Carbapenem Resistance Gene Profiling in *Klebsiella pneumoniae* Isolated from clinical and non-clinical (water and vegetables) samples

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology

Department of Mathematics and Natural Sciences
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Declaration

It is hereby declared that

1. The thesis submitted is 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.
4. We have acknowledged all main sources of help.

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Approval

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of Summer, 2019 have been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology in 14th December, Fall 2023.

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Ethics Statement

For the completion of this study, samples from selected venues were collected following all the necessary precautions. All the experiments were done at BRAC University Life Sciences Laboratory. It should also be noted that no animal or human models were used in this study.

Abstract

Klebsiella pneumoniae is a bacterium that has a rod-like structure and is classified as Gram-negative. It is a member of the Enterobacteriaceae family. This microorganism is recognized for its clinical importance, since it is frequently found in the human microbiome and has the capacity to cause respiratory, urinary, and bloodstream infections. *Klebsiella pneumoniae* is distinguished by its polysaccharide capsule, which enhances its ability to cause disease and common source of opportunistic infections that demonstrate resistance to antimicrobial treatments in hospitalized individuals. Carbapenem Resistance Gene Profiling in *Klebsiella pneumoniae* is conducted with the goal of comprehending the genetic foundation of resistance to carbapenem drugs and this focuses on the identification of distinct resistance genes found in strains of *Klebsiella pneumoniae*, thereby addressing the urgent problem of antibiotic resistance.

In this study, a total of 27 carbapenem resistant *Klebsiella pneumoniae* isolates were incorporated, with 25 isolates derived from non-clinical sources, comprising vegetables and water, and 2 isolates obtained from clinical sources. The major purpose of this study was to isolate *Klebsiella pneumoniae* from clinical and non-clinical sources, and to analyze their antibiotic resistance profiling as well as carbapenem resistant gene profiling. The aim of this targeted approach was to identify five distinct genes linked to carbapenem resistance, specifically NDM-1, blaKPC, blaOXA-48, blaVIM, and blaIMP. Additionally, PCR was performed and upon conducting gel electrophoresis and analyzing specific data, it was observed that the detection rate for the NDM-1 gene was notably higher, with approximately 11.11% showing positive results. Among the 25 carbapenem-resistant *Klebsiella pneumoniae* isolates, PCR analysis revealed 2 (8%) NDM-1 positive cases from non-clinical sources and 1(50%) producers were detected from clinical sources. During the analysis, no positive result was obtained for rest of those four genes, and no bands were found within their respective ranges.

Keywords: *Klebsiella pneumonia,* carbapenem resistance, antibiotic resistance, gene profiling.

Acknowledgment

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Table of Contents

List of Tables

List of Figures

List of Acronyms

KPC	*Klebsiella pneumoniae* Carbapenemase
ESBL	Extended-spectrum beta-lactamases
MDR	Multidrug resistant
BHI	Brain heart infusion
CLSI	Clinical and Laboratory Standards Institute
OD	Optical density
NDM-1	New Delhi metallo-ß-lactamase-1
blaKPC	beta-lactamase - *Klebsiella pneumoniae*
mm	Millimeter
%	Percentage
CDC	Centers for disease control and prevention

Chapter 1

1.1. Introduction

Klebsiella pneumoniae, initially identified in the late 19th century and originally referred to as Friedlander's bacterium, is a Gram-negative bacterium characterized by its encapsulated, non-motile nature. It is commonly found in the environment, including soil, surface waters, and on medical equipment (Paczosa & Mecsas, 2016). Furthermore, *Klebsiella pneumoniae* is a prevalent cause of opportunistic infections that exhibit resistance to antimicrobial agents in hospitalized patients. This bacterium inherently demonstrates resistance to penicillins, and a significant portion of its population typically harbors acquired resistance to multiple antimicrobial drugs (Wyres et al., 2020).

Klebsiella pneumoniae sources are frequently found in diverse environmental niches, such as soil and surface waters, as well as in sewage and wastewater. The environment serves as a significant reservoir of *Klebsiella pneumoniae*. It can be commonly found in the environment, particularly in soil and water, where it naturally occurs as a bacterial species. *Klebsiella pneumoniae* can occasionally be detected in water sources such as rivers, lakes, and reservoirs. Water that is contaminated has the potential to serve as a source of infection (Navon-Venezia et al., 2017). Another significant source of *Klebsiella pneumoniae* is through food and vegetable sources. Although it is not often linked to foodborne disease, there have been cases when this bacterium has been detected in vegetables, especially those cultivated in soil or irrigated with water that is contaminated. In addition, Animal Source is a significant factor for *Klebsiella pneumoniae*, as it can also be found in the gastrointestinal tracts of animals. Although it is not a predominant zoonotic pathogen, there have been instances of transmission occurring between animals and humans (Kobayashi et al., 2016). Furthermore, *Klebsiella pneumoniae* may also be found in the broader population. This encompasses locations such as residences, public areas, and communal water facilities. Also, it is a common component of the human gastrointestinal tract, typically without causing any harm. *Klebsiella pneumoniae* is frequently linked to urinary tract infections, respiratory tract infections, and wound infections in healthcare settings thereby establishing a robust presence in the natural world. Its ability to survive in these environments has raised concerns regarding its potential to cause community-acquired infections. Moreover, *Klebsiella* has emerged as a significant pathogen within hospital environments, giving rise to nosocomial infections with a substantial 20% incidence rate (Tsay et al., 2002). The issue of antibiotic resistance is particularly pronounced in these cases. Normally residing in the human intestines without causing any harm, *Klebsiella* is rarely found in the oropharynx of individuals with a healthy immune system, with a carrier prevalence rate of only 1-6%. Consequently, individuals who are immunocompetent typically do not contract *Klebsiella* infections unless they become critically ill and require mechanical ventilation or intravenous catheters, or unless they undergo extended courses of broad-spectrum antibiotics (Woldu, 2016). *Klebsiella* species are also associated with various infections in humans, including pneumonia, septicemia, meningitis, rhinoscleroma, ozaena, sinusitis, otitis, enteritis, appendicitis, and cholecystitis. Also, *Klebsiella pneumoniae* is notorious for its propensity to

