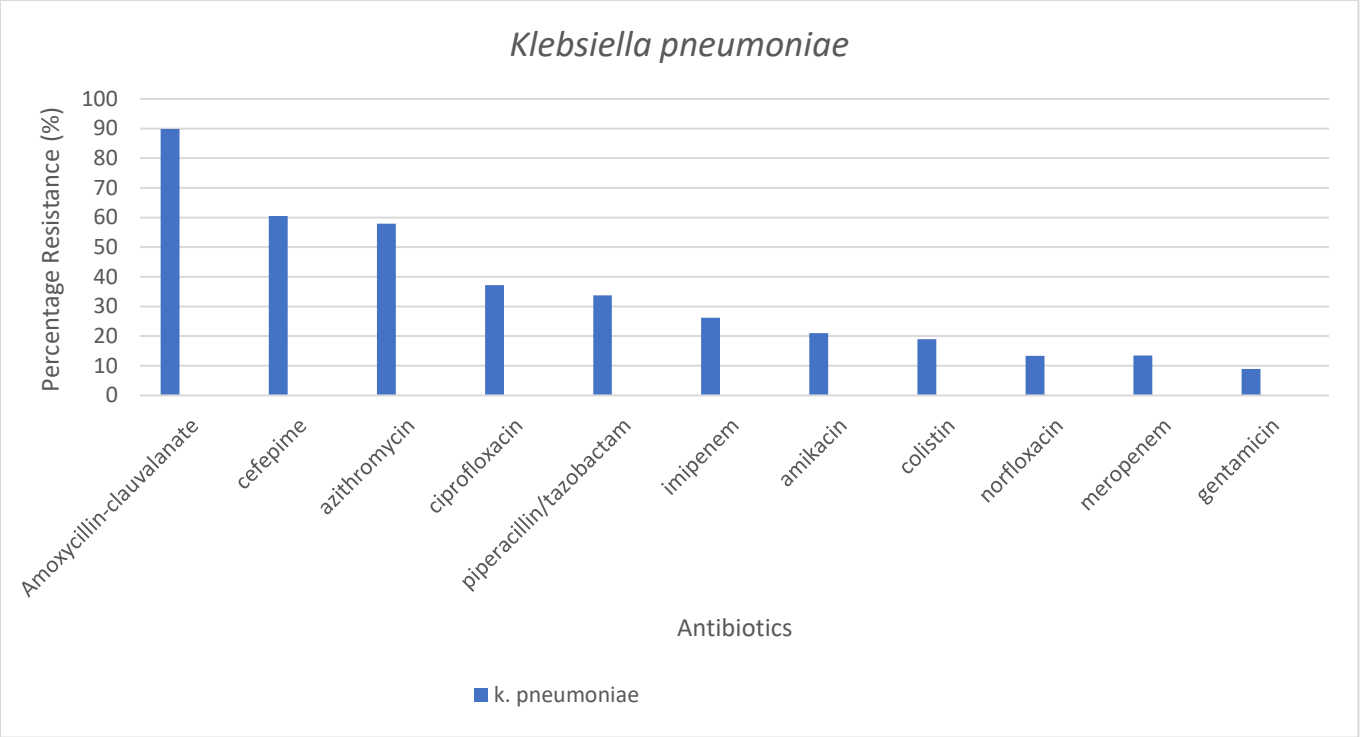

Figure 01: Percentage of resistant *Klebsiella pneumoniae* isolates for each antibiotic tested.

Antibiotics	*Klebsiella pneumoniae*		
	%R	%I	%S
Piperacillin/ Tazobactam	33.72	31.40	34.88
Imipenem	26.19	17.86	55.95
Meropenem	13.51	4.05	82.43
Amoxycillin-clavulanatae	89.86	4.35	5.80
Cefepime	60.49	22.22	17.28
Ciprofloxacin	37.25	47.06	15.69
Norfloxacin	13.33	13.33	73.33
Amikacin	20.99	27.16	51.85
Azithromycin	57.89	0.00	42.11
Colistin	19.0	40.5	40.5
Gentamicin	8.97	14.10	76.92

Table 04: Percentage of Resistant (R), Intermediate (I), and Sensitive (S) isolates from antibiotic disc diffusion testing for *Klebsiella pneumoniae*.

3.2. Distribution for MBL and ESBL genes

The isolates that displayed resistance to the medicines meropenem and imipenem were kept for further molecular analysis in order to assess the spread of carbapenem resistant bacteria. Among them 7.80% were blaNDM-1 positive, 5.87% were blaNDM positive, 6.90% were NDM positive, 6.90% were blaCTX-M positive and 8.05% were blaSHV positive, however, none of them found to be carrying blaIMP, blaVIM-2 and blaTEM.

