**The development of methods for the improved detection, identification and phenotypic characterisation of difficult-to-culture micro-organisms.**

**Olugbenga Samuel Oyeniyi**

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**ABSTRACT**

This project aimed to investigate the application of Matrix-Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS) for the identification and phenotypic characterisation of difficult to culture microbes. The main bulk of the work focussed on the rapid identification of *Acanthamoeba,* a parasite which can cause a severe eye infection (keratitis) that can lead to blindness if not quickly diagnosed. Through this project, a method for generating useful MALDI spectra for *Acanthamoeba* has been developed, with the identification of six peaks that are common to all 15 isolates used. These peaks do not co-identify with any other species in the SARAMIS (Spectral ARchive And Microbial Identification System) database. Currently there are no clinically useful MALDI spectra for the identification of *Acanthamoeba*. The sensitivity of generating useful MALDI spectra for Acanthamoeba have been determined to be 4x105 cells/ml; which is greater than the sensitivity required for bacterial identification via MALDI (1x104). Also, data generated during this project indicates that MALDI may differentiate to genotype level. This is important as the nomenclature and speciation of *Acanthamoeba* has moved away from species names to genotypes.

In addition, the potential of MALDI for determining bacterial isolate antibiotic sensitivity was investigated. This was focussed on the expression of β–lactamase enzymes, and for this a number of plasmid systems were utilised. Although, this part of the research work couldn’t achieve its initial aims of detecting the enzymes, however, the impact of plasmid acquisition on the MALDI spectrum of isolates was observed. Spectra changed with the level of plasmid induced expression in isolates and this is likely due to alteration in the profile of proteins expressed; this is the first observation of this effect. It is known that large scale industrial fermentation systems often show decreased product expression under stress and in some cases undergo plasmid ‘curing’. The cost of these events is so significant thus a rapid method for monitoring expression within cells would be a step forward in continuous process monitoring; MALDI may provide such a method.

**DECLARATION**

I declare that this PhD Project is solely mine and has not been submitted elsewhere.

**Name:**

**Signed:**

**Date:**

**DEDICATION**

This PhD Project dissertation is dedicated to my lovely wife - **Oluwadamilola Oyeniyi** and my two beautiful Princesses - **Ifeoluwadaju and Oluwadarasimi Oyeniyi**

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**CHAPTER 1**

**INTRODUCTION**

# 1.1 CLINICAL / DIAGNOSTIC MICROBIOLOGY

## 1.1.1 Historical Overview

The identification of microbes began with a Dutch cloth merchant, Anton Van Leeuwenhoek, who was the first to see bacteria (Lane, 2015). In the 1660s, he ground small glass beads to make better magnifying lenses so he could examine the cloth weave more easily to assess its quality. He used these magnified lenses to look at a sample of pond water and saw that it was teeming with tiny living things (including bacteria) that were not visible to the naked eye (Gest, 2004)). His report and drawings were sent to the Royal Society in London, although there were no scientific theories at the time that supported the existence of microorganisms. The theories of spontaneous generation (germs generated by decay) and miasmas (bad air) were the accepted theories explaining for infectious diseases (Bastian and Strick, 2001); this was later refuted by the proposal of the germ theory by few scientists including Louis Pasteur (Pasteur, 1878, Smith, 2012). This new discovery of the germ theory not only proved that microbes caused diseases but it also led to the development of antiseptics and vaccines (Schwartz, 2001). Another great scientist, Robert Koch, demonstrated in a scientifically sound manner that diseases are caused by microorganisms. This was confirmed in an experiment that showed that the bacterium, *Bacillus anthracis* caused the disease anthrax(Koch, 1876). The assignation developed by Robert Koch focussed on four basic criteria known as Koch’s postulates, which are:

i. The microbe must be present in every case of the disease

ii. The microbe must be isolated from the host with the disease and grown in pure culture.

iii. The specific disease must be reproduced when a pure culture of the microbe is inoculated into a healthy susceptible host.

iv. The microbe must be recoverable from the experimentally infected host.

These postulates are still in use today as a checklist for proving that a particular microbe is the actual cause of a specific disease (Blevins and Bronze, 2010). Although, he later discovered the microorganisms that caused tuberculosis, typhoid and cholera, without necessarily following the four postulates he invented (Pasteur, 1878; Berche, 2012), He also realised there were asymptomatic carriers of disease (which have become a common feature of many infectious diseases) who do not show the signs and symptoms of a disease on exposure to the causative agent. Examples of this includes viruses such as Polioviruses which cause paralysis in just a few infected people. This provided an exception for the second postulate, which stated that the cultured microorganisms should cause disease when introduced in to a healthy organism. This was further refined by Koch as he realised not all animals exposed to an infectious agent will acquire the infection, which may be due to the general health of the host, acquired immunity from previous exposure or excellent immune system.

In depth mechanistic details of the pathogenesis of infection have more recently been uncovered via advances in microbial genetics, proteomics and molecular cloning techniques, this has made it clear that the original Koch’s postulates might be inadequate to explain the occurrence, course and outcome of many serious infections (Segre, 2014). This has necessitated the adaptation of the molecular Koch’s postulates by showing that a gene, found in a microbial pathogen, encodes a product that contributes to disease caused by the pathogen i.e. is a pathogenicity factor (Falkow, 2004). Although not all genes from pathogenic microorganisms are involved in pathogenicity or virulence, however, just like Koch’s postulates serves as a guide to identify the casual relationship between an organism and a specific disease, molecular Koch postulates might be required to examine the potential roles of genes and their products in the pathogenies of infection and disease (Bryd and Segre, 2016). It is the dynamic expression of these genes that ultimately dictates whether the host-pathogen interaction result in clinical infection. The knowledge that microbes are constantly turning on and off virulence genes in a context dependent manner, and that they exchange genes with one another to acquire unique phenotypes indicates the need for more precise and robust information on causative agents of clinical infection beyond current definitions.

## 1.1.2 Earliest Conventional Methods for Microbial identification

Through the works of Pasteur and Koch, it has been established that pathogenic microorganisms cause infection and diseases. This now brings to focus the need to match each disease to its causative agent to find a quick and lasting probable cure. Leeuwenhoek microscopes and their descendants were the first available method for viewing bacteria (Lane, 2015). However, investigative microbiology requires more than the use of lenses for accurate definitive diagnosis due to the fact that other than lens-viewing, phenotypic and / or genotypic characteristics of the organisms needs to be ascertained within the laboratory environment.

Consequently, in the 19th century, the use of lenses and unsophisticated microscopes progressed and improved to the use of culture media (Brock, 1998). During this time, bacteriologists knew that the solution to diagnosing bacterial infection(s) was to grow the specific infecting organism(s) and thus they attempted with variable success to grow these organisms (Lagier *et al*, 2015). The subsequent growth of bacteria in solid/liquid media known as culturing, was one of the earliest widely accepted methods of viewing microorganisms, (Sakula, 1982). In 1860, Pasteur prepared a culture media made up of yeast, ash, sugar and ammonium salts which contained the basic requirements for microbial growth. (Lakhtakia, 2014)). Sugar provided the carbon source; ammonium salts gave nitrogen and vitamin was received from ash. Robert Koch further developed the existing culture media by inventing broths based on meat extracts and fresh beef serum which gave optimal growth (Brock, 1999) and later progressed by developing a culture media made up of meat extract with gelatine (a colourless substance obtained from collagen), which enabled the resulting mixture to be poured into a flat glass plate, the prototypical petri dishes (Blevins and Bronze, 2010). This is made possible due to the breaking of the weak non-covalent bonds that hold the collagen protein chains together when gelatine is dissolved in hot water. However, gelatine was found to be heat sensitive and could be degraded easily by a microbial enzyme, matrix metallopeptidase 2 (gelatinase) and thus was replaced with agar on the suggestion of German House wife Fannie Hasse (Pelczar, Chan and Krieg 2010). The superiority of agar (a polysaccharide) to gelatine was evident in agar’s resistance to digestion or hydrolysis by bacterial enzymes (McLachlan, 1985; Jain, Anjaiah and Babbar, 2005). Other properties of agar which makes it a gelling agent of choice, are its stability, high clarity, nontoxic nature and its stability in temperatures less than 90 0C (Atlas, 2010).

The growth of microbes in artificial solid environments such as on agar is often performed for the differentiation of organisms which allows the generation of single cell-derived colonies. This allows for identification based on morphological characteristics including colour, size, shape, opacity, growth characteristics and elevation. Also, there are wide-ranging biochemical assays for identification including catalase tests, coagulase test, indole test and urease test. In addition, the use of phenotypic tests gives an initial putative identification while their use in combination with one or more biochemical methods often leads to a definitive identification, most likely determining the genus and species of an organism. Another vital identification method (often used for classification) after culturing is the Gram staining. This ground-breaking milestone came in 1884 when a Danish Scientist named Hans Christian Gram developed a unique method for staining bacteria to make them more visible under the microscope (Hans, 1884; Coico, 2005).

## 1.1.3 Problems Associated with Conventional Methods

We are still ignorant of the complete bacterial life on earth (Wade, 2002). Environmental microbiologists estimate that less than 2% of bacteria can be cultured in the laboratory (Stewart, 2012). Of this ‘culturable’ group, they still require biochemical analysis for accurate identification. These further identification methods do have certain shortfalls such as the length of time required for definitive results, sometimes taking days or weeks (Ayling, 2000). Moreover, not all microorganisms can be identified by using biochemical methods due to the fact that some species exhibit unique biochemical traits which are completely different from those used as a characteristic standard of identifying the species (Pinsky *et al*, 2009). Other methods such as the Gram staining method cannot solely be used for definite identification as it can only indicate if a bacterium is Gram-positive or negative due to the retention of crystal violet staining (Coico, 2005).

## 1.1.4 Development of molecular diagnostic methods

The latter end of the 20th century saw the development and use of molecular and immunological methods of diagnosis in many clinical and research labs (Leven, 2000). Both of these methods have brought a greater understanding of the disease process and have also been vital in pathogen identification and diagnosis (Cho, 2007). Immunological methods relate to the production and use of antibodies to detect specific protein in biological samples (Peruski, 2003). Examples include agglutination assays, flocculation tests, western blots, immunofluorescence assays and ELISA (enzyme-linked immunosorbent assay). ELISA is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones (Shah, 2016). Both direct and indirect ELISA types have been used in the detection and measurement of antibodies against bacterial and viruses in human beings (Yang *et al*., 2016). Another method that revolutionised the field of bacteriology in the 20th century is the Analytical profile index (API), which classifies and identifies bacteria based on conventional experiments (Ferris *et al*, 2017). The API, invented in the 1970s, employs the use of standardized, miniaturized strips containing up to 20 biochemical tests, the result of which are converted to a 7-digit numerical index which is identifiable using comprehensive databases (Holmes, 1978). The method is economical to run, and is also user friendly which enables every laboratory to use the test kits (Ferris *et al*, 2017).

On the other hand, molecular techniques often involve the examination of a single gene or the overall genetic composition of an organism (Persing *et al,* 2016). Molecular methods currently employed in bacterial identification include polymerase chain reaction (PCR), reverse transcription (RT) PCR and random amplified polymorphic DNA (RAPD) PCR, microarrays, restriction fragment length polymorphism (RFLP) and plasmid fingerprinting. Amongst these relatively new molecular methods, one that stands out since its discovery is the polymerase chain reaction (PCR) due to importance in the field of molecular biology. PCR is mainly used in the amplification of a DNA sample (making multiple copies of a gene from few copies of the gene found in the sample). PCR amplification of a gene to make billions of copies allows for detection and identification of gene sequences using visual techniques based on size of the piece of DNA. (Mullis and Faloona, 1987). PCR uses a pair of primer sequences that aids in rapid identification at strain, species or genus level of specificity. A primer is a short, artificial strand of nucleotides that directs the start of DNA synthesis by hybridising to a specific target sequence. It should be noted that the advent of molecular techniques has revolutionized microbial identification and detection owing to the increased specificity, sensitivity and more importantly reduced identification times for bacteria (speed, accuracy and precision) (Persing *et al,* 2016).

However, these great improvements brought about by molecular methods have their own unique limitations. The use of API is hampered by the relative high rate of misidentification and also the identification of a limited number of Gram-negative species (Alexander, 2005). Another major disadvantage is that a presumptive knowledge or identification is required in order to tailor the use of the ‘strip’ to the appropriate groups of microbes (e.g. the API-20E strip for members of the family *Enterobacteriaceae*). With regards to PCR, the use of this method can be limited by its sensitivity to the presence of contaminants, which can produce misleading results (Smith and Osborn, 2009). Also, PCR can confuse diagnosis by detecting pathogens in a sample which may not be responsible for the symptoms presented by a patient (Baspinar *et al*., 2017). To design primers for PCR, some prior sequence data is required, which implies that PCR can only be used to identify the presence or absence of a known pathogen or gene. A typical example is in the detection of *Acanthamoeba* in the eye; PCR will always detect this organism because *Acanthamoeba* is a normal microflora in the eye however it might not be pathogenic at the time of testing (Kumar and Lloyd, 2002). This leads to the issue of sensitivity of assays that detect microorganisms, as they all have their unique lower limits of sensitivity (Lorenz, 2012). This factor plays a crucial role in the determining the best methods to be used for accurate, rapid and timely clinical diagnosis.

In summary, most immunological based methods are limited in their sensitivity and selectivity, compared to other methods as a result of difficulties in generating selective antibodies, and the need for large amounts of specific antigen to quantify the microbe. This limitation significantly affected the overall acceptance of immunological assays as the gold standard for rapid microbial detection.

## 1.1.5 The Present day

The search for improved microbial identification methods has led to improvements on the conventional, culture-based methods as well as the immunological and molecular methods. In particular, the last decade saw the development of mass spectrometry from an ordinary research tool to being at the forefront of clinical microbiology diagnostics (Warren, 2013). This paradigm shift for Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass spectrometry (MALDI-TOF MS) from research to clinical diagnosis has undoubtedly shown positive impact in infectious disease diagnostics in clinical laboratories and simultaneously on patient management (Singhal, 2015). MALDI-TOF is now fast replacing API strips and 16S rRNA gene sequencing (mostly used in reference/research labs) in bacterial identification in clinical diagnostic microbiology laboratories due to excellent species resolution and precision. (Suzuk *et al*, 2017). It should be noted that despite all these advances in rapid microbiological identification methods, culture media still remain a fundamental tool for clinical diagnosis, research or regular quality controls across the world (Emonet *et al*., 2010). Even with the new MALDI revolution, culturing is still an essential precursor to standard MALDI-TOF MS analysis.

# 1.2 THE CHANGING FACE OF MEDICINE & DIAGNOSTICS

## 1.2.1 Point of Care Technology (POCT)

Centralised laboratory testing has become the conventional model of diagnostic care in which analytical processes are automated to enable the analysis of large number of samples (Cohen-Bacrie *et al*, 2011). However, the healthcare system is changing rapidly, partly because of the need for care to be patient centred and more localised (Price, 2015). Although the central laboratory concept has worked for decades, it does not represent a convenient process for many patients particularly those in rural cities in developing countries (Jani and Trevor, 2013). Although, changing from central laboratory is expensive because the mass analysis of samples done in this huge laboratory environment often reduces cost per analysis. However, adopting a more localised approach gives an advantage of proximity, which often times leads to early rapid diagnosis and monitoring. A typical example of this approach is the development of Point of Care Technology (POCT), which provides almost instant diagnosis and care. This technology is based on the premises that healthcare should be organised more around the patient rather than the provider. This allows for direct monitoring of chronic conditions such as diabetes and improved clinic workflow as a result of a reduction in the number of follow-up calls for laboratory result. In microbiology, point of care laboratories deliver rapid diagnosis, mostly based on real-time PCR and immuno-chromatography (Drancourt *et al*, 2016). The use of POC tests in microbiology is boosted with the recommendation from the World Health Organisation (WHO), to use it as a diagnostic tool in all cases of suspected malaria before treatment of patients (WHO, 2013). A point of care testing for the malaria parasite called BinaxNOW can detect the four-plasmodium species infecting patients (Drancourt *et al*, 2016). POCT have also been involved in other areas of microbial diagnosis and care, they include genital tract infection, meningitis, digestive tract infection and community-acquired respiratory infection (Cohen-Bacrie, 2011).

In many cases, point of care technology may have been more expensive, however it provides a faster turnaround time than a centralised type of laboratory testing (Shaw, 2016). A healthcare system centred around the patient rather than the provider is a trend that should not be ignored, especially for patients with chronic diseases such as diabetes who require continuous and regular clinical monitoring.

## 1.2.2 The need for rapid microbial identification and detection

Although there is no single diagnostic method in microbiology that gives 100% accuracy, the search for improved microbial identification/detection methods is an ongoing process (van Belkum *et al.*, 2017). The new methods to be proposed should have the potential to overcome the limitations of the current methods, while also aiding better understanding of the disease process. One of the major reasons for rapid microbial diagnosis stems from the fact that the reduction, prevention and control of infectious diseases caused by microbial pathogens lies in the timely and accurate detection of the offending pathogen leading to correct disease diagnosis (Rutanga and Nyirahabimana, 2016). Correct and timely disease diagnosis leads to early administration of medication, which often results in positive treatment outcomes which invariably saves human lives. A typical example is sepsis, a life-threatening condition caused by a deregulated host response to the presence of harmful microorganism in the blood or tissues, potentially leading to the malfunctioning of various organs, shock and death (Rello et al, 2017). This often proves fatal if not diagnosed early. Sepsis affects more than 30 million people worldwide every year, potentially leading to 6 million deaths (Benz *et al*, 2016; WHO, 2018).

Another main reason for rapid definitive microbial identification is in the facilitation of the control of fatal disease outbreaks. The recent epidemic of the Ebola virus in West Africa was fuelled by the delay in timely laboratory confirmation of the virus due to the lack of proximity of the molecular laboratories (Stamm, 2015). Not only did this delay cause more human death but also it extended the opportunities for transmission and outbreak to other neighbouring countries (Broadhurst, Brook and Pollock, 2016).

Most bacterial and fungal species that are clinically relevant (due to their pathogenicity) can be grown in-vitro, however some protozoa strains are difficult to culture (Cold et al, 2016), while some virus species do not readily infect a mammalian cell culture. It should be pointed out that, ‘unculturable’ does not necessarily mean that the organism can never be cultured, but merely that the current knowledge of the biology, nutrition and growth is still lacking (Browne *et al*., 2016). The aim behind this research is to use *Acanthamoeba spp* as a typical example of an organism that is difficult to treat and manage when diagnosed late; thus, the proposed use of MALDI-TOF MS as a means to rapidly identify and detect the organism. *Helicobacter pylori* is an example of a bacterium known for its difficulty to grow in the laboratory, often requiring complex growth media and environmental conditions (Stewart, 2012). For viruses, their isolation in cell culture is slow, time and labour intensive and also devoid of the sensitivity needed to make appreciable clinical impact (Lednicky and Wyatt, 2012). This thus translates that many of the viruses that are clinically important are simply difficult to grow or cannot be grown at all in cultured cells (Hodinka *et al*., 2013). Another group of micro-organisms that is rarely cultured are parasitic protozoans, which causes some of the deadliest diseases in the world (Andrews *et al*, 2014). Successful rapid growth of these pathogenic parasites will quicken diagnosis, antigen and antibody production, drug screening, vaccine production and the differentiation of clinical isolates. However, culturing of protozoans in the laboratory presents a number of challenges such as the need for highly complex laboratory procedures due to the different morphological stages within their life cycle and the fastidiousness in their growth requirements (Visvesvara and Garcia, 2002). For the purpose of this project, *Acanthamoeba* spp will be the main focus and dealt with in detail in the next chapter.

The need for rapid microbial identification also impacts on other elements of clinical microbiology such as antibiotic sensitivity.

## 1.2.3 Antibiotic Management

Several authors have identified the relationship between delayed microbial identification/detection/diagnosis and antimicrobial resistance (Lee *et al*., 2016; Tillotson, 2015). The global antimicrobial resistance surveillance system (GLASS) 2017 report authored by the World Health Organisation have reported the alarming levels resistance to common bacteria have reached in many parts of the world. According to Fair and Tor (2014), there has been an increase in the frequency of dangerous, antibiotic resistance bacteria observed. One of the main factors causing this increase in the incidence of antibiotic resistance is the inappropriate use of antibiotics, often as a result of late identification of the causative agent. The summary of the review set up by the British Government on Antimicrobial resistance detailed that the global rise in drug-resistant infections or antimicrobial resistance (AMR) is mainly caused by inappropriate use of antimicrobials (antibiotics) on patients who do not need them (O’Neil, 2014). The report emphasised the need to reduce the demand for antimicrobials so that the current stock of drugs can last longer by proposing seven major interventions. The first intervention is about the improvement on global surveillance of drug resistance and antimicrobial consumption in humans and animals, while the second focusses on the promotion of new, rapid diagnostics to reduce unnecessary use of antimicrobials. Figure 1 shows the approximate number of deaths attributable to major causes, including antimicrobial resistant infections which is currently responsible for 700,000 deaths, while it is projected to rise to 10 million deaths by 2050.

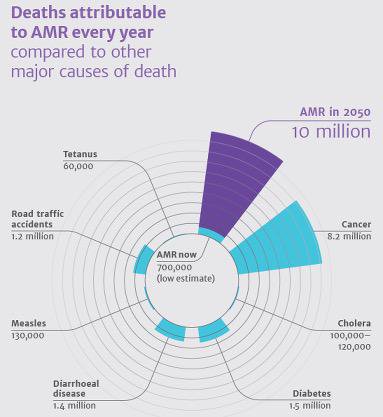


Figure 1: Deaths attributable to antimicrobial resistance every year compared to other major causes of death. (O’Neil, 2014).

A past Director General of the World Health Organisation (WHO), Dr. Margaret Chan, stated that “Antibiotics are rarely prescribed based on a definite diagnosis” (WHO, 2015). Diagnostic test can show whether or not an antibiotic is actually required, and which one precisely. Having rapid, low-cost and readily available diagnostics is an essential part of the solution to this urgent problem. This further suggest the importance of rapid diagnosis before prescription. However, this empirical decision-making on the part of Clinicians who are under time and financial pressure to address patients’ needs much faster are sometimes constrained to prescribe unnecessary antibiotics use (Webster, 2017). This unnecessary use of antibiotics is linked to the evolution of drug resistance over time, which is caused by organism mutation or horizontal gene transfer (Davies, 2010). An example *Mycobacterium tuberculosis*, a highly aerobic pathogenic bacterium, which is the causative agent for tuberculosis (TB) (Flynn and Chan, 2003). An estimated one third of the world’s population is infected with latent TB, although many will not have the disease progress to an active state, however a significant number do (Niederweis *et al*., 2010). The first line of treatment for TB infection is a combination therapy of rifamycin and isoniazid, however multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) is resistant to it (WHO, 2018). Another form of the organism known as the extensively drug resistant *Mycobacterium tuberculosis* (XDR-TB) is resistant to the first line of combination therapy and also further resistant to at least one-second-line combination therapy of fluoroquinolone and aminoglycoside (Chang and Yew, 2012). Sometimes XDR-TB requires a two-year course of antibiotics at a huge cost (approximately £350,000) and still can remain fatal even with proper treatment (Kim *et al*., 2008). These two highly resistant forms of TB provide an example of what could potentially happen to all microbes if adequate prevention steps are not taken.

Therefore, the continuous use of inappropriate antibiotics due to lack of rapid diagnostics remains a critical issue that needs to be urgently addressed. One of the key recommendations alongside the search for rapid diagnostic methods is the introduction of a reliable point-of-care technology, which can also facilitate patient treatment.

# 1.3 WHERE DO WE GO FROM HERE?

It has been established that the rapid identification of microorganisms in the clinical microbiology laboratory is of great value for the selection of optimal patient management strategies for infections. Aside from its importance in accurate diagnosis and treatment, rapid identification also enables expedient de-escalation from broad spectrum agents to targeted antimicrobial therapy which minimises the risks of antibiotics in the disruption of flora ad toxic side effects. The relatively new detection and identification methods are hampered by certain limitations such high sensitivity, time and cost constraints as well as pre-knowledge of microbe in certain situations. Thus, it has become necessary to narrow down the best detection/identification methods for each unique infectious agent in order to obtain accurate, rapid and timely clinical diagnosis.

That being said, it should be noted that there are a variety of complicated diagnostic scenarios other than earlier discussed which cannot be readily solved by a single method as described above. Although this does not readily relate to the project at hand, the knowledge is crucial in the overall understating of the infection/diagnosis topic. One of such is the existence of biofilms and their role in causing recurrent infections, resilient to stress such as lack of water, high or low pH and the presence of antibiotics or antimicrobials (Lebeaux, Ghigo and Beloin, 2014). Biofilms being a collection of microorganism that are packed so closely together that they are able to able to communicate by sending chemical signals which travel from microbe to microbe (Chandki and Banthia, 2011). This phenomenon is known as quorum sensing which only happens when bacteria are in close proximity like in a biofilm. The mechanism of antibiotic resistance in bacterial biofilms is quite different from those responsible for conventional antibiotic resistance. The detection and identification of infectious bacteria responsible for biofilm associated infections is challenging due to the complex and heterogeneous biofilm matrix (Li *et al*, 2014). The biofilm matrix accounts for over 90% of biofilm dry mass which provides a three-dimensional microenvironment that protects the bacteria (Flemming and Wingender, 2010). The physical structure of the biofilm and the composition of the extracellular polymeric substances (EPS) in the matrix, such as polysaccharides, proteins, nucleic acids and lipids vary among bacteria species (Flemming, Neu and Wozniak, 2007). These complex structure and composition could explain the increase in resistance to antimicrobial drugs in biofilms resulting in a rise in treatment failure rates (Ryder, Bryd and Wozniak, 2007). In addition, poor antibiotic penetration, nutrient limitation and slow growth as well as adaptive stress response and formation of persister cells have been suggested to constitute a multi-layered defence (Stewart, 2002)

Similarly, multifactorial disease i.e. the cause of a disease by more than one pathogen are often misdiagnosed after the huge investment of time, money and other resources. Examples of multifactorial disease are diabetes, obesity, high blood pressure, and some types of cancer, all of which are caused by a combination of small inherited variations in genes often acting in conjunction with environmental factors. In addition, the diagnosis of deep-seated infection is problematic because invasive methods are not easily applicable to critically ill patients (Naqvi, 2017). This therefore calls for non-invasive techniques such as X-ray, ultrasonography, computed tomography and magnetic resonance imaging (MRI) which are not inherently specific particularly at the early stages of infection. In addition, the use of quinolone-based imaging probes shows promise for the diagnosis of infections, however improvements must be made in terms of specificity and sensitivity. These improvements will require optimization of quinone structure which can be accelerated by refining microbiological assays (Naqvi and Drlica, 2017).

This mini review of existing and promising method for diagnosis suggest that more research needs to be done to reduce the limitations highlighted for each method. This will not only reduce diagnostic time, but also give accurate diagnostic analysis which ultimately save lots of human lives.

# 1.4 MOTIVATION FOR THE RESEARCH

The methods for identification and detection of microorganisms have evolved over time. The oldest and still the most commonly used methods of identifying micro-organism are the phenotypic approaches that require organisms to be cultured (Zapka *et al*, 2017). It is a fact that this sub-field of pathology is still dominated by culture-based methods after more than a century, despite the integration of serological and molecular methods (Willinger, 2017). This however shows that cell culture is still vital in the microbial diagnostics world and might remain the key reference for microbial identification for the next twenty years

Overall, the individual assays and techniques involved in pathogen identification particularly for clinical diagnosis still currently falls short of providing the necessary information (on time) which is required to produce a rapid, robust and accurate result. Although the advent of molecular and serological methods has shifted the paradigm slowly in diagnosis, however, microbial antibiotic susceptibility testing still needs more research. This PhD programme is borne out of the need to (i) improve detection technology and characterisation methods for microorganisms (ii) detect pathogens directly from clinical samples, and (iii) improve rapid MALDI-TOF methods for testing antibiotic resistance. For this PhD project, the improved identification and detection of *Acanthamoeba* will be the main focus. The organism has been chosen due to its unique traits; most especially the importance attached to its early detection to enable successful treatment. For effective treatment, *Acanthamoeba* infection needs to be detected and identified within seven days to avoid blindness of the eye; however, the current methods of detection take at least 2 weeks (Borin *et al*, 2013).

# 1.5 NOVELTY OF THE RESEARCH

The novel aspects of this research are that it investigated the use of MALDI-TOF MS in the detection and identification of a parasite (*Acanthamoeba*) and antimicrobial resistance. Although a paper published recently (Del Chierico, 2016) employed the use of MALDI-TOF MS to identify and type free-living Acanthamoeba, however, the MALDI-based assay used in the article is complex and complicated, while the identification was by visual inspection of the spectra acquired to show high similarities between the genotypes. Contrastingly, our MALDI-based assay is similar to the conventional MALDI assays employed for bacterial and fungal MALDI identification in hospitals all over the world. This assay can be adopted as the ‘gold standard’ due to its easy replicability and reproducibility within a short period of time and also opens an avenue for investigation of the potential different pathogenicity of the *Acanthamoeba* genotypes. Thus, it can be suggested that this research helped to contribute to knowledge on methods development for detecting and identifying a non-bacterial sample.