cause a wide range of illnesses, particularly in healthcare environments. It is prevalent in hospitals, nursing homes, and other healthcare settings and is a highly prevalent and other complications. Recent studies showed that the prevalence of *Klebsiella* colonization ranges from 18.8 to 87.7% in Asia and 5 to 35% in Western countries (Chang et al., 2021). Additionally, Hyper-virulent Klebsiella pertains to strains of the bacteria Klebsiella pneumoniae that exhibit heightened virulence, indicating their capacity to induce severe illnesses. Contrary to the usual Klebsiella pneumoniae, which is commonly linked to infections acquired in hospitals, hyper-virulent variants can lead to severe infections acquired in the community. These hyper-virulent strains possess distinct features, such as a stronger capsule that enables them to elude the host's immune system, and an enhanced capacity to propagate across the community. These microorganisms are recognised for their ability to induce illnesses, such as liver abscesses, pneumonia, and bloodstream infections, frequently impacting individuals who are in good health and not in healthcare environments. The appearance of highly virulent Klebsiella strains presents significant obstacles for public health because they have the potential to cause more severe and rapidly advancing infections.Consequently, they have become a crucial area of research and surveillance.

There are two main types of antibiotic resistance commonly observed in *K. pneumoniae*. One mechanism involves the presence of extended-spectrum β-lactamases (ESBLs), which make the bacteria resistant to cephalosporins and monobactams. The other mechanism, which is even more concerning, is the expression of carbapenemases by *K. pneumoniae*. These carbapenemases make the bacteria resistant to almost all available β-lactam antibiotics, including carbapenems. (Paczosa & Mecsas, 2016a).

The first case of *K. pneumoniae* expressing a carbapenemase was identified in North Carolina in 1996, and this specific type of carbapenemase is referred to as KPC. (Yigit et al., 2001). Subsequent carbapenemases genes, including MBL, NDM-1, IMP, and VIM, have been identified in *K. pneumoniae* strains. (Pitout et al., 2015). It is noteworthy that all these carbapenemases, including KPC, have been detected in other bacterial species as well, contributing to the global prevalence of carbapenem-resistant bacteria (Iredell et al., 2016) & (Munoz-Price et al., 2013). Irrespective of the specific carbapenemase they harbor, *K. pneumoniae* isolates that are resistant to carbapenems are collectively categorized as CRE, which stands for carbapenem-resistant Enterobacteriaceae. Infections caused by *K. pneumoniae* carrying ESBLs or demonstrating carbapenem resistance are associated with significantly increased morbidity and mortality rates in comparison to infections involving non-resistant bacteria. A report released by the CDC in 2013 underscores the frequency and severity of infections caused by these strains, based on a 2011 survey encompassing 183 hospitals in the United States. (Vital Signs: Carbapenem-resistant Enterobacteriaceae, 2013). Strains that produce ESBLs accounted for 23% of hospital-acquired *K. pneumoniae* infections, totaling 17,000 infections and resulting in 1,100 fatalities. In contrast, *Klebsiella pneumoniae* strains resistant to carbapenems caused 11% of hospital-acquired *K. pneumoniae* infections, amounting to 7,900 infections and leading to 520 deaths (Vital Signs: Carbapenem-resistant Enterobacteriaceae, 2013).

Klebsiella pneumoniae is recognized as a major multidrug-resistant (MDR) threat to global human health, as highlighted by the World Health Organization (WHO), the US Centers for Disease Control and Prevention (CDC), and Public Health England (PHE) (Derakhshan et al., 2016). Due to a history marked by the widespread and indiscriminate use of antibiotics in both environmental and hospital settings, Bangladesh is at risk of becoming a fertile breeding ground for MDR *Klebsiella pneumoniae* (Ahmed et al., 2019).

As a developing nation, Bangladesh has a notable prevalence of carbapenem-resistant organisms, with the majority of identified carbapenemase-producing strains belonging to the NDM category which is almost 55% (Begum & Shamsuzzaman, 2016). However, in recent years, strains producing OXA-48-like carbapenemases have also been identified. It is important to note that there is a lack of established surveillance systems in developing countries in this region, and only a limited number of reports exist concerning the molecular epidemiology of these resistant strains. (Hasan & Rabbani, 2019). So far, research pertaining to *Klebsiella pneumonia* in Bangladesh has predominantly been confined to hospital settings and clinical specimens. Investigations have centered on the isolation, molecular characterization, and resistance profiling of *Klebsiella pneumonia*, with a primary focus on strains exhibiting multidrug resistance. A limited number of studies have broadened their scope to encompass genetic characterization, delving into the prevalence and distribution of multidrug resistance genes, such as those related to carbapenemase genes like NDM-1, OXA-48, KPC, VIM and IMP (Okanda et al., 2021).

Therefore, the major aim of the study was to characterize the carbapenem resistant gene profiling in *Klebsiella pneumoniae* that are isolated from vegetable and clinical samples. Samples were collected over a period of five months from those sources to analyze and characterize carbapenem resistance mechanisms in *Klebsiella pneumoniae*.

1.2. Objective of the study

The specific aims and objectives of the study are as follows:

- To isolate *Klebsiella pneumoniae* from clinical and non-clinical (water and vegetables) samples in Dhaka city
- To analyze their antibiotic resistance profiling
- Carbapenem resistant gene profiling

Chapter 2

Method & Materials

2.1. Bacterial isolates

In this study, a total 27 carbapenem resistant *Klebsiella pneumoniae* isolates were included. Among them, 25 isolates were from non-clinical sources, which includes vegetables and water and 2 isolates were from a clinical sources.

Klebsiella pneumoniae isolates from clinical sources

A total of 12 clinical isolates of *Klebsiella pneumoniae* were collected from the Popular Diagnostic Center Ltd. These isolates were cultured from blood samples of patients diagnosed with bloodstream infections. Among these 12 isolates, 2 isolates were carbapenem resistant.

Klebsiella pneumoniae isolates from non-clinical sources

A total of 30 non-clinical isolates were collected. 15 isolates were from water sources, and 15 were from vegetable sources. These *Klebsiella pneumoniae* isolates were isolated by another research group from our lab. We collected 25 carbapenem resistant isolates from them.

