USE OF MATRIX ASSISTED LASER DESORPTION IONIZATION TIME OF FLIGHT MASS SPECTROMETRY (MALDI-TOF MS) TO DETECT CARBAPENEM-RESISTANT ENTEROBACTERIACEAE

2014

ANOU DIT MOISE SOMBORO
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ANOU DIT MOISE SOMBORO

2014

A thesis submitted to the School of Health Sciences, College of Health Science, University of KwaZulu-Natal, Westville, for the degree of Master of Medical Science.

This is the thesis in which the chapters are written as a set of discrete research publications that have followed the Journal of Microbiological Methods format with an overall introduction and final summary. Typically these chapters will have been published in internationally recognized, peer-reviewed journals.

This is to certify that the contents of this thesis is the original research work of Mr Anou Dit Moise Somboro, carried out under our supervision at the Catalysis and Peptide Research Unit, Westville campus, University of KwaZulu-Natal, Durban, South Africa and Biomedical Resource Unit, Westville campus, University of KwaZulu Natal, Durban, South Africa.

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ABSTRACT

Antimicrobial drug resistance is increasing worldwide at a frightening rate and is found among different species of bacteria causing nosocomial and community-acquired infections. Carbapenem-resistant Enterobacteriaceae (CRE’s) are one of the multi-drug resistant bacteria increasingly reported in the past decade, leaving the health care system unable to control the spread of antibiotic-resistant infections. A rapid and efficient method to identify drug resistant bacteria could advance the containment of this scourge.

We thus investigated the use of MALDI-TOF MS technology, recognized as an accurate method for microorganism identification, as a potential tool for the detection of CRE based on the protein profiling for specific fingerprints.

From 24 hours sub-cultured bacterial isolates known to carry and/or express single genes of a variety of carbapenemases, proteins were extracted using the formic acid ethanol extraction method and subjected to MS-based identification by MALDI-TOF MS Biotyper technology. The mass spectrometry spectra were then analyzed employing the programmed software flexAnalysis version 3.4 build 70 and ClinProTools version 2.2 build 83 to detect carbapenemases. The spectra were subjected to visual inspection for specific peaks characterization.

Despite their close relationship, the method was able to differentiate between CRE and susceptible bacteria. The differences within carbapenemases were better distinguishable by visual observation of specific biomarker peaks and the modified main spectrum (MSP) method demonstrated that these differences could be more obvious by focusing on matchless low intensity peaks.

Although the differentiation of CRE remains a challenge using MALDI Biotyper MS based on the protein profiling, according to our data and pending further investigation in clinical isolates of CRE, MALDI-TOF MS has potential as a diagnostic tool for the rapid detection of these multi-drug resistant bacteria.
DECLARATION - PLAGIARISM

I, Anou Dit Moise Somboro declare that

1. The research report in this thesis, except where otherwise indicated, is my original work.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other person’s data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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Signed

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List of publications

   Anou M. Somboro contributed to the design of the project, performed the microbiological, the analytical testing, and wrote the paper.

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Electronic copy of this thesis

Electronic copy of the submitted paper from this project
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CHAPTER 1

1 INTRODUCTION

In order to honor the title of this chapter, emphasis will be placed briefly on carbapenem-resistant bacteria. They have increased considerably in prevalence and incidence, appearing rapidly in several countries. Genes that encode them have jumped the species barrier. This work will thus focus on aspects of carbapenem-resistance enterobacteriaceae (CRE), including all reported classes of carbapenem hydrolyzing enzymes, then the application of mass spectrometry technology for the rapid diagnosis of these microorganisms.

1.1 CARBAPENEM-RESISTANT ENTEROBACTERIACEAE

Enterobacteriaceae are inhabitants of gastrointestinal flora and represent the most common of human pathogens, being responsible for several infections, such as cystitis and pyelonephritis with fever (Katsuta, Shoji, Watanabe, & Saitoh, 2013), septicaemia (Nordmann, Naas, & Poirel, 2011), pneumonia (von, Welte, Marre, Suttorp, & Ewig, 2010), peritonitis, meningitis and device associated infections (Andrade, et al., 2010). They are the cause of infections acquired in the community and hospitals, with the tendency to spread between humans and to obtain genetic material through horizontal gene transfer, intermediated by plasmids and transposons (Partridge, 2011; Toleman & Walsh, 2011). Due to the increased use and misuse of antibiotics, enterobacteriaceae have developed resistance to numerous antibiotics such as carbapenem class. Antimicrobial resistance is the focus of global attention and CRE pose the latest challenge in gram-negative bacterial infections (Gupta, Limbago, Patel, & Kallen, 2011). CRE have been reported worldwide, as a result of acquiring carbapenemase genes, making it difficult the control of the spread of contagion. Thereby CRE have created a global public health problem, for the infections caused by this resistant bacteria often fail to respond to the standard treatment, resulting in prolonged illness and greater risk of death.

In view of the difficulty of mastering these infections, faster susceptibility profiling is imperative for the early diagnosis, and containment of carbapenem resistance.
Furthermore rapid and efficient detection of carbapenemase production in clinical isolates is imperative to prevent the spread of drug resistance-associated deaths through early diagnosis of the causative organism. However, the detection methods for carbapenemase producers remain a challenge.

Currently, various methods are employed to detect carbapenemases. Most of these methods are phenotypic, such as E-test (Jorgensen & Ferraro, 2009), modified Hodge test (MHT) (Amjad, et al., 2011; K. Lee, et al., 2010), 3-dimensional extract (3-D) bioassay (Montealegre, et al., 2012), Carbapenemase Nordmann-Poirel test (Carba NP test) (Nordmann, Poirel, & Dortet, 2012), spectrophotometry-based assay (Bernabeu, Poirel, & Nordmann, 2012), and DNA-based tests (Xia, Liang, Su, & Xiong, 2012). The E-test and MHT are simpler to perform and have been widely used as screening methods but are phenotypic and unable to detect the presence of silent or poorly expressed carbapenemase genes. The limits of MHT, E-test and 3-D bioassay are that they time consuming because of the incubation duration and results are sometime difficult to interpret (K. Lee, et al., 2010). Spectrophotometric measurement is considered to be the reference method for the detection of carbapenemase production, but it is labor intensive and requires relatively skilled technical expertise (Bernabeu, et al., 2012). The Carba NP test is a biochemical method developed recently that uses a pH indicator for the detection of carbapenemase production, and is yet to be evaluated for some bacteria such as *Acinetobacter spp.* and it too is phenotypic (Dortet, Poirel, & Nordmann, 2012a, 2012b; Nordmann & Poirel, 2013). Polymerase chain reaction (PCR) of carbapenemase genes remains the gold standard and although reliable, it is more expensive, requires sophisticated equipment and a higher degree of expertise (Poirel, Walsh, Cuvillier, & Nordmann, 2011).

Four decades ago mass spectrometry (MS) has been applied to identify microorganisms (Anhalt & Fenselau, 1975; Bizzini, Durussel, Bille, Greub, & Prod'hom, 2010) and it has also proven its broad applicability in clinical epidemiology and infection control measures such as highlighting the dissemination of pathogens, in medical diagnostics, bio-defense, environmental monitoring, and food quality control. This study will use mass spectrometry technology specifically MALDI-TOF MS which is relatively simpler,
accurate, rapid and with lower consumable costs (Seng, et al., 2009), to address the above global problem.