The determination of antimicrobial susceptibility or resistance using the MALDI technique is not straight forward, due to the fact that species-specific proteins in the MALDI-TOF MS spectra are mainly unaltered by antimicrobial susceptibility status (Kostrzewa *et al*., 2013). Although, MALDI-TOF MS offers the potential to detect carbapenemase production, more improved and validated methods are required before antibiotic susceptibility testing becomes routine. This was the initial focus, until our results showed the effect of plasmid acquisition on the bacterial MALDI-TOF MS spectrum, which is not only important for healthcare but Biotechnology. The significance of method developing in microbial identification and rapid detection of antibiotic resistant organisms has been highlighted by several nations in the world (UK, USA, Germany and France), as antimicrobial resistance is sometimes predicted to kill more than 2 billion people by the year 2050 (O’Neil, 2014).

# 1.6 CHAPTER SUMMARY

Traditional methods for identification have served us well for approximately seven decades, however advances in our understanding of infections suggest that more research is needed into identification of pathogen due to its vital role in clinical management. Several methods have been highlighted and described in this chapter alongside their limitations which restrict clinical use. The work by Leeuwenhoek using simple lenses was only the start to identification in microbiology, the world still awaits the method/tool/technique that closes this chapter of Pathology.

**CHAPTER 2**

**LITERATURE REVIEW**

# 2.1 MASS SPECTROMETRY (MS)

The previous chapter gave an introduction into the significance of microbial identification in clinical microbiology. The problems associated with current clinical identification methods limits its efficiencies in producing a rapid and accurate diagnosis. This difficulty can be attributed to time constraints, cost implications, sensitivity issues and the need for more understanding on these infectious agents. However, the use of molecular methods of identification has now been complemented with the use of mass spectrometry in many diagnostic laboratories (Bailey, 2013). Mass spectrometry is a chemical analytical technique for measuring the mass of unknown molecules by ionizing, separating and detecting the ions according to their mass-to-charge ratios. Although mass spectrometry is making its mark on all facets of clinical laboratory medicine, arguably no field is witnessing its impact more than clinical microbiology (Bugni, 2017). The basic processes in mass spectrometry simply involve the conversion of the sample into gaseous ions (ionization) followed by the characterisation and separation according to their specific mass to charge ratio (m/z) and relative abundance of each ion type (Awad, Khamis and El-Aneed, 2014). The separation of ions is based on mass to charge ratio, detected in proportion to their abundance and displayed as a mass spectrum. The ions produced from the sample provides information regarding the nature and structure of their precursor sample or molecule via the peaks inside the spectrum. A typical example of what a mass spectrum looks like is shown in Figure 2, which is displayed in the form of a plot of ion abundance on the y-axis and the mass to charge ratio on the x-axis.



**10000 30000 50000 70000 m/z**

Figure 2: An example of a typical mass spectrum of bacterial isolate. The molecular weight of the fragments (m/z) is shown on the x-axis, while the relative intensity is shown on the y-axis.

## 2.1.1 Components of a Mass Spectrometer

The mass spectrometer comprises of three fundamental units which work together systematically (Boesl, 2017). These units as depicted in Figure 3 are: the ion source, mass analyser and the detector system.

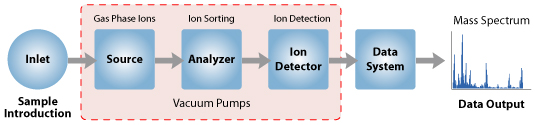


Figure 3: The basic components of a Mass Spectrometry system. (Sauer, 2010).

**Ionisation**

There would be no sample ionisation without the ion source. For analysis to occur in a mass spectrometer, samples must first be ionised (production of ions) before they can be measured or analysed (Dermatini, 2013). The process of ionisation varies widely depending on the thermolability of the sample involved and thus there are several types of ionisation techniques used in mass spectrometry. Hard ionisation techniques leave molecules in excited energy states which relax bond cleavage, thereby giving “daughter ion” fragments at a very lower m/z. On the other hand, soft ionisation techniques impart little residual energy onto the sample and thus minimise fragmentation resulting in spectra with fewer peaks. Examples of hard ionisation techniques are electron impact, chemical ionisation and fast atom bombardments, which are be effectively for samples that are not thermolabile.

Electron Impact ionisation works by volatizing a sample directly in the source that is contained in a vacuum system directly attached to the analyser. The gas phase molecules are bombarded by a beam of electrons which ejects an ion from the gas phase molecules to produce a radical ion (Wilm, 2011). Fast Atom Bombardment (FAB) works by mixing the sample with a matrix (glycerol) and bombarding the mixture in a vacuum with a beam of atoms (commonly used are inert gas - Argon or Xenon) which are accelerated to kilovolt energies (Schafer and Budzikiewicz, 2001). During this process, ions are formed which are adducts to the original sample. Also, with the atmospheric pressure chemical ionisation, a spray of solvent is heated to very high temperature (above 4000 C) and sprayed with high flow rates of nitrogen (Jin *et al*., 2016). This results in an entire aerosol cloud subjected to a corona discharge which creates ions with the evaporated solvent acting as the chemical ionisation reagent gas.

However, for thermolabile samples such as peptides, proteins and most biological samples, soft ionisation techniques such as electrospray ionisation (ESI) and matrix assisted laser desorption ionisation (MALDI) are generally employed. Electrospray ionisation techniques work by putting a high voltage on a flow of liquid at atmospheric pressure which creates a spray. This spray is then directed to an opening in the vacuum system of the mass spectrometer where droplets are de-solvated by a combination of heat, vacuum and acceleration into gas by voltages (Banerjee and Mazumdar, 2012). After a while, ions are ejected from the highly charged droplets and accelerated into the mass analysers in the presence of a high voltage (2.5 – 6.0 kV). In matrix assisted laser desorption ionization, the sample is initially mixed with a matrix and then bombarded with a laser (often nitrogen laser) in a vacuum (Rahi, Prakash, Shouche, 2016). The matrix absorbs the laser radiation and then transfers a proton to the sample to ionise the sample. The ions formed from the samples (mostly singularly positively charged) are separated by their charge to mass ratio for identification. Mass spectrometers measures mass of an ionised sample in m/z (m = mass and z = charge on the molecule). Dalton (Da) is a standard unit of mass that quantifies mass on an atomic or molecular scale and also a unit of measurement for mass spectrometer samples; Dalton (Da) is the standard unit of mass which has a value of equal to 1.661 x 10-27 kg (Chang, 2013). In summary, samples either in solid or liquid form are directly introduced into the mass spectrometer where they are subjected to ionisation via the ionisation source, which produces ions that acquire kinetic energy and leave the source into the mass analysing chamber.

**Mass Analyser (ion separator)**

After sample ionisation, the ions produced are analysed based on their masses via the mass analyser device which separates ion based on charge to mass ratio (Gross, 2017). Once the ions leave the ionisation source, they pass through a calibrated chamber which analyses the passing ions as a function of their mass to charge ratios. There are different kinds of mass analyser, using either static or dynamic fields, magnetic or electric fields. The mass analyser consists of a set of four conducting rods arranged in parallel with a space in the centre; the opposing pairs of the rods are connected to each other electrically (Syed Maher and Taylor, 2013). The separation of the ion is based on the stability of their flight trajectories through the oscillating electric field in the quadrupole. The electric field is generated when a radio frequency (RF) voltage is applied between one pair of opposing rods within the quadrupole, while the DC offset voltage is then applied to the other pair of opposing rods. Ions that will have a stable flight path through the quadrupole in the resulting electric field are those of certain m/z values, while other ions have unstable trajectories and thus will not reach the detector.

Furthermore, the ion trap mass analysers consist of a ring electrode and two end caps, similar to the quadrupole. It uses a combination of both electric and magnetic fields to ‘trap’ or capture the ions inside the mass analyser (Dermatini, 2013). There are several configurations of ion traps, they include 3D ion traps, a linear ion trap and electrostatic trap. In Fourier transform mass analyser, the frequency of the ions in a fixed magnetic field are used to determine the mass to charge ratio of ions. The ions are trapped in a penning trap, where they get excited to a large cyclotron radius by an oscillating electric field orthogonal to the magnetic field. The ions induce a charge which results in the superposition of sine waves, from where useful signal is extracted to obtain a mass spectrum (Scigelova *et al*, 2011). Lastly, the time of flight mass analyser uses an electric field to accelerate the ions through the tube and then measures the time it takes for each ion to reach the detector (El-Aneed, Cohen and Banoub, 2009). If the particles all have the same charge, the kinetic energies will be same and thus their velocities will depend solely on their masses. This then means that the ion with a lower mass will reach the detector fast as they can easily travel faster than other higher-mass ions.

**The Ion Mass Detector**

Once the ions have been separated via the mass analyser, the detection of the ion is made possible. The detection occurs when the detectors converts the energy of the incoming ions into a current signal that is registered by the electronic devices and transferred to the acquisition system of MS (Shrader, 2014). Examples are: photo plate, solid-state, image current and Faraday cup.

# 2.2 APPLICATION OF MASS SPECTROMETRY TO BIOLOGICAL CHARACTERISATION

The direct and hard ionisation of molecules have a major limitation on its use in diagnostic microbiology because it causes the analyte to split into tiny fragments (large degree of fragmentation) and thus the loss of the structure makes it impossible to study (Clarke, 2016). This necessitated the need for a softer ionisation technique, which does not break microbial molecules on impact leaving the structure shattered. Advances led to the discovery of soft ionisation techniques such as electrospray ionisation (ESI) and matrix-assisted laser desorption ionization (MALDI) that can transform biomolecules into ions (El-Aneed, Cohen and Banoub, 2009). The development of soft ionization techniques has made the application of mass spectrometry to biological materials unlimited, as samples need not be in vapour phase before ionisation.

This use of soft ionisation MS methods started with the discovery of alanine which could be easily ionized without fragmentation if mixed with another amino acid (tryptophan) and irradiated with a pulsed 266nm laser (Karas & Hillencamp, 1988). These two scientists first introduced the concept of soft ionisation methods into clinical analysis. Tryptophan could easily absorb energy from irradiation, which helps to ionise the alanine, which does not readily absorb energy. They attributed this effective and efficient ionisation to be due to the presence of a ‘matrix’ (highly absorbing substance) and the precision of the laser light source. This was detailed in a paper published in 1985 titled the “influence of the wavelength in high-irradiance ultraviolet laser Desorption Mass Spectrometry of Organic molecules” (Karas, Bachmann & Hillencamp, 1985).

Around the same time, a Japanese Engineer, Koichi Tanaka successfully ionised large macromolecules with no loss of structure by using ultra-fine metal powder mixed in glycerol (matrix) in the presence of a proper combination of laser wavelengths (Tanaka *et al*., 1988). He demonstrated this by irradiating cobalt particles in glycerol liquid with a 337 nm nitrogen laser which helped to ionise a carboxypeptidase A without affecting its structure. The metal and the glycerol served as the matrix which helped the ionisation process. He filed his work for patency application and in 1987, it became known as soft laser desorption. In the following year 1988, the two German scientists continued with their research and published a paper titled ‘Laser desorption of proteins with molecular masses exceeding 10,000 daltons’. In the paper, they reported the MALDI-TOF spectrum of four proteins namely, lysozyme from chicken egg white (molecular weight of 14,306), β-lactoglobulin A from bovine milk (molecular weight of 18,227), porcine trypsin (molecular weight of 23,463) and bovine albumin (molecular weight of 67,000). However, it should be noted that the most widely used mass spectrometry method for biological identification is MALDI-TOF, due to its ability to analyse whole biological cells directly, producing simple, reproducible spectral patterns over a broad mass range.

# 2.3 MALDI-TOF MS

One of the most widely used form of mass spectrometry particularly in biological systems is MALDI-TOF MS due to the soft ionizing properties. Although Electrospray ionization (ESI) - mass spectrometry, is also a soft ionising method, however it does not produce singly charged ions like MALDI-TOF MS, thus data interpretation is difficult (Singhal *et al*, 2015). Also, ESI-MS analysis requires a prior separation by chromatography for accurate results which is not needed for MALDI-TOF MS analysis (Everley *et al*, 2008). The MALDI-TOF MS system has presented a powerful platform for the analysis of a variety of different endogenous and exogenous molecules directly on cells or in tissues. MALDI-TOF MS has been reported in several journals to be accurately used in microbial identification and strain typing, epidemiological studies, detection of biological warfare agents, detection of water and food borne pathogens and antibiotic resistance detection (Greco, 2018). In MALDI-TOF MS, the sample for analysis is prepared by mixing it with a matrix (an energy-absorbent organic compound). The sample entraps with the matrix and crystallises with it on drying, followed by ionisation with a laser beam. Ionisation of the sample via the matrix generates singly charged ions which are then accelerated through the flight tube from where they are separated. During MALDI-TOF analysis, the m/z ratio of an ion is measured by determining the time required for it to travel the length of the flight tube (time of flight -TOF). The TOF information then generates a spectrum called the peptide mass fingerprint (PMF) which is unique for each sample (Singhal *et al*, 2015).

For species level identification of microbes, a typical mass range of 2-20 kDa is used because the majority of the masses of ribosomal proteins of micro-organisms are found within this range. (Croxatto Prod’hom, Gerub, 2012; Murray, 2012). According to Murray (2012), the unique pattern of highly abundant ribosomal proteins representing 60-70% of the dry weight of a microbial cell in the mass spectrum range of 2-20kDA is used to identify a particular microbe by matching its peptide mass fingerprint (PMF) pattern with the PMFs of the ribosomal proteins in the database. It is worthy of note that the use of matching biomarker masses with the molecular masses of proteins predicted from the genome sequence is not very common in microbiological diagnostic laboratories. This is because the complete genome sequence of an organism needs to be known, before a database of its predicted protein molecular masses could be developed (Croxatto Prod’hom, Gerub, 2012). In overall, databases are important in the MALDI-TOF MS systems particularly in the areas of identification and diagnosis.

So far, the application of MALDI-TOF MS has been successfully applied to bacteria and fungi, however its success in viruses and protozoans is yet to be confirmed. The reason for lack of success in viruses could be attributed to the relatively low protein content, higher molecular weight of viral proteins (>20,000 Da) and possibly the carryover of contaminants from the cell substrate in which viruses are cultured in vitro (Kliem and Sauer, 2012). Although not available in clinical settings yet, there have been reports of MALDI application to viruses (Calderaro *et al*, 2014; Cobo, 2013). On the other hand, parasites have several morphological forms they change to, which tends to make the routine method of bacterial identification not ideal for this class of micro-organism.

# 2.4 MALDI-TOF MS IN CLINICAL DIAGNOSIS

The last decade has seen the development of mass spectrometry as an ordinary research tool to being at the forefront of clinical microbiology diagnostics. This revolution brought about by MALDI-TOF MS has completely changed the practices for microbial identification and its establishment as the standard technology for cultivated microorganisms in many clinical laboratories in developed countries (Yssouf *et al*., 2014). This is due to the dramatically shortened time, from sample culturing to result, reduced consumable costs and improved quality of results. Before the advent of MALDI-TOF MS, other molecular methods were routinely used for microbial identification. However different studies have shown that MALDI-TOF MS has produced similar or better identification accuracy and quality of information at a relatively lower cost and a faster pace. For the first time in 1996, MALDI-TOF spectral fingerprints were obtained from whole bacterial cells such as *E.coli*. The unique mass spectra obtained for every micro-organism is largely based on differences and similarities in chemical markers, mostly ribosomal proteins. The resulting spectral patterns are then used to identify bacterial species by comparing the patterns with several library spectra ‘fingerprints’ or by using vital protein markers to directly identify the bacterial species. Within less than a decade, MALDI-TOF MS has swiftly become the new gold standard method for microbial identification in clinical microbiology laboratories (Schubert and Kostrzewa, 2017).

The first comprehensive microbial database of MALDI-TOF MS profiles was developed and published in 2004 using NCTC bacterial cultures (Keys *et al*.,2004). The use of MALDI-TOF MS in clinical microbiology has secured both in-vitro diagnostics (IVD) approval in Europe and also the Food and Drug Administration (FDA) approval in the United States. With constant updates to the database, MALDI has allowed the rapid identification of 90-95% of bacteria commonly found in clinical diagnostic laboratories (Emonet *et al*., 2010; Welker, 2011).

## 2.4.1 Microbial identification via MALDI-TOF MS

The acceptance of MALDI-TOF MS for bacteria identification started with several scientific research publications which concluded on the superiority of MALDI-TOF MS over all other available methods at the time, especially the conventional biochemical methods (Wang *et al*., 1998; Demirev, *et al*., 1999; Pineda *et al*., 2000). The identification of bacteria (particularly pathogenic ones) at least down to the species level is a key function of any microbiology laboratories. The bacterial identification process helps to provide knowledge to the cause of the bacterial infection, which guides the physicians in the course of treatment. The rapidity and reliability of the identification results is also of importance to clinical management, due to the rapid progression of some bacterial infection such as Meningitis, flesh eating bug or Tuberculosis.

The first comparison of bacterial identification via MALDI-TOF MS with other methods suggested MALDI superiority over other methods. (van Veen, Class and Kuijper, 2010). The paper details the use of MALDI-TOF MS in the evaluation of 327 clinical isolates which have been previously cultured from patients’ samples (blood, urine, pus, biopsy, swab from any site of the body, cerebrospinal fluid, respiratory tract and wound specimens) and identified by conventional techniques (biochemical tests - Vitek II, API). The identification result revealed that MALDI-TOF MS has higher overall performance in terms of micridentification which is significantly better than the conventional biochemical methods. Precisely, MALDI-TOF MS correctly identify the bacteria and yeast species (92.2% and 83.1% respectively), while it had fewer incorrect genus identification (0.1% and 1.6% respectively). According to individual species, 97.7% of *Enterobacteriaceae* were correctly identified by MALDI-TOF MS, 92% of non-fermentative Gram-negative bacteria, 94.3% of *Staphylococci*, 84.8% of *Streptococci*, 84% of miscellaneous group (*Haemophilus, Actinobacillus, Cardiobacterium, Eikenella* and *Kingells*) and 85.2% of yeasts. The paper reported that MALDI-TOF MS performed significantly better than conventional methods and concluded that MALDI-TOF MS can be implemented for routine identification of bacteria and yeasts. In addition, another research team in Hungary performed the first experiment on identifying *Bacteroides* using MALDI-TOF MS (Nagy *et al*., 2009). As it is widely known, *Bacteroides fragilis* are involved in mixed infections of different origins (such as in GI tract infections) and the isolates are phenotypically similar and grow more slowly than aerobic bacteria. This often causes misidentification using classical or automated phenotypical identification methods. However, MALDI-TOF MS was applied to 277 clinical isolates of the Bacteroides genus and compared to results obtained from conventional phenotypical identification of the isolates which served as the reference. From the 277 isolates, MALDI-TOF MS correctly and unequivocally identified 270 isolates by comparison to the reference strains present in the MALDI database. MALDI-TOF MS identified 23 species differently from the conventional phenotypic identification, 11 of these were sequenced. The sequencing data confirmed that MALDI-TOF MS was correct in ten cases, while the sequencing data could not lead to determination of species in the last one. The authors concluded that the power of discrimination and accuracy of identification of MALDI-TOF MS is superior to that of biochemical testing for Bacteroides species. Another review compared two methods of MALDI-TOF MS (VITEK MS and Bruker Microflex MS) with an API 20AN for identification of clinically relevant anaerobic bacteria (Jamal, Shahin and Rotimi, 2013). After the analysis of 274 isolates of clinically significant anaerobic bacteria which were recovered from clinical specimen cultures, the authors concluded that the two MALDI-TOF MS methods gave high confidence identification results. This suggests that MALDI-TOF MS is still a reliable method for anaerobic bacteria, compared to other genotypic methods and reduction in operation costs in an anaerobe laboratory.

## 2.4.2 MALDI-TOF- MS: the gold standard in bacterial identification

As of today, MALDI-TOF MS has gained an undisputed superiority over all other means of microbial species identification in the routine clinical laboratory (van Belkum *et al*., 2017). Despite its introduction into routine laboratories approximately ten years ago, it has become a leading method for rapid microbial species identification. Although, there is no single diagnostic method in clinical microbiology that shows 100% accuracy, however, MALDI-TOF MS shows a relatively higher level of accuracy (van Belkum, 2015). It is regarded as a powerful bacterial identification method owing to its reproducibility, speed, sensitivity of analysis and rapid results, which are available within minutes to a few hours rather than several days as obtained in other methods. In general, a minimum of approximately 104 cells per sample is required to yield a spectrum of sufficient quality for microbial species identification. Isolated bacterial colonies are picked from an agar medium and smeared in a thin film onto a MALDI stainless steel target plate, overlaid with a matrix of choice and introduced in the mass spectrometer for data acquisition. The resulting spectral patterns can be used to identify bacterial species by comparing the patterns with a library of known spectra ‘fingerprints’ (reference database) or by using vital protein markers to directly identify the bacterial species. These microbial spectra generated are often between 2,000 and 20,000 Daltons (m/z) as shown in Figure 4.

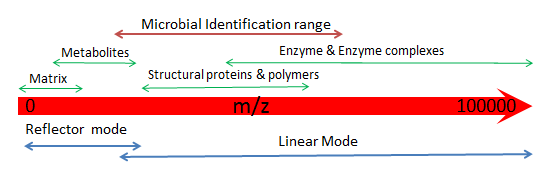


Figure 4: A mass-to-charge range of 0 to 100,000 m/z for different types of analyte in MALDI-TOF MS. Each analyte has most of its peaks within the arrow directly below it.

The use of MALDI-TOF MS in microbial analysis is aided by certain organic acids (matrix), which when co-crystallised (by mixing) with the analyte, allows for the facile ionisation of large biomolecules. Several matrices are used for microbial characterisation depending on the size of the sample, although the most common and widely used matrix is the α-cyano-4-hydroxycinnamic acid (CHCA) (Tsai *et al*., 2017). The nitrogen laser at a wavelength of 335nm or 337nm is fired at the matrix crystals which absorbs the laser energy making it ionised. The ionised matrix then transfers a proton to the analyte molecule thus charging the analyte. The chemical composition and the methods of preparation of these matrices have been shown to have a great influence on the quality of the MALDI mass spectra of peptides and proteins. The extent or the level of discrimination is highly dependent on several variable factors such as the sample-matrix solution compositions, sample-matrix preparation method, the pH, the rate at which the sample-matrix co-crystals are grown and the method of layout on the MALDI plate. As stated above, the sample-matrix ratio and preparation method has a great impact on the quality on the MALDI mass spectra. This is because the mixture of the sample and matrix forms crystals which gets ionised in the MALDI vacuum chamber by accepting a proton, however, the rate of formation of the crystals determines the ionisation process. A number of sample-matrix preparation methods have been developed, one of them is the dried-drop method (crystals forms directly on MALDI plate) which is the earliest and most widely used procedure. Another sample-matrix preparation method is the slow crystallisation method (crystals from slowly in a tube before it is transferred to the MALDI plate. The rapid crystallization is another sample-matrix preparation method, in which the sample-matrix mixture is put onto the MALDI target plate and immediately transferred to the MALDI-TOF chamber.

## 2.4.3 Non-bacterial identification via MALDI-TOF MS

Similar to bacterial identification, the use of conventional methods for identification are time consuming and also requires a considerable amount of expertise. One of the first trials of using MALDI-TOF MS for non-bacterial species was performed by a research team in Germany, which evaluated the use of MALDI-TOF MS for the rapid routine identification of clinical yeast isolates (Markelein *et al*, 2009). A total of 18 type collection strains and 267 recent clinical isolates of yeast made up of *Candida, Cyptococcus, Saccharomyces, Trichosporon, Geotrichum* and *Blastoschizomyces* species were put through MALDI-TOF MS for identification. The results were compared to those obtained by conventional phenotypic and biochemical tests. The result showed accurate species identification for MALDI-TOF MS was obtained for 247 of the clinical isolates (92.5%). The remaining 20 isolates could not be identified due to lack of a comprehensive database, but after database complementation, all isolates were unambiguously identified. The biochemical diagnostic system could only manage to identify 244 isolates.

Recently, the use of MALDI-TOF MS has been applied to identification of filamentous fungi of the order of Mucorales (Shao *et al.*, 2018). MALDI-TOF MS was able to identify a total of 111 isolates covering six species of Mucorales down to 100% genus level and 90% species level using the Bruker and BMU databases. In addition, the potential use of MALDI-TOF MS for detection and identification have been investigated in the field of entomology and parasitology. According to Laroche *et al*., (2017), MALDI-TOF MS has the ability to identify parasites down to the species level. With a running cost lower than $1 per sample, this technology is far less expensive than the current traditional methods employed, even despite the huge initial cost of the device (Lo *et al*., 2015). Also, MALDI-TOF MS have been successfully used in the identification of parasites like *Leishmania, Cryptosporidium, Entamoeba* and *Giardia* (Singhal, Kumar and Virdi, 2016). In summary, the authors concluded that MALDI-TOF MS was a better tool for identification of yeast and yeast-like fungi owing to its rapidity, reliability, lower costs, easy interpretation of results and a fast turnaround time.

## 2.4.4 Limitations of MALDI-TOF MS in microbial identification

The unique specificity of MALDI-TOF MS in microbial identification makes this method one of the most preferred in clinical diagnosis and often referred to as the current gold-standard diagnostic method. However, MALDI-TOF has its limitations. The first limitation of this method is the inability to be performed directly on clinical samples except the samples are first grown artificially in a laboratory to generate colonies (Kock *et al*., 2017). Most clinical samples have a relatively low number of microorganism present, which does not allow for accurate spectra acquisition. The number of micro-organism present in a clinical sample is boosted by enriching the sample in a solid or liquid culture phase before identification can be possible. In general, a microbial load of approximately 104 cells per sample (100 cells per µl) is the minimum biomass needed to produce a spectrum of sufficient quality for identification, which are not usually present in a typical clinical specimen (Hsieh *et al*., 2008). For positive blood cultures, the microbial number is sufficient for identification, often after it has been concentrated and purified by a lysis, centrifugation/filtration step to give pure microbial pellets (Fothergill *et al*, 2013). According to Kohling *et al*., (2012), bacteria can be directly detected from heavily infected urine samples by MALDI-TOF MS without an incubation step. Furthermore, reliable direct identification was obtainable in urine samples with at least 103cells per ml. Although, this direct identification from urine samples is often obscured due to the multiple microbial contamination or the presence of defensin peaks (anti-microbial peptides) which interfere with identification results (van Belkum, 2017).

Another major limitation of MALDI-TOF MS is the differentiation of closely related species. Although, the differentiation of *Shigella species* and *E. coli* by MALDI have been a problem in the past however novel methods have been proposed which are yet to be validated or approved (Paauw *et al*., 2015). Other closely related species which poses a distinction problem for MALDI-TOF MS include members of the genus *Streptococci* and *Pneumococci* as well as the members of the C*andida albicans* complex. In addition, species with a low rate of differences in their ribosomal protein sequences, such as *Stenotrophomonas maltophilia*, *Propionibacterium acnes* and members of the *Streptococcus oralis* group, can be misidentified by MALDI-TOF MS. The difficulty of differentiating down to species level of certain microbes, such as *Mycobacterium spp*, *Burkholderia* *spp*, *Acinetobacter spp* and *Corynebacteria*, has also been reported due to their high degree of genetic similarity (Girard *et al*., 2016). Another major limitation is the spectral interference due to the presence of spores in some organism species, e.g. *Clostridium spp.* Other problems associated with MALDI-TOF MS include difficulty in lysing cell wall structures of some organisms, particularly those that possess capsules, thus preventing efficient lysis of cells and resulting in weak extraction yield and poor spectral quality.

It is true that a larger portion of microbial species encountered in a routine clinical laboratory have MALDI-TOF MS databases which are fairly comprehensive. Thus, it is safe to suggest that the current available databases are sufficient enough to allow effective routine microbial identification via MALDI-TOF MS. However, only a small percentage of the global microbial diversity is well known and described which means MALDI-TOF MS microbial database can never be complete (Lagier *et al*., 2016). This means that the MALDI-TOF MS commercial databases needs to be updated on a regular basis to include species of sufficient clinical relevance to human health. The United States FDA’s guideline states that a species can be incorporated for routine IVD in an approved database if at least 30 unique isolates are collected during clinical trials. This poses a challenge due to the limitation involved in finding sufficient number of strains to build an accurate MALDI-TOF MS database for identification. Moreover, unlike bacterial with constant updated databases, non-bacterial species such as parasites and some yeasts do not have databases. This calls for the creation and continuous updates of such new databases.

In summary, the various techniques used for microbial identification earlier highlighted in the previous chapter and here often have advantages and disadvantages as pointed out. Table 1 below summaries the various microbial identification methods described above and highlights key aspects in the overall processes.

Table 1: Comparison of the several microbial identification methods available in clinical and diagnostic laboratories.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **METHOD TYPES** | **Traditional – Phenotypic** | **Molecular Methods** | | **Chemotaxonomic Methods** |
| Examples | Biochemical methods | RT-PCR | 16s rRNA gene sequencing | MALDI-TOF MS |
| Sensitive | Yes | Yes | Yes | Yes |
| Specific | Yes | Yes | Yes | Yes |
| Rapid | Yes/No | Yes | No | Yes/No |
| Labour intensive | Yes | No | No | No |
| Easy data interpretation | Yes | Yes | Yes | Yes |
| Cost effective | Yes | Yes | Yes | Yes |
| Automation | Can be semi-automated | Yes | Yes | Yes |
| References | (Becerra, 2016) | (Kralik, 2017) | (Marino, 2018) | (Calderaro *et al*., 2016) |

Table 1 shows the summary of the various identification methods obtainable in clinical microbiology with their respective low and high points. However, MALDI-TOF MS seems to currently be at the forefront of routinely identifying bacteria and yeast in clinical laboratories due to its relative rapid timing and low per sample costs. With the on-going research and updates in this field, the reach of MALDI-TOF MS in clinical microbial diagnosis is yet to be known. Interestingly, many papers have reported another potential revolution in clinical microbiology with regards to the use of MALDI-TOF MS in antimicrobial susceptibility testing (AST). This is because the application of MALDI-TOF MS for AST would provide further utility for this method and also allow rapid identification and susceptibility testing of pathogens with a single assay.

**2.5 Antimicrobial Resistance**

Antimicrobial resistance represents one of the most important challenges in clinical microbiology today, due to its potential to be a major problem in successful treatment. Antimicrobial resistance is capable of rendering all current antibiotics ineffective thereby facilitating the spread of all kinds of infections which can easily result to death (O’Neil, 2016). The challenges of antimicrobials in combatting infectious agents in modern day are on the increase, due to the rising level of resistance and the emergence of multidrug-resistant strains (O’Neil, 2016). The rise to optimum effectiveness of antimicrobials can only begin with the resolving and providing newer and faster diagnostic platforms. This is because antibiotic resistant bacterial infections often require rapid administration of appropriate and effective antibiotic therapy, which can be achievable only by rapid diagnostic means. The provision of rapid and accurate pathogen identification will help prevent the misuse of antimicrobials, which is why research is ongoing on the novel method for detection and identification of pathogens which can be inculcated into routine diagnosis. Therefore, diagnostic assays which are capable of rapid and correct determination of pathogen resistance profiles are critical in health care settings.

Few methods have been used routinely employed for AST of medically important organisms for a number of decades. One of these AST methods is the disk diffusion method invented by (Bauer *et al*., 1966) which is still in use in many diagnostic laboratories. Another method is the broth micro-dilution (BMD) testing which is commonly used due to the fact that other AST methods are compared to it during verification, validation and clinical trials (Stalons, 1975; van Belkum and Dunne, 2013). Another advantage of BMD is that it gives a numerical minimum inhibitory concentration (MIC) value, which is the lowest antibiotic concentration that completely inhibits the visible growth of a microorganism (Talaro and Chess, 2017).

Table 2 shows the comparison of current AST methods in use while Table 3 shows the future potentials or alternatives for AST. Although the conventional methods for AST have been certified for routine use across the globe, however the time-factor limitation has necessitated the need for newer methods. The newer technologies have to primarily improve the time factor limitation as well as compete with the current reference standards.

Table 2: Current antimicrobial susceptibility testing methods

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Current AST Methods** | **Principle** | **Requires growth of isolates** | **Cost** | **Automation (Manual or Automatic)** | **Test time in hours** | **References** |
| Agar dilution testing | Growth inhibition on solid medium with antibiotics | Yes | Low | Manual | >10 | (Haltalin, Markley and Woodman, 1973) |
| Automated testing (VITEK) | Monitoring of growth or substrate conversion in a dedicated machine using optics | Yes | Low | Automatic | <10 | (Mazzariol *et al*., 2008) |
| Broth dilution testing | Growth inhibition in liquid medium together with antibiotics. | Yes | Low | Both | >10 | (McDermott *et al*., 2006) |
| Disk diffusion | Growth inhibition measurement around an antibiotic-containing disk | Yes | Low | Both | >10 | (Gianecini *et al*., 2018) |
| E-test | Measurement of growth inhibition around a strip containing an antibiotic gradient. | Yes | Low | Both | <10 | (Liu *et al*., 2014) |
| Real-time microscopy | Looking and Filming division of bacterial at the single-cell level | No | Low | Manual | <1 | (Fredborg *et al*., 2015) |
| Live-dead fluorescent staining | Microscopy of (non) permeable cells in the presence of fluorescent stains. | No | Low | Manual | <1 | (Feng at al., 2014) |
| PCR Gene detection | DNA amplification | No | Low | Both | <1 | (Furlaneto-Maia *et al*., 2014) |

Table 3: Developed/alternative methods for routine antimicrobial susceptibility testing.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Future potentials for AST** | **Principle** | **Requires growth of isolates** | **Cost** | **Automation** | **Test time in hours** | **References** |
| Calorimetric | Detection of heat produced by stressed bacteria | Yes | Intermediate | Automatic | <5 | (Tunney *et al*., 2004) |
| RNA sequencing | Definition of gene expression differences by sequencing | No | High | Automatic | 10 | (Khaledi *et al*., 2016) |
| FACS | Sizing and measuring differential fluorescence between living and dead cells | No | Intermediate | Automatic | <5 | (Saint-Ruf *et al*., 2016) |
| Bacteriophage amplification | Detection of phage reproduction in living cells only | No | Intermediate | Manual | <10 | (Hemvani, Patidar and Chitnis, 2012) |
| Cell death markers | Detection of compounds produced upon bacteria cell death | Yes | Intermediate | Both | <1 | (Mlynárčik and Kolar, 2016) |
| Next generation sequencing | Sequencing of all cellular DNA and RNA | No | High | Automatic | **>**10 | (Ellington *et al.*, 2017) |
| MALDI- TOF MS | Detection of antibiotic degradation products | Yes | Intermediate | Automatic | **<10** | (Idelevich *et al*., 2018) |

From the list of potential future technologies for AST, MALDI-TOF MS is well positioned and closer to be the next globally accepted method for routine AST, due to emerging prospects and vast research currently going on. The application of MALDI-TOF MS for AST would not only provide further utility for this technique but also allow the rapid identification and susceptibility testing of pathogens with a single assay. Currently, there are four known developing categories of MS-based methods for rapid determination of resistance or susceptibility. First among them is the identification of resistant clones by MALDI-TOF MS, using the same spectrum used for identification for typing purposes. This approach only works when the proteins conferring resistance falls within the mass range used for WCMS identification. For example, some methicillin-resistant *Staphylococcus aureus* (MRSA) strains have the phenol-soluble modulin (Psm) protein at *m/z* 2,415 Da (Josten *et al*., 2014). Detection of an intense peak at *m/z* 2,414 Da indicates methicillin resistance although absence of the specific MALDI peak does not correlate to susceptibility. Another antimicrobial susceptibility testing (AST) method in MS is via the detection of microbial growth in the presence of antimicrobials by relating protein peaks to the internal standard peak and reaching conclusions based on the pattern of growth inhibition (Jung *et al*., 2016). The third approach is via detection of antibiotic modifications by degrading enzymes, through the observation of a mass shift due to the hydrolysis of the antibiotics resulting in significant mass shifts (Idelevich *et al*., 2018). The last approach is via the detection of proteins responsible for the resistance by MS/MS method. Although proteins are not directly detected by MS/MS approach, but subjecting the proteins extracted from a colony sample to tryptic digestion results in a mixture of a large number of peptides. These peptides can be easily analysed via LC-MS/MS method, this is done by identifying specific peptides based on their parent ion mass and fragmentation patterns which can then be used to ascertain the proteins of interest.

Recent studies suggested MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA) as an alternative MALDI-TOF MS based method for AST which could solve the limitations of the earlier methods (Jung *et al*., 2016). This technique works by utilising semi-quantitative MALDI-TOF MS to measure the relative growth rates of bacterial isolates exposed to antibiotic compared to untreated controls during a short incubation step. Samples are spiked with a reference standard from the database to allow normalization across spectra, while the total area under curve (AUC) of all peaks within a defined mass range is calculated to provide a measure of microbial growth. A significantly reduced AUC generated from antibiotic treated samples relative to the controls indicates susceptibility to the antibiotic. One major limitation of the assay is the necessary optimization of the concentration of antibiotic to be used and incubation time for each species (Sparbier, Schubert and Kostrzewa, 2016). Although it has been applied to a small range of clinically relevant gram-negative species with a great deal of success but not for Gram-positive species (Ceyssens *et al*., 2017). However, a recently published paper, demonstrated for the first time that the resistance profile of a Gram-positive species (*S. aureus)* can be determined using a semi-quantitative MALDI-TOF MS (MBT-ASTRA). Overall accuracy of approximately 95% across 35 strains for each antibiotic tested was obtained, which is marginally lower than the accuracies noted in previous studies for even Gram-negative bacteria (Maxson, Taylor-Howell and Minogue, 2017). Research into non-bacterial species such as protozoans are also vital due to the various number of clinically important infections they cause.

# 2.6 ACANTHAMOEBA

## 2.6.1 Background

The protozoan *Acanthamoeba* is the organism of interest for this research. *Acanthamoeba* is a free-living microscopic amoeba causing a rare but sight-threatening infection of the eye as well as infections of the skin and the central nervous system. The parasite is found worldwide, in diverse natural environments, mostly in lakes, swimming pools, fresh and seawater, pond water, hot spring resorts, heating air condition units, soil and the air (Marciano-Cabral, 2003). *Acanthamoeba* have been isolated from distilled bottled waters (Legarreta, 2013), nuclear power plants (Siddiqui, 2012), sewage (Schroeder, 2011), swimming pools (Caumo, 2009), contact lens equipment (Woodruff, 1999), medicinal pools (Kaji, 2005), compost and vegetables (Khan, 2006). Also, they have been recovered from human nostrils, throats, lung tissues, skin lesions, corneal biopsies, stool samples, cerebrospinal fluids and the brain necropsies (Ozpinar, Ozcelik and Tunlu, 2017; Liang *et al*., 2010) This shows that the parasite is ubiquitously present in the environment, which means humans are exposed to the organism on a daily basis as evidenced in the presence of anti-*Acanthamoeba* antibodies in healthy individuals (Brindley, Matin and Khan, 2009). The amoeba spreads to the eyes mainly through contact lenses, to the skin via cuts and skin wounds and to the lungs via inhalation. Although most people are exposed to the organism during their life time, however, very few are infected from the exposure. The factors that contribute to *Acanthamoeba* infection and severity include parasite biology, genetic diversity, environmental spread and host susceptibility (Siddiqui, 2012).

## 2.6.2 Cell Structure and Biochemistry

*Acanthamoeba* is from two Greek words; ‘acanth’ meaning ‘spikes’ and ‘amoeba’ meaning ‘to go’. The organism contains one or more prominent contractile vacuoles, whose function is to expel water for osmotic regulation (Siddiqui and Khan, 2012). Several other types of vacuoles are present in the cytoplasm and they are lysosomes, digestive vacuoles and a large number of glycogen-containing vacuoles. With regards to the composition, plasma membrane consists of proteins at 33%, phospholipids at 25%, sterols (13%) and lipophosphonoglycan at 29% (Dearborn, 1974). The main fatty acid chains in the organism are oleic acids (45-50%) and longer polyunsaturated fatty acids (20-30%). *Acanthamoeba* contains low level of glycolipids, while glucose accounts for about 60% of the sugars of the glycolipids of the whole cells and of the plasma membranes. The parasite possesses large numbers of mitochondria with a genome size of around 40,000 base pairs. The parasite normally possesses a single nucleus that is approximately the one sixth its total volume size, although multinucleate types have been observed (Burger, 1995). *Acanthamoeba* has long been studied as a model eukaryotic cell due to the actin cytoskeleton-based motility. It moves relatively fast compared to other related organisms, with a locomotory rate of around 0.8 µm per second.

With regards to scientific taxonomic classification of the protists, *Acanthamoeba* is grouped as follows (Visvesvara, 1991): DOMAIN: Eukaryota; KINGDOM: Protozoa; PHYLUM: Amoebozoa; CLASS: Lobosa; ORDER: Amoebida; FAMILY: Acanthamoebidae; GENUS: *Acanthamoeba*

## 2.6.3 Morphological Forms

*Acanthamoeba* exists in two stages in its life cycle: trophozoites and cysts. Trophozoites (Greek ‘tropho’ meaning ‘to nourish’) are the active and mobile stage that exhibits vegetative growth feeding on organic particles and other small organisms (microbes). Trophozoites are amoeboid in shape, very small and usually around 15 to 35 µm in length. During the trophozoite stage *Acanthamoeba* divides mitotically under optimum conditions of adequate food supply in addition to neutral pH, a temperature around 30 0C, and 50-80 milliosmols (mOsmol) (Siddiqui and Khan, 2012). Exposure to harsh conditions leads to cellular differentiation into a double-walled cyst form, the outer walls consists of proteins and polysaccharides, while the inner wall possesses cellulose. The dormant stage cysts are incredibly resistant to irradiation methods. The double walls are separated by a space, except at certain points where they form opercula in the centre of ostioles. According to Dudley, Jarroll and Khan (2009), the carbohydrate composition of cyst walls revealed a high percentage of galactose and glucose and small amount of xylose and mannose. Linkage analysis revealed several types of glyosidic linkages including the 1, 4-linked glucosyl conformation indicative of cellulose (Dudley, Jarroll and Khan, 2009). Figure 5 shows the scanning electron microscope (SEM) of the trophozoite form of *Acanthamoeba.*

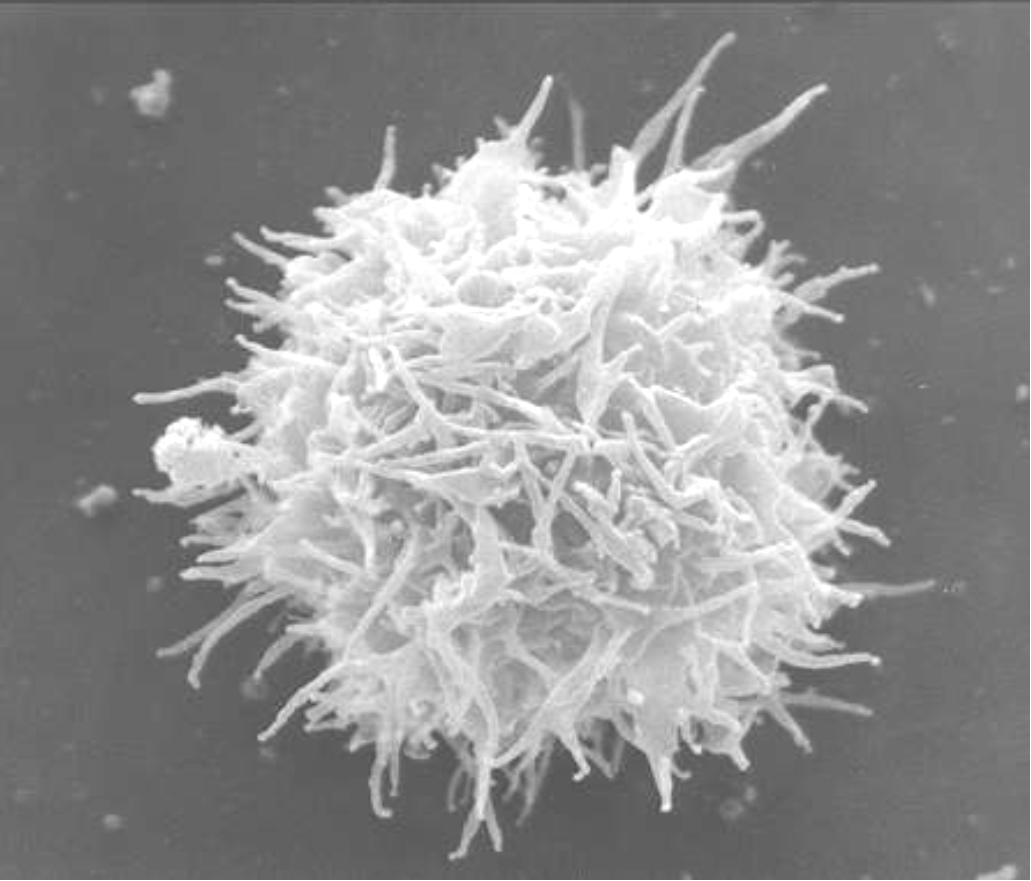


Figure 5: SEM of an *Acanthamoeba* trophozoites. Spiny surface structures called acanthopodia distinguish *Acanthamoeba* from other free-living amoebae that infect humans. (Bar is 1µm). (Siddiqui and Khan, 2012).

## 2.6.4 Associated diseases

*Acanthamoeba* causes three main diseases:

*Acanthamoeba keratitis* (AK): This is the most common disease the organism causes. It is an infection of the eye in a healthy person which can lead to blindness or permanent visual impairment if not treated (Figure 3). It was first identified in 1973, and first published in the UK in 1974 (Nagington *et al*., 1974). *Acanthamoeba keratitis* is an infection of the eye characterised by pain and the late clinical appearance of a stromal ring-shaped infiltrate (Jiang *et al.*, 2015). It can become so severe as to cause permanent visual impairment or blindness. The main risk factor is the contact lens and also low levels of anti-*Acanthamoeba* IgA in tears. Contact lens wearers are at risk of getting infected by the parasite by the combination of one of more of the following sequence in no particular order; wearing contact lens for extended period of time, exposure to contaminated water, biofilm formation on the contact lens, lack of personal hygiene, inappropriate cleaning of the contact lenses and exposure to the eye (Lorenzo-Morales, Khan and Walochnik, 2015). These sequences of events lead to the breakdown of the epithelial barrier; the invasion of the stromal of the eye by *Acanthamoeba*, keratocyte depletion, induction of an inflammatory response, photophobia and finally the necrosis of the eye stroma which leads to blindness (Panjwani, 2010).

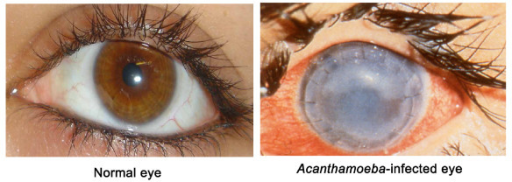


Figure 6: First image is a normal eye, while the second image is an *Acanthamoeba* infected eye that has led to loss of vision. (Siddiqui and Khan, 2012).

Granulomatous amoebic encephalitis (GAE): This is a rare but fatal infection of the central nervous system (CNS) (brain and the spinal cord). It is mainly common in immunocompromised persons, who are susceptible hosts such as individuals undergoing immunosuppressive therapy or individuals with diabetes mellitus, pneumonitis, renal failure, liver cirrhosis or hematologic disorders (Parija, Venugopal and Dinoop, 2015). *Acanthamoeba* entry into the CNS is most likely via the blood-brain barrier.

Disseminated infection: This is a systemic infection all over the body which is common in immunocompromised persons, affecting the lungs, sinuses, skin and other organs independently or in combination (Morrison, 2016).

## 2.6.5 Pathogenesis

The development of the infections caused by this parasite is due to the adhesion of the parasite to the host cell which is made possible mainly by a mannose-binding protein (MBP) (Garate *et al*., 2004). This protein, expressed on the surface of the organism consists of six exons and five introns which spans 3.6 kilo base pairs. The parasite also adheres to the surface of the host cell via other adhesins such as the 28.2 kDa laminin-binding protein, 55 kDa laminin-binding protein and a 207 kDa adhesin (Rocha-Azevedo *et al*., 2009). According to Sissons (2005), the MBP binding leads to production of toxins which leads to host cell death in a phosphatidylinositol 3-kinasedependent (PI3K) manner. Moreover, *Acanthamoeba* has been found to display plasminogen activator activity by catalysing the cleavage of host plasminogen to form plasmin, which activates the matrix metalloproteinase of the host causing degradation of basement membranes. In addition, *Acanthamoeba* possess hydrolytic enzymes such as phospholipases, glycosidases, elastases and a variety of metaloproteases (Omaña-Molina, 2013). These enzymes are secreted only by clinical isolates which indicates their role as a diagnostic target or a potent virulence factor (Huang, 2017). In addition, *Acanthamoeba*’s ability to persist in harsh environmental conditions and its resistance to chemotherapeutic drugs by differentiating into cysts contributes to its pathogenicity In summary, the mechanism in which *Acanthamoeba* breaches biological barriers is complex, most likely to involve the adhesins and enzymes of the parasite as well as the immune susceptibility of the host (Khan, 2006).

## 2.6.6 Symptoms

The symptoms of Acanthamoeba keratitis include a red and frequently painful eye infection that doesn’t improve with treatment, feeling of something moving in the eye, tearing and sensitivity to light. Other symptoms that can be observed are blurred visions, red irritated eyes that lasts longer than usual even after removal of contact lenses.

## 2.6.7 Detection and Identification

Effective treatment of all *Acanthamoeba* infections is based on its early detection. The diagnosis of *Acanthamoeba* keratitis is problematic due to its misdiagnosis with bacterial, fungal or viral keratitis. Although, the use of contact lenses by patients coupled with excruciating pain is strongly indicative of this infection, however this is not the generally accepted method for routine diagnosis. The use of *in-vivo* confocal microscopy to view/observe growth of *Acanthamoeba* trophozoites or cysts from the scrapings of the cornea has emerged as a valuable non-invasive tool for clinical diagnosis (Vaddavalli *et al*., 2011). This can be confirmed by evidence from other laboratory-based assays, it is worthy of note that the cultivation of *Acanthamoeba* from the biopsy of the corneal or from contact lenses is still the most widely used and accepted assay for laboratory diagnosis (Vaddavalli *et al*, 2011).

Other laboratory-based assays such as immunofluorescence assays and multiplex real-time PCR methods have also been developed for the simultaneous detection of the pathogenic free-living amoeba in the same sample. (Qvarnstrom *et al*., 2006). The use of real-time fast duplex Taqman PCR for the detection of 10 different genotypes of *Acanthamoeba* can be employed to detect 1 cyst/ 10 µl (Goldschidt *et al*., 2009). This research project investigates the potential use of mass spectrometry methods in the detection and diagnosis of this parasite, which has been shown to be of potential value in the rapid identification of the parasite in clinical samples.

## 2.6.8 Treatment

Early diagnosis and aggressive treatment is key to the successful management of the disease. According to Siddiqui and Khan, (2012), no single drug or medication is known to be effective against all the 17 isolates/genotypes of *Acanthamoeba.* There are several eye medication prescriptions available to combat the infection; the treatment regimen includes polyhexamethylene biguanide or chlorhexidine digluconate together with propamidine isethionate 0.1% or hexamidine 0.1% is sometimes often effective. The addition of antibiotics (neomycin or chloramphenicol) to the regimen might be necessary in case bacteria are present in the infection (Perez-Santonja *et al*., 2003). In extreme cases, surgical care, such as keratoplasty or corneal transplantation, may be needed in situations where the cornea is permanently scarred, or in cases refractory to the optimal medical treatment. In cases of GAE, there is no recommended treatment due to lack of anti-amoebic compounds which can penetrate the blood-brain barrier. The prognosis of GAE is relatively very poor, which means that most of the cases are diagnosed at the post-mortem stage.

It is worthy of note that the first cure of corneal infection was achieved in at Moorfields Eye Hospital in London in 1985 (Wright, Warhurst and Jones, 1985).

## 2.6.9 Genotyping

Based on rRNA gene sequences, the genus *Acanthamoeba* is divided into 17 different genotypes (T1 – T17) (Corsaro and Venditti, 2010; Nuprasert *et al.*, 2010). The majority of *Acanthamoeba* infections linked to human beings have been associated with the isolates of the T4 genotype. For instance, more than 90% of *Acanthamoeba keratitis* (AK) cases have been linked with this genotype (Nuprasert *et al.*, 2010). In addition, T4 has been the major genotype associated with the non-keratitis infections such as GAE and the cutaneous infections. The reason behind the abundance of human - *Acanthamoeba* infections linked to the T4 isolates is not yet understood. However, there are peer-reviewed publications which pointing towards their greater virulence and properties that enhance transmissibility and reduced susceptibility to chemotherapeutic agents (Maghsood, 2005).

A current list of *Acanthamoeba* genotypes and their links to human infection is documented in Table 4 below (Maghsood, 2005; Corsaro and Venditti, 2010).

Table 4: List of *Acanthamoeba* genotypes and their related human disease (Maghsood, 2005),

|  |  |  |
| --- | --- | --- |
| ***Acanthamoeba* genotypes** | **Pathogenic/ Non- pathogenic** | **Updates of diseases caused by each genotype after 2005** |
| T1 | Encephalitis |  |
| T2 | Keratitis, Encephalitis |  |
| T3 | Keratitis |  |
| T4 | Keratitis, Encephalitis |  |
| T5 | Keratitis, Encephalitis |  |
| T6 | Keratitis |  |
| T7 | Non-pathogenic |  |
| T8 | Non-pathogenic |  |
| T9 | Keratitis | (Hajialilo *et al*., 2016) |
| T10 | Keratitis, Encephalitis |  |
| T11 | Keratitis |  |
| T12 | Encephalitis |  |
| T13 | Non-pathogenic |  |
| T14 | Non-pathogenic |  |
| T15 | Keratitis |  |
| T16 | Non-pathogenic |  |
| T17 | Non-pathogenic |  |

## 2.6.10 Immune response to Acanthamoeba infections

The recurrence of *Acanthamoeba* infections is quite common which suggests that the corneal infection alone does not induce protective immunity against the antigens of the parasite. A study conducted by Garate *et al*., (2006), on experimental animals showed a significantly lower infection rate in subjects immunised orally with *Acanthamoeba* antigens mixed with the cholera toxin compared to the control group (21.4% v 72.6%). The study showed that oral immunization using recombinant MBP improved *Acanthamoeba keratitis* (AK)*,* while protection was associated with the presence of higher levels of anti-MBP sIgA in tears of the immunized animals which is corroborated by Alizadeh (2008). On examination, AK patients generally show decreased overall levels of sIgA as well as specific anti-*Acanthamoeba* sIgA.

Moreover, tears contain a complement made up of serum-borne molecules that acts in a cascade-like manner. *Acanthamoeba* directly activates the complement system via an alternative pathway; however pathogenic amoebae are resistant to complement-mediated lysis due to expression of complement regulatory proteins including the decay accelerating factor (Toney and Marciano-Cabral, 1998). According to van Klink *et al*., (1998), the presence of macrophages in corneas exposed to *Acanthamoeba*-infected lenses prevented the development of *Acanthamoeba keratitis in vivo*. This is made possible by the induction of an inflammatory response, particularly the secretion of macrophage inflammatory protein-2.

Immunization using *Acanthamoeba* antigens via intravenous, oral or intranasal administration routes had a protective effect on GAE type of *Acanthamoeba* infection. This validates the fact that GAE is common in persons of weakened or compromised immune systems. According to Walochnik, Scheikl and Haller Schober (2001), immunization stimulates the secretion of pro-inflammatory cytokines (interluekin-1-beta (IL-1β, interleukin-6 (IL-6) and tumour necrosis factor –alpha (TNF-α) which play a crucial role in the protective immunity.

# 2.7 RESEARCH QUESTIONS

The aim of this research is to investigate the application of MALDI techniques for rapid identification and phenotypic characterisation of ‘difficult to culture’ pathogens, especially directly from clinical samples.

The goals are as follows:

1. Explore the use of MALDI-TOF MS to detect the expression of plasmid-encoded genes.
2. The development of a MALDI-TOF MS method for routine use in the detection and identification of a non-bacterial organisms such as *Acanthamoeba*.
3. To investigate the specificity, sensitivity and accuracy of MALDI in detecting *Acanthamoeba*.
4. The genotyping of fifteen available *Acanthamoeba* isolates in order to compare the results with those obtained for MALDI-TOF MS.

**CHAPTER 3**

**GENERAL METHODOLOGY**

# 3.1 OVERVIEW OF THE CHAPTER

For this work, a range of micro-organisms were used. These included bacteria, yeast and protozoans. Although *Acanthamoeba* was the main organisms of interest, other organisms were used in the research either to validate the development of a method or as part of training tools for cell culturing and the use of the MALDI-TOF MS system. The methodology of this research work can be sub-divided in to four main parts according to the series of experimentation done throughout the research.

* Routine Culture
* MALDI-TOF MS
* Maintenance of clones for plasmid work
* Polymerase Chain Reaction

# 3.2 ROUTINE CULTURE

## 3.2.1 Culture of Bacteria

All bacteria species were obtained from the University of Sunderland’s microbial culture collection. The bacterial species were obtained from a -80 0C freezer and resuscitated on nutrient agar (NA) media. The NA medium was prepared according to the manufacturer’s recommendation and autoclaved at 121 0C for 15 minutes prior to use. The agar was then poured in to petri dishes and stored in the cold room at 4 0C. All bacterial isolates were grown on NA (unless otherwise stated) at 37 0C, except for *Citrobacter spp.* which was grown at 30 0C. Other media types were also used to grow bacteria for some experiments, they are Lysogeny broth (LB), MacConkey and cysteine lactose electrolyte deficient (CLED) agar. These media types were also prepared according to the laboratory standard operating procedures and autoclaved at 121 0C for 15 minutes prior to use. All plates from each batch of a bacterial species resuscitated was discarded after one month of use and new batches were reinstated from the -80 0C freezer. This routine culture of bacteria served as the source of each bacterium for all experiments conducted throughout this research. The bacteria used in the project are detailed below:

Table 5: Genus and Species names of bacteria used.

|  |  |
| --- | --- |
| **No** | **Species name (and strain where appropriate** |
| 1 | *Escherichia coli* K-12 |
| 2 | *Klebsiella aerogenes* |
| 3 | *Salmonella enterica serovar tinda* |
| 4 | *Proteus mirabilis* |
| 5 | *Enterobacter aerogenes* |
| 6 | *Citrobacter freundi* |
| 7 | *Staphylococcus aureus* |
| 8 | *Streptococcus mutans* |
| 9 | *Pseudomona putida* |
| 10 | *Pseudomonas aeruginosa* |
| 11 | *Micrococcus luteus* |
| 12 | *Bacillus subtilis* |
| 13 | *Staphylococcus epidermidis* |

## 3.2.2 Culture of Fungi

All yeast species were obtained from the University of Sunderland’s microbial culture collection. The yeast species stored in a -80 0C freezer, was resuscitated on agar and prepared according to the manufacturer’s recommendation of autoclaving at 121 0C for 15 minutes prior to use. All fungi/yeast isolates were grown at 37 0C, sub-cultured according to guidelines and discarded after one month of use. This routine culture of fungal isolates served as the source of fungi for all experiments conducted throughout this research. The fungi/yeast used in this project are *Candida albicans, Saccharomyces cerevisiae* and *Candida glabrata.*

## 3.2.3 Culture of Acanthameoba

All *Acanthamoeba* isolates used in this study were obtained from Dr Simon Kilvington at the University of Leicester. A total of fifteen isolates of *Acanthameoba* were available and used for this research. Each isolate was cultured, managed and treated the same way, which involved growing them in *Acanthamoeba* broth, also known as Peptone-Yeast Extract-Glucose (PYG) Medium (Health protection agency 2007). The formula for preparing *Acanthamoeba* broth is detailed in Table 6 below

Table 6: List of ingredients of the *Acanthamoeba* Broth (PYG Medium – 10% Glucose)

|  |  |
| --- | --- |
| ***Acanthamoeba* Broth Ingredients** | **Mass per litre of deionised water** |
| Peptone | 20.0 g |
| Yeast Extract | 2.0 g |
| Sodium citrate | 1.0 g |
| Magnesium sulfate | 0.98 g |
| Sodium phosphate | 0.355 g |
| Potassium phosphate | 0.34 g |
| Ferrous ammonium sulfate | 0.02 g |

These ingredients contained in the PYG medium enables it to supply nitrogenous and other necessary nutrients for the growth of *Acanthamoeba*. The cultures were maintained in flat plastic cell culture 25cm3 flasks at 30 0C, while they were being passaged regularly (bi-weekly) by transfer of a small aliquot of a densely grown culture to a new 25cm3 flask containing *Acanthamoeba* broth. 1 ml from a densely grown culture flask (at least 1 x 106 cells/ml) is added to a new flask containing 9 ml of *Acanthamoeba* broth for continuous growth.

Three days after a subculture, an *Acanthamoeba*-containing flask typically consists of equal amount of cyst and trophozoites, which is confirmed by observation via the light microscope. However, to obtain 90% cyst form of *Acanhamoeba* in a culture flask, the flask is left in the incubator without sub-culturing for more than five days. This is because as the nutrient in the medium declines and the environment becomes unfavourable the trophozoites inside the culture start changing to the rigid cyst form. Also, to obtain or harvest 90% of trophozoite in a cell culture, the cells from an ongoing/confluent culture is added to a flask containing fresh media. Within twenty-four hours, 80-90% of the cysts change to trophozoites because the condition is now favourable due to the new added nutrients. The *Acanthamoeba* isolates used for the research is tabulated in Table 7 below.

Table 7: List of *Acanthamoeba* isolates used in this research

|  |  |
| --- | --- |
| **Codes for the *Acanthamoeba* isolates** | ***Acanthamoeba* Genotype** (Genotype determined in this project**)** |
| SK14-4 | T4 |
| SK19-9 | T4 |
| SK19-56 | T4 |
| SK19-59 | T4 |
| PM19 | T4 |
| 20365 | T4 |
| 30234 | T4 |
| 30371 | T4 |
| 30461 | T4 |
| 30874 | T4 |
| 50575 | T4 |
| ROS | T4 |
| ISO/3G | T4 |
| Rad A | T11 |
| LSH TM 1630 | T4 |

## 3.2.4 Culture of modified bacteria (Plasmid-containing)

Some experiments were conducted using modified bacterial strains, which contained plasmids that carried antibiotic resistance genes as selective markers and thus confer antibiotic resistance to the bacteria carrying them. The agar plates used for the growth of these transformed bacteria were quite different from normal laboratory strains because they had the presence of antibiotics. Prior to pouring the agar media into petri dishes, pre-prepared antibiotics at the right concentration (Table 8) were added into the agar to sustain the continued conferment of the resistance in the bacteria. The plates were then subsequently stored in the cold room (4 0C) until required. All transformed bacteria isolates were grown at 37 0C, and were sub-cultured regularly.

Table 8: List of Antibiotic diluted in agar preparation and the working concentration used

|  |  |
| --- | --- |
| **Antibiotic** | **Working concentration** |
| Ampicillin | 100 µg/ml |
| Chloramphenicol | 25 µg/ml |
| Kanamycin | 50 µg/ml |
| Tetracycline | 10 µg/ml |
| Gentamycin | 10 µg/ml |

Some of the bacteria had undergone transformation elsewhere after which we took possession of them for research and just needed subsequent sub-culturing, while others were transformed in house as detailed in section 3.4. Plasmid carriage was confirmed for each of them by culturing with appropriate antibiotics to test if the bacteria had been successfully transformed. For example, a normal laboratory *E. coli* strain that has undergone transformation by the insertion of a plasmid which contained both the chloramphenicol and ampicillin genes will grow in the presence of ampicillin or chloramphenicol supplemented agar plates. This was done for all the transformed samples to confirm their successful transformation and expression. A negative control was set up to confirm antibiotic selection; this was tested and confirmed by the failure of a plasmid-free laboratory strain of *E. coli* to grow on the antibiotic supplemented agar plates.

# 3.3 MALDI-TOF MS

## 3.3.1 General Sample Preparation

The isolates used for this experiment were from the normal routine culture collections as described in section 3.2 which have been grown according to the specification of each organism. For bacterial isolates, they were collected from the routine cultures and then grown on fresh agar plates in the same requirement needed for routine growth for at least 18-24 hours. After the required 18-24 hours growth, the colonies formed were ready to be used for MALDI-TOF MS experiments. The summary of the mechanism for MALDI-TOF MS experiments as depicted in Figure 7 involved prior calibration of the MALDI system as recommended and detailed by the manufacturer. This is then followed by the application of a fresh thin film of sample (colonies of micro-organism grown 18-24 hours earlier) on the MALDI target plate via a pipette tip, this was then overlaid with a suitable matrix (pre-prepared separately). The mixture was then allowed to dry on the target plate at room temperature, afterwards the MALDI target plate was inserted in to the MALDI-TOF MS system for analysis, which in our case was done via a MALDI-TOF MS Shimadzu Microflex Mass Spectrometer. The result was the production of a spectrum displayed on a monitor screen. The spectrum result was then read, interpreted or underwent further analysis by matching it to an existing online database for sample (microbial) identification. The stages of the MALDI-TOF MS experiment done will be broken down for detailed analysis and reporting, they are: sample application on MALDI plate, sample preparation, matrix preparation, matrix application, setting machine/system parameters, database search, and display of results.

## 3.3.2 Sample application on MALDI plate

There are several ways of applying samples from colonies of bacterial, yeast or parasite onto the MALDI plate in order to yield the best reproducible and robust result. Some samples were obtained from microbial growth on solid agar plates, while others were in liquid culture. All bacterial and fungal samples used in this research were from solid culture plates, while protozoan samples (*Acanthamoeba*) were from cultures grown in liquid media. For samples to be deposited in an appropriate manner as described in Figure 7, it is crucial that application of the sample on to the target plate was precisely and carefully done. This entails applying correct amount of sample and matrix onto each well simultaneously followed by brief mixing, while the mixture remained inside the well. Finally, the samples were analysed within two hours of application and preparation to avoid degradation as instructed by the manufacturer.



Figure 7: A typical MALDI plate showing only rows J, K and L. Cell material has been applied to each of the wells in row L. In L1, too much material has been deposited, wells L2 and L3 show good deposition of cell material and well 4 possibly does not contain enough material. (Bar is 0.5mm).

## 3.3.3 Sample Preparation for MALDI

Most bacterial and fungal samples do not need extra preparation and extraction steps because the matrix can lyse the cell envelope open to make cytoplasmic contents available for analysis. However, some micro-organisms do not give good reproducible spectra patterns because the matrix cannot achieve complete cellular disruption on its own. Thus, extra protein extraction steps were needed apart from the matrix application to lyse the walls of the cells and expose content for analysis. For samples grown in solid media such as bacteria and yeast, the various methods that were used to prepare the samples is detailed below:

Direct smear method: This method was used for all normal laboratory bacteria strains involved in this project. It is the fastest and simplest method of sample preparation. It involved the application of a thin film of microbial sample approximately 0.5mg (using the tip of a pipette) from a single colony on the agar plate on to the target plate. This was then immediately overlaid by 1 µl matrix (See sub- section 3.3.4 for matrix preparation). The mixture on the spot was then allowed to dry at room temperature and then analysed on the system according to manufacturer’s guidelines.

Direct smear plus formic acid: This protocol was used for all fungi and yeasts, because they did not produce ‘enough peaks’ on the spectrum required for identification. This involved extra steps compared to the direct smear method. After a small amount of cell material from a single colony on the agar plate was smeared on the MALDI target plate, 0.5µl of 25% formic acid was added to the cell material on the target plate and left until almost dry. This was then followed by the application of 1 µl of matrix, the mixture was then allowed to dry at room temperature and then analysed on the MALDI system.

Protein Extraction method: The process for the protein extraction method involved taking a full loop of cell material from the agar plate/culture medium. This was then suspended in 300µl of deionised water in a micro centrifuge tube and vortexed for one minute. 900 μL of ethanol was added to the suspension to clear out the debris, this was vortexed for one minute. The resulting suspension was then centrifuged for 2 minutes at 113 x g. The supernatant was removed and then another 900 μL of ethanol was added to the suspension and vortexed for one minute to remove the ethanol solution completely. The resulting pellet was re-suspended in 50 μL of 70% formic acid, followed by vortexing and sonication of the suspension for 10 minutes. 50 μL of acetonitrile was added to the sample, and then vortexed and sonicated as previously described, this was followed by centrifugation for 2 min at 113 x g. 70 μL of the supernatant was transferred to 30 μL of purified water to make a final solution. Lastly, 0.5 µl of the final solution was deposited onto the target well, and then 0.5 µl of the matrix was added onto the sample immediately. This was then analysed under the MALDI-TOF MS system. This method was used once to see if the spectrum of a yeast - *Candida albicans* could be improved upon to produce an identification with better percentage probability.

Adapted extraction method for plasmid-containing bacteria: This method was obtained and used for the continuation of the phenotypic characterisation of AmpC β-lactamase enzyme work. The bespoke extraction method involved sample to be emulsified in 3ml plastic test tubes containing 2ml purified water and adjusted to an optical density of McFarland 4. Afterwards, the suspension was centrifuged at 3000 xg for 5 minutes and the supernatant discarded. The deposit was re-suspended in 100µl of 50% trifluoroacetic acid (TFA), 2.5% acetonitrile (ACN), and 47.5% purified water to give a final concentration equivalent to McFarland 80. 1.2 µl of the final bacterial suspension was applied to the target plate in the 3 separate wells in order to provide triplicate data, followed by 1.2 µl of the matrix CHCA, and thereafter left to air dry before MALDI-TOF system analysis.

Preparation of *Acanthamoeba* isolates for MALDI-TOF MS

For *Acanthamoeba* isolates, various methods used in this work are detailed below:

Direct smear method: This involved the application of a mixture of *Acanthamoeba* cysts and trophozoites samples to the MALDI target plate. The *Acanthamoeba* sample is obtained from the normal routine culture collections described in section 3.2.3. The culture from the flask was decanted in to a centrifuge bottle and centrifuged accordingly. The pellet was re-suspended in media and a cell count was done. A cell count of at least 1 x 106 cells/ ml was required to continue the experiment. 1µl of the re-suspended cell was added to the target plate which was followed by 1µl matrix (CHCA) before MALDI-TOF system analysis.

However, the direct smear method generated poor, inconsistent and non-reproducible spectral results, which necessitated the need for other sample preparation methods. *Acanthamoeba* presents in two life cycle stages: the dividing trophozoites and the quiescent cyst (Bowers, 1969). The cyst wall comprises of two layers: one with a fibrous matrix (exocyst), and another composed of fine fibrils forming a granular matrix (endocyst). The endocyst seems to be mostly composed of cellulose (Linder, Winjecka-Krusnell and Linder, 2002) and thus cannot be lysed easily even with the matrix, therefore there is need for a lysing agent or a mechanical way of disrupting the cell wall. Two main extraction methods have been reported in published journals to be effective in the extraction of proteins identifiable by MALDI-TOF MS (Matsuda *et al*., 2012): the standard extraction method and the on-plate extraction method, both explained below.

Standard protein extraction (Modified for liquid samples): The procedure for the standard protein extraction method started with the decanting 10 ml of cells growing in liquid media into centrifuge bottles. Cells were then prepared by centrifugation of cells in media at 1,000 xg for 10 minutes. The resulting pellets of cells were then suspended in 300µl of deionised water in a micro centrifuge tube and vortexed for one minute. Cells were counted using an automated cell counter; a minimum of 1 x 106 cells per ml was required to continue the experiment. 900 µl of ethanol was added to the suspension and vortexed for one minute. This was followed by centrifugation of the suspension for 2 minutes at 13,000 xg and then supernatant was removed. The suspension was centrifuged for the second time to completely remove the ethanol solution. The pellet was then re-suspended in 50 µl of 70% formic acid. The suspension was then vortexed and sonicated for 10 minutes. Lastly, 50 µl of acetonitrile was added to the suspension, after which it was vortexed and sonicated as describe above, then centrifuged for 2 minutes at 13000 x g. To the MALDI plate, 0.5 µl of the supernatant was deposited onto the target well and then 0.5 µl of the prepared matrix was immediately deposited onto the plate and mixed for few seconds using the pipette before allowing to dry. The target plate was then inserted into the MALDI-TOF system for analysis.

On-target protein extraction: The procedure for the on-target protein extraction also started with the decanting of 10 ml of cells growing in liquid media into centrifuge bottles. Cells were then prepared by centrifugation of cells in media at 1000 x g for 10 minutes. The cell pellet was reconstituted in 300 µl of deionized water in a micro centrifuge tube and vortexed for one minute. Cells were counted using an automated cell counter; for MALDI experiment, a minimum of 1 x 106 cells per ml was required. 1 µl of the reconstituted cell was deposited onto the MALDI target plate, followed by the addition of 0.5 µl of 70% formic acid and 0.5 µl of HPLC grade acetonitrile. This was then mixed together for few second using the pipette and then allowed to dry. Lastly, 1µl of the prepared matrix was deposited onto the dried well and mixed for few seconds using the pipette and then allow to dry before insertion into the MALDI-TOF system for analysis.

## 3.3.4 Matrix preparation

Matrices are the organic materials that ensure the ionisation of the sample in the gas chamber. There are several types of matrices as shown in Table 9 which are used for specific reasons and thus prepared differently. Examples of matrices are 1, 5- diaminonapthalene, α-cyano-4-hydroxycinnamic acid, 3, 5-dimethoxy-4-hydroxycinnamic acid, 9-aminoacridine and 3-hydroxypicolinic acid.

Table 9: Different types of MALDI matrices used in this research and their most suitable targets

|  |  |  |
| --- | --- | --- |
| **Matrix** | **Other Names** | **Suitability** |
| α-cyano-4-hydroxycinnamic acid. | CHCA | This matrix was the standard matrix for the research because it was effective in detecting peaks within 2 kDA and 20 kDa which is the usual range for detecting most micro-organism. |
| 2,5-di hrdoxybenzoic acid | DHB | This particular matrix was not routinely used for microbial identification. It is a good matrix for general MALDI-MS of protein digest, carbohydrates, oligosaccharides, glycopeptides and both proteins and peptides below 10 kDa. |
| 3,5-dimethoxy-4-hydroxycinnamic acid | Sinapinic Acid | This matrix was used to detect high-weight molecules like proteins that are larger than 20,000 Daltons. It was mostly used for calibration of the system which involved higher protein masses up to 150 kDA. |
| Trihydroxyacetophenone | THAP | It was used as part of the initial training into MALDI-TOF MS. |

In addition, twelve novel-in-house matrices were used in this project to compare their effectiveness in identifying bacteria with the use of CHCA. These in-house matrices were produced as part of a project by Dr. Peter Dawson. The identifiers for these novel matrices are PD 58, PD 64, PD 84, PD 85, PD 87, PD 110, PD 112, PD 115, PD 116, PD 118, PD 120 and PD 132. The preparation of the matrix starts with preparing the matrix solvent (which can often be used with any kind of matrix) and then the matrix itself.

Preparation of Matrix Solvent

To prepare the solvent solution, 3.3 ml of HPLC grade acetonitrile with 3.3 ml HPLC grade ethanol and 3.3 ml of deionised high purity water (Milli-Q or HPLC grade) were mixed together. To the mixture, 300 µl of TFA was then added and the solution was carefully mixed.

Preparation of CHCA

CHCA (40 mg) was dissolved in 1 ml of the matrix solvent prepared in 3.3.3.1. The solution was mixed using a vortex at room temperature which resulted in a standard solution. Any undissolved solid (pellet) was further centrifuged, while the clarified upper solution (supernatant) of the matrix is used for MALDI-TOF MS analysis.

Preparation of DHB

50mg of DHB was approximately weighed from the stock box and was dissolved in 1 ml of the matrix solvent prepared in 3.3.3.1 above. The solution was mixed using a vortex at room temperature which resulted in a standard solution.

Preparation of Sinapinic acid

20mg of Sinapinic was weighed from the stock box and was dissolved in 1 ml of the matrix solvent prepared in 3.3.3.1 above. The solution was mixed using a vortex at room temperature which resulted in a standard solution

Preparation of THAP

40mg of THAP was weighed from the stock box and was dissolved in 1 ml of the matrix solvent prepared in 3.3.3.1 above. The solution was mixed using a vortex at room temperature which resulted in a standard solution.

## 3.3.5 Application of matrix on sample

The manner of applying matrix to the sample on the MALDI target plate determines how the sample is prepared for MALDI-TOF MS analysis inside the system. The main method used in this project for preparing samples for analysis via MALDI-TOF MS is the dried droplet technique. This technique involved the application of the sample, which was immediately followed by the application of the matrix on the MALDI target plate. This was left to air dry which helps to remove residual contamination and provide crystallisation.

## Machine/system parameters

MALDI-TOF MS has general parameters which helps the successful analysis and the generation of reproducible results. The general MALDI-TOF MS parameters used for the project were: The system was in liner positive mode with a Power of 70%, Ion gate set at 1500, Pulse extractor at 8300, while there were 100 laser shots per sample. The mass range set for microbial identification was between 2000-20000 m/z or Dalton. The peaks were isotopically resolved with a smooth data while at subtract baseline. The Peak detection method was used and the threshold offset was at 10.0. There was monoisotopic peak picking, peak filtering was set at a tolerance of 100-0, Dalton minimum mass at 0.0 and there were overlapping distributions and minimum peak percent at 10.0.

## 3.3.7 Database searching

The analysis of the sample on the MALDI-TOF system generates a MALDI spectrum which was then matched to a database for identification. The generated spectrum from the sample known as the reference spectrum was compared to a super spectra references on the databases to identify the sample. The database available for the researcher at the University of Sunderland was the SARAMIS database which consists of clinically important bacterial and fungal profiles. The process for the database matching began by acquiring a MALDI-MS spectrum for the sample using the target manager in automated experiment. This unique spectrum generated was saved with a filename in the ASCII format. The ASCII file(s) was then copied from the MALDI-MS system and then transported into a Files/ASCII folder on the mapped SARAMIS server drive. ASCII files in this folder was automatically searched by SARAMIS, the results of SARAMIS searches were shown in the status window of the SARAMIS premium software. The result included identifying genus, species as well as percentage probability of each identification. 0% percentage probability represents identification not possible, while 100% means identification was certain. The colour code for result confidence values on the system also helps to monitor the probability of each identification. For our research lab, we have chosen a confidence level of a percentage probability of 80% which has been routinely used in clinical application and typical for positive identification in most clinical laboratories.

# 3.4 EFFECT OF PLASMIDS ON BACTERIAL MALDI-TOF MS SPECTRA

There were series of experiments involving the use of plasmids in bacteria. Our research work was a continuation of the work started by a Masters’ student in 2013. The previous work involved the collection of samples of *Escherichia coli* that were positive for ampicillin gene and randomly paired with those that are negative for the Ampicillin gene. The same samples which have been stored frozen since 2013 were transferred to the researcher and resuscitated as well as grown as explained in sub-section 3.2.4. The positive and negative strains were confirmed for positive expression of ampicillin and negative expression by growing the positive ampicillin strains on ampicillin agar plates which grew, while the negative ampicillin strains did not grow on ampicillin plates, but instead grew on normal agar plates. The formation of colonies from each strain enabled the continuation of the project into further analysis via MALDI-TOF MS as detailed in sub section. The second set of experiments was similar to the first, which is to determine the effect of *ampC* gene expression in *E. coli* K-12 clinical isolate via MALDI TOF MS spectra. Two different promoters were used for *ampC* gene insertion into the bacterium for high expression labelled as Opt1 and Opt 2. A normal laboratory strain of *E. coli* K-12 and a strain with an empty plasmid was added. MALDI TOF MS was employed to test whether the effect of *ampC* gene expression could be detected via the generated MALDI spectra.

The third set of experiments involving plasmid involved understanding of the impact of plasmid-borne *ampC* gene expression on bacteria species when analysed via MALDI-TOF MS. The experiment was designed to look at *ampC* insertions via plasmids in a standard *E. coli* laboratory strain, the samples were obtained from a researcher within the university, who did the first part of the experiment by working on a parent plasmid (pACYC184) carrying a chloramphenicol and tetracycline resistance gene. The parent plasmid was altered by deleting the tetracycline gene and naming that as empty plasmid (pACYC184ΔHN) (Mitchell, 2007), while they also replaced the tetracycline gene with an ampicillin resistance gene expressed from a either a wildtype promoter or mutant (optimised) promoter. In total, there were two optimised *ampC* expression plasmids with different promoters and one empty plasmid. The second part of the experiment involved the insertion of the plasmids into bacterial cells to express the genes as well as analysis on the MALDI-TOF MS. The protocol for the transformation of the plasmid into *E. coli* DH5α (a laboratory K-12) strain is detailed as follows: cells were thawed on ice for 10 minutes and then 1 pg -100 ng of plasmid DNA (1-5µl) was added to 50µl cells and mixed without vortexing. This was then placed on ice for 30 minutes, followed by heat shocking at 42 0Cfor 10-30 seconds and then placed on ice for 5 minutes. 950µl of room temperature super optimal broth with added glucose (SOC) was added and the samples placed in an orbital shaker at 37 0C, 200 rpm for 60 minutes. 50-100µl of each dilution was spread onto pre-warmed selection plates and incubate overnight at 37 0C. The work generated three different strains - two AmpC+ optimised plasmid transformations with different level of expressions and an empty plasmid. The three modified strains were compared to a control, a normal parent *E. coli* lab strain. These four were then grown accordingly in the laboratory as described in subsection 3.2.4, prepared for MALDI-TOF analysis as described in 3.4 prior to MALDI-TOF system analysis.

# 3.5 POLYMERASE CHAIN REACTION (PCR)

## 3.5.1 General Sample Preparation

Similar to MALDI-TOF MS, PCR experiments started with collecting microbial samples from the normal routine culture collection storage. PCR was used in this research for DNA sequencing of the fifteen isolates of *Acanthamoeba* used in this project.

## 3.5.2 DNA Preparation

The *Acanthamoeba* isolates were collected from routine cultures as described in 3.2.3 and then grown on fresh liquid media in 25 cm3 flask for 18-24 hours before being used for the PCR experiment. The aim of the PCR experiment is to amplify the *Acanthamoeba* genes which will be used for identification. The PCR method began with decanting all the cells from a 25 cm3 containing *Acanthamoeba* cells grown in PYG broth (see sub-section 3.2.3) (18-24 hours) in to a 50 ml centrifuge tube. The cells were washed three times in phosphate-buffered saline (PBS) and then re-suspended in 500 μL of autoclaved water. The new suspension was mixed by vortexing and cell number estimated via a digital automated cell counter. A minimum cell count of 1x 106 is required for the PCR analysis.

## 3.5.3 Lysis methods

Due to the hardness of the *Acanthamoeba* cell wall of as detailed in 3.3.2 above, an extra step is needed to break down the wall. Three methods were used initially (boiling, sonication and lysing buffer), out of the three, the boiling method is the only method which produced bands on the gel electrophoresis after DNA extraction. The extra lysing step continues after the cell count of the cell was made as detailed in sub-section 3.5.2.

Boiling method: The cells prepared in sub section 3.5.2 above were incubated at 90°C in a water bath for 30 minutes. The cells were then vortexed at high speed for 10 seconds, centrifuged at 10,000–12,000 xg for 2–3 minutes. The supernatant is collected and then used as the template DNA for PCR.

Sonication: The cells prepared in sub section 3.5.2 above were sonicated in a sonicator for 30 minutes. The cells were vortexed at high speed for 10 seconds and then centrifuged 10,000–12,000 xg for 2–3 minutes, then it is ready for the next stage of the experiment.

Lysis buffer: The cells prepared in sub section 3.5.2 above were subjected to a lysing buffer called Instagene matrix (from BioRad). A ratio of 1:1 is required for the cell lysis. This means 500 μl of the lysing buffer (instagene matrix) was added to 500 μl of the cells for 30 minutes. This was incubated at 56 0C, followed by vortexing and another incubation at 100 0C for 8 minutes. The cell solution was vortexed at high speed for 10 seconds and then centrifuged 10,000–12,000 xg for 2–3 minutes, then it is ready for the next stage of the experiment.

## 3.5.4 PCR Analysis

A 50 μl PCR reaction contained:

DNA template (supernatant from cell suspension) 2 μl

Forward Primer 1 μl (3.2pmol/μl)

Backward primer 1 μl (3.2pmol/μl)

Master mix reagent 25 μl

PCR grade deionised water 21 μl

Total 50 μl

NB: The negative control of the PCR micro tube contains all the above contents except the DNA template, the deionised water was made up to 23 μl to get a total of 50 μl for the PCR analysis.

The sequence of the 18S rDNA primers, used for the *Acanthamoeba* was obtained from JDT and consisted of forward primer JDP1 (5′- GGCCCAGATCGTTTACCGTGAA-3′) and reverse primer JDP2 (5′-TCTCACAAGCTGCTAGGGGAGTCA- 3′) (Schroeder, 2001). Depending on the genotype, the primers amplified 423 to 551 bp of 18S rDNA between reference bp 936 and 1402.

PCR was performed in the resulting 50 μl microtube with the PCR thermal cycler set at the following amplification profile: at 95°C for 3 minutes, 40 cycles of amplification at 95°C (30 seconds), 62°C (30seconds), and 72°C (30 seconds). After the PCR cycling, the PCR products were visualised by gel electrophoresis using 1.8% agarose gels containing a gel red staining. The purification of the DNA fragment was assessed using the Monarch DNA Gel Extraction kit (NEB#T1020). The DNA fragment was excised from the agarose gel and transferred to 1.5 ml microfuge tube. Excess agarose gel was removed from the DNA fragment, while minimising the exposure to UV light. The gel slice containing the DNA fragment was weighed so as to know the corresponding volume of dissolving buffer to be added. After weighing, 4 volumes of the Gel dissolving buffer was added to the gel slice and incubated at 55 0C for 5-10 minutes, while also vortexing periodically until the gel slice is completely dissolved. The resulting sample was loaded onto a column; the cap was closed and then centrifuged for 1 minute at 16,000 x g. The flow-through was discarded, while the column was re-inserted into a new collection tube. 200 μl of the DNA Wash Buffer was added to the column and then centrifuged for 1 minute at 16,000 x g. The flow-through was discarded and the column was re-inserted into a second new collection tube. 200 μl of the DNA Wash Buffer was added to the column again and then centrifuged for 1 minute at 16,000 x g. The flow-through was discarded again ensuring that the tip of the column does not come into contact with it. The column was then transferred in to a clean 1.5 ml microfuge tube, and then 20 μl of the DNA elution buffer was added to the centre of the matrix. This was left for approximately 1 minute before being centrifuged at 16,000 x g for 1 minute to elute the DNA. The DNA eluted was then sent offsite for sequencing at the Department of Bioscience at the University of Durham.

**CHAPTER 4**

**RESULTS**

# 4.1 OVERVIEW OF THE CHAPTER

This first chapter of the result section details the MALDI-TOF MS result obtained from various experiments involving bacterial and fungal isolates as well as a modified strain of *E. coli* K-12. The first set of results presented was to validate the SARAMIS identification process which had become routine use in most clinical diagnostic laboratories in Europe. This initial work apart from being a validation process for SARAMIS identification also doubles as a training and development course for the researcher in order to be able to use MALDI-TOF MS system in carrying out the research work. The aim of this work is to examine the impact of media types on the MALDI-TOF spectrum generated for an organism. Currently, manufacturers of MALDI-TOF MS do not specify the exact or precise media type to be used for culturing organisms, this does leave the media-type decision open to individual laboratories. It would be crucial and scientifically relevant to know and understand the effect the differing media types brings to not only MALDI spectral patterns but SARAMIS identification.

The next set of results is obtained from the application of ‘in-house developed’ matrices to the generation of MALDI-TOF MS spectra instead of the standard matrix (CHCA). A number of matrices were developed in the University of Sunderland for the identification of peptides and other related substances which have smaller molecular weights (typically less than 2 kDa). Although, these in-house matrices were developed for peptides with relatively lower molecular weight that microbial species, however it would be interesting to know if positive identification could be obtained for microbial species. This could be followed by the comparison and analysis of the spectral patterns obtained from each of these in-house matrices in relation to the standard matrix and the subsequent identification via the SARAMIS database.

This chapter ends with the results looking at the effect of plasmid acquisition on the bacterial MALDI-TOF MS spectrum. The novelty of this work goes hand in hand with its clinical significance which could revolutionise the detection of antibiotic resistance in bacteria. The objective behind this is to investigate the use of MALDI-TOF MS in the possible detection of organisms which produces the enzyme β-lactamase that provides multi-resistance to a wide variety of β-lactam antibiotics. However, as the work progressed and initial results were analysed, the objective shifted a little bit from identifying the enzyme to detecting the impact of the enzyme. The subsequent experiments centred on the effects/impacts of plasmids on the bacterial spectrum generated by MALDI-TOF MS. This is done by comparing the MALDI spectral patterns obtained from a normal parent organism to the same organisms that has become transformed via the acquisition of plasmids (resistance genes).

The aims of this first result section are:

1. To confirm the identities of ten known microbial species blinded (names not given) to the researcher, which can validate the SARAMIS ID process as well being used for training and development for the researcher.
2. To investigate the effect of using different solid-media types for culturing two organisms (*B. subtilis* and *S. epidermidis)* prior to MALDI analysis
3. To investigate the potential use of novel in-house matrices in the identification of microbial species via MALDI-TOF MS.
4. To explore the use of MALDI-TOF MS in detecting the impact/effect of plasmid acquisition by a bacteria on their MALDI-TOF MS spectrum.

# 4.2 VALIDATION OF THE SARAMIS IDENTIFICATION PROCESS

Ten different micro-organisms (composed of bacterial and fungal species) which were blinded to the researcher but known to the Microbiology technical staff of the University were analysed by MALDI-TOF MS and identified via the SARAMIS database. Using the standard mass range m/z of 2-20 kDa, a unique characteristic MALDI-TOF spectrum (peptide mass fingerprint - PMF) was generated for each organism. Our mass spectrometer (detailed in the previous chapter) utilises SARAMIS, which is a microbial identification database software routinely used in analytical and diagnostic-research laboratories. The MALDI-TOF spectrum (PMF) obtained was matched/compared to the PMFs already contained in the SARAMIS (**S**pectral **AR**chive **A**nd **M**icrobial **I**dentification **S**ystem) database. The matching results from the SARAMIS database showed identification of the blinded microbial species up to a genus level of 100% and species level of 90% accuracy as confirmed by the Microbiology technical staff. The microbial unknown group composed of bacterial and fungal species, each with their percentage probability. Figure 8 below shows the test reference result obtained from SARAMIS database for one out of the ten organisms. Table 10 is a summary from the ten test reference results showing the names (genus and species), microbial type and percentage probability of the ten microbial samples identified by MALDI- TOF MS SARAMIS via the SARAMIS database.

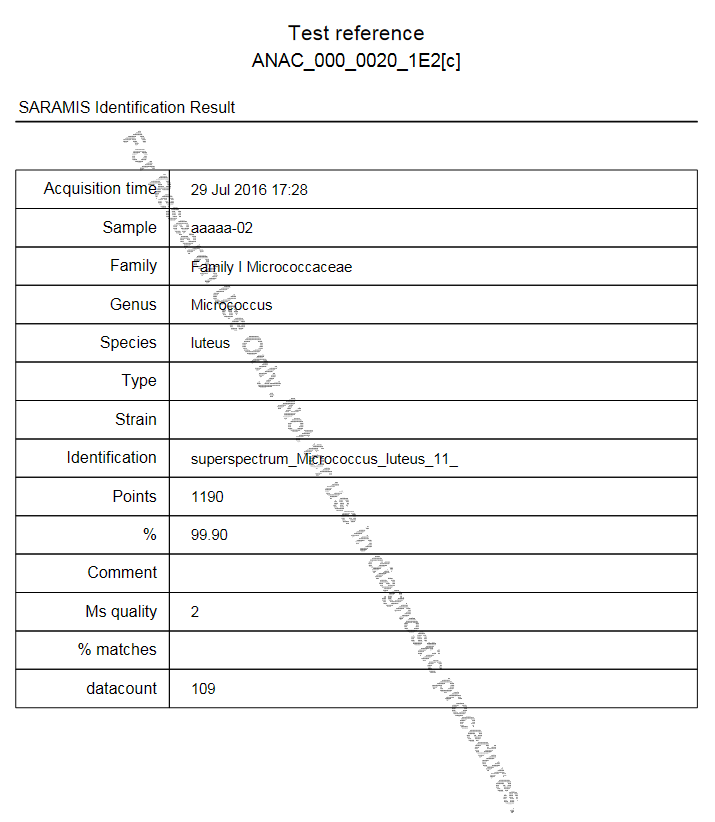


Figure 8: A typical example of a test reference result obtainable from the MALDI-TOF MS bioMerieux SARAMIS mass spectrometry. The percentage probability for this organism is 99.90%, which suggests the organism is most definitely *Micrococcus luteus*. The higher the probability percentage, the more definitive is the confirmation.

Table 10: A summary table showing the identification of the ten unknown microbial samples which were correctly identified by MALDI-TOF MS via the SARAMIS database.

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample** | **SARAMIS Suggestion** | **Microbial type** | **% ID Probability** |
| A | *Micrococcus luteus* | Gram-positive bacterium | 99.90 |
| B | *Pseudomonas aeruginosa* | Gram-negative bacterium | 99.90 |
| C | *Bacillus subtilis* | Gram-positive bacterium | 99.90 |
| D | *Pseudomonas putida* | Gram-negative bacterium | 96.30 |
| E | *Candida glabrata* | Yeast | 93.10 |
| F | *Escherichia coli* | Gram-negative bacterium | 99.90 |
| G | *Staphylococcus epidermidis* | Gram-positive bacterium | 99.90 |
| H | *Proteus mirabilis* | Gram-negative bacterium | 89.90 |
| I | *Enterobacter aerogenes* | Gram-negative bacterium | 99.90 |
| J | *Saccharomyces cerevisiae* | Yeast | 95.0 |

Figure 8 is a test reference result obtained which gives details of the micro-organism in question. The information contained in the test reference includes the date of acquisition, the sample name, while results are the name of Genus, Family and Species of the micro-organism in question in addition to the percentage probability and the MS quality number. The test reference results shown in Figure 9 represents the typical example of test reference results obtained from all the ten micro-organisms as identified by the SARAMIS database. Most diagnostic laboratories with similar MALDI-TOF MS systems often accept a percentage ID probability of 80% and above, while anything less than that is re-tested (Wang, 2014). In an hospital diagnostic laboratory or a medical research centre, this result establishes the basis for the next phase of treatment.

A summary of all the ten test reference results is detailed in Table 10. All the ten genus names were all correctly identified (100% genus level accuracy), while nine out of the ten species names were correct (90% species level accuracy), only missing out the species ‘mirabilis’ by naming it as ‘vulgaris’. Also, the percentage probabilities for all the ten isolates were obtained which was highest in five micro-organisms (99.9%) and lowest at *Proteus mirabilis* (89.9%). In general, the closer to 100% the percentage probability is, the certain the identification is, however MALDI analysis with percentage probabilities less than 80% are often repeated to confirm results (Wang, 2014).

# 4.3 EFFECTS OF SOLID-MEDIA TYPE ON MALDI SPECTRUM AND SUBSEQUENT IDENTIFICATION VIA THE USE OF SARAMIS DATABASE

This experiment investigates the effect of solid-media type on the MALDI spectrum and subsequent identification via the SARAMIS database. Two organisms namely - *B. subtilis* and *S. epidermidis* were grown in four different media types: lysogeny broth (LB) agar, nutrient agar (NA), trypticase soy agar (TSA) and cysteine lactose electrolyte deficient (CLED) agar. Colonies from each media type were chosen as detailed in the method section and analysed via MALDI-TOF MS using CHCA as the matrix. The resulting spectra from each of the four media types was subjected to the SARAMIS database for identification. Figure 9 (a, b, c, d) and Figure 10 (a, b, c, d) show the MALDI-TOF MS spectrum in triplicates of *B. subtilis* and *S. epidermidis* respectively grown on the four different media types.

****

**2000 4000 6000 8000 10000**

**m/z**

Figure 9a: Three overlaid MALDI-TOF MS Spectra of *B. subtilis* grown on TSA media prior to MALDI analysis with the use of CHCA as the matrix.

****

**2000 4000 6000 8000 10000 m/z**

Figure 9b: Three overlaid MALDI-TOF MS Spectrum of *B. subtilis* grown on NA media prior to MALDI analysis with the use of CHCA as the matrix.

****

**2000 4000 6000 8000 10000 m/z**

Figure 9c: Three overlaid MALDI-TOF MS Spectra of *B. subtilis* grown on CLED media prior to MALDI analysis with the use of CHCA as the matrix.



**2000 4000 6000 8000 10000 m/z**

Figure 9d: Three overlaid MALDI-TOF MS Spectra of *B. subtilis* grown on LB media prior to MALDI analysis with the use of CHCA as the matrix.

****

**2000 4000 6000 8000 10000 m/z**

Figure 10a: Three overlaid MALDI-TOF MS Spectrum of *S. epidermidis* grown on NA media prior to MALDI analysis with the use of CHCA as the matrix.

****

**2000 4000 6000 8000 10000 m/z**

Figure 10b: Three overlaid MALDI-TOF MS Spectra of *S. epidermidis* grown on TSA media prior to MALDI analysis with the use of CHCA as the matrix.

****

**2000 4000 6000 8000 10000 m/z**

Figure 10c: Three overlaid MALDI-TOF MS Spectra of *S. epidermidis* grown on CLED media prior to MALDI analysis with the use of CHCA as the matrix.

****

**2000 4000 6000 8000 10000 m/z**

Figure 10d: Three overlaid MALDI-TOF MS Spectrum of *S. epidermidis* grown on LB media prior to MALDI analysis with the use of CHCA as the matrix.

Figures 9 (a to d) shows the overlaid MALDI-TOF MS spectra analysis of *B. subtilis* *which* were grown on different media types prior to analysis, while Figures 10 (a to d) shows the overlaid MALDI-TOF MS spectra analysis of *S. epidermidis* *which* were also grown on different media types prior to analysis. The standard matrix (CHCA) was used while the mass range used for both analyses was between 2-20 kDa, although a shorter span of the spectrum (2- 10 kDa) is shown here to make the spectrum bolder and visible to the readers. In addition, this section of the spectrum (2- 10 kDa) shown in the figures above contained all the peaks generated during the MALDI analysis. The characteristic unique MALDI spectrum (PMF) generated for each organism and media type was matched to the SARAMIS database for identification. MALDI Spectrum generated from each of the media types correctly identified the two organisms when matched to the SARAMIS database, although with different levels of percentage probability. The result from the database shows that media type can affect the confidence scores generated or percentage probability when identifying the same bacterial isolate on different media types. Also, as observed in the trend of these two organisms, the peak masses and their respective intensities were not exactly the same in all the media type, causing a little variation in spectral pattern or protein expression profile, however identification in all media types via SARAMIS was possible and correct. This is because the spectral generated from the all the four media types contained the unique ‘identification peaks’ which made the identification of each organism possible.

The main peaks from each media types for *B. subtilis* and *S. epidermidis* are highlighted in tables 11 and 12 respectively, while the average percentage probability from the triplicates of each media type is calculated. The summary table also indicates that the percentage probability for correct identification is highest in LB media type and lowest in CLED media for both organisms.

Table 11: The main peaks from the MALDI-TOF MS spectrum of *B. subtilis* from each of the media types and the average percentage probability for the three replicates.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Individual peak acquired from the MALDI-TOF MS analysis. (*m/z*)** | **LB** | **NA** | **TSA** | **CLED** |
| 3045 |  |  |  |  |
| 4308 |  |  |  |  |
| 5258 |  |  |  |  |
| 6687 |  |  |  |  |
| 7740 |  |  |  |  |
| 8602 |  |  |  |  |
| 9647 |  |  |  |  |
| 12748 |  |  |  |  |
| Average % Probability | **99.63** | **95.12** | **93.43** | **85.46** |

Table 12: The main peaks from the MALDI-TOF MS spectrum of *S. epidermidis* from each of the media types and the average percentage probability for the three replicates.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Individual peak acquired from the MALDI-TOF MS analysis. (*m/z*)** | **LB** | **NA** | **TSA** | **CLED** |
| 2477 |  |  |  |  |
| 2837 |  |  |  |  |
| 4295 |  |  |  |  |
| 5117 |  |  |  |  |
| 5393 |  |  |  |  |
| 6403 |  |  |  |  |
| 6690 |  |  |  |  |
| 9643 |  |  |  |  |
| Average % probability | 99.30 | 94.41 | 90.36 | 88.55 |

# 4.4 COMPARISON OF SPECTRA GENERATED WITH CHCA AND NOVEL MATRICES

These experiments compare the spectral patterns obtained from MALDI-TOF MS analysis of *E. coli* using CHCA to the spectral patterns obtained via the use of thirteen different ‘in-house’ developed matrices. The sample preparation and MALDI-TOF MS parameters were all done the same way, the only difference is the matrix used for ionization. Figures 11a, b, c and d show the spectral patterns obtained from MALDI-TOF MS analysis of *E. coli* using CHCA and three out of the thirteen in-house developed matrices.



**4000 6000 8000 10000 m/z**

Figure 11a: Three MALDI-TOF MS Spectra of *E. coli* grown on LB agar prior to analysis with the use of CHCA matrix.



**4000 6000 8000 10000 m/z**

Figure 11b: Three MALDI-TOF MS Spectra of *E. coli* grown on LB media prior to analysis with the use of novel matrix PD 58.



**4000 6000 8000 10000 m/z**

Figure 11c: Three MALDI-TOF MS Spectra of *E. coli* grown on LB media prior to analysis with the use of novel matrix PD 64.



**4000 6000 8000 10000 m/z**

Figure 11d: Three MALDI-TOF MS Spectra of *E. coli* grown on LB media prior to analysis with the use of novel matrix PD 84.

The spectrum of MALDI analysis of *E. coli* with CHCA (Figure 11a) shows more than six clear distinct peaks with high signal to noise ratio. However, Figures 11b to 11d shows the MALDI spectra analysis of *E. coli* generated using three out of the thirteen tested in-house matrices. These spectral patterns had few clear distinct peaks with low signal to noise ratio especially in Figure 11c. The mass range used for both analyses was between the ranges of 2-20 kDa, which is the typical range for bacterial identification. However, a section of the spectrum 4-10 kDa is shown here to make the spectrum more visible to the reader and also it contains all the peaks generated during the MALDI analysis. Although, some spectra obtained from few in-house matrices do have similar peak masses compared to CHCA particularly PD 84. The structure of the PD 84 matrix closely matches the structure of the CHCA matrix as detailed in the previous chapter. Table 4 lists all the peaks acquired from all the analysis and matches each peak to the matrix responsible for it. Table 13 shows the similarities in peak masses from all the matrices employed in MALDI-TOF MS analysis of *E. coli* including CHCA.

Table 13 displays a summary of peak masses obtained from the CHCA matrix as well as three out of the thirteen in-house matrices. Table 13 further revealed that the spectrum from the standard matrix (CHCA) had more peaks than other spectra using the in-house novel matrices. However, some of the peaks seen in some of the in-house novel matrices spectra particularly PD 84 were also observed in CHCA spectrum. It is noteworthy that some of the masses of the ‘identification peaks’ unique to *E. coli*’s profile such as 4363, 5101, 5386, 6265, 7168 and 9078 m/z were not all seen in the spectra from the in-house matrices but all seen in the profile with CHCA as the matrix.

Table 13: Comparison of the masses of the main peaks in the spectrum generated using the CHCA matrix and three out of the thirteen in-house matrices.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Individual peak acquired from the MALDI-TOF MS analysis. (*m/z*)** | ***E. coli* with CHCA** | ***E. coli* + PD 58** | ***E. coli* + PD 64** | ***E. coli* + PD 84** |
| 2695 |  |  |  |  |
| 3413 |  |  |  |  |
| 4165 |  |  |  |  |
| 4363 |  |  |  |  |
| 4873 |  |  |  |  |
| 5101 |  |  |  |  |
| 5386 |  |  |  |  |
| 6253 |  |  |  |  |
| 6265 |  |  |  |  |
| 6516 |  |  |  |  |
| 7168 |  |  |  |  |
| 7185 |  |  |  |  |
| 7272 |  |  |  |  |
| 8320 |  |  |  |  |
| 9068 |  |  |  |  |
| 9777 |  |  |  |  |
| 10303 |  |  |  |  |
| 10481 |  |  |  |  |

# 4.5 BACTERIAL IDENTIFICATION VIA MALDI SARAMIS USING IN-HOUSE NOVEL MATRICES

As explained in the sub-section 4.4, the characteristic unique MALDI spectrum (PMF) generated for each of the matrices was matched to the SARAMIS database for identification. Although, the spectral patterns using the in-house novel matrices to analyse *E. coli* via MALDI-TOF MS did not exactly match the spectra generated using CHCA as the matrix. However, few of the peaks do match and thus it is imperative to find out if any of the thirteen in-house generated matrices originally made for peptide identification would be able to correctly identify *E. coli* via the SARAMIS database. Using the typical mass range m/z of 2-20 kDa for microbial species level of identification, the unique characteristic MALDI-TOF spectra generated for *E. coli* with the use of the matrices was matched to the SARAMIS database for identification.

The spectrum obtained via CHCA was correctly identified via SARAMIS while there was no identification for all the ‘in-house’ developed matrices. The percentage probability from the MALDI analysis of *E. coli* using the CHCA matrix is 99.9%, while the others are 0.00% which confirms that the organism cannot be identified. The novel matrices used could have commercial applications resulting in patents, thus details about them have not been provided. It is not surprising that none of the thirteen novel in-house matrices could correctly identify *E. coli* despite some of them having few peak masses which similar to the spectrum from the standard matrix. The reason for this could be due to their spectra patterns, which do not have the complete unique ‘identification’ peaks which are known to the SARAMIS database. In addition, the ratio of signal to noise is higher in the MALDI spectra and thus it would be impossible for accurate identification. This suggests that these novel matrices originally made for peptide identification were not suitable for the detection of proteins from bacteria samples as evidenced from the spectrum obtained from each matrix, which is quite different from the standard matrix (CHCA). The reverse is the case for CHCA spectra which had the unique peaks for identifying *E. coli.*

# 4.6 EFFECTS OF PLASMIDS ACQUISITION ON THE BACTERIAL MALDI-TOF MS SPECTRUM

This section details the result of three studies to determine the effect of *ampC* gene expression in *E. coli* K-12 clinical isolate via MALDI-TOF MS. The first set of results compared the MALDI-TOF MS spectrum of samples expressing (*ampC*+) and samples that did not (*ampC*-). Figure 12a is the MALDI spectra of *E. coli* K-12 which contains a plamsid expressing AmpC, while Figure 12b is the MALDI spectrum of *E. coli* K-12 containing a plasmid that do not have an *ampC* expression.



**2000 4000 6000 8000 10000 m/z**

Figure 12a: Three Overlaid MALDI spectra obtained for the lab strain *E. coli* K-12, *CHCA* as the matrix of choice



**2000 4000 6000 8000 10000 m/z**

Figure 12b: Three Overlaid MALDI spectra obtained for the lab strain *E. coli* K-12 expressing *ampC*+, *CHCA* as the matrix of choice

Table 14: Summary of the peaks in *m/z* obtained from normal lab strain of *E. coli* K-12 and another lab strain of *E. coli*  expressing *ampC*+

|  |  |  |
| --- | --- | --- |
| **Individual peak acquired from the MALDI-TOF MS analysis. (*m/z*)** | ***E. coli* K-12** | ***E. coli* K-12 expressing *ampC*+** |
| 3417 |  |  |
| 4165 |  |  |
| 4365 |  |  |
| 5096 |  |  |
| 5386 |  |  |
| 6265 |  |  |
| 7172 |  |  |
| 7283 |  |  |
| 8338 |  |  |
| 9078 |  |  |
| 9754 |  |  |
| 10481 |  |  |

For the second set of results, two different promoters for *ampC* gene expression in the bacterium which corresponds to wild-type and mutant ‘optimised’ versions of the promoters (Opt1 and Opt 2) were used, MALDI-TOF MS was employed to test whether *ampC* gene expression could be detected. In addition, two *E. coli* K-12 isolates were added to the experiment, one contained an empty plasmid, while the second was a normal laboratory strain which served as the control. To show successful *ampC* gene expressions, the two expressing *E. coli* K-12 isolates grew on ampicillin plates to test their resistance to ampicillin, while the laboratory strain (*E. coli* K-12) and the isolate with the empty plasmid did not grow on ampicillin plates, as shown in table 15.

Table 15: Result of the four isolates grown on ampicillin plates overnight

|  |  |  |
| --- | --- | --- |
| **Isolates** | **Ampicillin Concentration** | **Growth on Ampicillin plates** |
| *E. coli* Lab strain | 100 µg/ml | No |
| Empty plasmid (*ampC*-) | 100 µg/ml | No |
| *ampC*+ (Opt1) | 100 µg/ml | Yes |
| *ampC*+ (Opt2) | 100 µg/ml | Yes |

In addition, MALDI-TOF MS spectra was obtained for the isolate with the empty plasmid, Opt1 (high expressing), Opt2 (low expressing) and normal lab strain. Figure 13a shows the MALDI spectra of the normal lab strain of *E. coli* K-12*,* Figure 13b is the MALDI spectra of *E. coli* K-12 which contained an empty plasmid and Figure 13c is the MALDI spectra of *E. coli* K-12 containing a plasmid with a high *ampC* expression.



**2000 4000 6000 8000 10000 m/z**

Figure 13a: Three Overlaid MALDI spectra obtained for the lab strain *E. coli* K-12*,* using the direct Figure 13a: Three Overlaid MALDI spectra obtained for the lab strain *E. coli* K-12. The analysis was done using the direct smear method and *CHCA* as the matrix of choice*.*



**2000 4000 6000 8000 10000 m/z**

Figure 13b: Three Overlaid MALDI spectra obtained for the modified lab strain *E. coli* K-12 transformed with an empty plasmid (no AmpC expression). The analysis was done using the direct smear method and CHCA as the matrix of choice.



**2000 4000 6000 8000 10000 m/z**

Figure 13c: Overlaid MALDI spectra obtained for the modified lab strain *E. coli* K-12 transformed with AmpC+ plasmid (Opt1 - high AmpC expression). The analysis was done using the direct smear method and CHCA as the matrix of choice.

The spectral patterns generated from the MALDI-TOF analysis of cultures of *E. coli* carrying plasmids consisting of a high expressing *ampC* gene and one empty plasmid were compared for analysis with the original parent bacterium. Although, the spectral patterns do not show a distinct difference from each other which could be used for routine *ampC* detection as they follow similar trends even in intensities, however little differences were identified. Closer observation revealed that there are common peak masses unique to all the three isolates, which interestingly were ‘identification peak masses’ for *E. coli* via the SARAMIS database. The results from matching the spectra (PMF) of each isolate to the SARAMIS database showed correct identification as *E. coli.* In addition, visual observation also revealed that the MALDI spectra seem similar however lesser number of peaks was observed in the modified isolates. This led to the tabulation of the peak masses from each individual isolate for a closer inspection of the data as seen in Table 16.

Table 16: Summary of the peaks in *m/z* obtained from normal lab strain, empty plasmid (pACYC184ΔHN) and *ampC+* induced expression (pACYC-opt-*ampC*).

|  |  |  |  |
| --- | --- | --- | --- |
| **Individual peak acquired from the MALDI-TOF MS analysis. (*m/z*)** | **Normal lab strain** | **Empty plasmid** | **Opt1 (*ampC*+)** |
| 3417 |  |  |  |
| 4165 |  |  |  |
| 4365 |  |  |  |
| 5096 |  |  |  |
| 5386 |  |  |  |
| 6265 |  |  |  |
| 7170 |  |  |  |
| 7283 |  |  |  |
| 8338 |  |  |  |
| 9078 |  |  |  |
| 9754 |  |  |  |
| 10481 |  |  |  |

From observation of the results, the laboratory strain (*E. coli* K-12) had more peaks, while the modified strain with an empty plasmid had fewer peaks and lastly the isolates with plasmid containing high level of *ampC* expression had the least number of peaks. This peak-reduction hypothesis could however be useful in other MALDI research studies to differentiate a typical organism from its modified form. In other words, this result suggests that MALDI-TOF MS spectra could be potentially clinically significant in differentiating between parent organisms and its biologically modified/transformed form. This led to the comparison of the MALDI-TOF spectral patterns obtained for laboratory strain *E. coli* K-12 with its modified form containing plasmids with selective genes to observe the differences between the spectral patterns of the two isolates. The results are shown below.

A similar experiment to the MALDI spectrum comparison among plasmid and non-plasmid containing isolate was conducted to confirm the previous results. Using the MALDI-TOF MS, an *E. coli* K-12 isolate was compared to three transformed isolates of the same organism. The first modified form of the organism had the plasmid pACYC184ΔHN containing only the chloramphenicol gene, the second had the plasmid pUC18 which is a small high copy number cloning vector, in this case the ampicillin resistance gene and the third plasmid was pET 28a which carries the kanamycin resistance gene. The MALDI-TOF MS spectral patterns of the isolates were compared to each other for distinct differences particularly in relation to the number of generated peaks. Figure 14a shows the MALDI-TOF MS spectra obtained from lab strain *E. coli* K-12*,* while Figure 14b, 14c and 14d shows the MALDI-TOF MS spectra obtained from the three transformed forms of the lab strain *E. coli* K-12. Each isolate had three different wells, which means there were three runs and three different spectra were obtained for each isolate. These three MALDI-TOF MS spectra were overlaid on top of each other, while each colour designates a spectrum.



**2000 4000 6000 8000 10000 m/z**

Figure 14a: Three overlaid MALDI-TOF MS spectra of the laboratory strain of *E. coli* K-12 using the CHCA matrix

******

**2000 4000 6000 8000 10000 m/z**

Figure 14b: Three overlaid MALDI-TOF MS spectra of the laboratory strain of *E. coli* K-12 with plasmid pACYC184ΔHN carrying chloramphenicol gene



**2000 4000 6000 8000 10000 m/z**

Figure 14c: MALDI-TOF MS spectra of the laboratory strain of *E. coli* K-12 with plasmid pUC18 carrying the ampicillin resistance gene.

******

**2000 4000 6000 8000 10000 m/z**

Figure 14d: MALDI-TOF MS spectra of the laboratory strain of *E. coli* K-12 with plasmid pET 28a carrying the medium copy-number of the kanamycin resistance gene.

Table 17: Summary of the peaks in *m/z* obtained from normal lab strain and strains with different resistance genes.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Individual peak acquired from the MALDI-TOF MS analysis. (*m/z*)** | ***E. coli***  **K-12** | ***E. coli* K-12 with plasmid pACYC184ΔHN carrying chloramphenicol gene** | ***E. coli* K-12 with plasmid pUC carrying the ampicillin resistance gene** | ***E. coli* K-12 with plasmid pET 28a carrying the medium copy-number of the kanamycin resistance gene** |
| 3417 |  |  |  |  |
| 4165 |  |  |  |  |
| 4365 |  |  |  |  |
| 5096 |  |  |  |  |
| 5386 |  |  |  |  |
| 6265 |  |  |  |  |
| 7172 |  |  |  |  |
| 7283 |  |  |  |  |
| 8338 |  |  |  |  |
| 9078 |  |  |  |  |
| 9754 |  |  |  |  |
| 10481 |  |  |  |  |

Similar to what has been obtained in previous experiments involving modified strains, the MALDI spectra of the laboratory strain of *E. coli* K-12 had the highest number of peaks compared to the modified laboratory strain of *E. coli* K-12 which had acquired plasmids. This results further suggested that MALDI does not detect the ‘normal peaks’ in a modified laboratory strain of *E. coli*, possibly due to the presence of plasmids which contained selective genes. This is in line with previous sub-sections which showed reduction of peaks in MALDI spectra patterns of isolates with transformations compared to the parent isolate.

# 4.7 CHAPTER DISCUSSION

The validation of the MALDI-TOF MS system at the beginning of the research project is crucial to justify the results obtained via this machine. Since the beginning of the decade, MALDI-TOF MS has emerged as a routine tool for microbial identification and diagnosis particularly due to its speed and sensitivity. However, one of the limitations of the technology is that the identification of new isolates is only possible if the spectral database contains peptide mass fingerprints of the organism that is to be identified. This necessitated the selection of a broad range of micro-organisms (bacteria and fungi) that are not too common in research and diagnostic laboratories. For example, *Micrococcus luteus* used to be an example of an organisms with no reference on the spectral databases (Kooken, Fox and Fox, 2012), also *Pseudomonas* species are one of the most difficult species to identify due to high identification error rates as it is difficult to differentiate between the species (Hotta *et al*., 2010). In addition, yeast are problematic microbial species to be identified via MALDI-TOF MS due to the extra extraction steps required for MALDI analysis. According to Cassagne (2013), yeast organisms are identified more reliably with an extra step which usually involve a full formic acid/acetonitrile extraction technique. This modification in sample preparation protocols is required for yeast isolates, with the probability that the reference spectrum of the fungal species is available in the database.

Moreover, the experiment examining the effect of commonly used growth medium on bacterial identification using MALDI-TOF MS was also ascertained. Previous studies revealed that MALDI-TOF MS spectra of bacteria consists of signals derived mostly from ribosomal and other abundant proteins (Alispahic, 2014). This therefore translates to mean that, for proteins that are of ribosomal origin, the effect of growth conditions on the mass spectra and thus on the identification result is expected to be minimal (Cherkaoui *et al*., 2010). However, growth conditions can influence the expression pattern of other proteins and hence disrupt the normal mass spectrum (Valentine *et al*., 2005). According to Anderson *et al*., (2012), culture media contains a variety of differential and selective components particularly salts which are well known inhibitors and interferes with the process of mass spectrometry. This means that each media type can induce changes in the bacterial expression which can lead to a disruption in the normal profile and hence MALDI-TOF MS Spectra. In addition, growth medium compounds can interfere with the ionization of the bacterial biomolecules particularly when the bacterial cells tend to adhere to the culture medium surface. The result obtained from this experiment suggested that genus and species level differentiation is growth medium independent. It would be right to conclude that a well-constructed and robust MALDI-TOF MS identification database such as the SARAMIS would ideally comprise of mass spectra of multiple reference strains per species which have been grown on different culture media and conditions. Thus, as shown in this result section, all media types should possess the required identification peaks within the spectrum which makes it identifiable by a database at least to the species level of identification. However, we don’t have enough data to affirm the strong impact the growth medium might have on strain level differentiation.

The matrix α-cyano-4-hydroxycinnamic acid (CHCA) is known to enhance the ionisation of proteins or peptide standards and samples in MALDI mass spectrometers (Smirnov *et al*., 2004). This matrix is also very useful for the MS examination of intact proteins as it provides a better resolution making the proteins peak much sharper. The performance of the CHCA matrix in bacterial sample ionisation was found to be better than matrices in overall sensitivity and show better sequence coverage at low-digest levels (Beavis, Chaudhary and Chait, 1992). CHCA is one of the best universal matrices for analysing proteins and peptide digests due to the fact that it readily forms uniform co-crystals and also have an absorption maximum close to the wavelength of the typical nitrogen (N2) laser used in MALDI-TOF MS (337 nm).

Though CHCA is referred to as the ‘gold standard’ matrix, there is still a continuous search for improved laser desorption matrix for peptides and proteins (Zhang, 2009). The thirteen novel matrices developed in house were made in the laboratory by altering the functional groups on the CHCA matrix core as shown in Figure 15.

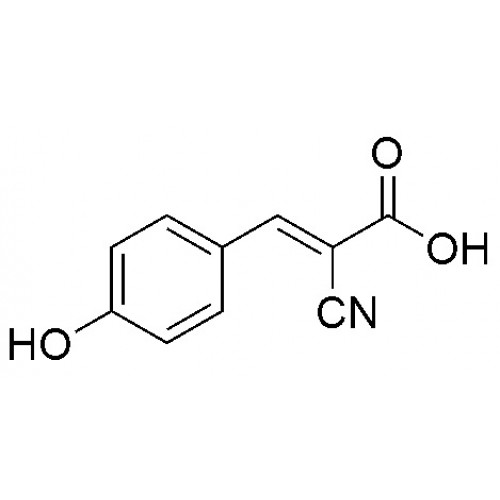


Figure 15: Chemical structure of the MALDI matrix CHCA

The summary behind the development of the ‘in-house matrices’ lies in the OH functional group on the benzene ring. The closest so far to CHCA is PD 84 which expectedly gave one of the best spectra among the thirteen matrices. It is however no surprise that the identification of the organisms with the use of the ‘in-house’ matrices fails to correctly identify the organisms.

The study of the effect of plasmid acquisition by bacteria is not entirely new. In 1986, Bailey reported the studies of host-plasmid interaction in recombinant microorganism which set a new foundation for this area of microbial research (Bailey *et al*., 1986). Introduction of plasmids into bacterial cells enables synthesis in those cells of valuable proteins which creates new manufacturing routes and sources of supply for numerous proteins of practical value. Mason and Bailey (1989) reported that plasmid presence mediates subtle changes in the net expression of host enzyme involved in carbon metabolism. Another similar research by Seo and Bailey (1985) concluded that the overall efficiency of plasmid gene expression, measured as the ratio of beta-lactamase specific activity to plasmid content, decreased significantly with increasing plasmid content in the LB medium. The impact of these plasmids on MALDI-TOF signals, peak masses and spectrum is yet to be reported which makes this wok novel. The summary from the results suggested that plasmid does have an impact on MALDI-TOF spectrum, causing a reduction in the number of peaks generated. Plasmids have been shown to be involved in repair, mutagenesis, replication and recombination of the host chromosome which undoubtedly would require extra resources from the cell, by extending DNA metabolism potential thereby promoting evolutionary transformations (Bettent, 2008). Some plasmids do confer antibiotic resistance to host bacterium, this shows that keeping a plasmid inside a bacterial cell would increases the metabolic burden on the cell. This is because replicating DNA already uses up energy and thus to replicate the plasmid DNA, the bacterial cell is stressed. (Mason and Bailey, 1998). In situations where plasmids have to protect the bacteria host from stress-related death (antibiotic), a plasmid maximises its chance of being retained. This involves continuous replication of the plasmid DNA at the expense of ribosomal proteins which are natural to the host cell (Birnbaum and Bailey, 1991). This corroborates with the result obtained when the impact of plasmids was tested via MALDI-TOF MS.

**CHAPTER 5**

**RESULTS: ANALYSIS OF *ACANTHAMOEBA* ISOLATES VIA MALDI-TOF MS & PCR.**

# 5.1OVERVIEW OF THE CHAPTER

This chapter focusses on MALDI-TOF MS identification of the protozoan parasite – *Acanthamoeba.* The first sub-section of this chapter is centred on the establishment of a routine method for the generation of useful reproducible spectra for this organism. The development of this method would be the initial step in its use as a potential diagnostic means in clinical microbiology. The standard method for bacterial and fungal species failed to generate reproducible result and thus the need for a more specific method is detailed below. After the establishment of an appropriate MALDI-TOF MS method that produces reproducible spectra, the following section details the results of the MALDI-TOF MS spectra of the fifteen *Acanthamoeba* isolates. Although, the MALDI-TOF MS spectra of three out of the fifteen isolates have been shown here, however a detailed table showing all the peak masses from each isolate have been manually commutated.

We also looked at difference between the MALDI-TOF MS spectra obtained from the cyst and trophozoite stages of the organism. Cultures of *Acanthamoeba* cyst and trophozoites were analysed under MALDI-TOF MS, the generated spectral as well as the peak masses were compared to each other and to the mixed cultures. The last MALDI-TOF MS sub-section details the result of the sensitivity limit test for generating good spectral pattern for *Acanthamoeba i*solates. As known for bacterial cultures, a sufficient number of cells (typically 104 cells per ml) are required to generate detectable MALDI –TOF MS ion signals, the same is required for *Acanthamoeba.* The final result sub-section is on the genotyping results of all the fifteen isolates of *Acanthamoeba*. The genotyping involves PCR, gel electrophoresis, DNA purification and sequencing. The result from the gel electrophoresis is shown in the last sub-section as well as the sequencing results that were obtained from an external source.

The aims of this chapter are:

* To establish a routine method for the generation of good reproducible spectra patterns for *Acanthamoeba* isolates which can be potentially used for diagnosis.
* To generate unique and reproducible MALDI spectra patterns for each of the fifteen clinical *Acanthamoeba* isolates.
* To identify the peaks from each of the two life stages of the organism as well as comparing it to the mixed culture.
* To identify the sensitivity limit in terms of minimum numbers required to produce consistent and reproducible spectral patterns from a mixed culture of *Acanthamoeba*
* To obtain the genotype of each of the fifteen *Acanthamoeba* isolates using PCR, gel electrophoresis and DNA sequencing.

# 5.2 ANALYSIS OF *ACANTHAMOEBA* SPECIES VIA MALDI-TOF MS

Although *Acanthamoeba* species have been tested via MALDI-TOF MS, none of the reported papers could identify the isolates due to the fact that there was no reference database available for this organism. The only reported paper (Del Chierico *et al*., 2016) also did not compare the relativity of the isolates to each other possibly via spectrum or peak masses but instead used PCR to confirm identification. The result in Figure 16 below showed the spectrum in triplicates obtained from the standard sample preparation method (direct smear) of *Acanthamoeba* species.



**2000 6000 10000 14000 18000 m/z**

Figure 16: Three overlaid MALDI spectra obtained from the standard sample preparation method (direct smear) analysis of a mixed culture of *Acanthamoeba* cysts and trophozoites.

Results from the standard method (direct smear) used in MALDI bacterial identification when applied to *Acanthamoeba* yielded poor non-reproducible spectra. The poor spectra (due to too much noise) obtained from this direct smear method necessitated the use of extraction methods to obtain consistent reproducible results. Thus, the need to try a few extraction methods (standard and on- plate extraction methods) and then evaluate the results afterwards.

## 5.2.1 Acanthamoeba MALDI-TOF MS result

Figure 17a and 17b shows the MALDI spectra in triplicates from the analysis of *Acanthamoeba* using the two extraction methods.



**2000 6000 10000 14000 18000 m/z**

Figure 17a: Three MALDI spectra overlaid on top of each other obtained from the standard extraction method for the analysis of a mixed culture of *Acanthamoeba* cysts and trophozoites.



**2000 6000 10000 14000 18000 m/z**

Figure 17b: Three overlaid MALDI spectra overlaid on top of each other obtained from the on-plate extraction method for the analysis of a mixed culture of *Acanthamoeba* cysts and trophozoites.

To further investigate the robustness and reproducibility of the results, further analysis is required. The peak masses from each spectrum obtained from the three methods of preparation/extraction were tabulated and compared against each other. This is to clarify which of the preparation/extraction methods gives a much more reliable and detailed result, which could be a standard for choosing the routine method for further experiments.

Table 18: Different *Acanthamoeba* peaks from the three preparation methods.

|  |  |  |  |
| --- | --- | --- | --- |
| **Individual peak acquired from the MALDI-TOF MS analysis. (*m/z*)** | **Direct Smear**  **(Figure 16)** | **MALDI Standard extraction**  **(Figure 17a)** | **MALDI On-plate extraction**  **(Figure 17b)** |
| 3183 |  |  |  |
| 3397 |  |  |  |
| 4251 |  |  |  |
| 4772 |  |  |  |
| 5615 |  |  |  |
| 6099 |  |  |  |
| 7172 |  |  |  |
| 7478 |  |  |  |
| 8515 |  |  |  |
| 9739 |  |  |  |
| 14335 |  |  |  |
| 14350 |  |  |  |

The results reveal that although two peaks (*m/z* 6099 and 14335) seem to be common to the three methods, the standard extraction and the on-plate extraction have similar peak values and spectra patterns. The on-plate extraction generated more peaks at 5615, 9739 and 14350 *m/z*. Based on its short protocol, rapidity and the extra peaks generated, the on-plate method closely followed by standard extraction, seems the best out of all the methods tested for *Acanthamoeba* extraction. For further analysis of the *Acanthamoeba* species mentioned in this thesis, the on-plate extraction method was employed unless otherwise stated.

# 5.3GENERATION OF MALDI SPECTRAL FOR FIFTEEN CLINICAL *ACANTHAMOEBA* ISOLATES USING ON-PLATE EXTRACTION METHOD.

A reproducible routine method for MALDI-TOF preparation of *Acanthamoeba* samples has been established using one of the isolates, thus all the remaining fourteen isolates were analysed via MALDI-TOF MS method. As mentioned and detailed in the previous chapter, fifteen isolates of *Acanthamoeba* were available and thus tested via MALDI-TOF MS using the same culture densities. Figures 18a, 18b and 18c shows the MALDI spectrum in triplicates obtained for three of the fifteen isolates of *Acanthamoeba* using the on-plate extraction method and the CHCA matrix. The data for all the fifteen isolates is shown in Table 19.



**2000 6000 10000 14000 18000 m/z**

Figure 18a: Three MALDI spectra obtained for the isolate SK-19-56 using the on-plate extraction method for the analysis of the mixed culture.



**4000 8000 12000 16000 m/z**

Figure 18b: Three MALDI spectra obtained for the isolate 30371 using the on-plate extraction method for the analysis of the mixed culture.



**4000 8000 12000 16000 m/z**

Figure 18c: Three MALDI spectra obtained for the isolate ROS using the on-plate extraction method for the analysis of the mixed culture.

For subsequent differential analysis of isolates, the masses of peaks from the fifteen *Acanthamoeba* isolates was compared by physical observation and via a table. This analysis further showed that there are six peak masses which are common to all the *Acanthamoeba* isolates. The common peak masses are 3400, 6100, 8515, 9691 10633 and 14100, these could be the potential ‘markers’ for identifying *Acanthamoeba* via MALDI-TOF MS, similar to that has been used for bacterial and fungal isolates. To identify each of these peaks, an extra experimentation involving MS/MS will have to be adopted which is not within the scope of this project.

Table 19: Comparing all the fifteen clinical isolates of *Acanthamoeba* with their corresponding peak masses in m/z.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Individual peak acquired from the MALDI-TOF MS analysis. (*m/z*)** | | | | | | | | | | | | | | | |
| **No** | ***Ac.* Isolates** | **2241** | **3400** | **4159** | **4871** | **5228** | **5943** | **6100** | **6928** | **7483** | **8512** | **8873** | **9691** | **10633** | **12751** | **14100** | **16849** |  |
| 1 | SK14-4 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 2 | SK19-9 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 3 | SK19-56 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 4 | SK19-59 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 5 | PM19 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 6 | 20365 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 7 | 30234 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 8 | 30371 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 9 | 30461 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 10 | 30874 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 11 | 50575 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 12 | ROS |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 13 | ISO/3A |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 14 | Rad A |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 15 | LSH TM 1630 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |

# 5.4 COMPARISON AND DIFFERENTIATION OF *ACANTHAMOEBA* CYST AND TROPHOZOITES SPECTRA PATTERNS

In an ongoing *Acanthamoeba* infection, both the cysts and trophozoites will be present. For example, in *Acanthamoeba* keratitis, both cysts and trophozoites are present in the eye infection, although the ratio might vary. This makes it clinically important to be able to distinguish the two phases of the organism using the MALDI-TOF MS spectra. Figure 19, 20 and 21 below shows the MALDI spectra obtained from a particular strain (SK14-4) from a mixed culture of *Acanthamoeba* cysts and trophozoites as well as individual culture of cysts and trophozoites.



**2000 6000 10000 14000 18000**

**m/z**

Figure 19: Three overlaid MALDI spectra in triplicates from a mixed culture (50:50) of *Acanthamoeba* cysts and trophozoites from the strain SK14-4.



**2000 6000 10000 14000 18000**

**m/z**

Figure 20: Three overlaid MALDI spectra in triplicates from a 95% culture of *Acanthamoeba* cyst from strain SK14-4.



**2000 6000 10000 14000 18000**

**m/z**

Figure 21: Three overlaid MALDI spectra in triplicates from an 80% culture of *Acanthamoeba* trophozoites from strain SK14-4.

For further analysis, the peak masses contained in each spectrum for each of the cultures have been tabulated for proper comparison.

Table 20: The different *Acanthamoeba* peaks of enriched (95%) cyst, enriched (80%) trophozoites and mixed culture (50-50) of SK14-4 strain resulting from on-plate extraction method. The result revealed three peaks with same *m/z* values.

|  |  |  |  |
| --- | --- | --- | --- |
| **Individual peak acquired from the MALDI-TOF MS analysis. (*m/z*)** | **95% enriched Cyst culture** | **80%enriched *Trophozoites* culture** | **Mixed culture** |
| 3183 |  |  |  |
| 3396 |  |  |  |
| 4442 |  |  |  |
| 6070 |  |  |  |
| 6099 |  |  |  |
| 6378 |  |  |  |
| 8503 |  |  |  |
| 10642 |  |  |  |
| 13627 |  |  |  |
| 14335 |  |  |  |
| 16248 |  |  |  |

From the Figures above, the cyst culture had a better spectral pattern in terms of low level of noise compared to the trophozoite culture. Also, there are more distinct peaks in cyst cultures than trophozoite culture which makes it more suitable for identification. The high noise level observed for trophozoite might be due to the sample preparation method which involved formic acid application to the cells. The use of the acid might be too harsh for trophozoites, thus degrading and losing all the cytoplasmic contents of the cells which may be the reason for high noise levels seen in the spectral pattern.

# 5.5 LIMIT OF SENSITIVITY FOR PRODUCING REPRODUCIBLE MALDI SPECTRA FOR *ACANTHAMOEBA I*SOLATES

Most biological methods have sensitivity limits by which they do not respond to analysis. For example, bacterial samples have MALDI sensitivity limits for accurate identification, which must be adhered to, in order to get an accurate result. Thus, it is imperative to know the sensitivity limit for *Acanthamoeba* at which it does not give a reproducible spectrum which is suitable for identification. Figures 22a to 22d shows the MALDI spectra patterns for different number of cells per ml using a particular strain (SK14-4) from a mixed culture of *Acanthamoeba* cyst and trophozoites.



**2000 6000 10000 14000 18000**

**m/z**

Figure 22a: Three overlaid MALDI spectra in triplicates of 1 x 105 cells per ml of a mixed culture of *Acanthamoeba* strain SK14-4

****

**2000 6000 10000 14000 18000**

**m/z**

Figure 22b: Three overlaid MALDI spectra in triplicates of 2 x 105 cells per ml of a mixed culture of *Acanthamoeba* strain SK14-4.

****

**2000 6000 10000 14000 18000**

**m/z**

Figure 22c: Three overlaid MALDI spectra in triplicates of 3 x 105 cells per ml of a mixed culture of *Acanthamoeba* strain SK14-4.

****

**2000 6000 10000 14000 18000**

**m/z**

Figure 22d: Three overlaid MALDI spectra in triplicates of 4 x 105 cells per ml of a mixed culture of *Acanthamoeba* strain SK14-4.

Figures 22a to 22d shows the spectral patterns for the *Acanthamoeba* strain SK14-4 at different cell densities. As with bacterial and fungal identification, a sufficient number of bacterial cells are required to generate detectable MALDI-MS ion signals, the same applies to *Acanthamoeba*. As seen in Figure 22a the overlaid MALDI-TOF MS spectra was not consistent, improved with increased cells in Figure 22b and also got better with more cells in Figure 22c. Figure 22d shows an overlaid MALDI-TOF MS spectra that is similar to each other as well as reproducible. Thus, it was suggested that the the detection limits for *Acanthamoeba* is 4 x 105 cells per ml, which equates to 400 cells per MALDI target plate.

# 5.6 PCR AMPLIFICATION AND DNA SEQUENCE ANALYSIS OF THE *ACANTHAMOEBA* 18S RRNA GENE

The detection of *Acanthamoeba* isolates via PCR has been reported in literature (Yera, *et al*, 2013). Genotyping of the *Acanthamoeba* isolates would enable the exploration of the genetic variants embedded in each strain, more importantly when this is compared to its MALDI-TOF results. This could lead to a deeper understanding of disease aetiology on a molecular level and better differentiation even down to the strain level. The process of genotyping starts from the extraction of the DNA from the isolates, followed by PCR amplification, then gel electrophoresis and then sequencing to obtain the precise genotype of each isolate. The first experiment sets out to find out how best to extract DNA from *Acanthamoeba* that can result in a band with gel electrophoresis. One strain (SK-14-4) was picked at random for this test. DNA was extracted in three different ways from this particular *Acanthamoeba* isolate. These were (1) Sonication, (2) Boiling and (3) buffer lysis as explained in the general methods section. The extracted DNA was amplified via PCR as detailed in the method section, this was followed by gel electrophoresis. The result obtained from GelRed-stained 2% agarose gel showing the PCR-amplified product of extracted *Acanthamoeba* (SK-14-4) DNA via the three methods is shown below:

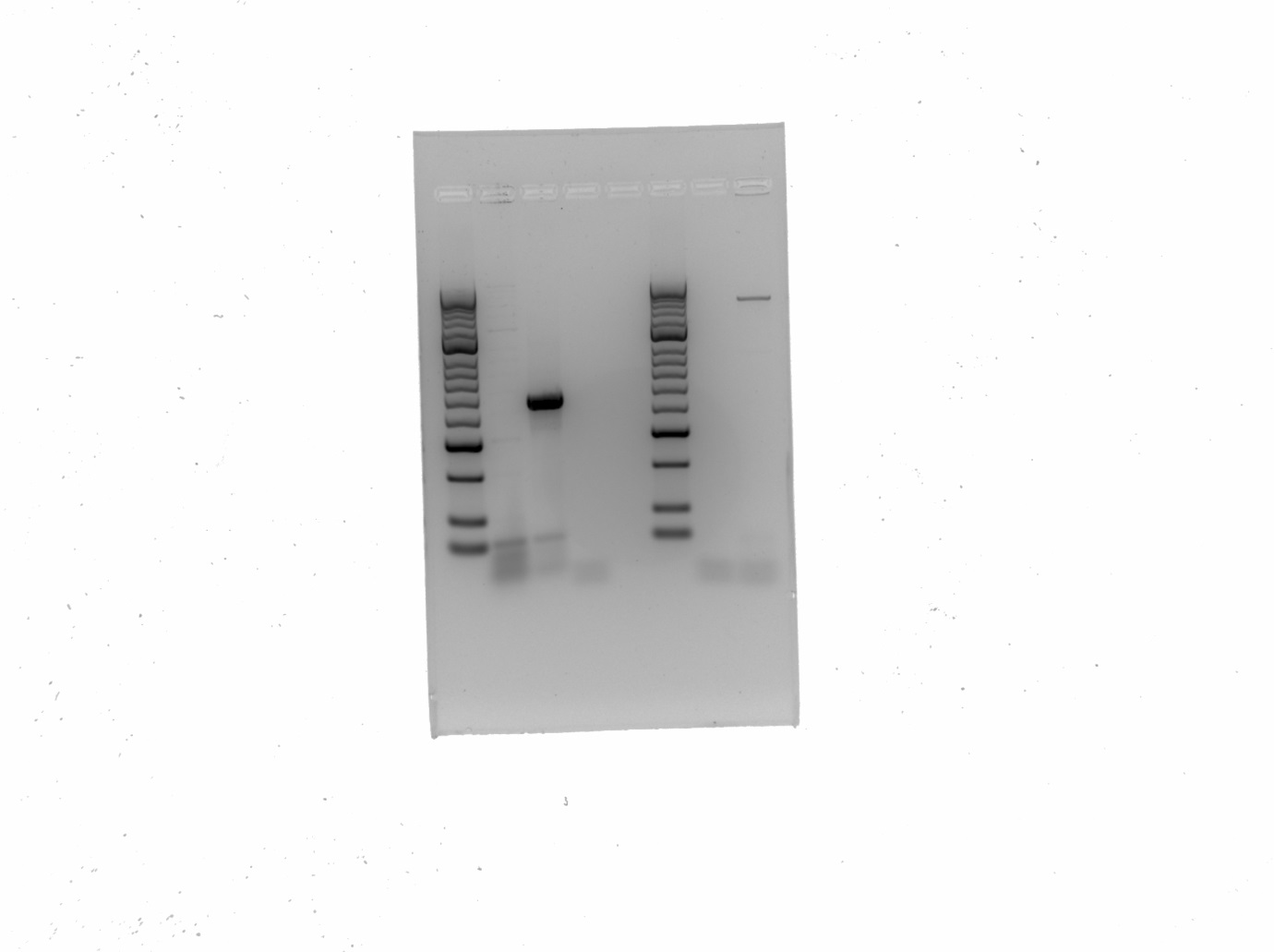


Figure 23: Gel electrophoresis results of the *Acanthamoeba* isolate SK-14-4. The first lane is the ladder, lane 2 is the sample from DNA extracted using sonication, lane 3 is a sample from DNA extracted by boiling, while lane 4 is a sample obtained from buffer lysis and lane 5 is the negative control while the sixth and last lane is the ladder.

Three DNA extraction methods as mentioned above were employed for the first five *Acanthamoeba.* Out of the three methods, boiling was the only successful method that could obtain a band on the gel electrophoresis after PCR amplification. The gel band obtained from the boiling sample was at 450 base pairs (bp) as expected for this organism. The portion of the band was cut, purified and sent for sequencing at the Department of Bioscience at the University of Durham. The result showed that the strain belongs to the *Acanthamoeba* T4 genotype.

The establishment of a DNA extraction method that works allows for the genotyping of the other strains of *Acanthamoeba.* The first five strain (including SK-14-4 which was analysed above) were subjected to boiling as a means of DNA extraction, followed by PCR amplification and gel electrophoresis. The result obtained from GelRed-stained 2% agarose gel showing the PCR-amplified products (450 – 500bp) of extracted DNA from the five *Acanthamoeba* isolates is shown below:

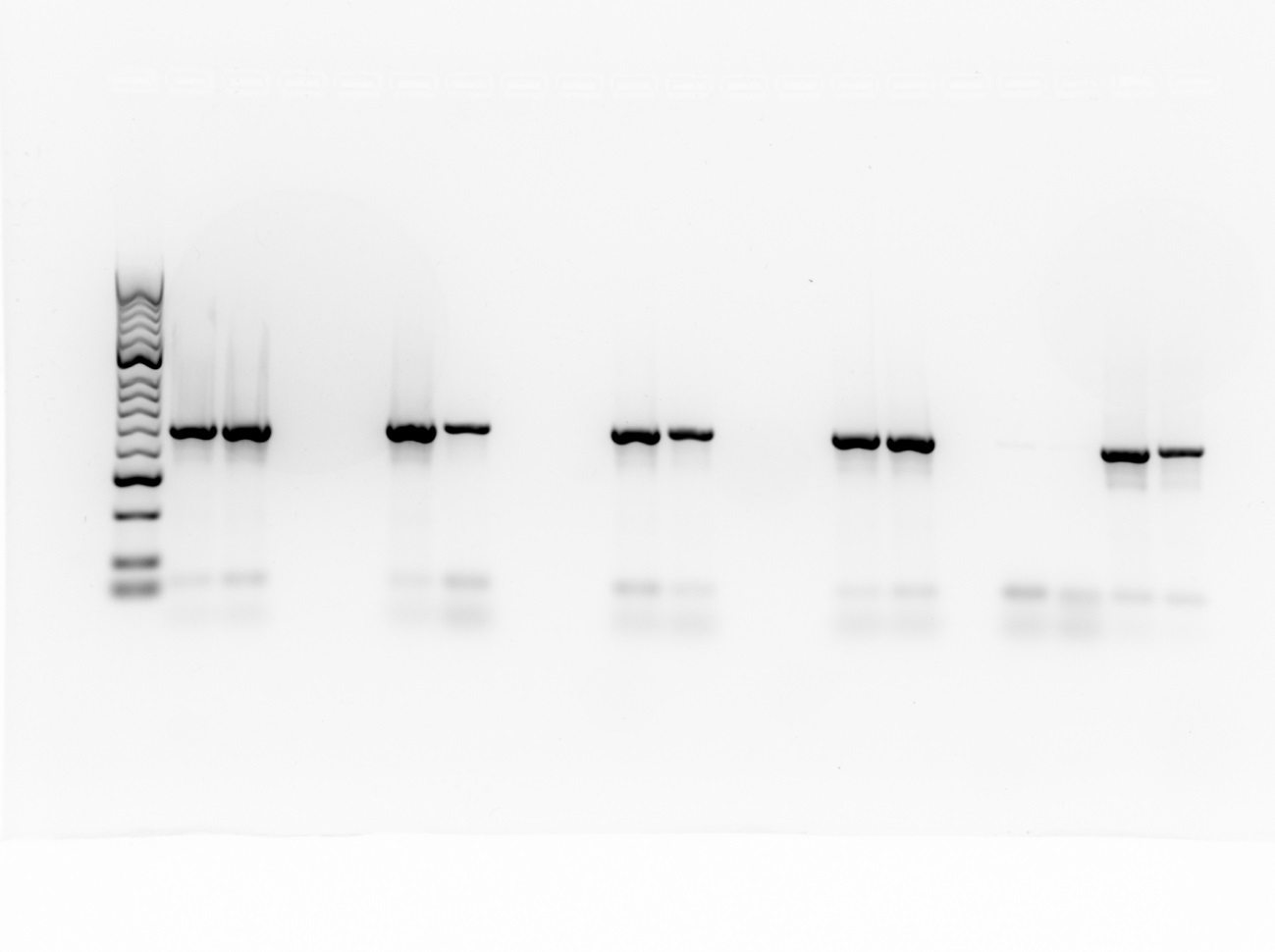


Figure 24: Gel electrophoresis results of the first five *Acanthamoeba* isolate done in duplicates using boiling as a means of DNA extraction. The first lane is the ladder, the second and third lane is SK-14-4, the next two lanes represents the isolate SK-19-9, followed by SK-19-56, SK-19-59, the negative control and the two last lanes are BM19.

From Figure 24, all the five isolates tested showed similar band sizes, when analysed on the gel electrophoresis. The gel bad obtained was at 450 – 500 base pairs (bp), this was cut, purified and sent for sequencing at the Department of Bioscience at the University of Durham. The result showed that all the isolates belonged to the *Acanthamoeba* T4 genotype. The final ten isolates were also subjected to DNA extraction, followed by PCR amplification and gel electrophoresis. The result obtained from GelRed-stained 2% agarose gel is shown below in Figure 25.

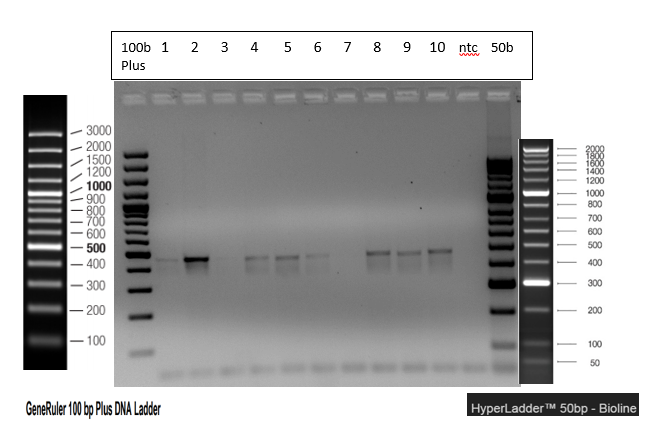


Figure 25: Gel electrophoresis results of ten *Acanthamoeba* isolates using boiling as a means of DNA extraction. The first lane is the ladder, followed sequentially by isolates ISO/3A, 50575, 30874, Rad A, ROS, LSH TM 1630, 30234, 20365, 30461, 30371, negative control and lastly is the ladder.

From Figure 25 above, it is clear that only eight isolates out of the ten tested had similar band width when analysed on the gel electrophoresis. The gel band obtained was between 450 -500 bp, this was cut, purified and sent for Sanger DNA sequencing at the Department of Bioscience at the University of Durham.

The 18S rRNA gene amplicon sequences obtained for all the *Acanthamoeba* isolates are detailed in appendix A. The analysis of the *Acanthamoeba* sequence was conducted using the nucleotide BLAST (Basic Local Alignment Search Tool) program in conjunction with the database, which is available for use online at the NCBI (National Centre for Biotechnology Information) website. BLAST can rapidly align and compare a query nucleotides sequence (s) to both single and large database of sequences and calculates the statistical significance for each sequence alignment result, known as the expect or probability value (E or P value) (Altschul *et al*, 1990). BLAST is an alignment program which uses rapid programming algorithms to finds regions of similarity between biological sequences. Simply put, BLAST is an algorithm which compares primary biological sequence information such as DNA sequences. It allows a researcher to compare a query sequence with a library of database of sequences and identify library sequences that resemble the query sequence above a certain threshold. It can also be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families.

From this sequence table (appendix A), we obtained genotype identification for all the 15 isolates via the BLAST software. Fourteen out of the fifteen isolates have the genotype T4, while one isolate had the genotype T11. This is not surprising as most *Acanthamoeba* obtained from clinical sources are often of the T4 genotype. To link the relationship between these isolates, an alignment is performed with the Clustal Omega using the sequences previously obtained (Sievers *et al*., 2014). A neighbour-joining phylogram tree was generated using the Omega Clustal algorithm program, which produces a multiple sequence alignment by firstly producing pairwise alignments and then the clustering of the sequences. The phylogenetic tree obtained from the sequence of the *Acanthamoeba* isolates is shown in Figure 26 below.

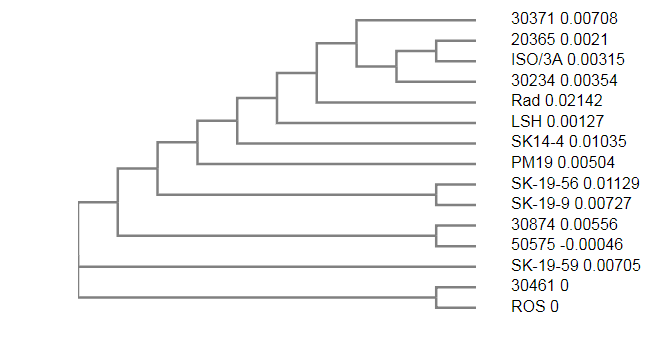


Figure 26: The phylogenetic tree of the *Acanthamoeba* isolates obtained by the neighbour-joining method of the Omega Clustal algorithm program. This is a cladogram (horizontal branch lengths are not informative and it only shows group not genetic distances between isolates).

To compare the results obtained from Sequencing and MALDI-TOF MS of the *Acanthamoeba* isolates, a simple phylogenetic denodgram-like diagram is obtained. This is done by grouping the isolates based on visual inspection of the peak masses similarity from their spectra.

30371

20365 ISO/3A

30234

Rad A

LSH TM

PM19

SK14-4

ROS

30461

30874

50575

SK-19-9

SK19-56

SK19-59 Figure 27: A dendogram-like diagram, grouping the *Acanthamoeba* isolates based on similarity in peak masses of the spectra.

The sequence alignment of the *Acanthamoeba* isolates is detailed below:

CLUSTAL O(1.2.4) multiple sequence alignment

30371 ---------------------------------ACCGAAGACGCCAA----------GGN 17

20365 ---------------------------------------------------------GGG 3

30234 -----------------------------------------------------------G 1

ISO/3A ---------------------------------------------------------AGG 3

LSH CGCGCATTTCAAGCGCCCGTGCCATCGGGTCAAACCGGTGGCTGCGTTGGCGTTGCGGGC 60

SK-19-59 ------------------------------------------------------------ 0

30461 ------------------------------------------------------------ 0

ROS ------------------------------------------------------------ 0

SK14-4 ------------------------------------------------------------ 0

Rad ------------------------------------------------------------ 0

SK-19-56 ------------------------------------------------------------ 0

PM19 ------------------------------------------------------------ 0

SK-19-9 ------------------------------------------------------------ 0

30874 ------------------------------------------------------------ 0

50575 ------------------------------------------------------------ 0

30371 ACGAACC--------GCACCGNATGGCTGGTGATTTTGTATTCA------ACGTCT---C 60

20365 AC-GACC--------GCACCGATGG--TGGT-GTTTTGTATTCA------ACGTCT---C 42

30234 AC-GACC--------GCGCCGATGG--TGGT-GTTTTGTATTCA------ACGTCT---C 40

ISO/3A AC-GACC--------GCACCGATGG--TGGT-GTTTTGTATTCA------ACGTCT---C 42

LSH TCGGTCCGTCGGTGCCCCACAAAGGGCTATCGGCGTGTCAACCGGCCCGCCCGTCCCCTC 120

SK-19-59 ------------------------------------------------------------ 0

30461 ------------------------------------------------------------ 0

ROS ------------------------------------------------------------ 0

SK14-4 ------------------------------------------------------------ 0

Rad ------------------------------------------------------------ 0

SK-19-56 ------------------------------------------------------------ 0

PM19 ------------------------------------------------------------ 0

SK-19-9 ------------------------------------------------------------ 0

30874 ------------------------------------------------------------ 0

50575 ------------------------------------------------------------ 0

30371 CTAATCG-----CTGGTCGGCAT---CGTTTATGGTTAAGACT-----------ACGACG 101

20365 CTAATCG-----CTGGTCGGCAT---CGTTTATGGTTAAGACT-----------ACGACG 83

30234 CTAATCG-----CTGGTCGGCAT---CGTTTATGGTTAAGACT-----------ACGACG 81

ISO/3A CTAATCG-----CTGGTCGGCAT---CGTTTATGGTTAAGACT-----------ACGACG 83

LSH CTTCTGGATTCCCGTTCCTGCTATTGAGTTAGTGGGGACGTCACAGGGGGTCCATCGTCG 180

SK-19-59 ------------------------------------------------------------ 0

30461 ------------------------------------------------------------ 0

ROS ------------------------------------------------------------ 0

SK14-4 ------------------------------------------------------------ 0

Rad ------------------------------------------------------------ 0

SK-19-56 ------------------------------------------------------------ 0

PM19 ------------------------------------------------------------ 0

SK-19-9 ------------------------------------------------------------ 0

30874 ------------------------------------------------------------ 0

50575 ------------------------------------------------------------ 0

30371 ----GT-----ATCTGATCGTCTTCGA---------TCCCCTAACTTTCGTTCTTGAT-- 141

20365 ----GT-----ATCTGATCGTCTTCGA---------TCCCCTAACTTTCGTTCTTGAT-- 123

30234 ----GT-----ATCTGATCGTCTTCGA---------TCCCCTAACTTTCGTTCTTGAT-- 121

ISO/3A ----GT-----ATCTGATCGTCTTCGA---------TCCCCTAACTTTCGTTCTTGAT-- 123

LSH TGCGGCGTCAAAACCGTGCGGCGGTGGGTCCCTGGGGCCCAGATCGTT-TACCGTGAAAA 239

SK-19-59 ------------------------------------------------------------ 0

30461 ------------------------------------------------------------ 0

ROS ------------------------------------------------------------ 0

SK14-4 ------------------------------------------------------------ 0

Rad ------------------------------------------------------------ 0

SK-19-56 ------------------------------------------------------------ 0

PM19 ------------------------------------------------------------ 0

SK-19-9 ------------------------------------------------------------ 0

30874 ------------------------------------------------------------ 0

50575 ------------------------------------------------------------ 0

30371 ---TAATGAAAACATCCTTGGCAGATGCTTTCGCAGAAGTTAATCTTTCATAAATCCAAG 198

20365 ---TAATGAAAACATCCTTGGCAGATGCTTTCGCAGAAGTTAATCTTTCATAAATCCAAG 180

30234 ---TAATGAAAACATCCTTGGCAGATGCTTTCGCAGAAGTTAATCTTTCATAAATCCAAG 178

ISO/3A ---TAATGAAAACATCCTTGGCAGATGCTTTCGCAGAAGTTAATCTTTCATAAATCCAAG 180

LSH AATTAGAGTGTTCAAAGCAGGCAGATCCA-T----------TTTCT-------------- 274

SK-19-59 --------GTTTTCAGGCATGCAGATCCA-T----------TTTCT-------------- 27

30461 ------------------------------------------------------------ 0

ROS ------------------------------------------------------------ 0

SK14-4 ---------GTTCAAAGCAGGCAGATCCAAT----------TTTCT-------------- 27

Rad ------------------------------------------------------------ 0

SK-19-56 --------AACTTCAAGCAGGCAGATCCA-T----------TTTCT-------------- 27

PM19 ---------GTTCAAAGCAGGCAGATCCAAT----------TTTCT-------------- 27

SK-19-9 ------TTGAGTCTCAGCATGCAGATCCA-T----------TTTCT-------------- 29

30874 ------------------------------------------------------------ 0

50575 ------------------------------------------------------------ 0

30371 AATTTCACCTCTGACAATTA--------AATATTAATGCCCCCAACTATCCCTATTAATC 250

20365 AATTTCACCTCTGACAATTA--------AATATTAATGCCCCCAACTATCCCTATTAATC 232

30234 AATTTCACCTCTGACAATTA--------AATATTAATGCCCCCAACTATCCCTATTAATC 230

ISO/3A AATTTCACCTCTGACAATTA--------AATATTAATGCCCCCAACTATCCCTATTAATC 232

LSH ---GCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTG-TCCTCCTATTTTCA 330

SK-19-59 ---GCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTG-TCCTCCTATTTTCA 83

30461 --------------------------ATAATGGAATAGGACCCTG-TCCTCCTATTTTCA 33

ROS ---------------------------TAATGGAATAGGACCCTG-TCCTCCTATTTTCA 32

SK14-4 ---GCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTG-TCCTCCTATTTTCA 83

Rad ---------------------ATGGGATAATGGAATAGGACCCTG-TCCTCCTATTTTCA 38

SK-19-56 ---GCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTG-TCCTCCTATTTTCA 83

PM19 ---GCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTG-TCCTCCTATTTTCA 83

SK-19-9 ---GCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTG-TCCTCCTATTTTCA 85

30874 ---------------TTAGCCATGGGATAATGGAATAGGACCCTGNTCCTCCTATTTTCA 45

50575 -------------------GCATGGGATAATGGAATAGGACCCTG-TCCTCCTATTTTCA 40

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30371 ATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGG--AGGACAGGGTCCTATT 308

20365 ATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGG--AGGACAGGGTCCTATT 290

30234 ATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGG--AGGACAGGGTCCTATT 288

ISO/3A ATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGG--AGGACAGGGTCCTATT 290

LSH GTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAA 390

SK-19-59 GTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAA 143

30461 GTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAA 93

ROS GTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAA 92

SK14-4 GTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAA 143

Rad GTTGGTTTTGGC-ACGCGAGGACCAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAA 97

SK-19-56 GTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAA 143

PM19 GTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAA 143

SK-19-9 GTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAA 145

30874 GTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAA 105

50575 GTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAA 100

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30371 ----CCATTATCCCATGCTAATGTATTCGGT----GGCAGAA----------AATTGGAT 350

20365 ----CCATTATCCCATGCTAATGTATTCGGT----GGCAGAA----------AATTGGAT 332

30234 ----CCATTATCCCATGCTAATGTATTCGGT----GGCAGAA----------AATTGGAT 330

ISO/3A ----CCATTATCCCATGCTAATGTATTCGGT----GGCAGAA----------AATTGGAT 332

LSH TATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCAT 450

SK-19-59 TATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCAT 203

30461 TATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCAT 153

ROS TATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCAT 152

SK14-4 TATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCAT 203

Rad TATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCAT 157

SK-19-56 TATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCAT 203

PM19 TATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCAT 203

SK-19-9 TATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCAT 205

30874 TATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCAT 165

50575 TATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCAT 160

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30371 CTGCCTGCTTTGAACACTCTAAT------------------------------------- 373

20365 CTGCCTGCTTTGAACACTCTAAT------------------------------------- 355

30234 CTGCCTGCTTTGAACACTCTAAT------------------------------------- 353

ISO/3A CTGCCTGCTTTGAACACTCTAAT------------------------------------- 355

LSH CTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATA 510

SK-19-59 CTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATA 263

30461 CTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATA 213

ROS CTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATA 212

SK14-4 CTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATA 263

Rad CTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATA 217

SK-19-56 CTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATA 263

PM19 CTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATA 263

SK-19-9 CTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATA 265

30874 CTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATA 225

50575 CTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATA 220

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30371 ------------------------------------------------------------ 373

20365 ------------------------------------------------------------ 355

30234 ------------------------------------------------------------ 353

ISO/3A ------------------------------------------------------------ 355

LSH CCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAA 570

SK-19-59 CCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAA 323

30461 CCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAA 273

ROS CCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAA 272

SK14-4 CCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAA 323

Rad CCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAA 277

SK-19-56 CCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAA 323

PM19 CCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAA 323

SK-19-9 CCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAA 325

30874 CCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAA 285

50575 CCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAA 280

30371 ------------------------------------------------------------ 373

20365 ------------------------------------------------------------ 355

30234 ------------------------------------------------------------ 353

ISO/3A ------------------------------------------------------------ 355

LSH CACCACCATCGGTGCGGT-CGTCCTTGGCGT----------------------------- 600

SK-19-59 CACCACCATCGGTTGCGGTCGTCCTTGGCGTCTCGGTTTCG------GC--CGGGGCGCG 375

30461 CACCACCATCGG-TGCGGTCGTCCTTGGCGTC--GGTTTCG------GC--C--GGCGCG 320

ROS CACCACCATCGG-TGCGGTCGTCCTTGGCGTC--GGTTTCG------GC--C--GGCGCG 319

SK14-4 CACCACCATCGGTTGCGGTCGTCCT-GGCGTCTCGTTTCGG--CCGGGC-GCGGGACG-- 377

Rad CACCACCATCGG-TGCGGTCGTCCTTGGCGCGTCGTG------GCTTGCTGCGGCGTGCG 330

SK-19-56 CACCACCATCGGTTGCGGTCGTCCTTGGCGTCTCGGTTTCG------GC--CGGGGCGCG 375

PM19 CACCACCATCGGTTGCGGTCGTCCTTGGCGTCTCGGTTTC------GGC--CGGGGCGCG 375

SK-19-9 CACCACCATCGGTTGCGGTCGTCCTTGGCGTCTCGGTTTCG------GC--CGGGGCGCG 377

30874 CACCACCATCGG-TGCGGTCGTCCTTGGCGTCTCGGTCCTTCACGGGGC--CGGGGCGCG 342

50575 CACCACCATCGG-TGCGGTCGTCCTTGGCGTCTCGGTCCTTCACGGGGC--CGGGGCGCG 337

30371 -------------------------------------------TTTTTCACGGTAAACGA 390

20365 -------------------------------------------TTTTTCACGGTAAACGA 372

30234 -------------------------------------------TTTTTCACGGTAAACGA 370

ISO/3A -------------------------------------------TTTTTCACGGTAAACAA 372

LSH ------------------------------------------------------------ 600

SK-19-59 GGGATGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCCTAGCAGCTTTGTGAGAA---- 431

30461 GGGGCGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCCTAGCACCTTTGTGAGAAGGGG 380

ROS GGGGCGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCCTAGCACCTTTGTGAGAA---- 375

SK14-4 --------TTAGCCCGGC----CCGAA--------------------------------- 392

Rad AGGGCGGTTTAGCCTGATGGCATCGGTGAATGACTCCCCTAGCACCTTTGTGAGAA---- 386

SK-19-56 GGGATGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCCTAGACCTTTGGTGAGAAA--- 432

PM19 GGGATGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCCTAGCAGCTTGT-GAGAA---- 430

SK-19-9 GGGATGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCCTAGCACTTTGGTGAGAA---- 433

30874 GGGGTGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCCTAGCACCTT-GTGAGAA---- 397

50575 GGGGTGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCCTAGCAGCTTTGTGAGAA---- 393

30371 TC-TGGGCCA-------------------------------------------------- 399

20365 TC-TGGGCCA-------------------------------------------------- 381

30234 NTCTGGGCCA-------------------------------------------------- 380

ISO/3A TCCTGGGCCA-------------------------------------------------- 382

LSH ------------------------------------------------------------ 600

SK-19-59 ------------------------------------------------------------ 431

30461 NCCGGGGCTAGCCGCCCCGCGCCGGCCGAAACCGACGCCAAGGACGACCGCACCGATGGT 440

ROS ------------------------------------------------------------ 375

SK14-4 ------------------------------------------------------------ 392

Rad ------------------------------------------------------------ 386

SK-19-56 ------------------------------------------------------------ 432

PM19 ------------------------------------------------------------ 430

SK-19-9 ------------------------------------------------------------ 433

30874 ------------------------------------------------------------ 397

50575 ------------------------------------------------------------ 393

30371 ---------------- 399

20365 ---------------- 381

30234 ---------------- 380

ISO/3A ---------------- 382

LSH ---------------- 600

SK-19-59 ---------------- 431

30461 GGTGTTTTGTATTCAA 456

ROS ---------------- 375

SK14-4 ---------------- 392

Rad ---------------- 386

SK-19-56 ---------------- 432

PM19 ---------------- 430

SK-19-9 ---------------- 433

30874 ---------------- 397

50575 ---------------- 393

Figure 28: The sequence alignment of the *Acanthamoeba* isolates via Clustal Omega.

# 5.7 CHAPTER DISCUSSION

It is well established that the rapid and accurate identification of a pathogenic organism is crucial for patient treatment and management. *Acanthamoeba* has gained increased attention from the scientific community in the last decade, due to increasing rate of incidence caused by its potential pathogenicity in humans (Trabelsi *et al*., 2012). *Acanthamoeba* Keratitis is one of the main infections caused by the organism, which is still hugely underestimated at both individual patient level and as a public health problem (Panjwani, 2010). This is because of the lack of standardized guidelines for clinical diagnosis, especially when the signs and symptoms closely mimic other infections. Although molecular methods have been applied to this organism (*Acanthamoeba*), which tend to be accurate and applicable, however, these methods are labour intensive, expensive and time consuming and sometimes difficult to interprete results (Wong *et al.*, 2014). Also, apart from the ‘Genus’ identification of the parasite, it will also vital to clinical pathology to be able to identify down to the species level. However, the current use of morphological characteristics for identifying the different species of *Acanthamoeba* has proven to be inadequate to distinguish the different life cycle forms of the organism (Smirnov *et al.*, 2011). In 2015, researchers introduced a new type of classification for *Acanthamoeba,* dividing it into 20 different genotypes from T1 to T20 on the basis of ribosomal RNA sequences (Fuerst, Booton and Crary, 2015).

MALDI-TOF MS is a well-established method for the identification of bacteria at both genus and species level, therefore its potential use in *Acanthamoeba* can only be imagined. Recently, in the last few years, MALDI-TOF MS have been incorporated in to clinical parasitology. Some studies have reported the use of MALDI-TOF MS for identification of parasites such as *Cryptosporidium, Entamoeba, Giardia* and *plasmodial* parasites (Laroche *et al.*, 2017), (Yssouf *et al.*, 2015), (Singhal, Kumar and Virdi, 2016). Current reviews on the use of MALDI-TOF MS in clinical parasitology suggests the potential of this high-throughput technique in analysing large number of samples rapidly and at limited costs compared to current methods (Murugaiyan and Roesler, 2017). The current challenges posed is mainly due to the generation of a robust and comprehensive database which could reduce misidentification (Rothen *et al.*, 2016).

Our results showed that the application of on-plate extraction method to a mixed culture of *Acanthamoeba* isolate generated good and reproducible MALDI spectra patterns. There were more peaks identified using this method and a higher signal to noise ratio was achieved, which made the peaks distinct. This on-plate extraction method is an improvement on the routine method used for bacterial identification, involving few extraction processes which diminishes the turnaround time by 10 to 15 minutes as detailed in the method section. Aside from the turnaround time, the method is superior in spectra quality and peak accuracy than the standard extraction method and routine direct smear method. The problems associated with the routine MALDI application method in obtaining good spectra results for *Acanthamoeba* could be due to insufficient cell wall disruption which might not be sufficient to adequately prepare proteins for detection by MALDI-TOF MS (Alatoom *et al.*, 2011).

Using the on-plate extraction method, all the fifteen available *Acanthamoeba* isolates were analysed via MALDI-TOF MS. Three out of the fifteen resulting MALDI spectra was shown in Figures 18a to 18c, while Table 19 shows all the peak masses for each isolate. From Table 19, it is clear and evident that six peak masses (3400, 6100, 8515, 9691 10633 and 14100) are consistent in all the fifteen isolates. The spectra obtained for each isolate was put through the SARAMIS database, the search on SARAMIS database gave no identification which made each spectrum unique. This increases the confidence in the uniqueness of the spectra and the possibility of the use of the six constant peak masses for identifying the organisms.

Moreover, the analysis performed on individual cultures of cyst and trophozoites gave an interesting result. This is because the cyst culture can be said to have a better spectra due to the high signal to noise ratio than the trophozoite culture. The spectra result were analysed in a tabular form (Table 20), which revealed the unique peak masses for trophozoite, cyst and mixed cultures. Although, there were similarity in peak masses between the cyst and the trophozoite, the trophozoite culture had fewer peaks in their spectra that cyst culture. This could be attributed to the extra extraction process involving formic acid that was employed in the sample preparation prior to MALDI analysis. The acid could have a more damaging effect on the trophozoites due to their lack of a tough and heavy cyst wall which could have disrupted the proteins detectable by MALDI-TOF MS. On the other hand, the cyst has undergone encystation and thus covered with a thick hard wall, the application of acid might just be enough to break the cyst wall, leaving the proteins intact.

According to Chiu (2014), the sensitivity limit for bacterial identification via MALDI-TOF MS is approximately at 104 cells per ml (Evason, Claydon and Gordon, 2001). With regards to *Acanthamoeba,* most of our work done with MALDI-TOF MS was performed at a cell density set at 106 cells per ml, which gave consistent and reproducible results. However, reducing the number to 105 cells per ml gave poor non-reproducible spectra. A serial dilution of 1 x 105, 2 x 105, 3 x 105, 4 x 105, up until 1 x 106 was subjected to the same protein extraction procedure as described in the method section, followed by analysis by MALDI-TOF MS. The first three dilutions were poor and inconsistent, until the *Acanthamoeba* cell number reached 4 x 105cells per ml, when the spectra were consistent and reproducible. Similar sensitivity level was also reported in a study of MALDI-TOF MS as a potential tool for another parasite identification – *Trichomonas vaginalis* (Calderaro *et al.*, 2016). This low sensitivity did not allow the identification of the parasite (*T. vaginalis),* directly in clinical samples as shown in the urine samples, which could be applicable to *Acanthamoeba* in the eye. This suggests that the technology might have a limitation for its potential application to the diagnostic work-flow of *Acanthamoeba,* due to the fact that samples presenting a very high concentration of the parasite might be required or after its culture in the laboratory. However, MALDI-TOF MS, unlike culture does not require the viability of the parasite and also is not limited in the application to corneal scrapings and eye secretions compared to the rapid antigen tests. Also, in comparison to nucleic acid amplification tests, which are not widely performed in parasitology laboratories, MALDI TOF MS is less cumbersome and expensive. This shows the current identification of parasites currently done by reference laboratories only could be extended for routine use in clinical laboratories, which might replace/augment molecular methods in clinical parasitology laboratories.

Lastly, to ascertain, the relationship between the fifteen isolates, PCR and sequencing were conducted for each of them, the sequence alignment obtained was compared to the MALDI spectra of each isolate. As mentioned above, we obtained MALDI spectra for all the fifteen isolates, likewise we also obtained fifteen gel bands and sequencing. The phylogeny tree obtained via the Omega Clustal website shows the relationship between the fifteen isolates. Comparing this result to the MALDI spectra interestingly shows similar ‘grouping’. All the T4 isolates are closely related based on the number of similar peak masses they share, same goes for other genotypes. This suggest that MALDI-TOF MS could be effective and routinely used in identification of *Acanthamoeba* isolates.

**CHAPTER 6**

**DISCUSSION & CONCLUSIONS**

# 6.1 GENERAL DISCUSSION

The general discussions of this thesis are centred around the findings from the results obtained as well as additional approaches beyond the scope of this project which could provide avenues of investigations for the direction of future work.

## 6.1.1 Potential detection/identification method for Acanthamoeba

The major contribution of this thesis to clinical practice is its investigation into the potential use of MALDI-TOF MS in the detection and identification of a non-bacterial species, in this example, a Protozoan (*Acanthamoeba*). Although an independent study has also been carried out during the current project to investigate and demonstrate this potential by identifying the organism through an in-house created database (Del Chierico *et al*, 2016). The present thesis has added to the existing knowledge by the generation of new spectral patterns which can be used as reference spectra in the build-up of an identification MALDI database for the individual *Acanthamoeba* genotypes. The optimization of the MALDI-TOF MS, particularly with the creation of a comprehensive database, has the potential to reliably identify *Acanthamoeba* spp.

Currently, the use of 18S rDNA amplification in conjunction with sequencing analysis allows for enhanced identification and diagnosis performance (Hajialilo *et al.*, 2016). However, there are limitations for the PCR system such as the requirement for specific primers for every species, which is not practicable, especially for *Acanthamoeba* which cause a rare eye infection. Although generic primers can work, however they could easily be contaminated because of the sensitivity of the PCR, which is why most labs don’t use generic primers because of negative result. On the other hand, MALDI is an existing technology which is common in most clinical laboratories because of its general acceptability for routine bacterial and fungal identification. Since MALDI-TOF MS is already in most clinical laboratories, readily used for identification of bacteria and fungus, it can thus be readily applied to other micro-organisms. Once MALDI-TOF MS can be adapted for the identification of non-bacterial and non-fungal organisms, it removes the need to set up a new PCR system unit for the identification of a few class of organisms including *Acanthamoeba*.

Another novelty of this thesis is the possible differentiation of the two stages of the organism (trophozoites and cysts). It is noteworthy from our results that the observable peak masses from the MALDI-TOF MS spectra can be used to differentiate the trophozoites and cysts form of the organism. This will be useful in the treatment of *Acanthamoeba* keratitis in relation to the therapy employed. For example, to determine the efficacy of the corneal cryosurgery (application of extreme cold to destroy abnormal or disease tissues) in the treatment of *Acanthamoeba* keratitis, *in-vitro* freeze-thaw-refreeze cryotherapeutic methods were tested on the trophozoite and cysts of *Acanthamoeba.* The concluding results showed that the trophozoites were killed but cysts survived suggesting cryosurgery might not be an effective means to kill *Acanthamoeba* cysts from the cornea (Khan, 2015). This shows that a pre-knowledge of the stage of the *Acanthamoeba* whether cyst or trophozoite can inform good choice for treatment and management Chierico *et al*., 2016).

## 6.1.2 Improved knowledge and better understanding of micro-organisms

Another novelty behind this thesis is the possible speciation of the *Acanthamoeba* genus*.* The identification of the organism up to the sub-species level provides a broader understanding of the infection, because more information other than specie and genotype identification is known about the organism. This helps in the accurate treatment and management of the infections caused by the organism. Studies have shown that host specificity exists in *Acanthamoeba* keratitis, for example, successful *Acanthamoeba* keratitis models that mimic the human form of disease were only produced in pigs and Chinese hamsters but not in rats, mice or rabbits, suggesting that the expression of specific molecular determinants may be limited to certain mammalian species (Niederkorn, 2002). Many of the current assays including PCR employed in the identification of *Acanthamoeba* do so up to the genus-level, while very few could differentiate up to the pathogenic and non-pathogenic *Acanthamoeba.* Our results have suggested the possibility of having subspecies-specific mass peak biomarkers, which has the potential for sub-species identification via an enhanced database and software. This also includes the method development for an optimized sample preparation/ extraction process of *Acanthamoeba* prior to MALDI analysis. This has generated reproducible spectra patterns with unique peak masses, which could be used to develop a robust database for identification of the organism.

In addition, the sensitivity test of the organism via MALDI-TOF MS reveals that 4 x 105 cells per ml of cells is enough to generate detectable MALDI ion signals resulting in reproducible MALDI spectra. Although, the sensitivity limit number obtained for *Acanthamoeba* in this thesis might not be readily seen in the corneal scrapings of the eye, however, a short-term laboratory growth of the cells obtained from the corneal scrapings or PCR can yield enough cells for MALDI-TOF MS analysis. *Acanthamoeba* cells can be obtained via appropriate DNA technology, a typical example is the use of PCR to amplify the DNA obtained from scrapings of the eye. The amplified DNA can be subjected to analysis via MALDI-TOF MS which is poised to give a more rapid outcome than current methods. The merger of these two identification methods might offer a better and improved outcome for the management and treatment of *Acanthamoeba.* This has the potential to reduce bad prognosis of the infection (which can lead to complete blindness of the eye) and hence a huge savings on NHS costs. Nonetheless, the advantage of MALDI-TOF MS over 18S rDNA sequencing lies in the established routine use of MALDI-TOF MS in clinical labs, which makes it more ideal and easier to adapt to other micro-organisms. Also, the appropriate expenditure of the techniques, excluding the machines and the personnel would also be higher in 18S rDNA than MALDI –TOF MS.

## 6.1.3 “Novel” Matrix for improved MALDI-TOF MS spectrum

It is possible that other types of matrix apart from CHCA could add more knowledge to the current understanding and knowledge obtained from MALDI spectra of micro-organisms. According to Smolira and Wessely-Szponder (2014), despite the increased knowledge of the MALDI process, matrix selection and optimization of the preparation protocol are still processes/procedures requiring more research. The selection of an appropriate matrix for the sample is a critical point in order to obtain high quality MALDI mass spectra. Sample impurity, solvent content, substrate surface and environmental conditions (temperature and humidity) all affect the rate of matrix-analyte co-crystallisation. Few studies have also suggested that matrix mixture often offers several advantages for sample preparation (Morman *et al*, 2000; Shahgholi *et al.*, 2001). This includes being simple, fast, and spectra acquired at any position on the sample surface, simplifying fully automated data acquisition. The importance of finding a better suited MALDI matrix led us to test some ‘novel in-house matrices’ in bacteria identification. Although, the use of novel matrices has not identified any of the known organisms, however it shows unique peak masses different from those obtained using CHCA. This might be crucial in the identification of non-bacterial species particularly protozoans and other parasites in the generation of unique peaks that could give more information than already known. CHCA is the most commonly used matrix for bacterial identification, however due to the different sample preparation involved, CHCA might not be ideal for protozoans. The result from the use of ‘our’ matrices have shown that more information could be known with each unique matrix, thus the successful identification of a ‘gold standard’ matrix for each microbial species is essential.

## Potential Application in Biotechnology

An unexpected finding from this work is the use of MALDI in the differentiation between normal laboratory strain and a modified-plasmid carrying strain via the use of MALDI-OF MS. This differentiation could be readily observed via the peak masses on the MALDI spectrum. The acquisition of plasmids particularly those containing resistance genes could have an effect on the normal MALDI spectra patterns. The effect can be observed in the significant reduction in the number of peaks as well as intensity. Further development of the method and validation could be of economic importance in biotechnology companies, because it can be used to check for presence of plasmids by spectra comparison (Russel *et al*, 2007). This unexpected outcome can also be used as a way of looking at the presence of plasmids in plasmid expression systems.

Specifically, this biotechnological application can be used for rapid screening method for recombinant and high level of expressions. Similarly, it could be clinically significant in the detection of modified forms of organisms either by mutation or acquisition of plasmid carried genes.

The use of recombinant protein expression systems in biotechnology is routine, vast and widespread, this is because several industries rely on the production of recombinant proteins, including pharmaceuticals, chemical and food production industry. Advances in protein engineering and biotechnology have led to the extensive use of recombinant proteins throughout the industry. Certain industries such as the dairy industry would not exist in its current form without the use of recombinant enzymes, a typical example is in cheese making, chymosin and other necessary enzymes are produced recombinantly by K*luyveromyces lactis* (Kumar and Lloyd, 2010)*.* Presently, the current methods for monitoring and detecting the expression of proteins in these systems are often lengthy and time consuming (Vincentelli *et al.*, 2002). This has led to the search for more efficient methods to be used for protein expression analysis and monitoring especially when used in high-throughput protein production. Other applications of recombinant protein expression in cell culture include production of bulk proteins for both industrial purposes and pharmaceutical drug screening. Since the use of expression systems can sometimes lead to absolutely no expression or misexpression, there is a need for rapid, sensitive, reliable methods which can detect and monitor protein production over time. Challenges normally encountered in this process include protein degradation, unexpected posttranscriptional and posttranslational modification as well as cloning artefacts.

Currently, the methods of choice for monitoring protein expression include gel electrophoresis with or without subsequent western blot analysis, enzymatic activity assays, ELISAs and others too (Yingfeng, 2001). However, all these methods are time consuming and labour intensive and do not lend themselves to repeated monitoring of protein production during growth of cell cultures. For instance, analysis using gel electrophoresis can take several hours, and the sensitivity of typical staining methods might not be adequate. Also, even though Western blotting can improve the sensitivity, the result obtained does not provide relative comparison to estimate the extent of expressions (Vincentelli *et al.*, 2002). Similarly, enzymatic actions in ELISA techniques are often sensitive to contamination from cell extracts therefore requiring time-consuming purification such as HPLC or FPLC. Screening for and monitoring of recombinant proteins expression directly from cell cultures and either from the whole cells or from cell culture supernatants, using mass spectrometry is feasible, time and labour efficient. Most MALDI sample preparation methods take approximately 5 – 15 minutes after culturing, except when there is need to disrupt the cell wall (Easterling *et al.*, 1998). This allows clinical information to be obtained in a relatively short amount of time in addition to easy data analysis compared to other methods. MALDI MS used in conjunction with the sample preparation described provides the ideal analysis method for monitoring intracellular and secreted recombinant protein expression in prokaryotes and eukaryotes. In summary, real-time high throughput and mass screening of these proteins can be done via MALDI –TOF MS (Gobom *et al.*, 2009).

# 6.2 CONCLUSIONS

# Mass Spectrometry (MS) has added positive value to the clinical laboratory due to its improved analytical performance, novel diagnostic approaches and the potential to develop new markers for a variety of organisms and diseases (Jannetto and Fitzgerald, 2015). It is one of the most important analytical tools of modern times due to its increasing roles, uses and capacity in clinical settings. It is now possible to obtain the mass spectra of a metabolites/drugs or cancer markers, which can all be measured on a patients’ skin or in his/her blood via MALDI-TOF MS analysis (Swiatly, 2017). It has also been suggested to arrive soon at the Physicians’ consulting room where it could aid diagnosis during complex surgeries within the operating theatre (Opota, 2017).

# Since its introduction by Karas in 1987 (Jannetto and Fitzgerald, 2016), MALDI-TOF MS has been substantially developed and it is now used for routine diagnostics in clinics, veterinary, pharmaceutical, food and environmental microbiology laboratories. Much more important to us, is its consideration as a real and valid alternative for microbial identification as of now. (Randell, 2014). MALDI-TOF MS’s evolvement into a powerful tool can be ascribed mainly due to its, provision of rapid and accurate results via its throughput capabilities with a greatly reduced technical hands-on time and the benefit of significant time and cost savings (Kostrzewa and Schubert, 2016). Apart from routine analysis of microbial contents leading to identification, its novel application has been extended to antimicrobial susceptibility/resistance testing (Idelevich, 2018), and also clinical imaging to identify proteins (Webster, 2012), peptides (Dave, 2011), drugs (Su, 2005), metabolites (Lee, 2018), lipids (Fuchs, 2010) and other analytes in tissues (Aicher and Walch, 2015).

# The use of MALDI-TOF MS in identifying microbes via proteomic analysis due to the unique ribosomal protein ‘fingerprints’ possessed by each micro-organism is revolutionary. At the moment, MALDI-TOF MS allows the identification of more than 10,000 micro-organisms with the impressive capability to differentiate closely related species from a genus (Greco, 2018). PCR is another technology which is sensitive and precise for quantitative detection of human pathogens via analysis of a target segment of an amplified DNA. PCR can only be used to identify organisms or provide information for which there are validated primer pairs, whereas MALDI can identify any organism as long as it is in the spectral database. In essence, PCR is better suited to address the questions “is it there” rather than “what is there”. Conversely, PCR might be better suited to quickly identify some microbes than MALDI-TOF MS due to the culturing process required in the latter. Without a doubt, MALDI-TOF MS does not yet have the capacity to achieve entirely what PCR does, although PCR has a longer step process than MALDI-TOF MS and also needs a good quality set up and technical know-how in the laboratory. It thus can be suggested that if MALDI-TOF MS is already routinely used in every clinical laboratory for bacterial identification, it could readily be adopted to identify all microbes which might cancel outs the need for PCR.

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# Figure 29: A cartoon showing the relative information you can obtain from the analysis of a clinical sample using different methods. (Walker, LeVine and Jucker, 2016).

The potential major impact of this work in relation to clinical practice is that it offers a prospective rapid diagnostic method for detecting and identifying *Acanthamoeba.* Although, its applicability for routine practice needs further research, due to certain major constraints. However, this promising technology might surely replace/augment molecular methods in clinical parasitology laboratories. Comparatively, the use of 18S rDNA in the amplification of *Acanthamoeba* has to a great extent enhanced diagnosis performance, nonetheless the attestation of genotyping requires supplementary sequencing-based procedures. Currently, diagnosis involves laboratory culturing of corneal scrapings or corneal swabs to check for *Acanthamoeba* DNA via PCR, which often takes days to get results. In addition, confocal microscope is a useful, non-invasive technique which can also be used in the diagnosis and treatment of *Acanthamoeba keratitis,* particularly in cases where the corneal scraping, cytological analysis and cultures are negative (Nakano *et al.*, 2004; Daas *et al.*, 2017).

The use of MALDI-TOF MS for identification has been shown in published papers to reduce the diagnostic waiting time particularly if the organisms could be identified directly from clinical samples (Moussaoui *et al.*, 2010). This same approach can be related to *Acanthamoeba* diagnosis directly from corneal scrapings or swabs. Although the correct diagnosis of *Acanthamoeba* keratitis is problematic due to the fact that it is often misdiagnosed for bacterial, viral or fungal keratitis, thus a correct diagnosis for *Acanthamoeba* keratitis requires awareness of its clinical symptoms as well as familiarity with the morphological characteristics of *Acanthamoeba* (Khan, 2015)*.* The mechanism of MALDI-TOF MS analysis is via the use of ribosomal protein profiles of organisms which will be analysed and matched against a database in a species-specific biomarker mass patterns called Super Spectra. This specifically reduces the misdiagnosis of *Acanthamoeba* keratitis for bacteria, viral or fungal infection of the eye because MALDI-TOF can discriminate differences in the ribosomal proteomic contents.

In addition, it is known that *Acanthamoeba* eyeinfection is challenging to treat due to resistance to many treatment options, mainly due to its dormant, long-surviving cyst form. Thus, the differentiation of the stages of the organism is suggested to assist with the treatment and management of the organism. Culture isolation of *Acanthamoeba* is considered as the gold standard for routine laboratory diagnosis (Khan, 2015). The most common method often used is the culture of corneal scrapings of patients on 2% non-nutrient agar plate overlaid with non-pathogenic strains of *E. coli, Klebisiella Pneumoniae or Enterobacter aerogenes.* The identification is complete with the use of electron microscopic procedures to study ultra-structural features in trophozoite and cysts forms of *Acanthamoeba.* Scanning electron microscope is used to specifically examine certain characteristics to differentiate between the two forms the organism (Fatimah *et al.*, 2011). This includes the examination of the surface alteration, the number of vacuoles, number of fat droplets and the position of the Golgi cisternae in both trophozoite and cyst forms of *Acanthamoeba* sp. However, MALDI-TOF MS is a prospective tool which offers potentially, improved knowledge on both trophozoites and cyst form of the organisms with reduced labour and time as shown in this thesis. Our results have shown the use of MALDI-TOF MS in the discrimination between cyst and trophozoites stages of the organism via the differences in observable MALDI-TOF spectra patterns. From the data so far from this project, it can be suggested that the introduction of this technology in clinical parasitology will improve the diagnosis of infections produced by parasites and the possible detection of antimicrobial resistance.

# 6.3 LIMITING FACTORS AND PROPOSALS FOR FUTURE WORK

There were several limitations during the course of the project, however the research team was able to overcome most of them. Firstly, since this project involves few genotypes of *Acanthamoeba,* larger prospective studies involving all genotypes from all possible samples sources are required for confirmation and validation of the results. Another limiting factor was timing in the course of this project study especially when it involves process in clinical diagnosis, management and treatment. This project would have significantly expanded an already existing reference database if there was one. The database creation would only be possible with the collaboration between major commercial MS companies and hospitals / laboratories in several countries. In retrospect, it took fourteen years for the first complete reference database for bacteriato be reportedand subsequently MALDI-TOF MS introduced in to routine microbiology (Keys *et al.*, 2004). Hopefully, parasitology would not follow similar pattern and MALDI-TOF MS could be introduced into routine practice more quickly. However, this project has hundreds of *Acanthamoeba* spectra which could eventually be added to a reference database for *Acanthamoeba*.

The next line of action for the *Acanthamoeba* work is the creation of a super reference database which could be universally used for identification of the organisms. However, one person/author cannot create such database and thus hundreds of authors have to combine spectra patterns for this to work. All the reported parasitology MALDI associated databases were developed as temporary in-house databases and usually not accessible by other researchers. This has made it difficult to create a robust MALDI database that could be universally used in the detection and identification of the organism. Another problem is the difference in instrumentation and software tools employed by researchers which has made it difficult to combine reference spectra, even if authors agreed to make them available. These factors have hindered progress on the creation of a universal accessible database for parasites. This slow progress underscores the possibilities of establishing an online public reference database which would be useful for major types of MALDI MS machines or spectra data formats. An online regulated public database for parasitology would speed up the process of a database for parasites. However, this would demand a great deal of standardization in terms of parameters and variables such as specimen storage conditions, developmental stage, matrix employed, sample preparation/extraction methods, variations due to spotting, system and software settings as well as other significant process that can affect sampling.

Another proposed future work is to investigate the ‘most suitable’ matrix for the identification of *Acanthamoeba* via MALDI-TOF MS. This might improve understanding of the organism in each of its dual-life cycle stages which might be critical to treatment and management. The validation of a universal sample preparation/extraction method for this organism would also help in the overall identification process via MALDI-TOF MS.

**LIST OF PRESENTATIONS**

**Poster Presentations**

* Annual University of Sunderland Research Conference, held at St. Peters Campus, University of Sunderland, United Kingdom. 7th January 2016
* 7th Academy of Pharmaceutical Society Conference held at the University of Strathclyde, Glasgow, Scotland, United Kingdom. 5th to 7th September, 2016.
* Great North Pharmacy Research Conference 2017 held at the University of Sunderland, Sunderland UK. 21st July, 2017
* 6th Early Career Scientist, Research Symposium of the Society for Applied Microbiology, held at the University of Westminster, London. United Kingdom. 19th April, 2017.
* Annual Applied Microbiology Conference of the Society for Applied Microbiology, held at the Baltic Centre, Gateshead, United Kingdom. 3rd to 6th July, 2017.

**Oral Presentation**

* Speaker, Teaching and Learning Conference, National Glass Centre, Sunderland, United Kingdom. 24th March, 2017
* Speaker, Great North Pharmacy Research Conference 2017 at the University of Sunderland, Sunderland UK. 21st July, 2017

**LIST OF PUBLICATIONS**

**In preparation**

* The potential use of MALDI-TOF MS in the detection and identification of *Acanthamoeba* isolates.
* MALDI-TOF MS: A potential system for discriminating the growth and level of plasmids in biotechnology use.

**Awards & Prizes.**

* First Prize in the North East Regional Three Minute Thesis Competition, held at the University of Newcastle, 15th June, 2016.
* Best Academic PhD Tutor 2018 , Brilliant Club, North East Zone, United Kingdom.
* Second place, Best Oral Presentation at the Great North Pharmacy Research Conference 2017 at the University of Sunderland, Sunderland UK. 21st July, 2017

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**Appendix A - Sequencing result**

Table 21: Summary result obtained from sequencing of the *Acanthamoeba* isolates and the resulting genotype when inserted into BLAST online software

|  |  |  |
| --- | --- | --- |
| Sample | Sequence | *Acanthamoeba Genotype* |
| SK19-9 | ----------------------------------------------------------TT  GAGTCTCAGCATGCAGATCCATTTTCTGCCACCGAATACATTAGCATGGGATAATGGAAT  AGGACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTA  ATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGA  AAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTT  AGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCG  ATTAGGAGACGTTGAATACAAAACACCACCATCGGTTGCGGTCGTCCTTGGCGTCTCGGT  TTCG------GCCGGGGCGCGGGGATGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCC  TAGCACTTTGGTGAGAA------------------------------------------- | T4 |
| SK-19-56 | -----------------------------------------------------------T  AACTTCAAGCAGGCAGATCCATTTTCTGCCACCGAATACATTAGCATGGGATAATGGAAT  AGGACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTA  ATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGA  AAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTT  AGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCG  ATTAGGAGACGTTGAATACAAAACACCACCATCGGTTGCGGTCGTCCTTGGCGTCTCGGT  TTCG------GCCGGGGCGCGGGGATGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCC  TAGACCTTTGGTGAGAAA------------------------------------------ | T4 |
| SK-19-59 | --------------------------------------------------------TTGG  GTTTTCAGGCATGCAGATCCATTTTCTGCCACCGAATACATTAGCATGGGATAATGGAAT  AGGACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTA  ATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGA  AAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTT  AGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCG  ATTAGGAGACGTTGAATACAAAACACCACCATCGGTTGCGGTCGTCCTTGGCGTCTCGGT  TTCG------GCCGGGGCGCGGGGATGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCC  TAGCAGCTTTGTGAGAA------------------------------------------- | T4 |
| SK14-4 | -------------------------TTGGCCCAGATCGTTTACCGTGAAAAAATTAGAGT  GTTCAAAGCAGGCAGATCCAATTTTCTGCCACCGAATACATTAGCATGGGATAATGGAAT  AGGACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTA  ATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGA  AAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTT  AGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCG  ATTAGGAGACGTTGAATACAAAACACCACCATCGGTTGCGGTCGTCCTGGCGTCTCGTTT  CGGCCGGGC-GCGGGACG------TTAGCCCGGCCCGAA--------------------- | T4 |
| PM19 | --------------------------TGGCCCAGATCGTTTACCGTGAAAAAATTAGAGT  GTTCAAAGCAGGCAGATCCAATTTTCTGCCACCGAATACATTAGCATGGGATAATGGAAT  AGGACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTA  ATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGA  AAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTT  AGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCG  ATTAGGAGACGTTGAATACAAAACACCACCATCGGTTGCGGTCGTCCTTGGCGTCTCGGT  TTC------GGCCGGGGCGCGGGGATGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCC  TAGCAGCTTGTGAGAA-------------------------------------------- | T4 |
| 20365 | GGNGCCGGGGCTAGCCGGTCCCGCCGCCGGCCCCGAAAGACCGAACGCCAAGGGACGACCGCACCGATGGTGGTGTTTTGTATTCAACGTCTCCTAATCGCTGGTCGGCATCGTTTATGGTTAAGACTACGACGGTATCTGATCGTCTTCGATCCCCTAACTTTCGTTCTTGATTAATGAAAACATCCTTGGCAGATGCTTTCGCAGAAGTTAATCTTTCATAAATCCAAGAATTTCACCTCTGACAATTAAATATTAATGCCCCCAACTATCCCTATTAATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGGATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAAACGATCTGGGCCA | T4 |
| 30234 | GGGCCGGGCTAACGCCCTCGCGCGCCCCGGCCGTGAAGCCGAGACACGCCAAGGACGACCGCGCCGATGGTGGTGTTTTGTATTCAACGTCTCCTAATCGCTGGTCGGCATCGTTTATGGTTAAGACTACGACGGTATCTGATCGTCTTCGATCCCCTAACTTTCGTTCTTGATTAATGAAAACATCCTTGGCAGATGCTTTCGCAGAAGTTAATCTTTCATAAATCCAAGAATTTCACCTCTGACAATTAAATATTAATGCCCCCAACTATCCCTATTAATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGGATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAAACGANTCTGGGCCA | T4 |
| 30371 | GGGTCCGGGGNCTAGCCACCCCCGGCGGCCCCGGGCCCCGGTGGAAGGGACCGAAGACGCCAAGGNACGAACCGCACCGNATGGCTGGTGATTTTGTATTCAACGTCTCCTAATCGCTGGTCGGCATCGTTTATGGTTAAGACTACGACGGTATCTGATCGTCTTCGATCCCCTAACTTTCGTTCTTGATTAATGAAAACATCCTTGGCAGATGCTTTCGCAGAAGTTAATCTTTCATAAATCCAAGAATTTCACCTCTGACAATTAAATATTAATGCCCCCAACTATCCCTATTAATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGGATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAAACGATCTGGGCCA | T4 |
| 30461 | AAGTNAGCAGGCNGATCCAATTTTCTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCACCATCGGTGCGGTCGTCCTTGGCGTCGGTTTCGGCCGGCGCGGGGGCGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCCTAGCACCTTTGTGAGAAGGGGNCCGGGGCTAGCCGCCCCGCGCCGGCCGAAACCGACGCCAAGGACGACCGCACCGATGGTGGTGTTTTGTATTCAA | T4 |
| 30874 | GTNAGCAGGCAGATCCATTTTTCTGCCACCGAATACATTAGCCATGGGATAATGGAATAGGACCCTGNTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCACCATCGGTGCGGTCGTCCTTGGCGTCTCGGTCCTTCACGGGGCCGGGGCGCGGGGGTGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCCTAGCACCTTGTGAGAA | T4 |
| 50575 | AAGTNAGCAGGCAGATCCATTTTCTGCCCCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCACCATCGGTGCGGTCGTCCTTGGCGTCTCGGTCCTTCACGGGGCCGGGGCGCGGGGGTGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCCTAGCAGCTTTGTGAGAA | T4 |
| ROS | ANNGTNAGCAGGCAGATCCATTTTCTGCCCCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCACCATCGGTGCGGTCGTCCTTGGCGTCGGTTTCGGCCGGCGCGGGGGCGGCTTAGCCCGGTGGCACCGGTGAATGACTCCCCTAGCACCTTTGTGAGAA | T4 |
| ISO/3A | TCNGGGGCCGGGGCTAGCCGCCCCGCGCCGGCCGAAACCGACGCCAAGGACGACCGCACCGATGGTGGTGTTTTGTATTCAACGTCTCCTAATCGCTGGTCGGCATCGTTTATGGTTAAGACTACGACGGTATCTGATCGTCTTCGATCCCCTAACTTTCGTTCTTGATTAATGAAAACATCCTTGGCAGATGCTTTCGCAGAAGTTAATCTTTCATAAATCCAAGAATTTCACCTCTGACAATTAAATATTAATGCCCCCAACTATCCCTATTAATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGGATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAAACAATCCTGGGCCA | T4 |
| Rad A | TACCAGTTNAGCAGGCAGATCCATTTTTCTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCACGCGAGGACCAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCACCATCGGTGCGGTCGTCCTTGGCGCGTCGTGGCTTGCTGCGGCGTGCGAGGGCGGTTTAGCCTGATGGCATCGGTGAATGACTCCCCTAGCACCTTTGTGAGAA | T11 |
| LSH TM 1630 | -----------------------GGTATTAAGCTCGTAGTTGGATCTAGGGACGCGCATT  TCAAGCGCCCGTGCCATCGGGTCAAACCGGTGGCTGCGTTGGCGTTGCGGGCTCGGTCCG  TCGGTGCCCCACAAAGGGCTATCGGCGTGTCAACCGGCCCGCCCGTCCCCTCCTTCTGGA  TTCCCGTTCCTGCTATTGAGTTAGTGGGGACGTCACAGGGGGTCCATCGTCGTGCGGCGT  CAAAACCGTGCGGCGGTGGGTCCCTGGGGCCCAGATCGTTTACCGTGAAAAAATTAGAGT  GTTCAAAGCAGGCAGATCCAATTTTCTGCCACCGAATACATTAGCATGGGATAATGGAAT  AGGACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTA  ATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGA  AAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTT  AGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCG  ATTAGGAGACGTTGAATACAAAACACCACCATCGGTGCGGT-CGTCCTTGGCGT------ | T4 |