2.2. Isolation of *Klebsiella pneumoniae* from clinical samples

Sample collection & processing

Blood samples were collected from patients diagnosed with bloodstream infections and then immediately taken to the hospital laboratory for further biochemical tests. After that, the culture plates were placed in a sturdy and secure container that provides protection against breakage during transportation. An insulated container was used to maintain the required temperature during transit. Collected clinical samples were taken to the university laboratory and spread on MacConkey media. By observing the colony morphology, suspected single colony was picked and citrate utilization test were conducted. Further confirmed by PCR and the isolates were stored on BHI media.

2.3. Isolation of *Klebsiella pneumoniae* from non-clinical samples

Sample collection & processing

This was done by another group of our lab. However, the procedure described as follows: The non-clinical isolates were obtained from the market using aseptic techniques to guarantee minimal contamination and to avoid the introduction of undesired microbes. After being gathered, the isolates were meticulously put in sterile containers to preserve their integrity and taken to the university laboratory. Collected isolates were suspended into 250 mL of Luria broth (LB). The suspended samples were incubated at 37°C for 24 h before being cultured over HiCrome™ KPC Agar Base. From HiCrome™ KPC Agar Base 5 to 6 colonies were picked

up and sub-cultured onto nutrient agar at 37°C for 24 h. Moreover, confirmed by PCR isolates were stored with 30% glycerol at −20°C for further use.

2.4. Bacterial culture on agar

The samples were spread on MacConkey agar plates and incubated at 44°C for 24 hours. Subsequent examination focused on identifying the presence of *Klebsiella pneumoniae* based on colony characteristics. The colonies exhibited a pinkish color, sliminess, and emitted an odor resembling burnt sugar or chocolate which identified the presence of *Klebsiella pneumoniae* (Figure1). On the other hand, if the colonies displayed different coloration, lacked sliminess and emitted a distinct scent unrelated to caramelized sugar, this identified the absence of *Klebsiella pneumoniae.*

Figure 1: *Klebsiella pneumoniae* culture on MacConkey Agar

Klebsiella pneumoniae colonies were identified as pink to dark pink on MacConkey agar plates, featuring a characteristic mucoid appearance that set them apart from other bacteria. The selective and differential properties of MacConkey agar aided in distinguishing lactose fermenters (with pink or red colonies) from non-lactose fermenters (displaying colorless or pale colonies). The pink coloration of *Klebsiella pneumoniae* colonies on MacConkey agar confirmed their ability to ferment lactose. Further tests were employed for additional identification and characterization to validate their classification as *Klebsiella pneumoniae.*

2.5. Citrate Utilization test

After the observation of colony morphology on selective media (MacConkey agar), a biochemical test was performed that was the Citrate Utilization test by Simmons Citrate Agar. For this test, *Klebsiella pneumoniae* isolates were inoculated onto a citrate agar tube and incubating it for approximately 24 hours. The isolates were able to utilize citrate as it exhibited growth and caused a colour transformation of the agar from green to blue (Figure 2). This signifies a favourable outcome for the use of citrate. If the isolates lacked the ability to utilize citrate, there was an absence of notable alteration in colour on the agar during incubation, thereby signifying a poor outcome in terms of citrate utilization.

Figure 2: Citrate Utilization Test

This observation highlights the considerable metabolic capacity of the bacteria that were tested to utilize citrate as the only source of carbon. Positive outcomes in the citrate utilization test suggest the presence of distinct physiological traits in *Klebsiella pneumoniae*. In addition, the consistent good outcomes observed in the citrate utilization test for all 27 samples emphasize the strong ability of the examined microorganisms to metabolize citrate.

2.6. DNA Extraction

The boiling method for DNA extraction is an easy and quick way to get DNA from biological data. The isolates were burned to a high temperature in this method, which breaks open the cells and lets the DNA out. After the DNA was released, it can be gathered and used for different genetic studies. At first, colonies that were presumed to be present were introduced into nutrient broth (NB) and placed in a shaker incubator for a duration of 24 hours. Upon the observation of growth, a 500 microliter solution was isolated and subjected to centrifugation at a speed of 12.5 X 100 picometers for a duration of 5 minutes. Next, the liquid portion was removed and 200ul of TE (Tris-EDTA) buffer was added to the solid residue. Subsequently, the tubes were subjected to boiling at a temperature of 100° C for a duration of 10 minutes. Following the boiling process, the solutions underwent an additional centrifugation for a duration of 5 minutes. Finally, the liquid portion was moved to fresh microcentrifuge tubes and kept at a temperature of -20° Celsius.

2.7. Polymerase chain reaction

PCR amplification of *Klebsiella pneumoniae* genes, reaction was performed in a total volume of 15 µL which included 7.5 µL of Master Mix, 2µL of DNA, reverse and forward primer of 0.5 µL and 4.5 µL NW (nuclease-free water). The cycling conditions were 10 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 20 s at 57 °C, and 20 s at 72 °C then 10-min hold at 72 °C. Later PCR products were stored at -20 °C (Liu et al., 2008).

Klebsiella pneumoniae isolates were confirmed by PCR (Yigremet al., n.d.). Pf (5′-ATT TGA AGA GGT TGC AAA CGA T3′)/Pr1 (5′-TTC ACT CTG AAG TTT TCT TGT GTT C-3′) and

Pf/Pr2 (5′-CCG AAG ATG TTT CAC TTC TGA TT-3′) were assigned as two pairs of *Klebsiella pneumoniae* specific primers. These two primer pairs were used to carry out PCR detection.

2.8 Agarose gel electrophoresis

10 µL of PCR products were transferred to a gel containing 2% agarose in TBE buffer (40mM Tris, 20mM boric acid, 1mM EDTA, pH of 8.3) and 0.5 µg/ml ethidium bromide dye. The gels were visualized under ultraviolet illumination using a gel image analysis system. Bands were visualized at the correct expected size for *Klebsiella pneumoniae*.