1.2 MASS SPECTROMETRY

Mass spectrometry is a qualitative and quantitative analytical method employed to reveal the composition of an unknown sample (Wieser, Schneider, Jung, & Schubert, 2012). Using this technology, trace amounts of material can be analysed to identify a specific molecule, in some cases where it is located and how much of the molecule is expressed. The principle of mass spectrometry is based on the acquisition and analysis of mass and charge values from individual ionized sample molecules (Kafka, Kleffmann, Rades, & McDowell, 2011). Mass spectrometry instruments consist of three components: the ionization chamber, the mass analyser and an ion detector. The unknown sample is pulsed in the ionization chamber with an energy source, and can be in different phases such as in gas, liquid or solid. The energy serves a double function, these being ionization of individual molecules, and the desorption of solid or liquid samples into the gas phase. The vaporized sample is then focused and accelerated through the mass analyser component that separates ions based on their mass to charge ratio. Acceleration of the charged atoms is achieved by applying a large voltage (Banerjee & Mazumdar, 2012). Upon emerging from the mass analyser, ionized elements bump with the ion detector, which measures both the mass and charge of each molecule as derived from their individual force and time to impact. These signals are converted to an electrical output and illustrated in a mass spectrum, which graphs the relative abundance of each detected ion on the y-axis plotted against its mass-to-charge ratio on the x-axis. The composition or structure of the unknown sample is subsequently derived from cautious interpretation and analysis of the ion peaks (El-Anned, Cohen, & Banoub, 2009).

Various mass spectrometry instruments are available, and are differentiated firstly by their method of sample ionization and secondly by the type of mass analyser used. The choice of instrument is based on the phase of the input sample, then on the physical and chemical properties of the analytics, such as their molecular weight, thermal stability and
side-chain modifications (Glish & Vachet, 2003).

Mass spectrometry technology was initially largely limited to the analysis of small, thermo-stable compounds that were able to support the harsh electric ionization techniques available at the time, and was not widely considered for routine applications in biological sciences. Larger polypeptides and other biomolecules were found to rapidly degrade under these conditions, which significantly hampered their characterization. In the past decade, with the beginning of the proteomics era, however, research into alternative mass spectrometry methods was accelerated, and resulted in the development of low energy or soft ionization techniques, such as electrospay mass spectrometry and Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) (Banerjee & Mazumdar, 2012). The major advantage of this method is its ability to ionize and desorb high molecular weight biomolecules into the gas phase, while preserving their intact state. Based on these general considerations, MALDI-TOF MS is used today as one of the most powerful tools to analyse and identify large polypeptides as well as whole microorganisms (Pavlovic, Huber, Konrad, & Busch, 2013; Pulcrano, et al., 2013; Webster & Oxley, 2012).

1.2.1 PRINCIPLES OF MALDI-TOF MS

The composition of the samples identified by the MALDI-TOF MS can vary greatly, from purified protein to whole-cell microorganisms. Unknown sample characterization occurs in three stages, beginning with sample preparation, followed by spotting on a solid target support plate, and lastly by being overlaid with a chemical matrix, depending on the composition, which must dry completely prior to analysis (El-Aneed, et al., 2009). The matrix is essential for the soft ionization process, and is selected for both its ability to effectively absorb the majority of pulsed ionizing energy, and its efficient desorption into the gas phase, thus protecting sample molecules from fragmentation. A number of matrices have been established and are each composed of small (<1000 Dalton), acidic molecules dissolved in an organic solvent. An approximate 10 to 1 ratio of matrix to sample is used for MALDI-TOF MS preparation to ensure the efficient dilution and protection of sample molecules from fragmentation. Once dried, the prepared target plate
is engaged into the ionization chamber, where each sample is irradiated with brief pulses of laser energy (in case of our Bruker instrument, 240 pulses are used from an ultraviolet nitrogen laser 337 nm). This process desorbs individual sample and matrix molecules from the target plate into a gas phase, with most of the energy being captivated by the matrix, which becomes ionized with a single positive charge. This positive charge is subsequently relocated from the matrix to native sample proteins through their arbitrary collision in the gas phase.

Figure 1. MALDI-TOF MS process (Elitza S. Theel, 2013)

MALDI-TOF MS has recently been initiated into routine laboratory methods as a novel approach for the accurate and rapid identification of bacteria, fungi and yeast (Jurinke, Oeth, & Van, 2004; Wieser, et al., 2012). This equipment generates distinctive unique signatures mass spectral fingerprints for each microorganism, and is therefore perfect for precise microbial identification at the genus and species levels. This method has the prospect of being expended for strain typing and identification with numerous successful reports on clinical isolates of bacteria (Patrick R. Murray, 2012). MALDI-TOF MS has also been used to demonstrate the diagnosis of some diseases such as tumours,
rheumatoid arthritis, and Alzheimer’s disease, through the characterization of particular biochemical markers (Marvin, Roberts, & Fay, 2003; Schoene, Hoefler, & Walch, 2013). While several studies have described the use of this method as a rapid detection tool for drug resistant bacteria, such as CRE, they were more focused on detecting the drug and their degradation products (Burckhardt & Zimmermann, 2011; Hrabak, et al., 2012; Sparbier, Schubert, Weller, Boogen, & Kostrzewa, 2012; L. Wang, Han, Sui, Wang, & Lu, 2013).

1.3 PROBLEM STATEMENT

MALDI-TOF MS has been tested for speed and accuracy on microorganisms for their identification at species and genus level, also for carbapenem resistant bacteria detection focusing on the characterisation of the drug and their degradation product, but no studies have been done to establish whether it can accurately and rapidly detect CRE based on protein profiling. The purpose of this study is therefore to apply MALDI-TOF mass spectrometry technology to accurately and rapidly diagnose drug resistant bacteria, such as CRE by characterisation of their protein profile. This will allow clinicians to accurately identify the identity and putative susceptibility profile of the causative bacteria informing the clinical management of the infection timeously.

1.4 AIM AND OBJECTIVES

The aim was to investigate the efficiency of MALDI-TOF MS as an accurate and rapid method to detect carbapenemase-producing bacteria.

The objective was to identify CRE based on rapid protein profiling for specific fingerprints using MALDI-TOF mass spectrometry.

1.5 CLASSIFICATION OF CABAPENEM HYDROLYSING ENZYMES

Not metalloenzyme carbapenemase (NMC-A) was identified in 1993 as the first class A carbapenemase produced by *enterobacteriaceae* (Naas & Nordmann, 1994), after which numerous others were identified in *enterobacteriaceae* belonging to three classes, these
being Ambler class A, B, and D beta-lactamases. In addition Ambler class C generated by *Enterobacteriaceae* may possess slight extended action toward carbapenems, although the clinical role of this class remains unknown.

### 1.5.1 AMBLER CLASS A CARBAPENEMASES

The Ambler class A serine carbapenemases can be broadly divided into subgroups based on phylogenetic data that include Guiana extend spectrum (GES), not metalloenzyme carbapenemase (NMC-A), *Klebsiella pneumoniae* carbapenamase (KPC), Imipenem hydrolysing beta-lactamase (IMI) and *Serratia marcescens* carbapenemase (SME). These enzymes have been identified in *Enterobacter cloacae*, *Serratia marcescens* and *Klebsiella pneumoniae* (Nordmann, Naas, et al., 2011). The clinically most important subgroup is the gradually emerging KPC enzymes (Nordmann, Picazo, et al., 2011). The gene that controls their production can be situated on the bacterial plasmid or the chromosome. KPC producers are generally multidrug resistant, and curative options for treating KPC related infections remain restricted (Nordmann, Cuzon, & Naas, 2009) as do rapid and efficient tools for their diagnosis (Birgy, et al., 2012).

### 1.5.2 AMBLER CLASS B CARBAPENEMASES

Metallo-beta-lactamases (MBLs), known as Ambler class B carbapenemases, contain zinc in their active sites. The enzymes Verona integron-encoded metallo-beta-lactamase (VIM), active on Imipenem (IMP), and New Delhi metallo-beta-lactamase (NDM) are members of this group. The subtype most widely disseminated was VIM (Queenan & Bush, 2007), and it was recently found that NDM-1 has spread worldwide, after initially only presenting in Asia, in *Enterobacteriaceae* and in the other gram-negative bacteria. The recurrent association of this resistant gene with a promiscuous plasmid enhances its proliferation (Nordmann, Poirel, Toleman, & Walsh, 2011; Yong, et al., 2009).

### 1.5.3 AMP C β-LACTAMASE

Numerous AmpC enzymes have an intrinsic but very weak ability to hydrolyse carbapenems. The first bacterial enzyme reported to destroy penicillin was the AmpC
beta-lactamase of *Escherichia coli* (Abraham & Chain, 1988), which belongs to the Ambler class C. In *P. aeruginosa* and in *enterobacteriaceae*, the genes responsible for the production of AmpC beta-lactamase can be located on the bacterial plasmids or chromosomes. The AmpC genes located on plasmids constitutively produce beta-lactamases, but they have slight activity against some classes of drugs, such as carbapenems and monobactam (aztreonam). However, once it is combined with reduced susceptibility due to efflux and porin mechanisms, clinically significant levels of resistance are accomplished (Marsik & Nambiar, 2011).

### 1.5.4 AMBLER CLASS D CARBAPENEMASES

The group of class D is of the oxacillinase (OXA) enzyme type, which is a penicillinase capable of hydrolysing oxacillin and cloxacillin. They present feeble activity against carbapenems, and are primarily found in *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, but rarely in *Enterobacteriaceae*. The location of these genes can be on bacterial plasmids or chromosomes (Mendes, Bell, Turnidge, Castanheira, & Jones, 2009). Activity of OXA carbapenemases can be increased by upstream elements that control gene expression. The main concern regarding OXA carbapenemases is their ability to rapidly mutate and enlarge their spectrum of activity (Queenan & Bush, 2007).

**Table 1**: Group of carbapenemases and AmpC beta-lactamases within β-lactamases classification (Marsik & Nambiar, 2011).

<table>
<thead>
<tr>
<th>Ambler group</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bush-Jacoby group</strong></td>
<td><strong>2f</strong></td>
<td><strong>3a</strong></td>
<td><strong>I</strong></td>
<td><strong>2df</strong></td>
</tr>
<tr>
<td><strong>Common name</strong></td>
<td>Serine carbapenemases</td>
<td>Metallo-carbapenemases</td>
<td>Serine carbapenemases</td>
<td>Carbapenemases</td>
</tr>
<tr>
<td><strong>Mediates Resistance to</strong></td>
<td>Carbapenems, Penicillins, Cephalosporins, Aztreonam</td>
<td>All beta-lactams except aztreonam</td>
<td>Penicillins, Cephalosporins including cefoxitin, cefotetan, ceftriaxone, cefotaxime</td>
<td>Carbapenems, penicillins, cephalosporins, aztreonam</td>
</tr>
<tr>
<td><strong>Sub-type example</strong></td>
<td>KPC, GES, SME</td>
<td>IMP, NDM, VIM, IND</td>
<td>AmpC</td>
<td>OXA</td>
</tr>
</tbody>
</table>
1.6 CURRENT THERAPEUTIC OPTIONS FOR TREATMENT OF BACTERIAL INFECTION BY CREs

The optimal treatment of infection due to carbapenemase producing organisms is uncertain, and antibiotic options are limited. The presence of KPC or metalloenzyme carbapenemase confers resistance to all penicillins, cephalosporins, and carbapenems (Arnold, et al., 2011). Due to the spread of carbapenemase producing bacteria, clinicians are becoming progressively dependent on colistin, polymyxins, aztreonan, fosfomycin, tigecycline and rifampicin for treatment of these infections (J. Lee, Patel, Huprikar, Calfee, & Jenkins, 2009; Livermore, et al., 2011; Zavascki, et al., 2006). Some experts reported that high-dose continuous infusion of a carbapenem might be helpful, although clear evidence of efficacy is lacking (Sakka, et al., 2007). There is a need for information concerning the optimal use of these antibiotics with or without other partially active antibiotics in the treatment of infections caused by carbapenemase producing bacteria.

Available beta-lactamase inhibitors, for example clavulanic acid, can restore activity of beta-lactams in vitro against carbapenemase producing bacteria, but such additions do not lower the MIC values of beta-lactam antibiotics to within the susceptible range and should not be used (Stachyra, et al., 2009). A novel beta-lactamase inhibitor (NXL104) is presently in development and has activity against the KPC enzyme (Livermore, Mushtaq, Warner, Miossec, & Woodford, 2008; Stachyra, et al., 2009), and an emphasis should therefore be also placed on the identification of new and innovative tool for diagnostic as well discovery of new beta-lactamases inhibitors.

Various methods are currently used to detect carbapenemases, most of them being phenotypic. Current methods used for bacterial identification and for drug susceptibility profile requires at least two days, or more for fastidious organisms. In addition, differentiation of isolates with a different taxonomic background but similar physiological characteristics is limited using these phenotypic methods. MALDI-TOF MS requires only overnight culture of bacteria. Reliable data are available within a total turnaround time of 16 hours. Due to short turnaround times, low sample volume requirements and low reagent costs, MALDI-TOF MS has recently emerged as a

1.7 OUTLINE OF THE THESIS

This project evaluated the potential of MALDI-TOF MS as a rapid and accurate detection tool for CRE producing different carbapenemases. Chapter 2 outlines the use of this tool to identify carbapenemase producing Escherichia coli. Chapter 3 reviews the characterisation of carbapenemase produced by different bacteria, such as K. pneumoniae, S. marcescens, E. cloacae. Chapter 4 presents the limitations and recommendations, establishes to what extent the aim was achieved, and indicates the significance of the study.

REFERENCES


CHAPTER 2

Rapid detection of carbapenemase-producing _Escherichia coli_ using Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

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**Keywords:** MALDI-TOF MS, Carbapenemase, _E. coli_

**ABSTRACT**

The method of direct mass spectrometry profiling is reliable and reproducible for the rapid identification of clinical isolates of bacteria and fungi. This study used the approach of MALDI-TOF mass spectrometry profiling for carbapenem-resistant _enterobacteriaceae_ (CRE), specifically the identification of carbapenemase-producing _Escherichia coli_. Proteins were extracted from sub-cultured _E. coli_ isolates known to carry and/or express single genes of a variety of carbapenemases and subjected to mass identification by MALDI-TOF MS Biotyper technology. The confidence score exceeding 2.3 suggested high quality of mass spectra and high significance of the data obtained. The MS spectra were subjected to visual peak picking and in all cases, there were significant differences in the presence or absence of peaks to differentiate isolates expressing different carbapenemases. The Biotyper MSP creation method and identification method could be optimized to provide a significantly high difference in score between _E. coli_ expressing different carbapenemases. Based on our data and pending further investigation in other CREs, MALDI-TOF MS has potential as a diagnostic tool for the rapid detection of even closely related carbapenemases in _E. coli_.

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1 INTRODUCTION

Antimicrobial resistance is the focus of global attention and carbapenem-resistant *enterobacteriaceae* (CREs) pose the latest challenge in gram-negative bacterial infections (Burckhardt & Zimmermann, 2011). The rapid and efficient detection of carbapenemase-production by clinical isolates is important to prevent drug resistance-associated deaths with early diagnosis of the causative organism as well as its susceptibility profile being imperative for the implementation of efficacious treatment as soon as possible (Nordmann, et al., 2012). However, the detection methods for carbapenemase producers remain a challenge. Currently, various methods are employed to detect carbapenemase and most of them are phenotypic, such as the minimum inhibitory concentration E-test (Jorgensen & Ferraro, 2009), modified Hodge test (MHT) (Amjad, et al., 2011; K. Lee, et al., 2010), 3-dimensional extract (3-D) bioassay (Montealegre, et al., 2012), Carbapenemase Nordmann-Poirel test (Carba NP test) (Nordmann, et al., 2012), spectrophotometry-based assay (Bernabeu, et al., 2012), and DNA-based tests (Xia, et al., 2012). The E-test and MHT are simpler to perform and have been widely used as screening methods. The MHT, E-test and 3-D bioassay are time consuming because of the incubation duration and the results can be difficult to interpret (K. Lee, et al., 2010). Spectrophotometric measurement is considered to be the reference method for the detection of carbapenemase production, but it is labor intensive and requires relatively skilled technical expertise (Bernabeu, et al., 2012). The Carba NP test is a biochemical method developed recently that uses a pH indicator for the detection of carbapenemase production, and is yet to be evaluated for some bacteria such as *Acinetobacter spp.* (Dortet, et al., 2012a, 2012b; Nordmann & Poirel, 2013). Polymerase chain reaction (PCR) of carbapenemase genes remains the gold standard and although reliable, it is more expensive and requires sophisticated equipment and a higher degree of expertise (Poirel, et al., 2011).

Over the last few years matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) has been recognized as a powerful tool for the identification of bacteria and yeasts in many clinical diagnostic laboratories (Bizzini, et al., 2010; Lindsay G. Stevenson, Steven K. Drake, & Murray, 2010; Seng, et al., 2009;
Stevenson, Drake, Shea, Zelazny, & Murray, 2010; van, Claas, & Kuijper, 2010; Wieser, et al., 2012). Spectral profiles obtained by MALDI-TOF MS are highly selective for each bacterial species, resulting in specific fingerprints that allow the differentiation at the genus, species and even strains levels (Bailey, et al., 2013; Balada-Llasat, Kamboj, & Pancholi, 2013; Marvin, et al., 2003; Wieser, et al., 2012). This method involves the desorption and ionization (thus the "D" and the "I" in the name MALDI) of individual proteins and other molecules that allow the resulting ions (typically singly charged) to be separated by the time-of-flight (TOF) analyzer and detected by the multichannel plate (MCP) or similar detector (Kafka, et al., 2011). MALDI-TOF MS has been evaluated by several investigators for the identification of carbapenemases, generally based on the detection of carbapenem drugs and their degradation products. They concluded that mass spectrometry is a useful tool for determining carbapenemase activity but was not able to differentiate between the carbapenemases expressed. (Burckhardt & Zimmermann, 2011; Hrabak, et al., 2012; Kempf, et al., 2012; W. Lee, et al., 2013).

Studies focusing on the protein MS profiling to identify antimicrobial resistance have also been published, such as for methicillin resistant *staphylococcus aureus* (MRSA) (Kornienko, et al., 2012; Y. R. Wang, Chen, Cui, & Li, 2013), which can be applied to the MALDI Biotyper as a conventional method for biotyping of drug susceptibility. Elucidation of mass spectra obtained by the method of direct MS profiling can consider not only species identity but also strain specificity of the analyzed mass spectra of *E. coli*, as different strains carry various virulence and pathogenicity factors. Since most virulence and pathogenicity factors are characterized by larger molecular masses than MS detection limits (Plata, Rosato, & Wegrzyn, 2009), we postulated that proteins may undergo some modifications during sample preparation.

The MALDI-TOF MS approach is relatively simple, rapid and reliable data are available within a total turnaround time of 16 hours also cost-effective method with significantly lower consumable compared to the current identification approaches (Pavlovic, et al., 2013; Seng, et al., 2009). The purpose of this study was to evaluate the potential of
developing MALDI-TOF MS as a rapid method to differentiate between *Escherichia coli* producing different carbapenemases.

2 MATERIALS AND METHODS

3.1 BACTERIAL ISOLATES

*E. coli* isolates expressing seven different carbapenemases enzymes (KPC-2, VIM-1, VIM-2, NDM-1, NDM-4, IMP-1, and OXA-48) belonging to the molecular classes A, B and D were purchased from France (Institut National de la Santé et de la Recherche Médicale, Paris, France). This group had previously characterized these isolates for their beta-lactamase content at molecular level (Nordmann, et al., 2012), and susceptibility test have been carried out for all the strains used in this study by our group prior to go for MALDI approach. Disc diffusion and broth dilution methods according clinical laboratory standard institute (CLSI) guidelines were followed for this experiment. *E. coli* ATCC (American Type Culture Collection) 25922 was used as drug susceptible strain in each set of tests for control. The designations (e.g. IMP-1) used here represent different classes of carbapenemases enzymes. All isolates were stored at -80 °C in tryptic soy broth with 10% glycerol and recovered at 37 °C in Nutrient Agar medium prior to use. Primary sub-cultures were used for MALDI-TOF MS analysis.

3.2 PREPARATION OF THE INTACT BACTERIAL CELL

All isolates were sub-cultured in Nutrient Agar medium (OXOID England) at 37 °C for 24 h. Fresh bacterial cells were transferred into protein LoBind tubes (eppendorf, Germany) containing sterile MilliQ water which was adjusted to an optical density of \( \text{OD}_{600} = 0.8 \), then 300 µL of this dilution was pipetted into a new eppendorf tubes and mixed with 900 µL of 100% ethanol (HPLC grade, Sigma Aldrich Germany). The mixture was centrifuged at 13000 rpm for 2 minutes; the supernatant was decanted and centrifuged once again to remove the ethanol completely. The pellet was re-suspended in 50 µL of 70% formic acid and 50 µL of acetonitrile mixed carefully and centrifuged at 13000 rpm for 2 minutes. An aliquot of 1 µL of the supernatant (intact bacterial cells)
was applied in duplicate directly onto a cell of the MSP 96 target polished steel plate (Bruker Daltonics, Germany). After drying for 1-2 minutes, 1 µL of the saturated matrix solution containing acetonitrile, trifluoroacetic acid and MilliQ water (HPLC grade respectively) with α-4-cyano-hydroxycinnamic acid (Bruker Daltonics, Germany) was applied onto the sample. The prepared target was air dried prior to MALDI-TOF MS analysis. Mass spectra were acquired in duplicate batches for each isolate. The same experiment has been repeated the following day for the reputability test.

3.3 MASS SPECTROMETRY TESTS

Mass spectra were acquired using a MALDI-TOF mass spectrometer (Bruker Daltonics) equipped with the nitrogen laser. Spectra were registered in the linear mode of positive ions (20 kV) in the range of molecular masses 2000-20000 Dalton (Da). Bruker Bacterial Test Standard (BTS) obtained from Bruker Daltonics Germany containing a carefully manufactured extract of \textit{E. coli} DH5 alpha with two additional proteins that extend the upper boundary of the mass range covered by BTS was selected as calibration standard for the instrument. The increase of detection sensitivity was achieved by matrix excess removal by 6 laser impulses at 40% power output followed by subsequent data acquisition at laser power from 30-40%. Each spectrum was acquired with 240 laser impulses (40 impulses with various spot points). The spectra with resolution exceeding 400 were acquired. The programmed software (flexControl version 3.4 build 119, flexAnalysis version 3.4 build 70 and ClinProTools version 2.2 build 83) was employed for acquiring, treatment, and analysis of mass spectra for differentiation of isolates at the strain level to differentiate drug resistant strains from the susceptible one investigated. This clustering version of mass spectra is an approach based on the use of algorithms of the ClinProTools software, which was initially developed for search of protein markers of various oncological diseases (Ketterlinus, Hsieh, Teng, Lee, & Pusch, 2005; Qiu, et al., 2009). Identification was performed using the MALDI Biotyper 3.1 software (Bruker Daltonics) by comparing experimental spectra with the reference library of the database containing 4613 spectra of various microorganisms. Mean spectra (MSP) was created by importing two suitable spectra into the MALDI Biotyper software to include all the peaks.
that were identified as exclusive between the species. More details about the exact settings chosen are presented in the results section. Species identification was considered reliable, when the score calculated exceeded 2.300. The score from 2.000 to 2.299 provided reliable genus identification only (Table 1).

**Table 1.** Biotyper score range and interpretation of values proposed by the manufacturer (Bruker Daltonics)

<table>
<thead>
<tr>
<th>Range</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.300-3.000</td>
<td>Highly probable species identification</td>
</tr>
<tr>
<td>2.000-2.299</td>
<td>Secure genus identification, probable species identification</td>
</tr>
<tr>
<td>1.700-1.999</td>
<td>Probable genus identification</td>
</tr>
<tr>
<td>0.000-1.699</td>
<td>Not reliable identification</td>
</tr>
</tbody>
</table>

3 **RESULTS**

3.1 **ANALYSIS OF MASS SPECTROMETRY DATA**

Primary bacteriological species identification of all selected *E. coli* (Nordmann, et al., 2012) was confirmed by MALDI-TOF MS profiling. The experiments were reputable and reproducible so well correlated. The database was reliable in identifying the *E. coli* species with the confident score of 2.4 to 2.5 when the standard methods for MSP creation and identification were employed (between the mass ranges of 2000 to 20000 Da). The carbapenemases enzymes IMP-1, NDM-1 NDM-4, OXA-48 and VIM-2-producing *E. coli* are best matched to *E. coli* DH5alpha, while KPC-2 and VIM-1 are best matched to *E. coli* MB11464 when analyzed on the current database of the Biotyper method. In all cases, *E. coli* ATCC 25922 was the second or third best matched with a score of 2.3 to 2.4.
3.2 Results of ClinProTools Analysis

Figure 1. Gel view comparing drug susceptible *E. coli* ATCC 25922 with other carbapenem resistant *E. coli* isolates producing different carbapenemases. The gel view shows the raw spectra of all loaded spectra files arranged in a pseudo-gel like style. The x-axis records the m/z value. The left y-axis displays the running spectrum number initiating from subsequent spectra loading. The peak intensity is expressed as a gray scale. The color bar and the right y-axis indicate the relation between the color with which a peak is displayed and the peak intensity in arbitrary units.

A virtual gel view representation of data derived from *E. coli* isolates is shown in (Fig.1). All individual spectra are shown on a density scale and this gel view shows the intensity distribution of the relevant signals in the different samples. The common bands generated by these isolates appearing at 4364, 5383, 6256 and 6313 Da were used for normalization of spectra.

The drug susceptible *E. coli* ATCC 25922 control strain was visually differentiated from the carbapenemase-producers bacteria and the most notable being the presence of bands at 4613 and 4769 Da, while there was an observed absence at 9063 and 9737 Da. These two latter bands were also absent in the isolate carrying the resistant enzyme VIM-2, but
could be distinguished by a band at 5096 Da with a higher intensity that is common to the carbapenemase-producers *E. coli*. NDM-4 was identifiable by high intensity bands at 6538 and 5755 Da. IMP-1 exhibited a distinct band at 3673 Da that is present in all of the other isolates but with much lower intensities. This procedure appears therefor to be capable of generating significantly different bands signals that may be employed in the differentiation of these isolates.

**Figure 2.** Spectra of the selected carbapenem resistant *E. coli* isolates producing different carbapenemases compared with the drug susceptible *E. coli* ATCC 25922. This Figure shows the generated spectra by the MALDI Biotyper method for each of the carbapenemase producing *E. coli* isolates compared to the sensitive ATCC 25922-type strain. It describes the presence and absence of various peaks and their corresponding m/z.
Table 2. Comparison of ClinProTools statistical versus visual peak picking randomly.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Number of peaks</th>
<th>ClinProtool Peak picking (Da)</th>
<th>Visual peak picking (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 25922</td>
<td>88</td>
<td>4769(I*); 4613(I)</td>
<td>3074; 3177; 3205; 4425; 6547; 6809; 6826</td>
</tr>
<tr>
<td>VS VIM-2*</td>
<td></td>
<td>6508(I); 5124(E); 3823(E*)</td>
<td>2370; 2405; 3084; 3509; 3823; 5124; 5459; 5538; 8326; 8431; 8458; 8793; 10463</td>
</tr>
<tr>
<td>KPC-2</td>
<td>96</td>
<td>10478(E); 5239(E)</td>
<td>5239; 5557; 8451; 10478; 11113</td>
</tr>
<tr>
<td>VS VIM-1</td>
<td></td>
<td>9717(I); 9067(I); 10562(E)</td>
<td>5283; 10562</td>
</tr>
<tr>
<td>NDM-1</td>
<td>78</td>
<td>7085(E)</td>
<td>7647; 7085</td>
</tr>
<tr>
<td>VS OXA-48</td>
<td></td>
<td>8430(E); 7393(E); 5238(I); 5858(E)</td>
<td>5858; 7085; 7393; 7525</td>
</tr>
</tbody>
</table>

*(I= Intensity, E= Exclusive, the designations for example, VIM-2 used here represent different classes of carbapenemases enzymes producing E. coli.)*

Three groups of isolates were chosen randomly to investigate the significance of the mass peaks by their close or distant relationship. ClinProTools provides a list of peaks selected according to statistical importance based on the intensity or the presence of one spectrum only, to differentiate between the selected isolates. The visual peak picking was based on the presence of identifying exclusive peaks between spectra, and this enabled the detection of significant differences amongst E. coli isolates producing different carbapenemases (Table 2). The comparison of drug susceptible E. coli ATCC 25922 and E. coli producing VIM-2 enzyme showed seven peaks distinctive to the standard strain while thirteen peaks unique to the carbapenemase-producing isolate. Within the carbapenemase-producing isolates, we compared VIM-1 to KPC-2 and NDM-1 to OXA-48 as an illustration. Five peaks were matchless to KPC-2 and two to VIM-1 by visualization, while NDM-1 versus OXA-48 showed two and four exclusive peaks respectively.
In order to differentiate the selected isolates, the ClinProTools specified a list of peaks arranged according to the statistical significance where the notified distinction was the changes of intensity in general Fig. 3 (left). Moreover the visual investigation was based only on the exclusive unique peaks Fig. 3 (right). This figure illustrates the text in table 2 showing presence/absence of peaks as well as differences in intensities among common peaks.

3.3 RESULTS OF MALDI BIOTYPER

We generated a main spectrum (MSP) of the standard control strain ensuring that the creation method included all of the peaks that were identified as exclusive between the species. An MSP is the basic of classification using MALDI Biotyper. It is a reference spectrum (normally an average of six to eight combined spectra) or more accurately a reference peak list that is assigned to a species or strain. Subtyping MSPs are used to distinguish between closely related species. In addition to extracting information on peak
frequency and peak intensity distribution, subtyping MSPs apply additional weighting to
distinguish unique peaks. The desired mass error for the MSP creation method was
changed from 250 ppm to 2000 ppm and the desired peak number from 70 to 100. The
mass error of the raw spectra for the identification method was restricted to be 250 ppm
as compared to the standard of 2000 ppm. The modifications of the mass error were
based on maximizing the inclusion of all relevant peaks. VIM-2 was scanned against the
existing Biotyper database to compare the standard methods with the modified version
and, with the inclusion of the newly created MSP of the \textit{E. coli} ATCC 25922- type strain.
The standard identification method gave a score of 2.5 indicating a false positive for the
species but modified version gave a score of 1.7. Since the modified method represented
a result closer to reality, these settings were used for the generation of the data in (Table
3). Database entries were created for all of the carbapenemase-producing \textit{E. coli} with the
new MSP creation method and found that this was warranted as more than 70 peaks were
identified for each carbapenemase-producing isolate.

\textbf{Table 3.} Evaluation of carbapenemase-producing \textit{E. coli} isolates by the modified MSP* creation and identification methods.

<table>
<thead>
<tr>
<th>Isolates (Carbapenemase)</th>
<th>First best matched</th>
<th>Log (Score)</th>
<th>Second best matched</th>
<th>Log (Score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMP-1</td>
<td>OXA-48</td>
<td>2.459</td>
<td>NDM-4</td>
<td>2.346</td>
</tr>
<tr>
<td>KPC-2</td>
<td>VIM-1</td>
<td>2.482</td>
<td>NDM-1</td>
<td>2.270</td>
</tr>
<tr>
<td>NDM-1</td>
<td>IMP-1</td>
<td>2.341</td>
<td>OXA-48</td>
<td>2.315</td>
</tr>
<tr>
<td>NDM-4</td>
<td>OXA-48</td>
<td>2.383</td>
<td>IMP-1</td>
<td>2.352</td>
</tr>
<tr>
<td>OXA-48</td>
<td>IMP-1</td>
<td>2.465</td>
<td>NDM-4</td>
<td>2.383</td>
</tr>
<tr>
<td>VIM-1</td>
<td>KPC-2</td>
<td>2.482</td>
<td>\textit{E. coli} DH5alpha</td>
<td>2.085</td>
</tr>
<tr>
<td>VIM-2*</td>
<td>IMP-1</td>
<td>2.129</td>
<td>\textit{E. coli} RV412</td>
<td>2.043</td>
</tr>
</tbody>
</table>

* Modifications of the mass error were based on maximizing the inclusion of all relevant peaks.
*The designations for example VIM-2 used here represent different classes of carbapenemases enzymes producing \textit{E. coli}.

We scanned the carbapenemase-producers against the extended MSP database and
ensured that the identification and MSP creation methods were matched to give full
scores for the isolates against their own entries (score of 3.000). The differences of
scores between the first matched strains of the carbapenemase-producing \textit{E. coli} were
0.518 to 0.871 and for the second matched strains were 0.617 to 0.957. VIM-1 and VIM-2, *E. coli* DH5 alpha and *E. coli* RV412 appeared as the second best matched with scores of 2.085 and 2.043 respectively, which are not considered as reliable in terms of strains identification. We therefore attempted to find a more accurate method to analyze these isolates.

4 DISCUSSION

Different data analysis tools were employed for the optimization in this study. The first phase of the analysis was the evaluation of the capacity of the MALDI Biotyper for isolate identification by MS profiling. This was accurate with respect to genus and species. Taking into consideration the advantages of this method of direct profiling, it is reasonable to conclude that it would be a potential tool for scientific and clinical laboratories studying various resistant *E. coli* strains provided that a significant difference in score is obtained between the analyzed isolates.

The classification by the mathematical models revealed peaks responsible for differentiation between different carbapenemase-producing isolates. However, it should be noted that while these models are very useful and rapid we could not totally rely on its analysis for the definitive identification of carbapenemases in *E. coli* based on results obtained. The visual peak picking showed clearly that several peaks were not captured by the ClinProTools software because the algorithm based its differentiation on both the presence and intensity differences of the spectra, while the visualization based its selection criteria on the exclusivity of peaks.

The second phase of this work was based on MSP creation method for carbapenemase detection. Carbapenemase-producing isolates were identified using Biotyper with inclusion of the cellular organism of the Bruker database and the created assigned MSP. Enterobacteriales from the cellular organism and the new MSP were selected to identify all of the tested strains. This method was able to give an improved difference between carbapenemase and non-carbapenemase producers, according to the score of best-matched identities. The first two best matched strains were mostly carbapenemase-
producers, except for the VIM-1 and VIM-2 enzymes, where the second best matched strains were *E. coli* DH5alpha and *E. coli* RV412 with unreliable scores. The significantly large difference in scores between isolates used for identification and the best matched indicates that a score above 2.5 could be used for “highly probable” subspecies identification.

Thus, based on our data, MALDI TOF MS has promising potential as a tool for rapid detection and diagnosis of carbapenemase-producing bacteria. This potential can be further enhanced if the software would allow for manual weighting of the desired biomarker peaks. Alternative, specific protein targets (biomarkers) can be used.

We also recommend that a much broader collection of clinical isolates is used to understand the significance of these findings in a follow up study. Subsequent studies should also identify the unique proteins observed for different isolates.
REFERENCES


CHAPTER 3

Evaluation of Maldi Biotyping as a tool for the characterization of carbapenemase-producing bacteria

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Keywords: Carbapenem-resistant enterobacteriaceae, MALDI-TOF MS

ABSTRACT

Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS), a tool that has been recently introduced in routine diagnostic microbiology laboratories for the identification of bacterial and yeasts isolated from clinical samples, also has potential for the characterization of antimicrobial resistance, such as conferred by beta-lactamases. In this study we investigated the capabilities of developing the MALDI-TOF MS based method as a rapid tool to characterize carbapenemase-producing enterobacteriaceae. Different types of bacteria (Klebsiella pneumoniae, Enterobacter cloacae, Enterobacter asburiae, Serratia marcescens) carrying carbapenemases were involved in this investigation and subjected to optimized methods described in our previous work on carbapenem resistant Escherichia coli. The characterization of carbapenemase-producing enterobacteriaceae using MALDI Biotyper MS based on the protein profiling appears promising by the visual observation of peaks that could be picked up from the MSP method by matchless low intensity peaks.
1 INTRODUCTION

Mass spectrometry has been applied four decades ago to identify microorganisms (Anhalt & Fenselau, 1975; Bizzini, et al., 2010). Recently, MALDI-TOF MS has been used for identification and a characterization method in many laboratories (Bailey, et al., 2013; Biswas & Rolain, 2013; Croxatto, Prod'hom, & Greub, 2012; L. Wang, et al., 2013), it has also proven its broad applicability in clinical epidemiology and infection control measures such as preventing dissemination of pathogens, medical diagnostics, environmental monitoring, bio-defense, and food quality control. Consequently, this technology is widely utilized and has recently become popular for bacterial, fungal and yeast detection (Theel, 2013). The short analysis time, accuracy, high sensitivity, minimal sample volume and large mass range (m/z) makes it a useful standard tool for bacterial identification (P. R. Murray, 2010).

Drug resistance detection is the most desirable and recent application of MALDI-TOF MS in microbiology. This assay has been described for characterization of drug resistant bacteria such as carbapenem-resistant enterobacteriaceae (CRE); various groups developed the method for the detection of the carbapenem antibiotics and their degradation products (Burckhardt & Zimmermann, 2011; Hrabak, Walkova, Studentova, Chudackova, & Bergerova, 2011; Sparbier, et al., 2012). Beta-lactamases can be monitored by mass spectrometry profiling because hydrolysis of beta-lactams by resistance bacteria results in disappearance of the primary mass peak through a molecular mass shift of 18 Da of the drug; in some cases the hydrolysis is directly followed by decarboxylation of the hydrolyzed product, conducting in a further mass shift of 44 Da from the hydrolyzed form (Sparbier, et al., 2012).

The spread of carbapenem-resistant bacteria, more specifically of carbapenemase-producing Enterobacteriaceae and Acinetobacter baumannii, is a threat to worldwide healthcare and patient safety and as a result, the microbiologists over the globe pay special attention to carbapenemase-producing resistant bacteria, specifically Gram-negative bacteria (Gupta, et al., 2011). Therefore, carbapenemase-resistant enterobacteriaceae (CRE) have indeed become a scourge of public health, locally and
Internationally, threatening the efficiency of all currently available antibiotics, save for colistin. Recently the emergence of NDM-1 and KPC-2 for the first time in South Africa, has been documented among clinical isolates of *Klebsiella pneumoniae* and *Enterobacter cloacae* in hospitalized patients in Johannesburg and Pretoria (Brink, et al., 2012). The Johannesburg and Cape Town Antimicrobial Resistance Reference Laboratories (AMRRL) of the Centre for Opportunistic, Tropical and Hospital Infections (COTHI) at National Institute of Communicable Diseases (NICD) of the National Health Laboratory Services (NHLS), since 2013, have been testing referred isolates of suspected carbapenemase-producing *enterobacteriaceae* for the presence of carbapenemase genes. The commonly referred isolates were *K. pneumoniae* (57/89, 64 %) and *E. cloacae* (22/89, 25 %). Of all these isolates, 65 % tested positive for selected carbapenemase genes (Centre for Opportunistic, 2013). There is thus a need for a rapid, accurate, sensitive and specific tool to detect these CRE.

There are various techniques employed to detect carbapenem resistance, such as E-test (Jorgensen & Ferraro, 2009), modified Hodge test (MHT) (Amjad, et al., 2011; K. Lee, et al., 2010), 3-dimensional extract (3-D) bioassay (Montealegre, et al., 2012), Carbapenemase Nordmann-Poirel test (CarbaNPtest) (Nordmann, et al., 2012), spectrophotometry-based assay (Bernabeu, et al., 2012). However, they are either dependent on phenotypic expression of the carbapenemases, time consuming or laborious, requiring much more technical skills. They can also give false positive results (Carvalhaes, Picao, Nicoletti, Xavier, & Gales, 2010). Genotype DNA/ RNA based PCR tests (Xia, et al., 2012) remains the gold standard for carbapenemase detection, it is a reliable method but expensive and become difficult to use with the increasing number of different types of carbapenemases (Poirel, et al., 2011).

The present study expands on the existing work on bacterial identification to carbapenemase producing bacterial characterization by rapid detection tools. Detection of methicillin resistant *Staphylococcus aureus* (MRSA) by MALDI-TOF MS based on protein profiling has been reported (Y. R. Wang, et al., 2013). The application of this method to MALDI Biotyper as a conventional tool for biotyping of drug susceptibility
can solve the problem of the need for rapid point of care drug resistance diagnostics. Illustration of mass spectra obtained by the method of direct MS profiling can reflect not only species identity but also strain specificity of the analyzed mass spectra of *enterobacteriaceae*, as different strains carry various virulence and pathogenicity factors.

The MALDI-TOF MS approach is quite easy to use, fast, relatively less laborious with significantly lesser consumable costs than traditional microbiological characterization methods (Seng, et al., 2009). The aim of this study was to thus explore the potential of developing MALDI-TOF MS based method as a rapid tool to characterize carbapenemase-producing *enterobacteriaceae*.

2 MATERIALS AND METHODS

2.1 COLLECTION OF BACTERIAL STRAINS

Bacterial isolates of *Klebsiella pneumoniae, Enterobacter cloacae, Enterobacter asburiae* and *Serratia marcescens* expressing different, well characterized carbapenemases, viz., KPC-2, KPC-3, GES-5, NMC-A, VIM-1, VIM-2, VIM-19, NDM-1, IMP-1, IMP-8, IMP-11, SME-1, SME-2, OXA-48, OXA-181) belonging to the molecular classes A, B and D were purchased from France (Institut National de la Santé et de la Recherche Médicale, Paris, France). This group had previously characterized these isolates for their beta-lactamase content at molecular level (Nordmann, et al., 2012) and susceptibility test have been carried out for all the strains used in this study by our group prior to go for MALDI approach. Disc diffusion and broth dilution methods according CLSI guidelines were followed for this experiment. *E. coli* ATCC (American Type Culture Collection) 25922 was used as the drug susceptible control. The designations (for example IMP-1) used here represent different classes of carbapenemases enzymes. All isolates were stored at -80 °C in tryptic soy broth with 10% glycerol and recovered at 37 °C in Nutrient Agar medium prior to use. Primary subcultures were used for MALDI-TOF MS analysis. Mass spectra were acquired in duplicate batches for each isolate. The same experiment has been repeated the following day for the reputability test.
2.2 PREPARATION OF THE INTACT BACTERIAL CELL

All isolates were sub-cultured in Nutrient Agar medium (OXOID England) at 37 °C for 24 h. Fresh bacterial cells were transferred into protein LoBind tubes (Eppendorf, Germany) containing sterile MilliQ water which was adjusted to an optical density of OD$_{600} = 0.8$, then 300 µL of this dilution was pipetted into a new Eppendorf tubes and mixed with 900 µL of 100% ethanol (HPLC grade, Sigma Aldrich Germany). The mixture was centrifuged at 13000 rpm for 2 minutes; the supernatant was decanted and centrifuged once again to remove the ethanol completely. The pellet was re-suspended in 50 µL of 70% formic acid and 50 µL of acetonitrile mixed carefully and centrifuged at 13000 rpm for 2 minutes. An aliquot of 1 µL of the supernatant was applied directly onto a cell of the MSP 96 target polished steel plate (Bruker Daltonics, Germany). After drying for 1-2 minutes, 1 µL of the saturated matrix solution containing acetonitrile, trifluoroacetic acid and sterile MilliQ water (HPLC grade respectively) with α-4-cyano-hydroxycinnamic acid (Bruker Daltonics, Germany) was applied onto the sample. The prepared target was air dried prior to MALDI-TOF MS analysis. Mass spectra were acquired in duplicate batches for each isolate.

2.3 MASS SPECTROMETRY TESTS

Mass spectra were acquired using a MALDI-TOF mass spectrometer (Bruker Daltonics) equipped with the nitrogen laser. Spectra were registered in the linear mode of positive ions (20 kV) in the range of molecular masses 2000 - 20000 Dalton (Da). The spectra were externally calibrated using the Bruker Bacterial Test Standard (BTS) obtained from Bruker Daltonics Germany containing a carefully manufactured extract of E. coli DH5 alpha with two additional proteins that extend the upper boundary of the mass range covered by this calibrant mixture. The increase of detection sensitivity was achieved by matrix excess removal by 6 laser impulses at 40% power output followed by subsequent data acquisition at laser power from 30-40%. Each spectrum was acquired with 240 laser impulses (40 impulses with various spot points). The spectra with resolution exceeding 400 were acquired. The programmed software (flexControl version 3.4 build 119, flexAnalysis version 3.4 build 70 and ClinProTools version 2.2 build 83) was employed.
for acquiring, treatment, and analysis of mass spectra for differentiation of isolates at the strain level to differentiate drug resistant strains from the susceptible one investigated. This clustering version of mass spectra is an approach based on the use of algorithms of the ClinProTools software, which was initially developed for search of protein markers of various oncological diseases (Ketterlinus, et al., 2005; Qiu, et al., 2009). Identification was performed using the MALDI Biotyper 3.1 software (Bruker Daltonics) by comparing experimental spectra with the reference library of the database containing 4613 spectra of various microorganisms. Species identification was considered reliable, when the score calculated exceeded 2.300. The score from 2.000 to 2.299 provided reliable genus identification only.

3 Results

3.1 MALDI Biotyper and new MSP results

Figure 1. MALDI-TOF mass spectra of *S. marcescens* IMP-1 (blue), *K. pneumoniae* IMP-1 (red), *E. coli* IMP-1 (green) and *E. cloacae* IMP-1 (pink) respectively.
Prepared samples from suspensions containing bacterial cells were grown on Nutrient Agar medium at 37 °C for 24 h. The final product that contained protein was applied onto a target and α-4-cyano-hydroxycinnamic acid was employed as the matrix solution. The acquired spectra by MALDI Biotyper were analyzed by flexAnalysis version 3.4; the inclusion of E. coli above was based on the fact that it was used to optimize the method in our previous work. Fig.1 shows the MALDI-TOF mass spectra of S. marcescens, K. pneumonia, E. cloacae and E. coli carrying IMP-1 enzyme illustrating the confidence of quality spectra obtained from the MALDI Biotyper (Table.1).

Table.1. Carbapenemase-producing enterobacteriaceae used in the study and their MALDI Biotyper scores for the standard MSP methods.

<table>
<thead>
<tr>
<th>Strains</th>
<th>MALDI Biotyper MS profiling</th>
<th>First best match</th>
<th>Score</th>
<th>Second best match</th>
<th>Score</th>
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<td>2.369</td>
<td>K. pneumoniae RV_BA_03_B LBK</td>
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</tr>
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<td>K. pneumoniae IMP-8</td>
<td>K. pneumoniae ssp pneumoniae 9295 CHB</td>
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<td>K. pneumoniae RV_BA_03_B LBK</td>
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<tr>
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<td>2.363</td>
<td>S. marcescens DSM 30122 DSM</td>
<td>2.359</td>
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</table>
The selected isolates for this study were identified using MALDI Biotyper software and compared with the reference library of the database that contains 4613 spectra of various microorganisms only. They were reliably classified with confident scores (Table.1). The MALDI Biotyper result confirmed the previous identity of all the isolates. Values between 2.000 to 2.299 shows that the isolate has secured genus identification and a probable species identification, while 2.300 to 3.000 gives highly probable species identification. Most of the *K. pneumoniae* strains were matched to *K. pneumonia ssp. pneumonia 9295 CHB* strain in the reference library, the *E. cloacae* to *E. cloacae MB 11506_1 CHB* and *S. marcescens* to *S. marcescens 13103_1 CHB*. The scores were, in most cases, less than 2.5 thereby providing enough room for subspecies generation.
Table 2. Comparative results of the standard and modified MSP methods for carbapenemase-producing *enterobacteriaceae* with the extended database.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Bruker standard MSP</th>
<th>Modified MSP</th>
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<td></td>
<td>First best match</td>
<td>Score</td>
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<td><em>k. pneumoniae</em> OXA-48</td>
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<td><em>E. cloacae</em> OXA-48</td>
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<td>2.468</td>
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<td><em>S. marcescens</em> VIM-2</td>
<td><em>S. marcescens</em> IMP-1</td>
<td>2.501</td>
</tr>
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</table>

The selected isolates were identified using the extended reference library that now contains the CREs from this study. The MSP of the isolate to be identified was excluded from the taxonomy after confirming that it gave a score of three for several runs with the optimized methods. The standard MSP creation and identification methods were in most cases able to match the CREs with at least one of the other carbapenemase producing *enterobacteriaceae* with high confidence to the species. Our previous work on carbapenemase producing *E. coli* has led to an optimization of the MSP creation and identification methods for gaining maximum inclusion of all relevant peaks, being the reason that the desired mass error for the MSP creation method was optimized from 250
ppm to 2000 ppm and the desired peak number from 70 to 100. The mass error of the raw spectra for the identification method was restricted to be 250 ppm as compared to the standard of 2000 ppm, the lower bound was extended from 3000 to 2000 and the upper bound from 15000 to 20000. All of the isolates of this study were included onto the existing MALDI Biotyper database that was subsequently used for identification. In the cases where the standard MSP methods were identified as non-CRE, there was no ambiguity with this approach. This is clearly illustrated by results obtained for *K. pneumoniae* IMP-1 and IMP-8, and *E. cloacae* KPC-2 and IMP-8 strains. The scores for the best-matched strains showed an overall decrease with most of the values being below 2.5 with exception to *K. pneumoniae* producing KPC-2, KPC-3, OXA-48 and VIM-1. In our previous study we concluded that it might be possible to get strain identification if the scores ranged between 2.5-3.0.
3.2 RESULTS OF CLINPROTOOLS ANALYSIS

The gel view representation of the acquired spectra from *K. pneumoniae*, *E. asburiae*, *E. cloacae*, and *S. marcescens* are shown in (Fig.2). All individual spectra are shown on a density scale and this gel view shows the intensity distribution of the relevant signals in the different samples. In each group of bacteria common bands were observed, at 7706 Da for *K. pneumoniae*, 7890 Da for *S. marcescens*, 7068 Da for *E. asburiae* and at 5474 Da for *E. cloacae*.

Within carbapenemase-producing *K. pneumoniae* distinguishable bands could be observed at 3060 Da with high intensity level for VIM-19 that was also present in NDM-1 and VIM-1 but easily separable by the presence of bands at 2505 Da for NDM-1 and
with a low intensity bands at 6450 Da in VIM-1. IMP-1 and IMP-8 were identifiable by the presence of bands at 8209 and 3843 Da in IMP-1. KPC-2 and KPC-3 exhibited a distinct band at 6011 Da. OXA-48 and OXA-181 could be distinguished at 5977 Da.

Specific bands could also be observed in the group of *E. asburiae* at 4685 and 2318 Da for GES-5, 2469 and 3392 Da for VIM-1. NMC-A was distinguishable by the presence of tinny bands at 10794 Da. In the group of *E. cloacae*, OXA-48 is observable by the band at 6484 Da, IMP-1 at 5993 Da, KPC-2 with a thin band at 8511 and NDM-1 at 10290 Da having low intensity.

*S. marcescens* carrying VIM-2 and IMP-1 enzyme were distinguishable by the bands at 15854 Da from others, and a particular band at 2015 Da, a tinny band also appeared at 1584 Da and absence of band at 9974 Da with IMP-11. Four consecutives bands were observable at 3046, 3112, 3177 Da for KPC-2. The differences between SME-1 and SME-2 could be observed at 6830 and 7501 Da. This procedure appears to be capable of generating significantly different bands signals that may be optimized and included in the differentiation of these isolates.

*Figure 3.* Comparative visual inspection of typical spectra for the standard and modified MSP methods showing distinctive low intensity peaks. Spectra of *K. pneumoniae* OXA-48 for the standard methods (left) and spectra of the same *K. pneumoniae* OXA-48 for the modified MSP methods (right). The visualization of peaks between the two methods revealed differences in terms of intensity and exclusivity. The standard method illustrates the matchless low intensity peaks and the modified method revealed that these significant peaks must be taken into account in the algorithm to make differences between the carbapenem-resistant enterobacteriaceae.
The inspection view (Figure 3) displays the graphical output of identification results obtained by comparing a loaded spectra or MSP (green and red) to be identified with a MSP used for characterization (blue). The upper part shows the adjusted normalized peak list of the unknown isolates spectra while the lower part represents the peaks list of the MSP used for identification that is selected in the MSP scores for biotyping. The x-axis records the m/z value (10^3) and the y-axis the relative peak intensity. The result of matching the peaks of the unknown isolates spectra MSP to be identified with those of the MSP used for identification is expressed by a traffic light coloring of the peaks of the unknown isolates spectra MSP. Peaks matching within the inner window (full match) were displayed in green, peaks matching within the outer window (partial match) in yellow and non-matching peaks in red. The standard MSP method used in our study showed more matchless low intensity peaks compared to the modified MSP method this suggest that differences between CRE producing different carbapenemases can be discriminated by consideration of these significant peaks in the algorithm.

4 Conclusion

The differentiation of carbapenemase-producing *enterobacteriaceae* remains a challenge using MALDI Biotyper MS based on the protein profiling due to the close relationship between the carbapenemases and the *Enterobacteriaceae*. The overall aim was to get the scores below 2.5 to leave enough room to develop the database to accommodate such these strains. The visual observation of peaks demonstrated that the differences within the carbapenemases could be improved with the modified MSP methods by focusing on matchless low intensity peaks since the non consideration of these peaks resulted in less significant scores. While many biomarker peaks may be identified, the software would need to allow for the user to determine the weighting of the peak and therefore its contribution to the final score.
Acknowledgments

The authors would like to thank the “Institut National de la Santé et de la Recherche Médicale, Paris, France” for supplying the isolates used in this study. We would also like to thank Usha Govinden for her support and suggestions. This work was made possible through the financial support from the College of Health Sciences, University of KwuZulu Natal (UKZN), Durban, South Africa.
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Campa, M, Fadda, G, Fortina, G, Gesu, G, Manso, E, Milano, F, Nicoletti, G,


CHAPTER 4

CONCLUSION

This study attempted to evaluate the potential of MALDI-TOF mass spectrometry Biotyper technology to discriminate between carbapenem-resistant *enterobacteriaceae* expressing a range of carbapenemases belonging to different Ambler classes. According to the data we found this tool to be promising as an accurate and rapid method for detection and diagnosis of carbapenemase-producing bacteria. This potential can be further enhanced if the software would allow for manual weighting of the desired biomarker peaks. Alternative, specific protein targets (biomarkers) can be also used.

We strongly recommend that a much broader collection of clinical isolates be tested in order to understand the significance of these findings in a follow up study. Subsequent studies should also identify the unique proteins observed for different isolates.