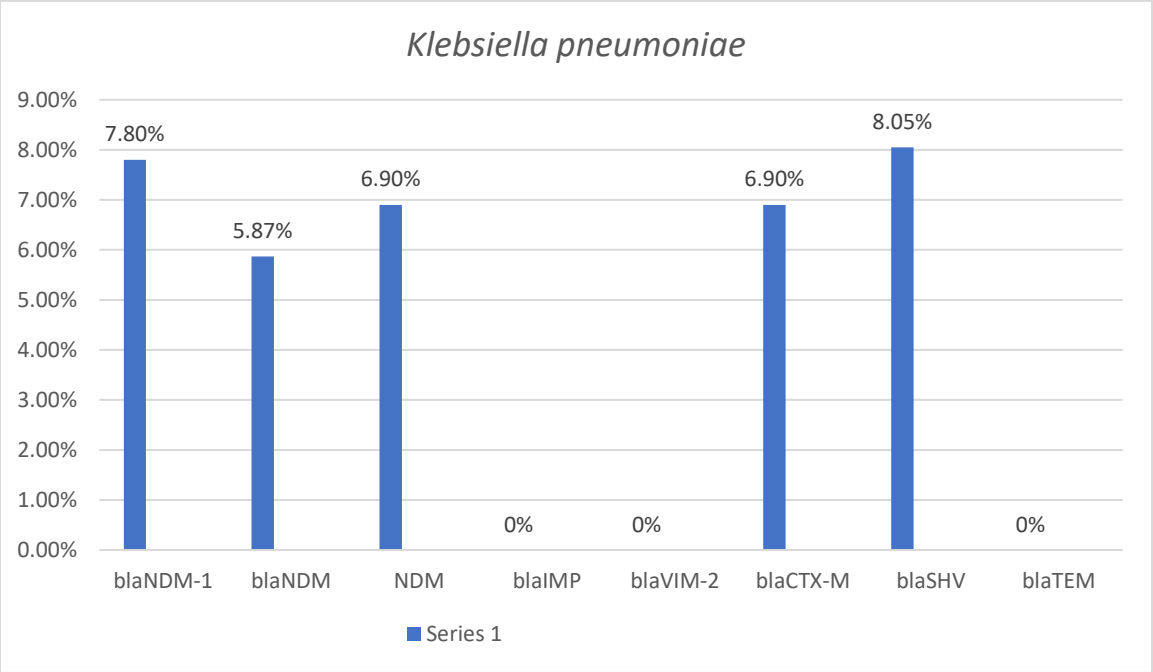

Figure 02: Prevalence of isolates positive for MBL & ESBL gene products

From this study it can be perceived that, 29.88% *Klebsiella pneumoniae* which were either imipenem or meropenem resistant or both of antibiotic resistant, most of them carried the blaNDM-1 and blaSHV gene which is the most common gene responsible for the carbapenemase enzyme known as metallo-beta-lactamase and ESBL that makes bacteria resistant to a broad range of beta-lactam antibiotics.

Chapter 4

Discussion

The number of impervious surfaces in Dhaka has dramatically increased recently, obstructing natural drainage patterns and limiting the amount of detention basins, that led to a shorter runoff concentration time and, as a result, waterlogging troubles. The Dhaka Water Supply and Sewerage Authority (WASA) begins digging up the roads during the monsoon every year in an attempt to solve these issues, but in the end, poor community drainage, sewage from overflowing sewers and latrines mix with rainfall runoff and drinking water lines, causing waterborne diseases. Through this work, it was discovered that samples of community sewage had numerous drug-resistant enteric bacteria that might potentially contaminate other bodies of water. In this study, multidrug-resistant strains of *K. pneumoniae* are particularly important since they are linked to a variety of gastrointestinal illnesses transmitted through polluted water. although antibiotics from the beta-lactam and carbapenem groups are frequently used to treat infections caused by Gram-negative group of bacterial pathogens, but developing countries like Bangladesh still lack thorough research on carbapenem producing Gram-negative bacteria. In one study, the percentage of *K. pneumoniae* resistant to carbapenems has been found to be 79.2% (Sakkas, H et.al 2019). In our Study we have also found out that, *K. pneumoniae* was the most prevalent amongst NDM-1 and blaSHV positive isolates. Given the serious risk that multidrug-resistant bacteria pose to the general public's health, prompt and urgent action is required to prevent their spread. Preventing contaminant exposure in the aquatic environment should be one strategy for attaining that aim. To conclude, the current study findings indicate a significant percentage prevalence and spread of and *Klebsiella pneumoniae* carrying different carbapenemase genes in Bangladeshi communities. Appropriate and adequate control measures should be adopted from awareness to prescription. And future research should establish the exposure risks linked to community water as well as the relative contributions of different types of contamination sources and factors influencing variation in the prevalence of enteric bacteria in community water, which may ultimately play a significant role in slowing the spread and transmission of antibiotic resistance.

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