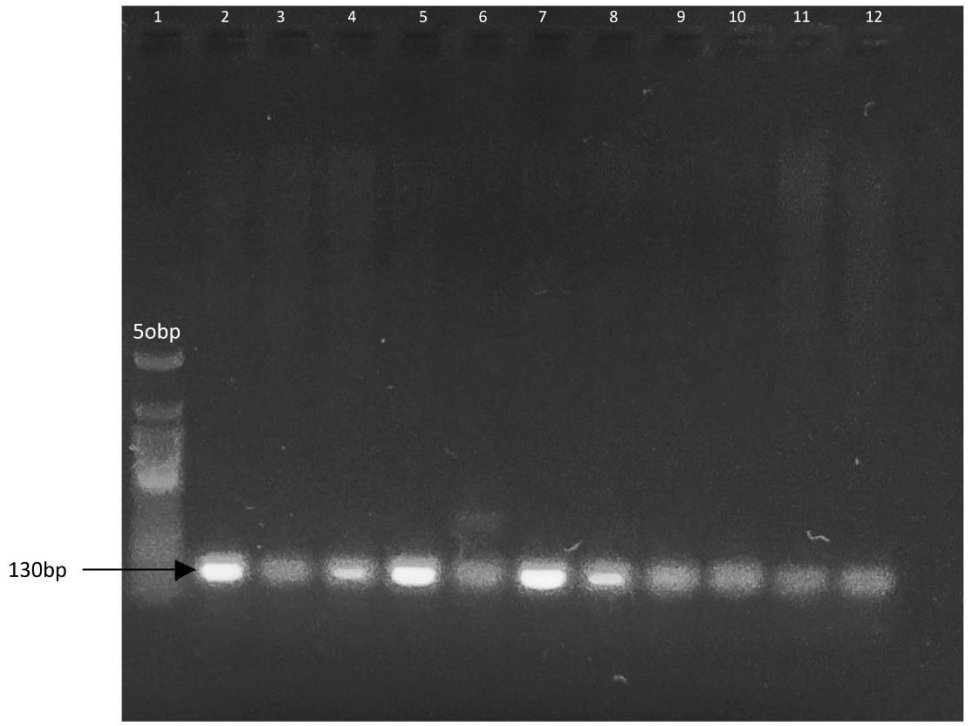

Figure 3 : Agarose gel electrophoresis of PCR assay of *Klebsiella pneumoniae.* Here, Lane 1: 50 bp Size marker; Lane (2-12) are some positive samples at 130bp.

2.9. Antibiotic Susceptibility Test

The Antibiotic Susceptibility Test (AST), also referred to as the Kirby-bauer disk diffusion method, was employed to assess the resistance or susceptibility of bacterial strains of *Klebsiella pneumoniae*.

In this study, antibiotic susceptibility test was majorly performed for 27 isolates of *Klebsiella pneumoniae* through 10 antibiotics (Table 1) and those are Amikacin, Gentamicin, Tetracycline, Tigecycline, Ciprofloxacin, Imipenem, Meropenem, Ampicillin, Ceftriaxone & Colistin. The isolates were mainly collected from non-clinical isolates which includes water and vegetable samples and some were clinical isolates that were collected from Popular Diagnostic Centre Ltd. All antibiotics have a unique range of susceptibility that is evaluated by CLSI. By using the susceptibility range, we can recognize the infections that are resistant and carry out additional steps. The procedure required, spectrophotometer and Mc-Farland 0.5 standard and Kirby-Bauer method was used. The single colony which was a 24-hours incubated sub-cultured isolate was inoculated in 5ml saline. Optical density was measured, here 0.5 McFarland was used as standard which is OD= 0.1. After confirming the desired value, the Kirby-Bauer method was carried out (Figure 4).

Table 1: Antimicrobial Susceptibility Testing - Zone Size Interpretative Chart

Antimicrobial agent	Symbol	Sensitive mm or more	Intermediate mm	Resistant mm or less
Amikacin	AK	17	15-16	14
Gentamicin	GE	15	13-14	12
Tetracycline	TE	15	12-14	11
Tigecycline	TGC	19	15-18	14
Ciprofloxacin	CIP	21	16-20	15
Imipenem	IMP	23	20-22	19
Meropenem	MRP	23	20-22	19
Ampicilin	AMP	17	14-16	13
Ceftriaxone	CTR	23	20-22	19
Colistin	CT	14	12-13	11

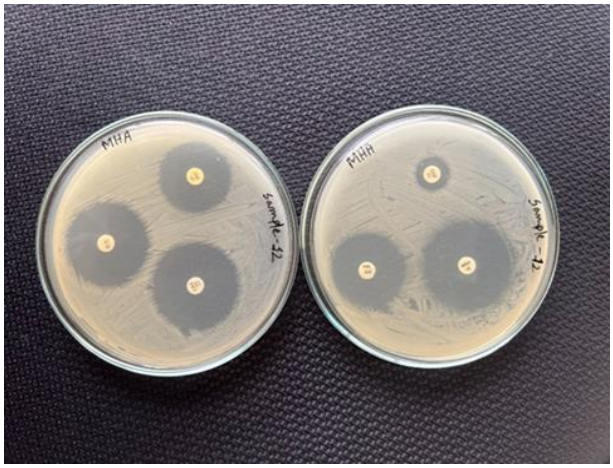

Figure 4: Antibiotic susceptibility test analysis

2.10. Carbapenem Resistance Gene Profiling

Carbapenem resistance gene profiling is an essential component in comprehending bacterial mechanisms of resistance. The NDM-1 gene, which encodes a metallo-beta-lactamase, contributes to resistance by breaking down carbapenem drugs by hydrolysis. Similarly, blaKPC generates a type A carbapenemase, which presents a notable obstacle in carbapenem treatment approaches. Conversely, the blaOXA-48 gene, which encodes an oxacillinase enzyme, and the metallo-beta-lactamases expressed by blaVIM and blaIMP, contribute to the increased variety of antibiotic resistance. Detecting the existence of these genes were crucial for customizing efficient antibiotic treatments and adopting strong infection prevention measures, particularly in healthcare environments where highly resistant bacteria present a significant risk.

