Prevalence of Carbapenem-resistant *Klebsiella pneumoniae* in vegetable and water samples in Dhaka South

By

Mugenyi Raymond

16226013

Bisoboka Claire Precious

16236012

Ashrin Haque

17126030

Mohammad Mushfiqur Rahman

17126032

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 Microbiology and Biotechnology

Department of Mathematics and Natural Sciences

BRAC University

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Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing a 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 that has been accepted or submitted, for any other degree or diploma at a university or other institution.

4. I/We have acknowledged all main sources of help.

Student's Full Name & Signature:

_____	_____
Mugenyi Raymond	Bisoboka Claire Precious
16226013	16236012
_____	_____
Ashrin Haque	Mohammad Mushfiqur Rahman
17126030	17126032

Approval

The thesis/project titled "Prevalence of Carbapenem-resistant *Klebsiella pneumoniae* in vegetable and water samples in Dhaka South." submitted by

1. Mugenyi Raymond (16226013)
2. Bisoboka Claire Precious (16236012)
3. Ashrin Haque (17126030)
4. Mohammad Mushfiqur Rahman (17126032)

of Spring, 2021 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology and Biotechnology on 28 March, 2022.

Examining Committee:

Supervisor:
(Member)

_____
Mahbubul Hasan Siddiqee, PhD
Assistant Professor, Coordinator Microbiology Program,
Department of Mathematics and Natural Sciences
BRAC University

Program Coordinator:
(Member)

_____
Mahbubul Hasan Siddiqee, PhD
Assistant Professor, Coordinator Microbiology Program,
Department of Mathematics and Natural Sciences
BRAC University

Departmental Head:
(Chair)

_____
A F M Yusuf Haider, PhD
Professor and chairperson
Department of Mathematics and Natural Sciences
BRAC University.

Ethics Statement

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

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Abstract

Carbapenems are commonly considered last-resort antibiotics in the treatment of severe infections caused by multidrug-resistant *Enterobacteriaceae*. The emergence of carbapenem-resistant isolates over the last decade has been a major concern as it has contributed to narrowing the current treatment palette, leaving few or in some cases optimal treatment options. The present research was done to isolate and assess the prevalence of carbapenem-resistant *Klebsiella pneumoniae* in vegetable and water samples from hospital adjacent areas in Dhaka South. In this study, 55 vegetable and 50 water samples were randomly collected from different local markets near hospitals in Dhaka South. These samples were collected from September 2021 to November 2021. Out of 606 strains isolated throughout the study (from both enrichment and without enrichment cultures), 77 (12.71%) were *Klebsiella pneumoniae*. These isolates were subjected to an antibiotic susceptibility test using the following 12 antibiotics; Meropenem+EDTA (MR+ED), Imipenem (IMI10), Kanamycin(K30), Gentamicin (CN10), Ceftazidime (CAZ30), Erythromycin(E15), Nitrofurantoin (NIT300), Ampicillin (AMP10), Amoxyclav (AMC30), Nalidixic acid (NA30), Streptomycin(S10), and Tetracycline (TE30). All the 77 *Klebsiella pneumoniae* isolates were found to be resistant to Meropenem + EDTA (100%), followed by Nalidix Acid and Erythromycin to which 72.70% and 63.60% were resistant, respectively. This was then followed by Kanamycin (57.10%), Ampicillin (55.80%), Ceftazidime (51.90%), Nitrofurantoin (49.40%), Tetracycline (33.80%), Streptomycin (18.20%), Imipenem (15.60%), Gentamicin (10.40%) and Amoxyclav (7.80%). 4 (5.19%) of the *K. pneumoniae* isolates were resistant to all the 12 tested antibiotics. When the Multiple Antibiotic Resistance (MAR) index was calculated, 5(6.49%), 39(50.65%), 29(37.66%), and 4(5.19%) were identified under MAR indices <0.2, 0.2-<0.5, 0.5-<1 and 1 respectively.

PCR was used to detect carbapenemase genes; $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-2}}$ in 59 DNA-extracted *K. pneumoniae* isolates. 5(8.47%) of the isolates were positive for the $bla_{\text{NDM-1}}$ gene, out of which, 3 and 2 came from samples collected from Anandabazar and Moulovibazar respectively. On the other hand, 9(15.25%) of the isolates were positive for the $bla_{\text{OXA-2}}$ gene, out of which, 5, 3, and 1 came from samples collected from Anandabazar, Moulovibazar, and Moghbazar respectively. The findings of the current study demonstrated the potential of vegetables and water from markets located in hospital adjacent areas of Dhaka South, to be reservoirs of carbapenem-resistant - multidrug-resistant bacteria.

Keywords: Carbapenem-Resistant *Enterobacteriaceae*, bla-NDM1, bla-OXA2, *Klebsiella pneumoniae*, Hospital Adjacent Area

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List of Acronyms

AMR	Antimicrobial resistance
ARB	Antibiotic-resistant bacteria
ARGs	Antibiotic resistance genes
AST	Antibiotic susceptibility testing
BHI	Brain heart infusion
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders.
BPW	Buffered Peptone Water
CDC	Centers for Disease Control and Prevention
CFU	Colony-Forming Unit.
CLSI	Clinical and Laboratory Standards Institut
CPE	Carbapenemase-producing Enterobacteriaceae
CRE	Carbapenem-resistant Enterobacteriaceae
DMCH	Dhaka Medical College Hospital
DSCC	Dhaka South City Corporation
EDTA	Ethylenediaminetetraacetic Acid
ESBL	Extended-spectrum b-lactamases
HAR	Hospital adjacent areas
MAR	Multiple Antibiotic Resistance
MBL	Metallo beta-lactamases
MDR	multidrug-resistant
MHA	Muella Hinton Agar
NDM	New Delhi metallo-ß-lactamase
NTC	No Template Control
OXA	Oxacillinase
PCR	Polymerase chain reaction
TSA	Tryptone Soy Agar
VE	Vegetable Enrichment
VP	Vegetable Without Enrichment
WE	Water Enrichment
WP	Water Without Enrichment
XDR	Extensively drug-resistant

Chapter 1
Introduction

1.1 Carbapenems

Carbapenems are the favored last-resort drugs for the treatment of multidrug-resistant (MDR) bacterial infections. They are also the mainstay of therapy for patients with serious and life-threatening infections caused by Enterobacteriaceae, which produce extended-spectrum b-lactamases (ESBL) (Potter et al., 2016).

Carbapenems (imipenem, meropenem, biapenem, ertapenem, and doripenem) have a penicillin-like five-membered ring, but the sulfur at C-1 in the five-membered ring is replaced with a carbon atom and a double bond between C-2 and C-3 is introduced (Jeon et al., 2015). The various carbapenems differ primarily in the configuration of the side chains at C2 and C6(Moellering RC Jr, 2018). This unique molecular structure confers remarkable stability against the majority of β-lactamases, including extended-spectrum β-lactamases (ESBLs) (Elshamy & Aboshanab, 2020).
In 1976, thienamycin, a naturally derived product of *Streptomyces cattleya*, was the first discovered carbapenem (Elshamy & Aboshanab, 2020). Unfortunately, thienamycin was found to be unstable in aqueous solution, sensitive to mild base hydrolysis (above pH 8.0), and highly reactive to nucleophiles, such as hydroxylamine, cysteine, and even thienamycin's own primary amine (J. S. Kahan et al., 1979). The chemical instability of thienamycin stimulated the search for analogous derivatives with increased stability. Due to the continued evolution of cephalosporin-resistant Gram-negative and Gram-positive pathogens, compounds derived from thienamycin were anticipated to have even greater value with time (Wright, 1981).

The first developed was the *N*-formimidoyl derivative, imipenem, which was a more-stable derivative of thienamycin and less sensitive to base hydrolysis in solution (Papp-Wallace et al., 2011). In 1985, imipenem (originally called MK0787) became the first carbapenem available for the treatment of complex microbial infections. Because of the lability of imipenem to hydrolysis by the human renal dehydropeptidase (DHP) causing inactivation of the drug (Kropp et al., 1982), it is dosed in combination with cilastatin, a DHP inhibitor that also acts as a neuroprotectant (F. M. Kahan et al., 1983).

Along the journey to the discovery of more-stable carbapenems with a broader spectrum, the other currently available compounds, meropenem, biapenem, ertapenem, and doripenem, were

developed (Papp-Wallace et al., 2011) with generally the same group of organisms included in their activity spectrum (Baughman, 2009). All these carbapenems are more stable chemically than imipenem, thus allowing for longer shelf life for the formulated drug and the potential for prolonged infusion times (Cielecka-Piontek et al., 2008; Prescott et al., 2011). Like imipenem, they are stable to most β-lactamases, other than the carbapenemases. (Bush, 2013). Meropenem, doripenem, ertapenem, and imipenem are the four most clinically used carbapenems (Yu et al., 2019). In terms of antibacterial activity, meropenem is generally twofold to fourfold more potent than imipenem against enteric bacteria (Lutgring, 2019).

Doripenem, a carbapenem with somewhat higher chemical stability than imipenem or meropenem, follows the antibacterial profile of meropenem but is slightly more potent against Gram-negative organisms. Ertapenem, recognized for its long elimination half-life in humans because of its high protein binding (95%), may be effectively administered once daily in contrast to the other carbapenems that are dosed most commonly two or three times a day. Although its antibacterial spectrum is similar to the other carbapenems against Enterobacteriaceae, ertapenem differs from imipenem, meropenem, and doripenem in that it has no useful activity against *P. aeruginosa* (Bush & Bradford, 2016).

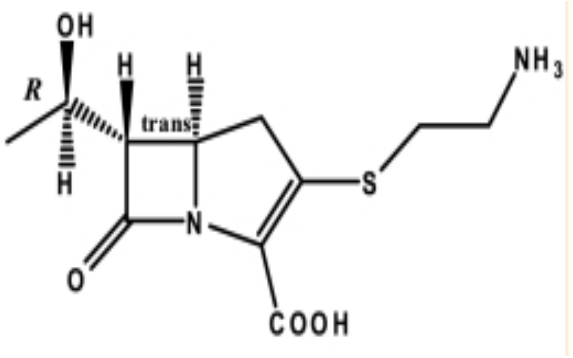


Figure 1.1.1 The chemical structure of thienamycin. (Papp-Wallace et al., 2011)

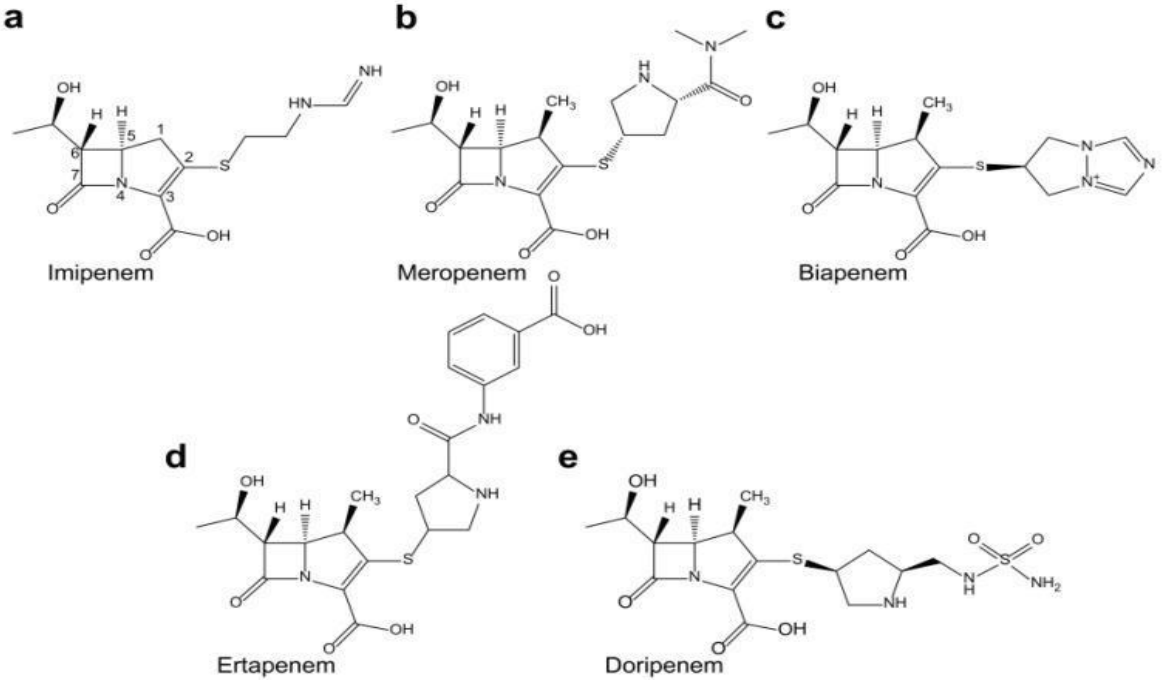


Figure 1.1.2: Chemical structures of (a) imipenem; (b) meropenem; (c) biapenem; (d) ertapenem; and (e) doripenem. The β-lactam nucleus is numbered. (Jeon et al., 2015)

1.1.1 Mechanism of action of carbapenems

As a class of β-lactams, carbapenems are not easily diffusible through the bacterial cell wall. Carbapenems enter Gram-negative bacteria through outer membrane proteins (OMPs), also known as porins. After traversing the periplasmic space, carbapenems "permanently" acylate the PBPs. PBPs are enzymes (i.e., transglycosylases, transpeptidases, and carboxypeptidases) that catalyze the formation of peptidoglycan in the cell wall of bacteria. Carbapenems act as mechanism-based inhibitors of the peptidase domain of PBPs and can inhibit peptide cross-linking as well as other peptidase reactions. A key factor of the efficacy of carbapenems is their ability to bind to multiple different PBPs. Since cell wall formation is a dynamic "three-dimensional process" with formation and autolysis occurring at the same time, when PBPs are inhibited, autolysis continues. Eventually, the peptidoglycan weakens, and the cell bursts due to osmotic pressure. (Papp-Wallace et al., 2011)

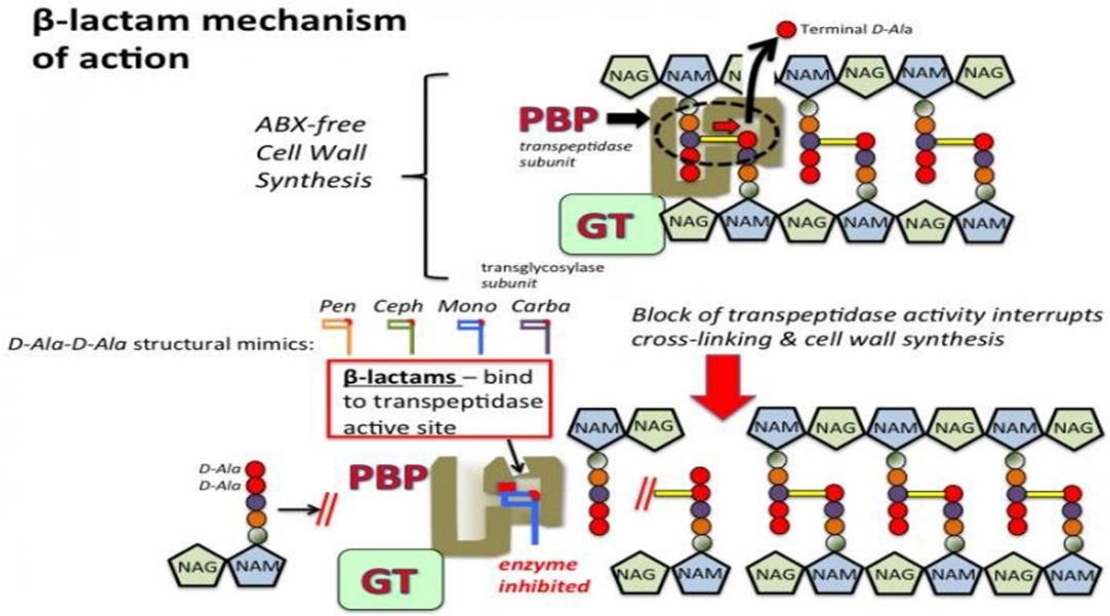


Figure 1.1.1: Mechanism of action of beta-lactam antibiotics (Carbapenems - Creative Biolabs, n.d.)

1.2 Carbapenem resistance

Over the past decade, the emergence of carbapenem-resistant isolates is very unnerving, since it contributes to the reduction of the current therapeutic pallet, therefore leaving few or, in some cases, no optimal therapeutic options (Andrade et al., 2020). Resistance to carbapenems may be attributed to three major mechanisms: porin-mediated resistance to reduce uptake of carbapenems, efflux pumps, which pump the carbapenem outside the cells, and enzyme-mediated resistance which is mediated via the acquisition of carbapenemase genes (Elshamy & Aboshanab, 2020).

1.2.1 Porin-mediated resistance

Bacteria can limit the entry of carbapenems into the periplasmic space where PBPs are located. This mechanism involves the modification of porin expression or alterations in the porin-encoding gene, leading to either complete loss or defects in the respective porin (Doumith et al., 2009).

1.2.2 Overproduction of efflux pumps

Efflux pumps are generally able to recognize numerous substrates, given that affinity is based on physio-chemical properties (e.g., electric charge, aromatic or hydrophobic properties) instead of chemical structures. This explains the presence of MDR efflux pumps which can expel many structurally unrelated antimicrobials (Lomovskaya et al., 2007). Gram-negative bacteria such as *P. aeruginosa* and *Acinetobacter* species are well known for their efflux-mediated β-lactam resistance (Quinn, 1972). The overexpression of efflux pumps active on carbapenems may lead to carbapenem resistance (G. Meletis et al., 2012; Georgios Meletis, 2016).

1.2.3 Enzyme-mediated resistance

In most cases, resistance is due to the production of β-lactamases capable of hydrolyzing carbapenems and other β-lactam antimicrobials, hence they are called carbapenemases. This resistance mechanism poses the greatest threat, as these enzymes can inactivate the majority of β-lactams and are encoded by genes carried on transposons, plasmids or other mobile genetic elements, which can be horizontally transferred to other bacterial species (Georgios Meletis, 2016).

