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Typing and Whole- Genome Sequencing of *Brucella* isolated from Clinical Samples in Lebanon

By

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To my loving mother I dedicate this thesis…

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Typing and Whole-genome Sequencing of *Brucella* isolated from Clinical Samples in Lebanon

Natalia Abou Zaki

ABSTRACT

Brucella is a Gram-negative intracellular bacterium that causes brucellosis characterized by several symptoms including fever, nausea and myalgia. Routes of infection include direct contact through skin or mucous membranes, inhalation, handling manure of infected animals and ingestion of infected dairy products. *Brucella melitensis* is the main causative agent of infection in Lebanon, with increasing incidence as a result of occupational contact and ingestion of infected meat and dairy products. Whole-genome sequencing (WGS) is essential for pathogenomics and evolutionary analysis of different species of *Brucella*. In this study, and as a follow-up to previous studies done in Lebanon, resistance profiles to common antibiotics used against *Brucella* by antibiotic disc diffusion method were obtained. The isolates were shown to be resistant to fluoroquinolones and co-trimoxazole. Typing of *Brucella* was performed using 16S rRNA sequencing, Bruce-ladder multiplex PCR, and PCR-RFLP of *omp2a* and *omp31* genes. 16S rRNA sequencing confirmed the genus *Brucella*, while PCR-RFLP of *omp31* and *omp2a* proved more accurate and practical in detecting the species than

Bruce-ladder PCR. Sixteen isolates were chosen for WGS and their subsequent genomes were analyzed for presence of virulence and resistance determinants, genomic islands, CRISPRs, prophages, insertion sequences and phylogenetic analysis. Several virulence genes vital for *Brucella* pathogenicity were detected in our isolates including type IV secretion system, flagellar and LPS genes, ureases and BvrR/S two component system. Additionally, antibiotic resistance genes against quinolones and efflux pumps mediating multidrug efflux were also found, indicating the emerging resistance patterns in Lebanon. Prophages, insertion sequences and genomic islands confirmed that lateral gene transfer occurred in our *B. melitensis* strains and further contributed to the genome evolution, diversity, and virulence. To our best knowledge, this is the first kind of study done on *Brucella* in Lebanon. It will provide an insight into brucellosis and its prevalence in addition to highlighting the importance of taking control measures to limit future spread of the disease.

Keywords: *Brucella*, *Brucella melitensis*, Brucellosis, Bruce Ladder, PCR-RFLP, Whole-genome sequencing, WGS, Virulence determinants

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LIST OF ABBREVIATIONS

16S rRNA: 16S ribosomal RNA

AHGT: anti-human globulin test

AMOS: *B. abortus*, *B. melitensis*, *B. ovis* and *B. suis*

***B. abortus*:** *Brucella abortus*

B. canis: *Brucella canis*

B. inopinata: *Brucella inopinata*

B. melitensis: *Brucella melitensis*

B. microti: *Brucella microti*

B. neotomae: *Brucella neotomae*

B. ovis: *Brucella ovis*

B. papionis: *Brucella papionis*

B. pinnipedialis: *Brucella pinnipedialis*

B. suis: *Brucella suis*

B. ceti: *Brucella ceti*

BBP: Brucella Bioinformatics Portal

BCV: *Brucella*- containing vacuole

Bp: base pair

Btp1: *Brucella* toll-like interacting protein

BvrRS TCS: BvrR/BvrS two component system

CNS: central nervous system

CRISPR: clustered regulatory interspaced short palindromic repeats

CRP: C-reactive protein

CSP: cold shock protein

dnaK: chaperone protein dnak

EEA-1: early endosome antigen

ELISA: enzyme-linked immunosorbent assay

ER: endoplasmic reticulum

ery: erythrulose-1-phosphate dehydrogenase

EtBr: ethidium bromide

GC: guanine cytosine

GI: genomic island

H_2S: hydrogen sulfide

IFN-γ: interferon gamma

IgG: immunoglobulin G

IgM: immunoglobulin M

Il-10: interleukin 10

Il-12: interleukin 12

Indel: insertion/deletion

IS711: insertion sequence IS711

IS: insertion sequence

LAMP-1: lysosome-associated membrane protein 1

LPS: lipopolysaccharide

BMAT: *Brucella* microagglutination test

MAT: microagglutination test

Mb: megabase

MLVA: multiple locus variable-number tandem repeat analysis

NF-κB: nuclear factor kappa B

NK: natural killer

NO: nitric oxide

omp2: outer membrane protein 2

omp22: outer membrane protein 22

omp25: outer membrane protein 25

omp2a: outer membrane protein 2a

omp2b: outer membrane protein 2b

omp31: outer membrane protein 31

PAMP: pathogen associated molecular pattern

PCR: polymerase chain reaction

PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism

PI3-kinase: phosphoinositide 3-kinase

PrpA: proline racemase protein A

PrPc: cellular prion protein

ROS: reactive oxygen species

SAT: standard agglutination test

SOD: superoxide dismutase

SP41: 41-kDa surface protein

T3SS: type III secretion system

T4SS: type IV secretion system

TAE: Tris-Acetic acid EDTA

TBE: Tris-Boric acid EDTA

Th1: T helper 1-Type

TLR2: toll-like receptor 2

TLR4: toll-like receptor 4

TNF-α: tumor necrosis factor alpha

US CDC: United States Centers for Disease Control and Prevention

VFs: Virulence factors

WHO: World Health Organization

WGS: Whole-genome sequencing

Chapter One

INTRODUCTION

Brucella is a Gram-negative, facultative intracellular bacterium belonging to α-proteobacteria (Moreno et al., 2002). They lack endospores, native plasmids, flagella, capsules and other classical virulence factors (Moreno et al., 2002). The twelve *Brucella* species are: *B. abortus, B. suis, B. ovis, B. melitensis, B. canis, B. neotomae, B. pinnipedialis, B. ceti, B. microti* and *B. inopinata* (Galinska & Zagórski, 2013), with two species recently discovered: *B. papionis* and *B. vulpis* (Scholz et al., 2016; Whatmore et al., 2014). Out of these, *B. melitensis, B. suis* and *B. abortus* mainly cause human infections, with some cases of *B. canis* infections (Wang et al., 2014). *B. abortus* causes the mildest form of brucellosis in humans, *B. suis* causes a more severe form, and the most pathogenic is *B. melitensis* (Wang et al., 2014). There is a high degree of genetic similarity between the different species as seen by DNA-DNA hybridization tests (Tiller et al., 2010).

Brucellosis is a zoonotic disease (Buzgan et al., 2010). The routes of transmission could be through consumption of infected meat, ingestion of raw dairy products or direct contact with animals carrying the disease (Buzgan et al., 2010). Animals that are infected with *Brucella* excrete the bacterium through body fluids including milk, sperm, urine and vaginal secretions (Xu et al., 2013). In the case of fetus abortion in animals such as cattle, *Brucella* is also excreted in amniotic fluid (Xu et al., 2013). Symptoms of

brucellosis include: fever, nausea, myalgia, arthralgia of large joints, headache, chills, malaise, and sweating (Fanni et al., 2013).

The disease is difficult to treat and its complexity resides in the fact that it is associated with bacteremia, toxemia, and allergy, in addition to the organism being able to invade many organs, and impenetrability of infected cells to antimicrobial agents (Ko & Splitter, 2003; Oliveira et al., 2010). The clinical forms of the disease could be acute, sub-acute, chronic, relapsed, non-active or active (Wang et al., 2014). Because of the nonspecific nature of the disease, diagnosis and confirmation with laboratory testing is a must (Wang et al., 2014). Antibiotic administration however, could cause and depending on the patient, relapses and treatment failure (Ariza et al., 2007). Hydrogen sulfide (H_2S) production, requirement of CO_2 or urease test can all be used to confirm the species after its isolation from blood, tissue specimens and body fluids (Al Dahouk et al., 2013). On the other hand, serological tests targeting specific *Brucella* antibodies in the host, or PCR based assays for detection of *Brucella* DNA can also be used (Sakran et al., 2006). Several drawbacks are associated with the isolation of *Brucella* species including the health hazard it presents to laboratory staff and time consumption (Pabuccuoglu et al., 2011). Serological tests work better with the risk of producing false positive results due to cross reactivity (Pabuccuoglu et al., 2011).

Regulatory programs have been implemented for the consequent eradication of brucellosis due to its prevalence in humans and in animals. These programs include vaccinations and sanitization procedures (Olsen & Stoffregen, 2005). Sanitization programs aim at implementing producers for decontamination and for the proper

discharge of contaminated material to decrease the dissemination of the infectious agent (Avila-Calderón et al., 2013).

Brucella pathogenesis lies in its ability to survive intracellularly and multiply within professional and non-professional phagocytic cells through lipopolysaccharides (LPS) and T4SS, which are thought to play a role in enhancing its virulence and aid in host evasion (Ben-Tekaya et al., 2013). Two phases constitute the *Brucella* lifecycle: acute infection which occurs in non-phagocytic cells and leads to abortion in animals, and chronic infection which happens in phagocytic cells leading to replication of the bacterium and its subsequent survival in the host (Yongqun, 2012). Lymph nodes, sex organs, spleen, liver, and bone marrow are all organs that can be affected by brucellosis and transmission to different organs and systems occurs via macrophages (Ko & Splitter, 2003).

Humoral immunity and production of antibodies by the host against the O-antigen on the LPS of the bacterium provides only partial protection, while cell mediated immunity is the main mode of defense used by the host against *Brucella* infection (Yongqun, 2012).

In Lebanon, brucellosis is mainly caused by *B. melitensis* (Araj & Azzam, 1996; Dajani et al., 1989) with a reported increase in disease incidence following contamination of meat and dairy products in addition to occupational risk (Young, 1991). Hence, the increase in the importance of studying this organism in order to better understand its mechanisms of infection and hopefully limit and control its spread in the future (Araj & Azzam, 1996)

Thirty-three *Brucella* isolates provided by Azm Center for Research and Biotechnology recovered mainly from blood, with only two from ascitic and synovial fluids were used in this study to (i) examine the population dynamics of pathogenic *Brucella* strains, (ii) sequence the 16S rRNA gene in order to identify *Brucella* on the genus-level and its differentiation from organisms which are closely related to it, (iii) type the isolates by Bruce Ladder multiplex PCR to discriminate between different species, (iv) identify the isolates' biotypes and biovars and determine DNA polymorphism from the restriction patterns through PCR-RFLP of *omp31* and *omp2a* genes, (v) study the functional genomics through whole-genome sequencing (WGS) of sixteen representative isolates, (vi) identify genes related to host adaptation and investigate variation in virulence potential in order to estimate the fitness of these pathogens, (vii) correlation between the virulence factors detected such as type IV secretion system, BvrR/S two component system, LPS- related genes, ureases and subsequent pathogenicity of *Brucella*, (viii) prediction and annotation of the components of the T4SS in *Brucella* spp. using the sixteen complete genomes and determine the physical and functional interactions between the corresponding proteins, (ix) screening the genomes for pathogenicity islands, clustered regularly interspaced short palindromic repeats (CRISPRs), and phage related proteins and correlate their role in virulence and resistance, (x) characterize the insertion sequences across the *Brucella* genomes and evaluate their roles, (xi) determine phylogenetic groups of isolated *Brucella*, and use genome data to construct a phylogenetic tree, (xii) and perform a comparative analysis of the genes present in the different phylogenetic groups.

Chapter Two

LITERATURE REVIEW

2.1. *Brucella*: An Overview

Brucellosis is a zoonotic disease that is caused by small aerobic Gram-negative bacteria that penetrate and proliferate in the lymphatic system before subsequently disseminating to various organs of the body causing infection in different tissues (Smits, & Kadri, 2005). *B. melitensis*, is the most pathogenic and was first isolated in Malta in 1887 (Galinska & Zagorski, 2013), *B. abortus* is associated with infections in cattle and humans (Kang et al., 2011), *B. suis* is linked to brucellosis in humans as well as swine (Kang et al., 2011), *B. canis* causes disease in dogs and possibly infections in humans (Iwaniak et al., 1999), *B. neotomae* infects rats (Szulowski, & Murat, 2008), *B. ovis* infects sheep and rams (Boryczko et al., 1985), *B. ceti* and *B. pinnipedialis* were isolated from whales and seals and other ocean mammals (Bricker et al., 2000; Tryland et al., 1999), *B. microti* was isolated from lymphatic systems of wild foxes and common vole (Scholz et al., 2009), and *B. inopinata* from breast implant of a woman that had brucellosis clinical signs (Scholz et al., 2010). Additionally, *B. papionis*, which was isolated in 2007 by Schlabritz-Loutsevitch et al., is a coccobacilli or short rod, non-motile, non-spore forming and aerobic. It is resistant to doxycycline, rifampicin, ciprofloxacin and streptomycin, and is catalase and urease positive in addition to being oxidase negative. It doesn't require CO_2 for growth and doesn't produce H_2S

(Schlabritz-Loutsevitch et al., 2009; Whatmore et al., 2014). A PCR assay (AMOS: abortus, melitensis, ovis, suis) can be used to confirm the *Brucella* genus through generating a specific amplicon of 180 base pairs (bp) (Schlabritz-Loutsevitch et al., 2009), however upon the application of Bruce-ladder PCR, RFLP on outer membrane protein 2 (*omp2*), and Multiple Locus Variable-number Tandem Repeat Analysis (MLVA) on *B. papionis*, the species was not identified (Whatmore et al., 2014).

Recently, Scholz et al. 2016 reported a new species of *Brucella*, named *B. vulpis* that was isolated from lymph nodes of red foxes and didn't fit the criteria of other species. Interestingly, 5% of the genome was attributed to non-*Brucella* origin, containing phages and insertion sequences that were not characterized yet in *Brucella*, and phylogenetic analysis showed that it was separated from other species and found in a distinct clade (Scholz et al., 2016).

2.2. Forms and Symptoms of Brucellosis

Since brucellosis affects many organs/systems, the clinical manifestations of the disease are very broad (Jiao et al., 2015). Abortion and sterility in animals is seen as a result of infection (Hashino et al., 2012). Clinical polymorphism of brucellosis causes difficulty in diagnosis and treatment, which leads to relapses, complications and misdiagnosis (Doganay et al., 2008; Young, 2005).