Table 2: PCR – Condition for Carbapenem Resistance Gene

PCR CONDITION													
Gene	**Initial Denaturation**		**Denaturation**		**Annealing**		**Extension**		**Final Extension**		**Cycle**	**Primer Sequence**	**Amplicon Size**
	Temp	**Time**	**Temp**	**Time**	**Temp**	**Time**	**Temp**	**Time**	**Temp**	**Time**			
NDM-1	94 °C	5 min	94 °C	30 sec	58 °C	30 sec	72 °C	30 sec	72 °C	7 min	30	F-GGTTTGGCGATCTGGTTTTC R-CGGAATGGCTCATCACGATC	621
blaKPC	94 °C	5 min	94 °C	30 sec	50 °C	30 sec	72 °C	30 sec	72 °C	7 min	30	F-CATTCAAGGGCTTTCTTGCTGC R-ACGACGGCATAGTCATTTGC	498
blaOXA-48	94 °C	5 min	94 °C	30 sec	50 °C	30 sec	72 °C	30 sec	72 °C	7 min	30	F-GCTTGATCGCCCTCGATT R-GATTTGCTCCGTGGCCGAAA	281
blaVIM	95 °C	5 min	94 °C	45 sec	60 °C	45 sec	72 °C	1 min	72 °C	7 min	30	F-GGTGTTTGGTCGCATATCGCAA R-ATTCAGCCAGATCGGCATCGGC	502
blaIMP	95 °C	5 min	94 °C	45sec	60 °C	45sec	72 °C	1 min	72 °C	7 min	30	F-TCGTTTGAAGAAGTTAACG R-ATGTAAGTTTCAAGAGTGATGC	568

Chapter 3

Results

3.1. Antibiotic resistance profiling of the 27 carbapenem resistant isolates

In this study, an analysis of Antibiotic Susceptibility Test (AST) results was conducted for Klebsiella pneumoniae isolates. Among the 30 samples examined, resistance to Carbapenem was demonstrated by 25 isolates from non-clinical (water and vegetable) sources. Surface water, drinking water, soil, plants, sewage, and industrial effluent are the environmental reservoirs of K. pneumoniae (Barati et al.). Those isolates were mainly collected from different areas of Dhaka city, such as Gulshan, Banani, Uttara, and several others. Additionally, by 2 out of 12 clinical carbapenem resistant isolates specifically collected from the Popular Diagnostic Center Ltd. After measuring the zone of inhibition (mm), the isolates were categorized into three groups: Resistant (R), Intermediate Resistant (I), and Sensitive (S) (Table 3).

Table 3: Resistant, Intermediate, and Sensitive isolates for all 10 antibiotics

Sample no	Aminoglycosides		Tetracyclines		Fluoroquinolones	Carbapenem		β- lactam		Sulphonamide
	AK	GEN	TE	TGC	CIP	IPM	MRP	AMP	CTR	CT
1	R	R	S	I	I	R	R	R	R	R
2	R	I	I	I	I	R	R	R	R	R
3	I	I	I	I	I	R	R	R	R	R
4	R	I	I	I	R	R	R	R	R	R
5	R	R	I	I	I	R	S	R	R	R
6	I	I	I	I	S	R	I	R	R	R
7	I	I	I	I	S	R	R	R	R	R
8	R	I	I	I	I	R	I	R	R	S
9	I	I	I	I	I	R	I	R	R	R
10	I	I	I	I	I	R	R	R	R	R
11	I	I	I	I	I	I	R	R	R	R
12	I	I	I	I	S	R	S	R	R	S
13	I	R	R	S	R	R	R	R	R	R
14	R	I	I	I	I	R	I	R	R	R
15	I	I	I	I	I	R	R	R	R	I
16	R	I	S	I	I	R	R	R	R	S
17	R	I	I	I	I	R	R	R	R	S
18	I	I	I	I	I	R	S	R	R	S
19	I	I	I	I	I	R	S	R	R	R
20	I	I	R	I	S	R	R	R	R	S
21	S	I	I	I	I	R	I	R	R	S
22	S	I	R	I	I	R	R	R	R	R
23	I	I	I	I	I	R	I	R	R	R
24	I	I	I	I	I	R	R	R	R	R
25	S	I	I	I	I	R	R	R	R	S
26	S	I	I	I	I	R	R	R	R	R
27	I	I	I	I	I	R	R	R	R	R

