



Effect of *in vitro* Zinc Depletion on Carbapenemase Activity in Enterobacterales Clinical Isolates

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science.

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DECLARATION

I, Froodia Ngondoh, hereby declare that this dissertation is my original and unaided work. It is submitted in fulfilment of the requirements for the Master of Science degree at the University of the Witwatersrand, Johannesburg. This dissertation has never been submitted previously for any degree or examination at another university.



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14 DAY OF APRIL 2025 IN JOHANNESBURG

ABSTRACT

Introduction: Metallo- β -lactamase (MBL)-producing carbapenem-resistant Enterobacterales (CRE) represent a serious clinical challenge due to limited treatment options, necessitating accurate testing to identify effective treatments and combat antibiotic resistance. Supraphysiological zinc levels in media can lead to skewed resistance profiles in MBL-producing CRE, complicating treatment decisions. This study investigates the effect of zinc depletion on carbapenem resistance.

Methods: Thirty MBL-producing CRE isolates (NDM, n=15; VIM, n=15) underwent testing before and after zinc depletion using broth microdilution, disk diffusion, and gradient diffusion testing. Zinc depletion from media was achieved through ethylenediaminetetraacetic acid (EDTA) addition.

Results: All isolates were resistant to at least one carbapenem by broth microdilution—100% (30/30) to imipenem and 93% (28/30) to meropenem—with both imipenem and meropenem having an MIC₅₀ of 32 μ g/ml and MIC₉₀ > 64 μ g/ml. Zinc depletion resulted in a notable shift towards susceptibility ($p < 0.0001$). MIC₅₀ values dropped to 0.25 μ g/ml for imipenem and < 0.12 μ g/ml for meropenem, with MIC₉₀ decreasing to 4 μ g/ml and 8 μ g/ml, respectively. NDM-producing isolates exhibited a higher MIC₅₀ (> 64 μ g/ml) compared to VIM-producing isolates (8-16 μ g/ml). Cohen's Kappa analysis showed substantial agreement ($k = 0.63$) between broth microdilution and gradient diffusion strip test in zinc-depleted media.

Conclusion: In MBL-producing Enterobacterales, zinc exacerbates *in vitro* carbapenem resistance, highlighting the potential for optimising antibiotic susceptibility testing by reducing zinc levels in media. While further clinical data would be required to support changes in testing protocols, lowered zinc levels may contribute to more accurate treatment decisions and expand carbapenem use in certain cases. These findings are valuable for improving CRE diagnostics and therapeutics, aiding in more effective antimicrobial stewardship.

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NOMENCLATURE

°C	Degree Celsius
µg	Microgram
µl	Microliter
AmpC	Type C ampicillinase
AMR	Antimicrobial resistance
AST	Antibiotic Susceptibility testing
ATCC	American Type Culture Collection
BDMIC	Broth Microdilution
caMHB	Cation-adjusted Mueller-Hinton Broth
CHDLs	Carbapenem-hydrolyzing class D β-lactamases
CLSI	Clinical and Laboratory Standards Institute
CP	carbapenemase producing
CRE	Carbapenem-resistant Enterobacterales
DPA	Dipicolinic acid
EDTA	Ethylenediaminetetraacetic acid
ESBLs	Extended-spectrum β-lactamase
E-test	Epsilometer test
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GES	Guiana extended-spectrum β-lactamase
GIM	German imipenemase
IMI	Imipenem-hydrolyzing β-lactamase
IPM	Imipenem
KPC	<i>Klebsiella pneumoniae</i> carbapenemase
MBL	Metallo-β-lactamase
MEM	Meropenem
mg	Milligram
MGEs	Mobile genetic elements
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimum Inhibitory Concentration
ml	Millilitre
NDM	New Delhi metallo-β-lactamase
OXA	Oxacillinase
SIM	Seoul imipenemase
SMP	SaoPaulo metallo-β-lactamase
SOP	Standard Operating Procedure
VIM	Verona integron-encoded MBL
Zn(II)	Zinc ion
ZOI	Zone of inhibition

GENERAL INTRODUCTION

Enterobacterales are implicated in numerous human infections. The widespread and regular administration of antibiotics in treating these infections has resulted in escalated antibiotic resistance, constraining treatment options to antibiotics such as carbapenems ¹. Carbapenem-resistant Enterobacterales (CRE) are emerging due to various carbapenemases, particularly the zinc-dependent metallo- β -lactamases (MBL) ². Of greater concern is the resistance exhibited by MBL-producing CRE strains to new agents targeting Enterobacterales, underscoring the necessity for alternative approaches ³.

A recent investigation indicates that *in vitro* MBL-harbouring Enterobacterales resistance does not necessarily equate to *in vivo* carbapenem inefficacy ⁴. This discordance between *in vivo* and *in vitro* outcomes is due to the heightened zinc levels in commercially available media ⁴. A comparative analysis has shown that the effectiveness of carbapenem *in vivo* aligns with *in vitro* susceptibility when utilising zinc-depleted media ⁵. This implies that zinc-depleted media most accurately simulate physiological conditions and that carbapenems can still be effective in treating patients infected with MBL-harbouring Enterobacterales ⁶.

Despite the considerable prevalence of MBL in Enterobacterales in Africa, there is an observable scarcity of published studies that specifically investigate the management of infections induced by MBL-producing CRE in this particular region. The recent findings on the *in vitro* carbapenems' efficacy against MBL-producing CRE isolates in the absence of zinc highlight the need for further research, especially in an African setting. In a review article, Asempa et al. emphasised the importance of additional investigations on carbapenem activity against MBL-producers in both standard and zinc-depleted media to strengthen understanding of the correlation between *in vitro* and clinical data ⁷.

Our study analysed the patterns in carbapenem susceptibility of MBL-producing Enterobacterales with and without zinc exposure. Considering the elevated zinc levels in cation-adjusted Mueller-Hinton broth and its influence on MBL-mediated β -lactam hydrolysis, evaluating the *in vitro* carbapenem susceptibility of MBL-producers in zinc-depleted media could present significant data supporting the use of these agents as viable therapeutic choices against these pathogens. This could have a significant impact considering the currently restricted treatment alternatives for MBL-producing Enterobacterales.

Moreover, the present study seeks to establish whether there are consistent outcomes in antibiotic susceptibility testing (AST) between zinc-depleted solid and liquid media. Should the susceptibility profiles of the organism remain consistent on solid media, it would offer substantial benefits to laboratories, as approaches like the E-test and disk diffusion are not only more economical but also entail less labour and time when contrasted with the broth microdilution technique^{8,9}. This investigation represents a novel aspect of this study, as solid zinc-depleted media for carbapenem AST has not been previously explored.

Chapter 1 outlines the research objectives and provides a comprehensive review of relevant literature on Enterobacterales, carbapenems, carbapenemases, and the role of zinc in modulating MBL activity. A thorough understanding of these fundamental concepts establishes the foundation for investigating the impact of zinc on antibiotic susceptibility.

Chapter 2 details our methodology, outlining the study design, sample processing procedures, and methods for AST using both conventional and zinc-depleted media. Additionally, statistical analysis methods utilised to interpret the results are outlined. These methodological insights are essential for understanding the experimental approach undertaken to address the research objectives.

In Chapter 3, we present the findings from AST using conventional media. This includes results from disk diffusion, broth microdilution, and gradient diffusion strip tests, offering insights into the susceptibility patterns observed under standard conditions. This data serves as a reference point for subsequent comparative analyses.

Chapter 4 extends our investigation by presenting results from AST using ethylenediaminetetraacetic acid (EDTA)-treated media. Through the chelation of zinc ions, EDTA treatment aims to disrupt MBL activity and restore antibiotic susceptibility. Consequently, this chapter sheds light on the involvement of this metal ion in modulating antibiotic resistance mechanisms.

Chapter 5 offers comparative analyses of results obtained from conventional and zinc-depleted media. It also assesses the agreement between different testing techniques and examines the impact of carbapenemase genotypes on antibiotic resistance trends. By means of these comparative analyses, trends and patterns in antibiotic susceptibility profiles emerge, offering key insights into the effectiveness of EDTA treatment and the reliability of various testing protocols.

In the final chapter, Chapter 6, we engage in a broader discussion on the effects of zinc depletion on antibiotic susceptibility, the effectiveness of EDTA treatment, the alignment between testing methodologies, and the correlation between carbapenemase genes and antibiotic resistance. This chapter delves into the broader implications of our research findings in the battle against antibiotic resistance among Enterobacterales. Finally, conclusive remarks are provided, and avenues for future research in this field are outlined.

CHAPTER ONE:

Introduction



1.1 Aim and Objectives

In this study, we aim to evaluate carbapenem *in vitro* activity against phenotypically confirmed carbapenem-resistant MBL-producing Enterobacterales in untreated media (i.e. with normal zinc levels) versus EDTA-treated media (with lowered zinc levels) using different AST methods.

Objectives:

- a. To evaluate the activity of meropenem and imipenem in untreated media versus EDTA-treated media using disk diffusion.
- b. To measure the minimum inhibitory concentrations of meropenem and imipenem in untreated media versus EDTA-treated media using broth microdilution.
- c. To measure the minimum inhibitory concentrations of meropenem and imipenem in untreated media versus EDTA-treated media using gradient diffusion strip testing.

1.2 Literature Review

1.2.1 Carbapenem Resistance in Enterobacterales and the Role of Metallo- β -Lactamases

1.2.1.1 Carbapenems

Carbapenems are β -lactam antibiotics known for their effectiveness against an extensive variety of both Gram-negative and Gram-positive bacteria ¹⁰. Key carbapenems include meropenem, imipenem, ertapenem, and doripenem ¹¹. Imipenem and meropenem possess a broad spectrum of activity and are crucial for treating severe infections ¹⁰⁻¹². Compared to ertapenem, they cover a broader range of pathogens, including non-fermenting Gram-negative bacteria and *Enterococcus* spp., which are not effectively targeted by ertapenem due to its narrower spectrum ¹⁰⁻¹³. Although doripenem exhibits lower Minimum Inhibitory Concentrations (MICs) against some pathogens and is less susceptible to hydrolysis by certain carbapenemases, its clinical relevance is limited by its less common use in practice ^{10,14,15}.

Carbapenems exert their antimicrobial effects by targeting the synthesis of the bacterial cell wall through interaction with penicillin-binding proteins (PBPs) ¹⁶. PBPs are crucial enzymes involved in the formation and maintenance of the peptidoglycan layer, which provides structural integrity to bacterial cells ¹⁷. Specifically, PBPs catalyse the polymerisation of glycan chains and the synthesis of cross-links in peptide side chains, essential processes for creating a robust cell wall ^{10,18}. Carbapenems mimic the natural substrates of PBPs due to their β -lactam ring structure. This allows them to bind covalently to the active sites of PBPs, acylating the serine residues and inhibiting the enzymes' activity. Consequently, the creation of cross-links between glycan chains is disrupted, weakening the peptidoglycan layer and rendering it unable to withstand internal osmotic pressure. This leads to cell wall instability, abnormal cell shapes, and ultimately, cell lysis and death ^{10,18,19}. The extensive antimicrobial activity of carbapenems is attributed to their ability to bind various types of PBPs with high affinity across various bacterial species, making them highly effective antibacterial agents ¹⁰.

Therapeutic options for infections that are unresponsive to carbapenems are limited, especially in resource-constrained settings. Recent advancements in antibiotic agents, primarily designed to target Enterobacterales, indicate a leap forward in the realm of developing new pharmaceuticals ²⁰. These novel agents comprise plazomicin, eravacycline as well as combination therapies such as relebactam-imipenem-cilastatin, meropenem-vaborbactam, and

ceftazidime-avibactam^{20,21}. As most antibiotic monotherapies have shown limited success, the use of combination therapeutic regimens is common to improve treatment outcomes^{22,23}. The combination of antibiotics with various modes of action holds the potential to effectively counter bacterial resistance mechanisms and substantially reduce the bacterial load²³. Given the challenges associated with managing carbapenem-resistant infections, effective infection control and prevention strategies have the potential to complement therapeutic interventions by decreasing the occurrence of new infections and the development of resistance mechanisms. Timely identification of the antibiotic resistance patterns exhibited by identified strains is crucial for guiding therapy and preventing the inappropriate use of drugs.

1.2.1.2 Enterobacterales and carbapenem resistance

Enterobacterales represent a cohort of Gram-negative bacteria associated with severe and often fatal infections such as bacteraemia, pneumonia, meningitis, skin and soft tissue infections, and urinary tract infections^{2,24,25}. There are various bacterial species in the Enterobacterales order, including *Enterobacter cloacae*, *Escherichia coli*, *Citrobacter freundii*, and *Klebsiella pneumoniae*²⁶. The dissemination of Enterobacterales in healthcare environments is mainly attributed to direct person-to-person transmission or indirect spread through contaminated materials and surfaces^{1,24}.

During the 1940s and later periods, patients diagnosed with bacterial infections were efficiently managed through the application of antibiotics^{27,28}. Antibiotics such as polymyxins, ceftazidime, aminoglycosides, and fosfomycin were employed against Enterobacterales²⁶. Nonetheless, the mishandling of antibiotics has brought about antibiotic resistance. In the year 2008, *K. pneumoniae* and *Enterobacter* species were pinpointed among the ESKAPE group of bacterial pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*) known for their ability to resist antibiotic therapies²⁹. Carbapenems were subsequently deemed as the optimal therapeutic solution for tackling multi-drug resistant Enterobacterales. However, the effectiveness of carbapenems against Enterobacterales is waning due to escalating carbapenem resistance².

As per the United States Centres for Disease Control and Prevention, carbapenem-resistant Enterobacterales (CREs) demonstrate resistance to one or more carbapenem antibiotics

(typically meropenem, imipenem, ertapenem, or doripenem) or produce a carbapenemase enzyme³⁰. CREs have shown resilience in harsh environments, even when exposed to a broad array of antibiotic agents, resulting in significant clinical implications such as treatment complexities and potential mortality arising from untreated infections, particularly affecting individuals with underlying health conditions^{31,32}. In essence, CRE infections are associated with extended hospitalisations, substantial economic impacts, and a significant upsurge in mortality and morbidity rates as opposed to carbapenem-susceptible bacterial infections²⁴. Within the United States alone, CRE leads to more than 13,100 hospitalisations and over 1,000 deaths every year³⁰. High mortality rates of 38% and 42.5% were noted in subjects with MBL-producing CRE bacteraemia in South Africa and India, respectively^{33,34}.

The surge of CREs is steadily increasing on a global scale, with certain regions facing higher levels of resistance compared to others^{22,32}. Recent data highlights a concerning increase of over 2.5 times in CRE cases between 2013 and 2020^{26,35}. In Japan, 2,289 cases of CREs were reported in 2018, with *Klebsiella aerogenes* (38%) and *E. cloacae* (28%) being the most common pathogens³⁶. The World Health Organisation classified CRE as of critical concern, emphasising the need for sustained surveillance and targeted interventions to address rising carbapenem resistance in Enterobacterales²⁵.

The rapid dissemination of CREs is propelled by an evolutionary force, enabling bacteria previously exposed to antibiotics to develop and propagate resistance genes^{37,38}. The hospital milieu offers a favourable context for the perpetuation and swift distribution of antibiotic-resistant strains, attributed to factors such as prolonged hospital admissions, extensive antibiotic administration, and invasive medical interventions. Healthcare facilities, especially tertiary medical centres, function as key focal points for the transmission of carbapenemase-producing Enterobacterales (CPE)³⁹. However, CREs have been detected not only in hospital environments but also in community contexts, with evidence indicating community transmission involving carriers lacking recent healthcare or drug exposure, suggesting alternative avenues of dissemination⁴⁰. Beyond clinical settings, CPE proliferate within communities through avenues like the food chain, person-to-person contact, and environmental contamination^{41,42}. The One Health Approach, which underscores the interconnections between environmental, animal, and human health, is pivotal in understanding and addressing the dissemination of emerging antibiotic resistance trends^{43–45}. Notably, the application of antibiotics in agriculture, aquaculture, and veterinary medicine contributes significantly to the selective pressure propelling the rise and dissemination of resistance^{46–49}. The interaction

among humans, animals, and the environment is particularly relevant for Enterobacterales, which demonstrate a widespread presence across species and ecosystems, allowing the transfer of resistance genes and mechanisms ^{46,49}. As a result, environmental reservoirs, such as water sources and livestock, also influence the propagation and sustained presence of carbapenemase-encoding genes in both hospital and community settings ^{49,50}. The review by Schneider et al.⁵¹ assessed the adoption of the One Health model to tackle the distinct challenges associated with the carriage of bacteria such as Enterobacterales.

In essence, the dynamic nature of the evolving carbapenem resistance patterns in Enterobacterales necessitates an innovative and multifaceted approach to uphold the effectiveness of carbapenem therapy. Continuous and comprehensive surveillance efforts, as articulated by Pitout and DeVinney ⁵², are critical for understanding the ever-changing landscape of antibiotic resistance and devising strategies to counteract the impact of Enterobacterales on public health.

1.2.1.3 Molecular basis of carbapenem resistance in Enterobacterales

Enterobacterales develop resistance to carbapenems through various mechanisms, such as mutation or modification of carbapenem targets like PBPs, depletion of outer membrane porins, heightened efflux pump activity, and the gain of resistance genes through horizontal gene transfer from mobile genetic elements (MGEs) (Figure 1) ^{2,22,32,53,54}. These resistance genes primarily include carbapenemases, while other β -lactam resistance determinants, such as extended-spectrum beta-lactamases (ESBLs), can contribute to carbapenem resistance when combined with porin loss ⁵⁵.

As the leading mechanism behind carbapenem resistance in Enterobacterales, carbapenemases have been detected in 85% of CRE cases worldwide ⁵³. Genes encoding carbapenemases are transferred from one bacterium to another through the dissemination of plasmids ⁵⁶. Plasmids containing carbapenemase genes often display diverse replicon types, enhancing their adaptability and ability to spread across different bacterial species ⁵⁷. More worrisome is that the enzymatic activity of carbapenemases extends beyond carbapenems to confer resistance against other beta-lactams ⁵⁸. Enterobacterales, such as *E. coli*, may display a nuanced interplay of resistance mechanisms, often harbouring multiple resistance genes or mechanisms that culminate in heightened levels of resistance against various classes of antibiotics ⁵⁹. An

example includes a strain containing genes encoding both ESBLs and carbapenemases. Managing infections caused by these bacteria becomes increasingly complex due to the interaction of resistance mechanisms, particularly in healthcare environments ⁵⁸.

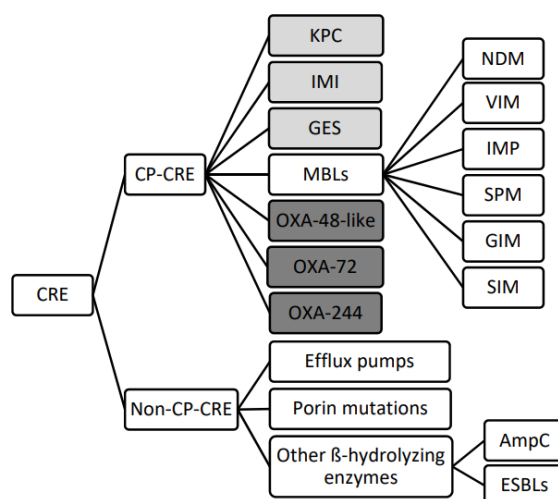


Figure 1 : Categorisation of Drug Resistance Mechanisms in Enterobacterales, sourced from Suay-García B and Pérez-Gracia ⁶⁰. (Ambler classes A, B, and D are indicated by light grey, white, and dark grey, respectively).

1.2.1.4 Different Classes of Carbapenemases in Enterobacterales

As per the Ambler classification, Enterobacterales present three groups of carbapenemases, referred to as Classes A, B, and D.

The Ambler class A carbapenemase known as *K. pneumoniae* carbapenemase (KPC) is predominantly present in Enterobacterales. From a structural perspective, KPC enzymes are serine-based and commonly spread through plasmids, which promote the transfer of genes horizontally. These enzymes interact with the carbapenem ring by leveraging their serine residue as a nucleophilic agent, allowing them to break down the antibiotic and consequently leading to the development of antibiotic resistance ⁵⁸.

The Ambler class B carbapenemases, referred to as metallo- β -lactamases (MBLs), function through a catalytic mechanism that relies on the presence of metal ions, particularly zinc ions, at their active site to break down antibiotics ⁶¹. The predominant MBLs found in Enterobacterales include Imipenemase (IMP), Verona integron-encoded metallo- β -lactamase

(VIM), and New Delhi metallo- β -lactamase (NDM) ^{2,53}. These MBL subclasses have similar zinc requirements, functioning as di-zinc enzymes that require two Zn^{2+} ions for their enzymatic activity ^{62–64}. The zinc-dependent hydrolytic activity involves the coordination of zinc ions with amino acid residues to enable the cleavage of the β -lactam ring ⁶¹. The impact of MBLs on healthcare settings is notable due to their role in spreading multidrug-resistant mechanisms, which in turn hinders the effective treatment of bacterial infections ³⁸. NDM, a highly potent carbapenemase, can break down many β -lactam antibiotics, including carbapenems, and is frequently linked to multidrug-resistant pathogens ³⁵.

Class D carbapenem-hydrolysing β -lactamases (CHDLs), commonly referred to as oxacillinases, are genetically determined by OXA-type genes, with OXA-48 being widely disseminated among Enterobacterales ³¹. While *Acinetobacter* spp. harbour most CHDLs variants, OXA-48 is distinctly found in Enterobacterales ⁶⁵. OXA-48 carbapenemases exhibit the ability to hydrolyse carbapenems to a certain degree while not affecting expanded-spectrum cephalosporins ^{65,66}. These enzymes are commonly harboured on plasmids, facilitating their transfer between various organisms ^{65,67}. Comparable to KPCs, they feature a serine-based active site and hydrolyse carbapenems using a serine nucleophile ⁶⁶. The varied substrate profiles of its variants present challenges in the identification and management of infections associated with OXA-48-producing strains ⁶⁶.

1.2.1.5 Epidemiology and Prevalence of Carbapenemases in Enterobacterales

The genes encoding carbapenemases can be located either on chromosomes or on MGEs like plasmids and integrons, which contribute to their dissemination among different bacterial species ⁶⁸. Chromosomal carbapenemases can be inherent to the bacterial genome or can be acquired and integrated into the chromosome through horizontal gene transfer. Examples of chromosomal carbapenemases include the Imipenem-hydrolysing beta-lactamase (IMI), which is chromosomally encoded in *E. cloacae*, conferring resistance to imipenem and other carbapenems ^{68,69}. Plasmid-mediated carbapenemases are encoded by genes located on plasmids, which are extrachromosomal DNA molecules. These plasmids can be transferred between bacteria, facilitating the rapid spread of resistance genes. KPC is typically plasmid-mediated and highly prevalent in Enterobacterales, initially identified in *K. pneumoniae* but now found in various species ⁶⁸. Integrons are genetic elements that can assimilate and express genes, especially those encoding antibiotic resistance. Integrons can be part of plasmids or

chromosomes and are key players in the horizontal transfer of resistance genes. The gene encoding the VIM is often part of class 1 integrons, found in *P. aeruginosa* and Enterobacterales⁷⁰.

The prevalence of carbapenemases in Enterobacterales displays variability across different species as well as geographical regions. Kazmierczak et al.'s⁵³ comprehensive study conducted over the period of 2012 to 2017 explored the worldwide prevalence and distribution of carbapenemases in meropenem-resistant Enterobacterales. According to these findings, *K. pneumoniae* emerged as the predominant carrier of KPC-type carbapenemases. OXA-48-like enzymes were regularly detected in *E. coli* and *K. pneumoniae*, whilst MBLs were widespread in species such as *Citrobacter* spp., *Enterobacter* spp., and *Proteaeae*. The study revealed a global distribution of carbapenemase types, with KPC prevailing in countries like the U.S., Argentina, and Brazil, and OXA-48-like being predominant in Turkey, Russia, and other regions. NDM-positive isolates were identified globally, with a more pronounced presence in Middle East/Africa and Asia/Pacific. The overall rise in meropenem-resistant Enterobacterales was correlated with an upsurge in carbapenemase-positive isolates, notably OXA-48-like and NDM types. This escalation is consistent with previous findings showing significant increases in rates of CRE in Europe and Asia^{23,31,60}.

Further research identified KPCs as the primary carbapenemases in Enterobacterales in Latin America and North America^{53,54}. Conversely, their occurrence is scarce in CRE isolates in the Middle East, with Israel being the only exception⁵⁴. In regions across Africa, including Morocco, Nigeria, and South Africa, it has been observed that MBLs are more prevalent than KPCs, making up over 40% of CRE isolates^{26,54}. Within South Africa, NDM and VIM are the most frequently identified MBL species in CREs³⁴.

The epidemiology of CREs in South Africa also reveals a concerning trend of rising resistance, particularly in healthcare settings. Since the implementation of molecular testing for CPE in 2011, a significant presence of these resistant organisms has been detected⁷¹. An analysis of 1,503 isolates from 2012 to 2015 confirmed that 68% contained carbapenemase-producing genes, with *K. pneumoniae* being the most frequent pathogen (60%)⁷¹. Among the most commonly identified resistance genes, NDM was predominant, followed by OXA-48 and its associated variants⁷¹. In 2016, another study reported a shift in the occurrence of these carbapenemase genes, with OXA-48 and variants becoming more common than NDM⁷². Further surveillance data from the National Institute for Communicable Diseases (NICD) for

2017 highlighted the continued prevalence of CRE, with *K. pneumoniae*, *E. coli*, and *E. cloacae* being the dominant organisms ⁷³. Resistance patterns varied among the different carbapenemase genes. NDM-associated isolates often exhibited high-level resistance, while VIM isolates mostly showed low-level resistance. OXA-48 variants exhibited comparable low and high resistance levels ⁷².

On a global scale, a significant and rapid dissemination of NDM in Gram-negative bacteria has been noted recently ⁷⁴. In contrast to other MBL types, NDM displays a unique characteristic as it is bound to the outer membrane rather than existing as a soluble periplasmic protein (Figure 1.3.3.3) ⁷⁵. This specific localisation has been recognised as a key element contributing to the clinical success of NDM ^{76,77}. The anchoring of NDM to the outer membrane confers protection against degradation even under hostile conditions imposed by the host immune system ⁷⁷. Particularly during infection, the host deploys a defence mechanism called "nutritional immunity", which results in the release of significant amounts of the metal-binding protein, calprotectin. This response results in a reduction of zinc levels within the bacterial periplasm,

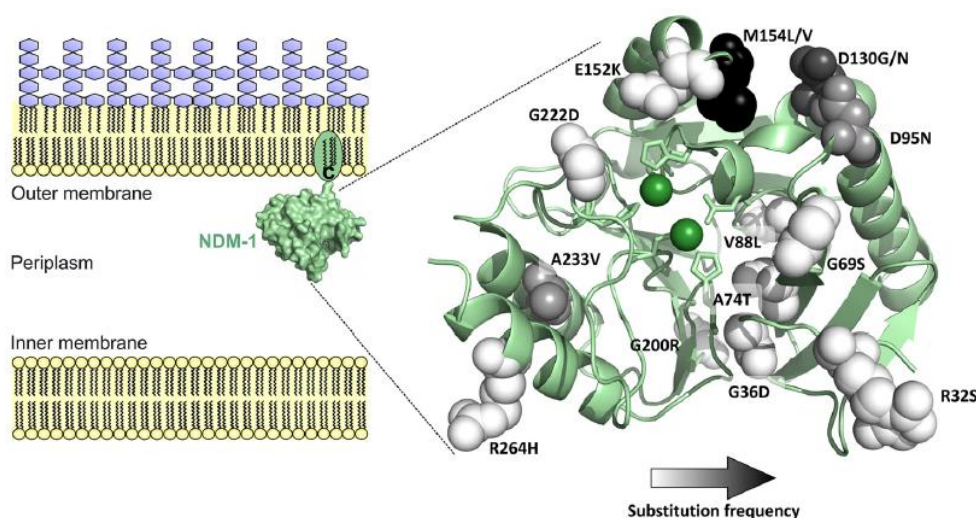


Figure 2 : An illustration of the intracellular localisation of NDM-1 in the inner layer of the outer membrane in Gram-negative bacteria, along with the NDM-1 crystal structure with focus on the specific amino acid residues that have undergone mutation in clinical variations (depicted as spheres). The colour spectrum of these spheres spans from white to black, indicating the growing incidence of substitutions at each position across alleles. Green spheres represent the active-site Zn(II) ions, while amino acid residues that act as metal ligands are depicted as sticks. This visual representation was sourced from Bahr et al. ⁷⁸.

leading to the aggregation of vulnerable apo (non-metalated) MBLs prone to enzymatic breakdown. Therefore, the outer membrane anchoring of NDM serves as a barrier, shielding it

from degradation during this zinc deficiency-induced process ⁷⁷. These findings shed light on the adaptive strategies harnessed by bacteria to withstand host defence mechanisms and underscore the importance of understanding the cellular localisation of NDM in the battle against antimicrobial resistance. Such insights not only advance our knowledge of bacterial survival tactics but also show promise for the development of targeted therapeutic interventions aimed at disrupting critical resistance mechanisms.

1.2.1.6 Treatment challenges and emerging carbapenemases

The growing prevalence of multidrug-resistant Enterobacterales brings about important clinical implications and treatment challenges ³². When diagnoses are inaccurate or delayed, clinicians frequently turn to empirical therapy to initiate treatment promptly. However, relying on empirical factors to guide treatment decisions carries risks, including treatment failure, prolonged hospitalizations, and higher mortality rates ⁷⁹. These risks are particularly pronounced in critically ill patients and those with weakened immune systems ⁸⁰. Given these circumstances, clinicians must advocate for cautious antibiotic use, stressing the importance of proper dosage, selection, and duration of therapy to mitigate the development and increasing prevalence of antibiotic resistance. The pressing need for alternative treatment options has spurred investigations into novel antibiotic agents, combination therapy, and innovative approaches like immunomodulation and phage therapy ^{81–83}.

Research efforts have been focused on improving therapeutic strategies through the integration of combination antibiotics as well as novel compounds. However, most of these newly introduced compounds are not effective in treating strains that produce MBLs ^{3,54,84}. Ceftazidime-avibactam, for instance, is a combination of a diazabicyclooctane-based inhibitor that effectively targets KPC and OXA-48-like producers but lacks efficacy against MBL producers ^{21,85}. Similarly, imipenem-relebactam and meropenem-vaborbactam (other combinations of β -lactam- β -lactamase inhibitors) exhibit activity against KPC resistance while showing limitations against MBL-mediated resistant strains ^{21,85}. Given the limited selection of novel antimicrobial agents available for MBL-harboring CRE, alternative strategies are undoubtedly required.

Treatment challenges are worsened as new carbapenemases variants continue to emerge³². The evolution of these carbapenemases is shaped by various factors such as horizontal gene transfer, recombination events, and genetic mutations, resulting in the emergence of distinct variants with unique enzymatic properties^{31,56}. These variants often demonstrate a wide range of genetic features and expanded substrate profiles, facilitating the breakdown of a more extensive array of antibiotics⁶⁰. This diversity in substrates reflects the adaptive nature of carbapenemases in response to the pressures exerted by antibiotic use in clinical settings. As a result, this variability in function poses significant challenges in anticipating susceptibility patterns and devising effective treatment plans. Continuous research and monitoring are crucial not only within Enterobacterales but also across other bacterial species, given the versatility that carbapenemases have acquired through evolution⁵⁷. Furthermore, newly emerging carbapenemases often coexist with other resistance mechanisms, resulting in intricate resistance profiles in bacterial strains³². The presence of multiple resistance mechanisms may synergistically enhance the overall resistance of bacterial strains^{58,86}. Therefore, understanding the interplay between carbapenemases and other resistance mechanisms like porin mutations and efflux pumps is vital for anticipating clinical outcomes and optimising treatment approaches.

1.2.2 In Vitro Zinc Modulation and Its Effect on Carbapenemase Activity

1.2.2.1 Zinc Acquisition and Regulation in Bacterial Resistance

Zinc, classified as a trace mineral, is essential in various biological processes such as DNA synthesis and enzymatic activities^{87,88}. Bacteria often come across environmental challenges, including alterations in metal ions availability. The upregulation of zinc uptake systems in reaction to these challenges represents an adaptive bacterial tactic to maintain optimal zinc levels for cellular functions. In scenarios where bacteria are exposed to antibiotics, this adaptive response becomes crucial as it enables them to meet the increasing requirement for zinc necessary for the synthesis and activation of resistance-associated metallo-enzymes⁸⁷.

The link between MBL bacterial resistance mechanisms and zinc status is a complex and dynamic process that has therapeutic relevance⁸⁹. Hood and Skaar⁸⁸ suggest that bacterial pathogens acquire zinc through energy-dependent mechanisms facilitated by transport systems like ZnuD, ZnuABC, and AdcBCA (Figure 3). These systems aid in transporting zinc ions across membranes, potentially in chelated forms. Zinc and manganese transporters are involved in pathogenesis, helping bacteria evade host immune responses, as observed in *Salmonella Typhimurium*'s and *A. baumannii*'s resistance to calprotectin-mediated zinc chelation^{88–90}. While specific zinc-chelating compounds similar to siderophores have not yet been identified, some bacteria may produce zinc chelators to obtain zinc⁸⁸.

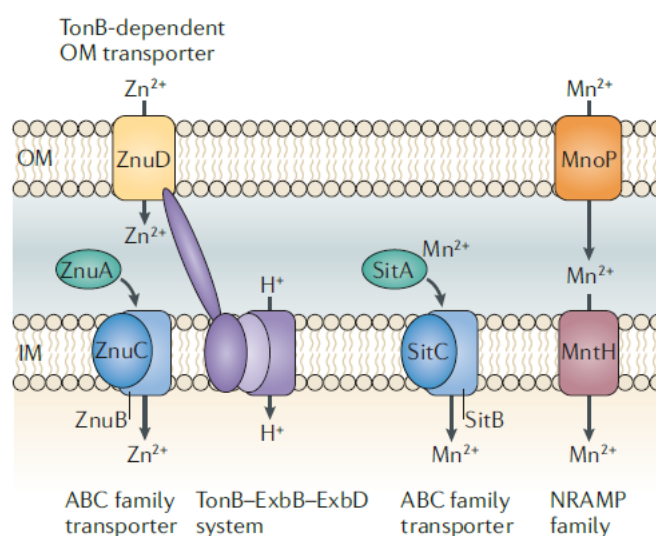


Figure 3 : Uptake processes of manganese ions (Mn²⁺) and zinc ions (Zn²⁺) within pathogenic Gram-negative bacteria, as sourced from Hood and Skaar⁸⁸.

1.2.2.2 Structural insights into zinc binding sites in metallo- β -lactamases

Zinc ions commonly act as cofactors in enzymes, playing a pivotal role in their catalytic function⁸⁷. The specific configuration and interaction of other molecules or atoms surrounding the zinc ion contribute significantly to the overall stability and proper functioning of the enzyme's active site⁷⁵. This coordination of elements often induces structural changes in the enzyme, enabling it to effectively execute its catalytic function^{77,78}.

In MBLs, this dual role of zinc ions is also displayed. The binding of zinc ions to MBLs aids in upholding the protein's structural integrity, creating a conducive environment for substrate binding and catalysis⁷⁷. Furthermore, zinc ions actively participate in the process of antibiotic hydrolysis, serving as vital components of MBLs' catalytic efficiency, thus directly influencing their resistance to denaturation and environmental stressors⁷⁷. Earlier investigations contrasting different mechanisms of MBL activity have unveiled the diversity of zinc-dependent catalytic processes. The coordinated interaction of one or two Zn (II) ions with specific amino acid residues, results in the creation of a stable complex, enables the nucleophilic attack on the β -lactam ring^{75,91}. Histidine and cysteine are the predominant amino acid ligands frequently engaged in the activity of MBLs, offering electron pairs for coordination with the zinc ions⁷⁵. The nucleophilic attack represents a critical phase in the catalytic process as it leads to the cleavage of the β -lactam ring, leading to the degradation of antibiotics and consequently rendering them ineffective^{75,91}.

MBLs demonstrate a fascinating balance between specificity and versatility. In contrast to certain other β -lactamases, MBLs possess the capability to interact with multiple categories of antibiotics owing to the distinctive structural features of their active site, which facilitate the binding of diverse substrates⁹². The flexible zinc-dependent catalytic function of MBLs enables them to break down drugs such as cephalosporins and carbapenems^{91,93}. However, MBLs, despite sharing common traits like zinc coordination, have distinct structural modifications that permit interactions with specific substrates, affecting both the effectiveness of hydrolysis and the array of antibiotics impacted⁹⁴. The biochemical processes involved in zinc-dependent catalysis within MBLs are dynamic rather than static, demonstrating the adaptation of MBLs for the effective breakdown of β -lactam antibiotics⁷⁸. Investigating the structural evolution of MBLs enhances our understanding of the selective pressures induced by exposure to antibiotics, and sheds light on how these enzymes have acquired the capacity to bind and utilise zinc for catalysis⁷⁸.

1.2.2.3 Inhibition strategies targeting zinc binding

The MBL-mediated antibiotic enzymatic catalysis, which relies on zinc, offers a unique target for therapeutic intervention. Innovative therapeutic strategies are aimed at disrupting zinc coordination within the MBL active site, thereby impairing the catalytic function of the enzyme^{7,94}. This approach may result in the reinstatement of β -lactam antibiotic efficacy against MBL-producing bacteria^{7,78}, except in cases where additional resistance mechanisms are present. Zinc chelators have been explored as potential inhibitors of MBLs^{78,87,95,96}. These metalloenzyme inhibitors are compounds or molecules that can bind to, and form coordinated bonds with zinc ions in the MBL active site, preventing a possible coordination of these zinc ions with their ligands⁹⁵. This interference hinders the zinc-dependent catalysis by MBLs, preventing them from hydrolysing β -lactam antibiotics⁹⁷.

The emergence of zinc-mediated resistance mechanisms in bacteria has sparked greater interest in zinc chelators as a potential solution to β -lactam resistance. Nevertheless, the development of zinc chelators as MBL inhibitors faces challenges, especially in achieving specificity for MBLs while maintaining essential zinc-dependent processes in the host. It is essential to thoroughly assess the likelihood of off-target effects when formulating these compounds⁹⁸. To this end, optimisation of the chelator's structure, pharmacokinetic properties, and selectivity is crucial^{99,100}. Designing effective zinc chelators requires a thorough understanding of the structural features of the MBL active site and catalytic mechanism to ensure successful binding and inhibition. The study by Lassaux et al.⁹⁷ aids in understanding the diverse resistance mechanisms exhibited by carbapenemases, particularly the variability in MBL active sites. The mechanistic diversity among MBLs highlights the need for customised therapeutic strategies designed to suit the specific features of different MBLs⁹⁶.

Given the evolving and multifaceted nature of MBLs, it is important to continuously monitor their resistance mechanisms and adjust therapeutic approaches accordingly¹⁰¹. Structural analyses play an essential role in guiding the creation of specific inhibitors that capitalise on the distinctive characteristics of individual enzymes. An interdisciplinary approach integrating medicinal chemistry, structural biology, and clinical studies will facilitate the development of therapeutics to address the complexities associated with MBLs. Future research should embrace multidisciplinary methodologies to tackle the challenges posed by MBLs effectively

1.2.3 Implications for Antibiotic Susceptibility Testing and Clinical Relevance

1.2.3.1 Nutritional Immunity and Transition Metals

Nutritional immunity entails the host sequestering essential nutrients like transition metals, such as zinc, to obstruct microbial growth and invasion during infection⁸⁸. Pathogens could evolve strategies to navigate around this limited nutrient availability and thrive in the host environment⁷⁸. The study conducted by Hood and Skaar⁸⁸ delved into the interplay of transition metals, particularly zinc, between the host and the pathogen, providing insight into the intricate dynamics that shape bacterial behaviour during infection. In human serum, zinc concentrations usually fall within the range of 0.6 to 1.4 $\mu\text{g/ml}$, with an important aspect being that a significant portion of zinc, around 80 to 90%, is bound to proteins^{4,103}. This binding substantially diminishes the quantity of zinc accessible for cellular interactions. In contrast to macrominerals like Ca^{2+} and Mg^{2+} , which typically remain at stable concentrations, the levels of free zinc tend to decrease even further during instances of acute infection or inflammation^{5,885,88}.

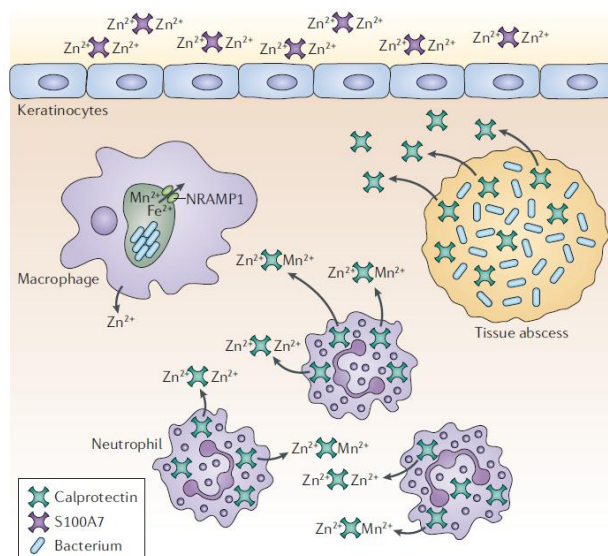


Figure 4 : Mechanisms through which S100 family proteins modulate the levels of Mn^{2+} and Zn^{2+} in the host. S100A7 at epithelial surfaces hinders bacterial invasion by binding to Zn^{2+} , while calprotectin (S100A8/S100A9) in tissue abscesses inhibits bacterial growth by binding to Mn^{2+} and Zn^{2+} . Macrophages engulfing bacteria reduce Zn^{2+} uptake and increase Zn^{2+} efflux, while the Natural resistance-associated macrophage protein 1 (NRAMP1) facilitates Mn^{2+} and Fe^{2+} release from phagosomes (Retrieved from Hood and Skaar⁸⁸).

The host employs multiple strategies to combat microbial invasion, one of which involves sequestering essential metallic elements such as Mn^{2+} and Zn^{2+} at the interfaces of mucosa and epithelium (as shown in Figure 1.3.5.1)⁸⁸. This process entails multiple proteins belonging to

the S100 family, among them being S100A7 (also known as psoriasin), S100A12 (or calgranulin C), S100A8 (or calgranulin A/MRP8), and S100A9 (or calgranulin B/MRP14) ⁸⁸. S100A7 secreted by keratinocytes impedes microbial growth by chelating Zn^{2+} . While S100A12, having an affinity for Zn^{2+} and Cu^{2+} , may generate superoxide species, the precise antimicrobial mechanism it utilizes remains ambiguous ⁸⁸. Calprotectin, a byproduct of S100A8 and S100A9, exhibits potent antimicrobial activity against various pathogens by sequestering Mn^{2+} and Zn^{2+} ⁸⁸. Moreover, these proteins display pro-inflammatory characteristics, indicating a potential correlation between metal binding and inflammation ¹⁰⁴.

1.2.3.2 Elevated zinc levels in conventional media: Implications for antibiotic resistance

Traditional laboratory media formulations, intended to support bacterial growth, often contain essential nutrients such as zinc (Table 1) ⁴. A number of research studies have pointed out that the levels of zinc in commonly utilised laboratory media exceed physiological thresholds ^{77,88}.

Table 1 : Zinc Levels in Different Commercial Lots of Cation-adjusted Mueller Hinton Broth, as sourced from Bilinskaya et al. ⁴

caMHB manufacturer and lot	Total zinc concn ($\mu\text{g/ml}$)
BD	
Lot A	1.181 ± 0.034
Lot B	1.318 ± 0.095
Lot C	1.262 ± 0.077
Oxoid	
Lot D	0.333 ± 0.007
Lot E	0.400 ± 0.005
Lot F	0.400 ± 0.002
Sigma	
Lot G	0.282 ± 0.013
Lot H	0.371 ± 0.005
Lot I	0.244 ± 0.007

caMHB: cation-adjusted Mueller Hinton Broth
concn: concentration

Elevated zinc concentrations found in laboratory media could have an impact on the behaviour and growth rates of bacteria ^{77,88}. Bacteria might react to excessive zinc exposure by regulating specific genes, thereby affecting their physiological condition ¹⁰⁵. When faced with an external stressor such as an abundance of zinc, bacteria might modulate their membrane permeability and efflux pump activity. Alterations in the properties of the membrane can have repercussions on the intake and outflow of antibiotics, potentially resulting in changes to susceptibility profiles. Pathogenic bacteria often utilise zinc as a coenzyme for the synthesis of virulence factors. Increases in zinc concentrations could affect the expression of virulence factors, potentially contributing to the pathogenicity of bacteria ¹⁰⁶.

Stress response and antibiotic tolerance

Elevated zinc levels have the potential to instigate changes in bacterial physiology, triggering stress response pathways that could lead to the development of antibiotic tolerance or persistence, ultimately reducing susceptibility to antibiotics^{107,108}. This stress-induced tolerance might cause inaccuracies in the determination of resistance rates in laboratory settings¹⁰⁸. Bilinskaya et al.⁴ compared meropenem MICs for MBL-harboursing Enterobacterales and control strains across different lots of cation-adjusted Mueller Hinton Broth (caMHB) from three manufacturers: Becton, Dickinson and Company, USA; Oxoid, Thermo Scientific, United Kingdom; and Sigma-Aldrich, USA (Table 2). Results indicated consistent meropenem resistance in BD and Oxoid broths, with MIC values ranging from 16 to >64 µg/ml and 4 to >64 µg/ml, respectively. In contrast, Sigma-Aldrich broths showed significant lot-to-lot variation, with MICs ranging from 0.25 to >64 µg/ml, which affected susceptibility categorisation. Control strains, including KPC-harboursing isolates and *P. aeruginosa* ATCC 27853, showed consistent results across all lots. Notably, zinc concentration was highest in BD broths, approximately 3-4 times more than in Oxoid and Sigma-Aldrich broths⁴.

Table 2 : Genotypic and Phenotypic Profiles of Serine and Metallo-β-lactamase Carbapenemase-Producing Isolates from Various Commercial Lots of cation adjusted Mueller Hinton Broth, as sourced from Bilinskaya et al.⁴

Isolate ID (β-lactamase[s]) ^a	MIC (µg/ml) of meropenem determined in caMHB from:								
	BD			Oxoid			Sigma		
	Lot A	Lot B	Lot C	Lot D	Lot E	Lot F	Lot G	Lot H	Lot I
EC 492 (NDM-1, CTX-M-3, TEM)	>64	>64	>64	>64	64	64	2	64	32
ECL 101 (NDM-1, LAP-2, ACT-17, TEM-1)	>64	>64	>64	>64	>64	64	8	64	64
KP 593 (NDM-1, SHV-11, CTX-M-15, OXA-1)	64	64	64	32	32	16	0.5	2	4
ECL 163 (NDM-6, SHV-12, TEM-OSBL, CTX-M-15)	>64	>64	>64	64	64	64	8	32	32
KP 667 (NDM-1, CTX-M-15, OXA-1, TEM-1B) ^b	>64	>64	>64	64	64	64	4	16	64
EC 677 (NDM-5, CTX-M-15)	>64	>64	>64	>64	>64	64	64	>64	>64
KP 880 (NDM-7, SHV-12, TEM-OSBL, CTX-M-15)	64	>64	>64	>64	>64	64	32	64	>64
KP 470 (VIM-1)	64	64	64	8	8	4	0.25	2	4
KP 655 (VIM-1, OXA-9, SHV-12, TEM-1A) ^c	32	32	64	16	8	8	0.5	8	16
KP 682 (VIM-1, SHV-30) ^d	32	32	32	16	16	8	1	16	16
KP 474 (IMP-26)	64	64	64	64	64	32	32	64	64
KP 684 (IMP-4, OKP-B-2, OXA-1, SFO-1, TEM-1B) ^e	32	32	16	16	16	16	16	16	32
KP 651 (KPC-2, TEM-1D) ^f	64	64	64	64	64	64	64	64	64
KP 652 (KPC-3, OXA-9, TEM-1B) ^g	64	64	64	64	64	32	64	64	64
EC 548 (KPC-3, OXA-9, TEM-1A) ^h	8	8	16	8	16	8	8	8	16

^aIsolate IDs begin with EC for *E. coli*, with KP for *K. pneumoniae*, and with ECL for *E. cloacae*. Isolate identification and corresponding meropenem MIC values as provided by the FDA-CDC Antimicrobial Resistance Isolate Bank for KP 667, KP 655, KP 682, KP 684, KP 651, KP 652, and EC 548 are listed in footnotes b to h.

^bAR Bank no. 0158 (MIC, >8 µg/ml).

^cAR Bank no. 0135 (MIC, 8 µg/ml).

^dAR Bank no. 0076 (MIC, 4 µg/ml).

^eAR Bank no. 0080 (MIC, 4 µg/ml).

^fAR Bank no. 0120 (MIC, >8 µg/ml).

^gAR Bank no. 0125 (MIC, >16 µg/ml).

^hAR Bank no. 0061 (MIC, 4 µg/ml).

Efflux pump regulation

The presence of zinc-induced stress can affect the regulation of efflux pump systems in bacteria¹⁰⁹. Increased activity of efflux pumps, which actively expel antibiotics from bacterial cells, can result in higher resistance rates during standard susceptibility testing^{110,111}.

Modulation of cell wall properties

Zinc is involved in maintaining the stability of bacterial cell walls⁷⁵. Variations in zinc levels can lead to alterations in cell wall properties, which can impact the ability of antibiotics to penetrate bacterial cells and affect their effectiveness, ultimately leading to changes in susceptibility patterns¹¹².

Selection of multidrug-resistant strains

Elevated zinc concentrations could potentially exert a bactericidal effect on microorganisms¹¹³. Under conditions of zinc toxicity, bacterial cells upregulate efflux systems to prevent an excess of zinc Enter. As a result, bacteria may undergo selection pressure favouring the survival of strains with altered membrane permeability and enhanced efflux pump activity, potentially causing the occurrence of multidrug-resistant phenotypes¹¹³. Research by Garvey and Piddock¹¹⁴ highlights how the use of the efflux pump inhibitor reserpine, intended to restore antibiotic susceptibility, may inadvertently promote the selection of multidrug-resistant strains in the presence of zinc. The ABC transporters PatA and PatB were identified as overexpressed in response to reserpine, leading to increased efflux of multiple antibiotics¹¹⁴.

1.2.3.3 Misinterpretation of Clinical Significance due to Higher Zinc Levels: Implications for antibiotic therapy and public health interventions

The accuracy of AST conducted *in vitro* holds significant importance in guiding clinical decision-making. Recent studies have drawn attention to the discrepancies in zinc levels between conventional laboratory settings and human physiological conditions, which may have implications for increased antibiotic resistance rates observed *in vitro*^{4,5}. Artificial induction of resistance phenotypes may confound the interpretation of test results, leading to inaccurate assessments of bacterial susceptibility to antibiotics. Inaccuracies in AST due to higher zinc levels may lead to the misinterpretation of the clinical significance of resistance¹¹⁵, which raises concerns about the reliability of surveillance data utilised to track antibiotic resistance patterns at both local and global levels. Essentially, the higher zinc concentrations in laboratory media contribute to the generation of data that may not authentically represent the actual

landscape of resistance ¹¹⁶. Consequently, public health efforts aimed at combating antibiotic resistance might be founded on flawed data, resulting in the implementation of ineffective strategies, affecting resource allocation, and potentially worsening the proliferation of resistant bacterial strains.

Clinicians depend on precise *in vitro* data to inform antibiotic selection, highlighting the significance of accurate resistance rates *in vitro* on treatment decisions ^{4,115}; and if resistance rates are artificially inflated due to higher zinc levels, it may lead to treatment decisions with suboptimal outcomes ¹¹⁷. When resistance rates are erroneously elevated because of increased zinc levels, treatment decisions suffer, resulting in the selection of less effective drugs or inappropriate treatment regimens. Therefore, overestimation of resistance rates due to heightened zinc levels poses a threat of treatment failures and complications. The repercussions of ineffective antibiotic therapy encompass prolonged infections, heightened complication risks, and the proliferation of drug-resistant strains. ²⁹.

1.2.3.4 Addressing the issue: Zinc depletion to restore *in vitro* antibiotic susceptibility

Given the impact of zinc on the *in vitro* antibiotic resistance rates of MBL-producing CRE, an important question arises about the effectiveness of carbapenems in the treatment of carbapenem-resistant infections. A study conducted in the United States of America demonstrated effectiveness of carbapenems in eliminating MBL-producing Enterobacterales isolates *in vivo*, even though the same isolates exhibited *in vitro* carbapenem resistance ^{4,5}. Comparative analyses in murine models suggest that the efficacy of carbapenems correlates with *in vitro* susceptibility under zinc-depleted conditions ^{4,5}. While *in vitro* studies using zinc-depleted media cannot conclusively determine carbapenem efficacy, the discrepancy between predicted and observed efficacy in animal studies indicates that treatments previously deemed ineffective based on high *in vitro* resistance may warrant further investigation as potential therapeutic options ⁶. Adjusting *in vitro* zinc levels to better reflect *in vivo* zinc levels could therefore improve the relevance of susceptibility data, aiding in more informed antibiotic selection ¹¹⁸.

1.2.3.5 Different ways of achieving zinc depletion

Various methodologies can be employed to effectively reduce the concentration of zinc in culture media, with notable examples being Chelex and EDTA ⁵. The application of these chelating agents in lowering zinc levels has significant implications for the accuracy and reliability of AST results, especially in bacteria with zinc-dependent resistance mechanisms. This could allow for a more precise evaluation of the effectiveness of antibiotics and trends in resistance, thus enabling well-informed therapeutic decisions in clinical settings. EDTA offers the advantage of forming stable metal chelates, ensuring the effective sequestration of zinc ions over a prolonged period, while Chelex serves as a swift and convenient approach for zinc removal ⁹⁹. Studies comparing the two methods have demonstrated comparable efficacy in zinc depletion and subsequent alterations in antibiotic susceptibility profiles ⁵.

As per the guidelines by the Clinical and Laboratory Standards Institute (CLSI), the Chelex method involves the initial preparation of standard ca-MHB. Subsequent steps encompass the chelation of cations utilising a resin, followed by resin filtration, pH verification, and adjustment if required, and ultimately, the reinstatement of non-zinc cations such as calcium and magnesium ¹¹⁹. The Chelex resin is equipped with iminodiacetate groups that selectively trap divalent metal ions like zinc, thereby aiding in their elimination from the culture medium ¹²⁰. While the sequestration of metals using Chelex is typically efficient, it could potentially lead to adverse outcomes. The application of Chelex treatment can introduce impurities or contaminants into the culture medium, consequently affecting bacterial growth or the performance of assays.

EDTA serves as a commonly used chelating agent and displays increased selectivity towards zinc as opposed to other cations at elevated pH levels, typically exceeding pH 7, where the deprotonated form of EDTA⁴⁻ prevails ^{4,121}. Despite its efficacy, using EDTA for zinc depletion may pose certain challenges. One potential challenge lies in the ability of EDTA to exhibit antibacterial properties, which could confound the interpretation of AST results ¹²¹. Therefore, careful optimisation of EDTA concentrations and experimental conditions is necessary to minimise any potential interference with AST outcomes. Another challenge linked to EDTA treatment pertains to its possible impact on bacterial growth patterns and metabolic functions. The reduction in zinc levels has been associated with alterations in bacterial physiology, including changes in gene expression patterns and cellular responses to stress ^{107,113}. Applying elevated amounts of EDTA in order to decrease zinc levels may have implications that extend beyond alterations in zinc levels, also impacting the levels of other cations like calcium and

magnesium¹²². Imbalances in the equilibrium of metal ions can interfere with the integrity of bacterial cell membranes, resulting in changes in bacterial physiological functions or growth rates¹²³, thereby impacting the assessment of susceptibility profiles.

In conclusion, the application of EDTA and Chelex methodologies represents effective strategies for zinc depletion in microbiological investigations, with each technique offering distinct advantages and considerations. Diligent adjustment and validation of experimental conditions are crucial to mitigate any biases or distortions introduced by EDTA or Chelex treatment and to ensure the accuracy and reliability of AST results.

It is with these factors in mind that we undertook this study to assess the effect of zinc depletion on the *in vitro* susceptibility of MBL-producing Enterobacterales to carbapenems. Given the discordance between *in vitro* and *in vivo* efficacy of carbapenems in the presence of MBLs, and the current lack of comprehensive studies focusing on MBL-producing CRE in Africa, this research aims to elucidate whether zinc-depleted media can more accurately reflect physiological conditions and thereby assess the true effectiveness of carbapenems against these pathogens. Furthermore, we seek to explore the consistency of AST results between zinc-depleted solid and liquid media, which could potentially streamline testing methods and enhance treatment strategies for managing MBL-producing Enterobacterales infections.

CHAPTER TWO:

Methods & Materials



2.1 Study design and sample processing

This study was designed as a laboratory-based investigation aimed at evaluating the antibiotic resistance profiles of CREs. Thirty isolates, consisting of 15 NDM and 15 VIM producers identified by multiplex PCR, were obtained from the NICD between May and November 2023 (Table 3). The decision to use a sample size of 30 isolates was driven by financial restrictions, however, it aligns with the CLSI-M52 2015 guideline's prescribed minimum for ensuring accuracy ¹²⁴. All 30 isolates, sourced from clinical samples, were harvested from purity plates, transferred to collection swabs, and transported to the Wits research laboratory. Upon receipt, they were plated on MacConkey agar (Thermo Fisher Scientific, USA) to confirm purity and stored at -70°C in microbanks for further analysis. Appendices A-B provides the isolate lab numbers and corresponding species names as supplied by NICD. Assistance with experimental setup and result interpretation was provided by the infection control laboratory staff.

Table 3 : Distribution of Organisms and Sample Size Based on Carbapenemase Genes

<i>Enterobacteriales</i> Species	NDM-Producing Isolates (n=15)	VIM-Producing Isolates (n=15)
<i>Klebsiella aerogenes</i>	13% (2/15)	0% (0/15)
<i>Enterobacter cloacae</i>	0% (0/15)	40% (6/15)
<i>Escherichia coli</i>	20% (3/15)	0% (0/15)
<i>Klebsiella pneumoniae</i>	67% (10/15)	60% (9/15)

Imipenem and meropenem were selected for their broad applicability and ongoing importance in clinical practice, ensuring our findings are directly applicable to current treatment practices. According to EUCAST guidelines, meropenem is recommended as a screening agent for detecting carbapenemase production in Enterobacteriales ¹²⁵, due to its breakpoint being sensitive enough to effectively identify carbapenemase-producing organisms, making it a reliable choice in clinical settings ^{126–128}. Conversely, ertapenem is less commonly used for this purpose because its narrower spectrum increases the risk of false-negative results, particularly with certain carbapenemases ¹²⁷. In South Africa, the clinical relevance of doripenem is limited by its infrequent use, largely due to market availability and competition from other carbapenems like meropenem and imipenem ^{15,129–131}.

Experiments, conducted from October 2023 to March 2024, began with untreated media, starting with disk diffusion testing. After overnight exposure to imipenem and meropenem

disks, the same isolates were used for broth microdilution and E-test experiments. The disk diffusion and E-test experiments were performed once. Broth microdilution was conducted in triplicate, yielding consistent MICs in most cases. In all instances, at least two of the three values were the same, and the repeated value was recorded. The MIC₅₀ and MIC₉₀ values—defined as the lowest concentrations of an antimicrobial agent required to inhibit 50% and 90% of bacterial isolates, respectively—were determined ¹³².

Testing was conducted sequentially, with all isolates first tested in untreated media media before proceeding to EDTA-treated conditions. The same operator (the current master's candidate) and laboratory were used for both conditions to ensure consistency. Results were interpreted using the same standardized protocols across both testing environments.

2.2 Antibiotic Susceptibility Testing using Untreated Media

2.2.1 Disk diffusion on Untreated Agar

Isolates were initially exposed to imipenem and meropenem disks to confirm that they were carbapenem-resistant, as these isolates were stored over a period of time. During this experimental procedure, isolated Enterobacterales were sub-cultured on MacConkey Agar (Thermo Fisher Scientific, USA) and subsequently allowed to incubate overnight at 35±2°C in ambient air (normal atmospheric conditions) . A single colony obtained from the freshly prepared overnight culture of Enterobacterales was inoculated into 4 ml of sterile 0.9% saline solution and thoroughly mixed. Standardisation of each bacterial suspension to a turbidity of 0.5±0.05 McFarland standard was achieved using the MicroScan Turbidity Meter (Beckman Coulter, USA). Following this, a sterile cotton swab was immersed in the bacterial suspension, and streaked on Mueller-Hinton agar (MHA; Thermo Fisher Scientific, USA). The agar plates with meropenem (10 µg) and imipenem (10 µg) disks were then incubated in ambient air at 35±2°C in ambient air for 16-18 hours to assess the inhibition zones as per CLSI M100 2023 guideline ¹³³. The diameters of inhibition zones (in millimetres) on media containing zinc were determined using Vernier calliper, and the isolates were categorised as susceptible, intermediate, or resistant to the respective antibiotics based on CLSI M100 2023 guideline ¹³³. To ensure quality control, the control strains *E. coli* ATCC 25922 (susceptible to meropenem and imipenem) and *K. pneumoniae* BAA-1705 (resistant to meropenem and imipenem) were included in the experiment ¹³³.

2.2.2 Broth Microdilution using Untreated Broth

The broth microdilution technique is broadly recognised as the optimal method for AST due to its precision and adaptability. This approach allows for the systematic dilution of antibiotics in liquid media, enabling precise determination of MICs essential for evaluating susceptibility levels in bacterial strains. Its versatility enables testing with a wide range of antibiotics and bacterial strains simultaneously, making it suitable for various clinical and research contexts¹³⁴.

The process of broth microdilution entails identifying the MIC required for an antimicrobial substance to impede bacterial growth¹³⁵. To prepare the antibiotic solution, the CLSI M100 2023-recommended diluent (distilled water for meropenem and Phosphate-Buffered Saline, pH 7.2 for imipenem) was added to the antibiotic powder in a 1:2 ratio (mass-to-diluent). Calculations were made to ascertain the volume of the initial antibiotic solution that needs to be combined with untreated caMHB (Thermo Fisher Scientific, USA) using the formula below, where specific concentrations and volumes were added accordingly:

$$C_1V_1 = C_2V_2$$

$$(500 \text{ mg/ml}) (V_1) = (128 \text{ mg/ml}) (10 \text{ ml})$$

- 500 mg/ml is the initial concentration of the antibiotic solution, formulated at a 1:2 ratio with the diluent.
- V_1 is the volume (in millilitres) of the initial antibiotic solution that we want to dilute.
- 128 mg/ml is the desired final concentration after dilution.
- 10 ml is the final volume of the diluted antibiotic solution.

This equation facilitates the determination of the amount of the concentrated antibiotic solution (initially at 500 mg/ml) required for mixing with the broth to achieve a final concentration of 128 mg/ml in a 10 ml volume.

Following the antibiotic solution preparation, 96-well microtiter plates were set up by dispensing 100 μ l of autoclaved ca-MHB (Thermo Fisher Scientific, USA) into each well. Subsequently, 100 μ l of the final antibiotic solution was added to the first wells, with serial dilutions performed up to the tenth well. The dilutions started at 64 μ g/ml and followed a doubling pattern across the 10 wells, resulting in a dilution of 0.12 μ g/ml in the final well.

NDM and VIM-producing isolates underwent exposure to imipenem and meropenem disks overnight to trigger the production of the MBL in accordance with the methodology described in section 2.2.1. These induced cultures were employed for conducting the broth microdilution assay. Quality control strains *E. coli* ATCC 25922 and *K. pneumoniae* BAA-1705 were utilised¹³³. Bacterial solutions were prepared by suspending a microdrop of cultured isolate in 4 ml sterile 0.9% saline, followed by brief vortexing to achieve homogenisation. Using the MicroScan Turbidity Meter (Beckman Coulter, USA), the bacterial solutions were standardised to a turbidity of 0.5 ± 0.05 McFarland standard. These standardised suspensions were subjected to a 10-fold dilution in distilled water prior to being inoculated into the 96-well microtiter plates along with serially diluted antibiotics. Negative controls consisted of wells without test strains but inoculated with the antibiotic dilution, while positive controls contained only the microbial suspension. The broth microdilution experiments were conducted in triplicate for each test strain using meropenem and imipenem. Incubating was done at $35 \pm 2^\circ\text{C}$ in ambient air for a period of 16-20 hours, as outlined in the CLSI M100 2023¹³³.

2.2.3 Gradient Diffusion Strip Test on Untreated Agar

The evaluation of carbapenem MIC in MBL-producing Enterobacterales isolates was also conducted through the utilisation of the gradient diffusion strip test (E-test; BioMérieux, France) procedure on MHA (Thermo Fisher Scientific, USA). The isolates underwent testing with meropenem (0.002-32 µg) and imipenem (0.002-32 µg). Quality control measures involved the use of *E. coli* ATCC 25922 (susceptible to meropenem and imipenem) and *K. pneumoniae* BAA-1705 (resistant to meropenem and imipenem)¹³³.

For the sake of working with MBL-induced isolates, the same microbial suspensions derived from disk diffusion overnight cultures were employed for the implementation of the E-test experiments. A sterile cotton swab was placed into the 0.5 ± 0.05 McFarland inoculum suspension and allowed to absorb for a brief period. Subsequently, surplus liquid was removed by delicately pressing the swab against the tube wall. Untreated MHA was inoculated by means of the streak lawn technique, with the plate being rotated at a 90° angle a minimum four times to ensure even distribution of the inoculum across the plate surface. The antibiotic-impregnated gradient diffusion strip, featuring a concentration scale (in µg/ml) on its surface, was placed on the MHA plates, following which they were subjected to a 16-18 hour incubation period (as per CLSI M100 2023, for Enterobacterales¹³³) at $35 \pm 2^\circ\text{C}$ in ambient air. The determination of

MIC values was carried out using magnifying glass to identify the point at which organism growth intersected with the strip, using the scale indicated on the strip, and then reporting the results in µg/ml. The results were interpreted as per the CLSI M100 2023 guidelines.

2.3 Zinc Concentration Testing

The primary stage of this experimental procedure entailed the evaluation of the traditional media to confirm the presence of zinc. To this end, inductively coupled plasma mass spectrometry (ICP-MS) provided by the Witwatersrand University School of Chemistry was employed for quantifying the zinc content in duplicate in ca-MHB. The ICP-MS analysis was conducted using an Agilent 7500 CE equipment (Agilent Technologies, USA) with a minimum limit of detection of 0.002 µg/ml ⁴. The assessment of zinc concentration was unfeasible on solid agar due to the intricacy of the experiment and inadequate resources.

2.4 EDTA treatment

The preparation of zinc-depleted ca-MHB (Thermo Fisher Scientific, USA) was done by adding EDTA (Sigma-Aldrich, USA) to autoclaved ca-MHB at a concentration of 300 µg/ml under sterile conditions ⁵.

To prepare the zinc-depleted MHA, 38 grams of agar powder (Sigma-Aldrich, USA) were thoroughly mixed with 1 Liter of purified water. The resulting solution was then autoclaved at 121°C for 15 minutes to ensure sterilization ¹³⁶. Subsequently, the MHA was allowed to reach a temperature of 45–50°C ¹³⁶, following which 300 µg/ml of EDTA was introduced ⁵ and allowed to dissolve in a water-bath set at 50°C for 2 hours. The zinc-depleted MHA was then transferred into sterile petri dishes and allowed to cool down to ambient temperature.

2.5 Antibiotic susceptibility testing using zinc-depleted media

Utilising the media depleted of zinc, a subsequent series of AST trials was conducted to establish the MICs and inhibition zones of meropenem and imipenem in the absence of zinc. Therefore, the methodologies of disk diffusion, broth microdilution, and gradient diffusion strip tests were replicated using the same experimental configuration as specified in section 2.2 after treating the broth and agar plates with EDTA.

2.6 Statistical analysis

Zinc concentrations observed in untreated MHB were displayed as mean $\pm$ standard deviation. Each experiment involved the measurement of mean $\pm$ standard deviation of MICs and zones of inhibition. The MIC₅₀ and MIC₉₀ of the antibiotics pre and post zinc depletion were also documented.

The evaluation of the variance between pre- and post-zinc depletion AST results was carried out by Fisher's exact test and the Wilcoxon matched-pairs signed-rank test using STATA software (version SE18, StataCorp LLC, Texas, USA, 2023). In the context of both analyses, the null hypothesis (H₀) suggests that there is no difference between the two variables under investigation. A *p*-value below 0.05 (*p* < 0.05) serves as compelling evidence to reject the null hypothesis, suggesting a significant difference between the variables. Fisher's exact test is tailored for categorical (e.g., resistant, susceptible, intermediate) data analysis, whereas the Wilcoxon matched-pairs signed-rank test is suitable for the examination of continuous data (MICs and zones of inhibition). Within the scope of this study, the Wilcoxon matched-pairs signed-rank test plays an important role in identifying whether there is a statistically significant difference between the AST data (untreated vs EDTA-treated) of the same group of isolates.

Cohen's Kappa analysis was employed to gauge the level of agreement among different techniques (broth microdilution, disk diffusion, and gradient diffusion strip test). With a confidence interval of 95%, kappa values are categorised as outlined in Table 4.

Table 4 : Levels of Agreement Interpretation Based on Kappa Value Ranges ¹³⁷.

Kappa Value Range	Level of Agreement	Description
< 0	No Agreement	<i>Indicates complete disagreement; the observed agreement is less than what would be expected by chance.</i>
0.01 - 0.20	Insignificant Agreement	<i>Slight agreement beyond chance; the agreement is barely better than random guessing.</i>
0.21 - 0.40	Fair Agreement	<i>Noticeable agreement; better than random chance but still weak.</i>
0.41 - 0.60	Moderate Agreement	<i>Moderate agreement; a moderate relationship between raters or measurements.</i>
0.61 - 0.80	Substantial Agreement	<i>Strong agreement; high level of consistency between raters or measurements.</i>
0.81 - 1.00	Almost Perfect Agreement	<i>Near-perfect agreement; very high consistency, almost no discrepancies between raters or measurements.</i>

The conclusive analysis aimed at determining whether one of the two MBLs under study (NDM or VIM) elicited higher MICs than the other in the untreated media. This was addressed through

quantitative analysis using the Mann-Whitney U test and the calculation of carbapenem MIC₅₀ and MIC₉₀ in each of the two groups.

CHAPTER THREE:

Outcomes of Untreated Media Testing



3.1 Disk Diffusion on Untreated Agar

As outlined in the experimental protocol, the disk diffusion test was carried out prior to other AST techniques to induce the expression of MBL genes in the isolates being studied. Table 5 presents a detailed breakdown of the antibiotic susceptibility profiles of both NDM- and VIM-producing isolates, assessed through the disk diffusion method on untreated agar. The interpretation of inhibition zones for imipenem and meropenem was conducted following the guidelines outline in the CLSI M100 2023 ¹³³.

Table 5 : Antibiotic Susceptibility Profile of NDM- and VIM-Producing Isolates in Untreated Media: Evaluation Using the Disk Diffusion Method

Bacterial Isolate	Imipenem		Meropenem	
ID	Zones of inhibition (mm)	Interpretation	Zones of inhibition (mm)	Interpretation
NDM-producing isolates				
19278	12	Resistant	11	Resistant
19275	15	Resistant	11	Resistant
19126	6	Resistant	6	Resistant
19122	13	Resistant	12	Resistant
19322	14	Resistant	11	Resistant
NDM 1	6	Resistant	6	Resistant
NDM 2	6	Resistant	6	Resistant
NDM 3	14	Resistant	13	Resistant
NDM 4	9	Resistant	10	Resistant
NDM 5	14	Resistant	15	Resistant
NDM 6	7	Resistant	10	Resistant
NDM 7	6	Resistant	6	Resistant
NDM 8	8	Resistant	6	Resistant
NDM 9	16	Resistant	15	Resistant
NDM 10	14	Resistant	10	Resistant
VIM-producing isolates				
18983	13	Resistant	18	Resistant
18893	12	Resistant	18	Resistant
18820	19	Resistant	22	Intermediate
17656	19	Resistant	21	Intermediate
18201	19	Resistant	23	Susceptible
16733	19	Resistant	22	Intermediate
16213	15	Resistant	20	Intermediate
16391	16	Resistant	19	Resistant
16381	17	Resistant	21	Intermediate
16615	16	Resistant	17	Resistant
VIM 1	11	Resistant	14	Resistant
VIM 2	19	Resistant	22	Intermediate
QF04104078	18	Resistant	21	Intermediate
VIM 4	21	Intermediate	21	Intermediate
HN06193535	15	Resistant	20	Intermediate
Control strains				
<i>E. coli</i> ATCC 25922	29	Susceptible	30	Susceptible
<i>K. pneumoniae</i> BAA-1705	12	Resistant	13	Resistant

Among NDM-producing isolates, all isolates were resistant, with inhibition zones ranging from 6 mm to 16 mm for imipenem and 6 mm to 15 mm for meropenem. The VIM-producing isolates demonstrated a distinct pattern of resistance, with zones of inhibition spanning from 11 mm to 21 mm for imipenem and 14 mm to 23 mm for meropenem. Nine VIM-producing isolate exhibited intermediate susceptibility to meropenem, while one isolate exhibited intermediate susceptibility to imipenem.

The control strain *K. pneumoniae* BAA-1705 demonstrated resistance to both antibiotics, displaying an imipenem inhibition zone of 12 mm and a meropenem inhibition zone of 13 mm. On the other hand, *E. coli* strain ATCC 25922 showed susceptibility to both imipenem and meropenem, with inhibition zones measuring 29 mm and 30 mm, respectively.

3.2 Broth Microdilution using Untreated Broth

By employing the broth microdilution method using untreated broth, the MIC for imipenem and meropenem were determined and are shown in Table 6. All NDM-producing strains displayed resistance to both imipenem and meropenem. Notably, among these strains, 60% (9/15) and 80% (12/15) demonstrated MIC values surpassing 64 µg/ml for imipenem and meropenem, respectively. In contrast, VIM-producing strains exhibited lower MIC values ranging from 8-64 µg/ml for imipenem and 2-32 µg/ml for meropenem. While all VIM-producing strains exhibited resistance to imipenem, two strains displayed intermediate susceptibility to meropenem.

The MICs recorded for the reference strain *E. coli* ATCC 25922 were 0.25 µg/ml for imipenem and < 0.12 µg/ml for meropenem, consistent with the CSLI ranges of 0.06-0.5 µg/ml for imipenem and 0.008-0.06 µg/ml for meropenem. Similarly, MICs of 16 µg/ml for imipenem and 32 µg/ml for meropenem were observed for our resistant control strain, *K. pneumoniae* BAA-1705, falling within the specified CSLI ranges (4-16 µg/ml for imipenem and 8-32 µg/ml for meropenem).

Table 6: Antibiotic Susceptibility Profile of NDM- and VIM-Producing Isolates in Untreated Media: Evaluation Using the Broth Microdilution Method

Bacterial Isolate		Imipenem		Meropenem	
ID	MIC (µg/ml)	Interpretation	MIC (µg/ml)	Interpretation	
NDM-producing isolates					
19278	> 64	Resistant	> 64	Resistant	
19275	> 64	Resistant	> 64	Resistant	
19126	> 64	Resistant	> 64	Resistant	
19122	64	Resistant	> 64	Resistant	
19322	64	Resistant	> 64	Resistant	
NDM 1	> 64	Resistant	> 64	Resistant	
NDM 2	> 64	Resistant	> 64	Resistant	
NDM 3	32	Resistant	64	Resistant	
NDM 4	64	Resistant	> 64	Resistant	
NDM 5	32	Resistant	32	Resistant	
NDM 6	> 64	Resistant	> 64	Resistant	
NDM 7	> 64	Resistant	> 64	Resistant	
NDM 8	> 64	Resistant	> 64	Resistant	
NDM 9	32	Resistant	64	Resistant	
NDM 10	> 64	Resistant	> 64	Resistant	
VIM-producing isolates					
18983	32	Resistant	32	Resistant	
18893	32	Resistant	32	Resistant	
18820	8	Resistant	2	Intermediate	
17656	8	Resistant	4	Resistant	
18201	16	Resistant	8	Resistant	
16733	16	Resistant	8	Resistant	
16213	64	Resistant	32	Resistant	
16391	16	Resistant	8	Resistant	
16381	16	Resistant	16	Resistant	
16615	8	Resistant	8	Resistant	
VIM 1	64	Resistant	32	Resistant	
VIM 2	8	Resistant	2	Intermediate	
QF04104078	8	Resistant	4	Resistant	
VIM 4	16	Resistant	8	Resistant	
HN06193535	16	Resistant	16	Resistant	
Control strains					
<i>E. coli</i> ATCC 25922	0.25	Susceptible	< 0.12	Susceptible	
<i>K. pneumoniae</i> BAA-1705	16	Resistant	32	Resistant	

3.3 Gradient Diffusion Strip Test on Untreated Agar

The gradient diffusion strip test, like the broth microdilution method, provides MICs as a measure of drug efficacy against the tested isolates. Table 7 presents the antibiotic susceptibility profiles of NDM- and VIM-producing strains derived from the gradient diffusion strip test on untreated growth media. In NDM-producing strains, the MIC values for imipenem

varied from 2 µg/ml to >32 µg/ml, with a predominant resistance trend noted, where 60% (9/15) of the strains displayed MIC values greater than 32 µg/ml. Similarly, for meropenem, MIC values ranged from 6 µg/ml to >32 µg/ml, indicating resistance across all strains.

Table 7 : Antibiotic Susceptibility Profile of NDM- and VIM-Producing Isolates in Untreated Media: Evaluation Using the Gradient Diffusion Strip Test

Bacterial Isolate		Imipenem		Meropenem	
ID	MIC (µg/ml)	Interpretation	MIC (µg/ml)	Interpretation	
NDM-producing isolates					
19278	> 32	Resistant	> 32	Resistant	
19275	8	Resistant	6	Resistant	
19126	> 32	Resistant	> 32	Resistant	
19122	6	Resistant	6	Resistant	
19322	6	Resistant	8	Resistant	
NDM 1	> 32	Resistant	> 32	Resistant	
NDM 2	> 32	Resistant	> 32	Resistant	
NDM 3	16	Resistant	16	Resistant	
NDM 4	32	Resistant	> 32	Resistant	
NDM 5	> 32	Resistant	> 32	Resistant	
NDM 6	> 32	Resistant	> 32	Resistant	
NDM 7	> 32	Resistant	> 32	Resistant	
NDM 8	> 32	Resistant	> 32	Resistant	
NDM 9	2	Intermediate	24	Resistant	
NDM 10	> 32	Resistant	> 32	Resistant	
VIM-producing isolates					
18983	> 32	Resistant	6	Resistant	
18893	16	Resistant	3	Intermediate	
18820	1.5	Intermediate	0.5	Susceptible	
17656	0.75	Susceptible	0.19	Susceptible	
18201	0.5	Susceptible	0.19	Susceptible	
16733	1	Susceptible	0.38	Susceptible	
16213	8	Resistant	0.75	Susceptible	
16391	1.5	Intermediate	0.75	Susceptible	
16381	1.5	Intermediate	0.38	Susceptible	
16615	1.5	Intermediate	1.5	Intermediate	
VIM 1	> 32	Resistant	12	Resistant	
VIM 2	0.5	Susceptible	0.5	Susceptible	
QF04104078	2	Intermediate	0.75	Susceptible	
VIM 4	0.75	Susceptible	0.5	Susceptible	
HN06193535	4	Resistant	0.5	Susceptible	
Control strains					
<i>E. coli</i> ATCC 25922	0.125	Susceptible	0.008	Resistant	
<i>K. pneumoniae</i> BAA-1705	8	Resistant	32	Susceptible	

Among the VIM-producing strains, both imipenem and meropenem displayed a wide range of MIC values, from 0.5 µg/ml to >32 µg/ml and 0.19 µg/ml to 12 µg/ml, respectively. The majority of VIM-producing strains showed susceptibility to meropenem (73%, 11/15). One third of the MIC values (5/15, 33%) correlated with susceptibility profiles for VIM strains,

with a comparable number of cases (5/15, 33%) of intermediate susceptibility or resistance being noted.

The *E. coli* ATCC 25922 strain displayed an MIC of 0.125 µg/ml for imipenem and 0.008 µg/ml for meropenem, while *K. pneumoniae* BAA-1705 exhibit an MIC of 8 µg/ml for imipenem and 32 µg/ml for meropenem. These MICs fall within the designated intervals set by the CLSI M100 2023 for imipenem (0.06-0.5 µg/ml; 4-16 µg/ml) and meropenem (0.008-0.06 µg/ml; 8-64 µg/ml).

CHAPTER FOUR:

Outcomes of EDTA-Treated Media Testing



4.1 Inductively Coupled Plasma Mass Spectrometry Results

An assessment was carried out using ICP-MS to confirm the presence of zinc in the ca-MHB, resulting in the identification of a zinc concentration of 2.76 mg/l (Table 8). EDTA was used to inactivate zinc ions functionally without completely removing them from the growth medium. As a result, zinc quantification in the liquid broth after EDTA treatment showed similar levels to those in the untreated MH broth.

Due to the unavailability of specialised equipment, determining zinc concentration in solid Mueller Hinton agar was challenging. Consequently, the presence of zinc in the untreated solid agar was inferred from the observed changes in resistance profiles after EDTA treatment, which correlated with results from the liquid medium.

Table 8 : Zinc Concentrations in Untreated cation-adjusted Mueller Hinton Broth Measured by ICP-MS

Sample	Units	Replicate 1	Replicate 2	Average	Std. Dev.
CA-MHB	mg/l	2.75	2.77	2.76	0.014

4.2 Disk Diffusion on EDTA-Treated Agar

The application of EDTA treatment on MHA led to a significant shift towards susceptibility among the examined isolates (Table 9). For imipenem, inhibition zones among the NDM-producing isolates ranged from 11 mm to 36 mm, with 93% (14/15) showing susceptibility. Similarly, 80% (12/15) of the previously resistant NDM-producing isolates exhibited susceptibility to meropenem. The remaining three isolates, which were also initially resistant on untreated agar, displayed intermediate susceptibility on EDTA-treated agar.

In the instances of VIM-producing isolates, imipenem zones of inhibition varied from 24 mm to 34 mm, while meropenem zones ranged from 28 mm to 38 mm, indicating susceptibility across all isolates. However, the antibiotic susceptibility profiles of *E. coli* ATCC 25922 and *K. pneumoniae* BAA-1705 isolates were unaffected by EDTA treatment on agar for both imipenem and meropenem. These results underscore the role of zinc availability in modulating the resistance patterns of MBL-producing isolates in untreated media.

Table 9 : Antibiotic Susceptibility Profile of NDM- and VIM-Producing Isolates in EDTA-treated Media: Evaluation Using the Disk Diffusion Method

Bacterial Isolate		Imipenem		Meropenem	
ID	Zones of inhibition (mm)	Interpretation	Zones of inhibition (mm)	Interpretation	
NDM-producing isolates					
19278	28	Susceptible	30	Susceptible	
19275	31	Susceptible	34	Susceptible	
19126	11	Resistant	20	Intermediate	
19122	30	Susceptible	33	Susceptible	
19322	28	Susceptible	30	Susceptible	
NDM 1	24	Susceptible	23	Susceptible	
NDM 2	27	Susceptible	23	Susceptible	
NDM 3	36	Susceptible	38	Susceptible	
NDM 4	26	Susceptible	27	Susceptible	
NDM 5	35	Susceptible	36	Susceptible	
NDM 6	29	Susceptible	30	Susceptible	
NDM 7	24	Susceptible	22	Intermediate	
NDM 8	25	Susceptible	22	Intermediate	
NDM 9	32	Susceptible	32	Susceptible	
NDM 10	24	Susceptible	23	Susceptible	
VIM-producing isolates					
18983	30	Susceptible	36	Susceptible	
18893	30	Susceptible	34	Susceptible	
18820	28	Susceptible	32	Susceptible	
17656	32	Susceptible	34	Susceptible	
18201	32	Susceptible	34	Susceptible	
16733	31	Susceptible	32	Susceptible	
16213	29	Susceptible	34	Susceptible	
16391	27	Susceptible	30	Susceptible	
16381	32	Susceptible	32	Susceptible	
16615	32	Susceptible	33	Susceptible	
VIM 1	30	Susceptible	34	Susceptible	
VIM 2	34	Susceptible	37	Susceptible	
QF04104078	34	Susceptible	38	Susceptible	
VIM 4	31	Susceptible	38	Susceptible	
HN06193535	24	Susceptible	28	Susceptible	
Control strains					
<i>E. coli</i> ATCC 25922	30	Susceptible	36	Susceptible	
<i>K. pneumoniae</i> BAA-1705	15	Resistant	12	Resistant	

4.3 Broth Microdilution in EDTA-Treated Broth

The results of the assessment of NDM- and VIM-producing bacterial strains in media with lowered zinc levels utilising the broth microdilution method is presented in Table 10. For NDM-producing isolates, imipenem showed MIC values ranging from < 0.12 µg/ml to 8 µg/ml,

as opposed to MICs of 32 µg/ml to > 64 µg/ml in untreated broth, with 60% (9/15) of strains identified as susceptible. Meropenem, similarly, exhibited MIC values ranging from < 0.12 µg/ml to 16 µg/ml, with susceptibility observed in 60% (9/15) of strains. A small subset of strains (27%, 4/15) showed resistance to both antibiotics in EDTA-treated broth, with two others indicating resistance to meropenem and intermediate susceptibility to imipenem.

Table 10 : Antibiotic Susceptibility Profile of NDM- and VIM-Producing Isolates in EDTA-treated Media: Evaluation Using the Broth Microdilution Method

Bacterial Isolate		Imipenem		Meropenem	
ID	MIC (µg/ml)	Interpretation	MIC (µg/ml)	Interpretation	
NDM-producing isolates					
19278	< 0.12	Susceptible	< 0.12	Susceptible	
19275	0.25	Susceptible	< 0.12	Susceptible	
19126	8	Resistant	8	Resistant	
19122	< 0.12	Susceptible	< 0.12	Susceptible	
19322	< 0.12	Susceptible	< 0.12	Susceptible	
NDM 1	8	Resistant	16	Resistant	
NDM 2	8	Resistant	8	Resistant	
NDM 3	< 0.12	Susceptible	< 0.12	Susceptible	
NDM 4	1	Susceptible	1	Susceptible	
NDM 5	< 0.12	Susceptible	< 0.12	Susceptible	
NDM 6	0.25	Susceptible	< 0.12	Susceptible	
NDM 7	4	Resistant	16	Resistant	
NDM 8	2	Intermediate	8	Resistant	
NDM 9	< 0.12	Susceptible	< 0.12	Susceptible	
NDM 10	2	Intermediate	4	Resistant	
VIM-producing isolates					
18983	0.5	Susceptible	< 0.12	Susceptible	
18893	0.5	Susceptible	< 0.12	Susceptible	
18820	0.25	Susceptible	< 0.12	Susceptible	
17656	< 0.12	Susceptible	< 0.12	Susceptible	
18201	< 0.12	Susceptible	< 0.12	Susceptible	
16733	0.5	Susceptible	< 0.12	Susceptible	
16213	0.5	Susceptible	< 0.12	Susceptible	
16391	1	Susceptible	< 0.12	Susceptible	
16381	0.25	Susceptible	< 0.12	Susceptible	
16615	0.25	Susceptible	< 0.12	Susceptible	
VIM 1	0.5	Susceptible	< 0.12	Susceptible	
VIM 2	< 0.12	Susceptible	< 0.12	Susceptible	
QF04104078	< 0.12	Susceptible	< 0.12	Susceptible	
VIM 4	0.25	Susceptible	< 0.12	Susceptible	
HN06193535	0.5	Susceptible	< 0.12	Susceptible	
Control strains					
<i>E. coli</i> ATCC 25922	0.25	Susceptible	< 0.12	Susceptible	
<i>K. pneumoniae</i> BAA-1705	16	Resistant	32	Resistant	

By comparison, VIM-producing strains demonstrated imipenem MIC values from < 0.12 µg/ml to 1 µg/ml, with all strains being categorised as susceptible. Meropenem exhibited an MIC

value lower than 0.12 µg/ml across all VIM-producing isolates, suggesting ubiquitous susceptibility. The overall analysis of MIC values indicates a notable shift towards susceptibility in EDTA-treated media for both NDM- and VIM-producing strains when compared to untreated media, underscoring the influence of zinc in modulating antibiotic resistance. The consistent MIC values of the control strains, *E. coli* ATCC 25922 and *K. pneumoniae* BAA-1705, across both media types (untreated and EDTA-treated) validate the experimental reliability.

4.4 Gradient Diffusion Strip Test on EDTA-treated Agar

The experimental phase of our research concluded with assessing the antibiotic susceptibility profiles of NDM- and VIM-producing isolates in zinc-depleted media using the gradient diffusion strip test, and the results can be found in Table 11. In most cases, the E-test yielded lower MICs compared to the broth microdilution method.

The NDM-producing isolates showed a variation in imipenem MIC values which ranged from 0.125 µg/ml to 12 µg/ml, with 60% (9/15) falling under the susceptible category and 27% (4/15) under the intermediate category. Meropenem exhibited MIC values within a range of 0.023 µg/ml to 16 µg/ml, with most isolates (53% - 8/15) identified as susceptible, while some displayed intermediate susceptibility (33% - 5/15) or resistance (13% - 2/15).

Regarding VIM-producing isolates, imipenem MIC values ranged from 0.094 µg/ml to 1 µg/ml, with all isolates classified as susceptible. Likewise, susceptibility to meropenem was detected in all VIM isolates, with MIC values varying from 0.012 µg/ml to 0.19 µg/ml.

In summary, the E-test results suggest that EDTA treatment induced a transition from predominantly resistant to mostly susceptible patterns, indicating the effect of zinc on resistance trends. These findings highlight the efficiency of the gradient diffusion strip test in evaluating antibiotic susceptibility, as the shift towards susceptibility seen in disk diffusion and broth microdilution is reinforced by this method. The consistent MIC values observed for the control strains, *E. coli* ATCC 25922 and *K. pneumoniae* BAA-1705, affirm that the standard experimental conditions were maintained.

Table 11 : Antibiotic Susceptibility Profile of NDM- and VIM-Producing Isolates in EDTA-treated Media: Evaluation Using the Gradient Diffusion Strip Test

Bacterial Isolate		Imipenem		Meropenem	
ID	MIC (ug/ml)	Interpretation	MIC (ug/ml)	Interpretation	
NDM-producing isolates					
19278	1	Susceptible	0.19	Susceptible	
19275	0.75	Susceptible	0.125	Susceptible	
19126	12	Resistant	1.5	Intermediate	
19122	0.75	Susceptible	0.064	Susceptible	
19322	0.75	Susceptible	0.094	Susceptible	
NDM 1	1	Susceptible	2	Intermediate	
NDM 2	8	Resistant	2	Intermediate	
NDM 3	1	Susceptible	1	Susceptible	
NDM 4	1.5	Intermediate	1.5	Intermediate	
NDM 5	0.125	Susceptible	0.023	Susceptible	
NDM 6	0.75	Susceptible	0.047	Susceptible	
NDM 7	2	Intermediate	16	Resistant	
NDM 8	2	Intermediate	2	Intermediate	
NDM 9	0.38	Susceptible	0.032	Susceptible	
NDM 10	2	Intermediate	4	Resistant	
VIM-producing isolates					
18983	0.38	Susceptible	0.032	Susceptible	
18893	0.25	Susceptible	0.023	Susceptible	
18820	1	Susceptible	0.032	Susceptible	
17656	0.19	Susceptible	0.016	Susceptible	
18201	0.25	Susceptible	0.032	Susceptible	
16733	1	Susceptible	0.19	Susceptible	
16213	0.5	Susceptible	0.064	Susceptible	
16391	1	Susceptible	0.125	Susceptible	
16381	0.38	Susceptible	0.047	Susceptible	
16615	0.38	Susceptible	0.094	Susceptible	
VIM 1	0.25	Susceptible	0.023	Susceptible	
VIM 2	0.19	Susceptible	0.016	Susceptible	
QF04104078	0.094	Susceptible	0.012	Susceptible	
VIM 4	0.5	Susceptible	0.032	Susceptible	
HN06193535	0.25	Susceptible	0.032	Susceptible	
Control strains					
<i>E. coli</i> ATCC 25922	0.125	Susceptible	0.012	Susceptible	
<i>K. pneumoniae</i> BAA-1705	8	Resistant	32	Resistant	

CHAPTER FIVE:

Comparative Analyses



5.1 Antibiotic Susceptibility Testing : Untreated vs EDTA-Treated Media

EDTA treatment was shown to be effective in its role as a chelating agent for zinc ions, as evidenced by the alignment of AST findings in EDTA-treated media with the anticipated outcomes. Figure 8 showcases a visual representation of the data obtained from our AST experiments.

In the disk diffusion assay, all isolates showed resistance to imipenem on untreated MHA, with the exception of one isolate (ID: VIM 4) exhibiting intermediate susceptibility (Figure 8). In contrast, all strains demonstrated susceptibility when tested in EDTA-treated media. Meropenem showcased greater variability, with an initial 67% (20/30) of strains being resistant, among which 17 transitioned to susceptible and 3 to intermediate post EDTA treatment. The remaining 33% (10/30) exhibited intermediate profiles when using untreated MHA, except for one strain (ID: 18201) which was susceptible (Figure 8). Following EDTA-treatment, all intermediate profiles shifted to susceptibility, and the single isolate that was initially susceptible maintained its status, albeit with an increased zone of inhibition (23 mm vs. 34 mm; Tables 5 & 9).

In the broth microdilution method, all isolates were initially resistant to imipenem when using the untreated media. Upon EDTA treatment, a shift was observed in 80% (24/30) of the strains towards susceptibility, 7% (2/30) towards intermediate susceptibility, and the remaining 13% (4/30) retained their resistance, albeit with notable decreases in MICs (from $> 64 \mu\text{g/ml}$ to $8 \mu\text{g/ml}$ for three strains and to $4 \mu\text{g/ml}$ for one strain) (Tables 6 & 10). With respect to meropenem, 93% (28/30) of the strains were found to be resistant in untreated media, with 22 transitioning to susceptible and 6 remaining resistant post zinc depletion, all displaying a notable decrease in MIC (with a minimum 4-fold reduction for the resistant profiles) (Tables 6 & 10). Two isolates initially displayed intermediate susceptibility to meropenem but subsequently became susceptible when exposed to EDTA-treated broth (Figure 8).

The gradient diffusion strip test revealed a resistance rate of 63% (19/30) to imipenem on untreated media, with 13 isolates becoming susceptible, 4 intermediate, and 2 persisting as resistant after EDTA-treatment with significant MIC reductions (from $> 32 \mu\text{g/ml}$ to $12 \mu\text{g/ml}$ for one strain, and from $> 32 \mu\text{g/ml}$ to $8 \mu\text{g/ml}$ for the other) (Tables 7 & 11). Among the 11 non-resistant strains on untreated MHA, 6 were intermediate, and 5 were susceptible, all of which presented a remarkable reduction in MIC on EDTA-treated MHA (Tables 7 & 11). Meropenem exhibited the highest number of susceptible strains when using the untreated

media, with 37% (11/30) being susceptible. All susceptible strains retained their status on EDTA-treated agar, with significant reductions in MIC values observed (Tables 7 & 11). Intermediate profiles (7% - 2/30) shifted to susceptible in the EDTA-treated media, with marked MIC reductions. Among the resistant profiles (56% - 17/30), five were intermediate on EDTA-treated MHA, and two remained resistant but with a decrease in MIC (Tables 7 & 11). Overall, an inclination towards antibiotic resistance was observed in the isolates when using untreated media, with the highest susceptibility rates noted in the EDTA-treated media.

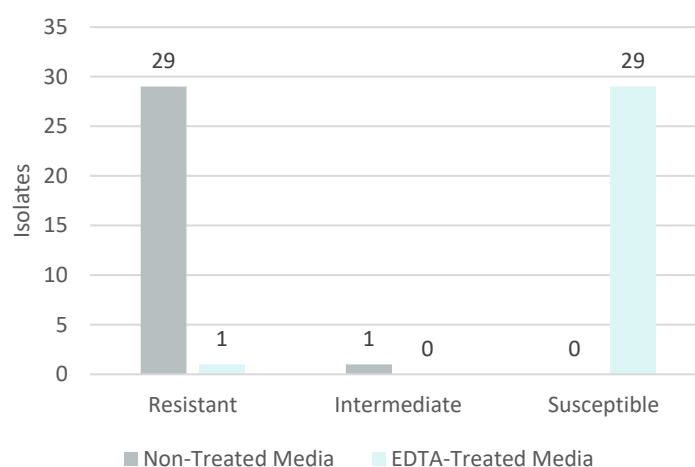


Figure 5 : Impact of High Zinc Levels in Untreated Media on Imipenem Susceptibility in MBL-Producing CRE Using Disk Diffusion (p -value < 0.0001).

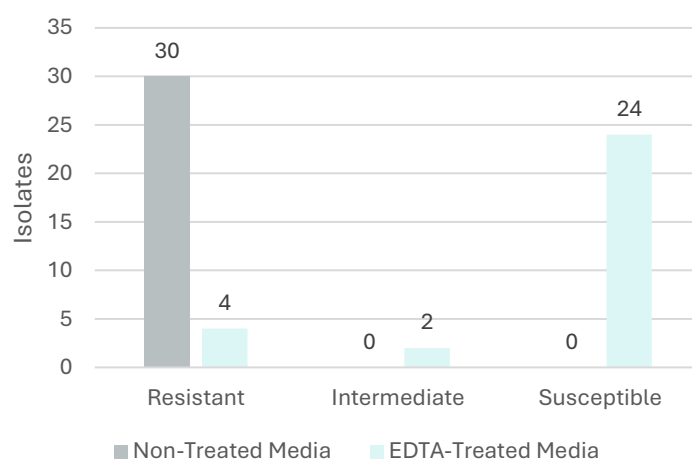


Figure 6 : Impact of High Zinc Levels in Untreated Media on Imipenem Susceptibility in MBL-Producing CRE Using Broth Microdilution (p -value < 0.0001).

			VIM														NDM															
Antibiotic	EDTA-Treated Media	Test	18983	18893	18820	17656	18201	16733	16213	16391	16381	16615	VIM 1	VIM 2	QF04104078	VIM 4	HN06193535															
Imipenem	NO	DD	13	12	19	19	19	19	15	16	17	16	11	19	18	21	15	12	15	6	13	14	6	6	14	9	14	7	6	8	16	14
	YES	DD	30	30	28	32	32	31	29	27	32	32	30	34	34	31	24	28	31	11	30	28	24	27	36	26	35	29	24	25	32	24
	NO	BDMIC	32	32	8	8	16	16	64	16	16	8	64	8	8	16	16	>64	>64	>64	64	64	>64	>64	32	64	32	>64	>64	>64	32	>64
	YES	BDMIC	0.5	0.5	0.25	<0.12	<0.12	0.5	0.5	1	0.25	0.25	0.5	<0.12	<0.12	0.25	0.5	<0.12	0.25	8	<0.12	<0.12	8	8	<0.12	1	<0.12	0.25	4	2	<0.12	2
	NO	E-test	>32	16	1.5	0.75	0.5	1	8	1.5	1.5	1.5	>32	0.5	2	0.75	4	>32	8	>32	6	6	>32	>32	16	32	>32	>32	>32	>32	2	>32
Meropenem	YES	E-test	0.38	0.25	1	0.19	0.25	1	0.5	1	0.38	0.38	0.25	0.19	0.094	0.5	0.25	1	0.75	12	0.75	0.75	1	8	1	1.5	0.125	0.75	2	2	0.38	2
	NO	DD	18	18	22	21	23	22	20	19	21	17	14	22	21	21	20	11	11	6	12	11	6	6	13	10	15	10	6	6	15	10
	YES	DD	36	34	32	34	34	32	34	30	32	33	34	37	38	38	28	30	34	20	33	30	23	23	38	27	36	30	22	22	32	23
	NO	BDMIC	32	32	2	4	8	8	32	8	16	8	32	2	4	8	16	>64	>64	>64	>64	>64	>64	>64	64	>64	32	>64	>64	>64	64	>64
	YES	BDMIC	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	8	<0.12	<0.12	16	8	<0.12	1	<0.12	<0.12	16	8	<0.12	4
	NO	E-test	6	3	0.5	0.19	0.19	0.38	0.75	0.75	0.38	1.5	12	0.5	0.75	0.5	0.5	>32	6	>32	6	8	>32	>32	16	>32	>32	>32	>32	>32	24	>32
	YES	E-test	0.032	0.023	0.032	0.016	0.032	0.19	0.064	0.125	0.047	0.094	0.023	0.016	0.012	0.032	0.032	0.19	0.125	1.5	0.064	0.094	2	2	1	1.5	0.023	0.047	16	2	0.032	4

DD: Disk diffusion

BDMIC: Broth microdilution

Antibiotic Susceptibility Profiles

Resistant	
Intermediate	
Susceptible	

Figure 7 : Antibiotic Susceptibility Profiles of 30 Isolates under Untreated and EDTA-Treated Conditions using Disk Diffusion (ZOI Interpretation: $R \leq 19$ mm, $19 \text{ mm} < I < 23$ mm, $S \geq 23$ mm) and MIC Methods (Broth Microdilution and E-test; MIC Interpretation: $R \geq 4$ $\mu\text{g/mL}$, $1 \mu\text{g/mL} < I < 4 \mu\text{g/mL}$, $S \leq 1 \mu\text{g/mL}$). CLSI M100 2023¹³³ was used to guide results interpretation. *R*: resistant; *I*: Intermediate; *S*: Susceptible.

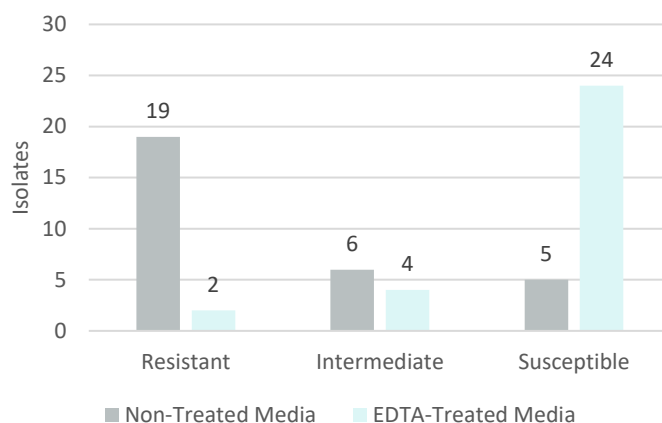


Figure 8 : Impact of High Zinc Levels in Untreated Media on Imipenem Susceptibility in MBL-Producing CRE Using Gradient Diffusion Strip Test (p -value < 0.0001).

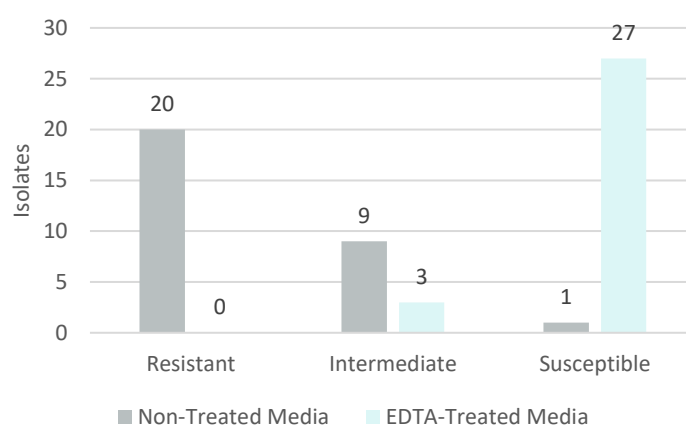


Figure 9 : Impact of High Zinc Levels in Untreated Media on Meropenem Susceptibility in MBL-Producing CRE Using Disk Diffusion (p -value < 0.0001).

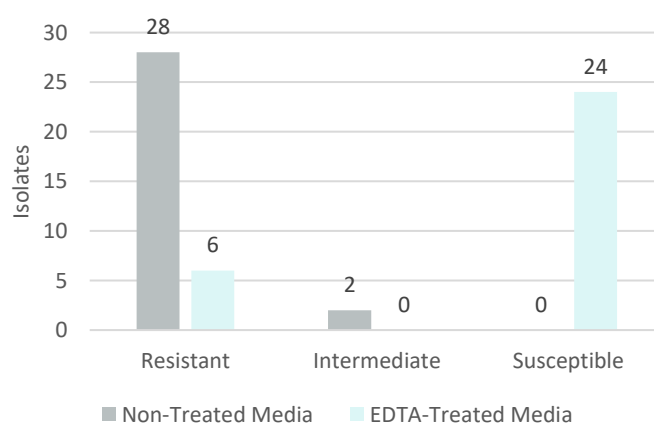


Figure 10 : Impact of High Zinc Levels in Untreated Media on Meropenem Susceptibility in MBL-Producing CRE Using Broth Microdilution (p -value < 0.0001).

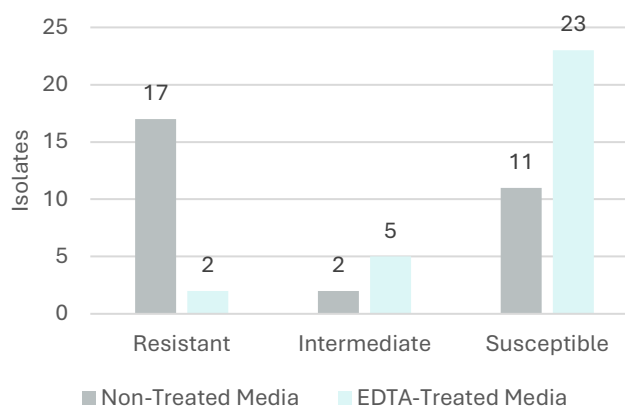


Figure 11 : Impact of High Zinc Levels in Untreated Media on Meropenem Susceptibility in MBL-Producing CRE Using Gradient Diffusion Strip Test (p -value < 0.0001).

A comparative analysis was carried out on MIC₅₀ and MIC₉₀ values to gain further insights into the influence of zinc on susceptibility profiles of MBL-harbouring Enterobacterales, as determined through broth microdilution and E-test techniques (Table 12).

Table 12 : Comparison of MIC₅₀ and MIC₉₀ Values for Antibiotic Susceptibility Testing in the Presence and Absence of Zinc Using BDMIC and E-TEST Methods

Antibiotic	Media	MIC ₅₀		MIC ₉₀	
		BDMIC	E-TEST	BDMIC	E-TEST
Imipenem	Untreated	32 µg/ml	8 µg/ml	>64 µg/ml	>32 µg/ml
	EDTA-treated	0.25 µg/ml	0.75 µg/ml	4 µg/ml	2 µg/ml
Meropenem	Untreated	32 µg/ml	6 µg/ml	>64 µg/ml	>32 µg/ml
	EDTA-treated	<0.12 µg/ml	0.064 µg/ml	8 µg/ml	2 µg/ml

Imipenem susceptibility testing in untreated media yielded MIC₅₀ values of 32 µg/ml for broth microdilution and 8 µg/ml for E-test, along with corresponding MIC₉₀ values of > 64 µg/ml and > 32 µg/ml, respectively. Conversely, EDTA treatment resulted in imipenem MIC₅₀ values of 0.25 µg/ml for broth microdilution and 0.75 µg/ml for E-test, while the MIC₉₀ values were 4 µg/ml and 2 µg/ml. Moreover, for meropenem, the presence of zinc in untreated media induced MIC₅₀ values of 32 µg/ml (broth microdilution) and 6 µg/ml (E-test), and MIC₉₀ values that exceeded 64 µg/ml and 32 µg/ml, respectively. However, when zinc levels were lowered with EDTA treatment, MIC₅₀ notably decreased to less than 0.12 µg/ml (broth microdilution) and 0.064 µg/ml (E-test), with MIC₉₀ values of 8 µg/ml and 2 µg/ml.

Using the Wilcoxon matched-pairs signed-rank test, a non-parametric statistical method suited for non-normally distributed data, an assessment was conducted to detect shifts between the untreated and EDTA-treated conditions. For all three AST methods, the analysis yielded a p -value of <0.0001 , indicating strong statistical evidence of a significant difference in MIC and/or ZOI values between the conditions with and without EDTA treatment. Since AST results show a tendency toward lower MIC values and higher ZOI when zinc levels are reduced in EDTA-treated media (Figure 8), this suggests that zinc depletion correlates with increased susceptibility to both imipenem and meropenem.

Taken together, the findings from the Wilcoxon matched-pairs signed-rank test underscore the impact of zinc presence or absence on antibiotic susceptibility outcomes across different AST methods, offering valuable insights into the relationship between environmental factors and microbial susceptibility.

5.2 Antibiotic Susceptibility Testing : Inter-Method Agreement

Having consistently observed heightened susceptibility of MBL-producing CRE to carbapenems in zinc-depleted media across all three AST techniques—disk diffusion, broth microdilution, and gradient diffusion strip test—the focus shifted to assessing the agreement among these methods. Cohen's kappa analysis was conducted by integrating observations from each pair of techniques to evaluate inter-rater agreement (Figure 15). Data from both imipenem and meropenem in zinc-depleted media were pooled. This approach improves the robustness of the analysis, as larger datasets generally provide more reliable statistical estimates.

The analysis revealed that E-test and broth microdilution demonstrated the highest level of agreement, with a kappa value (κ) of 0.63, indicating substantial agreement between these methods. The E-test and disk diffusion methods showed fair agreement, with a kappa value of 0.36. Similarly, a fair level of agreement ($\kappa = 0.26$) was observed between broth microdilution and disk diffusion techniques. These results not only confirm the consistency of the AST methods in detecting increased susceptibility of MBL-producing Enterobacterales to carbapenems in zinc-depleted media but also offer valuable insights into their comparative reliability and agreement.

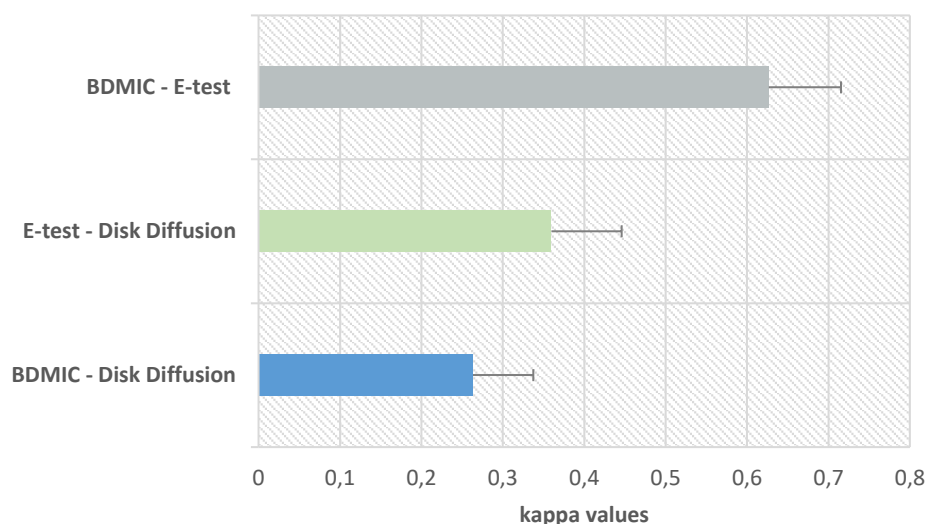


Figure 12 : Bar Chart illustrating Cohen's Kappa values for the assessment of inter-method agreement. The chart visually compares agreement levels among three methods: BDMIC (broth microdilution), E-test, and Disk Diffusion. Higher Kappa values indicate stronger agreement between raters.

5.3 Antibiotic Susceptibility Testing : NDM vs VIM

When examining the MIC₅₀ and MIC₉₀ values for NDM- and VIM-producing isolates in the presence of zinc using broth microdilution and E-test methodologies, notable distinctions arise between the two classes of carbapenemases. In particular, NDM-producing isolates exhibit significantly higher MIC₅₀ and MIC₉₀ values for meropenem in contrast to VIM-producing isolates across both techniques. This disparity is especially evident in the broth microdilution technique, where NDM-producing isolates exhibit MIC₅₀ and MIC₉₀ values exceeding 64 µg/ml, while VIM-producing isolates present markedly lower values, with an MIC₅₀ of 8 µg/ml and an MIC₉₀ of 32 µg/ml, suggesting higher susceptibility (Table 13).

Table 13 : Comparison of MIC₅₀ and MIC₉₀ Values for NDM- and VIM-producing isolates in the Presence of Zinc Using BDMIC and E-TEST Methods.

Antibiotic	Method	NDM		VIM	
		MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
Imipenem	BDMIC	>64 µg/ml	>64 µg/ml	16 µg/ml	64 µg/ml
	E-TEST	>32 µg/ml	>32 µg/ml	1.5 µg/ml	>32 µg/ml
Meropenem	BDMIC	>64 µg/ml	>64 µg/ml	8 µg/ml	32 µg/ml
	E-TEST	>32 µg/ml	>32 µg/ml	0.5 µg/ml	6 µg/ml

Similarly, for imipenem, NDM-producing isolates consistently display increased MIC₅₀ and MIC₉₀ values in both broth microdilution and E-test methods compared to their VIM-producing counterparts, except for their MIC₉₀ which remains unchanged when assessed using E-test (Table 13).

The Mann–Whitney U test offers additional clarification on the variances in antibiotic susceptibility profiles between NDM and VIM-producing isolates. For both imipenem and meropenem, disk diffusion indicates negative z-scores, suggesting larger inhibition zones for VIM-producing strains and thus implying increased susceptibility of VIM-producing isolates over NDM-producing ones (Table 14). Moreover, in both broth microdilution and E-test methods, positive z-scores indicate higher MICs for NDM-producing strains, further suggesting increased resistance to both antibiotics in this subset of isolates (Table 15). The *p*-values, nearing zero, denote statistical significance. These findings indicate a likely correlation between increased resistance to carbapenems and the presence of NDM carbapenemase genes when compared with strains possessing VIM carbapenemase genes.

Table 14 : Mann–Whitney Test Comparing Antibiotic Susceptibility Profiles between NDM and VIM-Producing Isolates using Broth Microdilution, Disk Diffusion, and E-test. *Disk Diffusion

	Method	z-score	p-value	Probability
Imipenem	DD*	-3.608	0.0003	$P\{zoi(gene==NDM) > zoi(gene==VIM)\} = 0.116$
	BDMIC	4.272	< 0.001	$P\{mic(gene==NDM) > mic(gene==VIM)\} = 0.947$
	E-test	3.494	0.0005	$P\{mic(gene==NDM) > mic(gene==VIM)\} = 0.864$
Meropenem	DD*	-4.606	< 0.001	$P\{zoi(gene==NDM) > zoi(gene==VIM)\} = 0.009$
	BDMIC	4.761	< 0.001	$P\{mic(gene==NDM) > mic(gene==VIM)\} = 0.991$
	E-test	4.596	< 0.001	$P\{mic(gene==NDM) > mic(gene==VIM)\} = 0.982$

The study as a whole emphasizes the importance of taking carbapenemase genotypes into account when assessing antibiotic susceptibility, given the potential disparities in resistance levels within different genotypes.

CHAPTER SIX:

General Discussion & Conclusion



6.1 Effect of Zinc Depletion on Antibiotic Susceptibility

MBLs represent a significant challenge with regards to antimicrobial therapy as a result of their ability to confer resistance to beta-lactam antibiotics through enzymatic breakdown³⁸. These enzymes rely on zinc for their functionality, thereby highlighting the crucial role of metal ions in bacterial resistance mechanisms⁶¹. Recent investigations have shed light on the involvement of zinc-dependent regulatory systems, such as the zinc uptake regulator (Zur), in modulating bacterial responses to antibiotics^{88,113,138}. Our study delves into the intricate connection between zinc availability and susceptibility to antibiotics, particularly in relation to carbapenems.

Our findings are in line with current literature that stresses the significant function of zinc in shaping MBL-harbouring Enterobacterales reactions to β -lactams^{4,7}. More specifically, we noted a significant decrease in MIC values subsequent to zinc depletion: the isolates harbouring MBLs predominantly exhibited resistance to meropenem and imipenem in media containing zinc, yet demonstrated susceptibility when the zinc was depleted. In a study by Asempa et al.⁵, MBL-harbouring isolates displayed MICs ranging from 16 to > 64 $\mu\text{g/ml}$ in conventional caMHB (Becton, Dickinson and Company, USA). However, these values decreased to a range spanning from < 0.06 to 1 $\mu\text{g/ml}$ upon evaluation in two distinct types of zinc-depleted broths – Chelex (Sigma–Aldrich, USA) broth and EDTA (Sigma–Aldrich, USA) broth.

Our current investigation involved assessing the susceptibility of our isolates on agar plates, either containing active zinc ions or lacking them. A noteworthy increase in inhibition zone diameters was observed under zinc-depleted conditions, suggestive of increased susceptibility to the antibiotics being tested. To the best of our knowledge, this study is the first to report alterations in the antibiotic susceptibility profiles of MBL-producing Enterobacterales on zinc-depleted solid agar.

Discrepancies in zinc content across different MHB brands have been observed^{4,139}. Considering the role of zinc in bacterial resistance mechanisms such as MBLs and its implications on antimicrobial susceptibility testing outcomes, it is crucial to establish standardised testing conditions to ensure accurate assessment of antibiotic susceptibility, especially in the context of MBL-producing Enterobacterales.

In conclusion, our research sheds light on the intricate interplay between zinc availability, antibiotic susceptibility, and MBL expression. A reduction in zinc levels leads to significant

changes in MIC values and zones of inhibition, highlighting notable gaps in antimicrobial susceptibility testing conditions. Clinical Microbiologists should factor in the potential effects of zinc concentration when analysing susceptibility test outcomes and selecting appropriate treatment regimens to optimise patient care and outcomes.

6.2 Effectiveness of EDTA Treatment in antibiotic susceptibility testing

EDTA forms complexes with a variety of metal cations, including zinc. However, it does not affect the total cation concentration in the solution. Consequently, quantification of zinc in EDTA-treated culture broth did not show any noticeable reduction in zinc concentrations. This is because the ICP-MS technique quantifies the overall zinc content present within the medium while lacking the ability to differentiate between zinc chelated by EDTA and zinc readily accessible to microorganisms^{140,141}. This contrast with the application of the Chelex approach, which effectively measures reductions in zinc levels to below detectable thresholds (< 0.002mg/l)⁵. Asempa et al.⁵ demonstrated that both Chelex treatment and EDTA supplementation at 300 mg/l were equally effective in reducing zinc availability, as evidenced by comparable reductions in carbapenem MICs. The depletion of zinc to undetectable levels in the Chelex-treated media, mirroring the untraceable zinc levels observed in the infected lungs of mice, further supports the effectiveness of these agents in disrupting zinc availability for bacterial pathogens. These findings suggest a potential correlation between carbapenem activity at the infection site in mice and in vitro conditions with zinc-depleted media.

Upon reviewing the work of Bilinskaya et al.⁴, Rennie¹²² raised an observation on the use of high concentrations of EDTA by the authors for depleting cations, a process that we replicated. This procedure has the potential to impact not only zinc but also other cations such as calcium and magnesium, thereby influencing the outcomes of susceptibility testing, as concluded by Rennie¹²². Our current investigation reveals that EDTA treatment has no impact on the susceptibility testing results for control strains, which serve as a standard for non-beta-lactamase validation, suggesting that the potential sequestration of calcium and magnesium by this chelating agent is not substantial enough to inhibit bacterial growth. This is in line with the findings of Bilinskaya et al.⁴, indicating that EDTA had no effect on the MIC values of non-MBL-harboursing strains (i.e., *P. aeruginosa* ATCC 27853 and three KPC isolates). The absence of MIC reduction in KPC-producing strains rules out the possibility that the observed

enhancement in *in vitro* killing of MBL-producing strains was a result of EDTA. Additionally, the *in vivo* efficacy of meropenem against MBL-harbouring Enterobacterales in an animal infection model correlates with the susceptible MICs determined in zinc-depleted broth (caMHB plus EDTA), providing further confirmation of the optimal levels of calcium and magnesium in the media treated with EDTA ⁵.

A plausible rationale for the limited impact of EDTA on calcium and magnesium in our study could be attributed to the fact that under alkaline pH conditions (pH greater than 7), the negatively charged EDTA molecule exhibits a stronger affinity for metal ions like zinc, rather than other divalent cations such as calcium and magnesium ¹²¹. The media used in our experiment has a pH of 7.3 ± 0.1 at 25°C, which is slightly above neutral and tends toward the alkaline range, creating an environment conducive to the selective binding of zinc cations by EDTA. While it has been acknowledged that chelating agents may possess direct antimicrobial characteristics ¹²¹, the reduction in meropenem and imipenem MICs observed in our analysis is primarily linked to reduced zinc availability rather than the inhibitory effects of EDTA. This is supported by the adequate growth of all microorganisms in the growth control wells and by the fact that the control strains' MICs remained within the anticipated ranges, despite the presence of EDTA.

Bilinskaya et al. ⁴ did not measure the levels of zinc or other cation in the EDTA-treated samples, a similarity noted in our own study, presenting a challenge in attributing variations in susceptibility profiles solely to zinc ¹²². As a result, Rennie hypothesised that the inconsistencies observed in antibiotic susceptibility patterns suggest a broader concern that may extend beyond variations in zinc levels in culture media ¹²². González et al. ⁷⁷ found that VIM-2 was undetectable in periplasmic extracts obtained from cells exposed to the metal-chelating compound dipicolinic acid (DPA), indicating a potential association with the loss of resistance conferred by VIM-2 under zinc-deficient conditions. Similar outcomes were observed when zinc sequestration was induced through the introduction of calprotectin into the medium, affirming that the impact stems from restricting the external availability of zinc, irrespective of the specific chelating agent utilised ⁷⁷.

While the levels of calcium and magnesium in culture media are regulated as per CLSI and EUCAST standards, with the medium named caMHB, inconsistencies in zinc concentrations have been documented in different sets of media ^{4,139}. The impact of EDTA on MICs across all isolates was observed to strongly correlate with the initial zinc availability, indicating a notable

dependency on zinc levels. Specifically, less reduction in MIC was noted with higher initial zinc levels⁴. For instance, at an EDTA concentration of 3 ug/ml, MIC values remained constant in BD lots (with zinc concentrations ranging from 1.181-1.318 ug/ml), whereas significant reductions in MIC were observed in Oxoid (zinc concentrations ranging from 0.333-0.400 ug/ml) and Sigma-Aldrich (zinc concentrations ranging from 0.244-0.282 ug/ml) broths⁴. This suggests that the effects of EDTA could be more accentuated if the initial zinc levels are lower, potentially leading to lower MIC values. However, Bilinskaya et al.⁴, demonstrated that the addition of EDTA at a concentration ≥ 300 mg/l was sufficient to create a zinc-restricted medium, thereby resulting in MICs that reflected the *in vivo* efficacy of meropenem. Therefore, we suggest that the MIC results obtained in zinc-depleted media with an EDTA concentration of 300 mg/l in the current study accurately mirror the *in vivo* performance of the antibiotics tested against the isolates.

6.3 Agreement Between Testing Techniques

The emergence of antimicrobial resistance (AMR) as a prominent global health issue underscores the need for timely and accurate identification of antimicrobial drug resistance, along with suitable therapeutic interventions and antimicrobial stewardship practices, to curb its spread. Recent investigations into the *in vitro* susceptibility of CRE strains, while evaluating different approaches to AST, are notably scarce. Our study sought to address this gap by conducting a comprehensive comparative analysis, assessing outcomes obtained from disk diffusion, broth microdilution, and gradient diffusion strip testing. The method of the broth microdilution has earned recognition as the gold standard for AST due to its potential to offer a quantitative assessment of antimicrobial efficacy *in vitro*, enabling the generation of data essential for surveillance and the precise determination of treatment regimens¹⁴². However, this technique poses challenges in regular application within hospital laboratories due to its labour-intensive nature¹⁴³. The gradient diffusion strip test serves as a practical alternative to traditional broth microdilution¹⁴³. Our study showed substantial agreement between gradient diffusion strip test and broth microdilution, suggesting its suitability for assessing changes in antibiotic susceptibility. Nonetheless, result interpretation may be subjective and reliant on the proficiency of the operator. Conversely, the commonly applied disk diffusion method is renowned for its simplicity and cost-effectiveness¹⁴³. Our investigations revealed fair agreement between the disk diffusion and broth microdilution methods, indicating that the

methods are somewhat aligned but not consistently reliable. This could affect treatment decisions and patient outcomes if the variability leads to inconsistent or incorrect susceptibility results. It should be noted, however, that the accuracy of the disk diffusion method could differ for specific antibiotics or bacterial strains due to variations in diffusion rates and growth conditions ¹⁴⁴.

By employing broth microdilution as the benchmark, a pattern was identified where both E-test and disk diffusion techniques appeared to underestimate the rates of resistance. Nana et al.¹⁴⁵ published findings on carbapenems and OXA-48-producing CREs, where E-tests also under-reported resistance. It was observed that the E-test method demonstrated a higher level of agreement ($k = 0.63$) with the established standard as opposed to the disk diffusion ($k = 0.26$). The results of our study indicate that while there may exist a satisfactory level of concordance in the qualitative data obtained from E-test and broth microdilution methods, discrepancies in MIC values have the potential to introduce inconsistencies in the prescription of drug dosage. The disparity in MIC values observed between these methodologies could be a result of suboptimal diffusion of the antibiotic within the agar medium ¹⁴³. Various environmental factors including humidity, temperature, and the depth of the agar medium can affect the efficiency of drug diffusion in the E-test ¹⁴⁶.

In essence, our findings reveal varying levels of agreement between the disk diffusion and E-test methods in relation to the broth microdilution method. However, all three methods consistently demonstrated a reduction in MIC values and an enlargement of inhibition zones as the transition was made from zinc-containing media to zinc-depleted media, underscoring their reliability in detecting the influence of zinc on bacterial growth.

6.4 Carbapenemase Genes and Antibiotic Resistance

Within the MBLs group, NDM has displayed the swiftest and broadest expansion across various geographic areas ³⁸. Unlike other MBLs of clinical importance that operate as soluble enzymes in the periplasm, NDMs are lipoproteins bound to the outer membrane of Gram-negative bacteria ⁷⁷. This particular localisation enhances the stability of NDMs in the face of zinc deficiency, which can be triggered by the immune response during infections ⁷⁷. Such findings bear implications for the overall virulence and adaptability of the bacterial strains.

Strains harbouring the NDM carbapenemase gene might gain a selective advantage in environments with high levels of antibiotic pressure, enabling them to persist and proliferate despite the presence of carbapenem antibiotics ¹⁴⁷. Consequently, this natural selection could foster the dissemination of antibiotic resistance, posing significant challenges for the effective treatment of infections stemming from strains carrying NDM. Following the initial identification of NDM-1, further investigation has revealed new allelic variants (totalling 16) ⁷⁸. Barh et al. ⁷⁸ demonstrated that these variants evolve through the accumulation of mutations that improve their stability or increase their affinity for Zn (II) binding *in vivo*, superseding the existing evolutionary pressures associated with catalytic efficiency.

In our investigation, various strains of MBL-producing Enterobacterales, predominantly VIM, displayed a susceptible phenotypic profile to carbapenems as determined by broth microdilution, disk diffusion, or E-test methodologies. Notwithstanding the presence of the MBL gene (NDM or VIM) and exposure to zinc-containing media, these strains demonstrated susceptibility to imipenem, meropenem, or both (with MIC values ≤ 1 mg/litre). This finding aligns with previous studies that have shown susceptibility of VIM-producing strains to imipenem, meropenem, and ertapenem ⁷.

Moreover, a tendency towards lower MICs was evident in strains with the VIM gene when compared to those with NDM. The higher MIC values detected in NDM-producing strains suggest that the presence of the NDM carbapenemase gene may confer a greater level of resistance to carbapenem antibiotics in comparison to the VIM gene. This finding is in line with prior research indicating a pattern of lower MICs among VIM-harbouring isolates as opposed to those carrying NDM and IMP subtypes, suggesting potential differences in zinc sensitivities or varying efficiencies of the respective carbapenemases in catalysing the hydrolysis of carbapenem antibiotics ^{4,7}. Since MIC values are significantly influenced by the presence of Zn(II), differences in susceptibilities could be attributed to the varying metal-binding affinities of each MBL, directly affecting their enzymatic functions ⁷⁷. Discrepancies in the genetic makeup of the strains might offer an explanation for the differences in their zinc-binding abilities. Consequently, gene expression holds significant implications in the context of antibiotic resistance.

The variation in MICs between NDM and VIM is not confined solely to these specific categories of MBL. Evidence suggests that plasmids encoding IMP-1 only confer marginal resistance to carbapenems in *E. coli*, even under conditions of high zinc concentrations ¹⁴⁸.

Conversely, the MICs of carbapenems against *E. coli* transconjugants containing NDM-1 plasmids are substantially higher ¹⁴⁸. Cheung et al. ¹⁴⁸ noted in their study that among 13 NDM Enterobacterales clinical isolate transformants that were examined, all displayed resistance to meropenem, whereas only one out of thirteen IMP transformants exhibited such resistance. These findings substantiate the assertion that NDM has a greater tendency to bestow carbapenem resistance compared to other MBLs like IMP or VIM within Enterobacterales. Nevertheless, in terms of meropenem catalytic efficacy, IMP and NDM are enzymes that are highly similar ¹⁴⁹. Thus, Cheung et al. ¹⁴⁸ undertook additional experiments with the aim of identifying plausible reasons for the variations in MIC between IMP and NDM. They discovered that NDM transformants of *E. coli*, *K. pneumoniae*, and *K. aerogenes* exhibited meropenem-hydrolysing activity that was 3 to 6 times more pronounced than that observed in IMP transformants. The heightened meropenem-hydrolysing activity was correlated with increased expression of NDM protein when compared to IMP, as assessed through liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteomics ¹⁴⁸.

Drawing from the findings of Cheung et al. ¹⁴⁸, it can be inferred that the differences in carbapenem MICs between NDM and VIM observed in our study may be attributed to variations in their expression levels in Enterobacterales. A relatively attenuated production of VIM in certain examined strains potentially accounts for their susceptibility to carbapenems (MIC $\leq$ 1 mg/l) when cultured in zinc-rich environments. However, while moderate expression of VIM does not confer resistance to carbapenems in Enterobacterales, it does lead to higher MICs when zinc is present, as indicated by observed MIC reductions in zinc-deficient settings. Reduced VIM production might be attributable to mutations or rearrangements to the promoter in these isolates, considering that the VIM-encoding gene is situated on a plasmid ¹⁴⁸. The authors further suggested that the heightened and consistent proficiency of NDM-associated strains in conferring resistance to carbapenems may play a key role in the predominance of NDM over other MBLs in carbapenem-resistant bacterial populations ¹⁴⁸. Kazmierczak et al. reported that 44%, 39%, and 17% of MBL-positive Enterobacterales isolates were found to carry NDM, VIM, and IMP, respectively ¹⁵⁰. The notable occurrence of NDM in CREs can be linked to the recurrent selective pressure arising from the utilisation of carbapenems ^{7,148}. These findings emphasise the crucial significance of considering the specific carbapenemase genotype in the assessment of antibiotic susceptibility. Differences in genotypes may result in varying degrees of resistance, underscoring the importance of targeted surveillance and efforts in antimicrobial stewardship to combat the spread of antibiotic resistance. Whole genome

sequencing and specifically plasmid sequencing would offer valuable information on the genotypes and expression.

6.5 Overall Interpretation and Conclusion

Our study delves into the intricate correlation between zinc availability and antibiotic susceptibility in MBL-harbouring Enterobacterales. The resulting findings consistently demonstrate a decrease in MIC values among MBL-harbouring Enterobacterales following zinc depletion, while the MICs of control strains consistently remain within the acceptable range prescribed by CLSI M100 2023. Furthermore, our comparative analysis indicates that the impact of EDTA extends beyond liquid broth to solid media, enhancing the reliability and precision of AST for well-informed clinical decision-making and improved patient care outcomes.

We advocate for the establishment of standardised guidelines aimed at implementing and regulating zinc-adjusted testing conditions. These guidelines are essential for promoting consistent interpretation of results across different laboratory settings, thereby enhancing the reliability of susceptibility testing outcomes. In spite of the successful reduction of zinc levels through EDTA treatment, the broad specificity of this method presents barriers to the specific elimination of this metal ion. As a result, future investigations will explore alternative methodologies, such as ion exchange chromatography or affinity chromatography, which offer the potential for targeted depletion of zinc ions without interfering with other essential cations. Media manufacturers should also consider these methods to enhance the effectiveness of their products. Additionally, the gradient diffusion strip test is recommended as a practical alternative to broth microdilution, as it closely aligns with broth microdilution results and can be easily implemented in routine microbiology laboratories.

The application of whole genome sequencing to uncover genotypic variations responsible for restricting the production of specific MBLs or reducing their affinity for zinc ions holds promise in unveiling innovative therapeutic approaches. By comprehensively analysing the genetic makeup of MBL-producing organisms, we can identify specific mutations or genetic factors that influence MBL expression and zinc-binding properties. This advanced understanding of the core molecular mechanisms can steer the development of novel therapeutic strategies aimed at targeting and counteracting MBL function, thereby addressing

antibiotic resistance. Hence, whole-genome sequencing emerges as a valuable instrument in addressing MBL-mediated antibiotic resistance and should be integrated into future investigations for a more thorough genomic evaluation.

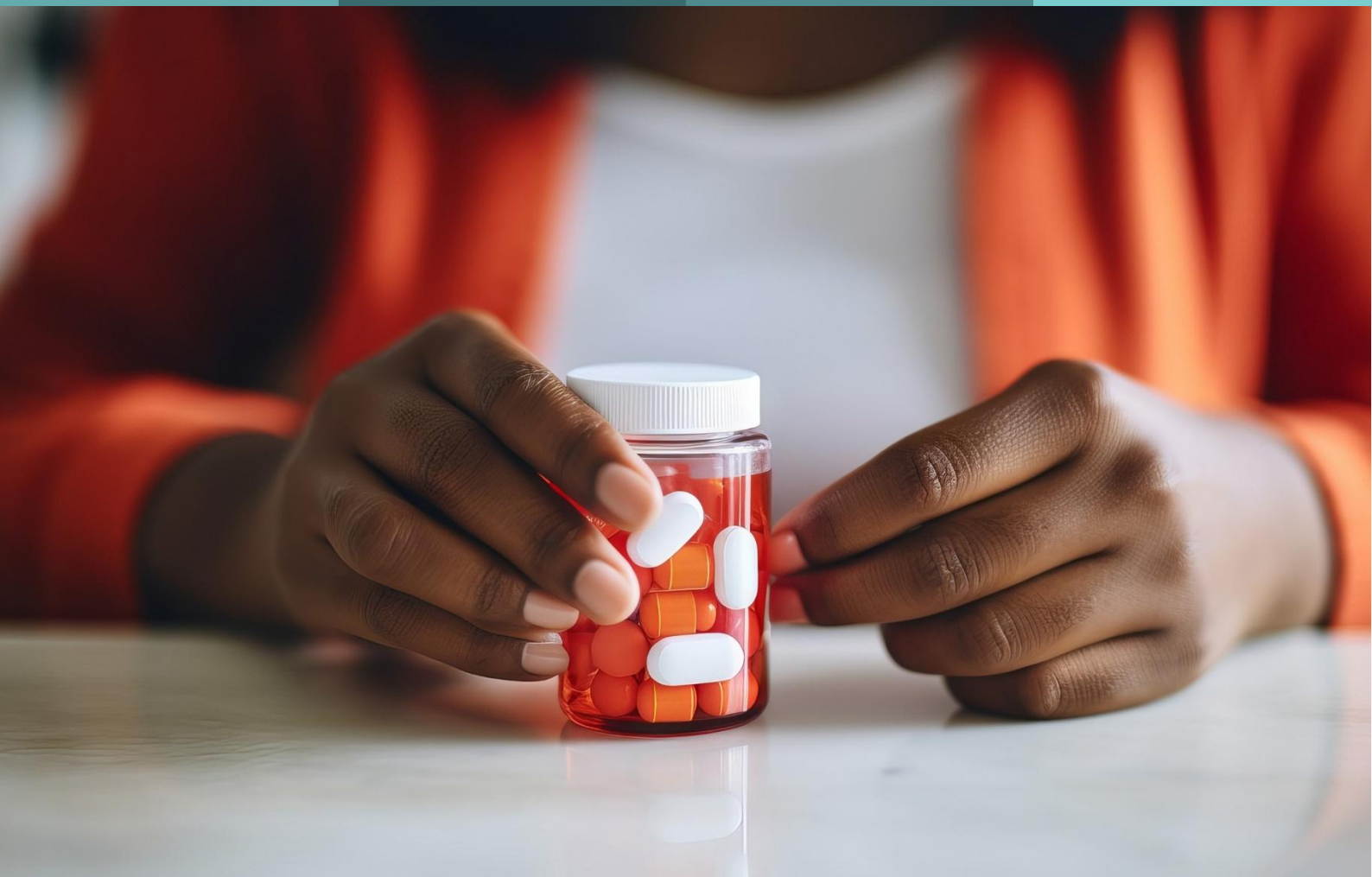
A significant limitation of our study is its exclusive focus on determining antibiotic resistance *in vitro*, which prevents us from conducting a comparative study that includes both *in vitro* and *in vivo* components. Additionally, the study's scope was constrained by a small sample size and a specific focus on carbapenems, limiting our ability to analyse β -lactams that may also be affected by zinc depletion. It would have been valuable to perform whole genome sequencing on isolates where the MIC did not decrease significantly, to identify other potential resistance mechanisms. Furthermore, the study did not include other MBLs such as IMP due to the lack of relevant isolates, a limitation imposed by local epidemiological factors. Future research should address these limitations by exploring a larger sample size, incorporating *in vivo* testing and whole genome sequencing, and evaluating the effects of zinc depletion on other antibiotics. These efforts will help advance our understanding of zinc-mediated antibiotic resistance and provide a foundation for developing targeted intervention strategies.

In conclusion, our research underscores the importance of zinc-dependent resistance to antibiotics. Having elucidated the impact of zinc on both MIC values and zones of inhibition, we have identified a critical need for guidelines to standardise testing conditions, ensuring accuracy of susceptibility testing results and facilitating informed treatment decisions. Optimising AST by reducing zinc concentrations in media is crucial for accurate CRE susceptibility testing, potentially enabling the use of carbapenems for patient management. In the current era of antimicrobial resistance, with limited treatment options, the use of carbapenems would be highly beneficial.

However, while carbapenems hold promise for treating MBL-producing Enterobacterales, this therapeutic approach may only offer a temporary solution in light of the adaptive nature of these strains, especially those harbouring the NDM gene. These strains are increasingly evolving to thrive under zinc-restricted conditions at infection sites⁷⁷. Thus, despite the possibility of resistance rates being inflated due to heightened zinc levels in growth media, there exists a looming risk of MBLs evolving to evade carbapenem therapies. Accordingly, there is an urgent need for the exploration of alternative treatment modalities that can effectively manage carbapenem resistance in response to evolving bacterial mechanisms.



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Appendix A: Raw Data for Isolate Identification and Imipenem AST Results

Lab Number	Gene	Organism	Untreated Media						EDTA-treated Media					
			Broth Microdilution		E-test		Disc Diffusion		Broth Microdilution		E-test		Disc Diffusion	
			MIC	Interpretation	MIC	Interpretation	Zone (mm)	Interpretation	MIC	Interpretation	MIC	Interpretation	Zone (mm)	Interpretation
IMIPENEM														
18983	VIM	<i>Enterobacter cloacae</i>	32	Resistant	> 32	Resistant	13	Resistant	0.5	Susceptible	0.38	Susceptible	30	Susceptible
18893	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	32	Resistant	16	Resistant	12	Resistant	0.5	Susceptible	0.25	Susceptible	30	Susceptible
18820	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	8	Resistant	1.5	Intermediate	19	Resistant	0.25	Susceptible	1	Susceptible	28	Susceptible
17656	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	8	Resistant	0.75	Susceptible	19	Resistant	< 0.12	Susceptible	0.19	Susceptible	32	Susceptible
18201	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	16	Resistant	0.5	Susceptible	19	Resistant	< 0.12	Susceptible	0.25	Susceptible	32	Susceptible
16733	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	16	Resistant	1	Susceptible	19	Resistant	0.5	Susceptible	1	Susceptible	31	Susceptible
16213	VIM	<i>Enterobacter cloacae</i>	64	Resistant	8	Resistant	15	Resistant	0.5	Susceptible	0.5	Susceptible	29	Susceptible
16391	VIM	<i>Enterobacter cloacae</i>	16	Resistant	1.5	Intermediate	16	Resistant	1	Susceptible	1	Susceptible	27	Susceptible
16381	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	16	Resistant	1.5	Intermediate	17	Resistant	0.25	Susceptible	0.38	Susceptible	32	Susceptible
16615	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	8	Resistant	1.5	Intermediate	16	Resistant	0.25	Susceptible	0.38	Susceptible	32	Susceptible
VIM 1	VIM	<i>Enterobacter cloacae</i>	64	Resistant	> 32	Resistant	11	Resistant	0.5	Susceptible	0.25	Susceptible	30	Susceptible
VIM 2	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	8	Resistant	0.5	Susceptible	19	Resistant	< 0.12	Susceptible	0.19	Susceptible	34	Susceptible
QF04104078	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	8	Resistant	2	Intermediate	18	Resistant	< 0.12	Susceptible	0.094	Susceptible	34	Susceptible
VIM 4	VIM	<i>Enterobacter cloacae</i>	16	Resistant	0.75	Susceptible	21	Intermediate	0.25	Susceptible	0.5	Susceptible	31	Susceptible
HN06193535	VIM	<i>Enterobacter cloacae</i>	16	Resistant	4	Resistant	15	Resistant	0.5	Susceptible	0.25	Susceptible	24	Susceptible
19278	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	> 64	Resistant	> 32	Resistant	12	Resistant	< 0.12	Susceptible	1	Susceptible	28	Susceptible
19275	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	> 64	Resistant	8	Resistant	15	Resistant	0.25	Susceptible	0.75	Susceptible	31	Susceptible
19126	NDM	<i>Enterobacter aerogenes</i>	> 64	Resistant	> 32	Resistant	6	Resistant	8	Resistant	12	Resistant	11	Resistant
19122	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	64	Resistant	6	Resistant	13	Resistant	< 0.12	Susceptible	0.75	Susceptible	30	Susceptible
19322	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	64	Resistant	6	Resistant	14	Resistant	< 0.12	Susceptible	0.75	Susceptible	28	Susceptible
NDM 1	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	> 64	Resistant	> 32	Resistant	6	Resistant	8	Resistant	1	Susceptible	24	Susceptible
NDM 2	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	> 64	Resistant	> 32	Resistant	6	Resistant	8	Resistant	8	Resistant	27	Susceptible
NDM 3	NDM	<i>Escherichia coli</i>	32	Resistant	16	Resistant	14	Resistant	< 0.12	Susceptible	1	Susceptible	36	Susceptible
NDM 4	NDM	<i>Escherichia coli</i>	64	Resistant	32	Resistant	9	Resistant	1	Susceptible	1.5	Intermediate	26	Susceptible
NDM 5	NDM	<i>Escherichia coli</i>	32	Resistant	> 32	Resistant	14	Resistant	< 0.12	Susceptible	0.125	Susceptible	35	Susceptible
NDM 6	NDM	<i>Enterobacter aerogenes</i>	> 64	Resistant	> 32	Resistant	7	Resistant	0.25	Susceptible	0.75	Susceptible	29	Susceptible
NDM 7	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	> 64	Resistant	> 32	Resistant	6	Resistant	4	Resistant	2	Intermediate	24	Susceptible
NDM 8	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	> 64	Resistant	> 32	Resistant	8	Resistant	2	Intermediate	2	Intermediate	25	Susceptible
NDM 9	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	32	Resistant	2	Intermediate	16	Resistant	< 0.12	Susceptible	0.38	Susceptible	32	Susceptible
NDM 10	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	> 64	Resistant	>32	Resistant	14	Resistant	2	Intermediate	2	Intermediate	24	Susceptible

Appendix B: Raw Data for Isolate Identification and Meropenem AST Results

Lab Number	Gene	Organism	Untreated Media						EDTA-treated Media					
			Broth Microdilution		E-test		Disc Diffusion		Broth Microdilution		E-test		Disc Diffusion	
			MIC	Interpretation	MIC	Interpretation	Zone (mm)	Interpretation	MIC	Interpretation	MIC	Interpretation	Zone (mm)	Interpretation
MEROPENEM														
18983	VIM	<i>Enterobacter cloacae</i>	32	Resistant	6	Resistant	18	Resistant	< 0.12	Susceptible	0.032	Susceptible	36	Susceptible
18893	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	32	Resistant	3	Intermediate	18	Resistant	< 0.12	Susceptible	0.023	Susceptible	34	Susceptible
18820	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	2	Intermediate	0.5	Susceptible	22	Intermediate	< 0.12	Susceptible	0.032	Susceptible	32	Susceptible
17656	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	4	Resistant	0.19	Susceptible	21	Intermediate	< 0.12	Susceptible	0.016	Susceptible	34	Susceptible
18201	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	8	Resistant	0.19	Susceptible	23	Susceptible	< 0.12	Susceptible	0.032	Susceptible	34	Susceptible
16733	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	8	Resistant	0.38	Susceptible	22	Intermediate	< 0.12	Susceptible	0.19	Susceptible	32	Susceptible
16213	VIM	<i>Enterobacter cloacae</i>	32	Resistant	0.75	Susceptible	20	Intermediate	< 0.12	Susceptible	0.064	Susceptible	34	Susceptible
16391	VIM	<i>Enterobacter cloacae</i>	8	Resistant	0.75	Susceptible	19	Resistant	< 0.12	Susceptible	0.125	Susceptible	30	Susceptible
16381	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	16	Resistant	0.38	Susceptible	21	Intermediate	< 0.12	Susceptible	0.047	Susceptible	32	Susceptible
16615	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	8	Resistant	1.5	Intermediate	17	Resistant	< 0.12	Susceptible	0.094	Susceptible	33	Susceptible
VIM 1	VIM	<i>Enterobacter cloacae</i>	32	Resistant	12	Resistant	14	Resistant	< 0.12	Susceptible	0.023	Susceptible	34	Susceptible
VIM 2	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	2	Intermediate	0.5	Susceptible	22	Intermediate	< 0.12	Susceptible	0.016	Susceptible	37	Susceptible
QF04104078	VIM	<i>Klebsiella pneumonia subsp pneumonia</i>	4	Resistant	0.75	Susceptible	21	Intermediate	< 0.12	Susceptible	0.012	Susceptible	38	Susceptible
VIM 4	VIM	<i>Enterobacter cloacae</i>	8	Resistant	0.5	Susceptible	21	Intermediate	< 0.12	Susceptible	0.032	Susceptible	38	Susceptible
HN06193535	VIM	<i>Enterobacter cloacae</i>	16	Resistant	0.5	Susceptible	20	Intermediate	< 0.12	Susceptible	0.032	Susceptible	28	Susceptible
19278	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	> 64	Resistant	> 32	Resistant	11	Resistant	< 0.12	Susceptible	0.19	Susceptible	30	Susceptible
19275	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	> 64	Resistant	6	Resistant	11	Resistant	< 0.12	Susceptible	0.125	Susceptible	34	Susceptible
19126	NDM	<i>Enterobacter aerogenes</i>	> 64	Resistant	> 32	Resistant	6	Resistant	8	Resistant	1.5	Intermediate	20	Intermediate
19122	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	> 64	Resistant	6	Resistant	12	Resistant	< 0.12	Susceptible	0.064	Susceptible	33	Susceptible
19322	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	> 64	Resistant	8	Resistant	11	Resistant	< 0.12	Susceptible	0.094	Susceptible	30	Susceptible
NDM 1	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	> 64	Resistant	> 32	Resistant	6	Resistant	16	Resistant	2	Intermediate	23	Susceptible
NDM 2	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	> 64	Resistant	> 32	Resistant	6	Resistant	8	Resistant	2	Intermediate	23	Susceptible
NDM 3	NDM	<i>Escherichia coli</i>	64	Resistant	16	Resistant	13	Resistant	< 0.12	Susceptible	1	Susceptible	38	Susceptible
NDM 4	NDM	<i>Escherichia coli</i>	> 64	Resistant	> 32	Resistant	10	Resistant	1	Susceptible	1.5	Intermediate	27	Susceptible
NDM 5	NDM	<i>Escherichia coli</i>	32	Resistant	> 32	Resistant	15	Resistant	< 0.12	Susceptible	0.023	Susceptible	36	Susceptible
NDM 6	NDM	<i>Enterobacter aerogenes</i>	> 64	Resistant	> 32	Resistant	10	Resistant	< 0.12	Susceptible	0.047	Susceptible	30	Susceptible
NDM 7	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	> 64	Resistant	> 32	Resistant	6	Resistant	16	Resistant	16	Resistant	22	Intermediate
NDM 8	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	> 64	Resistant	> 32	Resistant	6	Resistant	8	Resistant	2	Intermediate	22	Intermediate
NDM 9	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	64	Resistant	24	Resistant	15	Resistant	< 0.12	Susceptible	0.032	Susceptible	32	Susceptible
NDM 10	NDM	<i>Klebsiella pneumonia subsp pneumonia</i>	> 64	Resistant	> 32	Resistant	10	Resistant	4	Resistant	4	Resistant	23	Susceptible