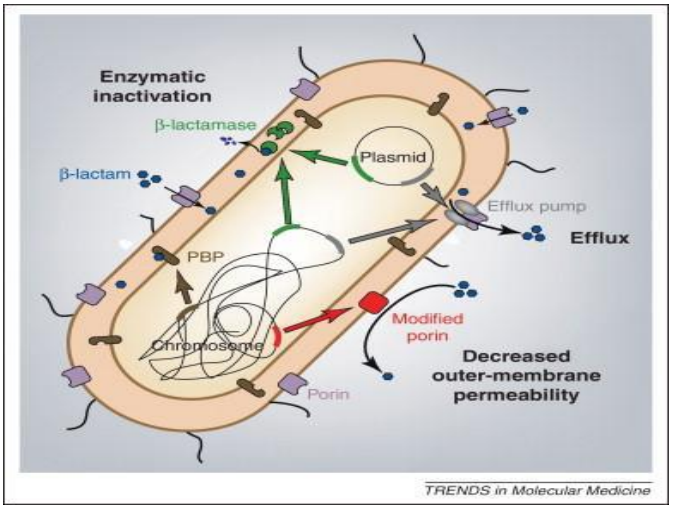


Figure 1.2: Primary mechanisms of carbapenem resistance. (Patrice Nordmann et al., 2012)

1.2.4 Other factors responsible for carbapenem resistance

Although not formally carbapenemases, AmpC b-lactamases, such as CMY (class C) and some extended-spectrum b-lactamase (ESBL), can also cause carbapenem resistance, especially when combined with other resistance mechanisms, such as porin loss or efflux mechanisms. Porin modifications can cause a decrease in the diffusion rate of the antibiotics across the Gram-negative outer membrane at a rate that allows ESBLs and AmpC enzymes to sufficiently break down the remaining antibiotic to result in a fully resistant phenotype. Modifications in efflux pumps can cause an increase in antibiotic expulsion from the cell before the drug is able to bind to PBPs, thus rendering it incapable of harming the cell. Finally, modifications within the PBPs themselves prevent the proper binding of the b-lactams to the targeted proteins, rendering them useless (Anderson & Boerlin, 2020).

1.3 Beta-lactamases

Beta-lactamases, (β-lactamases) are enzymes produced by bacteria that provide multi-resistance to beta-lactam antibiotics such as penicillins, cephalosporins, monobactams, and carbapenems (ertapenem), Beta-lactamase provides antibiotic resistance by breaking the antibiotics' structure. These antibiotics all have a common element in their molecular structure: a four-atom ring known as a beta-lactam (β-lactam) ring. Through hydrolysis, the enzyme lactamase breaks the β-lactam ring open, deactivating the molecule's antibacterial properties

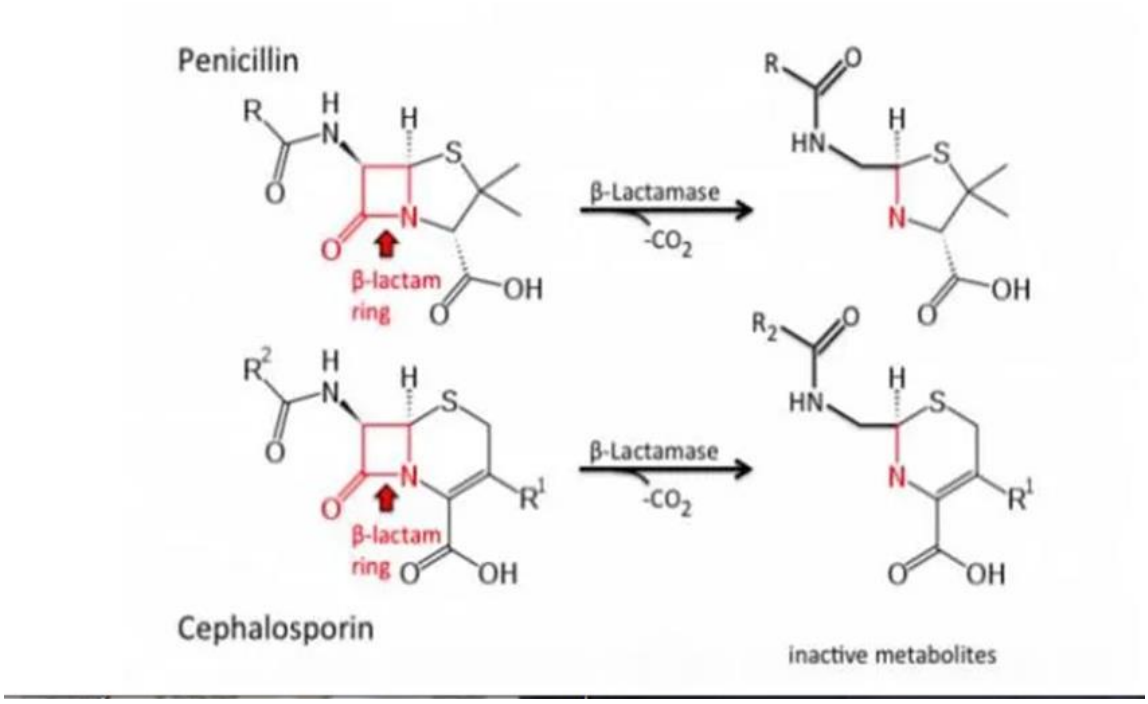


Fig 1.3.1: Mechanism of action of beta-lactamases (Zango et al., 2019)

Over 1,000 β-lactamases have been reported so far and they are grouped into four classes A to D based on sequence and structural similarities (Bush & Jacoby, 2010; Jacoby, 2006). Hydrolysis of β-lactam antibiotics by β-lactamases always involves a critical water molecule that, upon activation, carries out a nucleophilic attack on the β-lactam moiety to hydrolyze and "open" its ring structure (Drawz & Bonomo, 2010; Majiduddin et al., 2002; Wilke et al., 2005). Class A, C, and D β-lactamases are serine-based enzymes that execute the hydrolytic process in two steps. At the first acylation step, a conserved catalytic serine residue carries out a nucleophilic attack on the β-lactam substrate, leading to the formation of an acyl-enzyme adduct (ES*). At the subsequent deacylation step, an activated water molecule attacks ES*, leading to its hydrolysis and release from the active site with the β-lactam ring "opened". Two general base residues are required in this process, one to activate the catalytic serine residue in the acylation step, and the other to activate the hydrolytic water molecule in the deacylation step. Extensive studies have identified the individual residues that fulfill these catalytic roles in Class A, C, and D β-lactamases.

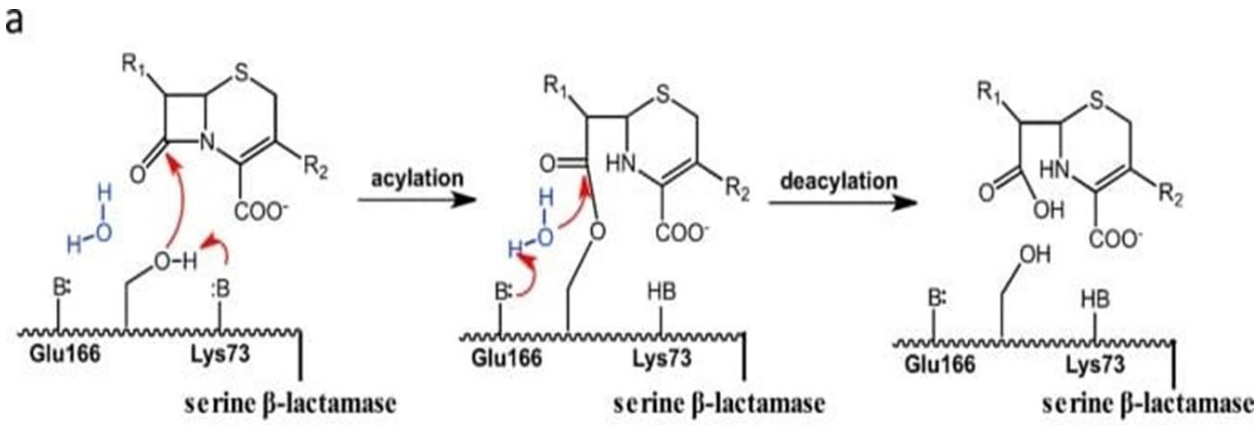


Figure 1.3.2a: The catalytic mechanism of serine-based β-lactamases of Class A, C, and D. (He et al., 2020)

In contrast to Class A, C, and D, Class B β-lactamases are metalloenzymes that employ Zn2+ or other metal ions to activate a water molecule for direct hydrolysis of the β-lactam substrate without the intermediate step of forming ES* (Drawz & Bonomo, 2010; Majiduddin et al., 2002). This distinct catalytic mechanism is partly responsible for the extreme promiscuity of Class B β-lactamases. In fact, these enzymes can readily hydrolyze all clinically available β-lactam antibiotics including the newest-generation carbapenems that are usually poor substrates for serine-based β-lactamases of Class A, C, and D (King, 2013; Papp-Wallace et al., 2011).

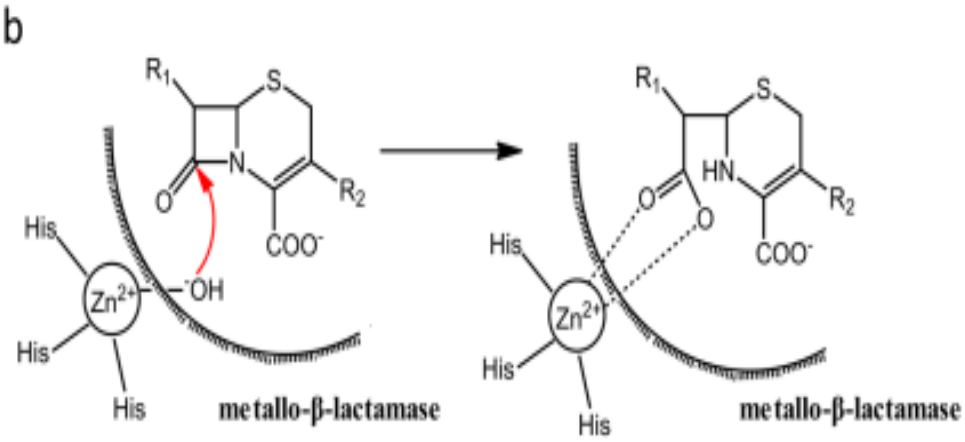


Figure 1.3.2b: The catalytic mechanism of Class B metallo-β-lactamases. Only one Zn2+ ion is shown for reference while two Zn2+ ions have been found in the active site of some Class B metallo-β-lactamases. (He et al., 2020)

1.4 Classification of carbapenemases

Based on their molecular structures, carbapenemases belong to three classes of β-lactamases; class A, B, and D.

1.4.1 Class A carbapenemases

These include *K. pneumoniae* carbapenemases (KPCs), imipenem-hydrolyzing β-lactamase (IMI), Guiana extended-spectrum carbapenemase (GES), *Serratia fonticola* carbapenemase, *Serratia marcescens* enzyme and non-metallo-carbapenemase-A (Patel & Bonomo, 2013). KPCs have the ability to hydrolyze all β-lactams and strains carrying the *blaKPC* gene are usually resistant to other antimicrobials, such as aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole, making them MDR. Thirteen KPC variants have been described so far (Djahmi et al., 2014). The most frequently reported of which are *KPC-2* and *KPC-3*. (Patrice Nordmann et al., 2009; Pfeifer et al., 2010). The *blaKPC* genes are plasmid-encoded and are thus prone to interspecies horizontal transmission (Cuzon et al., 2010).

Isolates that produce IMI might rarely be detected due to their unusual AMR profile, such isolates are usually resistant to imipenem but show intermediate resistance to ertapenem and are sensitive toward extended-spectrum cephalosporins. Moreover, the *blaIMI* gene is not included in the panel of genes targeted by commercially available molecular diagnostic kits. IMI-1 carbapenemases are chromosomally encoded and are thus considered clinically irrelevant (Miltgen et al., 2018).

1.4.2 Class B carbapenemases

In 1966, Sabbath and Abraham discovered the first-class B enzyme BCII, the *Bacillus cereus* MBL (Sabath & Abraham, 1966). By 1989, only four MBLs were discovered and were all chromosomally encoded, consequently, they were deemed clinically unimportant. Yet, in 1991, the plasmid-encoded imipenem-resistant *Pseudomonas*-type carbapenemases (IMP) were discovered in *P. aeruginosa* in Japan, which revived the clinical interest in this class of enzymes (Quinn et al., 1991). Today, MBLs are mainly plasmid-encoded, facilitating their transmission among microbial pathogens (Walsh et al., 2005). They are also the most molecularly diverse class of carbapenemases and can inactivate the majority of β-lactams, with the exception of monobactams (Walsh, 2010). New Delhi MBL (NDM) is an MBL that can confer resistance to enteric pathogens, such as *K. pneumoniae* and *E. coli*, making them resistant to β-lactams, including carbapenems (Yong et al., 2009) but not aztreonam (Doi & Paterson, 2015). Verona integron-encoded MBL (VIM) was first described in Verona, Italy, from a *P. aeruginosa* isolate in 1999. The hydrolytic profile of VIM is like other members of this class, hydrolyzing most β-lactams except for aztreonam (Walsh et al., 2005). It is worth mentioning that bacteria co-expressing SBLs and MBLs are usually able to hydrolyze the clinically relevant monobactam, aztreonam (Patrice Nordmann et al., 2011). Moreover, two MBLs, including German imipenemase and Sao Paulo MBL have been detected in the clinical isolates of *S. marcescens* and *P. aeruginosa*, respectively (Hong et al., 2015; Rieber et al., 2012).

1.4.3 Class D carbapenemases

These include the oxacillinase (OXA) enzymes, which have the ability to efficiently hydrolyze oxacillin, for which they were named (Majiduddin et al., 2002). The OXA-2 β-lactamase was the first discovered class D enzyme (Dale et al., 1985). The carbapenem-hydrolyzing OXA-48 enzyme

has high hydrolysis activity toward penicillins and low hydrolysis activity toward carbapenems (Logan & Weinstein, 2017). It is also not affected by β-lactamase inhibitors, which is why this enzyme has recently gained attention (Poirel et al., 2012). Other OXA β-lactamases such as OXA-23, OXA-24/40, and OXA-58, are frequently found in species of *Acinetobacter* but have a relatively weak carbapenemase activity (Tzouvelekis et al., 2012). One of the greatest threats posed by this class of enzymes is the lack of inhibitors for them (Majiduddin et al., 2002).

1.5 History, mechanism of action, and spread of NDM-1

New-Delhi-Metallo-beta-lactamase (NDM-1), a new type of MBL, was first detected in two K. pneumoniae and Escherichia coli strains isolated from a Swedish patient who was admitted to a hospital in New Delhi, India. In recent years, the emergence and dissemination of NDM-1-producing isolates have been reported in several countries, including the USA, Canada, Sweden, UK, Austria, Belgium, France, The Netherlands, Germany, Japan, Africa, Oman, and Australia (Baquero et al., 2008; Begum & Shamsuzzaman, 2016). NDM-producing bacteria are commonly resistant to almost all groups of antibiotics, including fluoroquinolones, aminoglycosides, and beta-lactams (especially carbapenems) but are susceptible to colistin and sometimes tigecycline. The NDM-1 gene has been detected on different large plasmids, which were readily transferable among bacteria, making NDM-1-producing bacteria a serious clinical and public health threat (van Duin & Doi, 2017). NDM-1 belongs to the Metallo-β-lactamase family containing Zn^{2+} and other divalent cations as cofactors. It inactivates almost all classes of β-lactams antibiotics including carbapenems by catalyzing the hydrolytic cleavage of the substrate amide bond. The active site contains two zinc ions. The reaction scheme itself is fairly straightforward: the beta-lactam is hydrolyzed by a water molecule that has been activated by NDM-1. The general beta-lactam ring gets hydrolyzed by a nucleophilic water molecule that has been activated by the metal Zinc ions in the active site of NDM-1.

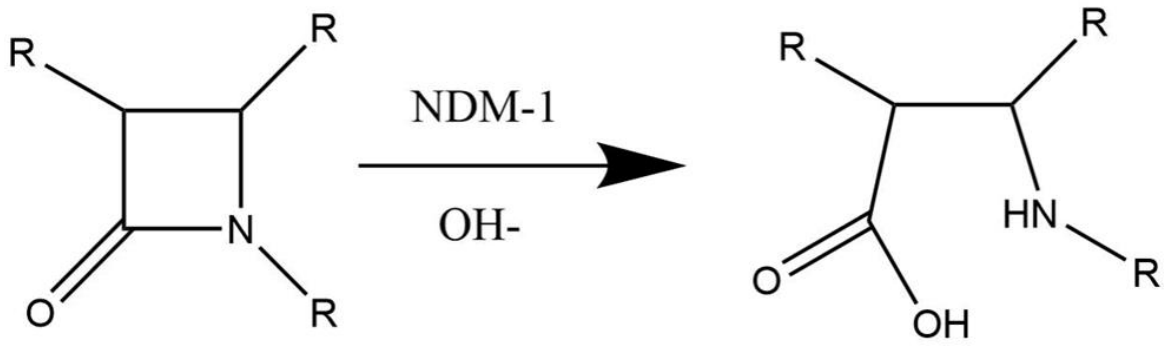


Figure 1.5: General beta-lactam ring being hydrolyzed by a nucleophilic water molecule that has been activated by the metal ions in the active site of NDM-1. (NDM-1: Metallo beta-lactamases (MBLs) and Antibiotic Resistance - Chemistry LibreTexts, n.d.)

The NDM-1 gene is located on self-transmissible plasmids that carry a considerable number of other antibiotic resistance genes. Widespread use of antibiotics in humans and in the food chain, and its spillover in the environment accelerate the development, selection, and/or horizontal transfer of antibiotic resistance plasmids in a given bacterial population. It is conceivable that the primary source of these bacteria is hospitals and other health care settings where severe cases of bacterial infections are present, and the volume of antibiotic use is high. Therefore, wastewater from hospital settings is likely to contain MDR bacteria unless these are treated adequately before discharge (P. Nordmann & Poirel, 2014).

Plasmids carrying the blaNDM-1 gene are diverse and can harbor a high number of resistance genes associated with other carbapenemase genes (OXA-48 types, VIM types), plasmid-mediated cephalosporinase genes, ESBL genes, aminoglycoside resistance genes (16S RNA methylases), macrolide resistance genes (esterase), and rifampin (rifampin-modifying enzymes) and sulfamethoxazole resistance genes as sources of multidrug resistance and pan drug resistance (Potter et al., 2016).

All NDM-producing enterobacterial species have been found to be involved in infections, but K. pneumoniae and *Escherichia coli* are the main causes of hospital and community-acquired infections, respectively (Sapugahawatte et al., 2022).

NDM producers show a more ubiquitous distribution and broader dissemination than other metallo carbapenemases, including in the environment, community, and goods. The prevalence of NDM carriers in endemic areas such as India and Pakistan reaches figures of 18% among hospitalized patients and 10% in the community. In this area, a high number of NDM-1 positive isolates were recovered from the Ganges River during seasonal pilgrimages (Andrade et al., 2020).

NDM-1 shares very little identity with other MBLs, the most similar being VIM-1/VIM-2, with only 32.4% amino acid identity. NDM-1 efficiently hydrolyses a broad range of b-lactams, including penicillins, cephalosporins, and carbapenems, but sparing monobactams such as aztreonam. Since the first description of NDM-1, eight variants of this enzyme have been published (NDM-1 to NDM-8), and 12 have been assigned most of them originated from Asia (Sapugahawatte et al., 2022).

The spread of NDM producers has been extensively identified not only among patients from the Indian subcontinent but also in the soil. Therefore, it is likely that the environment is already heavily contaminated with NDM producers. The prevalence of carriage is estimated to be 5–15% in that part of the world (Sapugahawatte et al., 2022).

1.6 History, mechanism of action, and spread of OXA-2

The OXA type of beta-lactamase is a plasmid-mediated enzyme which is found in a small proportion of ampicillin-resistant clinical isolates of Gram-negative bacteria (Dale et al., 1985). They belong to Ambler molecular class D and functional group 2d. These types of beta-lactamases are characterized by their high hydrolytic spectrum of activity against cloxacillin and oxacillin and are poorly inhibited by clavulanic acid. The presence of this gene was first reported in *Pseudomonas* in France, in *Escherichia coli* from Israel, and still in *E. coli* from India (Maurya et al., 2015).

According to (Antunes et al., 2014), the blaOXA-2 gene was previously considered a narrow spectrum class D beta-lactamase without carbapenemase activity. However, the same authors demonstrated that the OXA-2 beta-lactamase, an enzyme that was regarded as a non-

carbapenemase, has catalytic efficiencies against carbapenems similar to those of well-recognized CHDLs and is capable of conferring resistance to these last-resort antibiotics when expressed in *A. baumannii.* This was achieved when the catalytic properties and structural features of some CHDLs were elucidated and compared with those of class D enzymes that are considered non-carbapenemases (Antunes et al., 2014).

1.7 Carbapenem Resistant Enterobacteriaceae (CRE)

The World Health Organization has recognized antimicrobial resistance (AMR) as "a global health security threat that requires action across government sectors and society as a whole" (Han et al., 2020). Currently, carbapenem-resistant bacteria, especially Enterobacteriaceae (CRE) are a serious threat to global public health (Potter et al., 2016). Indeed, on the global priority list of antibiotic-resistant bacteria published by the World Health Organization in 2017, carbapenem-resistant pathogens, including carbapenem-resistant Enterobacteriaceae (CRE) are designated as being of critical priority for research and development of new antibiotics (Perilli et al., 2013). From the perspective of antimicrobial resistance, Enterobacteriaceae are especially important as they are a common cause of community-associated as well as healthcare-associated infections (Han et al., 2020). Enterobacteriaceae are a family of diverse Gammaproteobacteria which include common (*Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella enterica*) and rare (*Proteus mirabilis*, *Raoultella planticola*, *Citrobacter freundii*) human pathogens with increasing antibiotic resistance. The Clinical Laboratory Standards Institute defines Enterobacteriaceae as carbapenem-resistant if they have minimum inhibitory concentrations (MICs) of ≥2 μg/ml against ertapenem and ≥4 μg/ml against doripenem, meropenem, or imipenem [Patel 2015] (Potter et al., 2016).

In a 2013 US Centers for Disease Control and Prevention (CDC) report, carbapenem-resistant Enterobacteriaceae (CRE) were listed as one of the three most urgent antimicrobial-resistant threats. CREs received this highest threat level due to rapidly increasing global spread, propensity for multidrug resistance, and high mortality during bloodstream infections (Yu et al., 2019).
The first carbapenemase identified in Enterobacteriaceae was the chromosomally encoded NmcA from an Enterobacter cloacae clinical isolate in 1993. Since then, carbapenem-resistant Enterobacteriaceae have been reported worldwide, mostly as a consequence of the acquisition of carbapenemase genes (Benjamin Chun-Kit Tong, 2017).

The prevalence of CRE infections in the community is largely unknown, but a recent review found that percentages range from 0.04 to 29.5%. From a One Health perspective, the occurrence of CRE also poses a risk for public health. Notably, a prevalence of less than 1% among livestock and companion animals in Europe, but 2–26% in Africa, and 1–15% in Asia, has been documented (Bennett, 2008). Carbapenem-resistant Enterobacteriaceae (CRE) infections lead to longer lengths of stay, increased healthcare costs, and higher mortality than carbapenem-susceptible infections.

blaNDM-1 was the most common gene (36.8%, 60/163) in Enterobacteriaceae. The Study for Monitoring Antimicrobial Resistance Trends identified blaNDM-1 (96.3%, 130/135) as the most common variant in NDM-associated infections from 2008 to 2012 across geographically diverse countries (Andrade et al., 2020).

Three groups of carbapenemases – KPC, NDM, and OXA-48 – are currently considered to be the three major β-lactamases of epidemiological and clinical significance. Unfortunately, even carbapenems were not immune to the remarkable ability of Enterobacteriaceae to adapt to selective pressure. In the early 1990s, Enterobacteriaceae with resistance to carbapenems (carbapenem-resistant Enterobacteriaceae, or CRE) emerged in Japan, followed by neighboring countries. These strains produced Metallo-β-lactamase (MBL) IMP-1, which was capable of hydrolyzing carbapenems and was encoded on plasmids that could transfer from one species to another (López-Cerero & Almirante, 2014).

1.7.1 Detection of Carbapenem-Resistant Enterobacteriaceae (CRE)

The inability of classical antimicrobial susceptibility tests (ASTs) to clearly differentiate between carbapenemase-producing *Enterobacteriaceae* (CPEs) and other types of carbapenem-resistant *Enterobacteriaceae* (CREs) has emphasized the need for alternative methods for identifying CREs. Multiple methods have been developed in response to this need with variable success. The main approaches to date include i) tests based on carbapenemase inhibitors; ii) growth tests based on inactivation of carbapenems; iii) tests based on detection of carbapenem hydrolysis products; iv) immunochromatographic tests to detect carbapenemases, and v) Matrix-Assisted Laser

Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF) (Anderson & Boerlin, 2020).

1.8 Carbapenemase-producing Enterobacteriaceae (CPE)

Carbapenemase-producing Enterobacteriaceae (CPE), notably *Klebsiella pneumoniae*, produce serious infections (urinary tract infections, septicemia, pneumonia, and intra-abdominal infections) in debilitated and immune-compromised patients, in association with prolonged hospitalization and increased fatality ranging from 24% to 70%, depending on the study population (Patrice Nordmann et al., 2012). CPE are spreading globally as multidrug-resistant pathogens for which there are only a few treatment options available (Lutgring, 2019).

K. pneumoniae, the most commonly encountered CPE, is responsible for a dramatic increase in disease burden worldwide. One of the challenges among hospitalized patients is the asymptomatic gastrointestinal carriage of CPE which precedes, and significantly increases, the risk of developing infections caused by these pathogens (Bennett, 2008).

The ability of CPE to evolve and adapt rapidly due to antibiotic selective pressures is one of the biggest threats to medical care. An international, multi-disciplinary approach is urgently required to tackle this global threat. Pressing issues include improving surveillance to recognize the importance of mobile AMR elements and increasing the drive to move rapid, high-resolution diagnostics, such as whole-genome sequencing, from the research environment into routine clinical practice. A proactive approach involving all users of antimicrobials is imperative to prevent a return to the pre-antibiotic era (Oteo et al., 2014).

1.9 Carbapenem-resistant Klebsiella pneumoniae

Carbapenems have conventionally been used for treating infections caused by extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*, and are still considered as last resort antibiotics to date. According to the data from China Antimicrobial Surveillance Network (CHINET, www.chinets.com), the resistance rate of *K. pneumoniae* to meropenem and imipenem rapidly increased from 2.9 and 3.0% in 2005 to 26.3 and 25% in 2018, respectively. In Europe,

carbapenem-resistant *K. pneumoniae* are most widespread in the Mediterranean and Balkan countries with a prevalence of 60% in Greece and 40% in Italy, respectively (Han et al., 2020). In fact, antibiotic resistance occurring in community settings is, by definition, very difficult to contain, and diarrhea is the source of further spread of NDM producers in the environment, at least in Southeast Asia. It may therefore be expected that outbreaks caused mostly by NDM-producing *K. pneumoniae* will be increasingly reported worldwide (Sapugahawatte et al., 2022).

While in the past, studies have identified mainly environmental bacteria carrying chromosomal b-lactamase resistance genes, recent studies paint a very different picture, with a high prevalence of pathogenic bacteria (*Salmonella spp.*) and opportunistic pathogens such as *E. coli, Klebsiella pneumoniae, Citrobacter spp. and Enterobacter spp.;* bacteria that are not only able to cause community-acquired human infections but often carry resistance genes located on transmissible plasmids(Patrice Nordmann et al., 2012).

The most important mechanism of carbapenem resistance in the Enterobacterales order is the acquisition of plasmid-mediated carbapenemases, specifically three of the four Ambler classes. These are rapidly expanding globally, are proliferating at an unprecedented rate, are distributed in many species of Enterobacterales but are dominated by *K. pneumoniae* (Bennett, 2008).

1.10 Role of hospitals and nonmedical environments in the spread of antimicrobial resistance.

Due to the excessive use of antibiotics in hospitals, not only the clinically important bacteria but also the bacteria in the aquatic environment have become resistant to various antibiotics. Hospitals play an important role in the release and spread of antibiotic-resistant bacteria (ARB) in the environment. Studies revealed that hospital discharge is a highly selective environment, consisting of different types of bacteria, nutrients, and also antimicrobial agents that can thus provide close contact between bacteria and antibiotics (P. Nordmann et al., 2012).

Other studies have also reported that a substantial increase in the prescription of certain β-lactam antibiotics, such as ceftriaxone, meropenem, and ertapenem has led to the growth of ARB in hospital effluents (P. Nordmann et al., 2012).

While in past times, antimicrobial resistance was associated with hospital settings and medical care, nowadays, the dissemination of ARBs in non-medical environments is recognized and identified as an evolving problem (Patrice Nordmann et al., 2012). Due to the intense use of antibiotics in agriculture, fresh produce that is often consumed raw, such as fruits and vegetables, have been recognized as vectors for the transmission of pathogenic bacteria and ARBs (Patrice Nordmann et al., 2012). In addition, contamination of fresh produce can occur during harvesting, distribution, and in stores, as a result of non-hygienic human practices and incorrect handling (Patrice Nordmann et al., 2012). From surveillance of AMR, the knowledge of the associated bacteria and molecular elements is important in our aim to control the multidrug-resistant (MDR) Gram-negative pathogenic infection burden in the veterinary and public health sectors (Patrice Nordmann et al., 2012).

1.11 Research rationale

Reviewing the previous articles, the following research gaps were found;

- Food samples from the hospital adjacent areas, as potential reservoirs for carbapenemase genes, were not highlighted in any previous study in Bangladesh.
- The dissemination of the bla-OXA2 gene is not well understood in Bangladesh as this is not highlighted in any previous study.

1.12 Research hypothesis

The number of carbapenem-resistant Klebsiella pneumoniae strains will increase in proportion to the number of collected samples. High numbers of samples will contain increased *Klebsiella pneumonia*e strains resistant to Carbapenems and this carbapenem resistance is influenced by bla-NDM1 and bla-OXA2 genes.

1.13 Objectives of the Study

To isolate and determine the prevalence of carbapenem-resistant *Klebsiella pneumoniae* carrying carbapenemases from fresh vegetables and their wash water from local community markets located in hospital adjacent areas in Dhaka South.

The knowledge from this study highlights the role that hospitals play in spreading ARG to the environment by draining their untreated hospital wastewater into the same water and environmental resources that supply the community. This should encourage better management of hospital wastes to lower the risk of transmission of antimicrobial resistance genes to the environment.

Chapter 2
Materials and Methods

2.1 The work plan

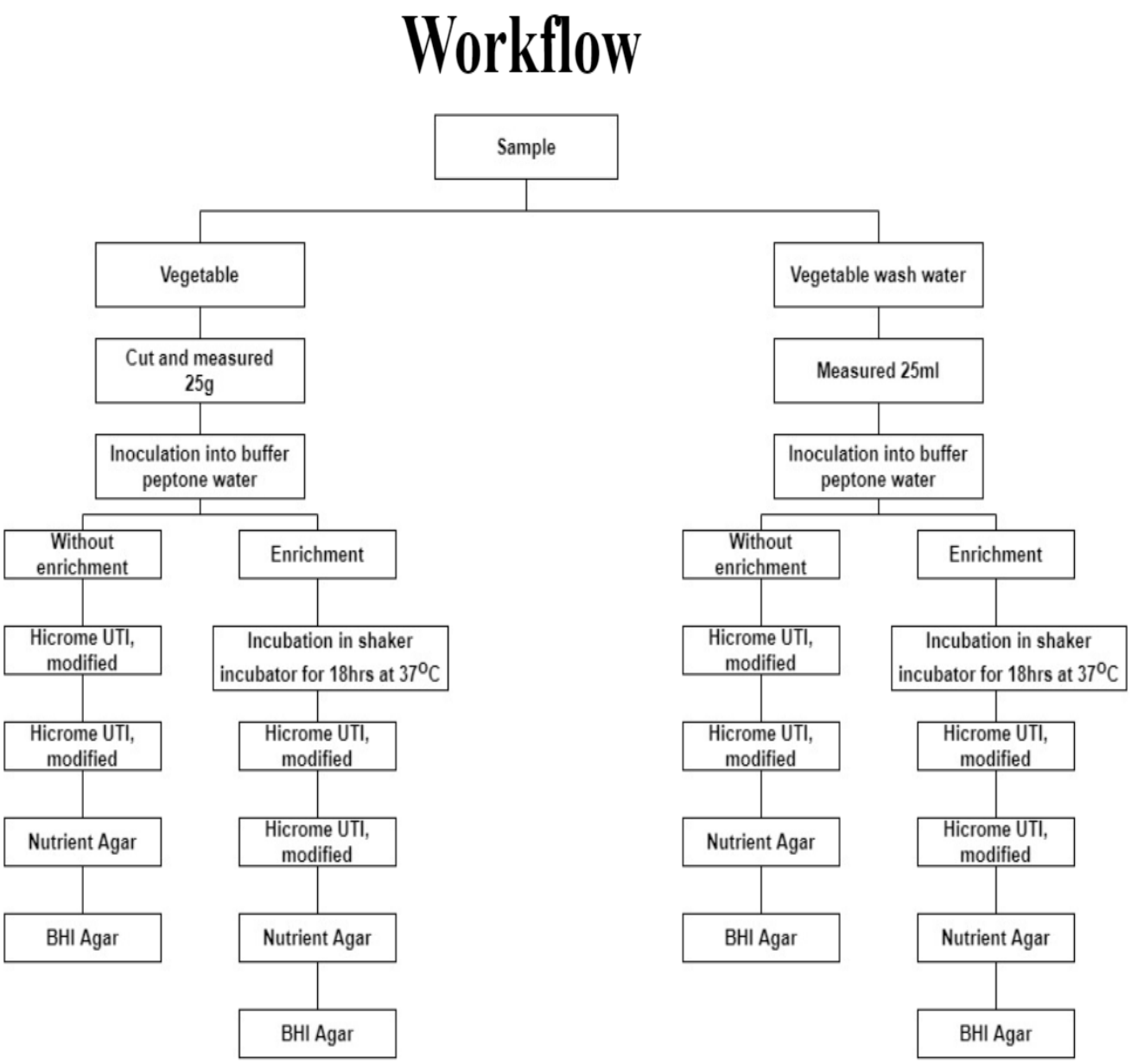
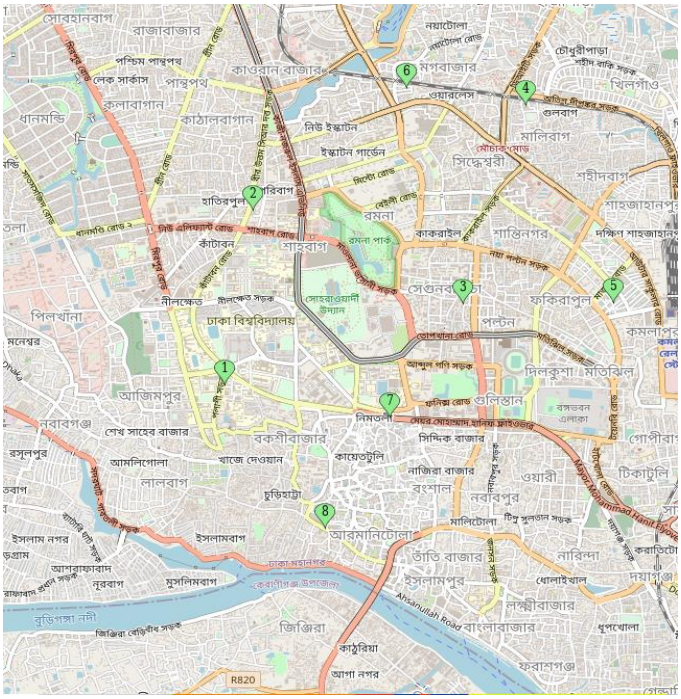


Fig 2.1: Workflow

2.2 Sampling Sites

The study site was Dhaka metropolitan area, especially southern Dhaka. We collected vegetable and water samples from local markets which are near the hospitals. These samples were collected from September to November 2021, mostly from hospital adjacent areas (HAR) of southern Dhaka city. Samples were obtained from community local markets near the large public/private hospitals or clinics which are located in the southern part of Dhaka city. From each market, we collected both vegetables and water samples. Samples were collected from the vendors, in case of water, we took the ones they use to wash vegetables with. We collected 50 water samples from HAR and an equal number of tap vegetable samples from the same areas. According to DSCC, in southern Dhaka which covers an area of approximately 127.63km2, 9 local markets were selected based on the presence of hospitals.



1	Palashi Bazar	23.727075,90.389618
2	Hatirpool Kachabazar	Hatirpool Kacha Bazar, Bir Uttam CR Datta Road, Hatirpool, Poribag, Kawran Bazar, Dhaka, Dhaka Metropolitan, Dhaka District, Dhaka Division, ১২০৫, Bangladesh
3	Shegun Bagicha Bazar	23.733639,90.409137
4	Malibag Bazar	23.749840,90.414310
5	Motijheel Ideal Colony Kacha Bazar	B-20, এজিবি কলোনি কাঁচাবাজার সড়ক, Arambag, Kamalapur, Dhaka, Dhaka Metropolitan, Dhaka District, Dhaka Division, ১০০০, Bangladesh
6	Mogbazar Kacha bazar	23.751251,90.404561
7	Ananda Bazar	Omor Akoshe Hall, Old Secretariat Road, Nimtali, Bongshal, Dhaka, Dhaka Metropolitan, Dhaka District, Dhaka Division, 1000, Bangladesh
8	Mowlvi Bazar	Gulbodon Super Market, 5, Mowlovi Bazar Road, Moulovibazar, Chawkbazar, Dhaka, Dhaka Metropolitan, Dhaka District, Dhaka Division, 1100, Bangladesh

Figure 2.2: Sampling sites

2.3 Sample collection

All the samples were randomly collected from community markets which were nearby hospitals in the morning between 9 am to 12 pm. There were two categories of samples where the first one was fresh vegetables and the second one was Vegetable wash water. To avoid cross-contamination while collecting samples one pair of gloves and a sterile ziplock bag were used. Moreover, for carrying the samples to the lab sterile ice boxes and ice packs were used.

As vegetable samples, 50g of coriander, green chili, betel leaf, cucumber, spinach, tomato, lady's finger, carrot, lemon, bean, etc were used and were collected from the community market. To avoid risk while collecting samples proper precautions such as wearing aprons and gloves were taken.

For collecting water samples autoclave falcon tubes of 50ml were used and for water samples, vegetable-washed water was used. To avoid leakage and cross-contamination the tubes were sealed with caps and placed in the falcon tube rack which was inside the icebox. After collecting all sorts of samples, the used gloves were kept in a separate Ziplock bag and the hands were sanitized with ethanol. Afterward, all the samples were transported to the lab within 2 hours of collection through a bike which maintained a speed of 30 to 40km/hr. to avoid accidents and sample damage.

2.4 Sample Processing

For sample processing 10 autoclaved scissors, 20 pairs of gloves, autoclaved Buffer Peptone Water (BPW), ethanol, and a weighing machine were used. For each vegetable sample, the items were cut into small pieces with sterile scissors and 25g of each sample was measured using the weighing scale. These measured samples were then dipped into conical flasks which contained 225ml of autoclaved Buffer Peptone Water (BPW). To properly mix the sample into autoclaved Buffer Peptone water the conical flask was shaken a little. On the other hand, for water samples, 25ml of each water sample was taken and poured into different conical flasks which contained BPW. After pouring, the flasks were shaken properly to get mixed and the top of the flask was sealed using foil paper and masking tape. Then the flasks were placed into the shaker incubator for 24 hours of incubation at 37°C and 100Rpm.

In this study there were two phases, the first one was without enrichment and the next one was enrichment. In both phases, Hicrome UTI modified agar, incorporated with meropenem and ZnSO4 were used. In the without enrichment phase, 1 loopful was taken from each conical flask carrying vegetable and water samples streaked on Hicrome UTI modified agar plate. The streaked plates were incubated for 24 hours at 37°C. After the without enrichment phase streaking, the conical flasks were put into a shaker incubator for 18 hours at 37°C,100Rpm.

Similarly, like without enrichment, in the enrichment phase, 1 loopful sample was taken from each conical flask containing vegetable and water samples streaked on Hicrome UTI modified agar plate. Then those plates were kept for 24 hours of incubation at 37°C. In this study, the motive behind the without enrichment and enrichment phase was to check differences in morphological characteristics and patterns of the growth. So, after these two phases, the colonies that were found on Hicrome UTI modified agar plates were again transferred to fresh Hicrome UTI modified agar plates to get more pure colonies with specific colors. Then from Hicrome UTI modified agar plates all types of colonies were stabbed into BHI agar stock media and incubated for 18 hours at 37°C. After incubation, paraffin oil was added to BHI media to keep the cell hydrated.

2.5 Antibiogram

71 Presumptive Klebsiella pneumoniae isolates that showed blue colony morphology on Hicrome UTI modified agar were tested for antibiotic resistance using the following antibiotics: Meropenem+EDTA(MR+ED), Imipenem (IMI10), Kanamycin(K30), Gentamicin (CN10), Ceftazidime (CAZ30), Erythromycin(E15), Nitrofurantoin (NIT300), Ampicillin (AMP10), Amoxyclav(AMC30), Nalidixic acid(NA30), Streptomycin(S10), Tetracycline(TE30).
Antibiotic Susceptibility Testing of the Isolates was performed using the Modified Kirby-Bauer disc diffusion test method.

A loopful of fresh pure culture was taken from a TSA plate and used to make a suspension by mixing it with 0.9%Saline in a test tube. The turbidity of the suspension was checked and compared with the 1.0 McFarland turbidity standard. Thereafter, a sterile swab was dipped into the suspension and spread on a fresh Mueller Hinton Agar (MHA) plate to make a lawn. The lid was slightly ajar allowing the plate to sit at room temperature. Then by using sterile forceps, each

antibiotic-impregnated disc was picked and placed onto the agar plate slightly pressing it to ensure complete contact with the agar surface. The MHA plates were then placed into the 37°C incubators for overnight incubation.

After 18-20 hours of incubation, the plates were examined for a zone of inhibition, and if present, the diameter of the zone of inhibition was measured using a ruler to the nearest millimeter. According to the CLSI guidelines, the zone diameters of each antimicrobial agent were then translated into susceptible, intermediate, or resistant categories.

2.6 DNA Extraction

Isolation of microorganism genomic DNA is an extremely vital step for identification and characterization. In our study, we followed the procedures described in (Kobayashi et al., 2009) with few necessary modifications to isolate bacterial DNAs.

Pure bacterial cultures grown on Tryptone Soy Agar (TSA) were inoculated in 5ml of Nutrient broth and incubated for 18-24 hours. Thereafter, the samples were centrifuged at 14000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended and washed in 400µL of distilled water. The bacteria suspension was centrifuged again at 14000 rpm for 5 minutes and a washed pellet was recovered. Then the pellet was resuspended in 400µL of distilled water and lysed at 100°C for 7 minutes. The lysed cells were centrifuged at 14000 rpm for 5 minutes and the supernatant was transferred to a new microcentrifuge tube. The supernatant contained extracted DNA.

2.7 Raw DNA gel run

The extracted DNA was checked by performing gel electrophoresis and then stored at -20°C for further use.

2.8 Preparation of primers for PCR (stock solution and working solution):

Primers that were used in this study were of 2x concentration. Stock solutions of both primers for blaNDM-1 and blaOXA-2 were present in the lab. Each time before performing PCR, working solutions were prepared from stock solutions.

To prepare the working solutions of blaNDM-1 primer (2x concentration), 2 microlitres of forward and reverse primers were taken in two different microcentrifuge tubes. 98 microlitres of distilled water were then put in each tube. After mixing the tubes were then vortexed and short spun for 20 seconds. The same procedure was repeated in the case of blaOXA-2 primers.

2.9 Sequences of primers used for amplification by PCR

Table 2.9: PCR primer with product size to identify blaNDM-1 and blaOXA-2 gene

Gene	*Primer sequence*	*Product size (bp)*	*Reference*
bla_{NDM-1}	Forward: 5'- ACCGCCTGGACCGATGACCA-3' Reverse: 5'- GCCAAAGTTGGGCGCGGTTG-3'	264	(Devi et al., 2020)
bla_{OXA-2}	Forward: 5'- TTCAAGCCAAAGGCACGATAG-3' Reverse: 5'- TCCGAGTTGACTGCCGGGTTG-3'	702	(Costa et al., 2006)

2.10 Preparation of controls for PCR

We had to prepare the positive control because the strains positive for *bla*$_{NDM-1}$ and *bla*-$_{OXA-2}$ were not available in the lab. So, we ran PCR to detect the presence of *bla*$_{NDM-1}$ and blaOXA-2 in four isolates that were resistant to all the tested antibiotics. Luckily enough, we were able to detect *bla*$_{NDM-1}$ in two of the isolates and *bla*$_{OXA-2}$ in three of the isolates. Positive controls for these two genes could therefore be picked. The positive control for the *bla*$_{NDM-1}$ gene was named R1NDM-1 and that for *bla*-$_{OXA-2}$ was named R2OXA-2. The negative control was *Acinetobacter baumannii* strain, obtained from the lab, which was negative for both genes. The No Template Control (NTC) used was distilled water.

2.11 PCR

The PCR was performed for Carbapenemase resistance genes bla NDM-1 and bla OXA-2 for 59 presumptive *Klebsiella pneumoniae* isolates.

PCR assay was performed in tubes with a total volume of 25µl. The reaction mixtures contained nuclease-free water 5µl, forward primer 2.5µl, reverse primer 2.5µl, template 5µl. And master mix 10µl. Re-pipetting and spinning were carefully done for proper mixing and also to get rid of bubbles. Afterwards, PCR was performed under the following conditions; 35 cycles with an initial denaturation at 94˚C for 3 minutes, denaturation at 95°C for 1 minute, annealing of primers at 55°C for 1 minute, extension at 72°C for 2 minutes, and final extension at 72˚C for 7 minutes. These were set in the thermal cycler. Distilled water was used as a negative control to monitor the contamination by any external DNA in the PCR reagents in the PCR reaction. These PCR conditions were applied in both cases of blaNDM-1 and blaOXA-2

2.12 Gel electrophoresis

Conventional agarose gel electrophoresis was performed to confirm the presence of the amplified product. The amplicon was separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide at 90 voltages for 1 hour. The gel was then visualized by UV transilluminator. The 100bp DNA ladder was used to determine the size of the amplicon. PCR assay was performed at least two times to check the reproducibility.

Chapter 3
Results

3.1 Growth, appearance, and isolation of strains on HiCrome UTI agar

HiCrome UTI agar was used to isolate organisms from vegetable and water samples. On this media, purple to magenta indicates *E. coli*, blue-green is *E. faecalis*, blue to purple is *K. pneumoniae*, light brown is *Proteus mirabilis*, colorless is *P. aeruginosa*, golden yellow is *S. aureus* and there was a white colony for which the identity was not known.

3.1.1 Isolates on HiCrome 1

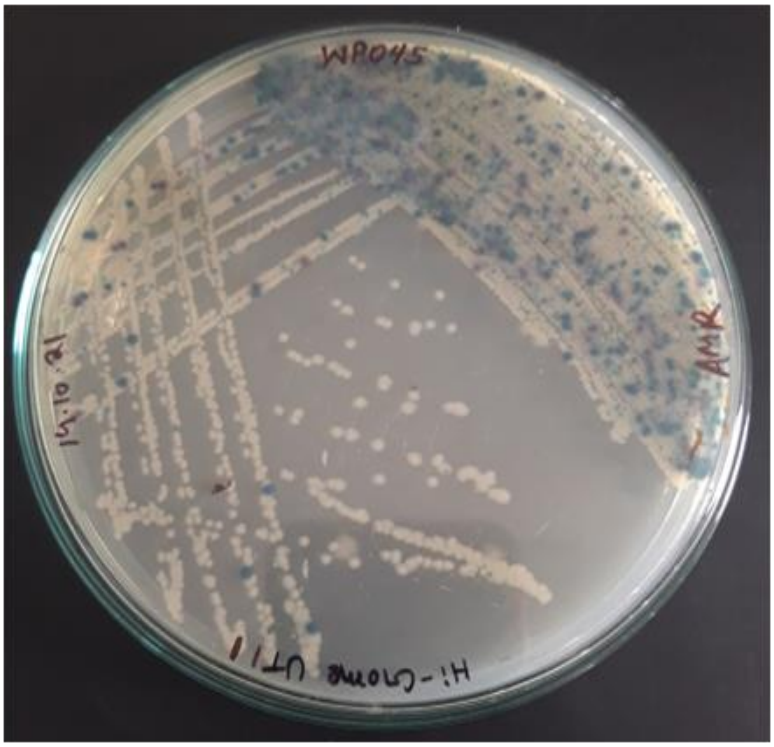


Fig 3.1.1: The appearance of different colored colonies on HiCrome 1. These were picked and transferred to HiCrome 2

3.1.2 Isolates on HiCrome 2

The different colored colonies from HiCrome 1 were transferred to HiCrome 2 in order to observe the consistency in colony morphology and also to get pure cultures.

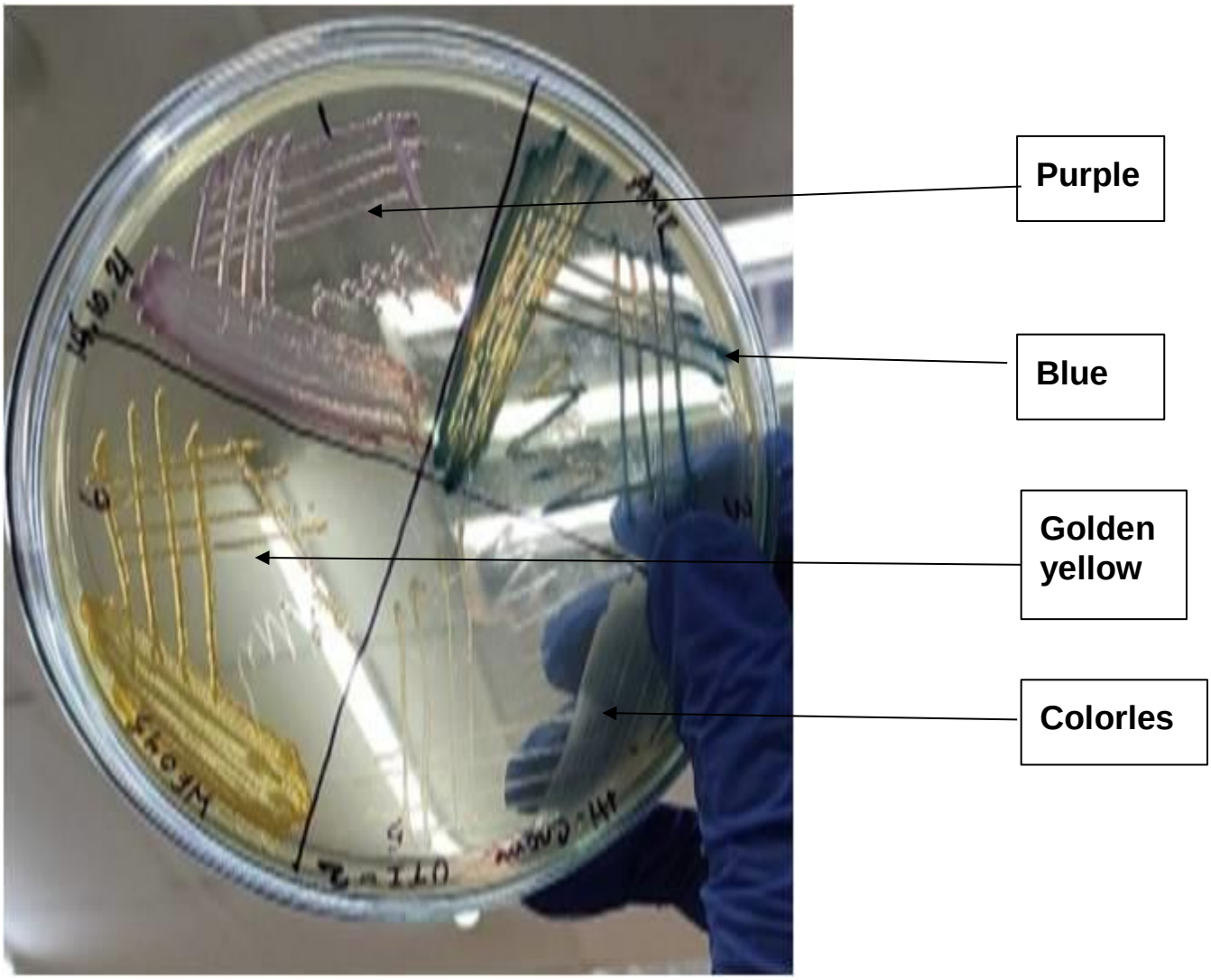


Fig 3.1.2: The appearance of different colored colonies on HiCrome 2.

3.1.3 The number of strains isolated throughout the study

Throughout the study, a total of 606 isolates were obtained. Among these, 337 were from vegetable samples and 296 were from water samples

Table 3.1.3: The number of isolates obtained from vegetable and water samples

Sample type	Number of isolates	Percentages
Vegetables	337	55.61%
Water	296	44.39%
Total	606	100%

3.1.4 The abundance of isolates obtained throughout the study

Among the 606 isolates, 71 were *Enterococcus faecalis*, 27 were *Escherichia coli*, 77 were *Klebsiella pneumoniae*, 6 were *Proteus mirabilis*, 204 were *Pseudomonas aeruginosa*, 98 were *Staphylococcus aureus* and 123 were that organism whose identity we could not determine on HiCrome UTI agar.

Pseudomonas aeruginosa was the most isolated organism followed by *Staphylococcus aureus* (if the unknown organism is to be disregarded) and then *Klebsiella pneumoniae*. The least isolated organism was *Proteus mirabilis.*

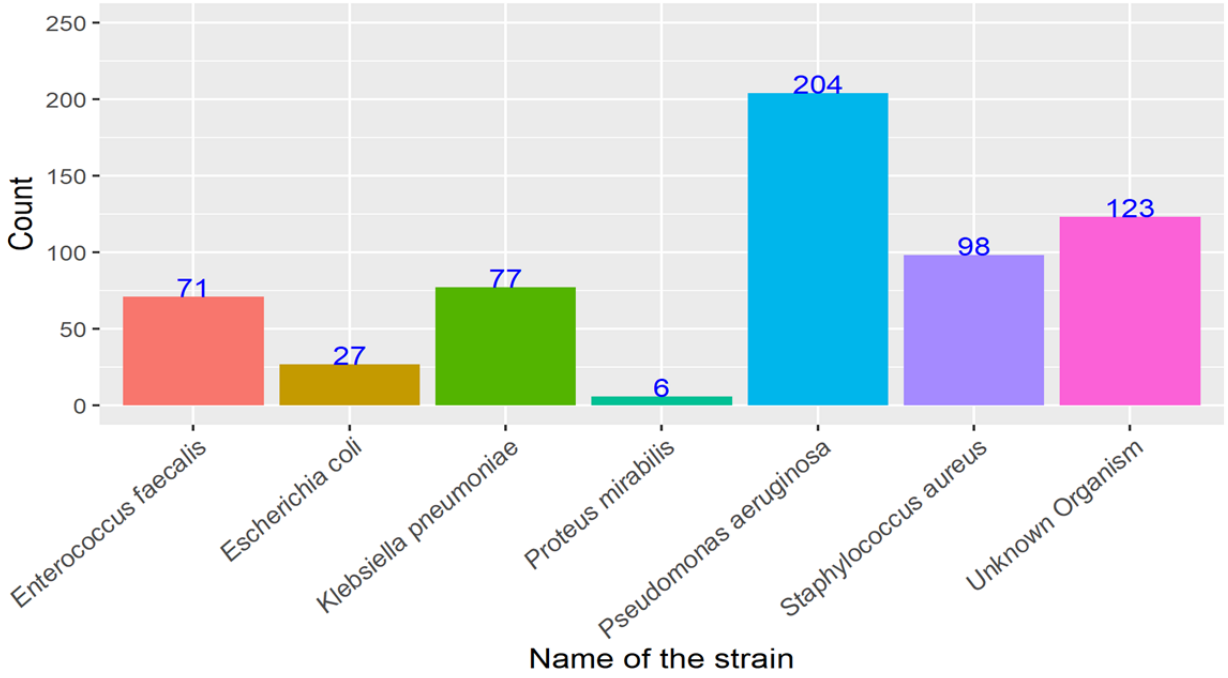


Figure 3.1.4: The abundance of isolated strains

3.2. Sample-wise distribution of isolates

269 isolates were obtained from water samples and 337 isolates from vegetable samples. Among vegetable samples, the highest number of isolates was obtained from Coriander (47) followed by Betel leaf (35), and the least number of isolates was obtained from Tomato (2) followed by Spinach (4) and Hog plum (4)

Table 3.2: The total number of isolates obtained per sample

Sample Type	Names of isolates (presumptive)	Number of isolates
Spinach	*Pseudomonas aeruginosa*(3)	04

	Unknown Organism(1)	
Red spinach	*Enterococcus faecalis* (1) *Escherichia coli* (2) *Klebsiella pneumoniae* (2) *Proteus mirabilis*(1) *Pseudomonas aeruginosa*(6) *Staphylococcus aureus*(5) Unknown Organism(3)	20
Coriander	*Enterococcus faecalis*(6) *Klebsiella pneumoniae*(3) *Proteus mirabilis*(1) *Pseudomonas aeruginosa*(14) *Staphylococcus aureus*(8) Unknown Organism(12)	47
Taro	*Enterococcus faecalis*(4) *Escherichia coli* (1) *Klebsiella pneumoniae*(3) *Pseudomonas aeruginosa*(11) *Staphylococcus aureus*(6) Unknown Organism(7)	32
Ladies finger	*Enterococcus faecalis*(2) *Klebsiella pneumoniae*(6) *Proteus mirabilis*(1) *Pseudomonas aeruginosa*(6) *Staphylococcus aureus*(3) Unknown Organism(7)	25
Tomato	*Pseudomonas aeruginosa(1)* Unknown Organism(1)	02
Cucumber	*Enterococcus faecalis*(4) *Escherichia coli*(2) *Klebsiella pneumoniae*(5) *Pseudomonas aeruginosa*(8) *Staphylococcus aureus*(5) Unknown Organism(9)	33
Betel leaf	*Enterococcus faecalis*(7) *Escherichia coli* (1) *Klebsiella pneumoniae*(2) *Pseudomonas aeruginosa*(18) *Staphylococcus aureus*(4)	35

	Unknown Organism(3)	
Water spinach	*Enterococcus faecalis* (2) *Klebsiella pneumoniae*(3) *Pseudomonas aeruginosa*(3) *Staphylococcus aureus*(2) Unknown Organism(2)	12
Radish	*Klebsiella pneumoniae*(2) *Pseudomonas aeruginosa*(4) *Staphylococcus aureus*(1) Unknown Organism(2)	09
Lettuce	*Enterococcus faecalis* (2) *Escherichia coli*(1) *Proteus mirabilis*(1) *Pseudomonas aeruginosa*(4) *Staphylococcus aureus*(3) Unknown Organism(2)	13
Yardlong bean	*Enterococcus faecalis*(2) *Escherichia coli*(1) *Klebsiella pneumoniae*(2) *Pseudomonas aeruginosa*(4) *Staphylococcus aureus*(3) Unknown Organism(5)	17
Hechi leaves	*Enterococcus faecalis*(2) *Escherichia coli*(1) *Pseudomonas aeruginosa*(2) *Staphylococcus aureus*(2) Unknown Organism(2)	09
Green chili	*Enterococcus faecalis*(3) *Escherichia coli*(1) *Klebsiella pneumoniae*(4) *Pseudomonas aeruginosa*(5) *Staphylococcus aureus*(2) Unknown Organism(3)	18
Lemon	*Escherichia coli*(1) *Klebsiella pneumoniae*(3) *Pseudomonas aeruginosa*(2) *Staphylococcus aureus*(1) Unknown Organism (3)	10
Bitter gourd	*Enterococcus faecalis*(1)	10

	Klebsiella pneumoniae(3) *Pseudomonas aeruginosa*(3) *Staphylococcus aureus*(1) Unknown Organism(2)	
Teasle gourd	*Enterococcus faecalis*(2) *Escherichia coli*(1) *Klebsiella pneumoniae*(5) *Pseudomonas aeruginosa*(4) *Staphylococcus aureus*(2) *Unknown Organism*(4)	18
Steam amaranth leaves	Enterococcus faecalis(2) *Klebsiella pneumoniae*(3) *Pseudomonas aeruginosa*(4) *Staphylococcus aureus*(2) Unknown Organism(2)	13
Cabbage	*Klebsiella pneumoniae*(2) *Pseudomonas aeruginosa*(1) Unknown Organism(3)	06
Hog plum	*Klebsiella pneumoniae*(1) *Pseudomonas aeruginosa*(1) Unknown Organism(2)	04
Water	*Enterococcus faecalis*(31) *Escherichia coli*(15) *Klebsiella pneumoniae*(28) *Proteus mirabilis*(2) *Pseudomonas aeruginosa*(99) *Staphylococcus aureus*(47) Unknown Organism(47)	269
Total		606 isolates

3.3 Distribution of isolates per location and culture type VE, VP, WE, and WP

From Anandabazar Kacha Bazar near Dhaka Medical College Hospital, 118 isolates (44 from VE, 38 from VP, 18 from WE, and 18 from WP) were obtained. From Hatirpul Bazar near

Bangabandhu Sheikh Mujib Medical University Hospital, 26 isolates (11VE, 4VP, 11WE) were obtained. From Malibagh Bazar near Khidmah hospital, 84 isolates (16VE, 20VP, 24WE, 24WP) were obtained. From Motijheel Bazar near Islami Bank Hospital, 58 isolates (15VE, 14VP,16WE, 13WP) were obtained. From Moulovibazar Kacha Bazar near Salimullah Medical College Hospital, 92 isolates (33VE, 30VP,22WE,7WP) were obtained. From Palashi Bazar near Dhaka Medical College Hospital, 44 isolates (11VE,10VP,11WE,12WP) were obtained. From Shegun Bagicha Bazar near BIRDEM General Hospital 2, 56 isolates (9VE, 16VP,14WE,17WP) were obtained. In general, the highest number of isolates was obtained from Malibagh Bazar near Khidmah hospital (128) followed by Ananda Bazar Kacha Bazar near Dhaka Medical College Hospital (118) and the least number of isolates was obtained from Hatirpul Bazar near Bangabandhu Sheikh Mujib Medical University Hospital (26) followed by Palashi Bazar near Dhaka Medical College Hospital (44)

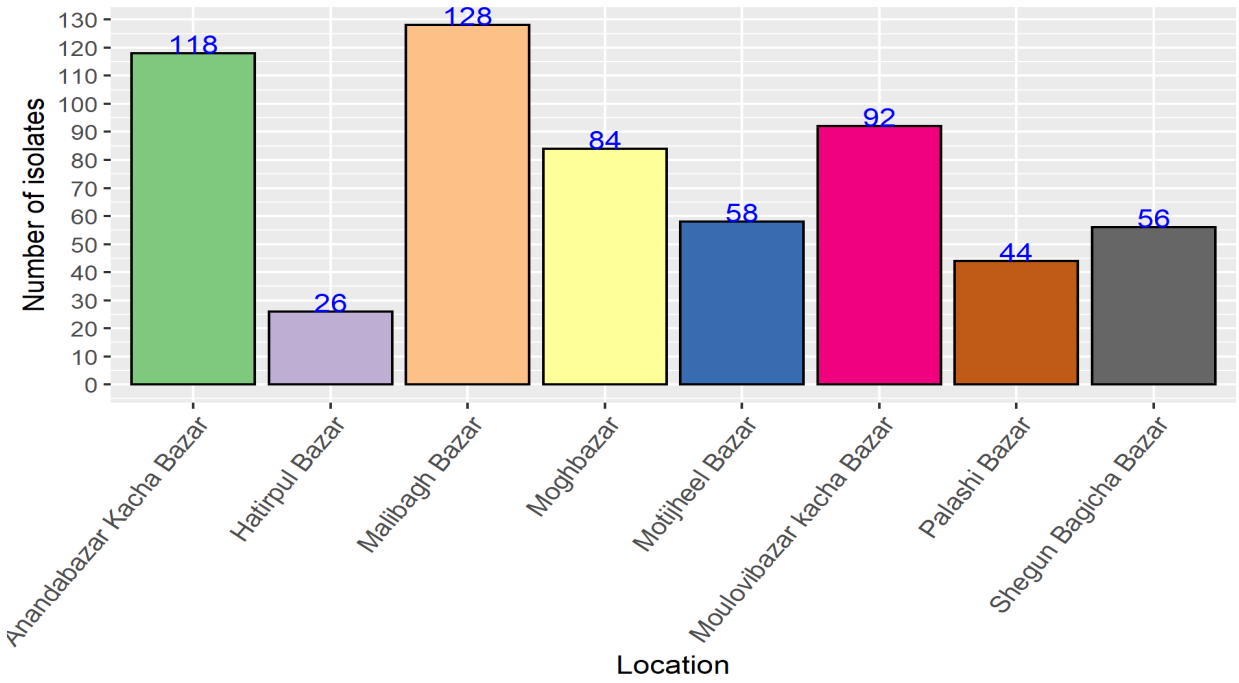


Figure 3.3.1: Location-wise distribution of isolates

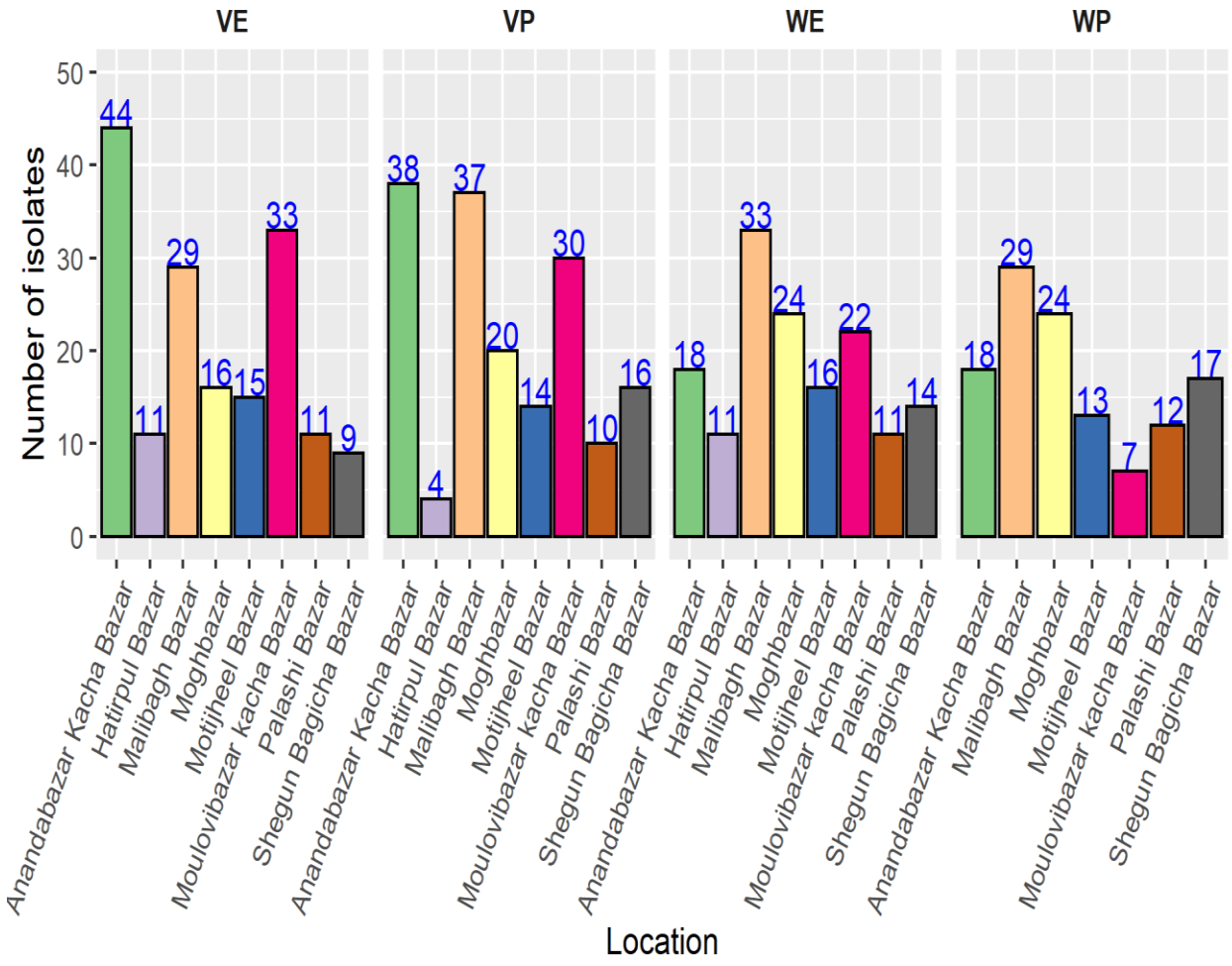


Fig 3.3.2: The number of isolates from VE, VP, WE, and WP

3.4 Klebsiella pneumoniae

Out of the 606 isolates obtained in the study, 77 were *Klebsiella pneumoniae*. These were considered for the rest of the analysis throughout the study.

3.4.1 Sample-wise distribution of presumptive Klebsiella pneumoniae isolates

Among the 77 presumptive *Klebsiella pneumoniae* isolates, 28 were obtained from water samples and 49 from Vegetable samples. Among the vegetables, the highest number of *K. pneumoniae* isolates was obtained from Ladies' finger (6) and the least number came from Hog plum (1)

Table 3.4.1: The total number of presumptive Klebsiella pneumoniae isolates obtained from each sample

Sample type	**Number of *Klebsiella pneumoniae* isolates**
Coriander	03
Bitter gourd	03
Betel leaf	02
Green chili	04
Ladies finger	06
Taro	03
Cabbage	02
Radish	02
Steam amaranth leaves	03
Cucumber	05
Teasle gourd	05
Water spinach	03
Yardlong bean	02
Hog plum	01
Red spinach	02
Lemon	03
Vegetable Wash Water	28
Total number of *K. pneumoniae* isolates	77

3.4.2 Comparison between Enrichment and Without Enrichment culture types among Klebsiella pneumoniae isolates

Among the 77 *Klebsiella pneumoniae* isolates, 31 were obtained from Vegetable Enrichment (VE), 18 from Vegetable Without Enrichment (VP), 15 from Water Enrichment, and 13 from Water without Enrichment (WP). With Vegetable samples, more *Klebsiella pneumoniae* isolates were recorded from Enrichment than from Without Enrichment culture (difference = 13 isolates). However, with water samples, there was no big difference between the number of isolates obtained from the two culture types(difference = 2)

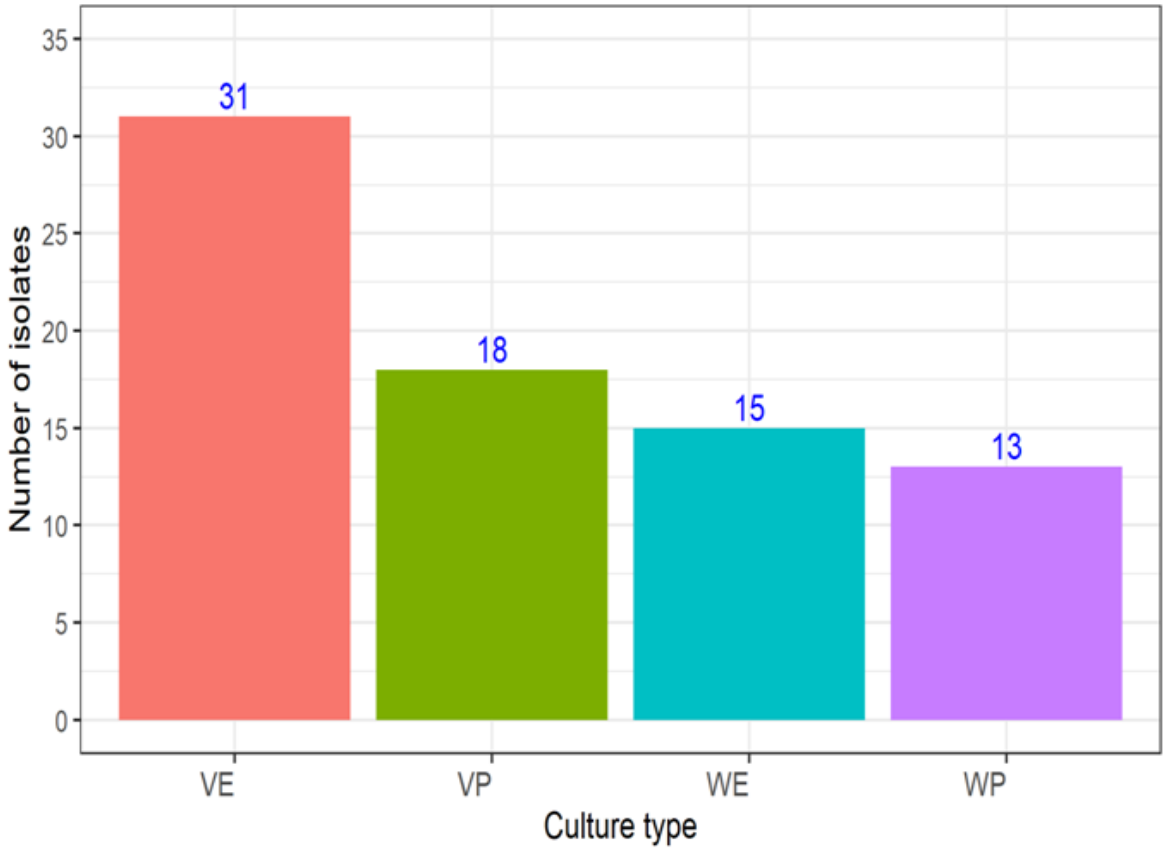


Figure 3.4.2: Enrichment versus Without enrichment among presumptive Klebsiella pneumoniae isolates.

3.4.3 Distribution of Klebsiella pneumoniae isolates per location and culture type

The Same number of *Klebsiella pneumoniae* isolates (30) was obtained from both Anandabazar Kacha Bazar near Dhaka Medical College Hospital and Moulovibazar Kacha Bazar near Salimullah Medical College Hospital. The remaining 17 isolates, out of the 77, were obtained from Moghbazar near Dhaka Community Medical College Hospital. Among the 30 isolates from Anandabazar, 16 were from VE, 7 from VP, 3 from WE, and 4 from WP. Then among the 30 isolates from Moghbazar, 5 were from VE, 3 from VP, 4 from WE, and 5 from WP. Lastly, among the 30 isolates from Moulovibazar, 10 were from VE, 8 from VP, 8 from WE, and 4 from WP.

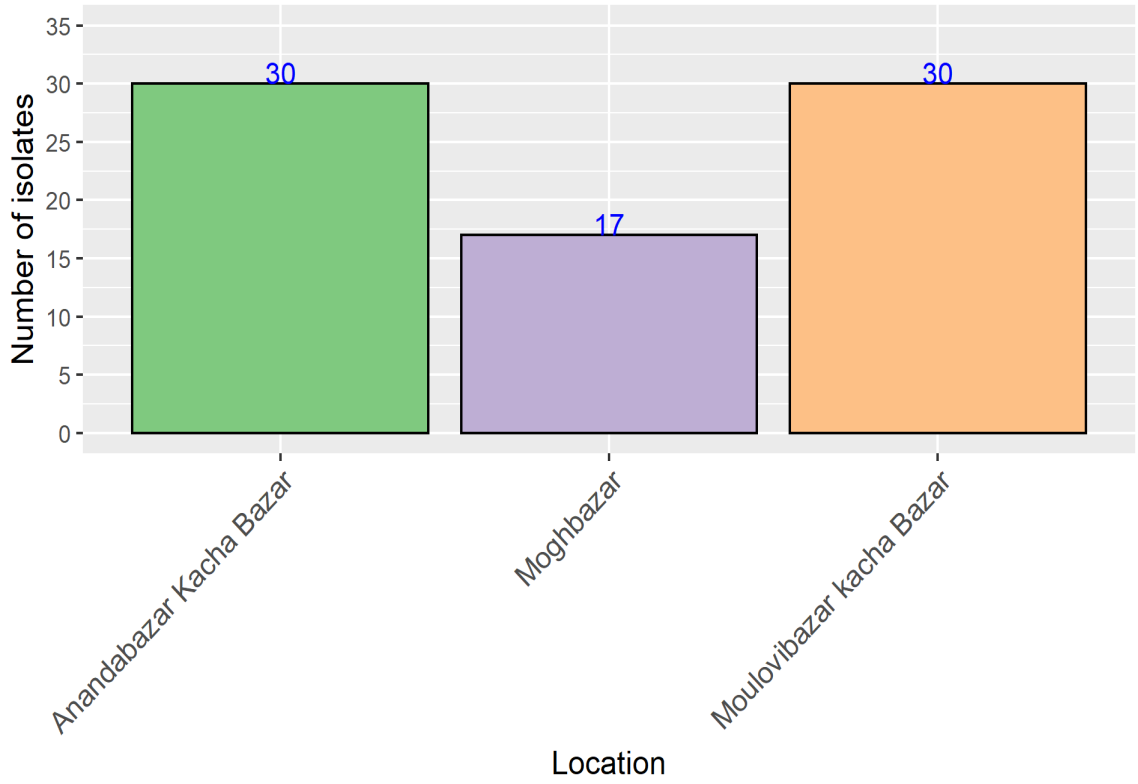


Fig 3.4.3.1: Location-wise distribution of Klebsiella pneumoniae isolates

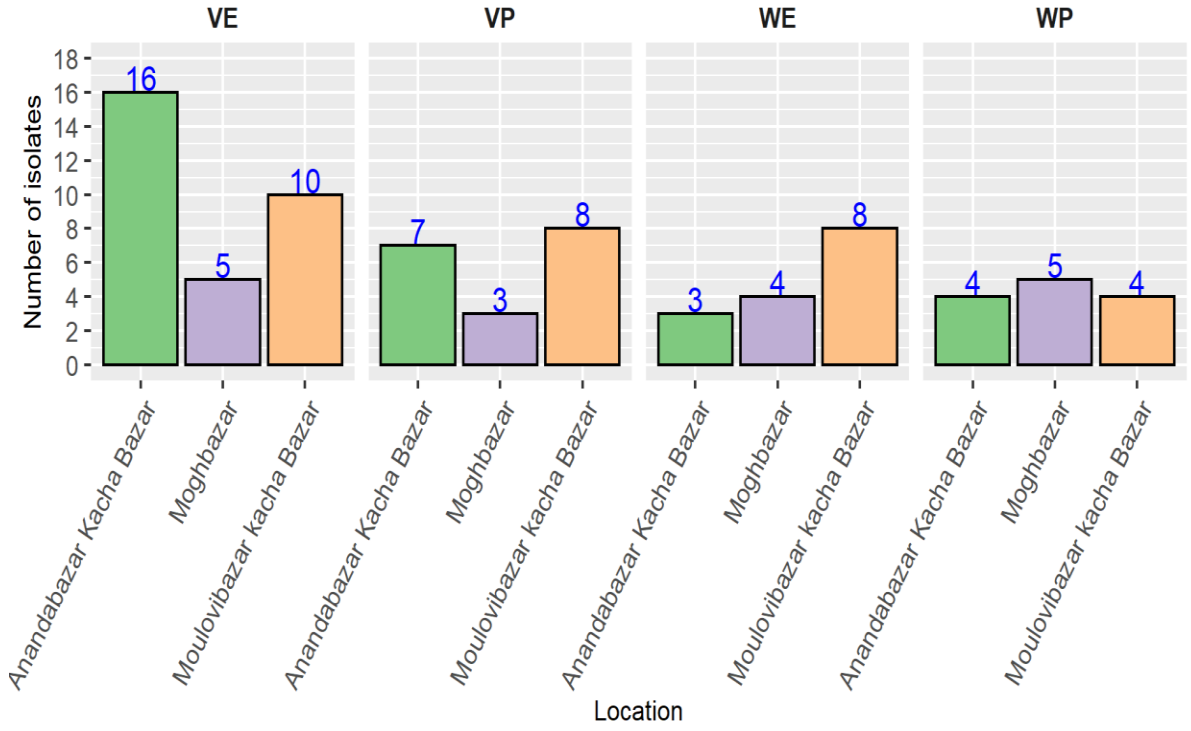


Fig 3.4.3.2: Number of Klebsiella pneumoniae isolates from VE, VP, WE, and WP.

3.5 Antibiogram

All the 77 presumptive *Klebsiella pneumoniae* isolates were considered for antibiogram

3.5.1 Antibiotic Susceptibility Test on MHA media- Modified Kirby-Bauer disk diffusion method

An antibiotic susceptibility test was done using the modified Kirby - Bauer disk diffusion method. After Incubation of the MHA plates, it was observed that some isolates were either resistant, intermediate, or sensitive to some of the antibiotics. This observation is represented by Fig 3.5.1.1.

It was also observed that some isolates were fully resistant to all the 12 tested antibiotics. This observation is represented by Fig 3.5.1.2.

NIT300, K30, TE30, CAZ30, NA30, AMP10	IMI10, E15, MR+ED, CN10,S10, AMC30

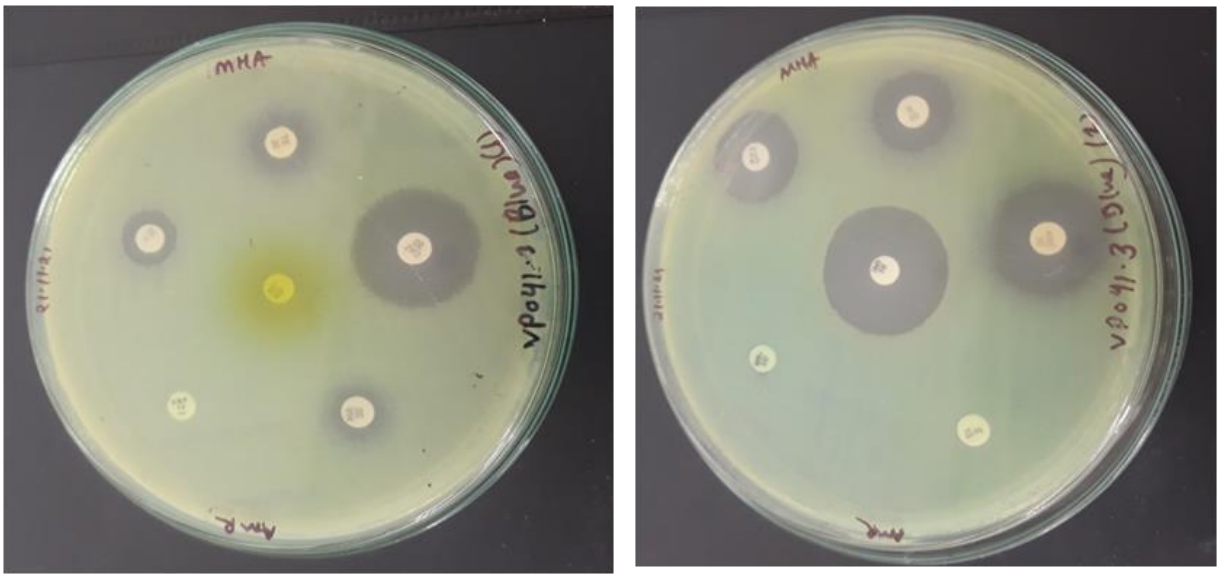


Figure 3.5.1.1: Resistant to some of the antibiotics

NIT300, K30, TE30, CAZ30, NA30, AMP10

IMI10, E15, MR+ED, CN10,S10, AMC30

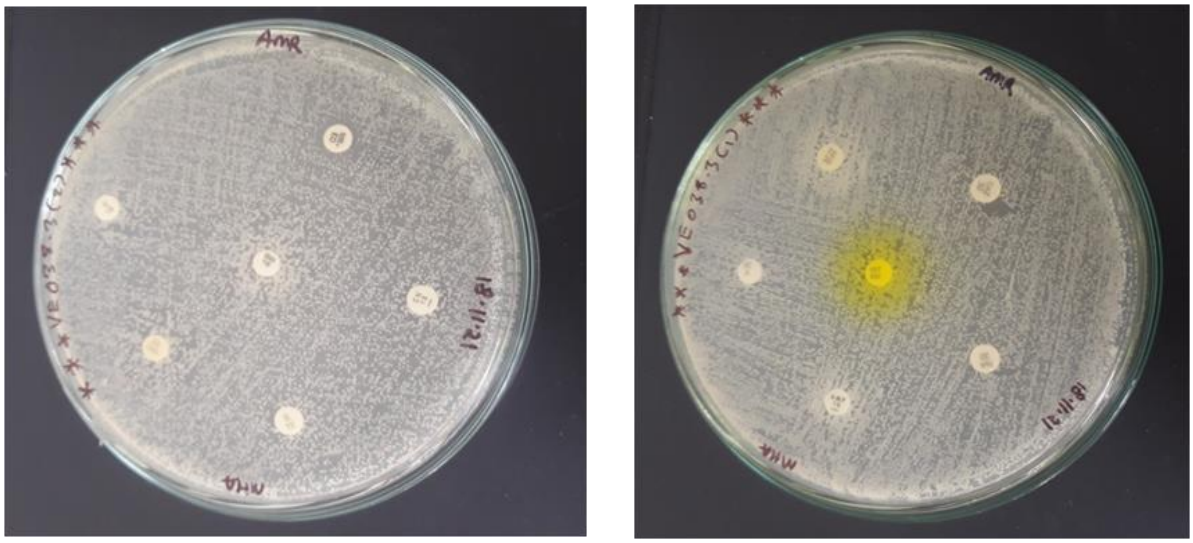


Figure 3.5.1.2: Resistant to all antibiotics

3.5.2 Antibiotic sensitivity pattern of presumptive Klebsiella pneumoniae isolates:

All the 77 presumptive *Klebsiella pneumoniae* isolates were resistant to Meropenem + EDTA followed by Nalidix Acid and Erythromycin to which 72.70% and 63.60% of the isolates were resistant, respectively. This was then followed by Kanamycin (57.10%), Ampicillin (55.80%), Ceftazidime (51.90%), Nitrofurantoin (49.40%), Tetracycline (33.80%), Streptomycin (18.20%), Imipenem (15.60%), Gentamicin (10.40%) and Amoxyclav(7.80%).

In terms of sensitivity, it is Amoxyclav to which most of the isolates (89.60%) were sensitive followed by Gentamicin (87.00%) and Imipenem (84.40%).

Table 3.5.2: Showing resistance pattern of presumptive Klebsiella pneumoniae isolates:

ANTIBIOTICS	RESISTANT	INTERMEDIATE	SENSITIVE
Meropenem + EDTA (MR+ED)	100.00%	0.00%	0.00%
Nalidixic Acid (NA30)	72.70%	6.50%	20.80%
Erythromycin (E15)	63.60%	22.10%	14.30%
Kanamycin (K30)	57.10%	15.60%	27.30%
Ampicillin (AMP10)	55.80%	0.00%	44.20%
Ceftazidime (CAZ30)	51.90%	10.40%	37.70%
Nitrofurantoin (NIT300)	49.40%	2.60%	48.00%
Tetracycline (TE30)	33.80%	10.40%	55.80%
Streptomycin (S10)	18.20%	10.40%	71.40%
Imipenem (IMI10)	15.60%	0.00%	84.40%
Gentamicin (CN10)	10.40%	2.60%	87.00%
Amoxyclav (AMC30)	7.80%	2.60%	89.60%

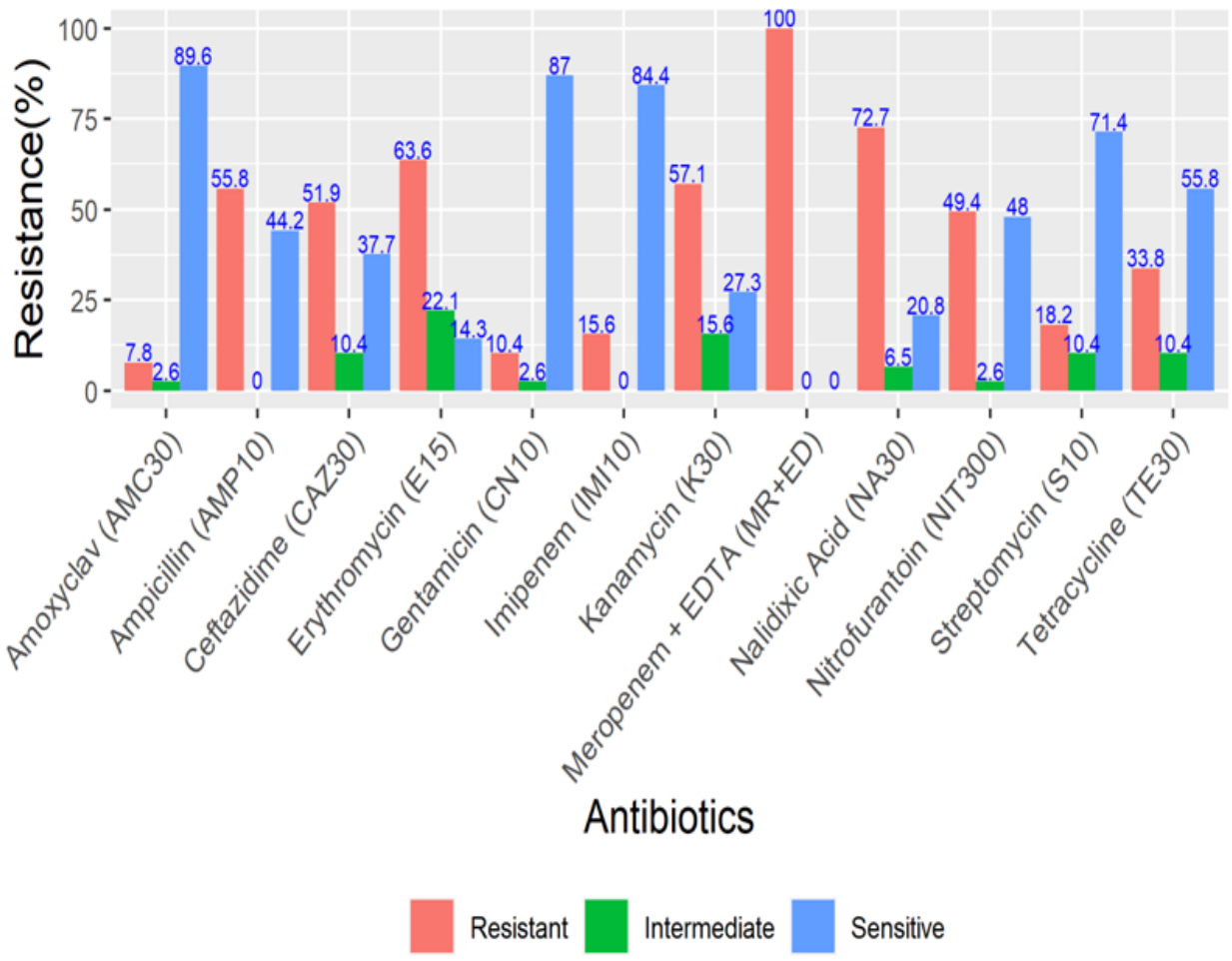


Figure 3.5.2: Resistance profile of presumptive Klebsiella pneumoniae isolates

3.5.3 Multiple Antibiotic Resistance (MAR) index

MAR index is calculated as the ratio between the number of antibiotics that an isolate is resistant to and the total number of antibiotics the organism is exposed to. A MAR index greater than 0.2 means that the high-risk source of contamination is where antibiotics are frequently used. (Ayandele et al, 2020).

MAR index was categorized into four groups; <0.2, 0.2-<0.5, 0.5-<1, and 1 which were respectively further categorized into four risk groups; Low, High, Higher, and Highest. A total of 12 antibiotics were tested. In the low-risk group, 5 isolates were found to be resistant to less than

2 antibiotics (MAR index <0.2), and among these, 2 were from Anandabazar, 2 from Moulovibazar, and 1 from Moghbazar. In the high-risk group, 39 isolates were found to be resistant to between 2 and 6 antibiotics (MAR index; 0.2-<0.5), and among these, 15 were from Anandabazar, 16 were from Moulovibazar, and 8 were from Moghbazar. Then in the higher risk group, 29 isolates were resistant to between 6 and 11 antibiotics (MAR index; 0.5-<1), and among these, 11 were from Anandabazar, 11 from Moulovibazar, and 07 from Moghbazar. Lastly, in the highest risk group, 4 isolates were found to be resistant to all the 12 antibiotics tested (MAR index =1), and among these, 2 were from Anandabazar, 1 from Moulovibazar, and 1 from Moghbazar. Most of the *Klebsiella pneumoniae* isolates were in the high-risk group (MAR; 0.2-<0.5)

Table 3.5.3: Multiple Antibiotic Resistance (MAR) index:

Risk	MAR index	Number of isolates	Location
Low	<0.2	05	Anandabazar (02) Moulovibazar (02) Moghbazar (01)
High	0.2-<0.5	39	Anandabazar (15) Moulovibazar (16) Moghbazar (08)
Higher	0.5-<1	29	Anandabazar (11) Moulovibazar (11) Moghbazar (07)
Highest	1	04	Anandabazar (02) Moulovibazar (01) Moghbazar (01)

3.6 PCR results:

Polymerase chain reaction (PCR) was done by using two primer pairs named; Bla-NDM1 and Bla-OXA2 to amplify $bla_{\text{NDM-1}}$ gene (product size: 264bp) and $bla_{\text{OXA-2}}$ gene (product size: 702bp) respectively. Out of 59 *Klebsiella pneumoniae* isolates from which DNA was extracted, 5 were positive for $bla_{\text{NDM-1}}$. and 9 were positive for blaOXA-2. In each of the two cases, other bands of different sizes were formed. For example, in the case of $bla_{\text{NDM-1}}$, bands below and above 264bp

were observed. Likewise, in the case of *bla*$_{\text{OXA-2}}$, bands below and above 702 bp were also observed.

3.6.1 PCR results for blaNDM-1 gene:

5 isolates (lanes: 3,20,35,56,59) formed the targeted band size of 264 bp and these were considered positive for the *bla*$_{\text{NDM-1}}$ gene. 11 isolates (lanes: 7,8,9,11,12,13,14,17,19,21,46) formed bands below 264 bp, 8 isolates (lanes: 2,27,29,36,37,38,50,55) formed bands above 264 bp.

a)

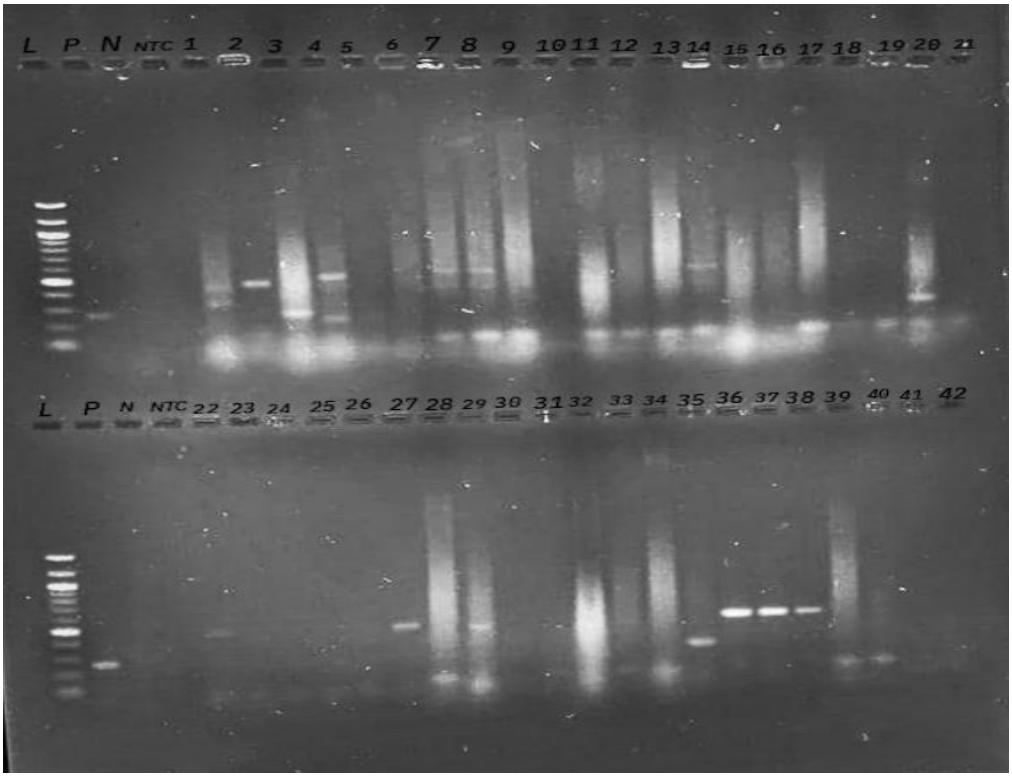


Fig 3.6.1a: blaNDM-1 PCR results for *K. pneumoniae* isolates (1-42). L represented the 100bp DNA Ladder, P represented the Positive Control (R1NDM-1), N represented the Negative control (Acinetobacter baumannii strain negative for the blaNDM-1 gene), and NTC represented the No Template Control (Distilled water). blaNDM-1 positive isolates are in lanes; 3,20, and35. The faint bands signify a low concentration of DNA

b)

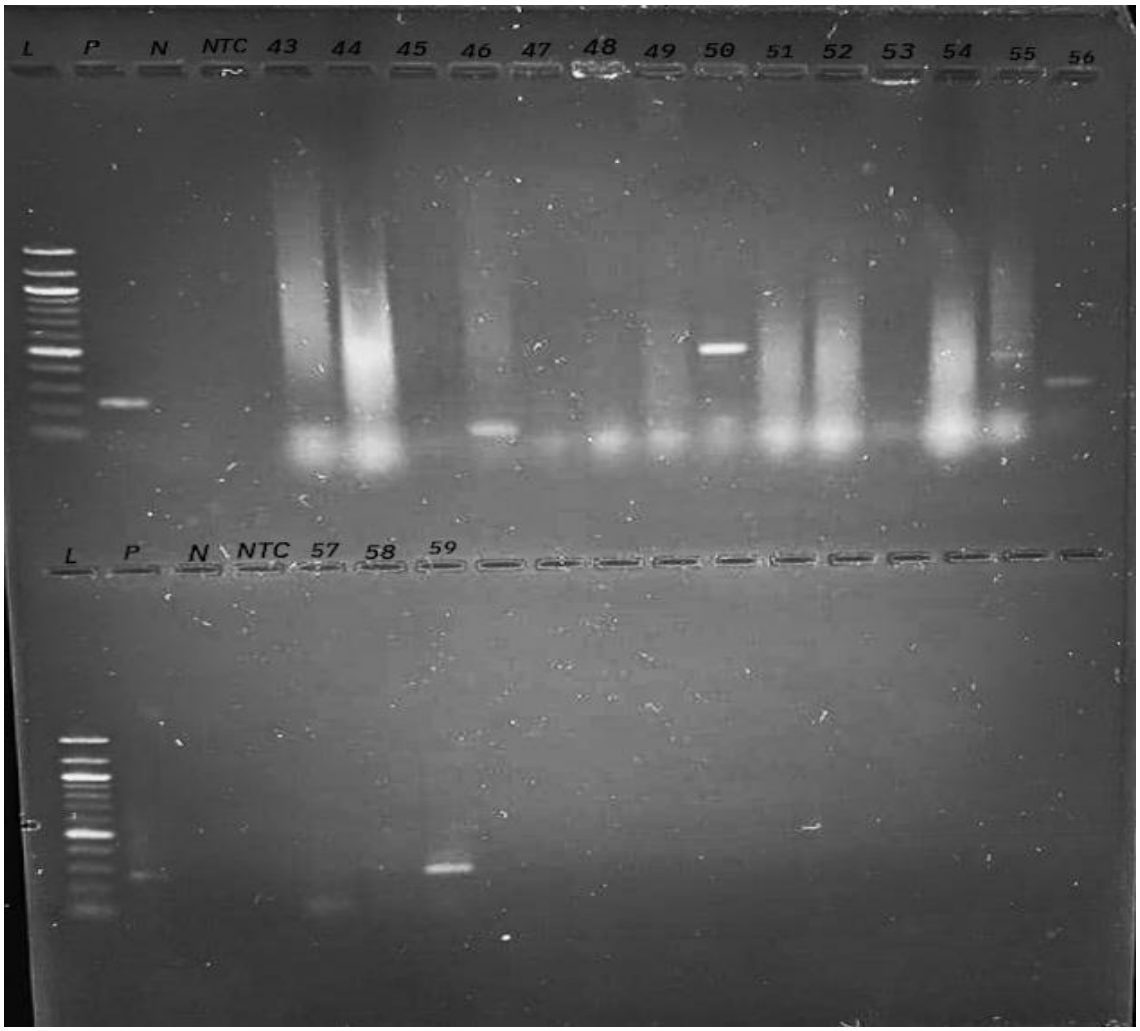


Fig 3.6.1b: blaNDM-1 PCR results for K. pneumoniae isolates (43-59). L represented the 100bp DNA Ladder, P represented the Positive Control (R1NDM-1), N represented the Negative control(Acinetobacter baumannii strain negative for the blaNDM-1 gene) and NTC represented the No Template Control (Distilled water).blaNDM-1 positive isolates are in lanes; 56, and 59. The faint bands signify a low concentration of DNA.

3.6.2 PCR results for blaOXA-2 gene

Nine isolates (lanes: 10, 27, 29, 33, 36, 45,48,50,55) formed the targeted band size of 702 bp and these were considered positive for the *bla*$_{\text{OXA-2}}$ gene. Two isolates (Lanes: 22 and 35) formed bands below 702 bp, and one isolate (Lane: 42) formed a band above 702 bp.

a)

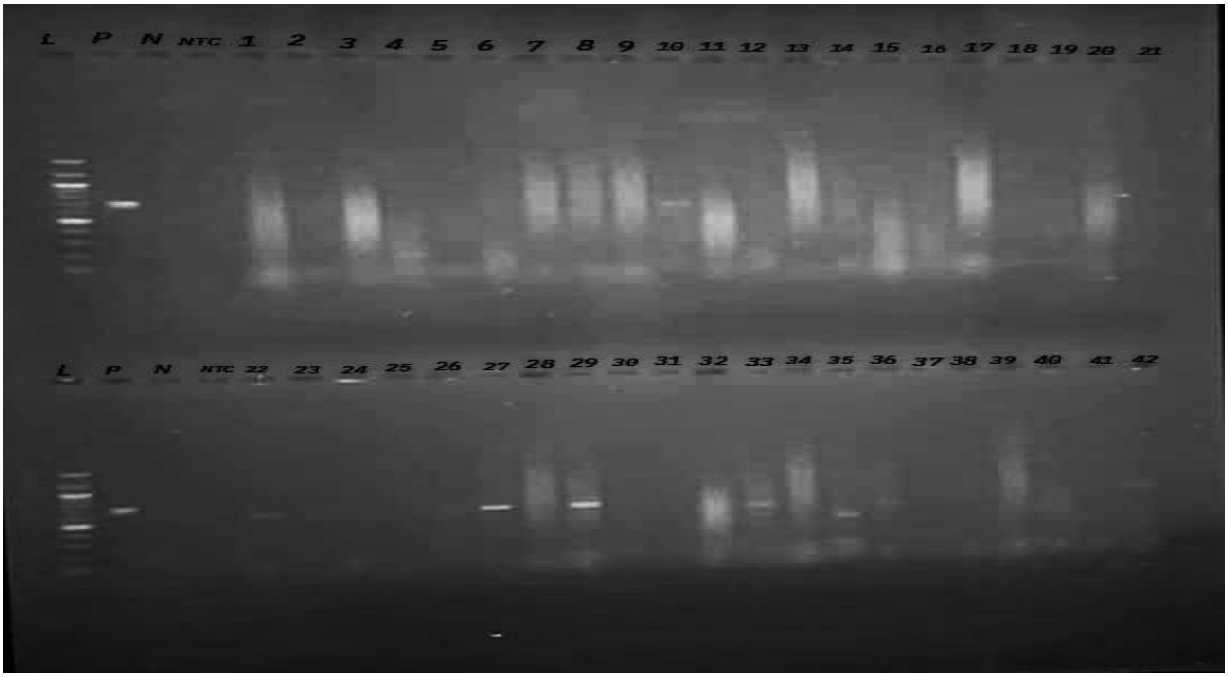


Fig 3.6.2a: blaOXA-2 PCR results for *K. pneumoniae* isolates (1-42). L represented the 100bp DNA Ladder, P represented the Positive Control (R2OXA-2), N represented the Negative control (Acinetobacter baumannii strain negative for the blaOXA-2 gene) and NTC represented the No Template Control (Distilled water). blaOXA-2 positive isolates are in lanes; 10, 27, 29, 33, and 36. The faint bands signify a low concentration of DNA.

b)

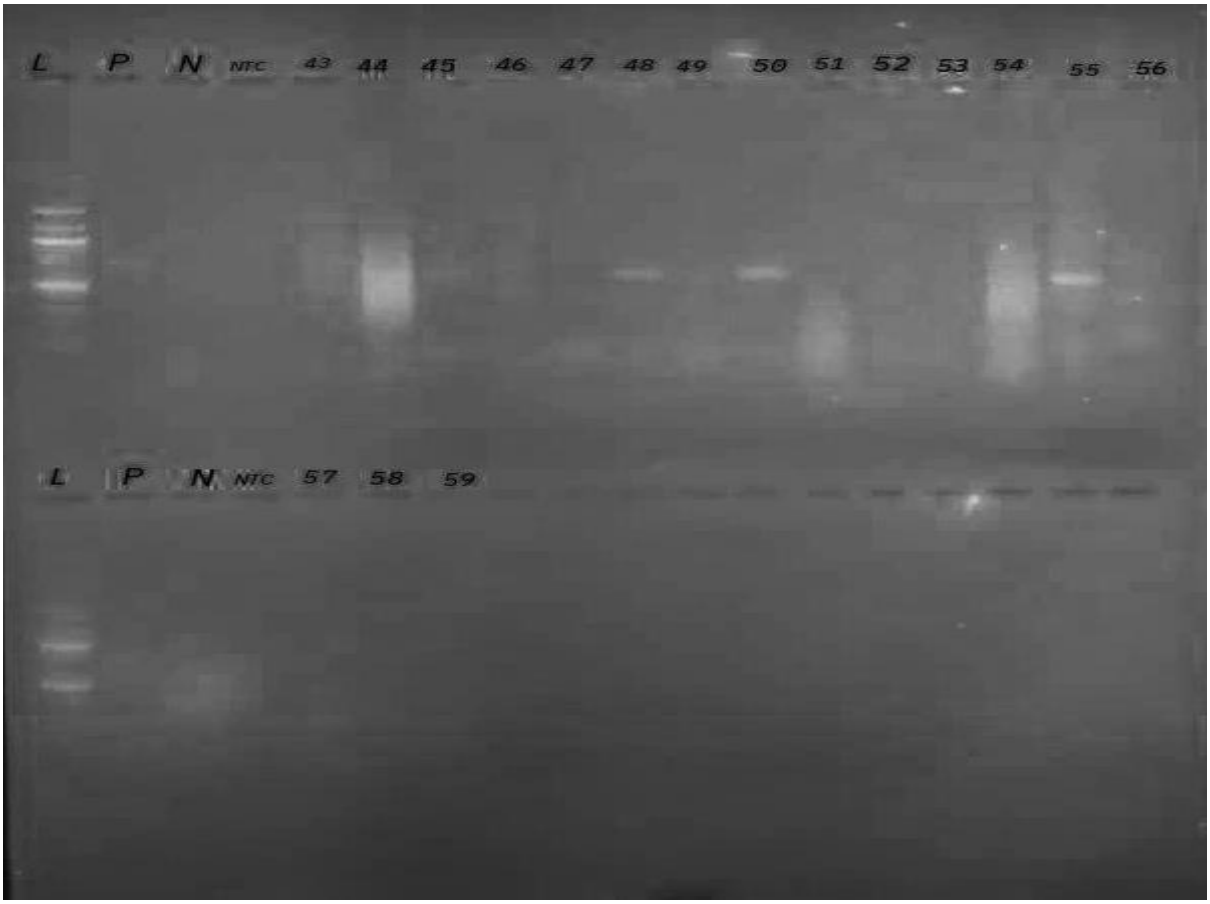


Fig 3.6.2b: blaOXA-2 PCR results for *K. pneumoniae* isolates (43-59). L represented the 100bp DNA Ladder, P represented the Positive Control (R2OXA-2), N represented the Negative control (Acinetobacter baumannii strain negative for the blaOXA-2 gene) and NTC represented the No Template Control (Distilled water). Here, the blaOXA-2 positive isolates are in lanes; 45, 48, 50, and 55. The faint bands signify a low concentration of DNA

3.7 Klebsiella pneumoniae isolates that were resistant to all the antibiotics:

Two isolates were obtained from Anandabazar, one of which contained $bla_{\text{OXA-2}}$ and it was from lemon, another one contained both genes ($bla_{\text{NDM-1}}$ and $bla_{\text{OXA-2}}$). 1 isolate was obtained from Moulovibazar, it contained $bla_{\text{NDM-1}}$ and it was from the lemon. The last isolate was from Moghbazar, it contained the $bla_{\text{OXA-2}}$ gene and it was from Yardlong bean.

Table 3.7: Details of the 4 presumptive Klebsiella pneumoniae isolates that were resistant to all the antibiotics

Location	Number of isolates	*bla*$_{NDM-1}$	*bla*$_{OXA-2}$	Sample type
Anandabazar	02	No	Yes	Lemon
		Yes	Yes	Lemon
Moulovibazar	01	Yes	No	Lemon
Moghbazar	01	No	Yes	Yardlong bean

3.8 Sample-wise distribution of the two carbapenemase genes among *K. pneumoniae* isolates

Lemon has the highest number of carbapenem-resistant *Klebsiella pneumoniae* isolates.

Table 3.8: Number of Klebsiella pneumoniae isolates in each sample harboring either blaNDM-1, blaOXA-2, or both

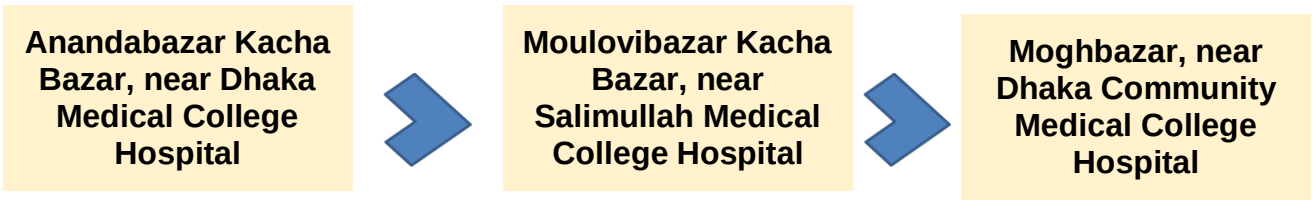
	Water	Green chili	Lemon	Bitter gourd	Steam amaranth leaves	Yardlong bean	Coriander
blaNDM-1	01+01	01	01+01	-	-	-	-
blaOXA-2	01	-	02	03	01	01	01
Total	03	01	04	03	01	01	01

3.9 Determination of the location at the highest risk of carbapenem resistance

Anandabazar is at highest risk of carbapenem resistance, followed by Moulovibazar and lastly Moghbazar.

Table 3.9: Details of the locations in regards to the number of K.pneumoniae strains isolated, MAR index, and presence of carbapenemase genes(blaNDM-1 and blaOXA-2)

	Anandabazar	**Moulovibazar**	**Moghbazar**
Isolation	30	30	17
MAR ≥ 0.2<1	26	27	15
MAR = 01	02	01	01
bla_{NDM-1}	03	02	0
bla_{OXA-2}	05	03	01
bla_{NDM-1} & $blaOXA_{-2}$	01	0	0



Chapter 4
Discussion

4.1 Discussion

Carbapenem-Resistant Enterobacteriaceae (CRE) are considered among the most urgent antimicrobial-resistant threats by US Centers for Disease Control and Prevention (CDC) (Potter et al., 2016).

Klebsiella pneumoniae causes a wide range of infections, including pneumonias, urinary tract infections, bacteremias, and liver abscesses. Furthermore, *K. pneumoniae* strains have become increasingly resistant to antibiotics, rendering infection by these strains very challenging to treat. (Paczosa & Mecsas, 2016). Although *K. pneumoniae* is considered to be an important opportunistic pathogen and a frequent cause of hospital-acquired infections, it is also found in non-clinical habitats, which include the mucosal surfaces of humans and animals, and environmental sources such as water, soil, sewage, and vegetation (Ferreira et al., 2019). The present research was done to isolate and assess the prevalence of carbapenem-resistant *Klebsiella pneumoniae* in vegetable and water samples in Dhaka South.

Rapid and reliable detection of viable *K. pneumoniae* in food samples is important for the prevention of its associated infections. The majority of the rapid genetic assays and even traditional techniques used to detect specific pathogens in foods require an enrichment step, which increases the analytical time. Although sample enrichment limits the assay speed, it provides benefits such as the increase of cell number to a detectable level, the dilution of the detection inhibitors, the differentiation of viable and non-viable cells, and the elimination of stressed or injured cells due to food processing (Rathnayaka, 2011). In this study, the enrichment cultures yielded more *K. pneumoniae* strains as compared to cultures without enrichment. A similar effect was observed in a study detecting *K. pneumoniae* from infant formula where the number of *K. pneumoniae* was increased from 1.5CFU/100g without enrichment to 10^5 CFU/1ml after enrichment (Y. Liu et al., 2008). Another study claimed that enrichment of their samples using BPW before selective media was their most suitable selective culture method for the recovery of *K. pneumoniae* species (Rodrigues et al., 2022). From Vegetable Enrichment (VE) and Water Enrichment (WE), 31 and 15 *Klebsiella pneumoniae* isolates were obtained respectively. The most likely reason for a more significant impact of enrichment in the vegetable samples as compared to enrichment in vegetable wash water samples is because the latter most probably got contaminated after being used to wash different vegetables. The vegetables which probably had a higher bacterial load from farms and

post-harvest practices were washed in the water hence contaminating it. In some cases, more turbid vegetable wash water was discarded and replaced with fresh and clear water as we observed different turbidity levels of the vegetable wash water that was collected from different stalls ranging from turbid to clear.

Rapid increases in the prevalence of CRE have been reported globally (Li et al., 2018). The last few years have brought a formidable challenge to the clinical arena, as carbapenems, until now the most reliable antibiotics against *Klebsiella* species, and other Enterobacteriaceae, are becoming increasingly ineffective (Perez & Van Duin, 2013). In this study, Antibiotic Susceptibility Test (AST) results revealed that 100% of the *K. pneumoniae* isolates were resistant to meropenem. This was understandable due to the selective pressure of meropenem antibiotics used in HiCrome selective media. However, only 15.6% of the *K. pneumoniae* isolates showed resistance to imipenem. In Bangladesh, a number of studies conducted in Dhaka Medical College Hospital have reported 37.33% (Sonia et al., 2020), 34.61% (Mostofa & Hasan, 2020), and 26.32% (Shamsuzzaman, 2015) imipenem resistance among clinical *K. pneumoniae* strains. These are higher than the 15.60% imipenem resistance observed among *K. pneumoniae* isolates in this research, suggesting a rise in imipenem resistance in Bangladesh. Resistance to these last-resort antibiotics can be attributed to the lack of strict policies and unprescribed use of antibiotics in Bangladesh (Ahmed et al., 2019).

The resistance of isolated *K. pneumoniae* strains to Nalidixic acid (72.70%) was comparatively higher than the resistance of 10% and 2% shown by foodborne *K. pneumoniae* isolated from China (Zhang et al., 2018) and Singapore (Hartantyo et al., 2020) respectively. Furthermore, all the *K. pneumoniae* isolates showed ceftazidime resistance (51.90%) which was much higher than the 19% and 0% reported by Junaid and Zhang. (Junaid et al., 2022; Zhang et al., 2018). The ampicillin resistance of *K. pneumoniae* isolates (55.80%) in this study is much lower than that observed in food-borne *K. pneumoniae* isolates; 82% and 98% in China (Zhang et al., 2018) and Singapore (Hartantyo et al., 2020) respectively.

The observed resistance trend ultimately shows the increasing surge of foodborne *K. pneumoniae* resistance towards common antimicrobials in Bangladesh. They should be carefully used in the treatment of antimicrobial infections to prevent the further spread of resistance. On the other hand,

K. *pneumoniae* isolates showed the highest susceptibility towards Amoxyclav (89.6%) and to Gentamicin (87.0%) which is slightly lower than the 95% and 100% respectively recorded by foodborne *K. pneumoniae* isolated in Singapore (Hartantyo et al., 2020). So, these Antibiotics may still be suitable treatment options for carbapenem-resistant *K. pneumoniae*.

According to Mishra et al. (2013), a MAR index of 0.2 or higher indicates high-risk sources of contamination, MAR index of 0.4 or higher is associated with the human fecal source of contamination. It is further stated that MAR index values > 0.2 indicate the existence of isolates from high–risk contaminated sources with frequent use of antibiotics while values ≤ 0.2 show bacteria from sources with less antibiotic's usage (Thenmozhi et al., 2014). High MAR indices mandate vigilant surveillance and remedial measures (Adenaike et al., 2016). In this study, 93.5% of the *K. pneumoniae* isolates had a MAR index ≥0.2. This is worrisome as it means that these isolates are multidrug-resistant. Compared to these findings, Hartantyo et al. (2020) report a lower incidence (12%) among *K. pneumoniae* isolates having a MAR index ≥0.2. On the contrary, a Bangladesh study by Safain et al. (2020) reveals that 76.2% of *K. pneumoniae* clinical isolates had a MAR index ≥0.2 which is also considerably lower than what is reported in this present research. The authors also noted that there was a yearly increase in the rate of MDR pathogens between 2015 and 2019 which is confirmed by the high MDR percentage of the K. *pneumoniae* isolates in this study. This shows a concern of heavy contamination of community food markets with MDR bacteria with the nearby hospitals as possible sources of contamination; this is a serious public health concern that needs food safety measures put in place.

The production of carbapenemases by Enterobacteriaceae is of great concern to public health as it threatens the use of carbapenem antibiotics (which are last-resort antibiotics) and also increases the mortality rate (Sugawara, Hagiya, et al., 2019). Among the Metallo-Beta-Lactamases (MBLs) mediated carbapenem resistance, that of bla_{NDM} type is the most widespread in the Indian subcontinent (Ahmad et al., 2018) with $bla_{\text{NDM-1}}$ being the major variant amongst the nineteen identified bla_{NDM} type carbapenemases (Khan et al., 2017). In a study by Kumarasamy et al. (2010), $bla_{\text{NDM-1}}$ producing bacteria were reported present in Bangladesh, India, Pakistan, and the UK. $bla_{\text{NDM-1}}$ producers have also been detected in Europe (Struelens et al., 2010), Kenya (Poirel et al., 2011), and Canada (Mulvey et al., 2011). This elaborates the prevalence of $bla_{\text{NDM-1}}$ harboring microorganisms worldwide and their raising alarm to global public health.

A number of studies have previously reported *bla*$_{\text{NDM-1}}$ producing *K. pneumoniae* of vegetable origin in Japan (Soliman et al., 2021), Pakistan (Junaid et al., 2022), and Myanmar (Sugawara, Hagiya, et al., 2019), but none has been reported in Bangladesh so far. Instead, *bla*$_{\text{NDM-1}}$ in Bangladesh has mostly been detected in clinical isolates. (Mohammad Aminul Islam et al., 2013; Rakhi et al., 2019).

In the present study, five *bla*$_{\text{NDM-1}}$ positive *K. pneumoniae* isolates were found which is comparatively higher than the two found by Sugawara et al. (2019) in vegetables from Myanmar. Moreover, all the *bla*$_{\text{NDM-1}}$ producing *K. pneumoniae* isolates showed a multidrug resistance phenotype which is similar to the findings reported by Sugawara et al. (2019) in Myanmar and M. A. Islam et al. (2012) in Bangladesh. The observed multidrug resistance nature of *bla*$_{\text{NDM-1}}$ harboring isolates is because of the broad-spectrum hydrolytic properties of *bla*$_{\text{NDM-1}}$ as it is found on diverse and conjugative plasmids that also harbor other resistance genes against a variety of antibiotics (Kalasseril et al., 2020). Plasmids carrying *bla*$_{\text{NDM-1}}$ can easily be transferred to other microorganisms via horizontal gene transfer and hence increasing the emergence of carbapenem-resistant pathogenic microorganisms (Khan et al., 2017).

A Bangladesh study illustrating the environmental spread of *bla*$_{\text{NDM-1}}$ producing multidrug-resistant bacteria found that 71% of their samples from hospital adjacent areas and 12% from community areas were positive for *bla*$_{\text{NDM-1}}$ with *K. pneumoniae* being the most prevalent bacteria species (Mohammad Aminul Islam et al., 2017). This continues to strengthen the hypothesis of this present study demonstrating that hospitals may be potential sources of carbapenem resistance to hospital adjacent areas.

Class D carbapenemases, also known as carbapenem-hydrolyzing class D beta-lactamases (CHDLs) are most problematic clinically, as they produce resistance to carbapenems, thus severely limiting therapeutic options (Antunes et al., 2014) (Antunes et al., 2014). The presence of the *bla*$_{\text{OXA-2}}$ gene, under this class, was first reported in *P.aeruginosa* in France (Bert et al., 2002), in *E. coli* in Israel (Chmelnitsky et al., 2005), and in *E.coli* from India (Bhattacharjee et al., 2007). Some global studies have reported the presence of *bla*$_{\text{OXA-2}}$ in Enterobacteriaceae. ie *E. coli* (Chmelnitsky et al., 2005; Mlynarcik et al., 2016) and *K. pneumoniae* (Mlynarcik et al., 2016) in

clinical samples. Unfortunately, specifically in Bangladesh, little is known about the spread of this gene in food and water samples from hospital adjacent areas.

The *bla*$_{\text{OXA-2}}$ beta-lactamases exemplify the narrow-spectrum enzymes capable of producing resistance to penicillins and some early cephalosporins (Poirel et al., 2010). It can extend its substrate profile to produce resistance to expanded-spectrum cephalosporins, such as ceftazidime (Antunes et al., 2014). This observation has been noted in the present study, where 51.90% of the *K.pneumoniae* isolates showed resistance to ceftazidime. It can be inferred that the *bla*$_{\text{OXA-2}}$ gene in some of these isolates might have contributed to ceftazidime resistance. A total of nine *bla*$_{\text{OXA-2}}$ positive *K. pneumoniae* isolates were identified in this study. Most of these isolates were distributed among vegetables; bitter gourd and lemon being the most contaminated.

Most studies worldwide, in South Africa (Ebomah & Okoh, 2020), Algeria (Chaalal et al., 2021), Columbia, Germany, Russia, Singapore, Switzerland, UK etc (Mairi et al., 2018) [Q] and also those in Bangladesh (Khatun & Shamsuzzaman, 2016; Rakhi et al., 2019) focused on Carbapenem Hydrolyzing Class D Beta-lactamases (CHDL's) mainly *bla*$_{\text{OXA-48}}$ as they were of more clinical significance and are widely disseminated in *K. pneumoniae* and other Enterobacteriaceae. This encourages the reason as to why we should also find the prevalence of *bla*$_{\text{OXA-48}}$ from the *K.pneumoniae* isolates in this study.

Despite all the isolated *K. pneumoniae* strains showing full resistance to meropenem, only a few of them showed a positive PCR result for the presence of carbapenem-resistance genes. This may suggest the presence of other mechanisms of carbapenem resistance such as modification of outer membrane permeability, e.g., porin loss, up-regulation of efflux systems, and hyperproduction of AMP-C beta-lactamases with increased capacity to hydrolyze carbapenems (Begum & Shamsuzzaman, 2016).

K. pneumoniae strains classified as XDR are rapidly emerging due to the dissemination of resistance toward aminoglycosides, β-lactams, fluoroquinolones, and carbapenems (Al-Marzooq et al., 2015). In the present study, it was observed that four K. pneumoniae isolates exhibited resistance to all antibiotics tested which was a comparatively higher number than that reported by Sugawara et al. (2019), who found only one foodborne *K. pneumoniae* isolate from chicken susceptible to only colistin out of all the antibiotics tested. Moreover, one Bangladesh study

reported that all the carbapenemase-producing isolates were resistant to all tested antibiotics except colistin (Begum & Shamsuzzaman, 2016) and this was observed in two carbapenemase-producing *K. pneumoniae* isolates found in the present study. Therefore, in addition to examining the XDR nature of isolates in the continuation of the present study, the colistin resistance pattern can also be found as this antibiotic is still a viable antibiotic alternative against Carbapenem-Resistant Enterobacteriaceae as seen in both of the mentioned studies.

In this study, K. *pneumoniae* isolates producing $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-2}}$ genes were found present in some vegetables that are often eaten raw, here in Bangladesh; such as green chili and lemon. Similarly, a study in China reported a high occurrence of carbapenem-resistant Enterobacteriaceae in different ready-to-eat vegetables mostly, curly endive, romaine lettuce, cucumber, and tomato (B. T. Liu et al., 2018). Consumption of such vegetables containing CRE poses a serious health risk to the body (Colosi et al., 2020) as they can facilitate the transfer of carbapenem-resistant genes to the gut microbiota (B. T. Liu et al., 2018) and complicated treatment. Therefore, measures that ensure food safety in vegetables that are often consumed raw should be put in place and implemented to promote consumer health.

Interestingly, lemons that have been proven to contain antibacterial potential (Ekawati & Darmanto, 2019; Verlekar & Chandak, 2018), had an unusually high number of carbapenemase-producing *K. pneumoniae*. The most likely reason for this could be that these lemons might have been contaminated during handling phases or when washing them in vegetable wash water previously used to wash other contaminated vegetables.

(Kalasseril et al., 2020) reports that warm temperatures and high relative humidity promote the emergence and survival of AMR bacteria in the environment. In line with this, the average temperature and humidity in the sample collection areas of our study were 32.78°C and 76.22 % respectively which puts the community at risk of the emergence of new community-associated bacterial infections. In fact, the IncX3 plasmid known to harbor $bla_{\text{NDM-1}}$ genes was capable of transferring more effectively to a recipient at temperatures as low as 25°C which may be one reason that explains its wide dissemination (Sugawara, Akeda, et al., 2019). A study is needed to compare the effect of temperature and humidity on the community resistance gene pool, conducted in the summer and winter seasons of Bangladesh.

Taking into consideration the MAR Index of the Isolates in the present research, *bla*$_{\text{NDM-1}}$, and *bla*$_{\text{OXA-2}}$ positive *K. pneumoniae* isolates, the location at highest risk for carbapenem resistance is Ananda Bazar near Dhaka Medical College Hospital. Dhaka Medical College Hospital (DMCH) is the largest and oldest tertiary hospital in Dhaka which means that it receives a high number of patients on a daily basis with infections that might require antibiotic treatment. Due to the fact that antibiotics are not fully metabolized, these active residues can continue to provide selective pressure for antimicrobial resistance in excreted waste leading to the emergence and survival of MDR pathogens and Superbugs (Founou et al., 2016) as shown by Begum & Shamsuzzaman (2016) and (Adnan et al., 2013) in the hospital waste discharge of DMCH.

Adnan et al. (2013) further pointed out the disposal of untreated hospital liquid wastes directly into the municipal sewage system and drains in Dhaka which increased the likelihood of emergence and distribution of antimicrobial resistance genes in environmental bacteria. So, as the distance between Ananda Bazar and Dhaka Medical College Hospital is very short (approximately 200m), there is a high probability that the source of contamination of the surrounding environment, water, and food with carbapenem-resistant Enterobacteriaceae is hospitals posing a serious threat to the hospital and adjacent areas and communities. A comparative study by Mohammad Mohammad Aminul Islam et al. (2017) which illustrated the relationship between distance from hospitals and the spread of carbapenem resistance showed that places nearer to hospitals had higher carbapenem and multidrug resistance profiles as compared to communities further away from hospitals. This clearly elaborates the need to increase the distance between hospitals and community markets here in Dhaka.

Bands that formed below or above 264 bp(blaNDM-1) and those which formed below or above 702 bp (blaOXA-2) were observed in this study. This could be due to primers used being nonspecific. Nonspecific primers will bind to unintended genes and result in nonspecific amplicons (Qu & Zhang, 2015). Additionally, PCR cannot necessarily identify the targeted carbapenemase gene to the variant level and for this reason, sequencing of the PCR products can generally be used to identify them (Anderson & Boerlin, 2020).

4.2 Conclusion and Future Direction

The findings of the current study demonstrated the potential of vegetables and water from markets located in hospital adjacent areas, to be reservoirs of carbapenem-resistant - multidrug-resistant bacteria. Therefore, strict food safety measures and public health regulations are necessitated to preserve consumer health. Market vendors also need to be sensitized and monitored on how they can lower the risk of the spread of carbapenem resistance. But most importantly, the government should implement regulations on the effective treatment of hospital wastes and disposal in the environment, and also look into increasing the distance between hospitals and community markets to prevent further dissemination of carbapenem-resistant microorganisms in the environment.

The present study was limited to detecting only two carbapenem resistance genes $bla_{\text{OXA-2}}$ and $bla_{\text{NDM-1}}$ so the presence of more genes such as bla_{KPC}, $bla_{\text{OXA-48}}$, bla_{VIM}, bla_{IMP} in the isolates could be investigated in the future. Furthermore, the relationship between clinical and environmental antimicrobial susceptibility patterns of carbapenem-resistant *K. pneumoniae* in hospital adjacent areas could be found. And also, a comparison between the summer and winter seasons' effect of temperature and humidity on the community resistance gene pool in Bangladesh would be an interesting finding.

Chapter 5
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