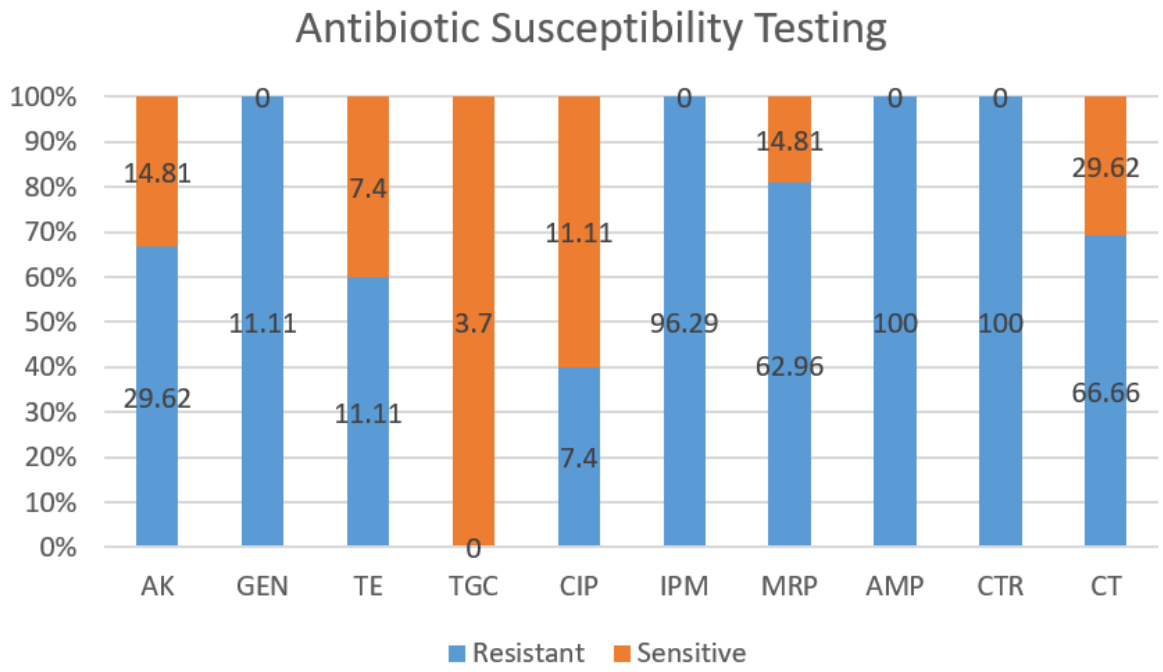

Figure 5: The number of Resistant and Sensitive isolates for all 10 antibiotics

3.2. Antibiotic resistance gene profiling

According to this study the phenotypic data for antibiotic susceptibility, a genotypic analysis was performed on 27 specifically chosen isolates that showed resistance to a majority of antibiotics. The objective of this focused strategy was to detect five specific genes associated with Carbapenem resistance, namely NDM-1, blaKPC, blaOXA-48, blaVIM, and blaIMP and PCR was conducted as well. The extensive genetic evaluation offered vital insights into the fundamental mechanisms of resistance, enabling a detailed comprehension of the interaction between genetic determinants and observed resistance patterns. The incorporation of phenotypic and genotypic data enabled a comprehensive assessment, facilitating the link between genetic factors and reported resistance profiles. The systematic methodology employed in this study facilitated a targeted examination, elucidating the precise genetic indicators responsible for the resistance seen in the analyzed isolates.

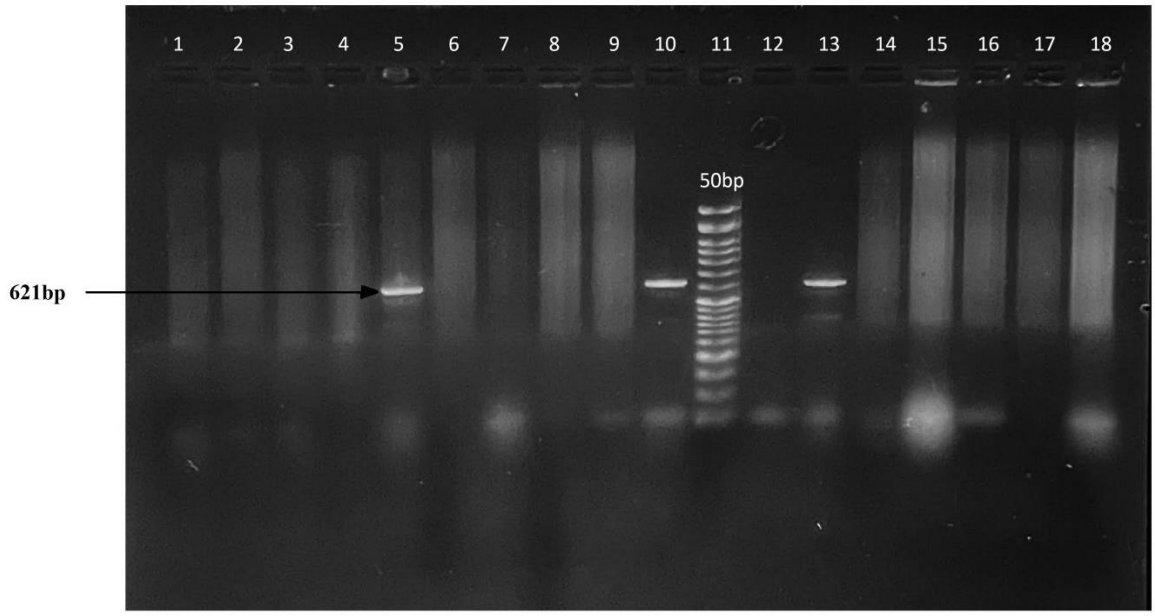

Figure 6: NDM-1 gene specific PCR. Here, Lane (10) is Positive control, Lane (11) is 50bp DNA marker and Lane (12) is Negative control. and Lane (5,10,13) are some positive samples at 621bp.

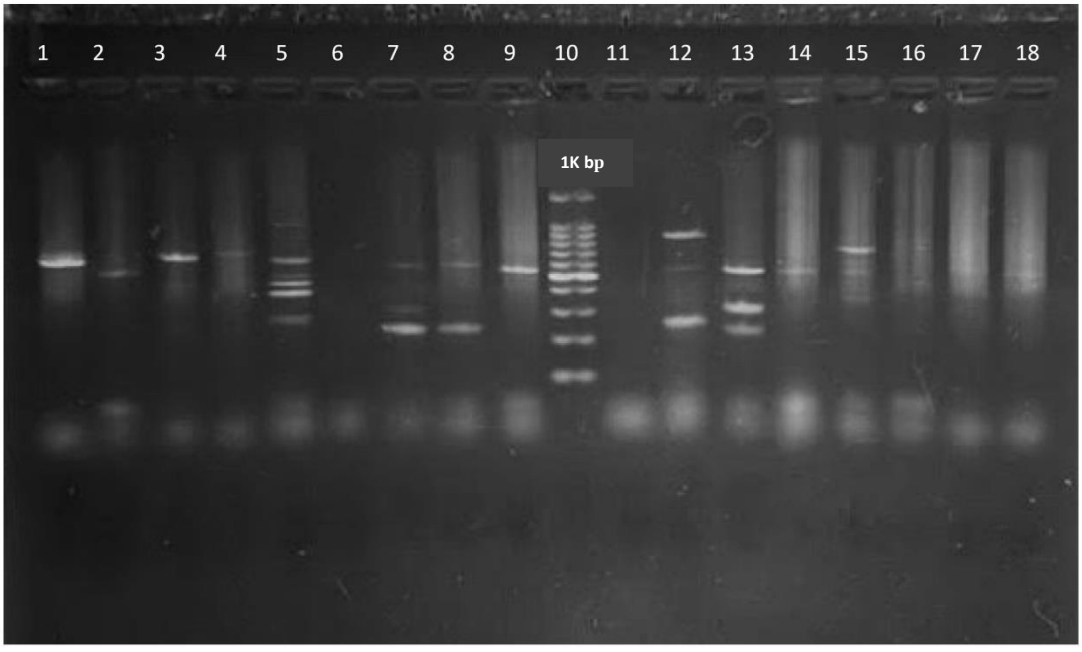

Figure 7: blaKPC gene specific PCR. Here, Lane (10) is 1k bp DNA marker and Lane (11) is Negative control.

