QATAR UNIVERSITY

Graduate Studies

College of Arts and Sciences

APPLICATION OF MALDI-TOF MASS SPECTROMETRY AS A TOOL FOR BIOTYPING OF *B. MELITENSIS*

A Thesis in

Department of Health Sciences

Biomedical Science Program

By

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Submitted in Partial Fulfillment of the Requirements for

The Degree of Master of Sciences / Arts

May 2015

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ABSTRACT

Background: Brucellosis is a global zoonosis caused by the bacteria of the genus *Brucella a* hazard group III pathogen. Typing of *Brucella* species is of great importance for understanding the epidemiology of the disease and an essential tool for the eradication program and vaccine development. The aim of the present study is to evaluate Matrix-assisted laser desorption ionization- time of flight spectrometry (MALDI-TOF MS), a proteomic based assay, for biotyping *B. melitensis* in Qatar.

Methodology: A total of sixty three *B. melitensis* isolated from clinical specimens, were biochemically identified by Vitek 2 Compact and serotyped using monospecific *Brucella* antisera. MALDI-TOF MS Identification was carried out against the newly constructed *Brucella* library. Subsequently, MALDI typing was performed by visual inspection of the generated spectra to determine potential biotype-specific marker peaks. Molecular typing was performed using *B. melitensis* biotyping PCR kit as a reference method.

Results: MALDI-TOF MS identified all the isolates as *B. melitensis* with a score of >2.3 indicating highly probable species identification. The visual inspection of the generated spectra revealed six promising marker peaks at m/z 4682, 5028, 5970, 6823, 7356, and 7326. The presence or absence of these marker peaks grouped the isolates into four groups with four distinct marker peak profiles. PCR typing results showed the presence of only two biotypes, *B. melitensis* biotype 2 (n=32) and *B. melitensis* biotype 3 (n=31). The mass spectral profiles that share the marker peak at m/z 7356 (n=32)

were confirmed as biotype 2 while the mass spectral profiles that share the marker peak at m/z 5970 (n=31) as biotype 3. No *B. melitensis* biotype 1 was detected in this study.

Conclusion: Human brucellosis in Qatar is exclusively caused by *B. melitensis* with equal distribution of biotype 2 and biotype 3. MALDI-TOF MS was found to be a promising tool to identify and differentiate *B. melitensis* biotypes 2 and 3. Peak at m/z 7356 was identified as biotype 2-specific marker peak and peak at m/z 5970 as biotype 3-specific.

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LIST OF APPREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AMOS-PCR	Abortus, melitensis, ovis, suis Polymerase Chain Reaction
AP-PCR	Arbitrary Primed Polymerase Chain Reaction
BCSP31	*Brucella* Gene Surface Protein 31
BTS	Bacterial Standard Strain
CDC	Center for Disease Control
ELIZA	Enzyme Linked Immunoassay
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus sequence PCR
H2S	Hydrogen Sulfide
IS711	Insertion Sequence 711
MALDI-TOF MS	Matrix-assisted Laser Desorption Ionization- Time Of Flight Mass Spectrometry
MBL	MALDI Biotyper Library
MLST	Multilocus Sequence Typing
MLVA	Multiple Locus Variable Number Tandem Repeats Analysis
MSP	Main Spectra Projection
M/Z	Mass to charge ratio

NCTC	National Collection of Type Cultures
NVSL	National Veterinary Service Laboratory
Biotyper OC	MALDI Biotyper Offline Classification
OMP	Outer Membrane Proteins
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RAPD-PCR	Random Amplified Polymorphic DNA Polymerase Chain Reaction
REP-PCR	Repetitive Intergenic Palindromic Sequence-PCR
RLPS	Rough lipopolysaccharide
Biotyper RTC	MALDI Biotyper Real time Classification
RT-PCR	Real Time Polymerase Chain Reaction
SAT	Serum Agglutination Test
SLPS	Smooth lipopolysaccharide
α-CHCA	α-Cyano-4- Hydroxycinnamic Acid

ACKNOWLEDGEMENTS

I take this opportunity to express my gratitude to everyone who supported me throughout the course of my Master study either by aspiring guidance, invaluably constructive criticism or friendly advice by which this thesis is made possible.

First and foremost, I would like to express my sincere gratitude to my advisors Dr. Marawan Abumadi and Dr. Anand Deshmukh for their continuous support, patience, motivation, enthusiasm, immense knowledge and guidance in all the time of research and writing of this thesis. I am sincerely grateful to them for sharing their truthful and illuminating views on a number of issues related to the project.

My sincere thanks also goes to Dr. Fikret Sahin, Ankara University, for being ever kind to show interest in my research and for facilitating the acquiring of molecular testing kit. I also express my warm thanks to Dr. Aarti Sharma from the Biomedical Research Center, Qatar University, Dr. Rajvir Singh from Medical Research Centre, Hamad Medical Corporation, Abdel-Salam Gomaa, Department of Mathematics, Statistics and Physics for their great help and assistance.

I would also like to thank Hamad Medical Corporation for providing the funds and the Biomedical Research Center, Qatar University for providing the facility for my research.

I would like to express my gratitude to the Department of Microbiology at Hamad Medical Corporation, in particular, my supervisor Mrs. Fatima Almohanadi to allow me to use the required facilities with conductive conditions for my project.

A special feeling of gratitude to my loving parents without whom I am nothing. Thanks for their faith in me and for their moral and emotional support.

Most importantly, I would like to thank my Husband. His support, encouragement, quiet patience and love were undeniably the bedrock upon which my success have been built.

I thank my loving sister, great brothers, and my wonderful and amazing kids, Shahed, Mohamed, Noor and Nada, who provided me with unending encouragement, support and love.

CHAPTER 1 INTRODUCTION

1.1 INTRODUCTION

Brucellosis, also named Mediterranean fever, Malta fever, undulant fever, and typhomalarial fever, (Bossi et al., 2004) is a re-emerging zoonosis that has resurfaced at the epicenter of scientific interest because of its evolving epidemiology (Pappas, 2010). Human brucellosis caused by an intracellular pathogen belong to the genus *Brucella*, is the most common zoonotic disease worldwide with more than 500,000 new cases annually (Buzgan et al., 2009). Although sporadic cases are reported from many parts of the world, it is hyperendemic in the Mediterranean Basin, Arabian Peninsula, India, Mexico, and Central and South America (Buzgan et al., 2009). In Western Europe and North America, brucellosis is rare since effective public health measures have been implemented. However, such countries are considering *Brucella* to be a potential bioterrorism threat leading to an increased interest (Cekovska et al., 2010). In addition to the public health importance, brucellosis constitutes a major economic problem and causes major economic losses due to time lost by patients from normal daily activities and losses in animal production (Dean et al., 2012).

Brucella species, a Category B agents, are highly infectious to mammals and humans. Currently the genus *Brucella* comprises ten genetically highly related species out of which *Brucella melitensis* is the species most frequently reported as a cause of human disease (WHO, 2006). In Qatar, *B. melitensis* is the

etiological agent of Brucellosis with 70-75 cases reported annually (unpublished data QNRF funded study # 5-746-3-176).

Brucella species, based on their phenotypic and genotypic differences, are further differentiated into biotypes (Alton et al., 1975). Typing is important for epidemiological purpose, public health and developing strategy for vaccination. Several conventional and molecular typing techniques are available. Conventional phenotypic typing techniques involve combination of metabolic, biochemical, and serological tests. However, these tests are not done in most clinical laboratories as they require biosafety level 3 facilities, time consuming, hazardous to laboratory workers and subjected to variable interpretation (Cekovska et al., 2010). On the other hand, molecular typing techniques such as Polymerase Chain Reaction (PCR) and Multiple Locus Variable number tandem repeats Analysis (MLVA) have been widely used for identification, classification and typing of *Brucella* species (Marianelli et al., 2007; Sayan et al., 2009). However these techniques are expensive, require molecular setup and possess variable discriminatory power (Ranjbar et al., 2014). In general, differentiation of the various species and biotypes of *Brucella* remains difficult and different results can be obtained by independent *Brucella* research groups (Scholz & Vergnaud, 2013). Therefore, development of new techniques that facilitates a reliable differentiation of the various species and biotypes of *Brucella*, is of great practical importance.

Matrix-assisted laser desorption ionization- time of flight spectrometry (MALDI-TOF MS), a proteomic based assay, is a promising technique in which

a unique mass spectrum with various peaks corresponding to the high-abundance soluble ribosomal proteins is produced for each organism (Dekker & Branda, 2011). This spectrum is compared to a library of spectra produced from known reference organisms and the organism's likely identification is provided based on the closest match (Lay, 2001). The advantage of MALDI-TOF-MS over other conventional and molecular typing methods is that it is fast, cost effective, and does not require high skilled personnel (Seng et al., 2009).

MALDI-TOF MS has been standardized for identification of microorganism but only recently studies were conducted for various bacterial typing by analysis of the peaks as exemplified for *Salmonella enterica* (Dieckmann & Malorny, 2011), and *methicillin-resistant Staphylococcus aureus lineages* (Wolters et al., 2011).

Ferreira et al (2010) and Lista et al (2011) have reported the reliability of MALDI-TOF MS in the identification of *Brucella* at genus and species level respectively. To our knowledge this is the first study that attempt to use MALDI-TOF MS for typing of *Brucella* isolates to the biotype level by identifying biotype-specific marker peaks.

1.2 HYPOTHESIS

Using biotyper software available in MALDI-TOF (Brucker Daltonics), along with standardized typing procedure, we will be able to generate a mass spectra with a biotype-specific peaks with reasonable signal intensities to biotype *B. melitensis*.

1.3 AIM

To evaluate MALDI–TOF MS as a safe, rapid, and reliable tool for biotyping of *B. melitensis*.

1.4 OBJECTIVES

The objectives of this thesis are:

1. to construct a *Brucella* reference library using *Brucella* reference strains,
2. to identify the clinical isolates against the newly constructed library,
3. to generate mass spectra for all clinical isolates and to use these spectra for typing, and
4. to evaluate the relatedness of the species using mass spectrum dendrogram.

CHAPTER 2 LITERATURE REVIEW

2.1 *BRUCELLA*

2.1.1 Microbiological Characteristics

Brucella species are facultative intracellular, gram negative coccobacilli or short rods that lack capsules, flagella, and endospores (Gwida et al., 2010). *Brucella* species grow on blood agar plate and chocolate agar but not on MacConkey, which allow them to be separated from some other Gram-negative coccobacilli (Young, 1995). They are slow growers and their growth is often improved by carbon dioxide which is essential for some strains. On suitable solid media *Brucella* colonies are visible after 2 days and are 0.5 to 1.0 mm in diameter with a convex and circular outline. Smooth strains are transparent and pale yellow while rough colonies are more opaque with a granular surface (Poester et al., 2010). *Brucella* species are positive for oxidase, catalase, and urease. The metabolism of the *Brucella* is mainly oxidative and they show little action on carbohydrates in conventional media (Al Dahouk et al., 2010).

2.1.2 Antigenic Components

The outer cell membrane resembles that of other Gram-negative bacilli with a dominant lipopolysaccharide component which is considered the target for many serological and immunological studies and the principal virulence factor of *Brucella* (Bossi et al., 2004). All *Brucella* species, except *Brucella ovis* and *Brucella canis*, contain smooth lipopolysaccharide (SLPS) in their outer cell

wall (Poester et al., 2010). Strains with SLPS are more virulent and more resistant to intracellular destruction by Polymorphonuclear leukocytes than the strains with rough lipopolysaccharide (Bossi et al., 2004). SLPS exist as antigenic epitopes A and M which have different quantitative distribution among the smooth *Brucella* strains and are absent, in the rough *Brucella* strains. This is of value in differentiating biotypes of the major species using absorbed monospecific A and M antisera (Alton et al., 1975). Outer membrane structural proteins (Omp25) are also useful in diagnostic tests. Others, such as ribosomal proteins (L7/L12) and fusion proteins, have demonstrated a protective effect against *Brucella* based on antibody and cell mediated responses (Araj, 2010).

2.1.3 Taxonomy

The genus *Brucella* belongs to the family Brucellaceae in the order Rhizobiales of the class Alphaproteobacteria (Ficht, 2011) and it contains highly infectious species that have been found to cause infections in a wide variety of mammals. Species and biotypes classification of *Brucella* is historically based on natural host preference and phenotypic traits (AlDahouk et al., 2010). However modern taxonomy practices have assisted in further delineating the significance of biotypes of the old species and a better understanding of the inter-relatedness of all currently described *Brucella* species (Pappas, 2010). Currently *Brucella* comprises ten species which are further sub-classified into several biotypes as shown in Table . The six well recognized species are *B. melitensis*, biotypes 1-3 (sheep and goats); *B. abortus*, biotypes 1-7 and 9 (cattle and other Bovidea);

B. suis biotypes 1-5 (biotypes 1-3 pigs, biotype 4 reindeer, biotype 5 small rodents); *B. canis* (dog); *B. ovis* (sheep) and *B. neotomae* (desert wood rats) (Lucero et al., 2008). Further, three novel species have been added to the genus, *B. pinnipedialis* (seals), *B. ceti* (dolphins and whales), and *B. microti* (common vole, red foxes and also from soil). Most recently *B. inopinata* isolated from a breast implant wound has been described as a new species with so far unknown animal reservoir (Al Dahouk et al., 2010). There are two other isolates, with typical *Brucella* characteristics but distinct from the currently described species, known to have caused individual incidences of diseases. These isolates still awaiting final taxonomical classification, one being referred to as Baboon type in the meantime (Pappas, 2010).

Human disease severity is to a significant extent determined by the type of *Brucella* to which an individual is exposed. *B. melitensis* is the type most frequently reported as a cause of human brucellosis and the most virulent type that is associated with severe acute disease (WHO , 2006).

Table 2.1: Provisional *Brucella* Taxonomy

	Species	**Animal host**	**Human disease**
Old species	*B. melitensis*	Sheep, goats, camels	The most common cause of human brucellosis
	B. abortus	Cattle, buffalo, elk, yaks, camels	The second most common cause of human infection
	B. suis	Domestic pigs, wild boar, reindeer, caribou, rodents	Of increasing interest in human disease, with hunters of wild boar at risk
	B. canis	Canines	Increasing reports, particularly from South America, possibly understudied elsewhere

	Species	Animal host	Human disease
	B. ovis	Sheep	Not reported
	B. neotomae	Rodents	Not reported
Novel species	*B. ceti*	Porpoises, dolphins, whales	Reports of complicated disease (neurobrucellosis, spondylitis) and one laboratory infection
	B. pinnipedialis	Seals	
	B. microti	Red foxes, common voles (also isolated from soil	Not reported
	B. inopinata	Unknown	Isolated from a human case (prosthetic breast implant infection)
Future species	BO2	Unknown	Isolated from a human case (chronic destructive pneumonia)
	Baboon isolate	Baboons	Not reported

Adapted from The changing *Brucella* ecology: novel reservoirs, new threats by Pappas, 2010. International Journal of Antimicrobial Agents.

2.1.4 Clinical Disease

Brucellosis is a highly transmissible zoonosis that causes a severely debilitating and disabling illness in human. Human infections arise through direct or indirect contact with infected animals or their products that are contaminated with these bacteria (Marianelli et al., 2007). The bacteria initially localize in the regional lymph nodes, then disseminate haematogenously to the organs of the reticuloendothelial system (Memish et al., 2000) leading to a variety of clinical presentations, such as recurrent fever that rise and fall in waves, weight loss, general malaise, muscle and joint pain and septicemia (Logan et al., 2011).

Signs and symptoms are similar in patients irrespective the route of transmission and are mostly non-specific which complicate the diagnosis (Bossi et al., 2004). Brucellosis is usually treated with doxycycline combined with rifampin or streptomycin (WHO, 2006). Without adequate and prompt antibiotic treatment, some patients develop a chronic brucellosis syndrome with multiple organs involved and with many features of the chronic fatigue syndrome (WHO, 2006). Morbidity depends largely upon the speed of diagnosis and the initiation of specific antimicrobial therapy (Al-eissa, 1999). Despite treatment including several antibiotic regimens, relapse is estimated to occur in 5–40% of patients with acute brucellosis in the following year, depending on antibiotic use, duration of treatment, and drug combination (Buzgan et al., 2009).

2.1.5 Geographic Distribution

At present, *B. melitensis* is by far the main cause of clinically apparent disease in human worldwide since bovine brucellosis has been successfully eradicated (Al Dahouk & Nockler 2011). *B. melitensis* is fairly widely distributed but is particularly occur endemically in the Mediterranean Basin, Arabian Peninsula, India, Mexico, and Central and South America (Buzgan et al., 2009). However, North America (except Mexico), Northern Europe, Southeast Asia, Australia and New Zealand are believed to be free (FAO/WHO, 1997).

Of the three different biotypes of *B. melitensis*, biotype 3 predominates almost exclusively in Mediterranean countries and Middle East, while biotype 1 seems to predominate in Latin America. The biotype 1 and 2 have also been reported in some southern European countries (European Commission, 2001).

2.1.6 Diagnosis

The diagnosis of brucellosis should be considered in a patient with fever of unknown origin and requires the combination of several approaches, including medical history, clinical examination, and several laboratory assays (Araj, 2010). At present, there are various assays for diagnosis of human brucellosis such as serological tests that detect anti-*Brucella* species antibodies, molecular methods that detect *Brucella* species DNA, and the standard microbiological tests that attempt to isolate *Brucella* species (Wang et al., 2014). Knowledge of the potentials and limitations of each test are warranted for their appropriate application and interpretation.

2.1.6.1 Serological Diagnostic Tests

The most commonly serologic tests used in the diagnosis of human brucellosis are Serum Agglutination Test (SAT), Rose Bengal test, Coombs' Test and Enzyme Linked Immunoassay (ELISA) (Al Dahouk & Nockler, 2011). SAT is the reference method in the serological diagnosis of human brucellosis that confirms the diagnosis when a single titre >1:160 is found or a four-fold rise in the antibody titre is noted between the onset of illness and convalescent-phase serum (Bossi et al., 2004). Serology is fast and non-hazardous, but the lack of standardization, cross-reactivity and long-term persistence of significant antibody titres after successful treatment may hamper laboratory diagnosis (Al Dahouk & Nockler, 2011). In addition adequate cutoff points have to be defined for different populations living in regions of varying endemicity to determine the significance of positive serologic test results (Al Dahouk & Nockler, 2011).

Serological diagnosis is presumptive evidence of infection; therefore, it is essential to correlate the serology findings with clinical signs and symptoms (Poester et al., 2010).

2.1.6.2 Molecular Diagnostic Assays

Molecular diagnostic assays are used for the direct detection of *Brucella* from clinical specimens, to monitor treatment response, and for the identification and differentiation of recovered *Brucella* species (Poester et al., 2010). These assays are based on the amplification of genomic targets through different PCR approaches. Genus-specific PCR assays that utilize one pair of primers that amplifies and targets a unique and highly conserved genetic loci in all *Brucella* species, like BCSP31gene, 16S rRNA, omp2, Outer membrane proteins (omp2b, omp2a and omp31) and IS711, are useful tools for diagnosis in human brucellosis (Yu & Nielsen, 2010). Although genus specific PCRs are usually adequate for diagnosis of human brucellosis, differential species-specific multiplex PCR like AMOS-PCR assay, Bruce-ladder multiplex PCR assay, Arbitrary Primed PCR (AP-PCR) or the Random Amplified Polymorphic DNA (RAPD-PCR) can also be used for confirmation (Poester et al., 2010). Molecular diagnostic assays are reproducible, rapid, and minimize the risk of infection among laboravatory workers. However, molecular methods are relatively expensive, with variable sensitivity (Al Dahouk & Nockler, 2011), and their efficiency is highly dependent on primers specificity (Wang et al., 2014). Furthermore, molecular detection of *Brucella* DNA does not necessarily indicate the presence of viable

organism. *Brucella* DNA remains detectable in the majority of brucellosis patients throughout treatment and treatment follow-up, as well as years after clinical cure (Al Dahouk & Nockler, 2011).

2.1.6.3 Standard Microbiological Tests

Diagnosis is definitively established by the isolation of *Brucella* from blood, bone marrow or body fluids (Al Dahouk & Nockler, 2011). Automated continuously monitored blood culture systems such as Bactec (BD Diagnostics, Sparks, MD, USA) and BacTAlert (bioMérieux, Durham, NC, USA) expedite the detection of bacterial growth (Araj, 2010). *Brucella* isolation rates are variable depending on the stage of disease, previous use of antibiotics, the clinical specimen and the culture methods (Al Dahouk & Nockler, 2011). Presumptive identification of *Brucella* species relies upon basic phenotypic tests such as colonial morphology, staining reaction, urease, catalase and oxidase tests. These tests can be done by most routine bacteriology laboratories and are the basis for a culture to be identified as belonging to the genus *Brucella* (Poester et al., 2010). Once a culture has been identified as *Brucella*, it is sometimes important to classify the species and the biotypes. This further classification should be done in specialized or reference laboratories since it is time consuming, hazardous, and required highly skilled personnel as well as biosafety level 3 facilities (Cekovska et al., 2010). However, bacteriological culture when positive, though hazardous and time consuming, is still considered the gold standard for human brucellosis diagnosis and is essential for subsequent strain typing for epidemiological studies (Hashim et al., 2014).

2.1.7 Typing

The process of differentiating strains based on their phenotypic and genotypic differences is known as "typing" (Belkum et al., 2007). Typing of *Brucella* strains is not required to establish a diagnosis but is useful in epidemiological studies, understanding the pathogenesis of infection and improving the outcomes of the national brucellosis eradication program and vaccine development (De Santis et al., 2011).

A wide variety of *Brucella* typing systems are currently in use that varies greatly with respect to cost, reliability, applicability and ability to discriminate between different strains. No one technique is optimal for all forms of investigation (Scholz & Vergnaud, 2013). Typing of *Brucella* strains can be carried out by phenotypic and genotypic characteristics analysis.