Chronic brucellosis is characterized by depression, emotional liability, and musculoskeletal pain such as: arthralgias, myalgia, and arthritis in addition to nervousness, malaise and a positive rheumatoid factor test (Colmenero et al., 2007;

Young 2005). On the other hand, clinical laboratory results in patients with acute and sub-acute forms include leukopenia, anemia, increased levels of C-Reactive Protein (CRP), elevated levels of liver enzymes and thrombocytopenia, high erythrocyte sedimentation rate, and high levels of monocytes and lymphocytes (Buzgan et al., 2010). Gastrointestinal, hematological, cardiovascular, central nervous system and respiratory are all systems that can be affected by the disease (Shapiro et al., 1999). Lymphadenopathy, which is not common to brucellosis, can only be seen in severe cases of the disease (Pappas et al., 2005). Severity of the disease is highly linked to the immune response especially the cell mediated, which is mostly involved in protection against brucellosis (Cannella et al., 2012).

2.3. Brucellosis Routes of Infection

Wild animals, rodents, cattle, sheep, and goats can all constitute a vector and a reservoir for the disease in humans since *Brucella* affects many animals including domesticated and wild, rodents and marine mammals (Godfroid et al., 2005). Incidence of brucellosis is highly related to occupation and the most susceptible individuals are the ones working in farms, meat processing enterprises, veterinary doctors, and zoo technicians (Galinska & Zagorski, 2013). *Brucella* can be transmitted by direct contact through breaks in skin and mucosal membranes (Corbel, 1997) or indirectly through the oral fecal route (Zhang et al., 2014). Inhalation of droplets from laboratory samples during specimen handling for laboratory technicians or inhalation as a result of close contact with infected animals also constitutes a common route of infection (Godfroid et al., 2005). Brucellosis could

also be transmitted through ingestion of food (meat, unpasteurized milk, or dairy products) as well as contaminated water (Ismayilova et al., 2013). *Brucella* is very potent and can survive 60 days in dairy products or meat and up to 120 days in soil and 150 in water (Jiao et al., 2009).

2.4. Pathogenesis and Virulence of *Brucella*

Brucella can evade the host immune response and invade cells to multiply either in the vacuole or in the cytoplasm (Gomez et al., 2013a). It uses fibronectin, vitronectin and SP41, which is a 41-kDa protein on its surface encoded by *ugpB* locus, to bind to receptors on surfaces and penetrate epithelial, phagocytic and non-phagocytic cells of mucosal membranes (Billard et al., 2005; Castaneda-Roldan et al., 2004; Kim et al., 2004).

Additionally, its LPS is composed of lipid A, O-antigen, and core oligosaccharide (Lapaque et al., 2005). LPS plays an important role in internalization of *Brucella* through interaction with lipid rafts, and Fc and complement receptors on the surface of host cells (Moreno & Moriyon, 2002). It also aids in evading the host immune response by protecting it from the complement cascade (Lapaque et al., 2006). As many other Gram-negative bacteria, *Brucella* grows as smooth or rough colonies, which depends on its LPS composition: smooth strains possess the O-antigen while rough strains lack it (Mancilla, 2015). The O-antigen has been classified as a virulence factor of *Brucella*, and hence rough *Brucella* strains are usually more attenuated (Pei & Ficht, 2004). Smooth strains however, are internalized at a lower rate than the rough ones although

they are more pathogenic, mainly because the uptake of smooth strains is through lipid raft dependent mechanisms, while rough strains are internalized by both lipid raft dependent and independent mechanisms (Gomez et al., 2013a; Jimenez de Bague et al., 2004). Lipid raft mediated uptake of *Brucella* involves two types of receptors: cellular prion protein (PrPC) and class A scavenger receptor (Kim et al., 2004).

Another system highly involved in internalization of *Brucella* is the BvrRS TCS, which is a two component regulatory system made of a histidine kinase sensor (BvrS) and cytoplasmic regulator (BvrR), involved in the control of structure of LPS and periplasmic protein expression thereby leading to the expression of outer membrane protein 22 (Omp22) and outer membrane protein 25 (Omp25) (Guzman-Verri et al., 2002; Manterola et al., 2007). A deletion of BvrRS TCS system in *Brucella* leads to altered uptake of the organism by host cells, in addition to attenuated virulence of *Brucella* and failure of the organism to replicate inside the host cell (Figueiredo et al., 2015).

On the other hand, the organism protects itself inside the host cell from bactericidal agents and harsh conditions such as lysozyme production. It does so by: the up regulation of virulence factors and structural components (Copin et al., 2012), and production of antigens, early endosome antigen 1 (EEA-1) and Rab5 antigens, following interaction with lysosomes and endosomes, which aid in *Brucella* internalization (Starr et al., 2008). For vacuole maturation, *Brucella* employs β-1,2-glucans that act through lipid raft control (Arellano-Reynoso et al., 2005). The *Brucella*-containing vacuole (BCV) then matures and expresses late endocytic markers such as lysosome-associated

membrane protein 1 (LAMP-1) and Rab7 (Boschiroli et al., 2002; Celli et al., 2005); a process through which T4SS encoded by *virB* operon is induced and activates effector proteins (Sieira et al., 2000).

Acidification of BCV and interaction with endoplasmic reticulum (ER) components allows *Brucella* to acquire ER-specific markers and only then it can efficiently replicate in the host (Celli et al., 2005; von Bargen et al., 2012). *Brucella* internalization and intracellular trafficking steps in mammalian cells of the host are briefly described in Figure 1, where it shows the mechanism of internalization mainly for smooth strains (Gomez et al., 2013a). Certain virulence factors including T4SS, BvrRS TCS and Omp25 proteins are upregulated in the intracellular stage of infection (Wang et al., 2009b).

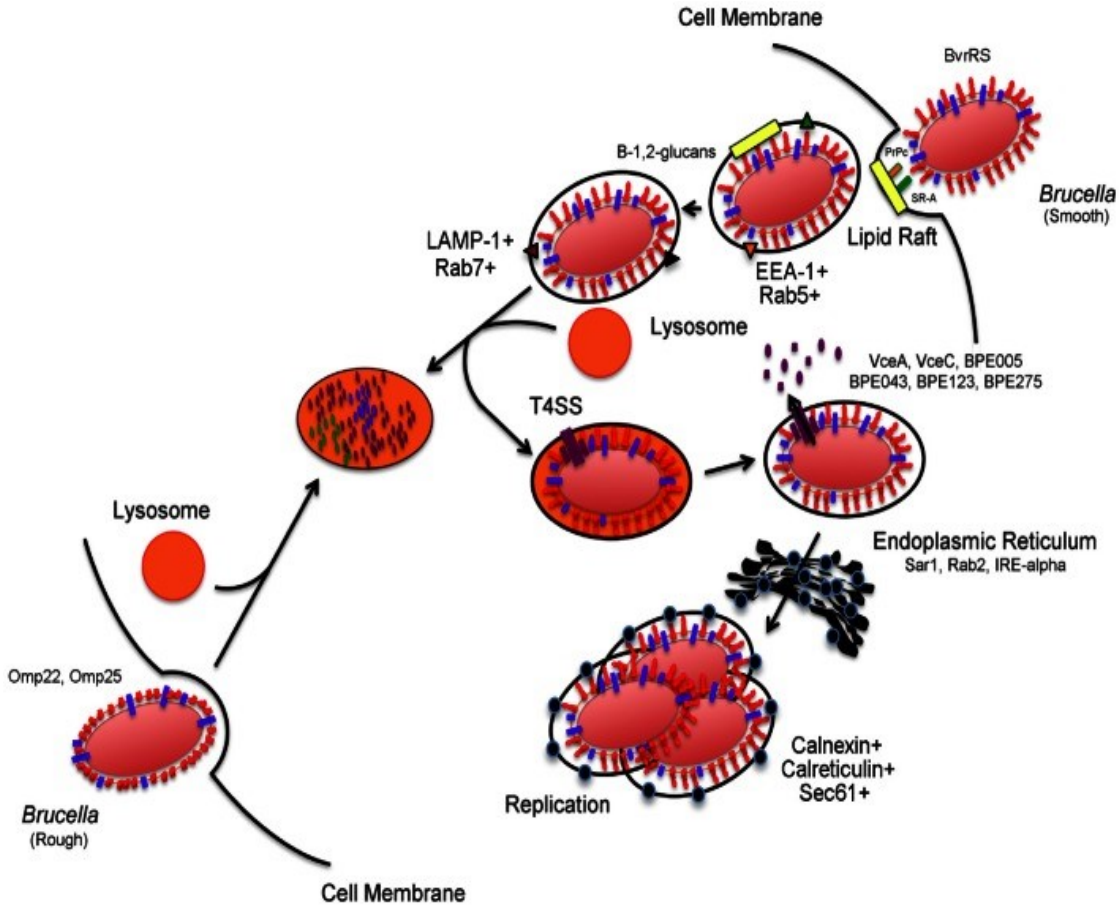

Figure 1. Schematic representation of steps in *Brucella* internalization and intracellular trafficking (Gomez et al., 2013a).

After internalization inside host cells, *Brucella* plays an important role in cell death by inhibiting apoptosis in monocytes and macrophages or by delaying apoptosis in lymphocytes in order to persist inside the host (Dornan et al., 2000; Hoover et al., 2003). This is mediated by the overexpression of anti-apoptotic proteins such as A1 of the Bcl-2 family and under expression of pro-apoptotic factors such as caspase-3 (Dornand et al., 2000). Other anti-apoptotic factors released by *Brucella* include outer membrane protein 2b (Omp2b) and Omp25 porins that regulate tumor necrosis factor alpha (TNF-α) secretion (Gross et al., 2000).

T4SS secretion system also has an important role in cell death as it is one of the most important virulence factors in *Brucella* having several effector proteins and 11 components (*virB1-11*) (Ke et al., 2015). Manipulation of vesicle trafficking in host cells, regulation of inflammatory response, inhibition of immune response, and enhancing intracellular survival in host cells are all ways through which T4SS enhances the virulence of *Brucella* (Ke et al., 2015). Its deletion leads to an increase in replication of host cells and a decrease in cytotoxic and cytopathic effects imposed on the cell as a result of *Brucella* infection (Ficht et al., 2008; Zhong et al., 2009).

Other virulence factors include the TpcB protein that inhibits the cytotoxic function of T-cells and subsequently protects from the cell-mediated immune response in addition to inhibition of dendritic cells maturation (Durward et al., 2012; Salcedo et al., 2008). Proline racemase protein A (PrpA), a protein that modulates the host immune response, induces B lymphocytes to produce the immunosuppressive cytokine interleukin 10 (IL-10), and serves as a virulence factor (Spera et al., 2006).

Other than the intracellular lifestyle, multiple metal homeostasis systems are found in *Brucella* that enhance its virulence (Roop, 2012). These include nickel, cobalt, zinc, manganese, iron, and copper transport systems that consist of translational and regulatory elements in addition to efflux proteins (Roop, 2012). Many metals play a role as cofactors that are needed for the activity of *Brucella* enzymes. Iron is needed for catalase and aldolase activity (Schroeder et. al, 2009), *Brucella* uses the oxygenase to break down heme to be used as a source of iron (Ojeda et al., 2012). Manganese is required too, and MntH is a manganese transporter, the deletion of which leads to

attenuation of *Brucella* (Anderson et al., 2009). On the other hand, zinc is a cofactor for the enzymes carbonic anhydrase I and II and has been also shown to increase virulence (Andreini et al., 2006; Lopez et al., 2012). Magnesium has a double function: it is a cofactor for erythritol kinase (Sperry & Robertson, 1975) and it maintains the integrity of the cell membrane (structural role) in *Brucella* (Moomaw & Maguire, 2008). Altogether this data indicate that *Brucella* can withstand a metal deprived environment due to these metal homeostasis systems, that help in the acquisition of the needed amounts of metals and sustaining its viability in the host (Roop, 2012).

2.5. Interaction with Host Immune System

The main mode of protection of the body against *Brucella* is through activated macrophages, in addition to the development of cell-mediated immune response and T helper 1-type (Th1) immunity (Mantur et al., 2007). Innate immunity against *Brucella* is mediated by phagocytosis, cytokine production, recognition of pathogen-associated molecular patterns (PAMPS), chemokine production and through the triggering and activation of the complement cascade (Ahmed et al., 2016). It utilizes neutrophils to reach lymphoid tissues and through the process employs different mechanisms to protect it from lysozymes, phospholipases, and reactive oxygen species (Nathan & Shiloh, 2000). Recognition of *Brucella* through Toll like receptors is down-regulated in the course of infection and is a mode by which the organism shields itself from host's immune response (Iwasaki & Medzhitov, 2004).

Moreover, down regulation of TNF-α associated with production of Omp25 leads to impairment in the activation and function of natural killer cells (NK) (Jubier-Maurin et al., 2001; Mantur et al., 2007). NK cells are activated by IL-2 and in turn secrete interferon gamma (IFN-γ) in order to exert a Th1 response against *Brucella* infection (Gao et al., 2011). *Brucella* down regulates Th1 immune response through bypassing IL-12 production (Salcedo et al., 2008), which leads to decreased maturation of dendritic cells and thus decreased antigen presentation to T cells (Salcedo et al., 2008).

It also uses phosphoinositide 3-kinase (PI3-kinase) pathway to facilitate entry into dendritic cells while using lipid rafts to internalize macrophages (Pei et al., 2008). Moreover, a fatty acid residue in LPS of *Brucella* (C_{28}) is longer than in other bacterial organisms and this decreases its endotoxic properties and helps it to evade recognition through toll-like receptor 4 (TLR4) (Lapaque et al., 2009). Additionally, C3 complement component has a reduced ability to bind to *Brucella* outer membrane surface during infection (Ahmed et al., 2016), whereas PrpA along with *Brucella* toll-like interacting proteins (Btp1/TpcB) and the organism's special structured LPS will regulate the immune response by decreasing levels of IFN-γ and increasing levels of IL-10 (Wang et al., 2012). Btp1 also has roles in decreasing nuclear factor kappa B (NF-κB) production, blocking TLR2 and TLR4 signaling and pro-inflammatory cytokine production in addition to causing inhibition of CD8+ cytotoxic T-cell killing capacity (Radhakrishnan & Splitter, 2010; Salcedo et al., 2008).

Humoral immunity does not play an effective role in the protection against *Brucella,* and antibodies are usually directed against *Brucella* LPS (Araya & Winter, 1990).

Antibodies do not protect against primary infection, but have a role in protection against re-infection (Casadevall & Pirofski, 2006). The role of antibodies is synergistic (Gomez et al., 2013b).

Thus, *Brucella* interplays between innate and adaptive immune response and produces several effector molecules that will modulate the immune response helping it to successfully evade it (Ahmed et al., 2016).

2.6. Diagnosis of Brucellosis

The United States Centers for Disease Control and Prevention (US CDC) reports that *Brucella* can be diagnosed either by bacterial isolation from different clinical samples including cerebrospinal and joint fluid but mainly from blood cultures and through detection of antibodies against *Brucella* through *Brucella* microagglutination test (BMAT) (CDC, 2012). In chronic brucellosis however, false negative results in standard agglutination (SAT) or microagglutination tests (MAT) should be taken into consideration (Araj, 2010).

Among the serological tests, the Rose Bengal agglutination test is very sensitive and is the method of choice for diagnosis in both humans and animals (Serra & Vinas, 2004). In order to confirm the result obtained by the Rose Bengal test, SAT and MAT tests are used, both having high specificity especially in the diagnosis of the acute form of brucellosis (Serra & Vinas, 2004). Enzyme-linked immunosorbent assay (ELISA) however, that can detect immunoglobulin G (IgG) and IgM antibodies against *Brucella* antigens, is sensitive but not specific so cannot be used to confirm the Rose Bengal

(CDC, 2008; Fadeel et al., 2011). On the other hand, rapid latex agglutination test and lateral flow assays are also sensitive and specific and can be used when laboratory resources are limited (Abdoel & Smits, 2007; Marei et al., 2011). Complement fixation test can also be an option for diagnosis, but suffers from several drawbacks including: expensive reagents, the need for expert trained staff, and requires a lot of standardization and reagent preparation (Poester et al., 2010). The major drawback of using serological tests is the cross-reactivity between antigens (Rubach et al., 2013).

At the molecular level, PCR is a sensitive and rapid method for detection of *Brucella* DNA, where multiplex PCR can be employed for confirmation and determination of the different species (Colmenero et al., 2011; Kumar et al., 2011). Several multiplex PCR assays were developed for diagnosis of species and biovars of *Brucella* (Yu & Nielsin, 2010). First, AMOS PCR was used to differentiate between *B. abortus*, *B. melitensis*, *B. ovis* and *B.suis* (Yu & Neilsin, 2010), while the Bruce-ladder PCR was used to differentiate between all *Brucella* species including marine species *B. ceti* and *B. pinnipedialis* in addition to vaccine strains (Lopez-Goni et al., 2008). Furthermore, several *Brucella* genes including *omp2*, *omp25*, *omp31*, chaperone protein Dnak *(dnaK)*, and erythrulose-1-phosphate dehydrogenase (*ery*) have shown to be polymorphic after performing restriction analysis preceded by PCR amplification of selected sequences (Cellier et al., 1992; Ficht et al., 1989). Finally, insertion sequence 711 (IS711) fingerprinting and MLVA typing are also common molecular techniques used for the detection and differentiation of *Brucella* species (Bounaadja et al., 2009; Le Fleche et al., 2006)

2.7. Treatment of Brucellosis

Antibiotic therapy is the method of choice for treatment of brucellosis through mono-combination therapy (Yousefi-Noorai et al., 2012). Antibiotic susceptibility testing is difficult to perform mainly because the organism is fastidious and biohazardous (Abdel-Maksoud et al., 2012). It requires biosafety level 2 or 3 and imposes risk on the laboratory personnel with many emerging cases of laboratory-acquired brucellosis (Abdel-Maksoud et al., 2012).

Rifampin, which blocks RNA and protein synthesis intracellularly (Gattringer et al., 2010), is the primary choice of drug in treatment of brucellosis and resulted in successful recovery in spite of having gastrointestinal side effects in certain patients (Xu et al., 2013). The World Health Organization (WHO) recommends the use of doxycycline with rifampin or streptomycin for combination therapy (Turkmani et al., 2006). Abdel-Maksoud et al. (2012) showed that patients who received streptomycin, doxycyline, and rifampin didn't show relapse. However, ototoxicity and nephrotoxicity were seen with the prolonged streptomycin treatment (Ronland & Wright, 2004).

On the other hand, levofloxacin, ceftazidime, amikacin, and sulfamethoxazole-trimethoprim are bactericidal, and were used in the treatment of brucellosis (Xu et al., 2013). Levofloxacin is a quinolone drug that inhibits the activity of DNA gyrase, prevents the DNA supercoiling, and inhibits bacterial replication (Aldred et al., 2014; Drlica et al., 2009). Ceftazidime on the other hand, is a β-lactam antibiotic that acts on the cell wall (Rawat & Nair, 2010). Successful treatment of brucellosis was the outcome

of using levofloxacin and ceftazidime in combination as a second choice after failing to treat with rifampin (Hashemi et al., 2012; Xu et al., 2013).

Co-trimoxazole was also used in the treatment of *Brucella*, and has shown to be effective in children and pregnant women (Khan et al., 2001; Shehabi et al., 1990). Within the aminoglycosides, gentamicin and amikacin are two popular drugs of choice for treatment (Ranjbar et al., 2007).

Due to many drawbacks associated with the use of antibiotics, studies have switched towards finding novel therapeutic strategies aimed at the treatment of brucellosis (de Figueiredo et al., 2015). The aim was to come up with anti-virulence compounds that will target specifically virulence factors without interfering with cellular functions and thus do not elicit any selective pressure on antibiotic resistance (de Figueiredo et al., 2015). *virB*8, which is a vital component of the T4SS system and essential for the virulence of *Brucella*, has been a target for high throughput small molecule screening targeting its inactivation (Paschos et al., 2006, 2011).

2.8. Brucellosis in the Middle East and in Lebanon

In the Middle Eastern and North African region, cases of brucellosis have re-emerged due to illegal transport of animals through open borders between countries and breakdowns in veterinary health systems (Gwida et al., 2010). Brucellosis was detected in almost all domestic animals in the Middle East especially camels and goats (Al-Shamahy, 1999). In cattle, sheep and goats, infection was mainly common in Jordan and Iraq, while in camels, which have the highest rate of infection; it was mainly

common in Saudi Arabia, Egypt and Iran (Gwida et al., 2010). Brucellosis can be found also in Oman among the Bedouin community (Scrimgeour et al., 1999), in addition to some incidences in Libya (Ahmed et al., 2015). Control measures include vaccinations for people with high risk occupations and surveillance and animal control (Zhang et al., 2014).

In developing countries, regulatory programs aiming to eradicate brucellosis still cannot be fully implemented due to limited resources and economic status (Godfroid et al., 2013). Mass vaccination particularly for sheep and goats was done in several countries, while test and slaughter policy in addition to vaccination of young females was implemented in other areas in the Middle East (Avila-Calderón et al., 2013).

In Lebanon, *B. melitensis* constitutes the main cause of brucellosis in humans (Dajani et al., 1989) being primarily linked to the consumption of unpasteurized milk or dairy products in endemic areas (Young, 1991).

Araj and Azzam (1996) studied the sero-prevalence of *Brucella*-specific antibodies in individuals with high-risk occupations in different areas in Lebanon (Tyre, Sidon, Shouf, Aley, Beirut, Biquaa, Kisrwan, Tripoli, Zgharta and Accar), and revealed that individuals with these occupations showed high sero-prevalence or antibody titers, which highlighted the need to take further protective measures to better control the disease and prevent its spread.

2.9. Genome Organization and Whole-Genome Sequencing

Over the past years, whole-genome sequencing (WGS) has been crossing the threshold between research and clinical diagnostics due to many improvements and advancements including low cost and rapid turnaround time of results (Koser et al., 2012a; Loman & Pallen, 2008). Applications of WGS include accurate determination of phylogenetic relations between bacteria and this is particularly important to trace the evolutionary history of pathogens. This evolution is attributed to horizontal gene transfer and recombination events that introduce changes in bacterial genomes over time. In addition, many bacteria are very conserved in their genome diversity and this is where WGS becomes valuable (Achtman, 2008; Pearson et al., 2009). Another utilization is epidemiological typing that includes outbreak studies and identification of cross contamination between different laboratories (Schurch & Siezen, 2010). For rapidly evolving organisms, WGS again proves useful in terms of transmission detection across facilities and between patients. This is critical for outbreak and infection control (Koser et al., 2012b; McAdam et al., 2012). When it comes to antimicrobial susceptibility testing, WGS is mostly useful when genotypic and phenotypic information can be correlated together and this might be challenging sometimes. Phenotypic tests will still be needed, but WGS can complement these results obtained by detecting mutations in resistance-related genes for example (Koser et al., 2012a & 2012b). Also, WGS is suitable for detection and identification of bacteria when routine laboratory methods suffer several drawbacks such as lack of success in pathogen identification, and fastidious growth requirements needed for bacterial growth, difficult bacterial culture and anaerobic organisms (Kuroda et al., 2012). WGS and genome studies therefore

provide a potential of adding a whole new dynamic and improve work done on pathogenic bacterial populations in addition to ultimately replacing routine phenotypic and laboratory methods (Loman & Pallen, 2015).

Sequencing of multiple *Brucella* species revealed that the average size of its genome is 3.3 megabases (Mb) and is composed of two chromosomes (Halling et al., 2005). *Brucella* has been classified as being a monospecific genus, mainly after performing DNA-DNA pairing studies and finding greater than 95% homologies between different species (Mantur et al., 2007). Moreover, comparing genomes of *Brucella* with closely related genera revealed that it was once a free living organism that underwent several evolutionary events including the loss and acquisition of genes to subsequently become an animal parasite (Moreno et al., 2002). Similarities are found in the sizes, nucleotide composition and synteny of genes in *Brucella*. This shows conservation between the different species (Paulsen et al., 2002) particularly in the distribution of housekeeping genes between chromosomes, and in the similarity in the number of coding regions and guanosine-cytosine content (GC) (Ficht, 2011). Moreover, natural plasmids were not detected in *Brucella* (Mantur et al., 2007). Neighboring genera also have the same property, which suggests that the original chromosome was separated into two units or that a megaplasmid was modified after its capture (Jumas-Bilak et al., 1998). Evolution of the organism was attributed to insertion elements, remnants of phages and presence of many transposons (Ficht, 2011).

Tevdoradze et al. (2015) performed whole-genome sequencing on ten brucellaphages and results revealed a high degree of homogeneity. However, genetic diversity was

detected in the phage collar protein that provided additional insight into host selection with a *Staphylothermus marinus* F1-like CRISPR spacer and the intergenic sequences encoded by *Ochrobactrum anthropi* being also identified.

Even though there is divergence when it comes to evolution, and despite presence of indels that have introduced certain variability in the virulence factors encoded between different species, these differences are not significant (Moreno et al., 2002). As a result, analysis of *Brucella* genomes will give an insight about the organism's metabolism, cell wall features, and virulence factors that will help in better understanding its pathogenicity (DelVecchio et al., 2002a).

Chapter Three

MATERIALS and METHODS

3.1. Clinical Isolates

Thirty-three *Brucella* isolates were kindly provided by Azm Center for Research and Biotechnology. Isolates were designated as Bru003-Bru011 and Bru013-Bru036 and were all recovered from blood except Bru016 which was recovered from articular fluid and Bru027 which was recovered from ascitic fluid. For whole-genome sequencing, sixteen isolates were chosen including: Bru003, Bru004, Bru008, Bru010, Bru013, Bru014, Bru015, Bru018, Bru025, Bru026, Bru027, Bru029, Bru030, Bru034, Bru035, and Bru036.

3.2. DNA Extraction

DNA was extracted using the Nucleospin Tissue kit (Macherey-Nagel, Germany) following the manufacturer's instructions.

3.3. 16S rRNA Sequencing

3.3.1. Pre-sequencing PCR

The 16S rRNA gene was sequenced in all the isolates (Becker et al., 2004). Initial PCR was done in 20 ul volume reaction using final concentrations of 2 mM dNTPs (Thermo Fisher Scientific), 10x Taq Buffer (Applied Biosystems), 25 mM $MgCl_2$ (Applied

Biosystems), 20 uM of primers SSU-bact-27F (5'- AGAGTTTGATCMTGGCTCAG - 3') and SSU-bact-519R (5'- GWATTACCGCGGCKGCTG- 3'), 5 U/ul of Hotstart Amplitaq Gold Taq Polymerase (Applied Biosystems), and 50 ng/ul of DNA. Cycling conditions were as follows: initial denaturation step at 95 ºC for 12 min, 30 cycles of denaturation at 94 ºC for 30 s, annealing at 53 ºC for 30 s, and extension at 72 ºC for 1min, followed by a final extension step of 72 ºC for 10 min. PCR products were separated for 30min at 80 V on 1% agarose gel in 1x Tris-Acetic Acid EDTA (TAE) buffer including 0.5 ug/mL of ethidium bromide (EtBr).

3.3.2. PCR Cleanup

Remaining PCR product was mixed with 0.5 ul of Exonuclease I (Thermoscientific, USA) and 1 ul of FastAP Thermosensitive Alkaline Phosphatase (Thermoscientific, USA). Cycling conditions were 37 ºC for 30 min followed by 80 ºC for 15 min.

3.3.3. Sequencing PCR

Sequencing PCR was performed in a 10 ul final volume using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). A total of 7 ul of 5X diluted BigDye v3.1 (Applied Biosystems) and 1.2 uM primer concentration was added to 3 ul of purified DNA. Cycling conditions were as follows: initial denaturation step at 96 ºC for 1 min, followed by 26 cycles of denaturation at 96 ºC for 10 s, annealing at 50 ºC for 5 s, and extension at 60 ºC for 4 min.

3.3.4.Purification

Purification was carried out using BigDye X-terminator Purification Kit (Applied

Biosystems, USA). Sequencing PCR product (10 ul) was pipetted into a well of a sequencing reaction plate. 45 ul of SAM solution (Applied Biosystems) and 10 ul X-Terminator (Applied Biosystems) were pre-mixed and added to the PCR products. Sequencing reaction plate was sealed with adhesive film and vortexed for 30 min at high speed (1800 rpm). Finally, reaction plate was centrifuged at 1000x g for 2 min in a swinging-bucket centrifuge.

3.3.5. Loading on Genetic Analyzer

The samples were ran on the Genetic Analyzer 3500 (Applied Biosystems, USA), using POP7 polymer in addition to 50 cm capillary array and RapidSeq_BDX_50_POP7 run module. Sequences were then extracted and their quality assessed using Sequencing Analysis v5.4 (Applied Biosystems, USA). Forward and reverse sequences were assembled using CLC Main Workbench (MWB) v7.0.2 (CLCBio, Denmark). Sequences were blasted against NCBI 16S ribosomal RNA database to determine the closest match.

3.4. Bruce-Ladder Multiplex PCR

Bruce-ladder multiplex PCR for species determination of *Brucella* isolates was performed following Garcia-Yoldi et al., (2006). In a final reaction volume of 25 ul, the following were added: 1x PCR Buffer (Thermoscientific, USA), 400 uM dNTPs (Thermo Fisher Scientific), 3 mM $MgCl_2$ (Thermoscientific, USA), 6.25 pmol of each primer (Table 1), 1.5 U of Taq DNA polymerase (Thermoscientific, USA) and 1 ul DNA. Cycling conditions were as follows: initial denaturation step at 95 ºC for 7 min, 25 cycles of template denaturation at 95 ºC for 35 s, primer annealing at 64 ºC for 45 s,

and primer extension at 72 ºC for 3 min, and finally extension at 72 ºC for 6 min. Samples were then electrophoresed at 120 V for 1 h on 1.5% agarose prepared in 1% TBE Buffer (89 mM Tris/HCL, 89 mM boric acid, 2.0 mM EDTA pH= 8.0) and stained with 0.5 ug/ul EtBr. 500 bp and 100 bp ladders were used as size markers. Bands were visualized under UV light.

Table 1. Primer sequences of forward and reverse primers used for Bruce-ladder PCR (Lopez-Goni et al., 2008)

Primer	5'-3' Sequence	Target Amplicon Size
BMEI0998f	ATCCTATTGCCCCGATAAGG	1682
BMEI0997r	GCTTCGCATTTTCACTGTAGC	
BMEI0535f	GCGCATTCTTCGGTTATGAA	450 (1320*)
BMEI0536r	CGCAGGCGAAAACAGCTATAA	
BMEII0843f	TTTACACAGGCAATCCAGCA	1071
BMEII0844r	GCGTCCAGTTGTTGTTGATG	
BMEI1436f	ACGCAGACGACCTTCGGTAT	794
BMEI1435r	TTTATCCATCGCCCTGTCAC	
BMEII0428f	GCCGCTATTATGTGGACTGG	587
BMEII0428r	AATGACTTCACGGTCGTTCG	
BR0953f	GGAACACTACGCCACCTTGT	272
BR0953r	GATGGAGCAAACGCTGAAG	
BMEI0752f	CAGGCAAACCCTCAGAAGC	218
BMEI0752r	GATGTGGTAACGCACACCAA	
BMEII0987f	CGCAGACAGTGACCATCAAA	152
BMEII0987r	GTATTCAGCCCCCGTTACCT	

*In marine isolates, the amplicon size is 1320 bp due to an insertion in *bp26* gene (Lopez-Goni et al., 2008).

3.5. Polymerase Chain Reaction -Restriction Fragment Length Polymorphism (PCR-RFLP) of *omp31* gene

PCR was performed in a 25 ul volume reaction containing 1x Taq Buffer (Thermoscientific, USA), 0.2 mM dNTPs (Thermo Fisher Scientific), 1 uM of primers

31sd (5'- TGACAGACTTTTTCGCCGAA- 3') and 31ter (5'- CATTCAGGACAATTCCCGCC- 3'), 1 mM $MgCl_2$ (Thermoscientific, USA), 1 U Taq Polymerase (Thermoscientific, USA), and 100 ng genomic DNA (Garcia-Yoldi et al., 2005; Vizcaino et al., 1997). Cycling conditions were as follows: initial denaturation at 94 ºC for 5 min, followed by 30 cycles of 58 ºC for 30 s, 70 ºC for 30 s, and 94 ºC for 30 s, then 58 ºC for 30 s, and finally extension at 70 ºC for 10 min. Each PCR product (5 ul) was cleaved in a 20 ul volume reaction with 5 U of restriction enzyme HaeIII (BsuRI) (Thermo Fisher Scientific) for 3 h at 37 ºC according to the manufacturer's instructions. Both PCR products and restriction fragments were run on 1.5% agarose gel in 1x TBE and stained with 0.5 ug/ul EtBr at 120 V for 1 h. As size markers, 500 bp and 100 bp ladders were used.

3.6. PCR-RFLP of *omp2a* gene

PCR was performed on sequenced isolates in a 50 ul final reaction volume containing 1x PCR buffer (Thermoscientific, USA), 2 mM $MgCl_2$ (Thermoscientific, USA), 0.15 mM dNTPs (Thermo Fisher Scientific), 2.5 U Taq polymerase (Thermoscientific, USA), 20 pmol of primers 2aA (5'- GGCTATTCAAAATTCTGGCG- 3') and 2aB (5'- ATCGATTCTCACGCTTTCGT- 3') and 0.5 ug of template DNA (Cloeckaert et al., 1995; Mirnejad et al., 2013). Cycling conditions were as follows: pre-denaturation step for 5 min at 94 ºC, followed by 35 cycles of denaturation at 94 ºC for 60 s, annealing at 50 ºC for 120 s, and extension at 72 ºC for 180 s, and finally an extension step at 72 ºC for 7 min. PCR products were ran on 1% agarose gel in 1x TBE and stained with 0.5

ug/ul EtBr at 80 V for 30 min. PCR product (7 ul) was cleaved in a 15 ul reaction volume containing 0.3 ul (10-20 U) of HinfI restriction enzyme (Thermo Fisher Scientific), 6 ul nuclease free water and 1.7 ul enzyme buffer at 37 ºC for 2 h. Restriction fragments were run on 2% agarose gel in 1x TBE and stained with 0.5 ug/ul EtBr at 120 V for 1 h. Size markers (500 bp and 100 bp ladders) were used.

3.7. Genome Sequencing

One ng of genomic DNA (gDNA) from each isolate was used as input for library preparation using the Nextera XT library prep kit (Illumina, Inc., San Diego, CA). The subsequent clean up steps were performed using the AMPure XP PCR purification beads (Agencourt, Brea, CA) following manufacturer's instructions. The resulting library was quantified by quantitative PCR in triplicate at 1:1000 and 1:2000 and using the Kapa library quantification kit (Kapa Biosystems, Woburn, MA) and as recommended by the manufacturer's protocol. The samples were pooled together and then sequenced on an Illumina Miseq for paired-end 250-bp reads.

3.8. Analysis of sequencing results

3.8.1. Assembly of the genome

After sequencing, *de novo* genome assembly was done using A5-miseq assembly pipeline keeping default parameters (Tritt et al., 2012). Data cleaning, error correction, contig assembly, scaffolding and quality control processes are all automated by this pipeline.

3.8.2. Annotation of the genome using RAST

The resulting *de novo* assemblies were annotated using the RAST server (http://rast.nmpdr.org) (Aziz et al., 2012). This service determines subsystems represented in the genome, protein-coding, tRNA, and rRNA genes and assigns their functions (Larsen et al., 2012).

3.8.3. Species Identification

KmerFinder tool was used to predict the species of bacteria based on the number of co-occurring k-mers in DNA (www.genomicepidemiology.org), which are substrings of k nucleotides found in the subsequent DNA sequence (Hasman et al., 2013). Genomes were also blasted on NCBI and RAST was used to determine closest neighbors (http://rast.nmpdr.org) (Aziz et al., 2012).

3.8.4. Detection of virulence and resistance determinants

To study virulence and resistance, the SEED viewer service from the RAST server was used in addition to the VFDB tool on the Online Analysis Tools website (http://molbiol-tools.ca/Genomics.htm) (Chen et al., 2012). The Brucella Bioinformatics Portal (BBP) which is a web-based portal for *Brucella* genome annotation was also used (Xiang et al., 2006).

3.8.5. Identification and localization of T4SS components and flagellar genes

T4SS and flagellar gene components were annotated using RAST and also, core components of the T3SS and T4SS were searched using the T346Hunter application (Martinez-Garcia et al., 2015).

3.8.6. Detection of phages and mobile elements

Phage Search Tool (PHAST) (http://phast.wishartlab.com/index.html) (Zhou et al., 2011) was used to detect phages, sites of integration and closest identity match.

3.8.7. Detection of CRISPRs

CRISPR loci were identified using CRISPRfinder program (http://crispr.u-psud.fr/Server/) (Grissa et al., 2007).

3.8.8. Detection of insertion sequences

Insertion sequences were detected using Biotoul IS-Finder (http://www-is.biotoul.fr/) (Siguier et al., 2006).

3.8.9. Detection of Genomic Islands

IslandViewer (http://pathogenomics.sfu.ca/islandviewer) (Dhillon et al., 2015) was used for the identification of pathogenicity islands. It predicts genomic islands by using three different tools: SIGI-HMM, IslandPath-DIMOB, and IslandPick.

3.8.10. Construction of circular genomes

Based on sequence analysis results, CGview Server V 1.0 (Grant & Stothard, 2008) was used to visualize the sequence feature information, and circular genome representation of sequenced isolates was obtained through comparison with *B. melitensis* 16M reference genome (accession #: NC_003317 and NC_003318) (DelVecchio et al., 2002b). DNAPlotter (Carver et al, 2009) was used for circular genome maps visualization.

3.8.11. Construction of phylogenetic tree

To determine the phylogenetic relationship of our strains, 37 concatenated marker genes were used to construct a maximum-likelihood tree using seven *B. melitensis* reference genomes (16M, ATCC 23457, Ether, NI, M5-90, M28, and strain 63/9), plus other *Brucella* species (*B. abortus* 2308 and strain 9-941, *B. canis*, *B. ceti*, *B. pinnipedialis*, *B. microti*, *B. suis*, *B. ovis* and *B. vulpis*). *Ochrobactrum anthropi* was used as an outgroup to root the tree. First, the genomes were processed with PhyloSift (Darling et al, 2014), then FastTree was used to construct the tree (Price et al, 2010), and Dendroscope (Huson & Scornavacca, 2012) was used to edit and visualize the tree. While constructing trees based on individual genes in Phylosift will give congruent results, their concatenation helps improve rare organism detection among microbial organisms and give a more accurate estimate of phylogenetic history (Darling et al., 2014).

Chapter Four

RESULTS

4.1. 16S rRNA Sequencing

16S rRNA gene was sequenced in all the isolates. 16S rRNA sequencing confirmed that all the isolates belonged to *Brucella* genus. Top three blast results for each isolate are represented in Table 2.

Table 2. Top three NCBI Blast results using 16S rRNA database for all thirty-three *Brucella* isolates.

Isolate #	Blast result
3, 4, 6, 8, 9, 10, 11, 13, 14, 15, 16, 17, 18, 19, 20, 21, 23, 24, 25, 26, 27, 29, 30, 31, 32, 33, 34, 35, 36	*Brucella ceti* 16S ribosomal RNA, complete sequence
	Brucella suis 1330 16S ribosomal RNA gene, complete sequence
	Brucella ovis strain ATCC 25840 16S ribosomal RNA gene, complete sequence
5, 7, 22, 28	*Brucella ceti* 16S ribosomal RNA, complete sequence
	Brucella suis 1330 16S ribosomal RNA gene, complete sequence
	Brucella melitensis biovar abortus 2308 strain 2308 16S ribosomal RNA, complete sequence

4.2. Bruce-Ladder Multiplex PCR

In all *Brucella* isolates a transcriptional regulator belonging to CRP family (152 bp), immunodominant antigen *bp26* (450 bp), erythritol catabolism gene *eryC* (587 bp) and polysaccharide deacetylase (794 bp) were amplified (Figure 2).

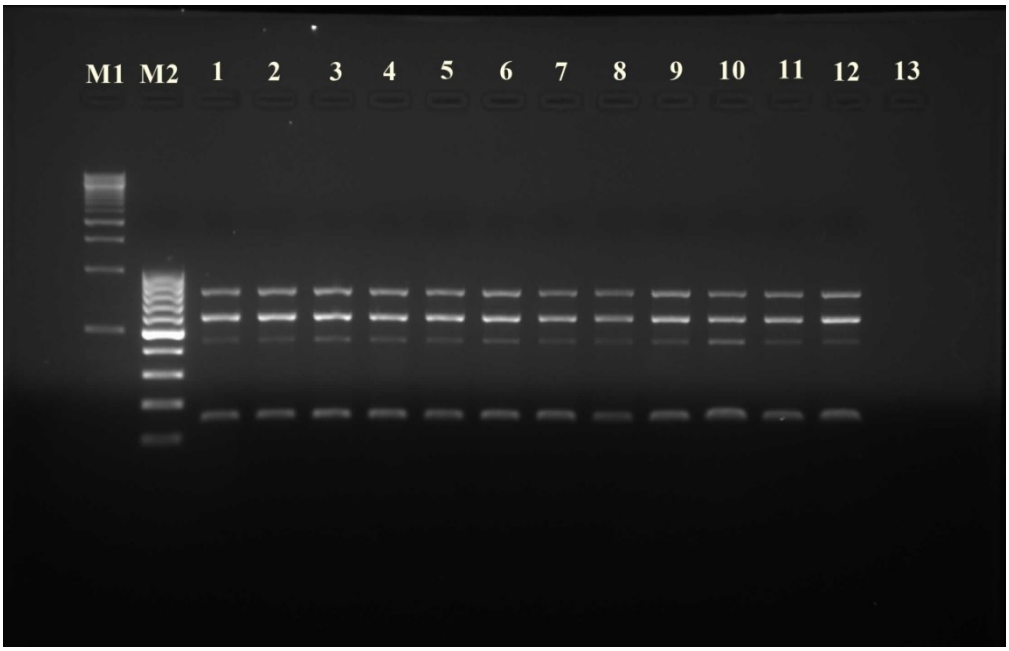

Figure 2. Agarose gel electrophoresis of PCR amplified products generated from DNA samples after Bruce-ladder multiplex PCR. Lanes M1 and M2 are 500 bp and 100 bp ladders respectively, lanes 1-12 show PCR products obtained with *Brucella* isolates, and lane 13 is negative control.

4.3. PCR-RFLP of *omp31* gene

omp31 gene (900 bp) was found in all isolates (Figure 3), and the P1 pattern was obtained as a result of enzymatic digestion with HaeIII enzyme. P1 pattern consisted of three bands with the sizes being around 210 bp, 280 bp, and 320 bp (Figure 4).

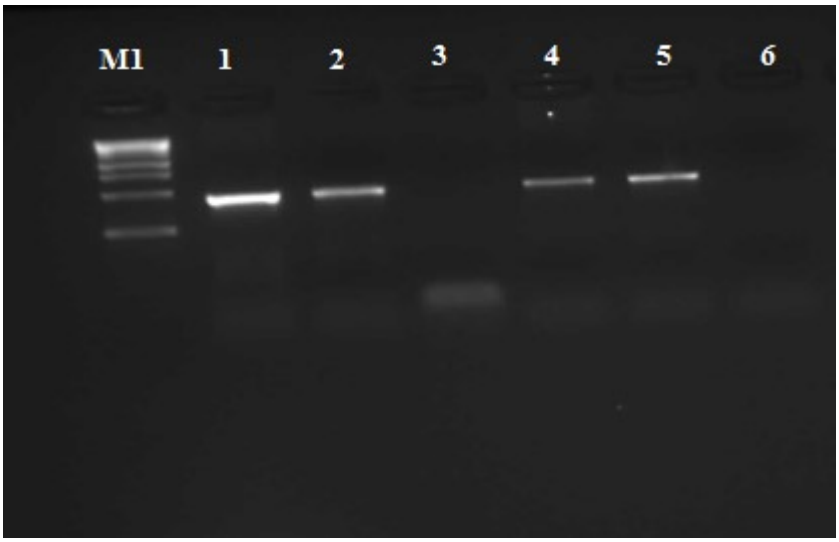

Figure 3. Agarose gel electrophoresis of PCR amplified *omp31* genes generated from DNA samples. Lane M1 is the 500 bp ladder, lanes 1, 2, 4, 5 show PCR products obtained with *Brucella* isolates, and lanes 3, 6 are negative controls.

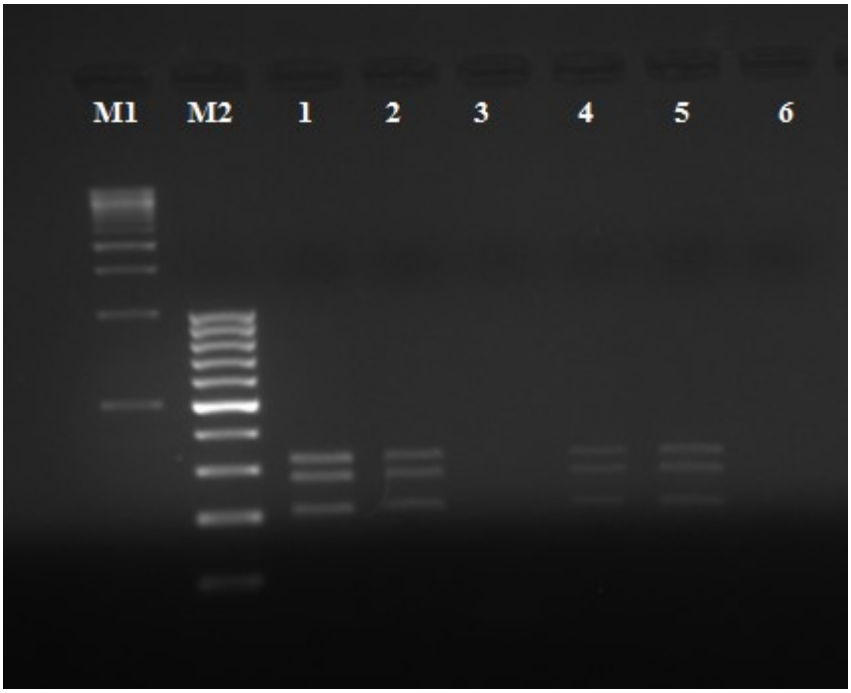

Figure 4. Restriction patterns of PCR amplified *omp31* genes digested by HaeIII enzyme. Lane M1 and M2 are the 500 bp and 100 bp ladders respectively, lanes 1, 2, 4, 5 show restriction products obtained with *Brucella* isolates, and lanes 3, 6 are negative controls.

4.4. PCR-RFLP of *omp2a* gene

In all sixteen sequenced isolates, the *omp2a* gene was detected (1100 bp) (Figure 5), and P2 pattern was obtained after enzymatic digestion with Hinf1 enzyme. P2 pattern consisted of three bands of sizes around 210 bp, 270 bp and 550 bp (Figure 6).

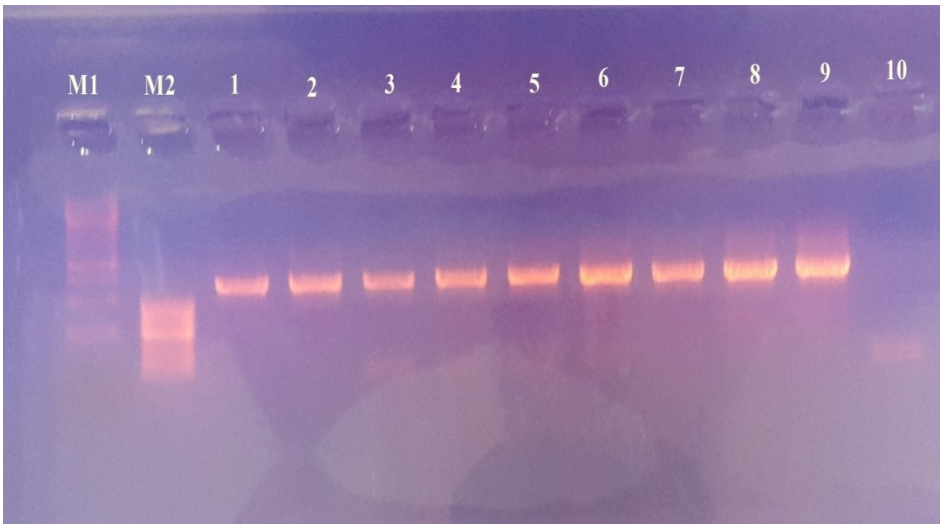

Figure 5. Agarose gel electrophoresis of PCR amplified *omp2a* genes generated from DNA samples. Lanes M1 and M2 are the 500 bp and 100 bp ladders respectively. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9 show PCR products obtained with *Brucella* isolates, and lane 10 is a negative control.

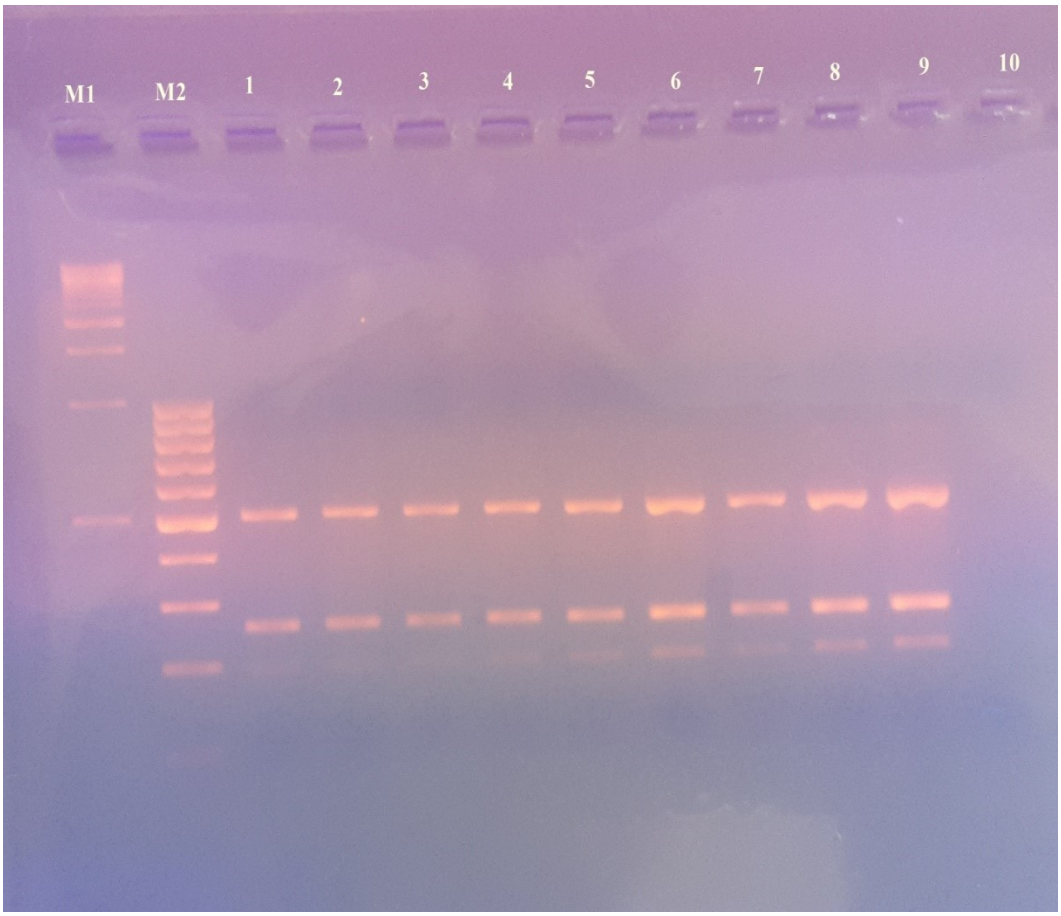

Figure 6. Restriction patterns of PCR amplified *omp2a* genes digested by Hinf1 enzyme. Lanes M1 and M2 are the 500 bp and 100 bp ladders respectively. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9 show restriction products obtained with *Brucella* isolates, and lane 10 is a negative control.

4.5. Whole-genome Sequencing

Bru003, Bru004, Bru008, Bru010, Bru013, Bru014, Bru015, Bru018, Bru025, Bru026, Bru027, Bru029, Bru030, Bru034, Bru035, Bru036 were chosen for whole-genome sequencing, all of which were recovered from blood except Bru027 recovered from ascitic fluid. The libraries were prepared using Nextera XT library prep kit (Illumina, Inc., San Diego, CA), and sequenced on an Illumina Miseq for paired-end 250-bp reads.

The A5-miseq assembly pipeline was used for *de novo* genome assembly (Tritt et al., 2012).

4.6. Genome Statistics

The average detected genome size was 3,297,621 bp (ranging from 3,290,179 bp to 3,322,963 bp). Number of contigs ranged from 34 to 80. The G + C content was 57.2 ± 0.1%, and the number of detected subsystems was 434 ± 4. The average number of coding sequences was 3327 (ranging from 3314 to 3339), with 56 RNAs being detected in all sixteen isolates (Table 3).

Table 3. Comparative genomic statistics of sixteen sequenced *Brucella* genomes.

Sample	Bru 004	Bru 008	Bru 010	Bru 013	Bru 018	Bru 026	Bru 029	Bru 034
Size (bp)	3298639	3298358	3296555	3305257	3300556	3296220	3294296	3296602
Number of contigs	58	59	55	58	70	53	41	53
G + C Content (%)	57.2	57.2	57.2	57.2	57.3	57.2	57.2	57.2
Number of subsystems	434	434	434	433	435	434	434	434
Number of coding sequences	3337	3329	3334	3335	3338	3325	3339	3323
Number of RNAs	56	56	56	56	56	56	56	56
Sample	**Bru 003**	**Bru 014**	**Bru015**	**Bru 025**	**Bru 027**	**Bru 036**	**Bru 030**	**Bru 035**
Size (bp)	3290766	3291305	3322963	3290178	3290853	3295865	3297707	3295809
Number of contigs	37	34	80	34	36	45	57	46
G + C Content (%)	57.2	57.2	57.1	57.2	57.2	57.2	57.2	57.2
Number of subsystems	436	436	438	436	436	436	434	434
Number of coding sequences	3327	3314	3329	3318	3317	3321	3320	3320
Number of RNAs	56	56	56	56	56	56	56	56

4.7. Determination of species of *Brucella*

All sixteen sequenced genomes were confirmed to be *B. melitensis* using KmerFinder tool along with NCBI Blast and RAST.

4.8. Genome annotation using RAST

Figure 7 represents the distribution of genes assigned to subsystems using RAST. Among SEED subsystem categories, genes coding for amino acids and derivatives (416 ORFs), carbohydrates (356 ORFs), cofactors, vitamins, prosthetic groups, and pigments (265 ORFs), and protein metabolism (248 ORFs) were abundant.

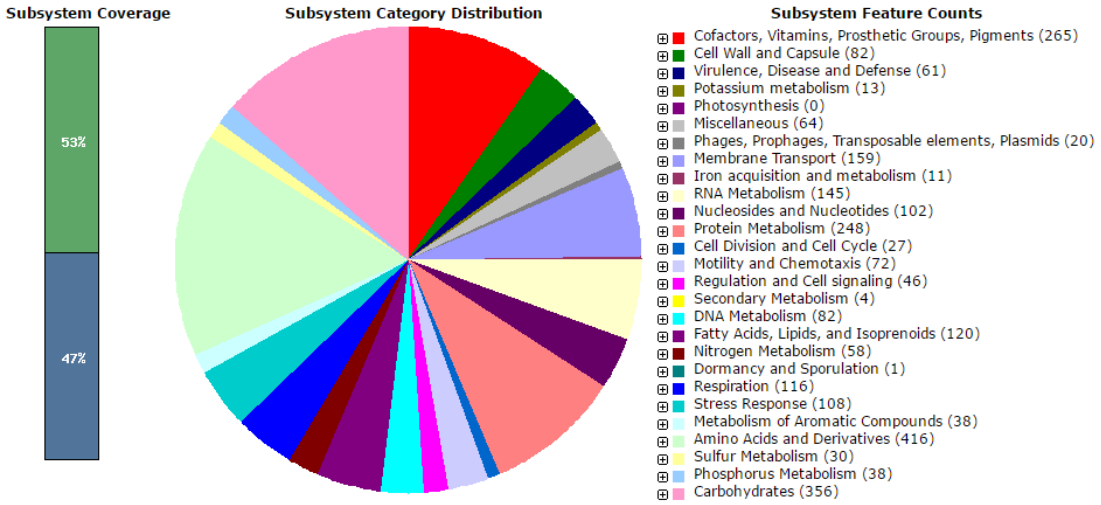

Figure 7. Distribution of subsystem categories of our genomes based on SEED databases.

The main virulence factors were classified into genes involved in secretion systems (T4SS and flagellar genes), LPS related genes involved in biosynthesis of O-antigen (genes encoding phosphomannomutase, mannose 6-phosphate isomerase, and mannose guanylyltransferase), ureases (*ureA*, *ureB*, *ureC*, *ureD*, *ureE*, *ureF*, and *ureG*) , BvrR/S two component system (encoded by *chvI* and *chvG*), regulation of gene expression (*hfq*), cyclic β-1,2-glucan *cgs* (*ndvb*), and stringent response (*spoT*/*rsh*).

4.9. Resistance profiles

The antimicrobial susceptibility testing was performed using the disk diffusion method for: ofloxacin, minocycline, rifampicin, tetracycline, gentamicin, ciprofloxacin, and sulfamethoxazole/trimethoprim (Hamzeh, personal communication). All isolates were susceptible to tetracyclines (minocycline and tetracycline), aminoglycosides (gentamicin), and rifamycins (rifampicin). Resistance to ofloxacin (fluoroquinolones) was detected in Bru007 and Bru028, whereas Bru029 was resistant to ciprofloxacin (Hamzeh, personal communication). The NorMI efflux pump which confers resistance to fluoroquinolones, in addition to *gyrA*, *gyrB*, *parC*, and *parE*, were all detected in the sequenced genomes. The former genes encode: DNA gyrase subunit A, DNA gyrase subunit B, DNA topoisomerase IV subunit A, and DNA topoisomerase IV subunit B which also play a role in fluoroquinolone resistance. Resistance to trimethoprim and sulfonamide was detected in 14 isolates (Bru003, Bru014, Bru015, Bru017, Bru019, Bru020, Bru021, Bru022, Bru023, Bru024, Bru028, Bru030, Bru031 and Bru032) (Hamzeh, personal communication), which carried the RND efflux pumps (confer resistance to trimethoprim).

4.10. Detection of phages and mobile elements

At least two prophages were detected per sequenced genome ranging in size between 6.1 and 60.4 kb. An intact 13.7kb phage (Paraco_vB_Pmas) and a 17.4kb incomplete phage (Acanth_moumouvirus) were present in all except for Bru013 and Bru018, which were negative for the Paraco_vB_Pmas phage and the Acanth_moumouvirus phage, respectively. Staphy_SPbeta_like phage was detected in isolates Bru003, Bru025 and

Bru036. However, phages Plankt_PaV_LD, Lactoc_jj50, Lactob_prophage_Lj965, Salmon_ST64B, and Pandor_inopinatum were only detected in Bru010, Bru015, Bru035, Bru013 and Bru018 respectively (Table 4).

Table 4. Phage associated regions in *Brucella* genomes.

Isolate	Related phages	Size (kb)	Status	Score	CDS	GC content (%)
Bru 003	Acanth_moumouvirus	17.4 kb	Incomplete	20	14	58.06%
	Paraco_vB_Pmas	13.7 kb	Intact	140	18	61.17%
	Staphy_SPbeta_like	38.8 kb	Intact	150	27	53.49%
Bru 004	Acanth_moumouvirus	22.6 kb	Incomplete	20	14	58.15%
	Paraco_vB_Pmas	13.7 kb	Intact	140	19	61.19%
Bru 008	Acanth_moumouvirus	17.4 kb	Incomplete	20	14	58.06%
	Paraco_vB_Pmas	13.7 kb	Intact	140	18	61.19%
Bru 010	Paraco_vB_Pmas	13.7 kb	Intact	140	18	61.19%
	Plankt_PaV_LD	6.1 kb	Incomplete	40	7	56.41%
	Acanth_moumouvirus	17.4 kb	Incomplete	20	14	58.06%
Bru 013	Acanth_moumouvirus	17.4 kb	Incomplete	20	14	58.05%
	Salmon_ST64B	24.1 kb	Intact	150	35	58.41%
Bru 014	Acanth_moumouvirus	17.4 kb	Incomplete	20	14	58.05%
	Paraco_vB_Pmas	17 kb	Intact	150	22	60.39%
Bru 015	Acanth_moumouvirus	17.4 kb	Incomplete	20	14	58.06%
	Paraco_vB_Pmas	13.7 kb	Intact	140	19	61.17%
	Lactoc_jj50	60.4 kb	Intact	150	62	47.21%
Bru 025	Acanth_moumouvirus	17.4 kb	Incomplete	20	14	58.06%
	Paraco_vB_Pmas	13.7 kb	Intact	140	18	61.19%
	Staphy_SPbeta_like	33.1 kb	Intact	140	30	53.20%
Bru 026	Acanth_moumouvirus	22.6 kb	Incomplete	20	14	58.15%
	Paraco_vB_Pmas	13.7 kb	Intact	140	18	61.19%
Bru 027	Acanth_moumouvirus	17.4 kb	Incomplete	20	14	58.06%
	Paraco_vB_Pmas	13.7 kb	Intact	140	19	61.19%
Bru 029	Acanth_moumouvirus	22.6 kb	Incomplete	20	14	58.15%
	Paraco_vB_Pmas	13.7 kb	Intact	140	18	61.19%
Bru 030	Acanth_moumouvirus	22.6 kb	Incomplete	20	14	58.15%
	Paraco_vB_Pmas	13.7 kb	Intact	140	18	61.19%
Bru 034	Acanth_moumouvirus	22.6 kb	Incomplete	20	14	58.14%
	Paraco_vB_Pmas	24.1 kb	Intact	150	32	58.44%
Bru 035	Acanth_moumouvirus	22.6 kb	Incomplete	20	14	58.15%

	Paraco_vB_Pmas	13.7 kb	Intact	140	18	61.19%
	Lactob_prophage_Lj965	9.7 kb	Incomplete	40	8	51.22%
Bru 036	Acanth_moumouvirus	17.4 kb	Incomplete	20	14	58.06%
	Paraco_vB_Pmas	13.7 kb	Intact	140	18	61.19%
	Staphy_SPbeta_like	20.4 kb	Intact	120	23	52.47%
Bru 018	Pandor_inopinatum	9 kb	Incomplete	30	12	54.30%
	Paraco_vB_Pmas	13.6 kb	Intact	140	19	61.11%

Figures 8 and 9 are graphical presentations for Acanth_moumouvirus and Paraco_vB_Pmas phages detected in Bru003, where the figures show the number of coding DNA sequences, GC content, location, and predicted prophage status in addition to a legend at the bottom representing the types of coding sequences identified. A circular graphical representation showing the location of the three detected phages in Bru003 is shown in Figure 10.

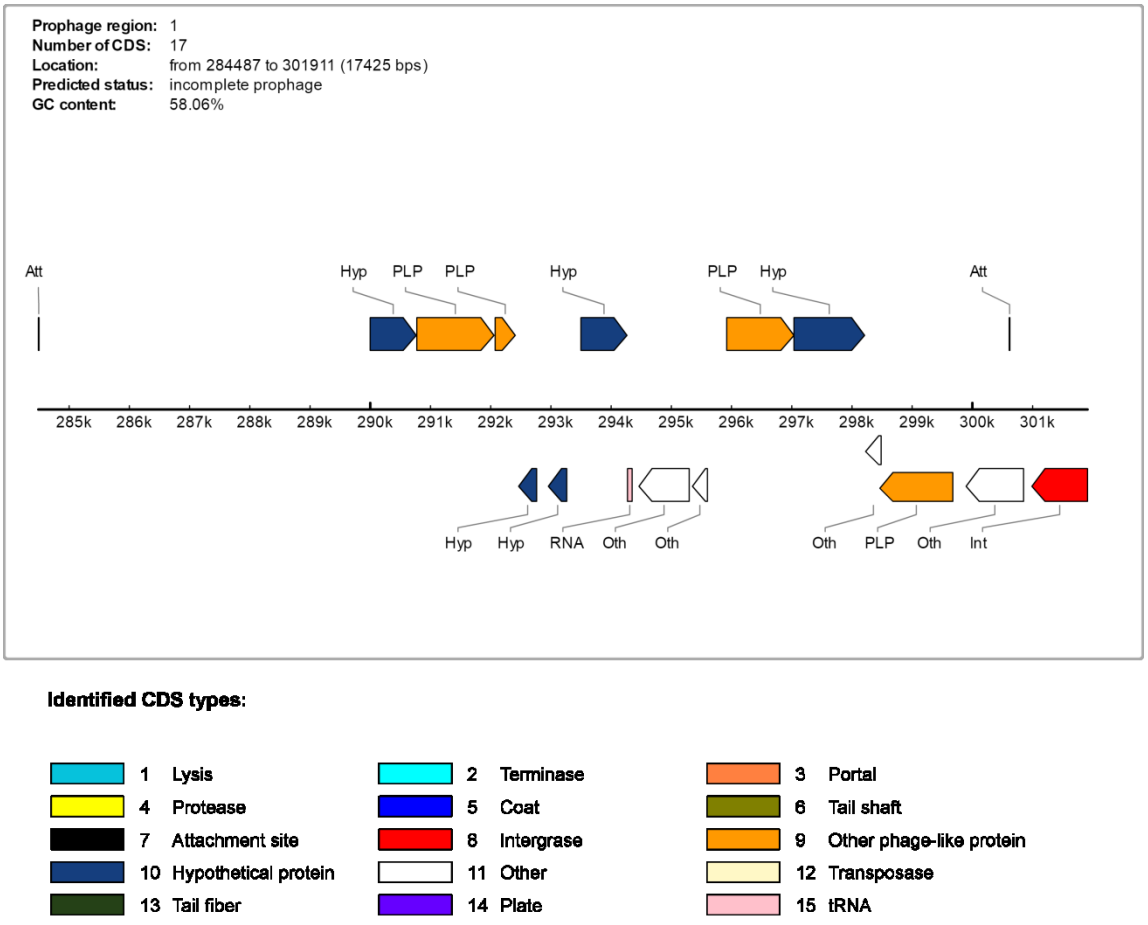

Figure 8. Linear graphical representation of Acanth_moumouvirus phage in Bru003.

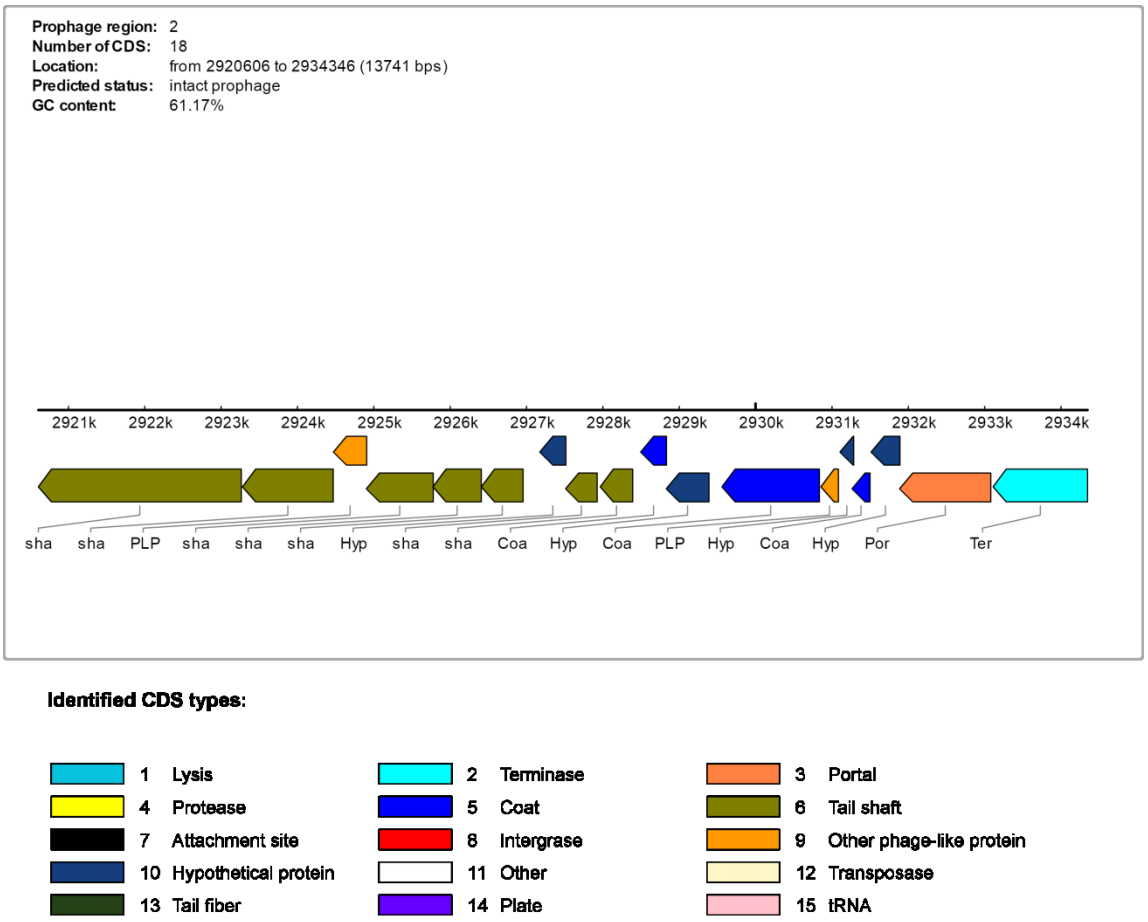

Figure 9. Linear graphical representation of Paraco_vB_Pmas phage in Bru003.

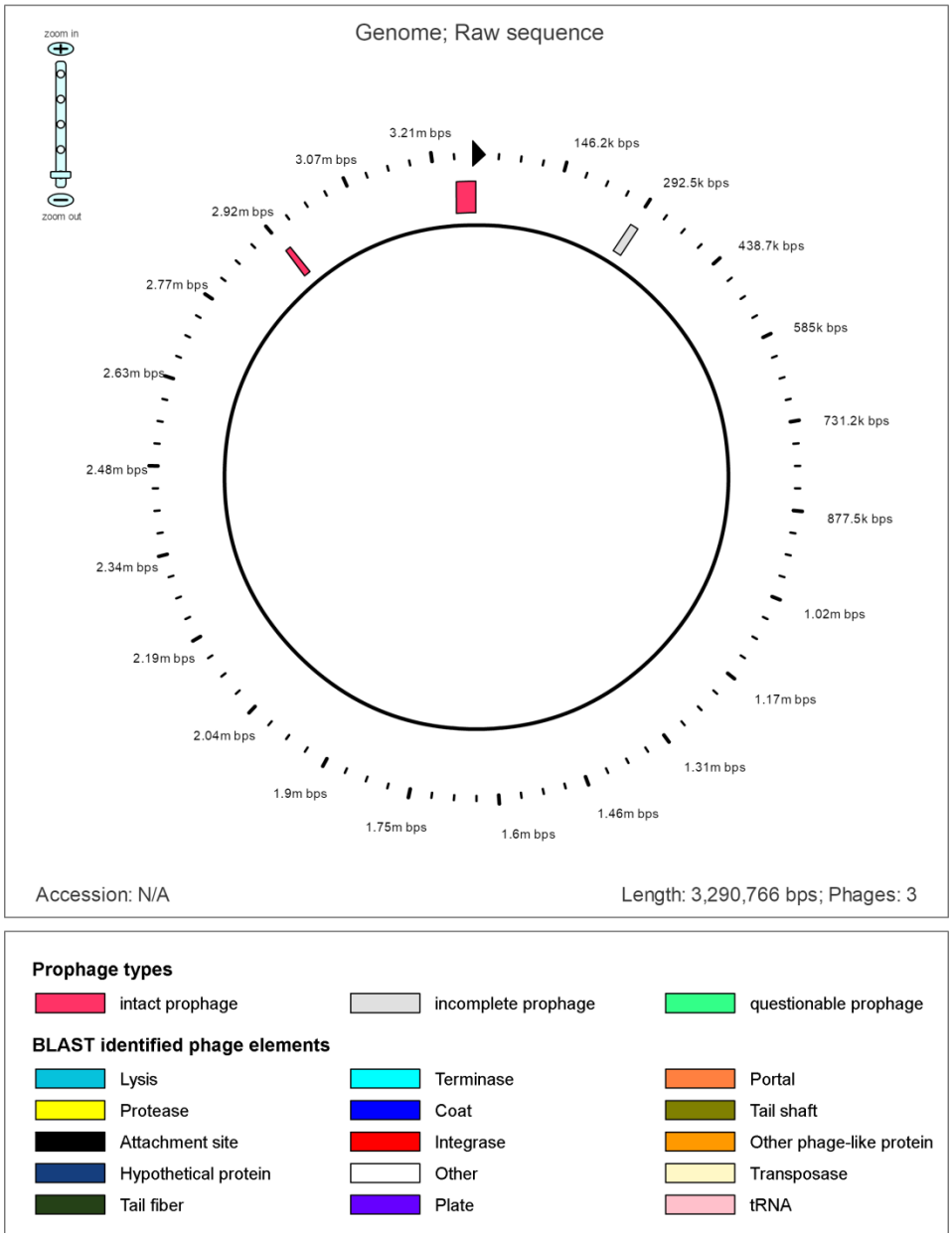

Figure 10. Circular graphical representation showing the location of the three detected phages in Bru003.

4.11. Detection of genomic islands

Islandviewer 3 was used to detect genomic islands aligning the genomes with reference strain *B. melitensis* 16M. LPS related genes, exonucleases, outer membrane proteins were found in several islands detected in the sequenced isolates (Table 5). Additionally, genes from *virB* operon (*virB2*, *virB3*, *virB4*, *virB5*, *virB6*, and *virB8*) involved in type IV secretion system were detected in Bru015.

Table 5. Genes found on genomic islands detected by Islandviewer3.

	wbkE	***per***	***gmd***	***wboA***	***wboB***	***wbkC***	***wbkA***	**Outer membrane protein**	**Exonuclease**
Bru003	-	+	+	+	+	+	+	-	-
Bru004	-	+	+	+	+	+	+	-	-
Bru008	+	+	+	+	+	+	+	+	-
Bru010	-	+	+	+	+	+	+	-	-
Bru013	-	+	+	+	+	+	+	-	+
Bru014	-	+	+	+	+	+	+	+	+
Bru015	-	+	+	+	+	+	+	+	+
Bru018	-	+	+	+	+	+	+	-	-
Bru025	+	+	+	+	+	+	+	-	-
Bru026	-	+	+	+	+	+	+	+	-
Bru027	-	+	+	+	+	+	+	-	-
Bru029	-	+	+	+	+	+	+	+	+
Bru030	-	+	+	+	+	+	+	-	-
Bru034	-	+	+	+	+	+	+	-	-
Bru035	-	+	+	+	+	+	+	-	+
Bru036	-	+	+	+	+	+	+	-	-

4.12. CRISPR detection

All sixteen sequenced isolates were shown to have one possible CRISPR with a size of 82 bp containing one spacer.

4.13. Identification and localization of T4SS and flagellar components

For T4SS components, *virB* 1-11 were detected in all the sixteen sequenced genomes using RAST and T346 Hunter. The components of T4SS were within one gene cluster. Table 6 shows the eleven core components of T4SS and their corresponding annotations. A linear graphical representation of T4SS core components of Bru014 was constructed using T346Hunter, where predicted ORFs of the core components are shown in red (Figure 11). Three gene clusters linked to flagellar components were also detected using T346Hunter. The core components comprising the three gene clusters and their corresponding annotations and functions are shown in Table 7. A linear graphical presentation of one of the three T3SS clusters is displayed in Figure 12, where predicted ORFs of core components are shown in red.

Table 6. Core components of T4SS.

Gene	Corresponding protein
virB1	Peptidoglycan hydrolase involved in T-DNA transfer
virB2	Major pilus subunit of type IV secretion complex
virB3	Inner membrane protein forms channel for type IV secretion of T-DNA complex
virB4	ATPase required for both assembly of type IV secretion complex and secretion of T-DNA complex
virB5	Minor pilin of type IV secretion complex
virB6	Inner membrane protein of type IV secretion of T-DNA complex
virB7	Lipoprotein of type IV secretion complex that spans outer membrane and periplasm
virB8	Inner membrane protein forms channel for type IV secretion of T-DNA complex
virB9	Outer membrane and periplasm component of type IV secretion of T-DNA complex, has secretin-like domain
virB10	Inner membrane protein of type IV secretion of T-DNA complex, TonB-like
virB11	ATPase required for both assembly of type IV secretion complex and secretion of T-DNA complex

Table 7. Flagellar genes detected using T346Hunter.

Gene	Corresponding protein
flip	Flagellar biosynthesis protein FliP
flgI	Flagellar P-ring protein FlgI
flgA	Flagellar basal-body P-ring formation protein FlgA
flgG	Flagellar basal-body rod protein FlgG
fliE	Flagellar hook-basal body complex protein FliE
flgC	Flagellar basal-body rod protein FlgC
flgB	Flagellar basal-body rod protein FlgB
fliI	Flagellum-specific ATP synthase FliI
flgF	Flagellar basal-body rod protein FlgF
motA	Flagellar motor rotation protein MotA
fliM	Flagellar motor switch protein FliM
fliN	Flagellar motor switch protein FliN
fliG	Flagellar motor switch protein FliG
flhB	Flagellar biosynthesis protein FlhB
fliR	Flagellar biosynthesis protein FliR
flhA	Flagellar biosynthesis protein FlhA
fliQ	Flagellar biosynthesis protein FliQ
flgD	Flagellar basal-body rod modification protein FlgD
flgL	Flagellar hook-associated protein FlgL
flgK	Flagellar hook-associated protein FlgK
flgE	Flagellar hook protein FlgE
fliK	Flagellar hook-length control protein FliK
motB	Flagellar motor rotation protein MotB
fliF	Flagellar M-ring protein FliF
fliC	Flagellin

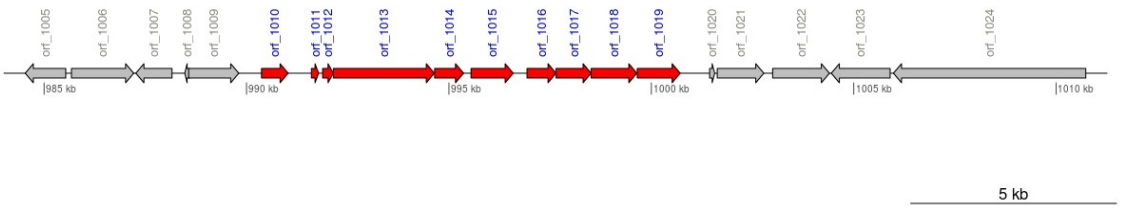

Figure 11. Graphical linear presentation of ORFs constituting the core components of T4SS in Bru014.

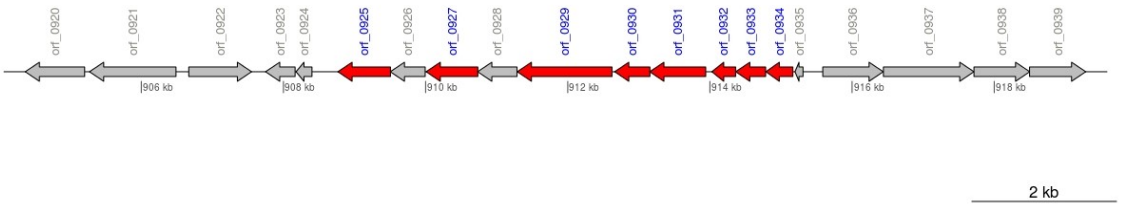

Figure 12. Graphical linear presentation of eight ORFs constituting the core components of T3SS cluster one in Bru014.

4.14. Detection of insertion sequences

ISL3, IS3, IS481, IS711 and IS5 were detected using IS Finder in all sixteen sequenced genomes. IS66 was also detected in Bru018, Bru029 and Bru035 and IS110 in Bru004, Bru008, Bru010, Bru014, Bru029, Bru030 and Bru034. Figure 13 is a representative of the pie chart for Bru004 showing the insertion sequences detected and their corresponding percentages.

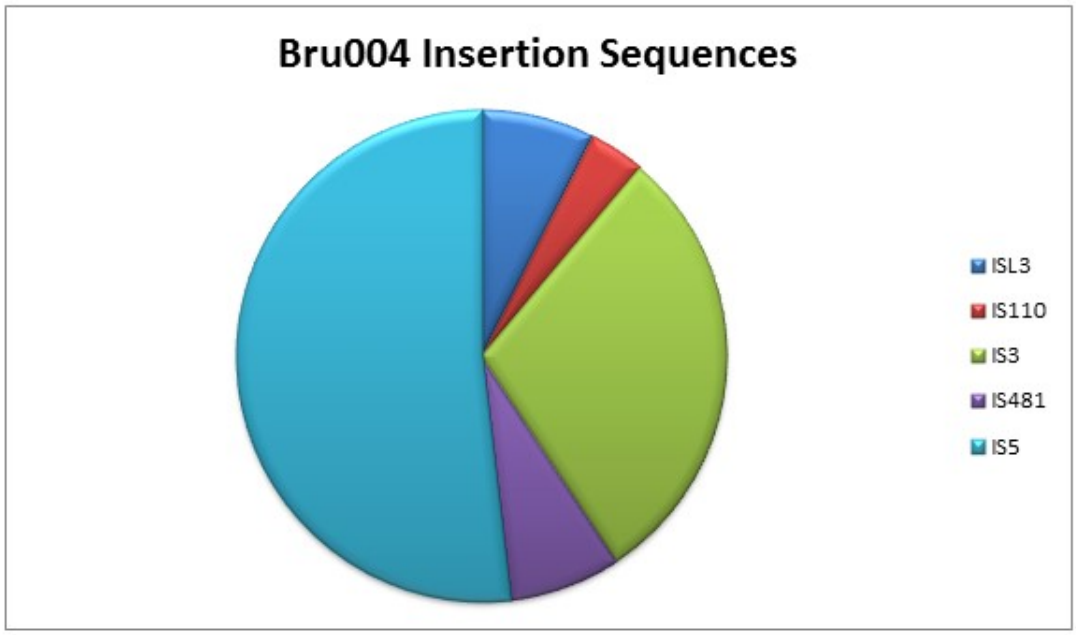

Figure 13. Representative figure of insertion sequences detected in sample Bru004 by IS Finder Tool.

4.15. Phylogenetic analysis

Phylogenetic analysis revealed the clustering of the sequenced isolates in five clades separately from other *B. melitensis* reference strains. Bru003, Bru015 and Bru034 were clustered in the same clade, and distant from the rest of the sequenced *B. melitensis* isolates used in this study, which in turn clustered into 4 clades (Figure 14).

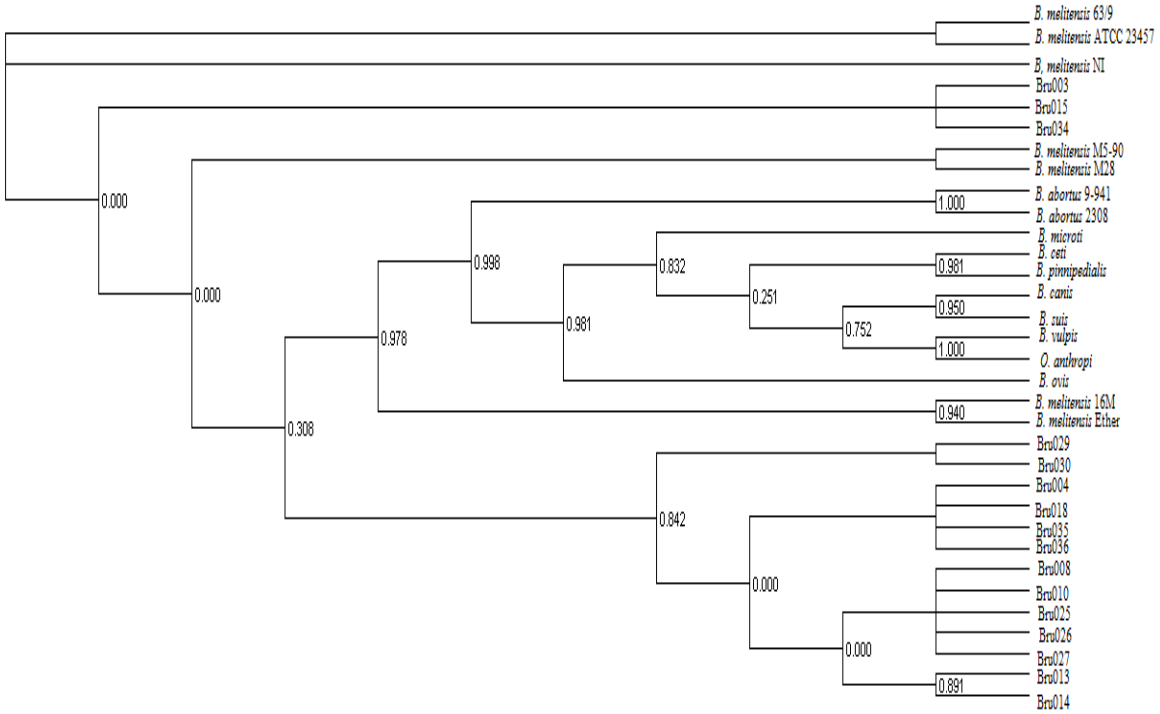

Figure 14. Maximum-likelihood concatenated marker tree of all *Brucella* strains used in this study. After downloading the genomes from the NCBI database, the filenames and sequences were reformatted for easier visualization. Phylosift was then used to screen the assemblies for core marker genes in search and align mode using isolate and best hit flags. PhyloSift concatenates and aligns the hits of interest. The sequences are then extracted from the PhyloSift output files and added to a single file to build the tree.

FastTree was used to infer the maximum-likelihood tree and Dendroscope was used for tree visualization.

4.16. Construction of circular genomes

DNAPlotter was used to generate a circular map of the genomes showing the sequences, tRNAs, and GC plot and skew (Figure 15). CGview on the other hand, aligned regions of the sequenced genomes to *B. melitensis* str. 16M which was used as a reference genome (Figure 16). The circular map shows the sequences, G+C content and skew, and BLAST results against the reference genome.

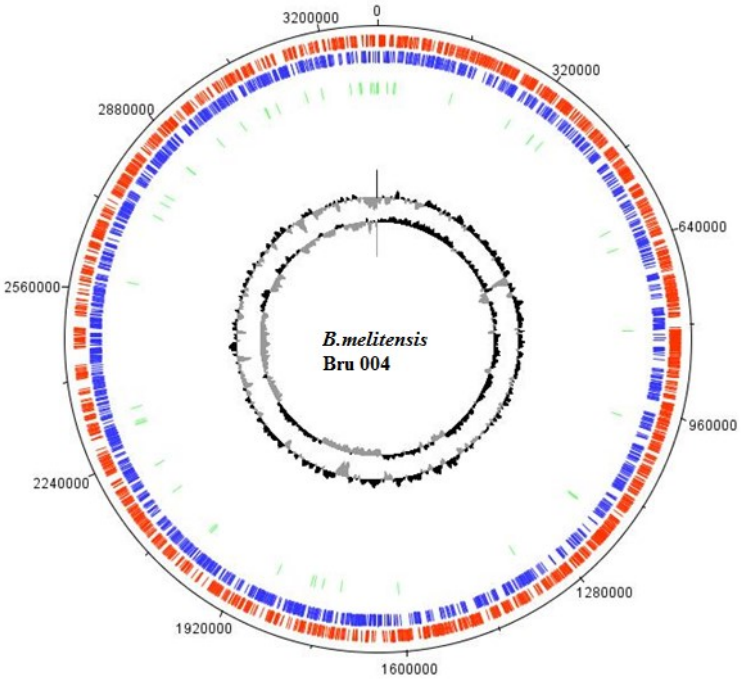

Figure 15. Circular map of Bru004 genome. From outside to inside, the tracks represent: Forward coding sequence (CDS) (red); reverse CDS (blue); tRNAs (green); G+C plot ; G+C skew (black above average and grey below average). DNAPlotter (Carver et al, 2009) was used to generate this image.

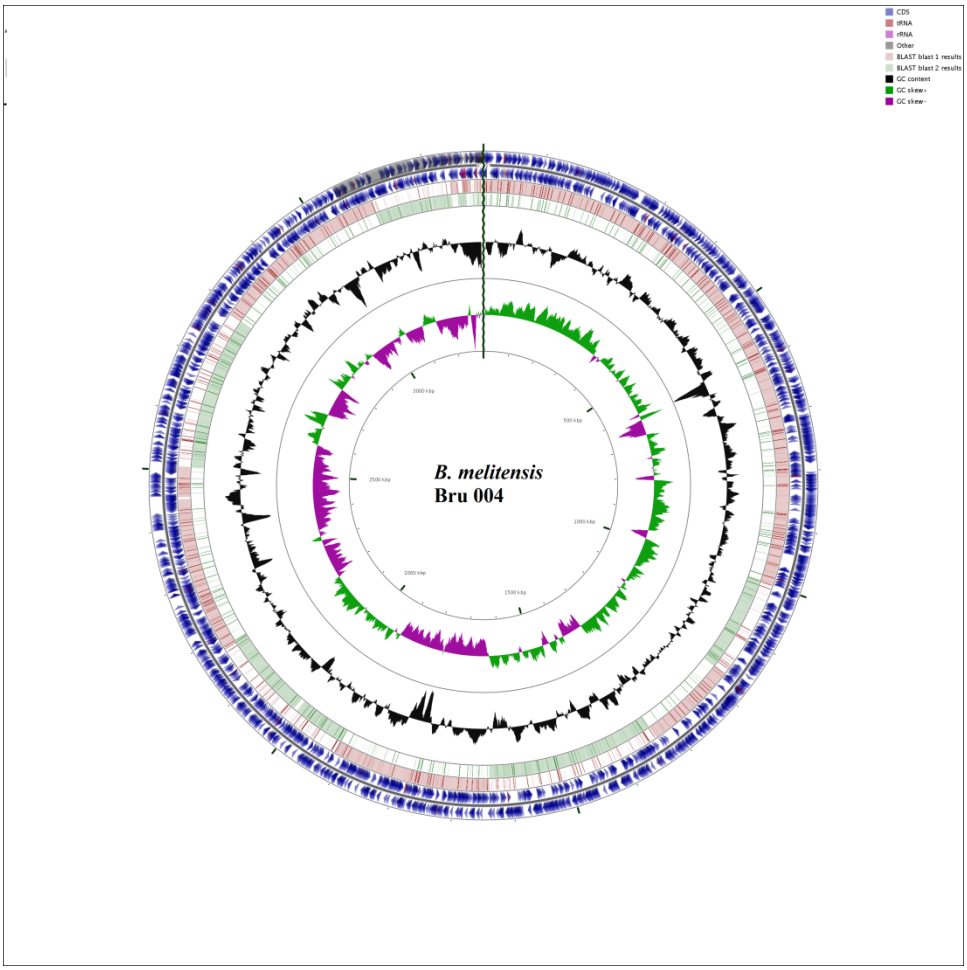

Figure 16. Circular map of Bru004 compared with *B. melitensis* str. 16M reference genome. From outside to inside the rings represent: Bru004 coding DNA sequence (CDS) on the forward and reverse strand (blue), *B. melitensis* 16M chromosome 1 and 2 Blast results (light pink and light green) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green) and G+C negative skew (purple). CGview Server V 1.0 (Grant & Stothard, 2008) was used to construct this image.

Chapter Five

DISCUSSION

B. melitensis is the major cause of brucellosis in the Middle East and Lebanon and mostly isolated from humans (Araj, & Azzam, 1996; Avila-Calderon et al., 2013). This study aimed at analyzing the genomic and functional attributes of sixteen sequenced *Brucella* genomes. In Lebanon, the virulence factors and genotypic characteristics of *Brucella* were not thoroughly identified and there was a lack of data on the pathogenomics of this important human pathogen.

5.1. Typing of *Brucella*

In this study, 16S rRNA sequencing, Bruce-ladder multiplex PCR and PCR-RFLP of *omp31* and *omp2a* gene were performed. 16S rRNA is a rapid test used to confirm *Brucella* at the genus level, but suffers from low accuracy in differentiating isolates at the species level due the low variability in the locus (Gee et al., 2004; Vizcaino et al, 2000). Using 16S rRNA sequencing all isolates were confirmed to belong to the genus *Brucella*. Bruce-ladder multiplex PCR was then performed to further type the organisms at the species level. This PCR assay can further differentiate between species including vaccine strains and ones infecting marine mammals (Lopez-Goni et al., 2008). Four genes were detected in all the isolates including a transcriptional regulator belonging to the CRP family, erythritol catabolism *eryC* gene, polysaccharide deacetylase gene and immunodominant gene *bp26* suggesting that the isolates could be either *B. abortus*, *B.*

melitensis, *B. ovis* or *B. suis*. However, based on the absence of specific genes (Lopez-Goni et al., 2008), we were able to eliminate the following species: *B. canis*, *B. ceti*, *B. pinnipedialis* or *B. neotomae*. The polysaccharide deacetylase gene is absent from *B. canis*, the *bp26* gene is absent from *B. ceti* and *B. pinnipedialis* and the transcriptional regulator belonging to the CRP family is missing in *B. neotomae*, all of which were detected in the isolates undertaken in this study. Accordingly, while the Bruce-ladder approach ruled out *B. canis*, *B. ceti, B. pinnipedialis* and *B. neotomae,* it was not conclusive.

Because of their polymorphism, *Brucella* outer membrane proteins have been used for species and biovars differentiation (Pishva et al., 2015; Vizcaino et al., 1997). Previously, PCR-RFLP analysis of *omp31* gene showed that it was deleted in *B. abortus*. This was confirmed by genome sequencing where a 25-kb deletion was found linked to the absence of *omp31* and other adjacent genes involved in synthesis of polysaccharide compared to other *Brucella* species (Vizcaino et al., 2001a). This method also differentiated *B. suis* biovar 2 from other *Brucella* species (Garcia-Yoldi et al., 2005; Vizcaino et al., 1997). The interest in *omp31* as a candidate for typing comes not only from its polymorphic characteristic but also from the fact that it is an immunodominant antigen and provides protective immunity against *Brucella* infection. It also possesses heme-binding properties and it is induced under conditions of limited iron availability (Delpino et al., 2006; Vizcaino et al., 2001b). Conducting the *omp31* PCR assay on all the isolates revealed that all carried the gene, thus confirming that they don't belong to *B. abortus*. In addition to that, the obtained restriction digestion pattern, P1, using HaeIII

enzyme ruled out *B. suis* biovar 2. *omp2a* is another candidate and target for PCR-RFLP analysis and differentiation of species and biovars of *Brucella* (Cloeckaert et al., 1995; Mirnejad et al., 2013). It is considered as an immunogenic antigen in addition to being a virulence factor (Cloeckaert et al., 1996). P2 pattern was obtained upon restriction digestion of *omp2a* using HinfI enzyme; a pattern usually associated with *B. melitensis* or *B. ovis*. However, through whole-genome sequencing and the use of different online tools (KmerFinder, BLAST and RAST), all sequenced genomes were confirmed to be *B. melitensis*.

5.2. Genome sequencing

5.2.1. Virulence determinants

Brucella lacks the classical virulence determinants usually detected in other pathogens such as cytolysins, exotoxins, fimbriae, plasmids and antigenic variation (Moreno & Moriyon, 2002). The most important virulence determinants that were detected in all our sequenced genomes can be classified into the following categories: genes involved in secretion systems (T4SS and flagellar genes), LPS related genes involved in biosynthesis of O-antigen (*manA*, *manB*, *manC*), ureases (*ureA*, *ureB*, *ureC*, *ureD*, *ureE*, *ureF*, and *ureG*) , BvrR/S two component system (encoded by *chvI* and *chvG*), cyclic β-1,2-glucan *cgs* (*ndvb*), regulation of gene expression (*hfq*), and stringent response (*spoT*/*rsh*).

Secretion systems in *Brucella*, specifically the T4SS and flagellar genes are considered key virulence factors (He, 2012). T4SS is needed for late stages of infection and for

chronic persistence (Hong et al, 2000), through upregulating *omp25* and *omp31* and adapting to a stressful environment (Wang et al., 2009a). The *Brucella* T4SS is intact (Celli et al., 2003), and mediates inflammatory response in addition to vesicle trafficking in the host cell (Ke et al., 2015). These features distinguish *Brucella* from other Gram-negative bacteria, with the T4SS being used for other purposes such as transformation and DNA uptake similar to *Helicobacter pylori* and *Neisseria gonorrhoeae* (Wallden et al., 2010). Components of the *Brucella* T4SS are encoded by the *virB* operon composed of 11 genes (*virB*1-11), which was detected in all sequenced isolates in this study except for *virB7*, encoding lipoprotein of T4SS that spans the outer membrane and periplasm. *virB7*, *virB9*, and *virB10* are part of the outer membrane complex, while the inner membrane complex is composed of *virB3*, *virB4*, *virB6*, *virB8*, and N-terminus of *virB10*. The energy center is composed of *virB4* and *virB11*, while *virB2* is the stretching needle complex and finally remnants of *virB5* and *virB10* constitute the linking stalk (Low et al., 2014, Trokter et al., 2014). *virB1* and *virB7* do not play an important role in *Brucella* virulence as opposed to the other proteins in the system (Ke et al., 2015), but *virB7*'s importance lies in the fact that even though the T4SS is conserved among all *Brucella* spp., the *virB7* in particular is dynamic (Sankarasubramanian et al., 2016). This variability in the presence/absence of *virB7* was observed in different species of *Brucella*; absent in some strains of *B. abortus*, *B. ovis*, *B. canis* and *B. suis* and absent in two strains of *B. melitensis*: strain M28 and M5-90. This however, could additionally indicate that our isolates were closely related to M5-90 and M28 *B. melitensis* strains (Sankarasubramanian et al., 2016).

Despite being non-motile, remnants of genes required for flagellar assembly were detected in all our sequenced isolates distributed into three gene clusters as was previously reported by Fretin et al. (2005). Among those detected were: *fliF* gene encoding a monomer of the MS- ring and regulated by FtcR, which controls flagella expression (Leonard et al., 2007). The hook encoded by *flgE* and the filament by *fliC* gene, which is also in turn responsible for production of flagellin in *B. melitensis* and is regulated by *flbt* (Ferooz et al., 2011). The *motB* encodes a motor rotation protein, *fliN* encodes a motor switch protein, *flhA* and *flhB* encode the export apparatus, *flgD* encodes a hook capping protein, *flgF* encodes for a basal body rod protein and *flgL* encodes a hook-associated protein (Fretin et al., 2005). Chemotactic proteins however, such as CheA, CheB, CheW, CheR, were all absent from the sequenced genomes in this study, which further confirms that the flagellar system in *Brucella* is cryptic and only contains remnants of genes (Pallen & Matzke, 2006). What distinguished this flagellar apparatus in *Brucella* from other non-motile organisms is the fact that the latter don't utilize it for infection, whereas in *Brucella* it is considered as a key virulence factor (Fretin et al., 2005). In *B. melitensis*, the distinguishing feature is that those set of genes code for a sheathed flagellum, which is expressed transiently under certain conditions and causes persistent infection, unlike its use in other species (Leonard et al., 2007). This all illustrates that although *Brucella* has lost motility due to its evolution from inhabiting soil to one that survives intracellularly, but the system is not only important for motility but also for persistence and infection (Pallen & Matzke, 2006).

Another aspect of *Brucella* virulence is the BvrR/S two-component system, where *chvI* codes for a response regulator and *chvG* encodes for the sensor histidine kinase (Rossetti et al., 2011). