EVALUATION OF THE SIMPLIFIED CARBAPENEM INACTIVATION METHOD (sCIM) FOR DETECTION OF CARBAPENEMASE-PRODUCING GRAM-NEGATIVE BACILLI

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A research report submitted to the faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Medicine in Microbiology and Infectious Diseases

Johannesburg 2021

DECLARATION

I Dr Bheki Zethy Masango declare that this research report is my own, unaided work. It is being submitted for the Degree of Master of Medicine in Microbiology and Infectious Diseases at the University of the Witwatersrand, Johannesburg. It has not been submitted before any degree or examination at any other University.

15 March 2021

Signature of Candidate Date

ABSTRACT

Title

Evaluation of the simplified carbapenem inactivation method (sCIM) for detection of carbapenemase-producing Gram-negative bacilli

Background and Objectives

Currently, prompt phenotypic detection of carbapenemase-producing Enterobacterales by clinical laboratories is a challenge. Available techniques are expensive, strenuous, delay results and are unsuitable for high throughput laboratories. This study evaluates the performance of the sCIM against the Modified Hodge and Imi-EDTA tests as an alternative method to detect carbapenemase enzymes in Gram-negative bacilli.

Methodology

A prospective laboratory-based study, comprising of 137 well characterised stored isolates. These included 96 Enterobacterales, 23 *Acinetobacter baumannii*, and 18 *Pseudomonas aeruginosa* isolates tested with the sCIM. The performance of the sCIM was compared to the Modified Hodge test and Imi-EDTA with the multiplex PCR used as a reference gold standard.

Results

Overall, we report 95.8% accuracy of the sCIM when testing Enterobacterales, 95.6% for *Acinetobacter baumannii*, and 77.8% for *Pseudomonas aeruginosa* using PCR as the gold standard. There was a significant correlation of 95.4% sensitivity and 100% specificity in testing both Enterobacterales and *Acinetobacter baumannii* isolates with the sCIM. Contrary to expectation both Enterobacterales and *Acinetobacter baumannii* showed low NPV of 69.8% and 50% respectively, whilst the PPV was 100% on both. *Pseudomonas aeruginosa* isolates showed 40% sensitivity, 92.3% specificity, 66.6% positive predictive value, and 80% negative predictive value.

Conclusion

Notwithstanding the lack of agreement with our low NPV, the sCIM demonstrated an acceptable performance as described in previous studies. It is inexpensive, less tedious and suitable for use in high throughput laboratories with reliable and consistent results.

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ABBREVIATION LIST

AMRL-CC/CHARM: Antimicrobial Resistance Laboratory and Culture Collection, Centre for Healthcare Associated infections, Antimicrobial Resistance and Mycoses.

AST: Antimicrobial Susceptibility Testing

ATCC: American Type Culture Colony

CHARM: Centre for health-associated infections, Antimicrobial Resistance and Mycoses

CHBAH: Chris Hani Baragwanath Academic Hospital

CLSI: Clinical and Laboratory Standards Institute

CMID: Clinical Microbiology and Infectious Diseases

CPE: carbapenemase-producing Enterobacterales

EUCAST: European Committee for Antimicrobial Susceptibility Testing

GNB: Gram-negative bacilli

CRE: Carbapenem-Resistant Enterobacterales

Imi-EDTA: Imipenem- Ethylenediaminetetraacetic acid

KPC: *Klebsiella pneumoniae* carbapenemase

MALDI-TOF: Matrix-Assisted Laser Desorption -Time of Flight

MBL: Metallo-β-Lactamase

mCIM: modified carbapenemase inactivation method

MHA: Mueller Hinton Agar

MHB: Mueller Hinton Broth

MHT: Modified Hodge Test

MIC: Minimum Inhibitory Concentration

NHLS: National Health Laboratory Services

NICD: National Institute of Communicable Diseases

NPV: Negative predictive value

PCR: Polymerase chain reaction

PPV: Positive predictive value

sCIM: simplified carbapenemase inactivation method

1. CHAPTER ONE - RESEARCH PROPOSAL AND LITERATURE REVIEW

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RESEARCH PROPOSAL AND LITERATURE REVIEW ABSTRACT

Title

Evaluation of the simplified carbapenem inactivation method (sCIM) for detection of carbapenemase producing Gram-negative bacilli

Introduction

Globally, the increase of carbapenemase-resistant Gram-negative bacilli (GNB) in healthcare facilities poses a public health threat with high rates of morbidity and mortality. Carbapenems are commonly used to treat resistant infections like extended spectrum beta-lactamase (ESBL) and Amp C producing organisms. This leaves us with limited treatment options for treating carbapenemase-producing GNB.

The Group for Enteric, Respiratory and Meningeal Diseases Surveillance in South Africa (GERMS-SA) conducted sentinel surveillance in four provinces between 2015 and 2017 using the National Health Laboratory Services (NHLS) data. They recorded a 24% increase in the carbapenem resistant Enterobacterales (CRE) bacteraemia. The Gauteng province had the highest number of CRE contributions of 73% followed by Kwa-Zulu Natal at 20% respectively.

Justification

Early detection of the carbapenemase-producing organisms in the laboratory allows for appropriate patient treatment; improved antimicrobial stewardship practices and infection control interventions.

Aim

This study aims to evaluate the sCIM performance in a routine diagnostic laboratory by assessing the sensitivity, specificity, negative and positive predictive value, repeatability, reproducibility and ease of use.

Proposed methodology

This prospective laboratory-based study will test one hundred and fifty well-characterised stored isolates comprising of Enterobacterales, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. We will comparatively evaluate the sCIM to the currently used Modified Hodge and Imi-EDTA tests, using PCR as reference gold standard.

Expected outcome

Good assay performance of the sCIM, for implementation in a routine microbiology diagnostic laboratory.

1.1 INTRODUCTION

The emergence and rise of Gram-negative bacilli that produce carbapenemase enzymes is a global notable public health threat (1–3). Infections caused by carbapenem-resistant Enterobacterales are a growing concern both locally and internationally (2). They are associated with high rates of morbidity and mortality (4). In 2016, the Centre for Healthcare-associated infections, Antimicrobial Resistance and Mycoses (CHARM) reported 72% of carbapenem-producing *Klebsiella pneumoniae* that were identified from significant sites (blood and urine) of which 34% and 63% were carrying bla_{NDM-1} and bla_{OXA-48} respectively (5). Carbapenems are the cornerstone for treating Gram-negative bacilli producing extended-spectrum β-lactamases (ESBLs), therefore the rise in carbapenemases poses a major challenge in health care settings (1). To curb the spread of carbapenemase-producing strains, quick detection of these enzymes is indispensable in our public health laboratories (1).

Most microbiology laboratories within the National Health Laboratory Services (NHLS) use the Modified Hodge Test (MHT) and Imipenem-Ethylenediaminetetraacetic acid (Imi-EDTA) as phenotypic screening methods for detection of carbapenemases. The MH and Imi-EDTA tests are no longer recommended by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) due to shortcomings (6,7).

1.2 BACKGROUND

The aptness of all contemporary clinical microbiology methods to reliably detect Enterobacterales that are carbapenemase-producing has limitations and hinders the effort in curbing the spread of drug-resistant pathogens (3). A novel, straightforward, affordable, sensitive and specific phenotypic method for the detection of carbapenemase production is essential in the effort to treat, prevent and contain the spread of carbapenemase-producing, carbapenem-resistant Enterobacterales (CP-CRE) (3). It enhances and plays a pivotal role in antimicrobial stewardship programs.

In the past 10 years, the prevalence of carbapenemases in carbapenemase-producing organisms including Enterobacterales, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* have substantially increased (7). This is attributed to plasmids found in bacteria that carry genes encoding carbapenemases and share these genes.

Apart from genes encoding carbapenemases due to production of carbapenem-hydrolyzing β-lactamases, Gram-negative bacilli exhibit other mechanisms of carbapenem resistance. These may be due to porin loss, efflux pump and alterations in chromosomally located genes (3,6,8). The distinction between carbapenem-resistance mediated by carbapenemases versus other mechanisms is of great importance in infection control and antibiotic stewardship programs (3,4,6,9). Currently, CLSI does not endorse molecular testing for clinical purposes (10).

Carbapenemases are classified based on molecular and functional classification schemes, Ambler and Bushy Jacoby respectively. Ambler classification is commonly used. It is based on the amino acid sequence and divides beta-lactamases into class A, B and D (9). Class A, and D utilize serine for β-lactam hydrolysis and class B metalloenzymes (MBLs) require divalent zinc ions for substrate hydrolysis (9). The most common in Class A is KPC enzymes, Class B IMP, VIM and NDM enzymes and class D OXA-23 like OXA-24 like, OXA-48 like and OXA-58 like enzymes (9).

Plethora's of phenotypic and non-phenotypic tests for the detection of carbapenemase-producing organisms are available (6). Phenotypic methods comprise of culture/growth based methods: MHT, Imi-EDTA, carbapenem inactivation method (CIM), modified carbapenem inactivation method (mCIM), and simplified carbapenem inactivation method (sCIM), chromogenic plate method (Chromagar), colorimetric assays (Carba NP) and lateral flow assays that detect OXA-48, KPC, NDM and VIM enzymes (O.K.N.V). Non-phenotypic methods encompass molecular methods (e.g. PCR) and spectrometry tests (UV spectrometry and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (6,10). Choosing an appropriate test requires consideration of multiple elements which include amongst other performance characteristics, workload, turnaround times (TAT), cost, equipment, reagents, ease of use and regulatory status of the test (11).

The MHT demonstrates acceptable sensitivity to class A carbapenemases, particularly the KPC enzyme, but has low sensitivity to MBLs (4). In one study, its detection rate of NDM-producing Enterobacterales was found to be around 50%. This limitation in detecting NDMs and Class D carbapenemase has dire consequences in the clinical setting.

The Imi-EDTA is an inhibitor-based assay for detecting MBLs. It is used in conjunction with the MHT and fails to detect class A and D carbapenemases (4,12). Although these methods are relatively user-friendly and cost-effective, there is delayed turnaround time (TAT),

difficulty in interpretation and a requirement of an additional 24 hours after antimicrobial susceptibility testing results become available.

The chromogenic plate, a phenotypic method, works on the principle of colour change to the medium used if a carbapenemase-producing organism is cultured. It gives a presumptive identification of carbapenemase producers within 18-24 hours. Multiple studies evaluating this method have reported acceptable specificity but poor sensitivity ranging from 53%- 100% (12). Other drawbacks include the difficulty in interpretation when multiple colours are present as well as short shelf life of the agar plate (12).

Carba NP, a colorimetric assay developed in 2012 uses the principle of enzymatic hydrolysis of the β-lactam ring of the carbapenem in the presence of enzymatic activity (13). This leads to a pH shift causing a colour change. The method is simple and easy to perform with rapid results. Studies assessing the performance of this test have been showing 100% specificity and 90%- 100% sensitivity (12). Some authors reported suboptimal results in detecting carbapenemases like OXA-48 and GES with low hydrolytic activity as they noted that mucoid isolates yielded false-negative results due to incomplete lysis (12).

Genotypic assay platforms such as PCR and DNA microarray for the detection of carbapenemase genes also have limitations as they can only detect known target genes (3,6,13,14) These platforms are expensive, require special expertise and equipment. In the South African context, these platforms are accessed at the National Institute of Communicable Diseases (NICD) and private laboratories. Hence, are not readily available for all public health laboratories.

The MALDI-TOF MS platforms are increasingly gaining momentum in genus and species identification of microbes but still lacks in carbapenemase detection (4). Currently two major applications are under evaluation, which includes rapid identification of carbapenemases by determining the degradation products and detection of carbapenemase carrying plasmid-associated protein peak (4). These platforms are very expensive and therefore not feasible in a routine diagnostic laboratory.

Lateral flow immunoassays are antibody-based methods used to identify the presence of carbapenemases. Recently, a new version called NG-Test Carba 5 targeting five carbapenemases (KPC, NDM, VIM, IMP and OXA-48 like carbapenemase) has been evaluated (4,15). Limitation of the assay lies in its inability to detect carbapenemases in non-fermenters.

In 2015, a carbapenem inactivation method (CIM), which allows for phenotypic detection of carbapenemase, was described (14). This test is based on the principle that when a carbapenem disk is incubated for two hours in a suspension comprising of a carbapenemase-producing organism; the carbapenem in the disk will be hydrolysed rendering the disk inactive.

The method is simple, easy to perform and inexpensive as most of the resources required for testing are conveniently available in the laboratories. When it was first introduced, three independent comparison studies were done comparing it to Carba NP test which yielded accurate detection of CP-CRE with acceptable sensitivity for the detection of OXA-48 type in Enterobacterales. However, other studies found it to have poor sensitivity in detecting OXA-48 type carbapenemases (15).

The CIM was further modified using tryptic soy broth (TSB) and extending the incubation time to four hours as opposed to two hours to improve the detection of OXA-48 type carbapenemases. The sensitivity of the mCIM was found to be 99% with a specificity of 100%. In 2017, CLSI recommended the modified inactivation method (mCIM) for use in clinical laboratories to detect carbapenemases of Enterobacterales and *P. aeruginosa*. (CLSI, 2018).

The simplified carbapenem inactivation method (sCIM) is simple and accurate, based on the mCIM, however eliminates the need to incubate the antibiotic disk in the TSB (9). Recent studies have found that the sensitivity and specificity of the sCIM and the mCIM are comparable. Nevertheless, the results showed that both had similar rates of detection for carbapenemase-producing Enterobacterales (CPE) and *Pseudomonas aeruginosa* but the sCIM performed better in the detection of carbapenemase in *Acinetobacter baumannii* (9). In our setting, *A. baumannii* is endemic and very challenging to treat and hence it will be of great benefit to have a phenotypic tool that detects carbapenemases.

This study aims to evaluate the performance of the sCIM in the detection of carbapenemase-producing Enterobacterales, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, compared to the MHT and Imi- EDTA using the multiplex PCR as a gold standard reference method.

1.3 STUDY OBJECTIVE

To verify the sensitivity, specificity, positive predictive value, negative predictive value, repeatability, reproducibility, accuracy and simplicity of the sCIM method in detecting

carbapenemase-producing Enterobacterales, *Pseudomonas aeruginosa* and *Acinetobacter baumannii.*

1.4 METHODS

i) Type of research

This is a prospective laboratory-based study that will test 150 well-characterized isolates comprising of 100 Enterobacterales, 25 *Pseudomonas aeruginosa* and 25 *Acinetobacter baumannii* that have been banked at Chris Hani Baragwanath Academic Hospital microbiology laboratory.

These isolates were identified using the analytical profile index (API) and Microscan systems. Epsilometer tests (E-tests) were used to determine the minimal inhibitory concentration (MIC) for all isolates. Carbapenem-resistant isolates were further subjected to the MHT and Imi-EDTA to look for the presence of carbapenemases. The Enterobacterales had been characterized using PCR at NICD except for the *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates which were sent prospectively.

All these isolates will be tested for carbapenemase production using the sCIM. The multiplex PCR results from the Antimicrobial Resistance Laboratory and Culture Collection, based at the National Institute of Communicable Diseases (NICD) in South Africa, Centre for Healthcare-Associated infections, Antimicrobial Resistance and Mycoses (AMRL-CC/CHARM) will be used as the reference gold standard. This molecular method confirms the presence of carbapenemases genes such as bla $_{KPC,}$ bla $_{OXA-48}$, bla $_{NDM}$, bla $_{VIM}$ and bla $_{IMP-1}$. Testing of each isolate will be performed in duplicate to assess repeatability. Reproducibility testing will be performed by four technologists using 15 isolates.

ii) Equipment and reagents

These are available at the NHLS microbiology laboratory at Chris Hani Baragwanath Academic Hospital.

iii) Methods to be compared

a) Modified Hodge test

The MHT applies the streaking of a suspected carbapenemase-producing isolate in a line away from a disk that has been impregnated with ertapenem or meropenem using a Mueller Hinton Agar (MHA) that has been lawned with a susceptible *Escherichia coli* strain (4). This

testing method relies on the carbapenemase producer to hydrolyze the carbapenem antibiotic, thus enabling the carbapenem susceptible *E. coli* isolate to grow around the streak line towards the carbapenem disk producing a cloverleaf image (4).

b) Imi-EDTA method

This method uses a disk with imipenem and EDTA placed on a Mueller Hinton agar (MHA) plate that has been lawned with the organism to be tested. The disk is compared with another imipenem disk that has no EDTA added. The test is positive if the zone around the Imi-EDTA disk is double, compared to the imipenem disk alone. This test is suitable for the detection of MBL producing Gram-negative bacilli.

c) sCIM

The sCIM was derived from the mCIM with innovation in the testing technique. In this version the antibiotic disk is not incubated in the organism culture media, however, the organism is smeared directly onto the antibiotic disk (12). The sCIM methodology tested on various bacteria differs slightly. The Enterobacterales uses 0.5 McFarland standard of the *E. coli* ATCC 25922 inoculated onto the MHA, whilst the *Pseudomonas aeruginosa* and *Acinetobacter baumannii* uses 0.5 McFarland standard, which is further diluted into 1: 10 in saline and thereafter inoculated onto the MHA plate. Plates are allowed to dry for 1-10 minutes. The organism to be tested is directly smeared on the imipenem disk, which is then directly placed onto the MHA (*E. coli* lawned) and incubated at 35ºC for 16-18 hours in ambient air. Bacteria that produce carbapenemase will have a zone less than ≤ 22 mm around the disk and those that do not will have a zone ≥ 26 mm around the disk. Those that have zones between 23-25 mm are indeterminate.

d) PCR detection of carbapenemase genes

A multiplex PCR will be used to detect genes responsible for carbapenemase production (14). The *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates will be tested prospectively whereas the Enterobacterales have already been molecularly characterised.

1.5 DATA ANALYSIS

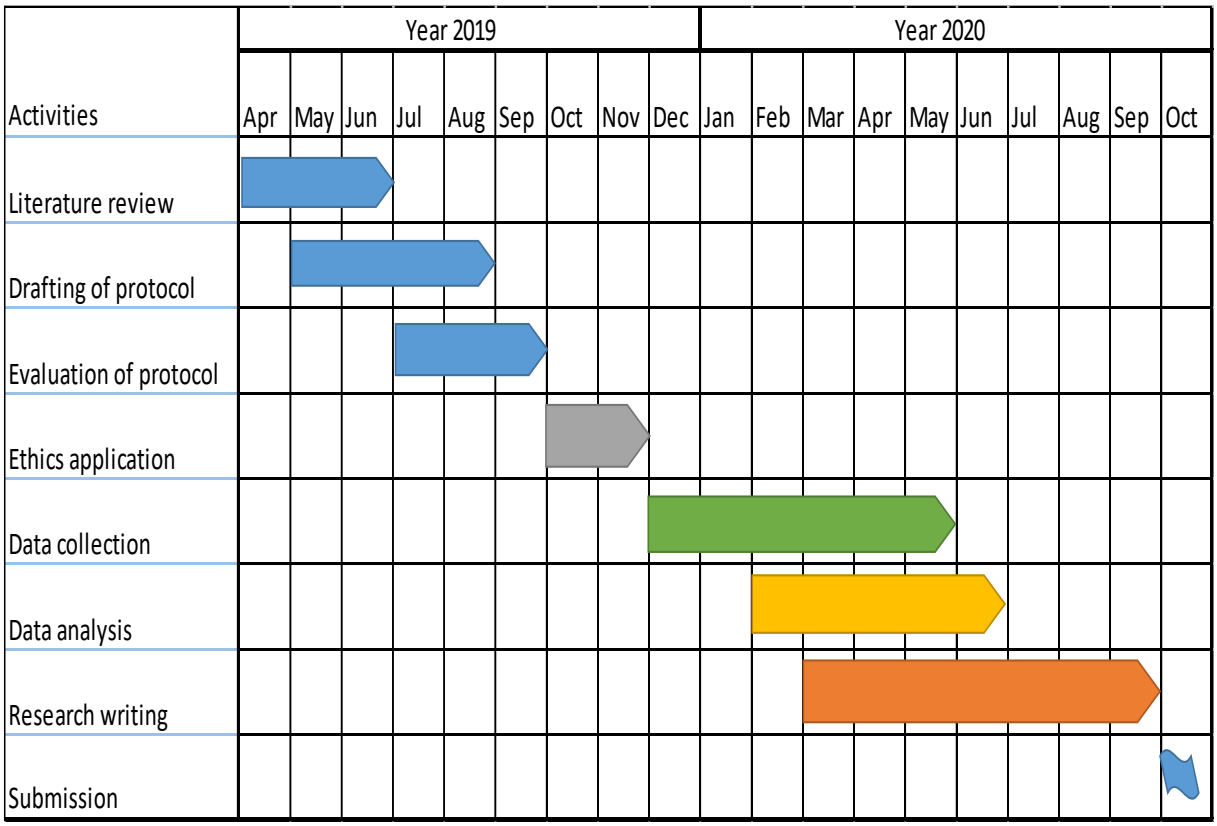
Once testing is complete, the results of each isolate tested will be captured on an excel data spreadsheet. The results will be compared with the phenotypic and genotypic findings. Data

analysis will be done using the excel spreadsheet. The analysis will specifically concentrate on the sensitivity, specificity, positive predictive value, negative predictive value, repeatability and reproducibility of the testing method. The ease of using the test will be compared with the MHT and Imi-EDTA tests that are currently being used.

1.6 ETHICS

The study will utilize well-characterized isolates that have been previously collected and banked at Chris Hani Baragwanath Academic Hospital microbiology department. No patient identifiers and clinical details shall be included. The study will be submitted to the Witwatersrand's Human Research Ethics Committee for research ethical considerations before commencement.

1.7 TIMELINE OF ACTIVITIES



1.8 FUNDING

The study will use resources of the National Health Laboratory Services, Chris Hani Baragwanath Academic Hospital site. The AMRL-CC/CHARM offered to support with banked and already characterized isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii.*

1.9 PROBLEMS

The study anticipates the following possible challenges:

- Contaminated isolates
- And non-viable isolates

The following solutions will be implemented:

- Contaminated isolates will be discarded
- Non-viable isolates will be revived in an enrichment broth and excluded from the study if isolates remain non-viable

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2. CHAPTER TWO – SUBMISSABLE ARTICLE

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Keywords:

sCIM; simplified carbapenem inactivation method; Enterobacterales*; Acinetobacter baumannii.*

2.1 INTRODUCTION AND BACKGROUND

The emergence and surge of carbapenemase-producing Enterobacterales (CPE) is a serious clinical and global health concern (5–9). Infections caused by carbapenem-resistant Enterobacterales (CREs) are increasing both locally and internationally (2). These infections are analogous to high rates of morbidity and mortality (2,5). What perturbs us is the development of resistance to a class of drugs called the carbapenems, which are appraised as compounds of last resort in the treatment of infections attributed to resistant Gram-negative pathogens (10,11). Resistance to carbapenems is for the most part, owing to the production of hydrolysing enzymes, the carbapenemases (11). Discerning these enzymes on time can have a remarkable role in patient care and antimicrobial stewardship strategies.

The aptness of a clinical microbiology laboratory to promptly detect CPEs by either a phenotypic or genotypic method is essential in developing strategies to curb their spread (11,12). A nimiety of phenotypic methods exist for the detection of carbapenemases; however, most methods have limitations (12). Consequently, clinical microbiology laboratories have struggled for years in unearthing a method that will be accurate, objective, affordable, user friendly, and feasible to implement in clinical microbiology laboratories of all sizes (13). Besides, the Clinical and Laboratory Standards Institute (CLSI) does not endorse molecular testing for clinical purposes (6). A rapid phenotypic method can aid the detection of CPEs timeously, thus help in guiding both therapeutic options for clinicians and antimicrobial stewardship strategies (6).

Currently, there is no single phenotypic test that meets all specifications of a definitive test to detect the presence of all carbapenemases (5,13). The choice of a carbapenemase detection test is subject to numerous factors, including cost, diagnostic performance traits, local carbapenemase prevalence, turnaround time (TAT), ease of use, workflow, regulatory status, and local molecular epidemiology (5). The Modified Hodge test (MHT) has been for years one of the phenotypic methods coupled with imipenem-Ethylene diamine tetra acetic acid test (Imi-EDTA) method used in most clinical laboratories for the detection of carbapenemases. This assay has proven to have an acceptable sensitivity to Class A carbapenemases, especially *Klebsiella pneumoniae* carbapenemase (KPC) enzymes, but low sensitivity to Metallo-β-lactamases (MBLs) (5). To complement this shortcoming, the addition of the Imi-EDTA method enhances sensitivity towards MBLs.

In 2015 van der Zwaluw et al. conceived a novel technique of detecting carbapenemases that is highly sensitive and relatively easy to operate (11,13). The method is the carbapenem inactivation method (CIM) and further modification lead to the modified carbapenem inactivation method (mCIM). The CLSI 2017 document recommend that the mCIM be based on the CIM method for use in clinical laboratories as it is capable and effective in detecting a variety of carbapenemases (11,13).

Considerable improvement of the mCIM resulted in the birth of sCIM method, ensuring user-friendliness. The sCIM technique involves smearing the organism directly onto the carbapenem disk, whilst in the mCIM the disk is immersed in tryptic soy broth (TSB) containing the test organism for 4 hours.

This study aimed to compare the performance of the novel sCIM with the MHT coupled with Imi-EDTA method. The multiplex polymerase chain reaction (PCR) was used as the gold standard in detecting the carbapenemase genes amongst the Enterobacterales, *Acinetobacter baumannii,* and *Pseudomonas aeruginosa* isolates (13). Our hypothesis expects the sCIM to be highly sensitive in detecting carbapenemases in carbapenemase-producing Gram-negative bacilli compared to the MHT and Imi-EDTA method combined.

2.2 MATERIALS AND METHODS

2.2.1 Ethical considerations

The Human Research Ethics Committee (Medical) of the University of the Witwatersrand in Johannesburg South Africa (HREC) granted permission to pursue the study, with clearance certificate no. M190840.

2.2.2 Study design and aim

We used a prospective laboratory-based study design in comparing the performance of the sCIM with the MHT coupled with Imi-EDTA method using the PCR as a reference standard. The study aimed to evaluate the performance of the sCIM in terms of sensitivity, specificity, negative and positive predictive value, repeatability, reproducibility, accuracy and ease of use.

2.2.3 Study setting

The study was conducted at Chis Hani Baragwanath Academic Hospital (CHBAH); National Health Laboratory Services (NHLS), in the Department of Clinical Microbiology and Infectious Diseases (CMID).

2.2.4 Sample size

A total of 137 isolates were collected for the study and comprising of 96 Enterobacterales, 23 *Acinetobacter baumannii,* and 18 *Pseudomonas aeruginosa*. The Enterobacterales isolates were composed of 59 strains of *Klebsiella pneumoniae*, 18 strains of *Enterobacter cloacae*, eight strains of *Escherichia coli*, three strains of *Enterobacter species*, three strains of *Citrobacter freundii*, three strains of *Providencia rettgeri*, two strains of *Klebsiella oxytoca*, and one strain of a *Klebsiella species.* All 96 Enterobacterales samples were tested with MHT and Imi-EDTA method. NICD had previously molecularly characterised these isolates as part of the laboratory surveillance and routine diagnostic program of CRE Enterobacterales.

The non-fermenting Gram-negative bacilli (GNB) comprised of 23 *Acinetobacter baumannii* and 18 *Pseudomonas aeruginosa* isolates from NICD.

2.2.5 Sampling technique

Isolates used incorporated all CRE's stored at CHBAH, NHLS laboratory from January 2014 to December 2019. These included isolates from various specimen types irrespective of ward, age group, health background and race. Specimens' acceptance criteria for Enterobacterales included the availability of results for MHT, Imi-EDTA, and PCR characterisation.

The Antimicrobial Resistance Laboratory and Culture Collection based in NICD, Centre for Healthcare-Associated infections, Antimicrobial Resistance, Mycoses (AMRL-CC/CHARM) made available the *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates for the research. These were isolates previously collected from CHBAH microbiology as part of surveillance.

2.2.6 Methods

The analytical profile index (API) method and the automated MicroScan® Walkaway system 96SI (Siemens, USA) had identified the organisms used.

Method 1: The simplified carbapenem inactivation method (sCIM)

The sCIM emanates from the mCIM with changes in the modus operandi (14). The organism to be tested is smeared directly on the Imipenem disk instead of incubating it in tryptic soy broth (TSB) culture media for 4 hours as in the mCIM (12) . To execute the sCIM, the procedure is slightly different for Enterobacterales compared with non-fermenters. For Enterobacterales, a lawn of *Escherichia coli* ATCC 25922 strain is made on Mueller Hinton Agar (MHA) plate using 0.5 McFarland standard suspensions (direct colony suspension method) (14). With non-fermenters, the 0.5 McFarland standard of the *E. coli* ATCC strain is further diluted to 1: 10 saline before it is inoculated on the MHA plate (14). Plates are allowed to dry for at least 3-10 minutes before the smeared disk is placed on the agar surface (14). Organisms to be tested should have been cultured overnight on blood agar plates.

After 18 hours of incubation all MHA plates are read. Isolates that produce carbapenemase hydrolyse imipenem and grow more towards the disk. Organisms that do not produce carbapenemase are susceptible to the imipenem and grow away from the disk. The zone of inhibition $\leq$ 22 mm is an indication that the isolate is capable of producing the carbapenemase enzyme and alternatively a zone of inhibition $\geq$ 26 mm is considered not capable of producing the enzyme (14). A positive and negative report is generated based on these findings. A zone of inhibition of 23-25 mm is reported as a carbapenemase indeterminate result (Figure 1) (14).

Figure 1: The simplified carbapenem inactivation method

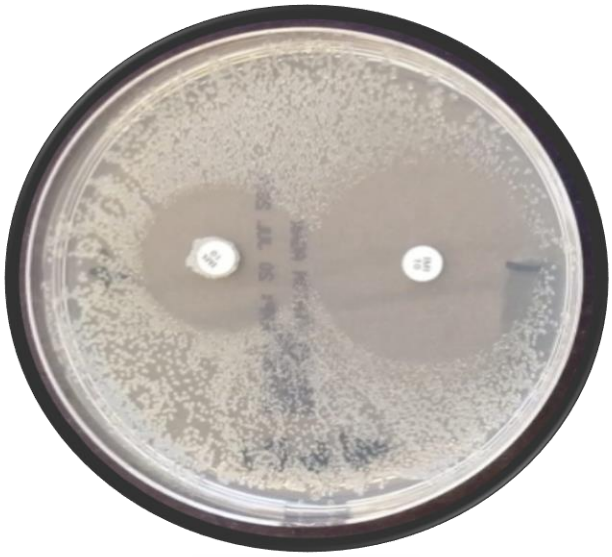


Figure 1: Results of sCIM testing of the Enterobacterales. The left demonstrates a positive isolate with reduced zone diameter ≤ 22mm compared to the negative control on the right with zone diameter ≥ 26mm. An imipenem disk was used (Courtesy of NHLS at CHBAH)

Method 2: The Modified Hodge Test

The MHT is based on the hydrolysis of a carbapenem by carbapenemase-producing strains (15). The procedure entails preparing a 0.5McFarland dilution of the *E.coli* ATCC 25922 (Indicator organism) in 5ml of broth or saline (15). Further dilution of 1: 10 by adding 0.5ml of the 0.5 McFarland to 4.5 ml of MHB or saline is performed (15). A lawn of the 1: 10 diluted *E.coli* ATCC 25922 is then streaked across the MHA and allowed to dry for 3-5 minutes thereafter, a 10µg disk of meropenem is placed in the centre of the MHA (15). The organism to be tested is then streaked in a straight line from the disk to the edge of the plate (15). A repeat with quality control strains is done the similar way. Once completed, the MHA is then incubated in ambient air at 35ºC for 18-24 hours. The expected positive result is seeing cloverleaf appearance post-incubation (Figure 2) (15).

Figure 2: The Modified Hodge Test

Figure 2: Results of MHT testing of Gram-negative bacilli showing cloverleaf image

P= Positive control streak, N= Negative control streak and T= Test organism (MHT positive)

A meropenem disk was used (Courtesy of NHLS at CHBAH)

Method 3: Imipenem – Ethylenediaminetetraacetic acid test

The MHT relies on Imi-EDTA to detect MBL enzymes. The Imi-EDTA method uses two imipenem disks placed side by side on a MHA plate that has been lawned with the organism to be tested (16). The two-imipenem disks 10µg each by (Davies Diagnostics) lie on the surface of the MHA at a distance of 4-5cm from each other. A 5µl of 750 µg/ml EDTA solution is then added to one of the imipenem disk (16). The inhibition zone found around the imipenem and Imi-EDTA disks are compared after 14-16 hours of incubation at 37ºC in ambient air (16). The difference of > 5mm between the inhibition zone diameter of the Imi-EDTA disk and that of imipenem only disk is considered to be a positive for the presence of MBL (Figure 3) (16). This test is suitable for the detection of MBLs producing GNB.

Figure 3: Imipenem – Ethylenediaminetetraacetic acid test

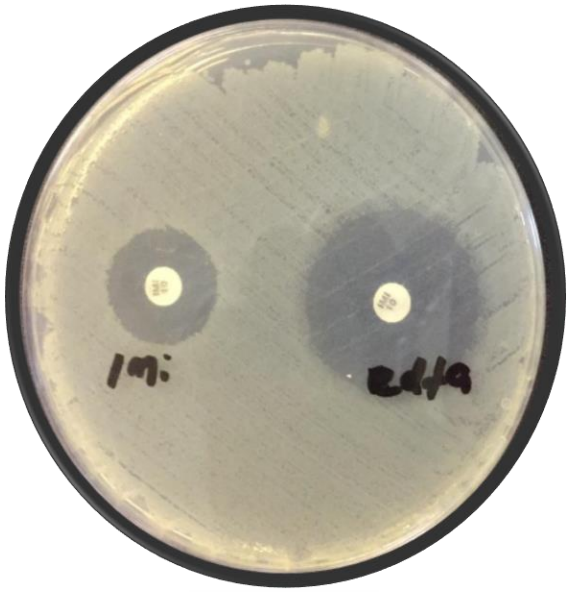


Figure 3: Results of Imi-EDTA test for MBL producing Gram- negative bacilli

Left disk with Imipenem only and Right disk with Imipenem +EDTA

(Courtesy of NHLS at CHBAH)

Method 4: Multiplex PCR for detection of carbapenemase genes

AMRL-CC/CHARM performed the molecular detection of the carbapenemase genes. Automated systems including the (VITEK® II (bioMèrieux France) and Microflex MALDI-ToF (Bruker Daltonik, GmbHA) identified isolates before characterisation. The automated MicroScan Walkaway system (Siemens, USA) was thereafter used for antimicrobial susceptibility testing (AST). Once confirmed as CRE, the multiplex PCR was then used to detect the specific type of carbapenemase/s gene each isolate carried.

The AMRL-CC/CHARM used multiplex PCR Light Cycler 480 II, Light Cycler 480 Probes, master kit and the individual Light Mix Modular kits supplied by Roche Diagnostics and applied science USA, to detect carbapenemase genes. The procedure involves DNA extraction

from the target isolate by the crude boiling method at 95ºC for 25 minutes for the cell to lyse (4).

2.2.7 Repeatability of the sCIM

To determine the repeatability of the sCIM, testing of all isolates were performed in duplicate.

2.2.8 Reproducibility of the sCIM

Four laboratory technologists performed the sCIM on 10 Enterobacterales, three *Acinetobacter baumannii*, and three *Pseudomonas aeruginosa* isolates for reproducibility. The scoring system involved the following:

1. Preparation of testing material
2. Performing the procedure
3. Reading of the results
4. Interpretation of results
5. Rating the sCIM when compared to the MHT and Imi-EDTA

2.2.9 Data management and data analysis

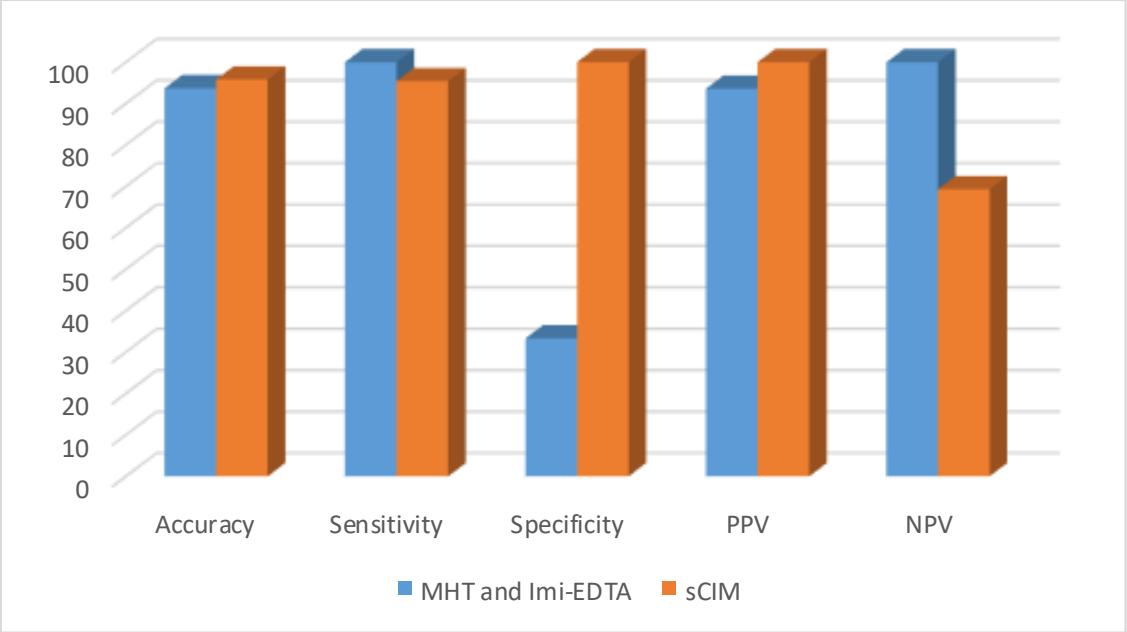
Microsoft Excel version 2016 for data entry and STATA 14 statistical software for analysis was used. Statistical significant was analysed using the OpenEpi tool to estimate sensitivity, specificity, PPV, NPV, and accuracy with 95% confidence interval.

2.3 RESULTS

2.3.1 Results of sCIM versus MHT and Imi-EDTA on Enterobacterales

The sCIM technique shows an advantage in its performance characteristics over the MHT and Imi-EDTA combined in sensitivity, specificity, accuracy and positive predictive value (Figure 4). Of note, the specificity was 100% and the sensitivity 95.8%. Apart from the low NPV of 69.8%, our results agree with our hypothesis, when testing Enterobacterales using the sCIM.

Figure 4: Performance characteristics of sCIM versus MHT and Imi-EDTA on Enterobacterales

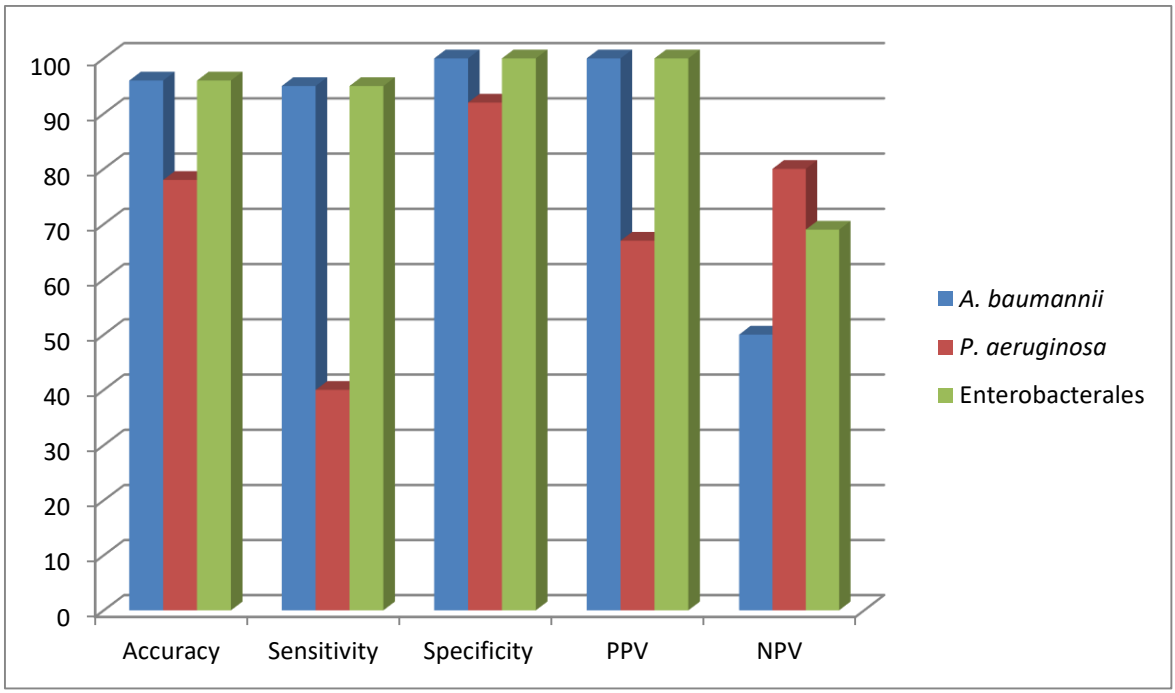


2.3.2 Performance characteristics of the sCIM on *A. baumannii, P. aeruginosa* and Enterobacterales

Collectively, the sCIM reported 95.8% accuracy when testing Enterobacterales, 95.6% for *Acinetobacter baumannii* and 77.8% for *Pseudomonas aeruginosa* (Figure 5). Remarkably, there was correlation of 95.4% sensitivity and 100% specificity in testing both Enterobacterales and *Acinetobacter baumannii* isolates (Figure 5). Contrary, both the Enterobacterales and *Acinetobacter baumannii* showed low NPV of 69.8% and 50% respectively, whilst the PPV was 100% on both (Figure 5). Further analysis revealed that

Pseudomonas aeruginosa isolates performed poorly when using the sCIM reporting 40% sensitivity, 92.3% specificity, 66.6% PPV and an 80% NPV (Figure 5).

Figure 5: Performance characteristics of the sCIM on *A. baumannii*, *P. aeruginosa* and Enterobacterales



2.3.3 Repeatability of the sCIM

The sCIM was performed in duplicates to ensure repeatability of the test under the same conditions tested by the same personnel in the same run. Only three isolates were initially found to be discordant, one Enterobacterales and two *Pseudomonas aeruginosa* isolates. The Enterobacterales was a *Klebsiella pneumoniae* mixed with an *Acinetobacter baumannii*, and after plating them out with re-testing, the results were identical. The two *Pseudomonas aeruginosa* isolates had insufficient colonies on culture plates, and upon repeat testing, both yielded the same results.

2.3.4 Reproducibility of the sCIM

There was a 100% concordance of the sCIM results performed by the four technologists with those previously tested by the author. The majority found it easy to very easy regarding the

preparation of materials and performing the test. Seventy-five percent of the technicians rated the sCIM above average compared to the MHT and Imi-EDTA and only 25% rated it easy. These results further strengthen our confidence in the user friendliness of the test.

2.4 DISCUSSION

Gram-negative bacteria that produce carbapenemases pose an undisputed threat to public health due to their resistance to antimicrobials. There is a need in both public and private microbiology laboratories for a robust and efficient carbapenemase detection method to identify and curb the spread of carbapenemase-producing organisms. Most sophisticated techniques like molecular assays and spectrometry have questionable use in a routine laboratory setting due to cost, availability, expertise and feasibility. A rapid phenotypic test that holds the advantage for use in a high throughput laboratory with minimal cost is urgently required.

The MHT has been the most widely used phenotypic test in public clinical microbiology laboratories for the screening of carbapenemase producers in Enterobacterales (5). It has the advantage of being a phenotypic test that is relatively cheap to perform and very sensitive to most carbapenemases like KPC and OXA-48 (5). Previous studies have demonstrated high sensitivity of MHT (100%) in detecting Enterobacterales that produce Class A (KPC) and Class D (OXA- 48) enzymes.

Unfortunately, the CLSI document M100-S22 has highlighted that not all carbapenemase-producing isolates of Enterobacterales are MHT positive as false negative results can be found in testing MBLs (17). Isolates with carbapenem-resistant mechanisms other than carbapenemase production can also be false positive. The Imi-EDTA usually compensates for this shortcoming for MHT; however, its specificity of (33%) is ill-starred by the sCIM with (100%) specificity. In this, study the presence of other resistant mechanisms such as extended-spectrum beta-lactamase (ESBL) and hyper production of AMP-C beta-lactamases and porin loss could be the cause of low specificity.

Furthermore, the MHT has low sensitivity in detecting NDMs enzymes. The low sensitivity can have severe repercussions in settings where NDMs are endemic. Interestingly, the multiplex PCR results confirmed the presence of NDMs in our setting, especially for *Acinetobacter baumannii* and Enterobacterales.

Our experimental set up of the sCIM bears a close resemblance to the MHT on the type of media used, indicator strain of *E. coli* and incubation needs. The sCIM however, has minimal

steps, is easy to perform and requires no special reagents or media. The sCIM is a more convenient alternative for high throughput laboratories (1,14).

This study has revealed that the sCIM has good performance characteristics, except for the low NPV. The low NPV could likely be due to the use of stored isolates leading to poor expression of the carbapenemase enzymes and/or loss of resistance genes carried on plasmids.

Some studies suggest degradation of plasmids are possible if exposed to freeze and thaw cycles (19). Hence, we postulate the possibility that prolonged storage, freeze and thaw cycles could have degraded the plasmids resulting in a loss of carbapenemase genes suggesting the need for prospective studies on fresh samples.

Another reason for the low predictive value could be low-level carbapenemase activity, whereby the multiplex PCR would pick up the gene, however, the gene would not be expressed phenotypically. The expression of carbapenemases depends on the resistant cassette found in the intergron (20,21). Expression of gene cassette in class 1 integrons is not uniform, and most class 1 integrons are found in GNB's (21). Subsequently, the sCIM detection threshold might not have been able to pick up poorly expressed genes. Extended incubation is suggested for the future probe to allow sufficient time for the genes to be expressed. Extended incubation had positive results with the mCIM (1).

Of note, our reference standard (PCR) could not detect one false-positive result of a *Pseudomonas aeruginosa* isolate. The isolate was positive on sCIM and remained positive on repeat testing. This could be attributed to the fact that PCR only detects targeted genes of interest and that the isolates could have had a gene outside of the target primers of the multiplex PCR. Whole-genome sequencing could have clarified this discordance; however, funding constraints did not permit this.

The cumitech 31 guide on verification and validation of procedures in the clinical microbiology laboratory requires that a screening test should satisfy both sensitivity and negative predictive values to be greater than 95% (22). The sCIM has met the standards on sensitivity for both Enterobacterales and *Acinetobacter baumannii* of 95.4%. The NPV was 69.8% and 50% for Enterobacterales and *Acinetobacter baumannii* respectively. Hence, we recommend prospective studies on fresh isolates for future studies. The performance of the sCIM when testing *Pseudomonas aeruginosa* isolates did not meet the cumitech 31 requirements.

2.5 STUDY LIMITATIONS

Potential source of limitations of the study include: use of stored samples, plasmid degradation and improper storage. There is evidence to suggest that long-term storage on ambient air can affect the stability of the plasmids carrying the carbapenemase genes, especially if not stored at temperatures below -20ºC (18). These could have been the reason for the low negative predictive value of 69.8% for Enterobacterales, 50% for *Acinetobacter baumannii,* and 80% for *Pseudomonas aeruginosa.*

2.6 CONCLUSION

This study provides the first comprehensive evaluation of the sCIM in South Africa. Notwithstanding the lack of agreement with the NPV, we believe our findings compare well with previous studies. There is little information about the performance of the test, with regards to the negative and positive predictive values on the literature review. The sCIM can be an innovative solution for high throughput laboratories, especially for the detection of carbapenemases amongst Enterobacterales and *Acinetobacter baumannii* in fresh samples. Future studies are still warranted to elucidate on low predictive values of the assay.

2.7 DISCLOSURE STATEMENT

The authors declared no potential conflict of interest surrounding the subject.

2.8 AUTHOR CONTRIBUTIONS

Dr Bheki Zethy Masango contributed by collecting isolates, performing the test, data collection, data analysis, and writing-original draft preparation. Dr's Jeannette Wadula and Prenika Jaglal made substantial contributions to conception, study design, methodology, and supervision of the study.

2.9 FUNDING

All laboratory resources used in the study belonged to the National Health Laboratory Services, Department of Clinical Microbiology and Infectious Diseases (CMID), University of the Witwatersrand at CHBAH and supported by AMRL-CC/CHARMS, Johannesburg, South Africa.

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3. CHAPTER THREE - APPENDICES

Appendix A: Ethical clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Dr Bheki Masango

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M190840 MED19-08-060

NAME: (Principal Investigator)	Dr Bheki Masango
DEPARTMENT:	Clinical Microbiology and Infectious Diseases Chris Hani Baragwanath Academic Hospital National Health Laboratory Services
PROJECT TITLE:	Evaluation of the simplified carbapenem inactivation method (sCIM) for simple and accurate detection of cabapenemase producing gram negative bacilli
DATE CONSIDERED:	30/08/2019
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Jeannette Wadula and Dr Prebinika Jaglal
APPROVED BY:	Dr C Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	08/11/2019

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed August and will therefore be due in the month of August each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

12/11/2019.
Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B Figures and tables

Figure 6: Distribution of carbapenemase genes in Enterobacterales detected by multiplex PCR

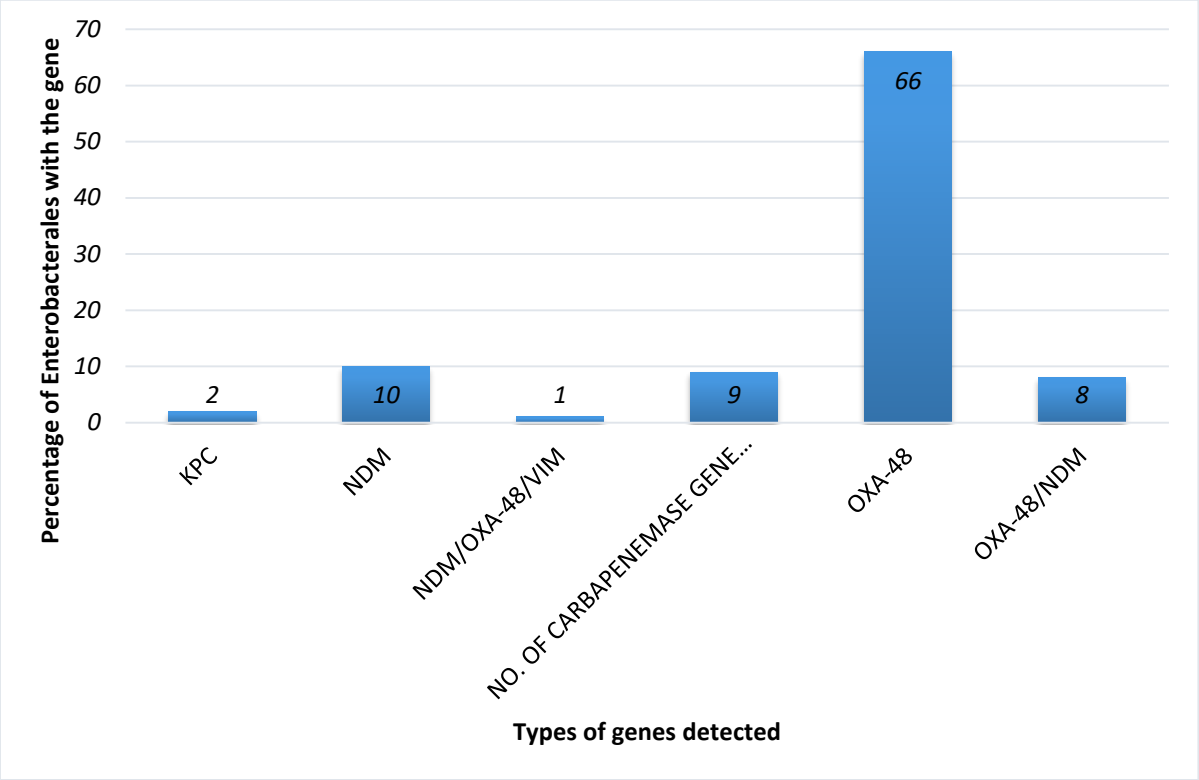

Figure 7: Distribution of carbapenemase genes in *Acinetobacter baumannii* detected by multiplex PCR

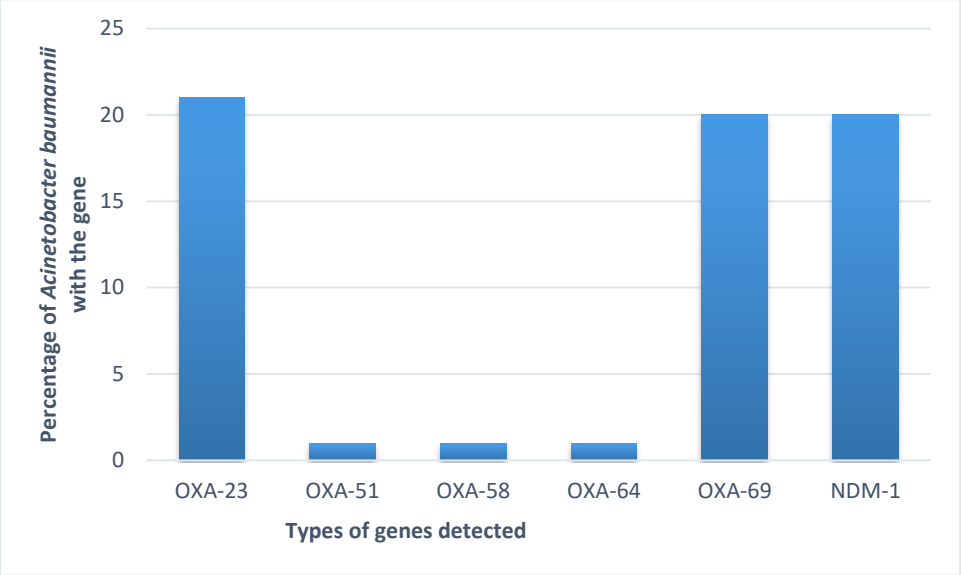

Figure 8: Distribution of carbapenemase genes in *Pseudomonas aeruginosa* detected by multiplex PCR

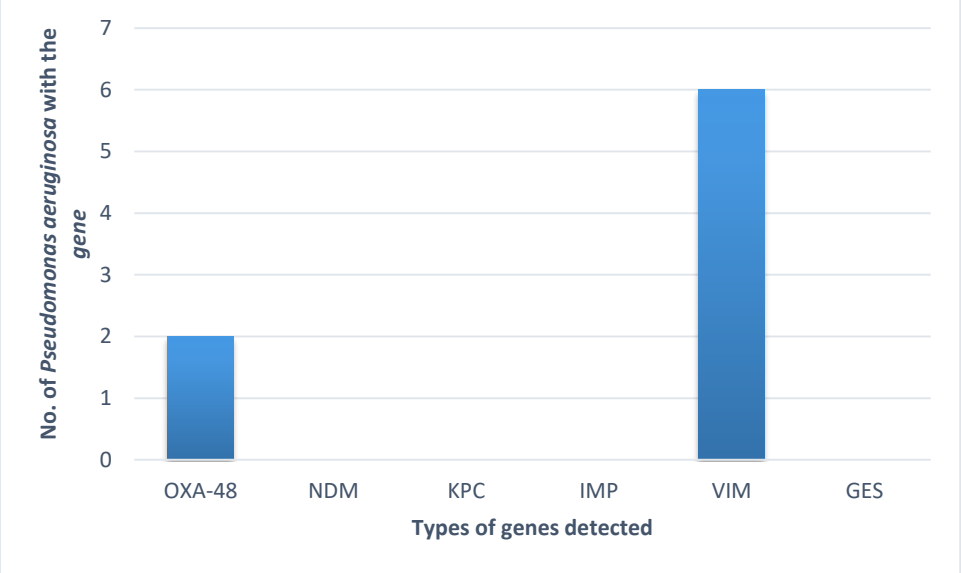

Table 1: Comparing performance characteristics of Modified Hodge and IMI- EDTA tests versus PCR for Enterobacterales

	Reference Test or Gold standard (PCR) n = 96			
Diagnostic		Positive	Negative	Total
Test (combined test)	Positive	87 (TP)	6 (FP)	93
	Negative	0 (FN)	3 (TN)	3
	Total	87	9	96

TP= True positive

FP= False positive

FN=False negative

TN=True negative

Sensitivity= TP/TP+FN= 87/87= 100.0%, 95% CI (95.7% -100.0%)

Specificity= TN/TN+FP =3/9 = 33.33%, 95% CI (20.2% -64.6.0%)

Positive predictive value =TP/TP+FP= 87/93=93.6% 95% CI (86.6% -97.0%)

Negative predictive value = TN/TN+FN=3/3= 100.0%, 95% CI (48.3% - 100.0%)

Accuracy= TP+TN/Total population =87+3/96 =93.7%, 95% CI (87.0% -97.1%)

Table 2: Comparing performance characteristics of sCIM versus PCR for Enterobacterales

	Reference Test or Gold standard (PCR) n=96			
Diagnostic		Positive	Negative	Total
Test (SCIM)	Positive	85 (TP)	0 (FP)	85
	Negative	4 (FN)	7 (TN)	11
	Total	89	7	96

Sensitivity= TP/TP+FN= 85/89= 95.4%, 95% CI (88.8% -98.2%)

Specificity= TN/TN+FP =7/7 = 100.0%, 95% CI (80.2% -100.0%)

Positive predictive value =TP/TP+FP= 85/85=100% 95% CI (95.6% -100.0%)

Negative predictive value = TN/TN+FN=7/11= 69.3%, 95% CI (48.3% - 87.3%)

Accuracy= TP+TN/Total population =85+7/96 =95.8%, 95% CI (89.8% -98.4%)

Table 3: Comparing performance characteristics of sCIM versus PCR for *Acinetobacter baumannii*

	Reference Test or Gold standard (PCR) n = 23			
Diagnostic		Positive	Negative	Total
Test (combined test)	Positive	21 (TP)	0 (FP)	21
	Negative	1 (FN)	1 (TN)	2
	Total	22	1	23

Sensitivity =TP/TP+FN = 21/22= 95.45%, 95% CI (88.2%-99.1%)

Specificity = TN/TN+FP = 1/1= 100%, 95% CI (64.5%-100.0%)

Positive predictive value = TP/TP+FP = 21/21= 100%, 95% CI (84.5%-100.0%)

Negative predictive value = TN/TN+FN = 1/2= 50%, 95% CI (33.7%-68.9%)

Accuracy =TP+TN/Total population = 22/23, 95.6%, 95% CI (79.0%-99.2%)

Table 4: Comparing performance characteristics of sCIM versus PCR for *Pseudomonas aeruginosa*

	Reference Test or Gold standard (PCR) n = 18			
Diagnostic		Positive	Negative	Total
Test (combined test)	Positive	2 (TP)	1 (FP)	3
	Negative	3 (FN)	12 (TN)	15
	Total	5	13	18

Sensitivity =TP/TP+FN = 2/5 = 40%, 95% CI (28.4%-50.6%)

Specificity = TN/TN+FN = 12/13 = 92.3%, 95% CI (85.6% -98.6)

Positive predictive value = TP/TP+FP = 2/3 =66.6%, 95% CI (50.5%- 81.3%)

Negative predictive value = TN/TN+FN =12/15 = 80%, 95% CI (77.5%-92.5%)

Accuracy =TP+TN/Total population = 14/18 =77.8%, 95% CI (70.5%-91.0%)

Appendix C Turnitin originality report

Turnitin Originality Report

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1. CHAPTER TWO – SUBMISSABLE ARTICLE Authors: Bheki Zethy Masango1 Jeannette Wadula2 Prenika Jaglal2 Affiliations: 1University of the Witwatersrand, Department of Clinical Microbiology and Infectious Diseases, National Health Laboratory Services, South Africa 2University of the Witwatersrand, Department of Clinical Microbiology and Infectious Diseases, Chris Hani Baragwanath Academic Hospital, National Health Laboratory Services, South Africa 2University of the Witwatersrand, Department of Clinical Microbiology and Infectious Diseases, Chris Hani Baragwanath Academic Hospital, National Health Laboratory Services, South Africa Keywords: sCIM; simplified carbapenem inactivation method; Enterobacterales; Acinetobacter baumannii. 2.1 INTRODUCTION AND BACKGROUND The emergence and surge of carbapenemase-producing Enterobacterales (CPE) is a serious clinical and global health concern (5–9). Infections caused by carbapenem-resistant Enterobacterales (CREs) are increasing both locally and internationally (2). These infections are analogous to high rates of morbidity and mortality (2,5). What perturbs us is the development of resistance to a class of drugs called the carbapenems, which are appraised as compounds of last resort in the treatment of infections attributed to resistant Gram-negative pathogens (10,11). Resistance to carbapenems is for the most part, owing to the production of hydrolysing enzymes, the carbapenemases (11). Discerning these enzymes on time can have a remarkable role in patient care and antimicrobial stewardship strategies. The aptness of a clinical microbiology laboratory to promptly detect CPEs by either a phenotypic or genotypic method is essential in developing strategies to curb their spread (11,12). A nimiety of phenotypic methods exist for the detection of carbapenemases; however, most methods have limitations (12). Consequently, clinical microbiology laboratories have struggled for years in unearthing a method that will be accurate, objective, affordable, user friendly, and feasible to implement in clinical microbiology laboratories of all sizes (13). Besides, the Clinical and Laboratory Standards Institute (CLSI) does not endorse molecular testing for clinical purposes. A rapid phenotypic method can aid the detection of CPEs timeously, thus help in guiding both therapeutic options for clinicians and antimicrobial stewardship strategies (6). Currently, there is no single phenotypic test that meets all specifications of a definitive test to detect the presence of all carbapenemases (5,13). The choice of a carbapenemase detection test is subject to numerous factors, including cost, diagnostic performance traits, local carbapenemase prevalence, turnaround time (TAT), ease of use, workflow, regulatory status, and local molecular epidemiology (5). The Modified Hodge test (MHT) has been for years one of the phenotypic methods coupled with Imi-EDTA method used in most clinical laboratories for the detection of carbapenemases. This assay has proven to have an acceptable sensitivity to Class A carbapenemases, especially KPC enzymes, but low sensitivity to MBLs (5). To complement this shortcoming, the addition of the Imi-EDTA method enhances sensitivity towards MBLs. In 2015 van der Zwaluw et al. conceived a novel technique of detecting carbapenemases that is highly sensitive and relatively easy to operate (11,13). The method is the carbapenem inactivation method (CIM) and further modification lead to the modified carbapenem inactivation method (mCIM). The CLSI 2017 document recommend that the mCIM be based on the CIM method for use in clinical laboratories as it is capable and effective in detecting a variety of carbapenemases (11,13). Considerable improvement of the mCIM resulted in the birth of sCIM method, ensuring user- friendliness. The sCIM technique involves smearing the organism directly onto the carbapenem disk, whilst in the mCIM the disk is immersed in tryptic soy broth (TSB) containing the test organism for 4 hours. This study aimed to compare the performance of the novel sCIM with the MHT coupled with Imi-EDTA method. The multiplex polymerase chain reaction (PCR) was used as the gold standard in detecting the carbapenemase genes amongst the Enterobacterales, Acinetobacter baumannii, and Pseudomonas aeruginosa isolates (13). Our hypothesis expects the sCIM to be highly sensitive in detecting carbapenemases in carbapenemase-producing Gram-negative bacilli compared to the MHT and Imi-EDTA method combined. 2.2 MATERIALS AND METHODS 2.2.1 Ethical considerations The Human Research Ethics Committee (Medical) of the University of the Witwatersrand in Johannesburg South Africa (HREC) granted permission to pursue the study, with clearance certificate no. M190840. 2.2.2 Study design and aim We used a prospective laboratory-based study design in comparing the performance of the sCIM with the MHT coupled with Imi-EDTA method using the PCR as a reference standard. The study aimed to evaluate the performance of the sCIM in terms of sensitivity, specificity, negative and positive predictive value, repeatability, reproducibility, accuracy, and ease of use 2.2.3 Study setting The study was conducted at Chis Hani Baragwanath Academic Hospital (CHBAH); National Health Laboratory Services (NHLS), in the Department of Clinical Microbiology and Infectious Diseases (CMID). 2.2.4 Sample size A total of 137 isolates were collected for the study and comprising of 96 Enterobacterales, 23 Acinetobacter baumannii, and 18 Pseudomonas aeruginosa. The Enterobacterales isolates were composed of 59 strains of Klebsiella pneumoniae, 18 strains of Enterobacter cloacae, eight strains of Escherichia coli, three strains of Enterobacter species, three strains of Citrobacter freundii, three strains of Providencia rettgeri, two strains of Klebsiella oxytoca, and one strain of a Klebsiella species. All 96 Enterobacterales samples were tested with MHT and Imi-EDTA method. NICD had previously molecularly characterised these isolates as part of the laboratory surveillance and routine diagnostic program of CRE Enterobacterales. The non-fermenting Gram-negative bacilli (GNB) comprised of 23 Acinetobacter baumannii and 18 Pseudomonas aeruginosa isolates from NICD. 2.2.5 Sampling technique Isolates used incorporated all CRE's stored at Chris Hani Baragwanath Hospital, NHLS laboratory from January 2014 to December 2019. These included isolates from various specimen types irrespective of ward, age group, health background and race. Specimens' acceptance criteria for Enterobacterales included the availability of results for MHT, Imi- EDTA, and PCR characterisation. The Antimicrobial Resistance Laboratory and Culture Collection based in NICD, Centre for Healthcare-Associated Infections, Antimicrobial Resistance, Mycoses (AMRL-CC /CHAIRM) made available the Acinetobacter baumannii and Pseudomonas aeruginosa isolates for the research. These were isolates previously collected from CHBAH microbiology as part of surveillance. 2.2.6 Methods The analytic profile index (API) method and the automated MicroScan® Walkaway system 96SI (Siemens, USA) had identified most of the organisms used. Method 1: The simplified carbapenem inactivation method (sCIM) The sCIM emanates from the mCIM with changes in the modus operandi (14). The organism to be tested is smeared directly

is an indication that the isolate is capable of producing the carbapenemase enzyme and alternatively a zone of inhibition ≥ 26 mm is considered not capable of producing the enzyme (14). A positive and negative report is generated based on these findings. A zone of inhibition of 23-25 mm is reported as a carbapenemase indeterminate result (Figure 1) (14). Figure 1: The simplified carbapenem inactivation method (sCIM) Figure 1: Results of sCIM testing of the Enterobacterales. The left demonstrates a positive isolate with reduced zone diameter ≤ 22mm compared to the negative control on the right with zone diameter ≥ 26mm. An imipenem disk was used (Courtesy of NHLS at CHBAH) Method 2: The Modified Hodge Test (MHT) The MHT is based on the hydrolysis of a carbapenem by carbapenemase-producing strains (15). The procedure entails preparing a 0. 5McFarland dilution of the E.coli ATCC 25922 (Indicator organism) in 5ml of broth or saline (15). Further dilution of 1: 10 by adding 0. 5ml of the 0.5 McFarland to 4.5 ml of MHB or saline is performed (15). A lawn of the 1: 10 diluted E.coli ATCC 25922 is then streaked across the MHA and allowed to dry for 3-5 minutes thereafter, a 10ug disk of meropenem is placed in the centre of the MHA (15). The organism to be tested is then streaked in a straight line from the disk to the edge of the plate (15). A repeat with quality control strains is done the similar way. Once completed, the MHA is then incubated in ambient air at 35°C for 18-24 hours. The expected positive result is seeing cloverleaf appearance post-incubation (Figure 2) (15). Figure 2: The Modified Hodge Test (MHT) Figure 2: Results of MHT testing of Gram-negative bacilli showing cloverleaf image Method 3: Imipenem – Ethylene diamine Tetra Acetic acid test (Imi-EDTA) The MHT relies on Imi-EDTA to detect MBLs enzymes. The Imi-EDTA method uses two imipenem disks placed side by side on a MHA plate that has been lawned with the organism to be tested (16). The two-imipenem disks 10ug each by (Davies Diagnostics) lie on the surface of the MHA at a distance of 4- 5cm from each other. A 5µl of 750 µg/ml EDTA solution is then added to one of the imipenem disk (16). The inhibition zone found around the imipenem and Imi-EDTA disks are compared after 14-16 hours of incubation at 37°C in ambient air (16). The difference of > 5mm between the inhibition zone diameter of the Imi -EDTA disk and that of imipenem only disk is considered to be a positive for the presence of MBLs (Figure 3) (16). This test is suitable for the detection of MBLs producing GNB. Figure 3: Imipenem – Ethylene diamine Tetra Acetic acid test (Imi-EDTA) Figure 3: Results of Imi-EDTA test for MBL producing Gram- negative bacilli Left disk with Imipenem only and Right disk with Imipenem +EDTA (Courtesy of NHLS at CHBAH) Method 4: Detection of carbapenemase genes (multiplex PCR) The Antimicrobial Resistance Laboratory and Culture Collection based in the National Institute of Communicable Diseases in South Africa, Centre for Healthcare-Associated Infections, Antimicrobial Resistance and Mycoses (AMRL-CC/CHARM) performed the molecular detection of the carbapenemase genes. Automated systems including the (VITEK® II (bioMérieux France) and Microflex MALDI-ToF (Bruker Daltonik, GmbHA) identified isolates before characterisation. The automated MicroScan Walkaway system (Siemens, USA) was thereafter used for antimicrobial susceptibility testing (AST). Once confirmed as a CRE, the multiplex PCR was then used to detect the specific type of carbapenemase/s gene each isolate carried. The AMRL-CC/CHARM used multiplex PCR (Light Cycler 480 II) supplied by Roche Applied Science together with Light Cycler 480 Probes Master kit and the individual Light Mix Modular kits by Roche Diagnostics, in, the USA to detect carbapenemase genes. The procedure involves DNA extraction from the target isolate by the crude boiling method at 95°C for 25 minutes for the cell to lyse (4). 2.2.7 Repeatability of the sCIM To determine the repeatability of the sCIM, testing of all isolates were performed in duplicate. 2.2.8 Reproducibility of the sCIM Four laboratory technologists performed the sCIM on 10 Enterobacterales, 3 Acinetobacter baumannii, and 3 Pseudomonas aeruginosa isolates for reproducibility. The scoring system involved the following: 1. Preparation of testing material 2. Performing the procedure 3. Reading of the results 4. Interpretation of results 5. Rating the sCIM when compared to the MHT and Imi-EDTA 2.2.9 Data management and data analysis We used Microsoft Excel version 2016 for data entry and STATA 14 statistical software for analysis. Statistical significant was analysed using the OpenEpi tool to estimate sensitivity, specificity, PPV, NPV, and accuracy with 95% confidence interval. 2.3 RESULTS 2.3.1 Results of sCIM versus MHT and Imi-EDTA on Enterobacterales The sCIM technique shows an advantage over the MHT and Imi-EDTA combined in its sensitivity, specificity, accuracy and positive predictive value (Figure 4). Of note, the specificity was 100% and the sensitivity 95.8%. Apart from the low NPV of 69.3%, our results agree with our hypothesis, when testing Enterobacterales using the sCIM. Figure 4: Performance of sCIM versus MHT and Imi-EDTA on Enterobacterales Perfomance of MHT and Imi-EDTA versus sCIM for Enterobacterales 100 80 60 40 20 0 Accur acy Sensitivity Specificity PPV NPV MHT and Imi-EDTA sCIM 2.3.2 Performance of the sCIM on A. baumannii, P. aeruginosa and Enterobacterales Collectively, the sCIM reported 95.8% accuracy when testing Enterobacterales, 95.6% for Acinetobacter baumannii and 77.8% for Pseudomonas aeruginosa (Figure 5). Remarkably, there was correlation of 95.4% sensitivity and 100% specificity in testing both Enterobacterales and Acinetobacter baumannii isolates (Figure5). Contrary, both the Enterobacterales and Acinetobacter baumannii showed low NPV of 69.8% and 50% respectively, whilst the PPV was 100% on both (Figure 5). Further analysis revealed that Pseudomonas aeruginosa isolates performed poorly when using the sCIM reporting 40% sensitivity, 92.3% specificity, 66.6% PPV and an 80% NPV (Figure 5). Figure 5: Performance of the sCIM on A. baumannii, P. aeruginosa and Enterobacterales 100 90 80 70 60 A. baumannii 50 P. aeruginosa 40 Enterobacterales 30 20 10 0 Accuracy Sensitivity Specificity PPV NPV 2.3.3 Repeatability of the sCIM The sCIM was performed in duplicates to ensure repeatability of the test under the same conditions tested by the same personnel in the same run. Only three isolates were initially found to be discordant, one Enterobacterales and two Pseudomonas aeruginosa isolates. The Enterobacterales was a Klebsiella pneumoniae mixed with an Acinetobacter baumannii, and after plating them out with re-testing, the results were identical. The two Pseudomonas aeruginosa isolates had insufficient colonies on culture plates, and upon repeat testing, both yielded the same results. 2.3.4 Reproducibility of the sCIM There was a 100% concordance of the sCIM results performed by the four technologists with those previously tested by the author. The majority found it easy to very easy regarding the preparation of materials and performing the test. Seventy-five percent of the technicians rated the sCIM above average compared to the MHT and Imi-EDTA and only 25 per cent rated it easy. These results further strengthen our confidence in the user friendliness of the test. 2.4 DISCUSSION Gram-negative bacteria that produce carbapenemases pose an undisputed threat to public health due to their resistance to antimicrobials. There is a need in both public and private microbiology laboratories for a robust and efficient carbapenemase detecting method to identify and curb the spread of carbapenemase-producing organisms. Most sophisticated techniques like molecular assays and spectrometry have questionable use in a routine laboratory setting due to cost, availability, expertise, and feasibility. A rapid phenotypic test that holds the advantage for use in a high throughput laboratory with minimal cost is urgently required. The MHT has been the most widely used phenotypic test in public clinical microbiology laboratories for the screening of carbapenemase producers in Enterobacterales (5). It has the advantage of being a phenotypic test that is relatively cheap to perform and very sensitive to most carbapenemases like KPC and OXA-48 (5). Previous studies have demonstrated high sensitivity of MHT (100%) in detecting Enterobacterales that produce Class A (KPC) and Class D (OXA- 48) enzymes. Unfortunately, the CLSI document M100-S22 has highlighted that not all carbapenemase- producing isolates of Enterobacterales are MHT positive as false negative results can be found in testing MBLs (17). Isolates with carbapenem-resistant mechanisms other than carbapenemase production can also be false positive. The Imi-EDTA usually compensates for this shortcoming for MHT; however, its specificity of (33%) is ill-starred by the sCIM with (100%) specificity. In this, study the presence of other resistant mechanisms such as extended-spectrum beta-lactamase (ESBL) and hyper production of AMP-C beta-lactamases and porin loss could be the cause of low specificity. Furthermore, the MHT has low sensitivity in detecting NDMs enzymes. The low sensitivity can have severe repercussions in settings where NDMs are endemic. Interestingly, the multiplex PCR results confirmed the presence of NDMs in our setting, especially for Acinetobacter baumannii and Enterobacterales. Our experimental set up of the sCIM bears a close resemblance to the MHT on the type of media used, indicator strain of E.

the gene, however, the gene would not be expressed phenotypically. The expression of carbapenemases depends on the resistant cassette found in the intergron (20,21). Expression of gene cassette in class 1 integrons is not uniform, and most class 1 integrons are found in GNB's (21). Subsequently, the sCIM detection threshold might not have been able to pick up poorly expressed genes. Extended incubation is suggested for the future probe to allow sufficient time for the genes to be expressed. Extended incubation had positive results with the mCIM (1). Of note, our reference standard (PCR) could not detect one false-positive result of a Pseudomonas aeruginosa isolate. The isolate was positive on sCIM and remained positive on repeat testing. This could be attributed to the fact that PCR only detects targeted genes of interest and that the isolates could have had a gene outside of the target primers of the multiplex PCR. Whole-genome sequencing could have clarified this discordance; however, funding constraints did not permit this. The cumitech 31 guide on verification and validation of procedures in the clinical microbiology laboratory requires that a screening test should satisfy both sensitivity and negative predictive values, be greater than 95% (22). The sCIM has met the standards on sensitivity for both Enterobacterales and Acinetobacter baumannii with the same margin of 95.4%. The NPV was 69.8% and 50% for Enterobacterales and Acinetobacter baumannii respectively. Hence, further prospective studies on fresh isolates for future studies. The performance of the sCIM when testing Pseudomonas aeruginosa isolates did not meet the cumitech 31 requirements. 2.5 STUDY LIMITATIONS Potential source of limitations of the study include: use of stored samples and improper storage. There is evidence to suggest that long-term storage on ambient air can affect the stability of the plasmids carrying the carbapenemase genes. Plasmids are prone to degradation, especially if not stored at temperatures below -20°C (18). These could have resulted in to the low negative predictive value of 69.3% for Enterobacterales, 50% for Acinetobacter baumannii, and 80% for Pseudomonas aeruginosa. 2.6 CONCLUSION This study provides the first comprehensive evaluation of the sCIM in South Africa. Notwithstanding the lack of agreement with the NPV, we believe our findings compare well with previous studies. High throughput microbiology laboratories can benefit from this phenotypic test. There is little information about the performance of the test, with regards to the negative and positive predictive values on the literature review. The sCIM can be an innovative solution for high throughput laboratories, especially for the detection of carbapenemases amongst Enterobacterales and Acinetobacter baumannii in fresh samples. Future studies are still warranted to elucidate on low predictive values of the assay. 2.7 DISCLOSURE STATEMENT The authors declared no potential conflict of interest surrounding the subject. 2.8 AUTHOR CONTRIBUTIONS Dr Bheki Zethy Masango contributed by collecting isolates, performing the test, data collection, data analysis, and writing-original draft preparation. Dr's Jeannette Wadula and Prenika Jaglal made substantial contributions to conception, study design, methodology, and supervision of the study. 2.9 FUNDING The test media and laboratory resources used belonged to the National Health Laboratory Services, at CHBAH. The study was also supported by NICD, AMRL-CC department, and the Department of Clinical Microbiology and Infectious Diseases (CMID), University of the Witwatersrand, Johannesburg, South Africa.