2.1.7.1 Phenotyping Methods

There are no single definitive phenotypic test that can identify individual *Brucella* species or biotype. The phenotypic identification of *Brucella* is based on a range of phenotypic tests; by the analysis of the combined results obtained from these tests, a fairly confident determination of the *Brucella species* and to a lesser extent the biotype can be made (Appendix C). The most common applied phenotypic tests are biotyping, serotyping, phage typing and antibiotic susceptibility typing (Miljković et al., 2009).

Biotyping tests include the determination of CO_2 requirements, H_2S production, urease activity, oxidative metabolic pattern, and dye sensitivity (inhibition of growth on media containing dyes such as thionin or basic fuchsine) (Poester et al., 2010).

Serotyping is based on A and M cell wall lipopolysaccharide antigens detection. Both antigens are simultaneously expressed on all smooth *Brucella* strains. Quantitative differences in the amounts of these antigen are thought to account for the three phenotypes, A+M-; A-M+; and A+M+, identified by agglutination (Bundle et al., 1989). Serotyping is a very subjective test and its cross-reactivity is well recognized due to the close structural similarities of the A and M antigens. In addition, strains converting to the rough phenotype on subculture may lose the characteristic agglutination pattern (Dawson et al., 2008).

Phage typing uses set of phages that allows definitive identification of smooth and rough strains of different *Brucella species* depending on the characteristic lysis of *Brucella* cultures with these phages (Filippov et al., 2013). The *Brucella* phages currently used for *Brucella* typing are Tbilisi(Tb), Weybridge (Wb), Izatnagar (Iz) and R/C (Filippov et al., 2013). However, Phage typing of *Brucella* is a multi-day procedure, does not provide differentiation of biotypes and the characteristic lysis pattern of smooth *Brucella* strains may be lost if the strains become rough on subculture (Dawson et al., 2008).

The use of commercially available systems such as Vitek 2 (bioMérieux, Durham, NC, USA) for *Brucella* typing is not recommended because of their lack of accuracy, danger of aerosols , and due to the fact that only *B. melitensis*

from the genus *Brucella* could be identified without biotype differentiation (Cekovska et al., 2010).

Phenotypic methods in general are time consuming, technically difficult subjected to variable interpretation and most importantly hazardous. Phenotypic tests require the isolation of highly infectious pathogen that carries the risk of intra-laboratory infection and hence biosafety level 3 precautions must be observed during testing procedure (Cekovska et al., 2010). Another limitation of phenotypic typing is the inability to get beyond a partial resolution between biotypes (Araj, 2010).

2.1.7.2 Molecular Genotyping methods

The genus *Brucella* is highly homogeneous with all members showing >95% homology in DNA–DNA pairing studies (Lucero et al., 2008). Therefore stable and specific molecular markers are needed for efficient discrimination between different *Brucella* strains. Most molecular typing methods are based on the use of a restriction enzyme such as ribotyping; pulsed field gel electrophoresis (PFGE); and amplified fragment length polymorphism (AFLP). Other methods are PCR-based such as RAPD-PCR; repetitive intergenic palindromic sequence-PCR (REP-PCR); AP-PCR ; enterobacterial repetitive intergenic consensus sequence-PCR (ERIC-PCR); polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP); and multilocus sequence typing (MLST) (Bricker et al., 2003). All these techniques possess different discriminatory powers, and their use depends on the final objective to be achieved (Whatmore, 2009).

MLVA is a powerful tool for epidemiological studies of closely related strains and has been used very successfully for *Brucella* genotyping (Sayan et al., 2009). MLVA based on eight locus scheme, a PCR assay called Hoof-Prints (Hypervariable Octameric Oligonucleotide Fingerprints), is highly discriminating and very efficient in distinguishing strains within an outbreak but is unable to predict the biotype of an isolate and yet cannot replace conventional biotyping methods (Poester et al., 2010). Other assays such as, MLVA-21; MLVA-15; and MLVA-16 with higher discriminatory power, were published (Tiller et al., 2009; De Santis et al., 2011) MLVA-16 assay, for example, consists of eight moderate variable minisatellite (panel 1) and eight highly discriminatory microsatellite (panel 2). The different alleles, amplified by standard PCR techniques, can be then analyzed by several electrophoretic techniques as agarose gel, or capillary electrophoresis sequencing (De Santis et al., 2011). However, the lack of an international database makes the MLVA typing method inappropriate for routine implementation in diagnostic laboratories worldwide (Sayan et al., 2009).

Single-nucleotide polymorphism analysis method for *Brucella* genotyping on the other hand, is found to be promising as the most genetic differences among different *Brucella* species and biotypes consist of single nucleotide polymorphisms (Sayan et al., 2009). In general, molecular applications are highly expensive, time consuming, and sometimes present a low level of reproducibility (Whatmore, 2009).

2.2 MATRIX-ASSISTED LASER DESORPTION IONIZATION- TIME OF FLIGHT SPECTROMETRY (MALDI-TOF-MS)

2.2.1 Overview

MALDI-TOF-MS has existed for a long time but it was 1996 when MALDI-TOF spectral fingerprints could be obtained from whole bacterial cells for the first time (Holland et al., 1999). The same year, Krishnamurthy et al. obtained spectral fingerprints of pathogenic species such as *Bacillus anthracis, B. melitensis, Yersinia pestis,* and *Francisella tularensis* using MALDI TOF (Carbonnelle et al., 2011). Ever since, the number of publications concerning not only bacterial but also mold and yeast identification increases exponentially. However, use of MALDI-TOF in clinical microbiology as a routine first-line identification method started just during the past five years (Kostrzewa et al., 2013).

MALDI-TOF MS technology has fundamentally altered well established diagnostic testing methods because of its significant advantages over other conventional and molecular identification methods (Murray, 2012). It is rapid, and reliable, it takes only few minutes for correct identification (Fenselau, 2012). In addition, MALDI technique is simple, does not require high skilled personnel and cost effective (Seng et al., 2009). MALDI TOF MS works well for many bacterial species hence has the potential to replace conventional phenotypic identification for most bacterial strains isolated in clinical Microbiology laboratories (Biswas & Rolain, 2013).

2.2.2 Principle

The intrinsic property of MALDI-TOF MS is to detect the mass-to-charge ratio (m/z) of bacterial ribosomal proteins, providing a unique mass spectrum of the microorganism within minutes (Carbonnelle et al., 2011). Importantly, MALDI approach do not rely on actual identification of the biomarker ion peaks in an MS spectrum but on the characteristic mass profile generated by a set of ion peaks that constitute a bacterial "fingerprint"(Dieckmann & Malorny, 2011).

This method requires that the biopolymer molecules be converted into isolated ionized molecules in the gas phase. These ions are then separated according to their molecular weight after migration in an electric field. Each molecule detected is characterized by the molecular mass, the charge, and the relative intensity of the signal (Carbonnelle et al., 2011). Amass spectrum unique to the organism is produced, get compared to a library of spectra obtained from known reference organisms, and the organism's likely identification is provided based on the closest match (Dekker & Branda, 2011).

MALDI-TOF MS utilize a soft ionization technique that allows the gentle ionization and vaporization of large and delicate biological macromolecules into the gas phase without extensive sub-fragmentation and without prior purification (Dekker & Branda, 2011). Soft ionization of MALDI-TOF have overcome the problem of harsh ionization by which only molecules of low molecular masses are analyzed (Carbonnelle et al., 2011).

MALDI-TOF MS may be used to analyze samples of many types, including solutions of organic molecules, nucleic acids, proteins, and whole microorganisms, with the last two being the most useful in present clinical Microbiology applications (Dekker & Branda, 2011). Fatty acids were evaluated early on as biomarkers for bacterial identification, but rejected as they are too dependent on growth and storage condition (Fenselau, 2012). However, the most reliable MS biomarkers for bacterial identification are considered to be the major proteins, mainly ribosomal proteins. Ribosomal proteins are abundant, basic, and of medium hydrophobicity, all biochemical traits that favor efficient ionization (De Carolis et al., 2014). A sufficient number of stable mass signals of these proteins(between 2000 Da and 20000 Da) can be detected, yielding profile spectra consisting of a series of peaks that are conserved at genus, species and subspecies as well (Barbuddhe et al., 2008).

2.2.3 Sample Preparation

Two principal bacterial treatment protocols can be used. The first is Direct Transfer protocol; based on introducing intact cells onto the MALDI target directly from agar plates using sterile loops or after harvesting by centrifugation from liquid media. The second protocol relies on extraction of bacterial proteins from the cells using different solvents (Sedo et al., 2010). The Direct Transfer can be used for 90-95% of routine samples. Extraction is especially recommended if very high spectra quality is needed and for very hard to measure microorganisms where the cell walls are hard or thick and the matrix is

not strong enough to break up the cells (Bruker Daltonics, Microflex LT Biotyper operating system). In addition, the extraction protocol inactivates a broad range of microorganisms. Different solvents are used in extraction approach with ethanol/formic acid the most used for bacterial identification (Bruker Daltonics, Microflex LT Biotyper operating system).

2.2.4 Technical Description

The MALDI-TOF MS instrument is composed of three principal units. The first is the specimen ionization chamber, within which the laser-based vaporization of the specimen takes place; transferring the sample molecule ions into a gas phase. The second unit is the time of flight mass analyzer that allows ion separation according to m/z. The last unit is the particle detector device that monitor the separated ions (De Carolis et al., 2014).

The first step is the formation of a crystal between the sample and an organic matrix (co-crystallization). The sample is spotted onto a MALDI-TOF sample target with an appropriate matrix and allowed to air dry at room temperature The matrix is believed to serve two major functions; absorption of energy from the laser, and isolation of the analyte molecules from each other (Carbonnelle et al., 2011). The matrix is selected for certain properties, including strong absorbance at laser wavelengths used for ionization, ability to ionize the clinical specimen, solubility in solvents that are compatible with the clinical specimen, and a complete lack of any chemical reactivity with the clinical specimen to avoid unwanted alterations or damage to the measured proteins (Clark et al., 2013a). A number of such matrix compounds have been developed for use in

MALDI-TOF MS, with 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), α-cyano-4-hydroxycinnamic acid (α-CHCA) and 2,5-dihydroxybenzoic acid (DHB) being the most commonly used in bacterial identification (De Carolis et al., 2014). Each matrix has a unique initial velocity when exposed to a pulsed laser beam under vacuum. For example, α-CHCA, often referred to as a hot matrix, has a high initial velocity (Olsen & Macek, 2009).

After co-crystallization of the sample and matrix, the target is loaded into the specimen ionization chamber, where the sample-matrix mixture is pulsed with a laser. Ultraviolet nitrogen lasers (337 nm) is usually used in MALDI-TOF applications, although other types of lasers can be used as well (Dekker & Branda, 2011). The supplied laser energy is so high that the matrix and the analyte are sublimated directly from the solid phase into the gas phase (Bruker Daltonics). The matrix absorbs energy from the laser resulting in vibrational excitation of the matrix and the ejection (desorption) of analyte molecules surrounded by clusters of matrix molecules, water, and ions (Clark et al., 2013b). Once desorbed, the matrix molecules transfer protons to the analyte, resulting in positively charged analyte cations in the gas phase. The most common MALDI ionization format is for analyte molecule to carry single positive charge (Dekker & Branda, 2011). The gas phase analyte cations are then accelerated across an electric field within the ionization chamber to a velocity that depends on the mass-to-charge (m/z) ratio of the analyte (De Carolis et al., 2014).

The ions leave the ionization chamber and enter the high vacuum flight tube (TOF) with smaller ions traveling faster than larger ones before hitting the detector located at the other end of the tube. Thus, the time of flight (TOF) required to reach the detector is dependent on the mass and charge of the ion (De Carolis et al., 2014). The impact of ions at the detector generates an electrical signals. These signals are analysed by software and displayed as a spectrum that is characterized by both the m/z and the intensity of the ions, which is the number of ions of a particular m/z that struck the detector (Croxatto et al., 2012). The time of ions transmission is measured precisely by their arrival at the mass detector and their m/z is determined with the help of a calibration curve that blot the flight time against m/z (Bruker Daltonics, Microflex LT Biotyper operating system). Mass spectra are then searched for in the appropriate database for the identification of the microorganism, comparing with values provided by database (Cobo, 2013).

2.2.5 Biotyper Software and Database

MALDI-TOF MS Biotyper software has three main components. MALDI Biotyper Real time Classification (RTC) for quick classification of unknown samples, MALDI Biotyper Offline Classification (OC) for advanced classification process and investigate the relationships between groups of organisms, and Flex Control Used to control the MALDI-TOF instrument. MALDI Biotyper database (Bruker Daltonics, Leipzig, Germany) used for routine identification of microorganism in clinical Microbiology contains database for wide variety of

clinically relevant microorganisms, involving bacteria and fungi, but some important pathogens like *Brucella*, have not been yet included in the database (Ferreira et al., 2010). However, the Biotyper software is an open platform allowing the user to save runs to expand the database of stored spectra by utilizing tools included in the software. The user also has the ability to create main spectra with the assistance of the software (Clark et al., 2013a).

2.2.6 Microorganism Identification

For microorganism identification, mass spectra are generated and analyzed with regard to spectrum peak frequency, position, and intensity against a data base of reference spectra referred to as the MALDI biotyper library (MBL) in a real time manner via the Biotyper RTC software (Clark et al., 2013a). MBL include species-specific fingerprints of several bacterial and yeast isolates. Through a pattern matching procedure, mass peaks in the unknown spectra are matched with reference spectra included in the database. Level of similarity between the acquired unknown spectra and MBL entries' is calculated as a score ranging from 0.00 indicating no similarity to 3.00 indicating a perfect match (De Carolis et al., 2014). A log score ranging from 2.3 to 3.000 is interpreted by the software as a highly probable species-level identification., log scores between 2.00 and 2.299 , genus identification and probable species-level identification and log scores ranging from 1.70 to 1.999 represent a probable genus identification. Log scores below 1.699 to 0 are not considered to be a reliable identification (Clark et al., 2013a). De Carolis et al (2014) found that the overall MALDI-TOF identification performance (93.2%) is significantly

better than that of BD Phoenix's (75.6%) (Becton Dickinson Diagnostic Systems, France) and Vitek 2's (75.2%) (BioMérieux, Marcy L'Etoile, France). The accuracy of the identification depends greatly on the number of database entries. An update of the reference database is needed to improve the identification performance of MALDI-TOF (De Carolis et al., 2014). The reproducibility of mass patterns is sensitive to sample culture, preparation, storage, and the kind of mass matrix used (Fenselau, 2012).

2.2.7 Microorganism Typing

MALDI-TOF spectrometry method has been recently used as a tool for classification and subtyping of bacteria. While MALDI-TOF identification of bacteria at genus and species levels has been shown clearly to be rapid and effective, the utility of this approach at the strain level has not been completely explored and lack approved guidelines for data interpretation (Sandrin et al., 2012). Identification to the more specific "strain" requires higher resolution approaches and tends to be more challenging, because strains within a single species are quite often extremely similar, genotypically and phenotypically (Sandrin et al., 2012). There are few studies published on potential use of MALDI for epidemiological typing and it is not clear if MALDI-TOF MS typing will be as successful as identification. Strain typing by MALDI-TOF MS was reported in recent studies on *S. enterica* (Dieckmann & Malorny, 2011), *methicillin-resistant S. aureus lineages* (Wolters et al., 2011), *Streptococcus agalactiae* (Lartigue et al., 2009) *,and Yersinia enterocolitica* (Rizzardi et al., 2013).

MALDI-TOF MS Bacterial typing at the strain level utilizes different approaches. One is strains categorization without providing discrimination of single strains using mass spectrometry-based dendrograms, in which closely related strains are separated hierarchically according to their mass signals and intensities (De Carolis et al., 2014). In contrast, strain differentiation based on distinguishing single strains from one another by the presence and/or absence of one or more discriminating peaks (Sandrin et al., 2012). However, this approach is subjected to analytical error, biological, and technical variation(Spinali et al., 2014). A third approach perform strain identification by comparing profiles of unknown strains to those in the reference strains (Sandrin et al., 2012).

2.2.8 MALDI TOF MS and *Brucella*

There has been considerable interest in using MALDI-TOF MS for the identification of fastidious organisms and potential agents of bioterrorism. Ferreira et al (2010) and Lista et al (2011) has reported the reliability of MALDI-TOF in the identification of *Brucella species* at genus and species level respectively. An important problem for the routine use of MALDI-TOF MS for identification of *Brucella* species is that there is no reference library for *Brucella* incorporated to the main databases, because of problems derived from their potential bioterrorist use (Ferreira et al., 2010). A recent study reported that MALDI could reliably identify 92% of the *Brucella* isolates at species level, but incorrect biotype assignments were frequently found (Karger et al., 2013).

CHAPTER 3 MATERIALS AND METHODS

3.1 List of Reagents

1. *B. melitensis* biotype 1 strain 16M (NCTC10094).
2. *B. melitensis* biotype 2 strain 63/9 (NCTC10508).
3. *Brucella abortus* biotype 1 (NCTC10093).
4. *B. abortus* monospecific antiserum, 1 ml/vial (USDA, National Veterinary Service Laboratory [NVSL], Ames; catalog #A12 Center for Disease Control) , reconstitute with 1 ml of distilled water and store 2-8° C.
5. *B. melitensis* monospecific antiserum, 1 ml/vial (NVSL; catalog #M12-CDC), reconstitute with 1 ml of distilled water and store 2-8° C.
6. Qiagens mericon DNA Bacteria Kit.
7. *B. melitensis* biotype typing PCR kit (Ankara University Biotechnology Institute, Ankara Turkey).
8. Absolute Ethanol (Fisher Scientific, Loughborough, UK).
9. 70% Formic Acid.
10. 100% Acetonitrile.
11. α-cyano-4-hydroxycinnamic acid (α HCCA, Bruker Daltonics)

3.2 Ethical Approval

Ethical approval was obtained from the Medical Research Center at Hamad Medical Corporation, protocol number 14383/14 (Appendix A & B).

3.3 Safety Precautions

All cultures were processed in a Class III biological safety cabinet in a negative pressure room using the appropriate personnel protective equipment. All tests were performed with attention to avoid the creation of dangerous aerosols, which is very important for laboratory safety. Recommended extraction method was performed to ensure safety of testing personnel and instrument contamination.

3.4 Bacterial Strains and Sample Size

3.4.1 Reference Strains

Three reference *Brucella* strains were used in this study as shown in Table 3.1. The strains were obtained from National Collection of Type Cultures (NCTC, Public Health England, UK).

B. melitensis Ether- biotype 3 strain (NCTC10509) was not available with NCTC and was not included in the study.

Table 3.1: Reference strains included in the study

Species/Subspecies	Type Strain
B. abortus biotype 1	NCTC10093
B. melitensis 16M biotype 1	NCTC10094
B. melitensis 63.9 biotype 2	NCTC10508

3.4.2 Clinical Isolates

A total of 63 *Brucella* isolates were obtained from Microbiology Biobank, Department of Microbiology at Hamad Medical Corporation. *Brucella* strains were originally isolated from positive cultures of patients attending Hamad Medical Corporation in the period of 2012-2013 (Patient demographics are shown in Table.2). All strains were presumptively identified at genus level in the clinical Microbiology laboratory on the basis of colonial morphology, microscopic appearance, showing Gram-negative coccobacilli, and biochemical properties like oxidase and catalase production after which they were cryopreserved in cryovials with beads & glycerol (mastcryobank, Mast Group Ltd, UK) and stored at -80° C.

Table 3.2: Demographic data of the study population

Characteristic	Number of Patients
All subjects	63 (100%)
Age group	
<12	15 (23%)
12–60	41 (66%)
>60	7 (11%)
Gender	
Male	54 (86%)
Female	9 (14%)
Nationalities	
Qatari	31 (49%)
Arabian Peninsula (Saudi Arabia, Yemen)	4 (6%)
Asians (Bangladesh, India, Sirilanka, Nepal)	10 (16%)
Egypt	5 (8%)
Sudan	5 (8%)
Others (Iraq, Jordan, Syria, Mauritania, Eritrea)	8 (13%)

3.5 Bacterial Reviving

All the clinical isolates from frozen cryovials as well as the three reference strains were cultured on sheep blood agar plates and incubated in aerobic conditions at 35°C in the presence of 5% CO_2 for 48 hours. The growing bacteria were then sub-cultured on sheep blood agar, incubated for 48 hours at 35° C in the presence of 5% CO_2. The sub-cultured bacteria were numbered as BRUC (1-63) and used for the subsequent testing.

3.6 Identification with Vitek 2 Compact

Identification of all clinical isolates to species level was done with Vitek 2 compact (bioMérieux, Durham, NC, USA) using gram negative (GN) cards. Bacterial suspensions were prepared in normal saline and adjusted to 0.5 McFarland. Tubes were then sealed to avoid the creation of dangerous aerosols, cards were inserted and the tube-card sets were loaded into Vitek 2 compact for identification.

3.7 Serological Typing

All the clinical isolates that have been identified with Vitek 2 compact were further classified into different biotypes using A and M *Brucella* monospecific antisera provided by National Veterinary Service Laboratory (NVSL), USA.

Actively growing cultures of reference strains and clinical isolates were used to prepare heavy suspension (equivalent to 2 McFarland) in 1 ml of phenolized saline in screw-cap tubes. Tubes were then incubated for 1 h in a 65° C water bath to deactivate the bacteria.10 µl of each A and M monospecific *Brucella*

antiserum was placed on a microscope slide and equal volume of organism suspension was added to each and mixed with an inoculating loop. After gentle shaking, agglutination reaction was examined within 1 minute. Positive results were obtained when agglutination of bacterial inoculum with antiserum occurred within 1 minute of mixing. Negative result on the other hands were considered when bacterial inoculum failed to agglutinate within 1 minute of mixing with antiserum. Identification of biotypes were made using Table 3.3 in Appendix C. Controls used were as follows: *Brucella abortus* biotype 1 (NCTC10093) agglutinates only with A antisera and *B. melitensis* biotype 1 agglutinates only with M antisera.

3.8 MALDI-TOF MS

Microflex LT Biotyper operating system (Bruker Daltonics, Bremen Germany) and Bruker Biotyper 3.0 software were used in the study.

3.8.1 Sample Preparation and Formic Acid Extraction

All the three reference strains as well as the 63 clinical isolates were prepared using Formic Acid Protein extraction method, which reliably killed the bacteria, in accordance with the company guidelines. Two extracts were generated from independent subcultures. Around 10 colonies were suspended in 300 μl of water and mixed well by vortexing to generate a homogeneous suspension. 900 μl of absolute ethanol was added and the suspension was mixed carefully. The suspension was left for 90 minutes at room temperature to inactivate all

of the bacteria after which it was centrifuged for 2 min at 13,000×g. The supernatant was removed. The spinning step was repeated and all the residual ethanol was removed by pipetting. The pellet was kept at room temperature for drying. Next, 50 µl of 70% formic acid was added to the pellet, and the pellet was mixed thoroughly for 2 minutes. Subsequently, an equal volume of 100% acetonitrile was added and mixed carefully. This allows the release of the cells contents into the supernatant. The particulate matter that could not be dissolved was spun down by centrifugation for 2 min at 13,000×g. The supernatant was then spotted onto a MALDI-TOF target plate and used for main spectra projection (MSP) creation, real time classification (RTC) and typing.

3.8.2 Target Plate Preparation:

Spots were created, using 0.1 µl of the supernatant per spot, onto a MALDI-TOF target plate (MTP 96 target, Bruker Daltonics) and air-dried. Subsequently, the spots were overlaid with 0.1 µl of α-cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltonics) and dried at room temperature. The number of spots created differs as per the analysis performed (Table).

Table 3.3: Number of spots per Analysis

Analysis	Number of spots
MSP creation	8 spots
Real time Classification RTC (Identification)	2 spots
Typing	2 spots

3.8.3 Mass Spectra Acquisition

All of the mass spectra were automatically acquired on a Bruker flex control in linear, positive mode at laser frequency of 60 Hz and laser intensity of 35%. The acceleration voltage was 20 kV and 18.7 kV for source voltage. For each spectrum, 240 laser shots in 40-shot steps from different areas of the sample spot were accumulated and analyzed (in automatic mode using default settings). The mass spectra were recorded at a mass/ charge range of 2000-20,000 Da for MSP creation and between 0 and 30,000 Da for typing.

3.8.4 Calibration

The instrument was calibrated using Bruker bacterial test standard BTS (Bruker part no. #255343), an extract of *Escherichia coli* DH5a which has been spiked with two additional proteins. Calibration was performed with each target slide following manufacturer's instruction. The Err/ppm column for each calibrant was checked, for the value which must not exceed ±300ppm. Once the calibration was achieved, the calibration values were saved and were further applied to the measurement of clinical experimental strains.

3.8.5 Quality Control

Positive and negative controls were included in each run. BTS was used as positive control while blank spot was used as negative controls. Furthermore, by including reference strains during each extraction procedure, the complete procedure was validated. Quality control criteria to qualify or disqualify a run

was set. BTS should yield an identification of *Escherichia coli* with log scores >2.30 and blank should yield no peak.

3.8.6 MSP Creation and Library Construction

Extracts from reference strains were used to create Main Spectra Projection (MSP). Eight spots were created using the procedure explained in target preparation (see 3.8.2 Target preparation). Target plate was loaded into the mass spectrometer. Spectra were generated using Bruker FlexControl (see 3.8.5 Mass spectra acquisition). Toward the goal of improving reproducibility, the eight spots were run three times and a total of 24 MS spectra were obtained for each strain. To ensure accuracy, two spots of freshly prepared BTS were included in each run (one BTS spot was used for calibration and the other was used as control for the standard curve). The obtained raw spectra for the three reference strains were manually processed using FlexAnalysis software. Smoothing and baseline subtraction were performed. The quality of individual raw spectrum measurements was carefully checked. Peaks of very weak intensity or too intense peaks were considered outliers and were excluded. Peaks deviation were checked by calculating the difference between the highest and lowest signal of particular peak. If the difference was more than 500 ppm, the spectrum of the deviated peak was discarded. Maximum of four spectra can be discarded. The processed spectra were then used to create MSP using the automated MSP creation functionality of the MALDI Biotyper 3.0 software. For each MSP, a minimum of 20 independent processed peaks were used to create main spectrum containing the information about mean peak masses, mean

peak intensities, and mean peak frequencies. The created MSPs were used to construct the *Brucella* library following the manufacturer's instructions. Each individual spectrum was then run against the MSP for that isolate to ensure that the identification scores were >2.3 for all spectra incorporated into the MSP. As a validation step, each created MSP was run later against a database consisting of 34 *Brucella* species mass spectra profiles that was obtained from the Special Pathogen Branch at CDC, Atlanta, USA.

3.8.7 MALDI-TOF MS Identification

Extracts from clinical isolates were used in this step. 2 spots were created, using the procedure explained in Target preparation (see 3.8.2 Target preparation). Target plate was loaded into the mass spectrometer. MALDI Biotyper RTC was started and MALDI Biotyper Real time Classification Wizard was launched. A new project was created and sample data were entered.

The obtained spectra were analyzed against three libraries:

- The Standard Bruker Reference Library (>5600 entries) to check if the obtained *Brucella* spectra matched any other bacterial spectra.
- The newly constructed *Brucella* library (3 reference MSPs) to validate the newly constructed library.
- The *Brucella* database provided by the Special Pathogen Branch at CDC, Atlanta, USA to validate the classification results.

The degree of spectral concordance was expressed as a logarithmic identification score ranging from 0 to 3 and was interpreted according to the manufacturer's instructions.

3.8.8 MALDI-TOF MS Typing

Extracts from reference strains as well as clinical isolates were used to create two spots using the procedure explained in Target preparation (see 3.8.2 Target preparation). Target plate was loaded into the mass spectrometer. Spectra were generated using Bruker flex control (see 3.8.5 Mass spectra acquisition). The two spots were run three times and a total of six MS spectra were obtained for each bacterium. Toward the goal of improving reproducibility, two independent runs were performed (the whole typing procedure was repeated twice). To ensure accuracy, one spots of freshly prepared BTS were included in each run as a control.

The obtained raw spectra were manually processed using FlexAnalysis software. After smoothing and baseline subtraction, potential biomarker peaks were identified by visual inspection of the mass spectra by FlexAnalysis (overlaid view). As the actual biotypes of the clinical isolates were unknown at the time of analysis, rules for selecting a biomarker peaks were set and an individual spectral analysis was performed. A Mass signal to be considered as biotype-specific peak should be recorded consistently in all spectra of a particular isolate with reasonable intensity (>1000 au) to avoid false interpretation (Spinali et al., 2014). Consistency of a peak recording means that the peak either present or absent in the biotype of choice within a m/z of ±5 range (Clark et al., 2013a), and exhibiting the opposite condition in all other biotypes. Typically, when two different biotypes were compared, several potentially biotype-discriminating peaks per spectrum were observed, of which usually several were

present in more than one biotype. Therefore, common peaks were excluded and the spectra were carefully inspected for reproducible biotype-specific markers. Promising markers were selected accordingly and evaluated for their usage to discriminate biotypes. Attempts to identify the different acquired peak profiles was carried out by comparing the marker peak profiles of the study isolates with the reference strains.

3.8.9 MSP Dendrograms Creation

MSPs were created for all the clinical isolates following the same procedure described in MSP creation for reference strains. The generated MSPs were used to perform a hierarchical clustering of strains using the MSP dendrogram tool of the MALDI Biotyper 3.0 software to infer the relationships of *Brucella* species or strains, the closeness of which is reflected by an arbitrary distance level calculated by the software.

3.9 Molecular Typing

3.9.1 DNA Extraction

DNA was extracted from the clinical isolates as well as the reference strains using Qiagens mericon DNA Bacteria Kit following the manufacturers recommended procedure. Colonies were suspended in 1 ml RNAase free water in 1.5 ml microcentrifuge tube. The suspension was then centrifuged at 13,000×g for 5 minutes. The supernatant was discarded ensuring that all liquid is completely removed without disrupting the pellet. The pellet was resuspended with the 400 µl of lysis buffer and vortexed vigorously for at least one minute.

The samples were then heated using heat block at 100° C for 10 minutes. Tubes were centrifuged at 13,000×g for 5 minutes.100 µl of the supernatant was transferred to fresh 1.5 ml microcentrifuge tube.

3.9.2 DNA Concentration and Purity Determination

The DNA concentration of the extracted DNA was determined using Nano Drop Spectrophotometer; all assays were performed according to the manufacture instructions using 1 µl of extracted DNA for each measurement. The concentration of DNA was calculated based on the approximation that an absorbance reading of 1 µl of the purified DNA at 260nm was taken to correspond to 50 ng/µl. DNA purity was estimated by determination of the A260/A280 ratio and reference value for purity was found to be1.8. Samples were stored at 4° C until analysis.

3.9.3 PCR Amplification and Gel Visualization

Samples identified as *B. melitensis* were analyzed using a *B. melitensis* biotype typing PCR kit (Ankara University Biotechnology Institute, Ankara Turkey). For this purpose, 2.5 µl of DNA extract was added to 17.5 master mix (9 µl SYBER Green, 9.5 µl water and 2 µl primer mix reverse and forward primers). Following polymerase activation (94° C for 2 min), 40 cycles were run with 45 s denaturation at 94° C, 45 s annealing at 58° C, and 45 s extension at 72° C (Table).The PCR products (10 µl) were separated by 1.5% agarose gel electrophoresis and visualized with the BIO-RAD Gel Doc XR+ system. Reference strains for biotype 1 and 2 were included in each run.

Table 3.4: RT-PCR Cycling Conditions

Step	Time	Temperature	Number of Cycles
Initial polymerase activation	2 min	94° C	-
Denaturation	45 sec	94° C	40 x
Annealing	45 sec	58° C	
Extension	45 sec	72° C	

3.10 Statistical Analysis

Descriptive statistics in the form of frequency and percentages were performed. Associations between the marker peaks and biotypes were assessed using Pearson chi-square test and Fisher's Exact Test. The measure of association phi and Cramer's V were computed to compare the strength of association between the marker peaks and biotypes.

Kappa measure of agreement was computed to assess the agreement between MALDI-TOF -typing, serotyping and PCR. All Statistical analyses were done using statistical packages IBM SPSS and P values (0.05) two tailed were considered as statistically significant.

CHAPTER 4 RESULTS

4.1 Identification with Vitek 2 Compact

All the 63 clinical isolates were identified as *B. melitensis* with a probability of 99% and excellent confidence level using Vitek 2 compact (Appendix D).

4.2 Serological Typing

Of the 63 isolates, 42 (66.6%) were serotyped as *B. melitensis* biotype 1, 14 (22.2%) were *B. melitensis* biotype 2, and 7 (11.11%) were *B. melitensis* biotype 3 as shown in Table . *Brucella abortus* biotype 1 (NCTC10093) and *B. melitensis* 63.9 biotype 2 gave agglutination reaction only with M while *B. melitensis* biotype 1 (NCTC10094) gave agglutination reaction only A antisera as shown in Table 4.2. Serotyping results of the isolates are shown in Appendix D.

Table 4.1: Summary of Serotyping Results of *B. melitensis* Isolates

Biotype	Agglutination with Mono-specific antisera		Number of isolates
	A	M	
B. melitensis biotype 1	-	+	42 (66.6%)
B. melitensis biotype 2	+	-	14 (22.2%)
B. melitensis biotype 3	+	+	7 (11.11%)

Table 4.2: Serotyping Results of the controls

Analyte Name	Serology	
	A	M
B. abortus biotype 1 (NCTC10093)	+	-
B. melitensis 16M biotype 1 (NCTC10094)	-	+
B. melitensis 63.9 biotype 2 (NCTC10508)	+	-

4.3 MALDI-TOF MS

4.3.1 Quality Control

BTS used as positive control yield an identification of *Escherichia coli* with log scores >2.30. Blank spot used as negative controls yield no peak.

4.3.2 MSP Creation and Reference Library Construction

MSPs were created successfully for all reference strains, and new *Brucella* MSP library was constructed as shown in Table.

When each individual spectrum was run against the MSP for that isolate, an identification scores of >2.3 for all spectra incorporated into the MSP were obtained (Table).

When each created MSP was run against a database obtained from the Special Pathogen Branch at CDC, all spectra were correctly identified with a scores of >2.00.

Table 4.3: The Identification of Reference Strains against the Newly Constructed *Brucella* MSP Database

AnalyteName	Organism(best match)	Score Value
B. abortus biotype 1 (NCTC10093)	*B. abortus*	2.485
B. melitensis 16M biotype 1 (NCTC10094)	*B. melitensis*	2.494
B. melitensis 63.9 biotype 2 (NCTC10508)	*B. melitensis*	2.432

4.3.3 MALDI-TOF MS Identification

When the spectra for all the clinical isolates were analyzed against the Standard Bruker Reference Library, that lack *Brucella* reference MSP, no reliable identification was obtained for any of the spectra.

However, when the spectra were analyzed against the newly constructed *Brucella* library and the database provided by the CDC, correct identifications at species level were obtained. All were identified as *B. melitensis* with a score of >2.3 indicating highly probable species identification (Appendix E).

Conversely, identification to biotype level gave inconsistent results and different biotype assignments were frequently found. Identification to biotype level was carried against the acquired CDC library but not against the limited newly constructed library that lack biotype 3 reference strain spectral profile.

4.3.4 MALDI-TOF MS Typing

After careful visual inspection of the obtained spectra (six for each isolate) using FlexAnalysis (overlaid view), several potential marker peaks per spectrum were observed. After excluding the common peaks, ten promising marker peaks (m/z values) in a range of m/z of 2500 to 7500 were identified. Four of the ten identified m/z values were found to represent doubly-charged ions of molecules, whose singularly-charged counterparts were also detected and do not add additional information for strain differentiation. These doubly-charged ions at m/z 2512, 3411, 3663, and 3678 were excluded from the analysis. The remaining six potential marker peaks were of m/z 4682, 5028, 5970, 6423, 7326, and 7356 within a range of ±5 m/z differences (Figure 4.1) and (Figure 4.2). These peaks were with intensity >1000 au, reproducible in the sense that the signal was consistently present or absent in all the six spectra obtained for the same isolate, and discriminating in the sense that they were not detected in all isolates and thus were incorporated into the typing scheme.

Analysis of all spectra for the presence or absence of the selected potential marker peaks revealed four distinct marker peak profiles dividing the isolates into four groups as seen in Table . Group 1(n=24) had only one marker peak at m/z 7356; Group 2 (n=8) had combination of three marker peaks at m/z 7356, 5028 and 4682; Group 3 (24) had two marker peaks at m/z 6823 and 5970; and Group 4 (n=7) had two marker peaks at m/z 7326 and 5970.

The marker peak profiles of the four groups were compared with the marker peak profiles of the reference strains (Table 4.5). It was observed that, marker peak at m/z 7356, present in *B. melitensis* biotype 2 reference strain, was present in groups 1 and 2. However, the other two extra marker peaks (m/z 4682 and 5028) seen in group 2 were not present in *B. melitensis* biotype 2 reference strain. Marker peaks at m/z 5970 and 7326, present in *B. melitensis* biotype 1 reference strain, were present in group 4. However, marker peak at m/z 5970 was also present in group 3 along with marker peak at m/z 6823.

Based on the comparison of the presence or absence of peaks in groups (1-4) with the peaks present in reference strains, three MALDI-types were identified. Groups 1 (showing complete peak match with biotype 2 reference strain) and group 2 (showing extra but not less peaks than biotype 2 reference strain) were considered of one type as both shared the marker peak at m/z 7356 that represent *B. melitensis* biotype 2 (NCTC10508), and were referred as MALDI-type2. Group 4 was comparable to *B. melitensis* biotype 1 (NCTC10094) and was referred as MALDI -type1. The lack of reference strain *B. melitensis* biotype 3 mass spectral profile hampered the analysis of the third group but as it

showed different peaks combination, it was considered a third type and was referred as MALDI-type 3 (Appendices F-J).

The different marker peak profiles of the four groups were evident on gel view created using Biotyper 3.0 software (Figure 4.3).

Table 4.4: The Four Distinct groups obtained by MALDI-typing

Marker Peaks (m/z)	Group 1 (n=24)	Group 2 (n=8)	Group 3 (n=24)	Group 4 (n=7)
4682	-	+	-	-
5028	-	+	-	-
5970	-	-	+	+
6823	-	-	+	-
7326	-	-	-	+
7356	+	+	-	-
MALDI-type	MALDI-type 2	MALDI-type 2	MALDI-type 3	MALDI-type 1

Table 4.5: Marker peak profiles for the Reference Strains

Peaks (m/z)	***B. melitensis* biotype 1 (NCTC10094)**	***B. melitensis* biotype 2 (NCTC10508)**	***B. melitensis* biotype 3 (NCTC10509)**
4682	-	-	Not Available
5028	-	-	
5970	+	-	
6823	-	-	
7326	+	-	
7356	-	+	

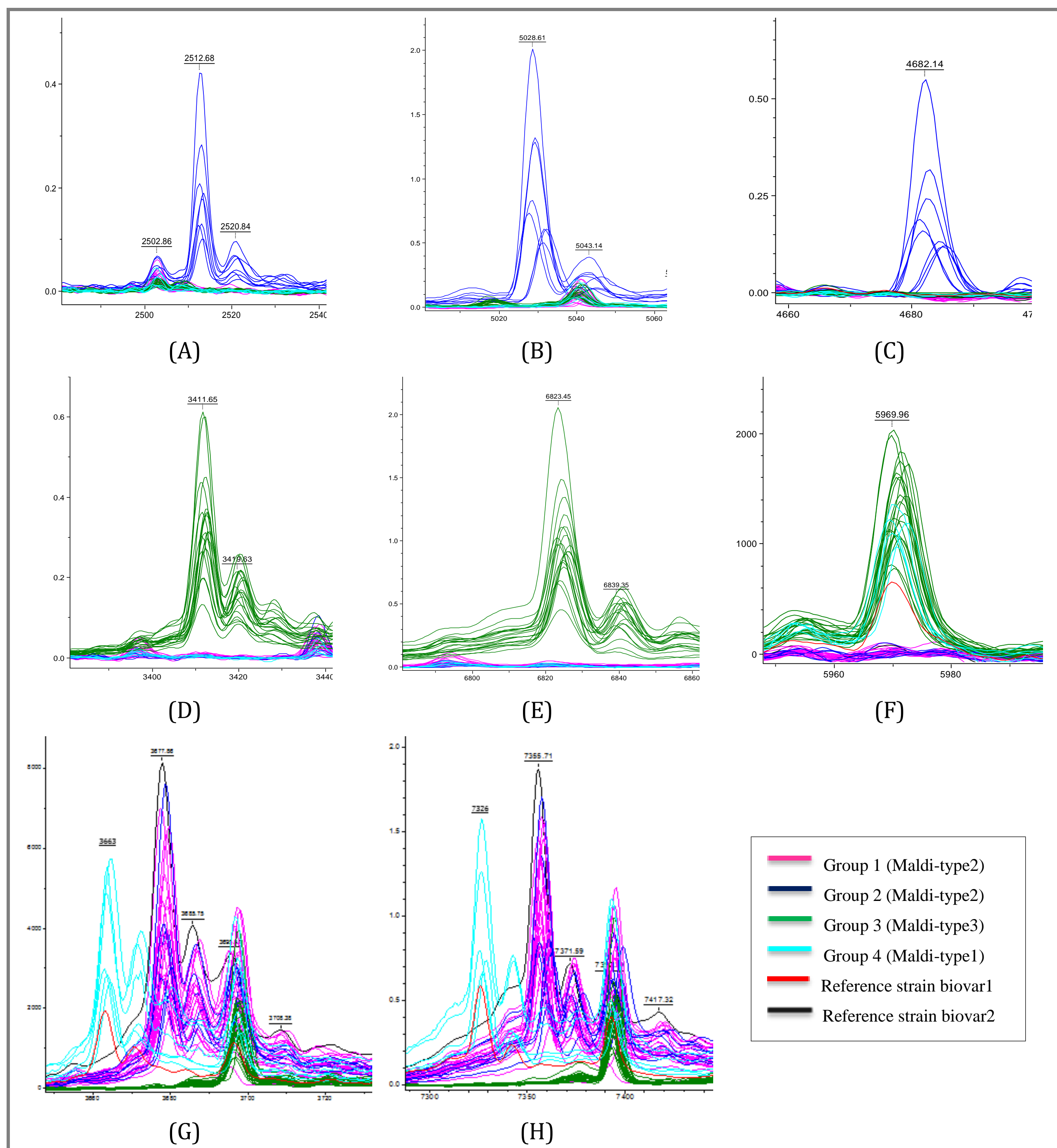

Figure 4.1: The Ten Potential Biotype-specific peaks.

Peaks at m/z (A) 2512(B) 5028, and (C) 4682 were detected only in group 2 isolates. Peaks at m/z (D) 3411, and (E) 6823 were detected only in group 3 isolates. Peaks at m/z (F) 5970 were detected in group 3 isolates, group 4 isolates and reference strain biovar1. Peaks at m/z (G) 3663 and (H) 7326 were detected in group 4 isolates, and reference strain biovar1.Peaks at m/z (G) 3678 and (H) 7356 were detected in group 1 isolates, group 2 isolates, and reference strain biovar2.

Figure 4.2: Representative Sections of the mass spectral profiles. Representative Sections of the mass spectral profiles for the reference strains and clinical isolates. Four distinct marker peak profiles showing marker peaks at m/z 4682, 5028, 5970, 6823, 7326 and 7356.

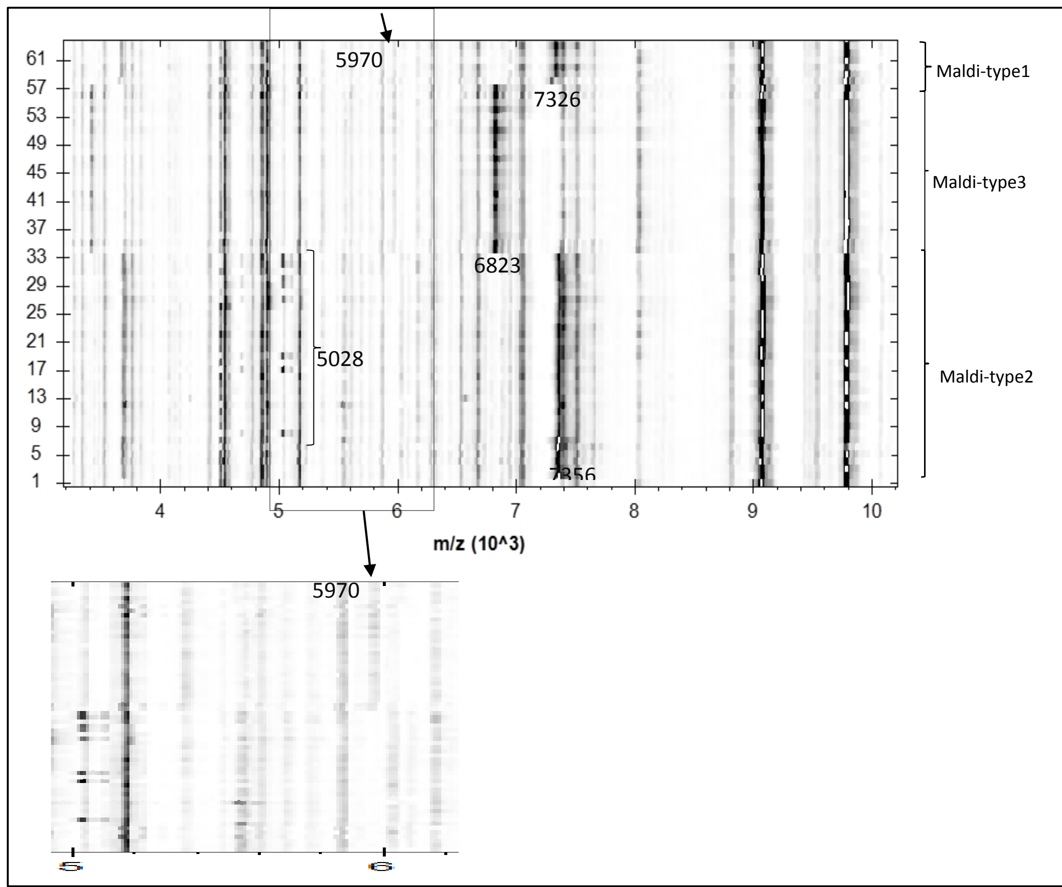

Figure 4.3: Gel view representation.

Gel view representation of the 63 B. melitensis isolates and the two reference stains (NCTC10094 and 10508).The mass spectral profiles of the four groups were evident on gel view created with the peaks of the strains using Biotyper 3.0 software. For example, the peak at m/z 7356 was consistently present in the spectra of MALDI-type 2, while it was absent in the others and was therefore incorporated into the typing scheme

4.4 Molecular Typing

4.4.1 PCR amplification and Gel Visualization

Three different band profiles (Figure 4.4) were obtained and were interpreted as per manufacturer's guidelines. Biotype 1 amplified one fragments of 564 bp; biotype 2 amplified three fragments of 176, 302 and 564 bp; and biotype 3 amplified two fragments of 176 and 564 bp.

Of the total; 63 clinical isolates, 32 isolates (50.8%) were identified as *B. melitensis* biotype 2 and 31 (49.2%) as, *B. melitensis* biotype 3 (Table 4.6). Standard strains for biotype 1 and 2 were included in each run.

Agarose gel electrophoresis images for all *B. melitensis* isolates included in the study are shown in Appendix K.

Table 4.6: Summary of Molecular Typing Results

	Gel electrophoresis bands			
Biotype	176	302	564	Number of isolates
B. melitensis biotype 1	-	-	+	-
B. melitensis biotype 2	+	+	+	32 (50.8%)
B. melitensis biotype 3	+	-	+	31 (49.2%)

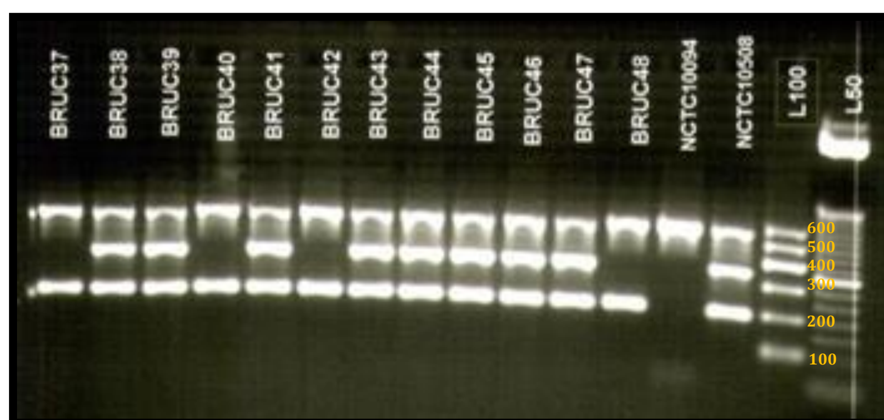

Figure 4.4: Representative image of PCR products of B. melitensis biotypes resolved on agarose gels.

Lanes 1-12, clinical isolates from BRUC (37_48).Lane 13, B. melitensis biotype 1 (NCTC10094), Lane 14, B. melitensis biotype 2 (NCTC10508).

4.4.2 Correlation between Serotyping, MALDI-typing and PCR:

MALDI-types were correlated with serotypes. Within MALDI-type1; 71.42% were found to be serotype 1 and 28.58% serotype3. Within MALDI-type 2, 40.6% were found to be serotype 1; 43.7% serotype 2; and 15.6% serotype 3. All isolates within MALDI-type 3 were found to be serotype1 (Table 4.7).

Table4.7: Correlation between Serotyping and MALDI-typing results

	MALDI-type As per matching with reference strains	**Biotype as per serology**
Clinical isolates (n=63)	MALDI-type1 (n=7)	71.42% Biotype 1 (n=5)
		28.58% Biotype 2 (n=2)
	MALDI-type2 (n=32)	40.6% Biotype 1 (n=13)
		15.6% Biotype 2 (n=14)
		15.6% Biotype 3 (n=5)
	MALDI-type3 (n=24)	100% Biotype 1 (n=24)

Results from MALDI-TOF MS typing were compared to reference typing by PCR. Isolates with MALDI-type 2 (n=32) were confirmed as *B. melitensis* bio-type 2, while isolates with MALDI-type 3 (n=24) and MALDI-type1 (n=7) were *B. melitensis* biotype 3(Table 4.8).

Table 4.8: Correlation between PCR and MALDI-typing results

	MALDI-type As per matching with reference strains	Biotype as per PCR
Clinical isolates (n=63)	MALDI-type1 (n=7)	Biotype 3
	MALDI-type2 (n=32)	Biotype 2
	MALDI-type3 (n=24)	Biotype 3

Table 4.9: Summary of PCR, MALDI-typing and Serotyping Results

	Biotype as per PCR	MALDI-type As per matching with reference strains	Biotype as per serology
Clinical isolates (n=63)	Biotype2 (n=32)	MALDI-type2 (n=32)	Biotype 1 (n=13)
			Biotype 2 (n=14)
			Biotype 3 (n=5)
	Biotype3 (n=31)	MALDI-type3 (n=24)	Biotype 1 (n=24)
		MALDI-type1 (n=7)	Biotype 1 (n=5)
			Biotype 3 (n=2)

4.5 MSP Dendrogram Creation

Using the MSP dendrogram tool of the MALDI Biotyper 3.0 software, all 63 *Brucella* spectral profiles were clustered into 2 groups corresponding to *B. melitensis* biotype 2 and *B. melitensis* biotype 3. The eight MALDI-type 2 isolates that showed extra peaks at m/z 4682 and 5028 were ranked among the biotype 2 and the seven MALDI-type1 isolates were ranked among the biotype 3 (Figure 4.5).

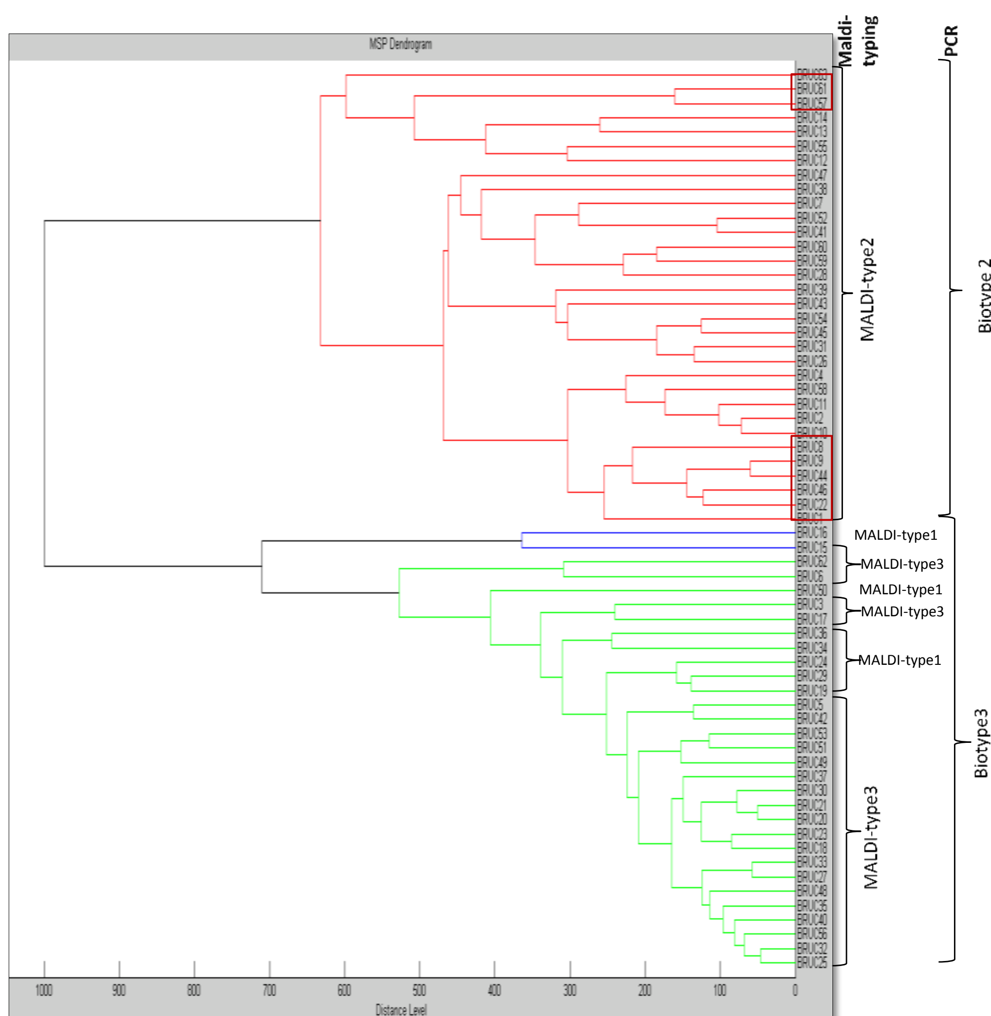

Figure 4.5: Dendrogram representation of hierarchical cluster analysis of the recorded MALDI-types.

4.6 Statistical analysis

4.6.1 Mean, standard deviation and coefficient of variation

Table 4.10: Mean, standard deviation and coefficient of variation of bi-omarker peaks

	Peaks of m/z					
	4682	**5028**	**5970**	**6823**	**7326**	**7356**
N	8	8	31	24	7	32
mean	4682.22	5028.75	5970.62	6823.38	7326.12	7356.08
SD	1.487	1.6809	1.6763	2.1015	1.2788	2.5756
CV	0.0317%	0.0334%	0.0280%	0.0308%	0.0175%	0.0350%

4.6.2 Marker Peaks Frequencies

Peak at m/z 7356 was present in 100% of biotype 2 spectra while peaks at m/z 5028 and 4862 were present only in 25% of biotype 2 spectra. None of these peaks were present in biotype3 spectra. Peaks at m/z 5970, 6823, and 7326 were present in biotype 3 in frequencies of 100%, 77.4% and 22.6% respectively and were absent in biotype 2 (Figure 4.6).

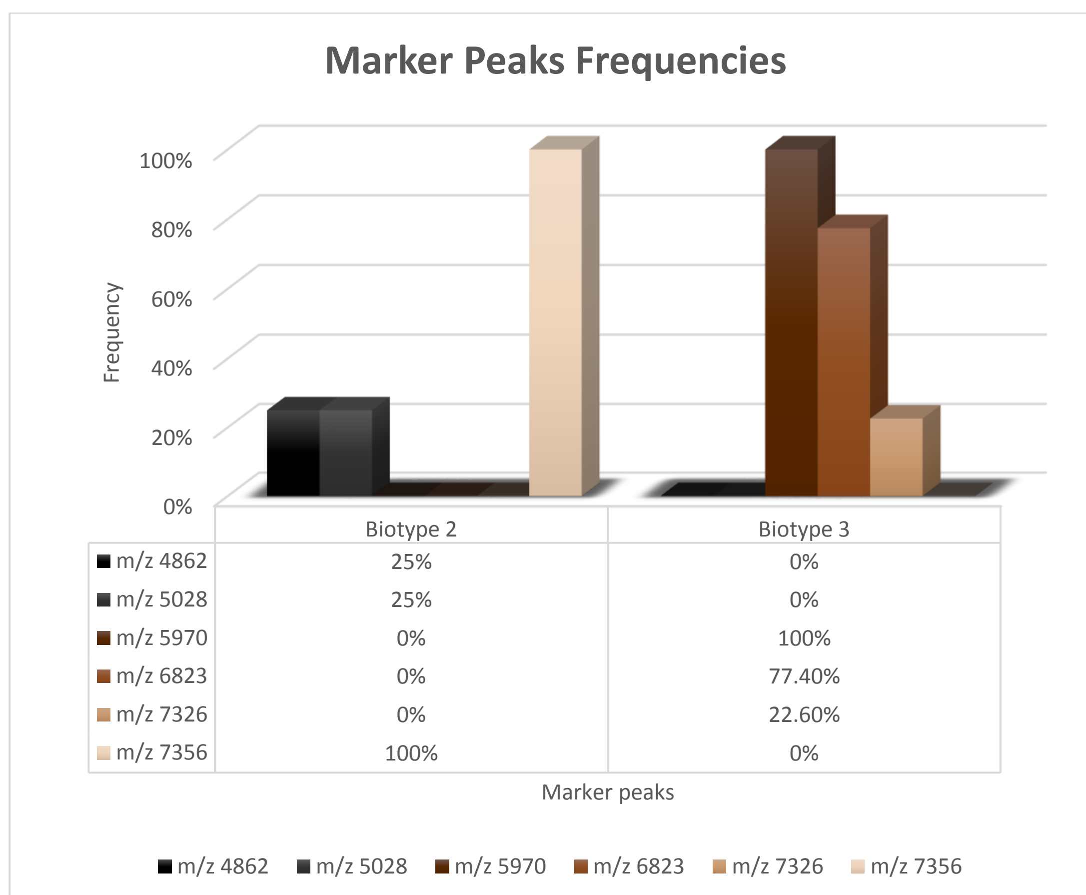

Figure4.6: Marker peaks frequencies in B. melitensis biotype 2 and 3.

4.6.3 Kappa measure of agreement

When agreement between results of serotyping, MALDI-typing and molecular typing were computed using Kappa measure of agreement, a very low level of agreement (Kappa 0.076) was found between Maldi-typing and serotyping results. However, a strong agreement (Kappa 0.8) was found between MALDI-typing and molecular typing, the reference methodology (Appendix M and N).

4.6.4 Pearson chi-square test, Phi and Cramer's V

When the associations were calculated using Pearson chi-square test and Fisher's Exact Test, the marker peaks at m/z 7356 (χ^2 63, n= 32, df1, P<0.001), at m/z 4682 and 5028 ($\chi^2$8.877, n = 8, df1, P 0.003) showed strong evidence of association for biotype 2. Strong evidence of association was also found for the presence of the marker peak at m/z 5970 for biotype 3 (χ^2 63, n= 31, df1, P<0.001). In addition, the marker peak at m/z 6823 (χ^2 40, n = 24, df1, P<0.001) and peaks at m/z 7326 (χ^2 8.129, n = 7, df1, P = 0.003), showed evidence of association for biotype 3.

When Phi coefficient and Cramer's V were computed to compare the strength of association between the marker peaks and biotypes, complete association was found between the marker peaks at m/z 7356 and biotype 2and between the marker peak at m/z 5970 and biotype 3 with Phi coefficient and Cramer's V equal to1 (Appendix O).

CHAPTER 5 DISCUSSION

5.1 Discussion

The aim of this study was to evaluate MALDI–TOF MS as a safe, rapid, and reliable tool for typing of *B. melitensis*. A total of 63 clinical isolates recovered from positive cultures collected between 2012 and 2013 and presumptively identified as *Brucella* species by conventional methods were included in the study. The study isolates were identified as *B. melitensis* by Vitek 2 Compact with a probability of 99% and further classified with monospecific *Brucella* antisera into biotype 1 (66.66%), biotype 2 (22.22%), and biotype 3 (11.11%).

The 63 isolates were then identified by MALDI-TOF MS against the newly constructed *Brucella* library as well as the *Brucella* database provided by the Special Pathogen Branch at CDC (Atlanta, USA). All *Brucella* isolates were identified as *B. melitensis* with a log values of >2.30, indicating highly probable species identification. Inconsistent results were obtained at the strain level. Our findings were consistent with previous studies that infer the reliability of MLADI-TOF MS for genus and species level identification of *Brucella* but not at strain level (Ferreira et al., 2010 and Lista et al., 2011). A more recent study reported that MALDI could reliably identify 92% of the *Brucella* isolates at species level, but incorrect biotype assignments were frequently found (Karger et al., 2013). Characterization at the strain level has proved to be more elusive. Several studies have reported an inability to characterize bacteria below the species level (Lasch et al., 2014 , Rim et al., 2015 & Lista et al., 2011).

However, the approach of identifying unique and reproducible peaks (m/z values) for different strains was proven to be successful in strain typing in *Campylobacter* and *Salmonella* (Zautner et al., 2013 & Dieckmann & Malorny, 2011).

In this study, we attempted strain level identification by screening the generated spectra for biotype-specific marker peaks. We were able to identify six promising unique marker peaks at m/z 4682, 5028, 5970, 6823, 7326, and 7356. The different combinations of the presence or absence of these peaks grouped the study isolates into four groups, each with distinct marker peak profile. After the comparison of these profiles with the reference strains, three MALDI-types were identified. These findings were revealed in gel separation view (MALDI-TOF MS) that showed the different mass spectral patterns.

MALDI-types were correlated with serotypes obtained earlier in the study. Results were found to be inconsistent with a very low Kappa agreement (0.076). Within MALDI-type1; 71.42% were found to be serotype 1 and 28.58% serotype3. Within MALDI-type 2, 40.6% were found to be serotype 1; 43.7% serotype 2; and 15.6% serotype 3. All isolates within MALDI-type 3 were found to be serotype1.

In order to better resolve the conflicting results, we turned to molecular approaches. Molecular typing was carried out using *B. melitensis* biotype typing PCR kit. PCR showed the presence of only two biotypes, *B. melitensis* biotype 2 (n=32) and *B. melitensis* biotype 3 (n=31). When genotype correlation of the observed MALDI-types was carried out, it was found that isolates with MALDI-

type 2 (n=32) were substantially interchangeable with *B. melitensis* biotype 2 while isolates with MALDI -type 3 (n=24) as well as MALDI-type1 (n=7) were genotyped as *B. melitensis* biotype3. Kappa agreement between PCR and MALDI-typing was found to be 0.8 indicating a strong agreement between the two methods.

Our findings determines that MALDI-typing is comparable to PCR, the reference methodology, but not to serotyping. The inconsistence between the serotyping and other typing methods (MALDI typing and PCR) might be related to the well-known cross reactivity of serotyping antisera. Difficulties in conventional biotyping characterization of *B. melitensis* strains have already been reported (Sayan et al., 2009 & European Commission, 2001).

Discrepancies between MALDI-typing and PCR were found only in 7 out of the 63 tested *Brucella* isolate. These seven isolates were identified as *B.melitensis* biotype 3 by PCR but were assigned to MALDI-type 1 as per the observed similarity of their marker peak profiles with the profile of biotype 1 reference strain (NCTC 10094). Similarity was based on the presence of marker peaks at m/z 5970 and 7326 in both profiles and their absence in others except for MALDI-type 3 that showed the presence of peak at m/z 5970. However , Sandrin et al. (2012) infer that the approach of strain identification by comparing its peak profile to a reference strain often requires analysis of the entire spectrum rather than the analysis of the presence or absence of one or a few biomarker peaks (Sandrin et al., 2012). Thus, marker peak based approach can be used to differentiate strains into different groups rather than identifying

them. Furthermore, the presence of peak at m/z 7326 in the spectrum of biotype 1 reference strain (NCTC 10094) doesn't necessarily prove that it is biomarker peak for biotype 1 unless we study more biotype 1 isolates which could not be done in this study since all the isolates were either biotype 2 or 3. On the other hand, the presence of peak at m/z 7326 in the spectrum of biotype1 reference strain doesn't rule out the possibility of being common peak for biotype 1 and 3. This possibility could not be assessed in this study since we lack biotype 3 reference strain. We believe that if we had the peak profile of biotype 3 reference strain and if we had the chance to analyze biotype 1 isolates, presumably specific marker peaks might be identified making the discrimination between biotype 1 and 3 more prominent. However, by refereeing to the results obtained by Ferreira et al (2010), the peak at m/z 7326 was found to be present in both *B.melitensis* biotype 1 and 3 reference strains indicating that it is a common peak rather than biotype1-specific peak. Considering this finding, genotype correlation of MALDI-TOF MS typing was reassessed as per the four marker peak profiles rather than the three MALDI-types. An interesting result was obtained when a detailed comparative analysis of the biomarkers corresponding to different biotypes was performed. It was observed that the mass spectral profile that share the marker peak at m/z 7356, present in groups 1 and 2, were genotyped as biotype 2. the mass spectral profile that share the marker peak at m/z 5970, present in groups 3 and 4, were genotyped as biotype 3. These findings indicate that the peak at m/z 7356 is biotype2-specific and 5970 is biotype3-specific.

Using the MSP dendrogram tool of the MALDI Biotyper 3.0 software, all study isolates were accurately clustered into 2 groups corresponding to *B. melitensis* biotype 2 and *B. melitensis* biotype 3. The eight MALDI-type 2 isolates that showed extra peaks at m/z 4682 and 5028 were accurately ranked among the biotype 2 and the seven MALDI-type1 isolates were accurately ranked among the biotype 3. This suggest that marker peaks at m/z 4682 and 5028 could be used for further classification of biotype 2 variants and marker peaks at 6823 and 7326 for further classification of biotype 3 variants.

Based on the findings in this study, we suggest that MALDI-TOF MS is a very promising tool for biotyping of *B. melitensis*. Using MALDI-TOF MS we were able to differentiate between two *B. melitensis* biotypes, biotype 2 and 3, based on the identification of their biotype-specific peaks, but unfortunately we were unable to define the marker peak (or peaks) for biotype 1 since none of the clinical isolates were of biotype 1 and due to the fact that we cannot rely on the analysis of a single spectra (the reference strain) to define the marker peaks.

Further studies are still needed to fully explore the utility of MALDI-TOF MS for identification at strain level. As per our findings in this study, MALDI-TOF was able to differentiate two different patterns of each biotype indicating that MALDI might have the differentiation power beyond the strain level identification. Future studies to define the potential biotype 1-specific marker peak (or peaks) are to be conducted. Studies should address the evaluation of interlaboratory reproducibility. Whether these biotype-specific peaks, detected

in this study, are specific for Qatar isolates only and related to the geographic character of the *Brucella* strains should be clarified with greater numbers of *B. melitensis* isolates from all over the world.

Our findings infer that B*. melitensis* biotype 2 and biotype 3 are equally distributed in Qatar. Studies about the prevalence of brucellosis in neighboring countries like Saudi Arabia revealed that the *B. melitensis* biotype 3 is the prevalent serotype (Memish, 2001). However Prevalence of *B. melitensis* biotype 1 was reported from Oman (Adam & El-Rashied, 2013).

5.2 Limitations

There are some limitations to this study

- We didn't include *B. melitensis* Ether biotype 3 (NCTC10509) standard strain in our study, as it was not available with Culture Collections at Public Health England, UK and they are the only supplier of standard reference strains (NCTC) of *Brucella*.
- Due to unavailability of biotype 1 isolates (clinical) in Qatar, we were not able to generate biotype 1 specific MALDI-type marker peak profile.
- Low sample size: We believe that in order to be able to provide a robust typing tool, more number of strains with different *Brucella* species should be analyzed and MALDI-type profiles generated, should be incorporated into the typing scheme.

CHAPTER 6 CONCLUSION

Human brucellosis in Qatar is exclusively caused by *B. melitensis* with equal distribution of biotype 2 and biotype 3. The detection of predominant species and biotype is the major step to develop control strategies for brucellosis.

We established a preliminary MALDI-TOF-based *B. melitensis* typing scheme that allows accurate and reproducible discrimination of the 2 prevalent biotypes. Therefore, our results indicate that MALDI-TOF has the potential to become a rapid first-line screening tool for prediction of biotypes and can be used as tool for epidemiological studies and outbreak investigation. MALDI-TOF MS Strain identification using the unique protein profiles can be achieved with minimal time, labor and cost, making it an attractive alternative to the relatively high investment required for other molecular settings.

To our knowledge, this is the first study employing the MALDI-TOF MS technique for the in-depth analysis of *B. melitensis.*

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APPENDIX A: ETHICAL APPROVAL

مركز البحوث الطبية
Medical Research Center

Ref No: MRC/1680/2014
Date: 22nd October 2014

Ms. Ola Asaad Alsharabasi
Senior Technologist
Lab, Medicine & Pathology
HGH

Dear Ms. Ola,

Research Protocol # 14383/14: "Application of MALDI-TOF Mass Spectrometry as a tool for biotyping of Brucella"

The above titled Research Proposal submitted to the Medical Research Center has been reviewed and classified as 'Non- Human Subject Research' and approval is granted from 22nd October 2014.

This research study should be conducted in full accordance with all the applicable sections of the rules and regulations for research at HMC and you should notify the Medical Research Center immediately of any proposed protocol changes. It is the Principal Investigator's responsibility to obtain review and continued approval of the proposal if there is any modification to the approved protocol.

Documents reviewed by the Research Center:

- Research Proposal
- Consent form: Waiver of Informed Consent
- Data Collection sheet

A study progress report should be submitted bi-annually and a final report upon study's completion.

We wish you all success and await the results in due course.

Yours sincerely,

Ms. Angela Ball,
Asst. Executive Director of Research and Business Development

Cc:

1. Dr. Anand Deshmukh, Specialist, Lab, Medicine & Pathology, HGH
2. Dr. Marwan Abumadi, Assistant Professor, Qatar University
3. Dr. Sanjay H. Doiphode, Consultant & Head of Microbiology DLMP, HGH
4. D. Yousef Al Maslamani. Medical Director, HGH

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APPENDIX B: FUNDING APPROVAL

مركز البحوث الطبية
Medical Research Center

Ref. No: MRC/1681/2014
Date: 22nd October 2014

Ms. Ola Asaad Alsharabasi
Senior Technologist
Lab, Medicine & Pathology
HGH

Dear Ms. Ola,

Research Protocol # 14383/14: "Application of MALDI-TOF Mass Spectrometry as a tool for biotyping of Brucella"

The Research Center reviewed the budget request of the above research proposal and approved the budget as follows:

Journal Publication	Approved standard Publication Charges up to **QR. 5,000/-** for the journal as reimbursement upon submission of supporting documents in original. Reimbursement of publication charges will be only for manuscripts accepted for publication in peer reviewed journals with a Journal Impact Factor 0.5 and above.
Conference	Medical Research Center has approved the conference budget for only one study personnel if the abstract is accepted for presentation and subject to the conditions mentioned in the conference sponsorship application form. Please contact Medical Research Center for conference application and for further information once the abstract is accepted for presentation.

Yours sincerely,

Ms. Angela Heather Ball
Asst. Executive Director
Business Development & Research
Medical Research Center

Cc:

1. Dr. Anand Deshmukh, Specialist, Lab, Medicine & Pathology, HGH
2. Dr. Marwan Abumadi, Assistant Professor, Qatar University
3. Dr. Sanjay H. Doiphode, Consultant & Head of Microbiology DLMP, HGH
4. D. Yousef Al Maslamani, Medical Director, HGH

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APPENDIX C: INTERPRETATION OF SEROTYPING

Table 1: Characters Differentiating the Species and Biotypes of *Brucella.*

Species	Biotype	CO_2	H_2S	Urease	Growth on Media Containing		Agglutination with Monospecific Anti-sera		
					thionin*	fuchsin*	A	M	R
B. melitensis	1	-	-	+	+	+	-	+	-
	2	-	-	+	+	+	+	-	-
	3	-	-	+	+	+	+	+	-
B. abortus	1	(+)	+	+	-	+	+	-	-
	2	(+)	+	+	-	-	+	-	-
	3	(+)	+	+	+	+	+	-	-
	4	(+)	+	+	-	(+)	-	+	-
	5	-	-	+	+	+	-	+	-
	6	-	-	+	+	+	+	-	-
	9	-	+	+	+	+	-	+	-
B. suis	1	-	+	+	+	(-)	+	-	-
	2	-	-	+	+	-	+	-	-
	3	-	-	+	+	+	+	-	-
	4	-	-	+	+	(-)	+	+	-
	5	-	-	+	+	-	-	+	-
B. neotomae	-	-	+	+	-	-	+	-	-
B. canis	-	-	-	+	+	(-)	-	-	+
B. ovis	-	+	-	-	+	(-)	-	-	+

*Concentration = 1/50 000 w/v
(+) = Most strains positive (-) = Most strains negative

Adapted from Brucellosis in humans and animals.2006.Publications of the World Health Organization.

APPENDIX D: RESULTS OF VITEK AND SEROTYPING

Table 2: Identification and typing of *Brucella* isolates by conventional methods.

Analyte Name	Vitek			Serology		
	Identification	**Probability**	**Confidence**	**A**	**M**	**Serotype**
BRUC01	*B. melitensis*	99%	Excellent	+	-	Serotype2
BRUC02	*B. melitensis*	99%	Excellent	+	-	Serotype2
BRUC03	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC04	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC05	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC06	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC07	*B. melitensis*	99%	Excellent	+	-	Serotype2
BRUC08	*B. melitensis*	99%	Excellent	+	-	Serotype2
BRUC09	*B. melitensis*	99%	Excellent	+	-	Serotype2
BRUC10	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC11	*B. melitensis*	99%	Excellent	+	-	Serotype2
BRUC12	*B. melitensis*	99%	Excellent	+	-	Serotype2
BRUC13	*B. melitensis*	99%	Excellent	+	+	Serotype3
BRUC14	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC15	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC16	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC17	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC18	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC19	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC20	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC21	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC22	*B. melitensis*	99%	Excellent	+	-	Serotype2
BRUC23	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC24	*B. melitensis*	99%	Excellent	+	+	Serotype3
BRUC25	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC26	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC27	*B. melitensis*	99%	Excellent	-	+	Serotype1

Analyte Name	Vitek			Serology		
	Identification	Probability	Confidence	A	M	Serotype
BRUC28	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC29	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC30	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC31	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC32	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC33	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC34	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC35	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC36	*B. melitensis*	99%	Excellent	+	+	Serotype3
BRUC37	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC38	*B. melitensis*	99%	Excellent	+	-	Serotype2
BRUC39	*B. melitensis*	99%	Excellent	+	+	Serotype3
BRUC40	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC41	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC42	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC43	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC44	*B. melitensis*	99%	Excellent	+	-	Serotype2
BRUC45	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC46	*B. melitensis*	99%	Excellent	+	-	Serotype2
BRUC47	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC48	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC49	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC50	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC51	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC52	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC53	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC54	*B. melitensis*	99%	Excellent	+	+	Serotype3
BRUC55	*B. melitensis*	99%	Excellent	+	+	Serotype3
BRUC56	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC57	*B. melitensis*	99%	Excellent	+	-	Serotype2
BRUC58	*B. melitensis*	99%	Excellent	+	-	Serotype2

Analyte Name	Vitek			Serology		
	Identification	Probability	Confidence	A	M	Serotype
BRUC59	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC60	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC61	*B. melitensis*	99%	Excellent	+	-	Serotype2
BRUC62	*B. melitensis*	99%	Excellent	-	+	Serotype1
BRUC63	*B. melitensis*	99%	Excellent	+	+	Serotype3

APPENDIX E: MALDI-TOF MS IDENTIFICATION

Table3: MALDI-TOF MS Identification Results.

Bruker Daltonics MALDI Biotyper Classification Results against the newly constructed library.

Analyte Name	Organism (best match)	Score Value	Organism (second best match)	Score Value
BRUC1	*B. melitensis*	2.582	*B. melitensis*	2.494
BRUC2	*B. melitensis*	2.537	*B. melitensis*	2.428
BRUC3	*B. melitensis*	2.385	*B. melitensis*	2.381
BRUC4	*B. melitensis*	2.6	*B. melitensis*	2.57
BRUC5	*B. melitensis*	2.626	*B. melitensis*	2.62
BRUC6	*B. melitensis*	2.578	*B. melitensis*	2.524
BRUC7	*B. melitensis*	2.559	*B. melitensis*	2.479
BRUC8	*B. melitensis*	2.537	*B. melitensis*	2.459
BRUC9	*B. melitensis*	2.505	*B. melitensis*	2.448
BRUC10	*B. melitensis*	2.596	*B. melitensis*	2.568
BRUC11	*B. melitensis*	2.568	*B. melitensis*	2.454
BRUC12	*B. melitensis*	2.373	*B. melitensis*	2.352
BRUC13	*B. melitensis*	2.511	*B. melitensis*	2.48
BRUC14	*B. melitensis*	2.542	*B. melitensis*	2.519
BRUC15	*B. melitensis*	2.671	*B. melitensis*	2.578
BRUC16	*B. melitensis*	2.654	*B. melitensis*	2.633
BRUC17	*B. melitensis*	2.589	*B. melitensis*	2.558
BRUC18	*B. melitensis*	2.614	*B. melitensis*	2.589
BRUC19	*B. melitensis*	2.646	*B. melitensis*	2.507
BRUC20	*B. melitensis*	2.676	*B. melitensis*	2.673
BRUC21	*B. melitensis*	2.624	*B. melitensis*	2.596
BRUC22	*B. melitensis*	2.585	*B. melitensis*	2.498
BRUC23	*B. melitensis*	2.641	*B. melitensis*	2.602
BRUC24	*B. melitensis*	2.636	*B. melitensis*	2.597
BRUC25	*B. melitensis*	2.609	*B. melitensis*	2.568
BRUC26	*B. melitensis*	2.662	*B. melitensis*	2.518

Analyte Name	Organism (best match)	Score Value	Organism (second best match)	Score Value
BRUC27	*B. melitensis*	2.629	*B. melitensis*	2.607
BRUC28	*B. melitensis*	2.647	*B. melitensis*	2.554
BRUC29	*B. melitensis*	2.616	*B. melitensis*	2.539
BRUC30	*B. melitensis*	2.602	*B. melitensis*	2.582
BRUC31	*B. melitensis*	2.622	*B. melitensis*	2.561
BRUC32	*B. melitensis*	2.662	*B. melitensis*	2.557
BRUC33	*B. melitensis*	2.639	*B. melitensis*	2.587
BRUC34	*B. melitensis*	2.605	*B. melitensis*	2.54
BRUC35	*B. melitensis*	2.619	*B. melitensis*	2.585
BRUC36	*B. melitensis*	2.604	*B. melitensis*	2.544
BRUC37	*B. melitensis*	2.636	*B. melitensis*	2.544
BRUC38	*B. melitensis*	2.54	*B. melitensis*	2.46
BRUC39	*B. melitensis*	2.638	*B. melitensis*	2.625
BRUC40	*B. melitensis*	2.627	*B. melitensis*	2.598
BRUC41	*B. melitensis*	2.622	*B. melitensis*	2.562
BRUC42	*B. melitensis*	2.622	*B. melitensis*	2.615
BRUC43	*B. melitensis*	2.601	*B. melitensis*	2.55
BRUC44	*B. melitensis*	2.591	*B. melitensis*	2.528
BRUC45	*B. melitensis*	2.627	*B. melitensis*	2.598
BRUC46	*B. melitensis*	2.546	*B. melitensis*	2.494
BRUC47	*B. melitensis*	2.617	*B. melitensis*	2.565
BRUC48	*B. melitensis*	2.633	*B. melitensis*	2.633
BRUC49	*B. melitensis*	2.563	*B. melitensis*	2.556
BRUC50	*B. melitensis*	2.535	*B. melitensis*	2.503
BRUC51	*B. melitensis*	2.601	*B. melitensis*	2.576
BRUC52	*B. melitensis*	2.579	*B. melitensis*	2.454
BRUC53	*B. melitensis*	2.604	*B. melitensis*	2.564
BRUC54	*B. melitensis*	2.637	*B. melitensis*	2.548
BRUC55	*B. melitensis*	2.588	*B. melitensis*	2.558
BRUC56	*B. melitensis*	2.582	*B. melitensis*	2.541
BRUC57	*B. melitensis*	2.627	*B. melitensis*	2.556

Analyte Name	Organism (best match)	Score Value	Organism (second best match)	Score Value
BRUC58	*B. melitensis*	2.638	*B. melitensis*	2.555
BRUC59	*B. melitensis*	2.635	*B. melitensis*	2.604
BRUC60	*B. melitensis*	2.632	*B. melitensis*	2.542
BRUC61	*B. melitensis*	2.647	*B. melitensis*	2.566
BRUC62	*B. melitensis*	2.647	*B. melitensis*	2.566
BRUC63	*B. melitensis*	2.614	*B. melitensis*	2.571

APPENDIX F: MALDI-TOF MS TYPING RESULTS

Table 4: MALDI-TOF MS Typing Results.

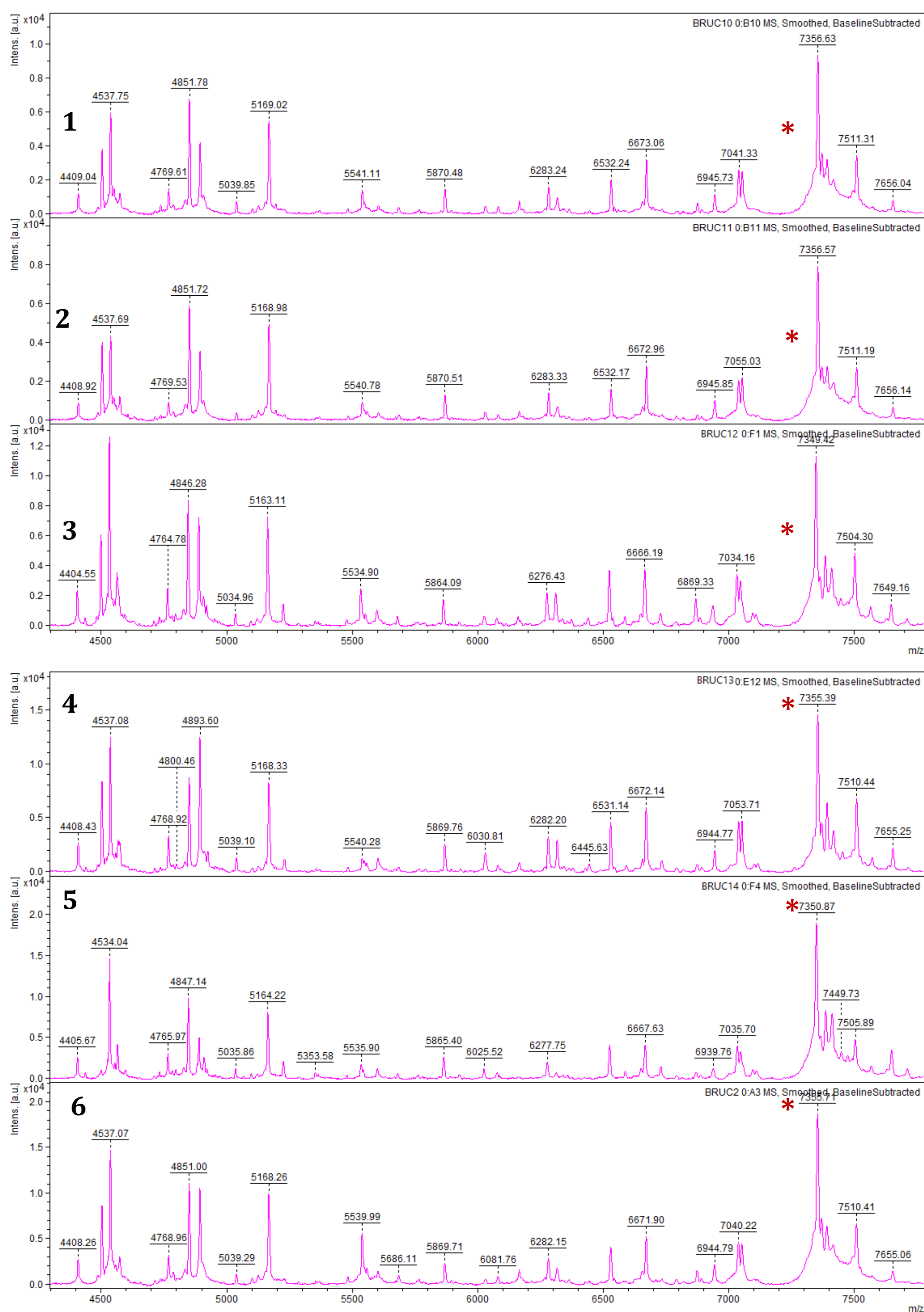
MALDI- typing Results. As per the marker peaks. The study isolates were divided into four distinct marker peak profiles and three main MALDI-types.

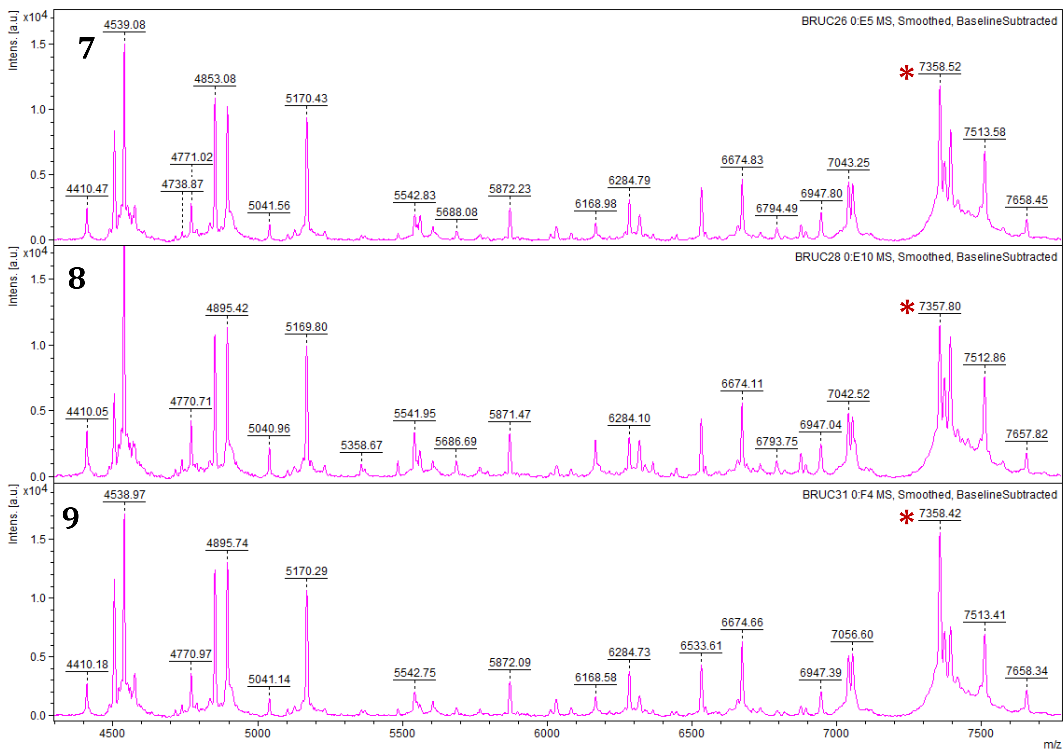
Isolate #	Peaks (m/z)						MALDI-type
	4682	5028	5970	6823	7326	7356	
NCTC10094 Biotype 1	-	-	+	-	+	-	MALDI-type1
NCTC10508 Biotype 2	-	-	-	-	-	+	MALDI-type2
BRUC02	-	-		-	-	+	
BRUC04	-	-	-	-	-	+	
BRUC07	-	-	-	-	-	+	
BRUC10	-	-	-	-	-	+	
BRUC11	-	-	-	-	-	+	
BRUC12	-	-	-	-	-	+	
BRUC13	-	-	-	-	-	+	
BRUC14	-	-	-	-	-	+	
BRUC26	-	-	-	-	-	+	
BRUC28	-	-	-	-	-	+	
BRUC31	-	-	-	-	-	+	
BRUC38	-	-	-	-	-	+	
BRUC39	-	-	-	-	-	+	MALDI-type2
BRUC41	-	-	-	-	-	+	
BRUC43	-	-	-	-	-	+	
BRUC45	-	-	-	-	-	+	
BRUC47	-	-	-	-	-	+	
BRUC52	-	-	-	-	-	+	
BRUC54	-	-	-	-	-	+	
BRUC55	-	-	-	-	-	+	
BRUC58	-	-	-	-	-	+	
BRUC59	-	-	-	-	-	+	
BRUC60	-	-	-	-	-	+	
BRUC63	-	-	-	-	-	+	
BRUC01	+	+	-	-	-	+	

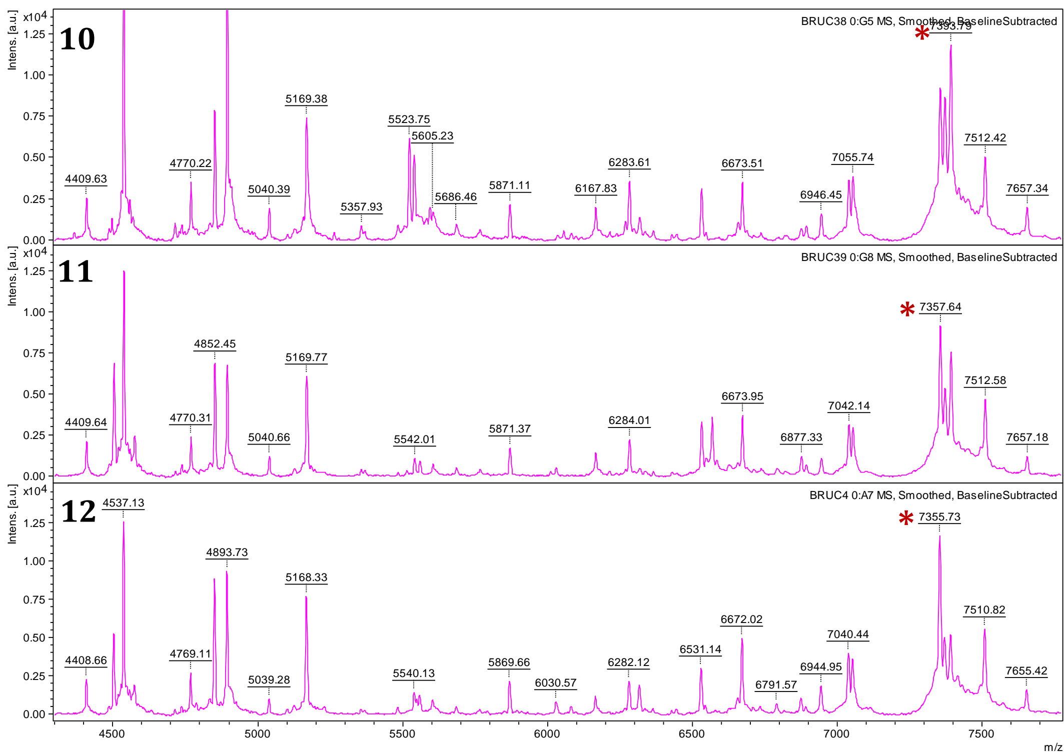
Isolate #	Peaks (m/z)						MALDI-type
	4682	5028	5970	6823	7326	7356	
BRUC08	+	+	-	-	-	+	
BRUC09	+	+	-	-	-	+	MALDI-type2
BRUC22	+	+	-	-	-	+	
BRUC44	+	+	-	-	-	+	
BRUC46	+	+	-	-	-	+	
BRUC57	+	+	-	-	-	+	
BRUC61	+	+	-	-	-	+	
BRUC03	-	-	+	+	-	-	MALDI-type3
BRUC05	-	-	+	+	-	-	
BRUC06	-	-	+	+	-	-	
BRUC15	-	-	+	+	-	-	
BRUC17	-	-	+	+	-	-	
BRUC18	-	-	+	+	-	-	
BRUC20	-	-	+	+	-	-	
BRUC21	-	-	+	+	-	-	
BRUC23	-	-	+	+	-	-	
BRUC25	-	-	+	+	-	-	
BRUC27	-	-	+	+	-	-	
BRUC30	-	-	+	+	-	-	
BRUC32	-	-	+	+	-	-	
BRUC33	-	-	+	+	-	-	
BRUC35	-	-	+	+	-	-	
BRUC37	-	-	+	+	-	-	
BRUC40	-	-	+	+	-	-	
BRUC42	-	-	+	+	-	-	
BRUC48	-	-	+	+	-	-	
BRUC49	-	-	+	+	-	-	
BRUC51	-	-	+	+	-	-	
BRUC53	-	-	+	+	-	-	
BRUC56	-	-	+	+	-	-	
BRUC62	-	-	+	+	-	-	
BRUC16	-	-	+	-	+	-	MALDI-type1
BRUC19	-	-	+	-	+	-	
BRUC24	-	-	+	-	+	-	

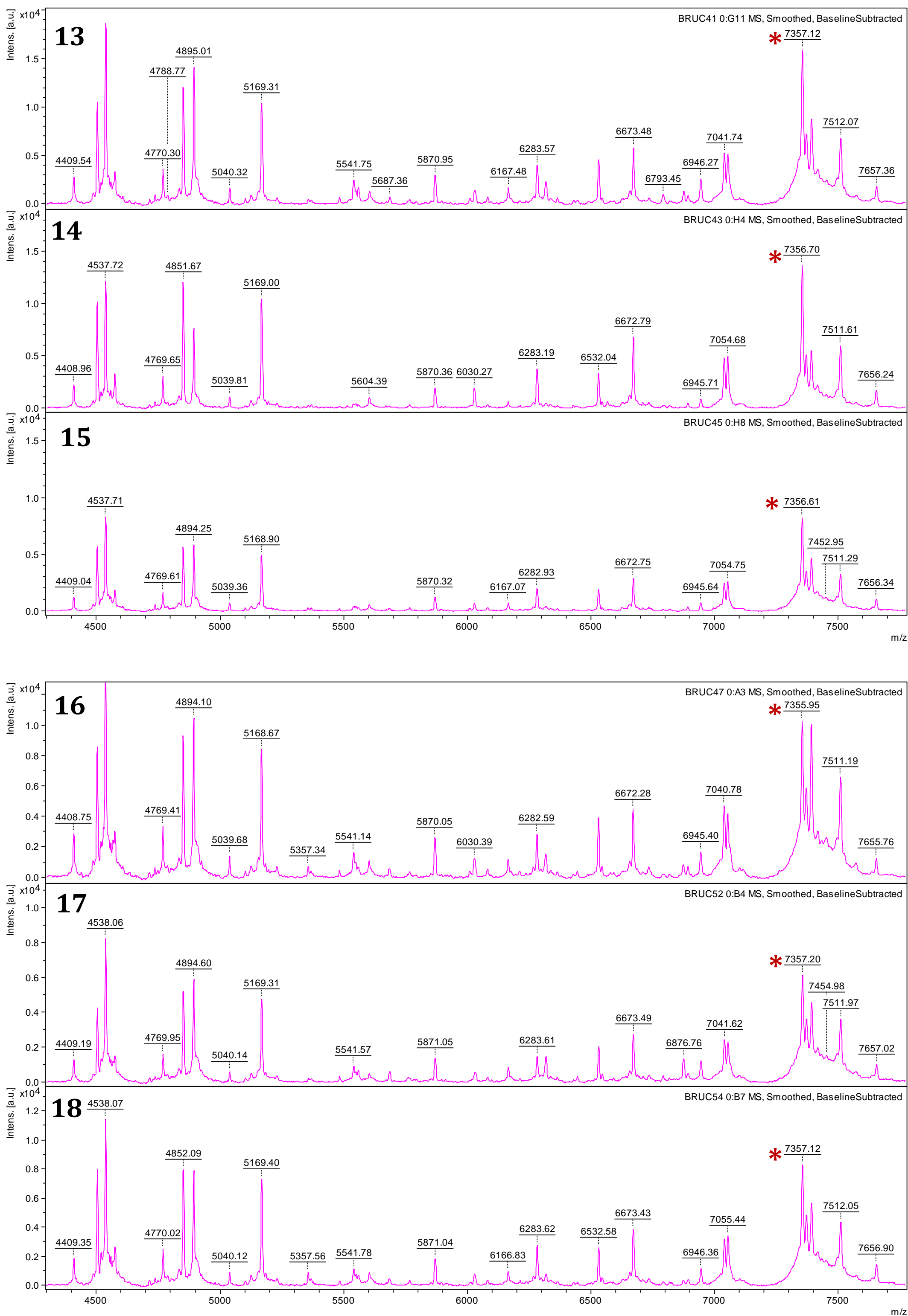
Isolate #	Peaks (m/z)						MALDI-type
	4682	5028	5970	6823	7326	7356	
BRUC29	-	-	+	-	+	-	
BRUC34	-	-	+	-	+	-	MALDI-type1
BRUC36	-	-	+	-	+	-	
BRUC50	-	-	+	-	+	-	

APPENDIX G: REPRESENTATIVE SECTIONS OF GROUP (1) MASS SPECTRAL PROFILE




7
BRUC26 0:E5 MS, Smoothed, BaselineSubtracted
4410.47
4539.08
4738.87
4771.02
4853.08
5041.56
5170.43
5542.83
5688.08
5872.23
6168.98
6284.79
6674.83
6794.49
6947.80
7043.25
7358.52
7513.58
7658.45
8
BRUC28 0:E10 MS, Smoothed, BaselineSubtracted
4410.05
4770.71
4895.42
5040.96
5169.80
5358.67
5541.95
5686.69
5871.47
6284.10
6674.11
6793.75
6947.04
7042.52
7357.80
7512.86
7657.82
9
BRUC31 0:F4 MS, Smoothed, BaselineSubtracted
4410.18
4538.97
4770.97
4895.74
5041.14
5170.29
5542.75
5872.09
6168.58
6284.73
6533.61
6674.66
6947.39
7056.60
7358.42
7513.41
7658.34
Intens. [a.u.]
x10^4
m/z
4500
5000
5500
6000
6500
7000
7500


10
BRUC38 0:G5 MS, Smoothed, BaselineSubtracted
4409.63
4770.22
5040.39
5169.38
5357.93
5523.75
5605.23
5686.46
5871.11
6167.83
6283.61
6673.51
6946.45
7055.74
7512.42
7657.34
11
BRUC39 0:G8 MS, Smoothed, BaselineSubtracted
4409.64
4770.31
4852.45
5040.66
5169.77
5542.01
5871.37
6284.01
6673.95
6877.33
7042.14
7357.64
7512.58
7657.18
12
BRUC4 0:A7 MS, Smoothed, BaselineSubtracted
4408.66
4537.13
4769.11
4893.73
5039.28
5168.33
5540.13
5869.66
6030.57
6282.12
6531.14
6672.02
6791.57
6944.95
7040.44
7355.73
7510.82
7655.42
Intens. [a.u.]
x10^4
m/z
4500
5000
5500
6000
6500
7000
7500



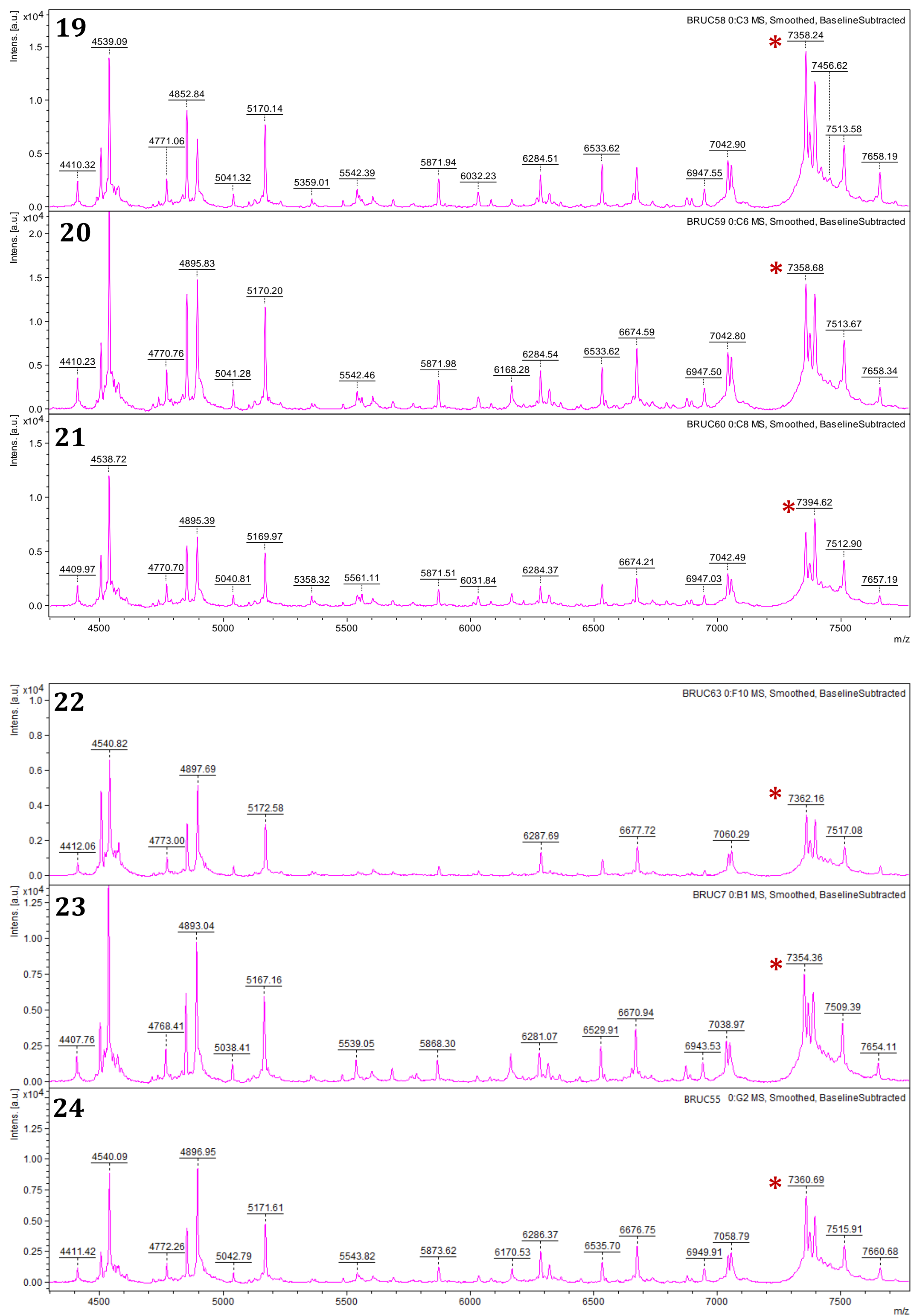


Figure 1: Representative Sections of Group 1 mass spectral profile.

Group 1(n=24) had only one marker peak at m/z 7356.

APPENDIX H: REPRESENTATIVE SECTIONS OF GROUP (2) MASS SPECTRAL PROFILE

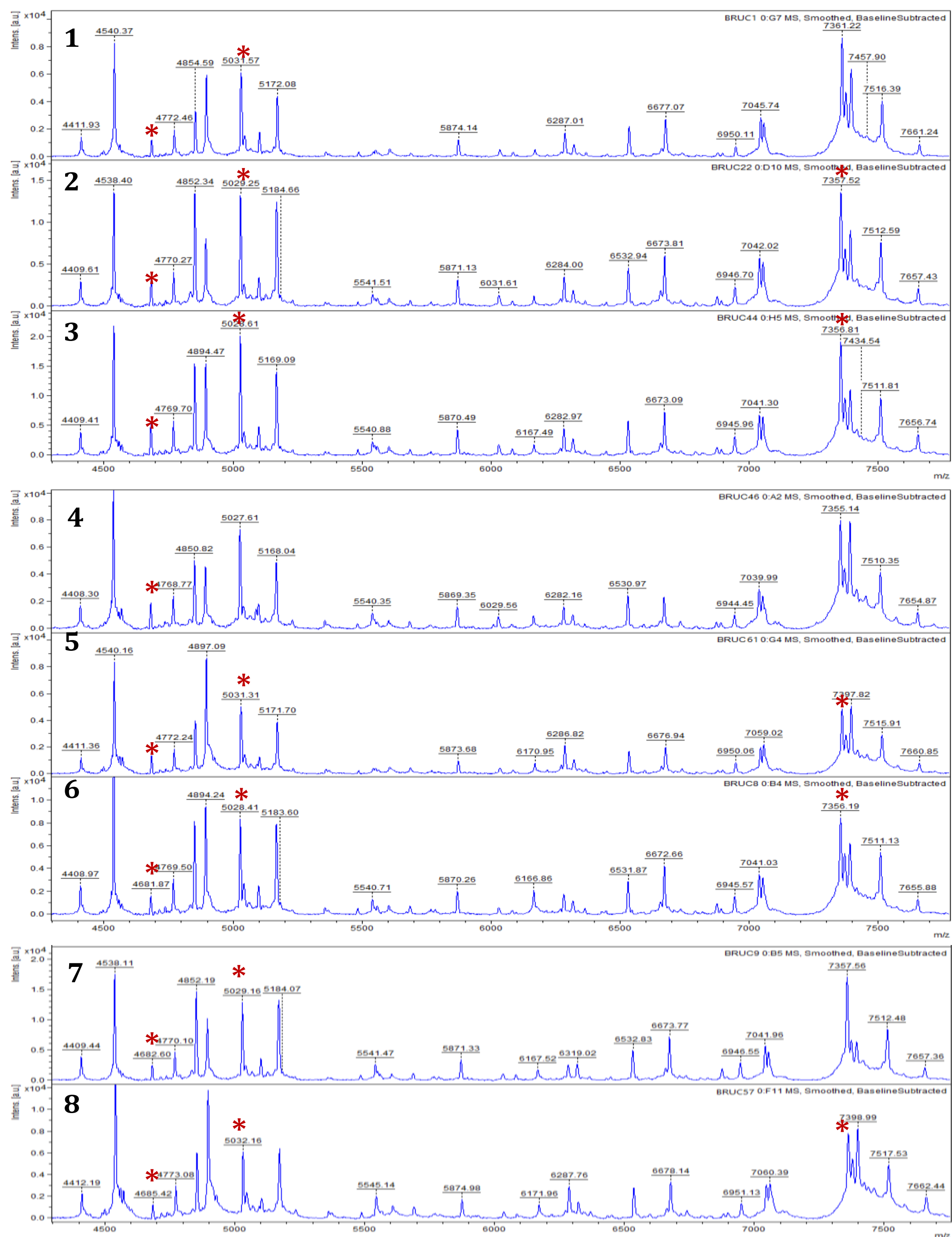
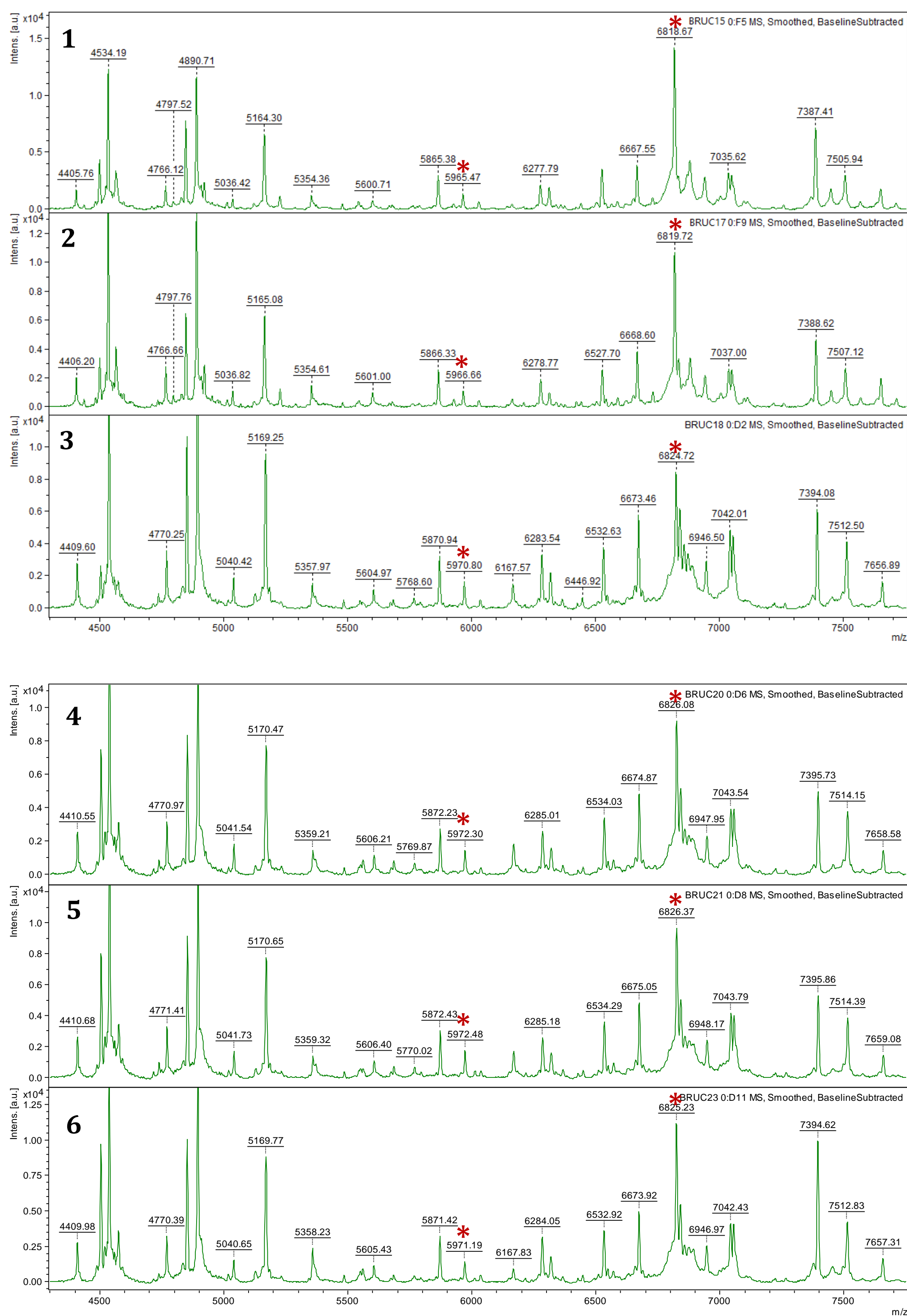
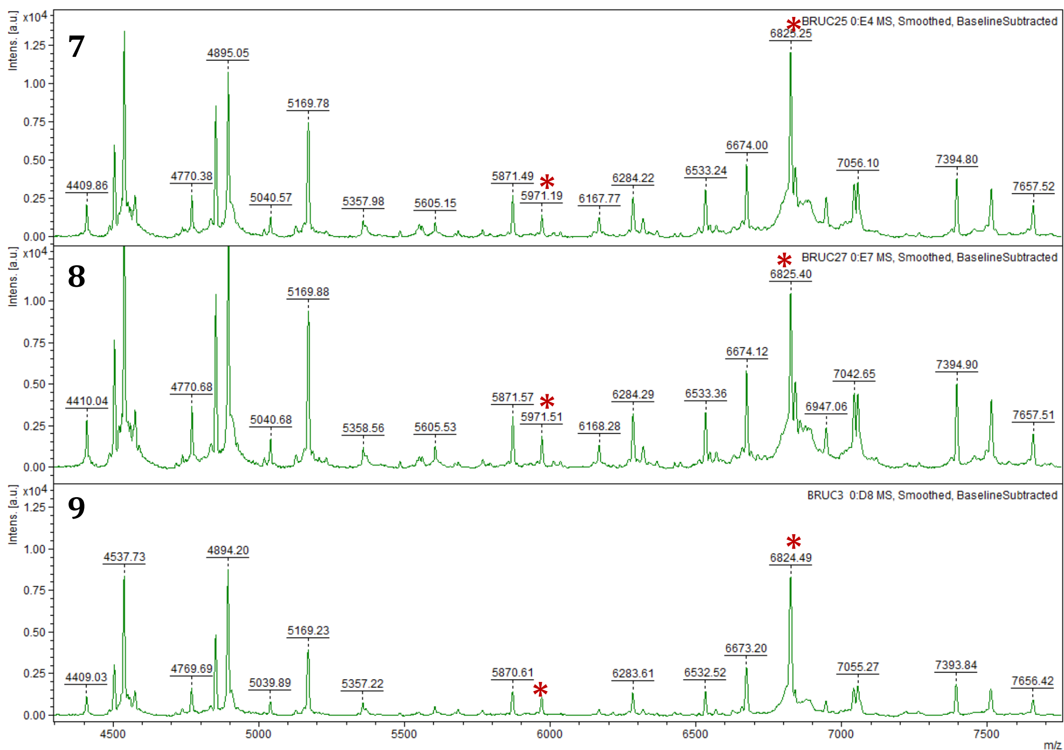


Figure 2: Representative Sections of Group 2 mass spectral profile.

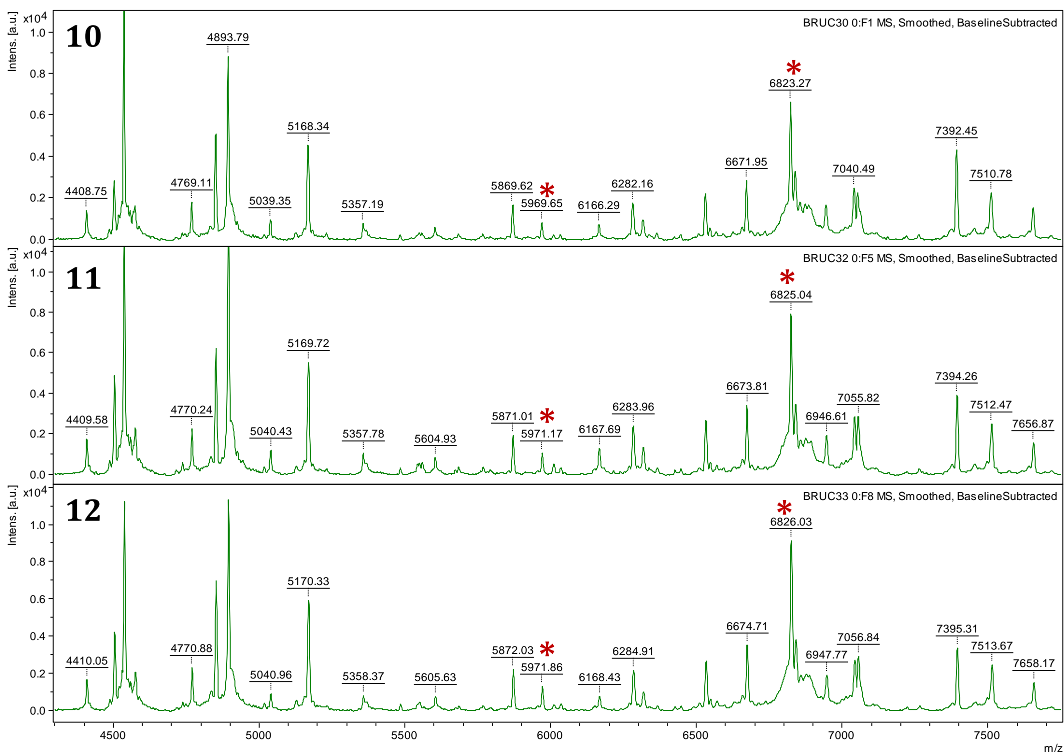
Group 2(n=8) had marker peaks at m/z 4682, 5028 and 7356.

APPENDIX I: REPRESENTATIVE SECTIONS OF GROUP (3) MASS SPECTRAL PROFILE

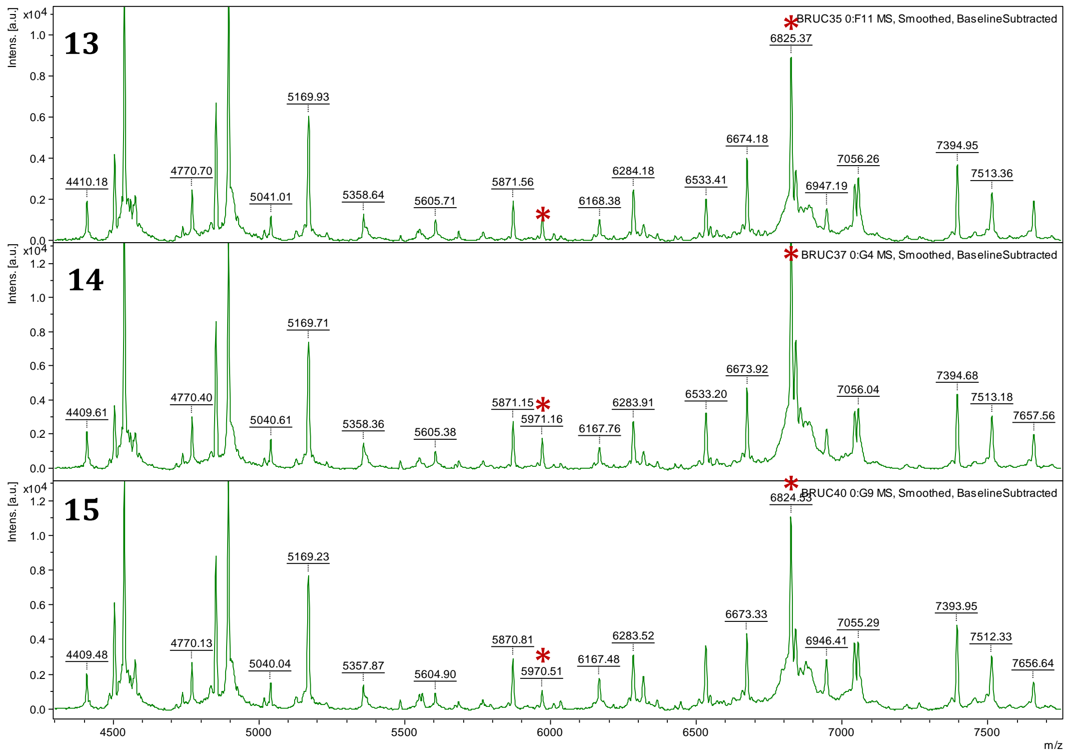


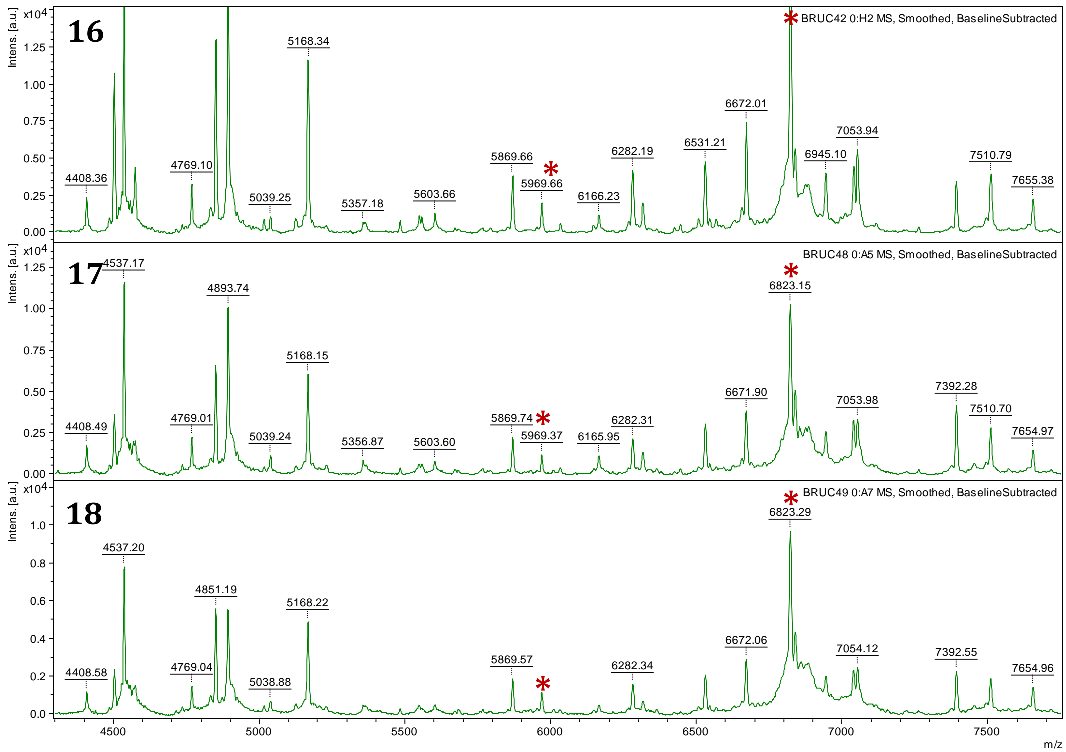


7
BRUC25 0:E4 MS, Smoothed, BaselineSubtracted
6825.25
4895.05
5169.78
6674.00
4409.86
4770.38
5040.57
5357.98
5605.15
5871.49
5971.19
6167.77
6284.22
6533.24
7056.10
7394.80
7657.52
8
BRUC27 0:E7 MS, Smoothed, BaselineSubtracted
6825.40
5169.88
6674.12
7042.65
7394.90
4410.04
4770.68
5040.68
5358.56
5605.53
5871.57
5971.51
6168.28
6284.29
6533.36
6947.06
7657.51
9
BRUC3 0:D8 MS, Smoothed, BaselineSubtracted
4537.73
4894.20
6824.49
5169.23
6673.20
4409.03
4769.69
5039.89
5357.22
5870.61
6283.61
6532.52
7055.27
7393.84
7656.42
Intens. [a.u.]
x10^4
m/z
4500
5000
5500
6000
6500
7000
7500



10
BRUC30 0:F1 MS, Smoothed, BaselineSubtracted
4893.79
6823.27
5168.34
7392.45
6671.95
7040.49
7510.78
4408.75
4769.11
5039.35
5357.19
5869.62
5969.65
6166.29
6282.16
11
BRUC32 0:F5 MS, Smoothed, BaselineSubtracted
6825.04
5169.72
7394.26
6673.81
7055.82
7512.47
4409.58
4770.24
5040.43
5357.78
5604.93
5871.01
5971.17
6167.69
6283.96
6946.61
7656.87
12
BRUC33 0:F8 MS, Smoothed, BaselineSubtracted
6826.03
5170.33
6674.71
7056.84
7395.31
7513.67
4410.05
4770.88
5040.96
5358.37
5605.63
5872.03
5971.86
6168.43
6284.91
6947.77
7658.17
Intens. [a.u.]
x10^4
m/z
4500
5000
5500
6000
6500
7000
7500


13
BRUC35 0:F11 MS, Smoothed, BaselineSubtracted
6825.37
5169.93
6674.18
7394.95
7056.26
4770.70
6284.18
6533.41
7513.36
4410.18
6947.19
5871.56
5041.01
5358.64
5605.71
6168.38
14
BRUC37 0:G4 MS, Smoothed, BaselineSubtracted
5169.71
6673.92
7394.68
7056.04
7513.18
4770.40
5871.15
6283.91
6533.20
4409.61
5040.61
5971.16
7657.56
5358.36
5605.38
6167.76
15
6824.55
BRUC40 0:G9 MS, Smoothed, BaselineSubtracted
5169.23
6673.33
7393.95
7055.29
4770.13
5870.81
6283.52
6946.41
7512.33
4409.48
5040.04
5357.87
5604.90
5970.51
6167.48
7656.64
Intens. [a.u.]
x10^4
m/z
4500
5000
5500
6000
6500
7000
7500


16
BRUC42 0:H2 MS, Smoothed, BaselineSubtracted
5168.34
6672.01
7053.94
6531.21
4408.36
4769.10
5869.66
5969.66
6282.19
6945.10
7510.79
5039.25
5357.18
5603.66
6166.23
7655.38
17
4537.17
BRUC48 0:A5 MS, Smoothed, BaselineSubtracted
4893.74
6823.15
5168.15
6671.90
7392.28
7053.98
7510.70
4408.49
4769.01
5039.24
5356.87
5603.60
5869.74
5969.37
6165.95
6282.31
7654.97
18
BRUC49 0:A7 MS, Smoothed, BaselineSubtracted
6823.29
4537.20
4851.19
5168.22
6672.06
7054.12
7392.55
4408.58
4769.04
5038.88
5869.57
6282.34
7654.96
Intens. [a.u.]
x10^4
m/z
4500
5000
5500
6000
6500
7000
7500

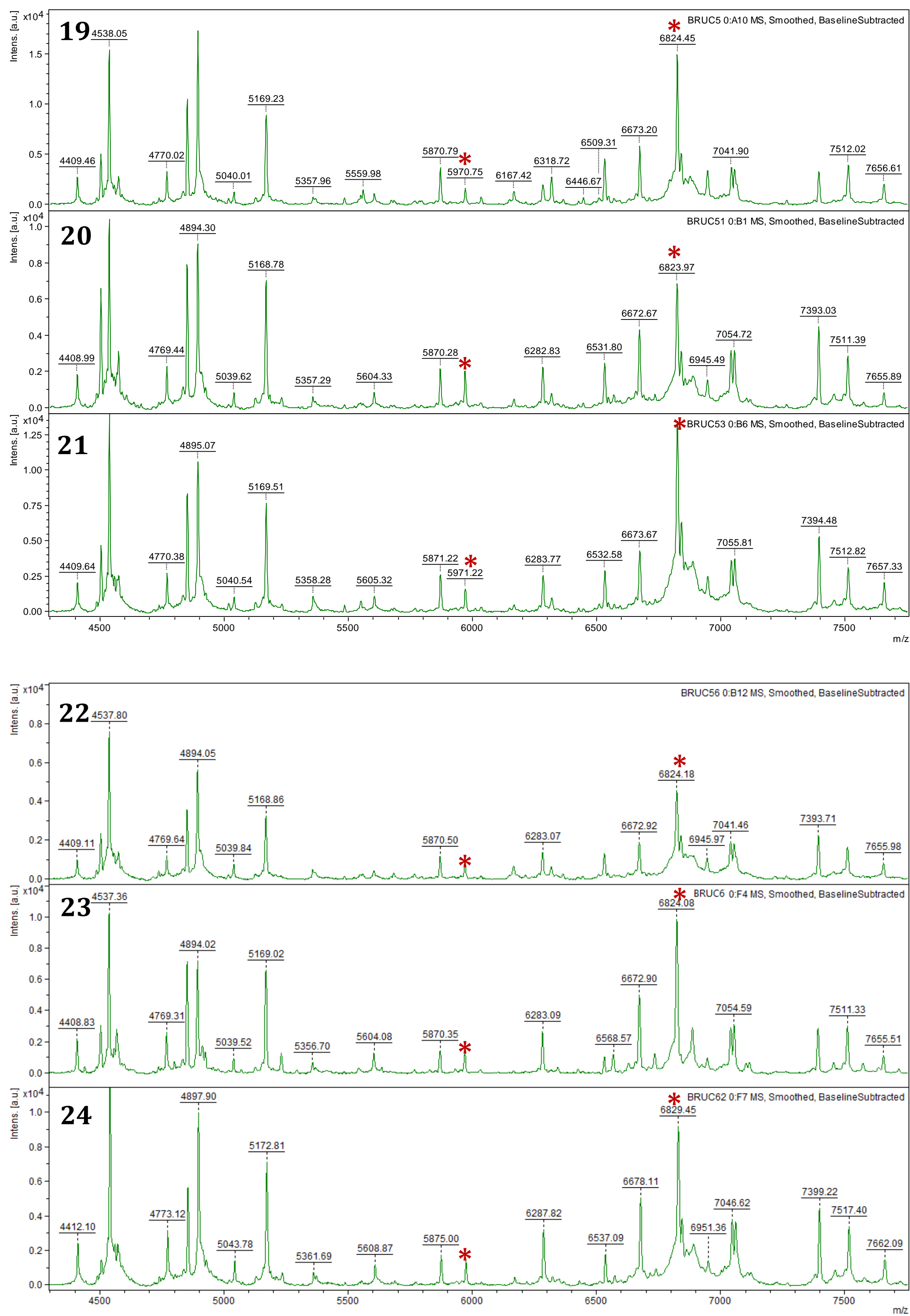


Figure 3: Representative Sections of Group 3 mass spectral profile.

Group 3(n=24) had marker peaks at m/z 5970 and 6823.

APPENDIX J: REPRESENTATIVE SECTIONS OF GROUP (4) MASS SPECTRAL PROFILE

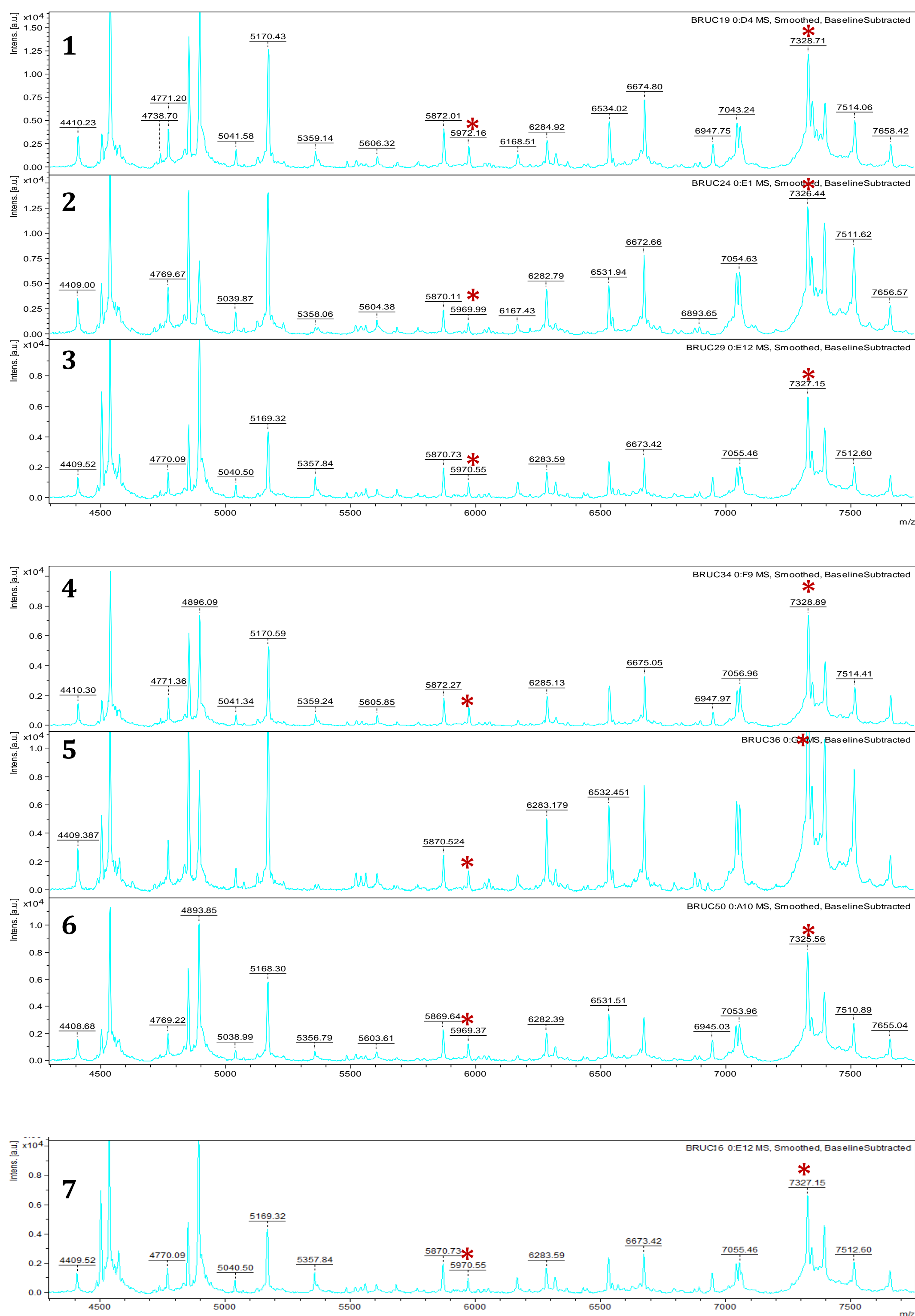


Figure 4: Representative Sections of Group 3 mass spectral profile. Group 4(n=7) had marker peaks at m/z 5970 and 7326.

APPENDIX K: PCR PRODUCTS OF *B. MELITENSIS* BIOTYPES RESOLVED ON AGAROSE GELS

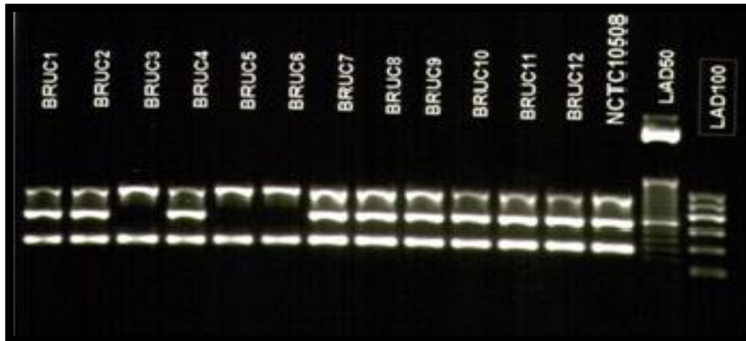


Figure 5-1

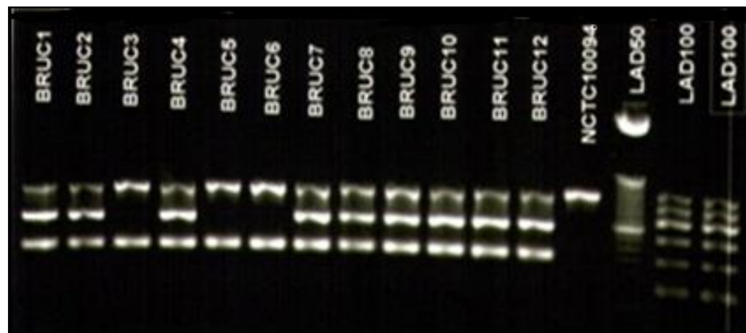


Figure 5-2

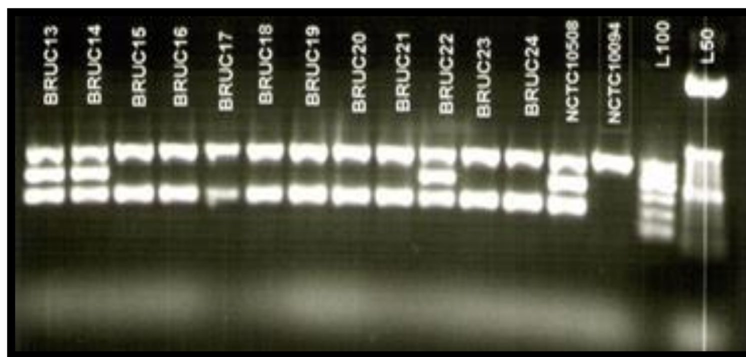


Figure 5-3

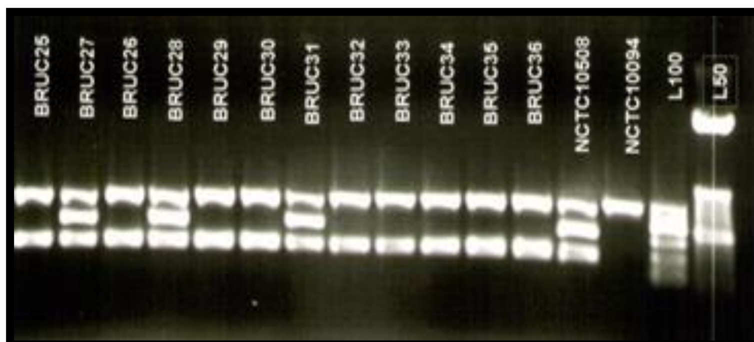


Figure 5-4

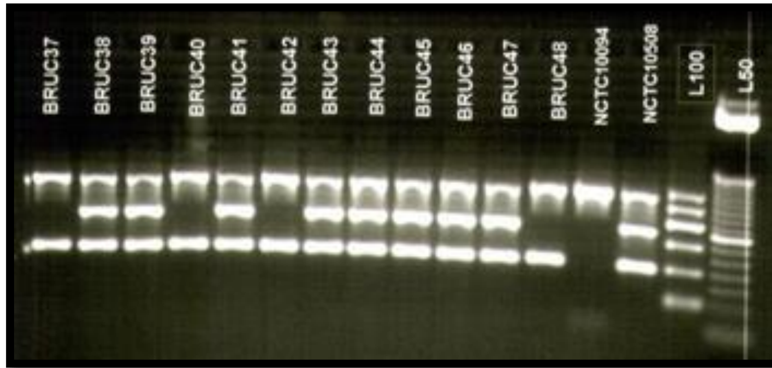


Figure 5-5

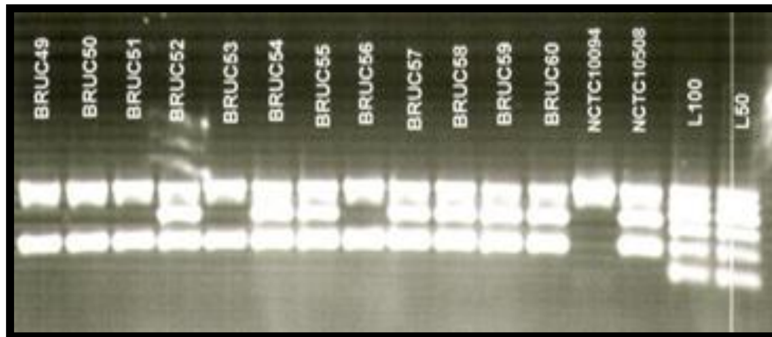


Figure 5-6

Figure 5-7

Figure 5: PCR products of B. melitensis biotypes resolved on agarose gels.

K1 to K7: For all images, Lanes 1-12 represent the clinical isolates BRUC (01 -63), Lane 13, *B. melitensis* biotype 1 (NCTC10094), Lane 14, *B. melitensis* biotype 2 (NCTC10508). 5-1 and 5-2 represent the same samples run in duplicate, each time with different reference strain. 5-7 represent repeated results for MALDI-type 1 as well as BRUC61-63.

APPENDIX L: COROLATION OF MALDI-TYPING, SEROTYPING, AND MOLECULAR TYPING

Table 5: Correlation of MALDI-typing, Serotyping, and Molecular typing.

Isolate #	Peaks (m/z)						MALDI	PCR	Serotype
	4682	5028	5970	6823	7326	7356			
NCTC10094 Biotype1	-	-	+	-	+	-	Maldi-type1	**biotype1**	Serotype1
NCTC10508 Biotype2	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype2
BRUC02	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype2
BRUC04	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype1
BRUC07	-	-		-	-	+	Maldi-type 2	biotype 2	Serotype2
BRUC10	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype1
BRUC11	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype2
BRUC12	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype2
BRUC13	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype3
BRUC14	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype1
BRUC26	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype1
BRUC28	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype1
BRUC31	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype1
BRUC38	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype2
BRUC39	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype3
BRUC41	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype1
BRUC43	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype1
BRUC45	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype1
BRUC47	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype1
BRUC52	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype1
BRUC54	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype3
BRUC55	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype3
BRUC58	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype2
BRUC59	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype1
BRUC60	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype1
BRUC63	-	-	-	-	-	+	Maldi-type 2	biotype 2	Serotype3
BRUC01	+	+	-	-	-	+	Maldi-type 2	biotype 2	Serotype2
BRUC08	+	+	-	-	-	+	Maldi-type 2	biotype 2	Serotype2
BRUC09	+	+	-	-	-	+	Maldi-type 2	biotype 2	Serotype2
BRUC22	+	+	-	-	-	+	Maldi-type 2	biotype 2	Serotype2
BRUC44	+	+	-	-	-	+	Maldi-type 2	biotype 2	Serotype2

Isolate #	Peaks (m/z)						MALDI	PCR	Serotype
	4682	5028	5970	6823	7326	7356			
BRUC46	+	+	-	-	-	+	Maldi-type 2	biotype 2	Serotype2
BRUC57	+	+	-	-	-	+	Maldi-type 2	biotype 2	Serotype2
BRUC61	+	+	-	-	-	+	Maldi-type 2	biotype 2	Serotype2
BRUC03	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC05	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC06	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC15	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC17	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC18	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC20	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC21	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC23	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC25	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC27	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC30	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC32	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC33	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC35	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC37	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC40	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC42	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC48	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC49	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC51	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC53	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC56	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC62	-	-	+	+	-	-	Maldi-type3	biotype 3	Serotype1
BRUC16	-	-	+	-	+	-	Maldi-type1	biotype 3	Serotype1
BRUC19	-	-	+	-	+	-	Maldi-type1	biotype 3	Serotype1
BRUC24	-	-	+	-	+	-	Maldi-type1	biotype 3	Serotype3
BRUC29	-	-	+	-	+	-	Maldi-type1	biotype 3	Serotype1
BRUC34	-	-	+	-	+	-	Maldi-type1	biotype 3	Serotype1
BRUC50	-	-	+	-	+	-	Maldi-type1	biotype 3	Serotype1
BRUC36	-	-	+	-	+	-	Maldi-type1	biotype 3	Serotype3

APPENDIX M: KAPPA MEASURE OF AGREEMENT (MALDI-TYPING vs SEROTYPING)

Table 6: Kappa measure of agreement; MALDI-typing vs serotyping.

SERO * MALDI Cross tabulation						
			Maldi			Total
			1	2	3	
sero	1	Count	5	13	24	42
		% within Maldi	71.4%	40.6%	100.0%	66.7%
	2	Count	0	14	0	13
		% within Maldi	0.0%	43.7%	0.0%	20.6%
	3	Count	2	5	0	8
		% within Maldi	28.6%	15.6%	0.0%	12.7%
Total		Count	7	32	24	63
		% within Maldi	100.0	100.0	100.0	100.0%

Symmetric Measures					
		Value	Asymp. Std. 1Error[1]	Approx. T[2]	Approx. Sig.
Measure of Agreement	Kappa	.076	.053	1.329	.184
N of Valid Cases		63			

[1] Not assuming the null hypothesis
[2] Using the asymptotic standard error assuming the null hypothesis

APPENDIX N: KAPPA MEASURE OF AGREEMENT (MALDI-TYPING vs PCR)

Table 7: Kappa measure of agreement; MALDI-typing vs PCR.

MALDI * PCR Cross tabulation						
			PCR			Total
			Biotype 1	Biotype 2	Biotype3	
MALD	Type1	Count	0	0	7	7
		% within PCR	0.0%	0.0%	22.6%	11.1%
	Type2	Count	0	32	0	32
		% within PCR	0.0%	100.0%	0.0%	50.8%
	Type3	Count	0	0	24	24
		% within PCR	0.0%	0.0%	77.4%	38.1%
Total		Count	0	32	31	63
		% within PCR	0.0%	100.0%	100.0%	100.0%

Symmetric Measures					
		Value	Asymp. Std. Error[3]	Approx. T[4]	Approx. Sig.
Measure of Agreement	Kappa	.800	.063	7.536	.000
N of Valid Cases		63			

[3] Not assuming the null hypothesis
[4] Using the asymptotic standard error assuming the null hypothesis

APPENDIX O: CHI SQUARE, PHI AND CRAMER'S V

Associations between the potential marker peaks and *B. melitensis* biotypes as per PCR calculated using Pearson chi-square test.

Table 8: Associations between Marker Peak at m/z 4682 and *B. melitensis* biotypes 2& 3

Crosstab					
			PCR		**Total**
			Biotype 2	**Biotype3**	
4682	**0**	Count	24	31	55
		% within PCR	75.0%	100.0%	87.3%
	1	Count	8	0	8
		% within PCR	25.0%	0.0%	12.7%
Total		Count	32	31	63
		% within PCR	100.0%	100.0%	100.0%

Chi-Square Tests				
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)
Pearson Chi-Square	8.877[a]	1	.003	
Continuity Correction [b]	6.765	1	.009	
Fisher's Exact Test				.005
N of Valid Cases	63			

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.94.
b. Computed only for a 2x2 table

Symmetric Measures			
		Value	Approx. Sig.
Nominal by Nominal	Phi	-.375	.003
	Cramer's V	.375	.003
N of Valid Cases		63	

Table 9: Associations between Marker Peak at m/z 5028 and *B. melitensis* biotypes 2& 3

Crosstab					
			PCR		**Total**
			Biotype 2	**Biotype3**	
5028	**0**	Count	24	31	55
		% within PCR	75.0%	100.0%	87.3%
	1	Count	8	0	8
		% within PCR	25.0%	0.0%	12.7%
Total		Count	32	31	63
		% within PCR	100.0%	100.0%	100.0%

Chi-Square Tests				
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)
Pearson Chi-Square	8.877[a]	1	.003	
Continuity Correction b	6.765	1	.009	
Fisher's Exact Test				.005
N of Valid Cases	63			

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.94.

b. Computed only for a 2x2 table

Symmetric Measures			
		Value	Approx. Sig.
Nominal by Nominal	Phi	-.375	.003
	Cramer's V	.375	.003
N of Valid Cases		63	

Table 10: Associations between Marker Peak at m/z 5970 and *B. melitensis* biotypes 2& 3

Crosstab					
			PCR		Total
			Biotype 2	Biotype3	
5970	0	Count	32	0	32
		% within PCR	100.0%	0.0%	50.8%
	1	Count	0	31	31
		% within PCR	0.0%	100.0%	49.2%
Total		Count	32	31	63
		% within PCR	100.0%	100.0%	100.0%

Chi-Square Tests				
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)
Pearson Chi-Square	63.000[a]	1	.000	
Continuity Correction [b]	59.063	1	.000	
Fisher's Exact Test				.000
N of Valid Cases	63			

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 15.25.

b. Computed only for a 2x2 table.

Symmetric Measures			
		Value	Approx. Sig.
Nominal by Nominal	Phi	1.000	.000
	Cramer's V	1.000	.000
N of Valid Cases		63	

Table 11: Associations between Marker Peak at m/z 6823 and *B. melitensis* biotypes2& 3

Crosstab					
			PCR		**Total**
			Biotype 2	**Biotype3**	
6823	**0**	Count	32	7	39
		% within PCR	100.0%	22.6%	61.9%
	1	Count	0	24	24
		% within PCR	0.0%	77.4%	38.1%
Total		Count	32	31	63
		% within PCR	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)
Pearson Chi-Square	40.020[a]	1	.000	
Continuity Correction[b]	36.804	1	.000	
Fisher's Exact Test				.000
N of Valid Cases	63			

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 11.81.

b. Computed only for a 2x2 table

Symmetric Measures

		Value	Approx. Sig.
Nominal by Nominal	Phi	.797	.000
	Cramer's V	.797	.000
N of Valid Cases		63	

Table 12: Associations between Marker Peak at m/z 7326 and B. melitensis biotypes 2& 3

Crosstab					
			PCR		**Total**
			Biotype 2	**Biotype 3**	
7326	**0**	Count	32	24	56
		% within PCR	100.0%	77.4%	88.9%
	1	Count	0	7	7
		% within PCR	0.0%	22.6%	11.1%
Total		Count	32	31	63
		% within PCR	100.0%	100.0%	100.0%

Chi-Square Tests				
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)
Pearson Chi-Square	8.129[a]	1	.004	
Continuity Correction[b]	6.003	1	.014	
Fisher's Exact Test				.005
N of Valid Cases	63			

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.44.

b. Computed only for a 2x2 table

Symmetric Measures			
		Value	Approx. Sig.
Nominal by Nominal	Phi	.359	.004
	Cramer's V	.359	.004
N of Valid Cases		63	

Table 13: Associations between Marker Peak at m/z 7356 and B. melitensis biotypes

Crosstab					
			PCR		Total
			Biotype 2	Biotype 3	
7356	0	Count	0	31	31
		% within PCR	0.0%	100.0%	49.2%
	1	Count	32	0	32
		% within PCR	100.0%	0.0%	50.8%
Total		Count	32	31	63
		% within PCR	100.0%	100.0%	100.0%

Chi-Square Tests				
	Value	df	Asymp. Sig. (2-sided)	
Pearson Chi-Square	63.000[a]	1	.000	
Continuity Correction[b]	59.063	1	.000	
Fisher's Exact Test				.000
N of Valid Cases	63			

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 15.25.
b. Computed only for a 2x2 table

Symmetric Measures			
		Value	Approx. Sig.
Nominal by Nominal	Phi	-1.000	.000
	Cramer's V	1.000	.000
N of Valid Cases		63	

جامعة قطر

قسم الدراسات العليا

كلية الآداب والعلوم

تطبيق قياس الطيف الكتلي (MALDI TOF) للتعرف على الفصائل المختلفة لجرثومة الحمى المالطية (*B. melitensis*)

قسم العلوم الصحية

برنامج العلوم الحيوية الطبية

مقدمة من

علا أسعد الشرباصي

علا أسعد الشرباصي 2015 ©

مقدمة لاستيفاء متطلبات درجة الماجستير في العلوم / آداب

مايو / آذار 2015

خلاصة البحث

تبين في هذه الدراسة أن المسبب الرئيسي لمرض الحمى المالطية أو ما يعرف بحمى البحر المتوسط في قطر هي البكتيريا المسماة *B. melitensis* وبالأخص فصيلين منها أثبت وجودهما بنسب متساوية هما

B melitensis biotype 2 and 3. و حيث أن عملية التعرف عن الفصائل السائدة من *B. melitensis* تمثل حجر الزاوية لتطوير استراتيجيات السيطرة على هذا المرض ، كان هدف هذه الدراسة والتي تعد الأولى من نوعها استخدام تقنية قياس الطيف الكتلي MALDI-TOF MS للتعرف على الفصائل المختلفة لجرثومة *B. Melitensis* . و قد تمكنا من الوصول الى نتيجة وان كانت مبدئية لكنها على درجة عالية من الدقة والقدرة على التمييز وبشكل واضح بين الفصيلين الأكثر انتشارا في قطر وذلك باكتشاف اختلافات في الطيف الكتلي لهذه الفصائل. هذاالاكتشاف يؤهل تقنية MALDI-TOF MS بما تتميز به من سرعة الأداء والدقة مع انخفاض سعر التكلفة لأن تكون بديلا ممكنا للتقنيات الجزيئية التي تتميز بتكلفتها المرتفعه نسبيا والمستخدمة في الدراسات الوبائية.