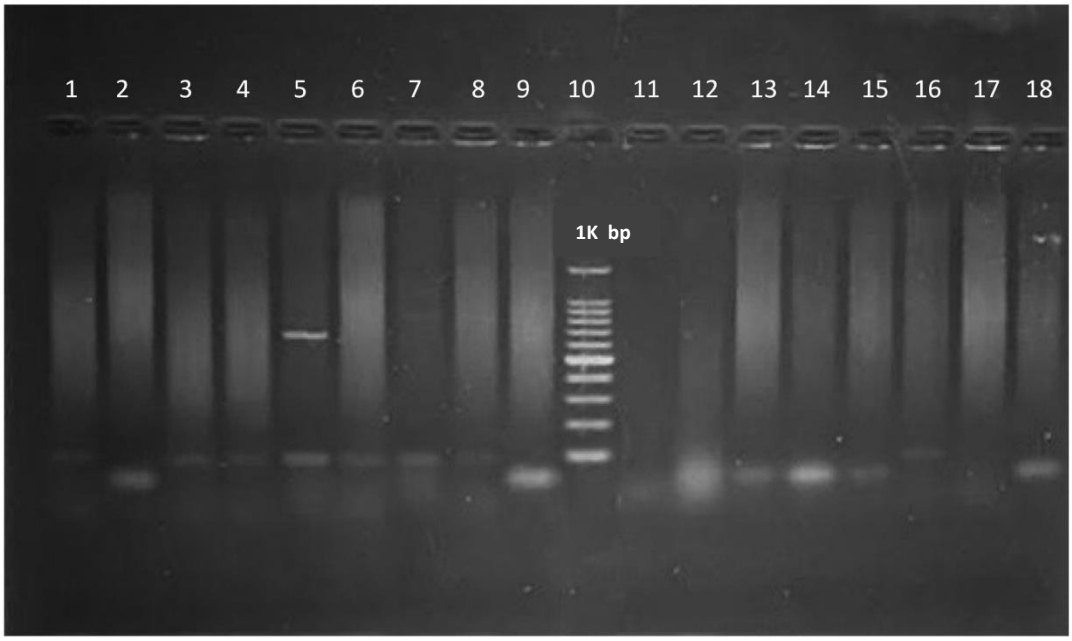

Figure 8: blaOXA-48 gene specific PCR. Here, Lane (10) is 1k bp DNA marker and Lane (11) is Negative control.

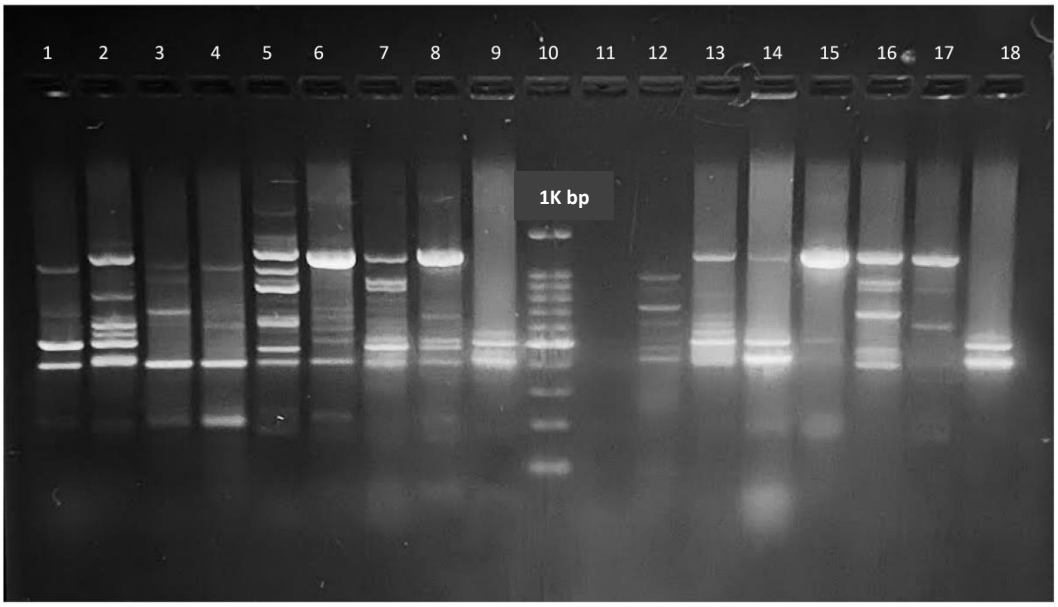

Figure 9: blaVIM gene specific PCR. Here, Lane (10) is 1k bp DNA marker and Lane (11) is Negative control.

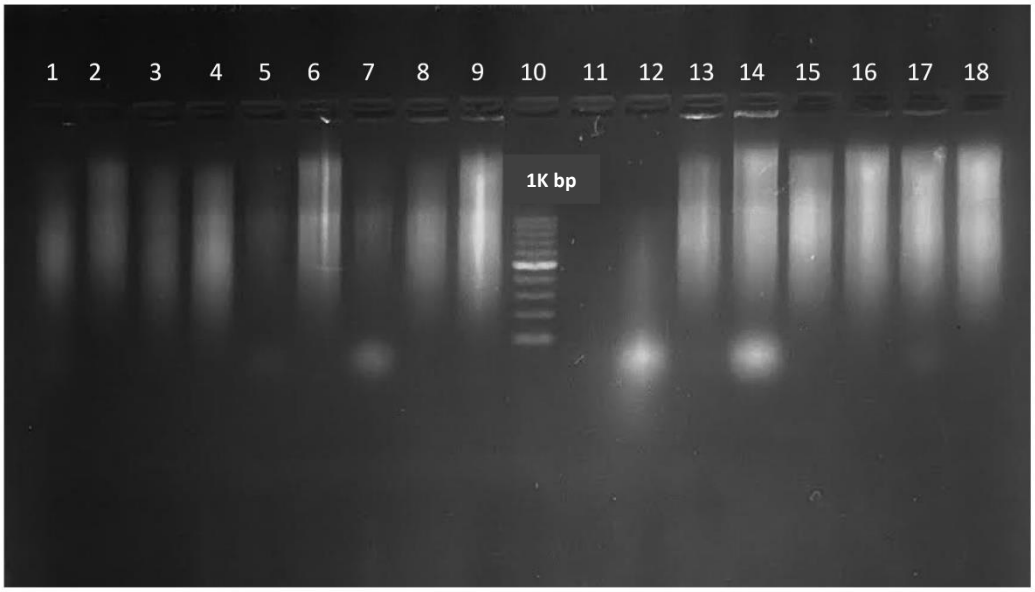

Figure 10: blaIMP gene specific PCR. Here, Lane (10) is 1k bp DNA marker and Lane (11) is Negative control.

The PCR product sizes for the five carbapenem-resistant genes were as follows such as NDM-1(621 bp), blaKPC(498 bp), blaOXA-48 (281 bp), blaVIM(502 bp) and for blaIMP(568 bp).After conducting gel electrophoresis and analyzing specific data, it was observed that the detection rate for the NDM-1 gene was notably higher, approximately 11.11% showing positive results. This finding was based on the examination of 27 isolated samples, out of which 3 yielded positive results for the presence of NDM-1. The ladder used for comparison was 50 base pairs (bp), and the positive results were evident upon comparing the ladder bands with the samples. Furthermore, it should be noted that for the genes blaKPC, blaOXA-48, blaVIM, and blaIMP, their expected size on the ladder was 1K base pair (kbp). However, during the analysis, no positive result was obtained for any of these four genes as well as no bands found within their range.

Chapter 4

Discussion

The widespread development and spread of acquired carbapenemases in Gram-negative bacteria are regarded as a significant global health issue. Although the incidence and type of carbapenemase enzyme produced by organisms varies from one region to the other, but when there is an emergence of resistance to a new antibiotic in one part of the world, it transmits to the other parts so rapidly that it knows no boundary (Kumarasamy et al., 2010). The important aspect of the global understanding of carbapenemases is recognizing their wide distribution among different bacterial species, such as *Klebsiella pneumoniae* whereas the emergence of carbapenemase-producing strains of *Klebsiella pneumoniae* represents a significant threat for the successful treatment and implementations of pneumonia.

NDM-1 gene is the prominent member of the carbapenemase family, and is a major global problem in the perspective of antibiotic susceptibility. In 2008, NDM-1 was initially identified. NDM, owing to its expeditious global distribution and its capacity to be expressed by a multitude of Gram-negative pathogens, is positioned to emerge as the most frequently isolated and distributed carbapenemase on an international scale (Patel and Bonomo). This NDM-1 gene, initially discovered in New Delhi, India, rapidly spread across national boundaries, highlighting the complex network of contemporary healthcare systems and worldwide travel. On an article, Abdul Ghafur highlights the widespread non-prescription use of antibiotics in India, leading to huge selection pressure, and predicts that the NDM-1 problem is likely to get substantially worse in the foreseeable future (Karthikeyan et al.). The extensive occurrence of carbapenemases in various bacterial species poses a significant obstacle, highlighting the crucial function they have in the overall context of antibiotic resistance. Gaining a comprehensive understanding of the ramifications of NDM-1 is essential for formulating successful tactics in the ongoing fight against germs that are resistant to several drugs.

Moreover, the global concern surrounding antibiotic resistance is accentuated by genes like blaKPC, blaOXA-48, blaVIM, and blaIMP. These genes, prevalent in bacteria such as Klebsiella pneumoniae and Enterobacteriaceae, encode enzymes that deactivate crucial carbapenem antibiotics (Castanheira et al., 2011). Their widespread presence poses a serious threat to public health, limiting effective treatment options and heightening the risk of untreatable bacterial infections on a global scale. A point mutant variation of OXA-48, exhibiting comparable carbapenemase activity, has been detected in strains originating from India and strains with Indian roots. According to a study conducted by Castanheira in India, 25.64% of carbapenem-resistant strains (10 out of 39) were found to carry the blaOXA-48 gene. The current study indicates that organisms producing the blaOXA-48 carbapenemase are emerging as a significant contributor to carbapenem resistance in Gram-negative bacteria in Bangladesh, alongside organisms producing blaNDM-1. (Poirel et al., 2012)

NDM-1 positive bacteria can destroy carbapenem antibiotics which have been serving as the basis for the treatment of antibiotic-resistant bacterial infections. The spread of pathogenic microorganisms carrying NDM-1 gene now becomes potentially a major global health problem. In this study, out of 25 carbapenem resistant *Klebsiella pneumoniae* isolates, 2(8%)

NDM-1 positive were detected by PCR from non clinical sources of water and vegetable samples. A journal published in the Lancet Infectious Diseases finds that the NDM-1 gene is prevalent in the water used for cooking, washing, and drinking in Delhi (Boseley). Moreover, out of 2 carbapenem resistant *Klebsiella pneumoniae* isolates, 1(50%)NDM-1 producers were detected from clinical sources specifically from the blood samples. In an article, it finds that the NDM-1 positive strains present major health problems as these strains show resistance against commonly used antibiotics, leaving the clinicians with limited options (Nahid et al.).

Limitations

While conducting PCR for the genes NDM-1, blaKPC, blaOXA-48, blaVIM, and blaIMP, only positive and negative controls were available for NDM-1. Consequently, proper bands and results were observed for NDM-1. However, for the remaining four genes, no positive control was available in our laboratory, resulting in uncertainty in result confirmation. Additionally, no bands were observed, and no results were obtained for these four genes and positive controls for these genes would enable further confirmation.

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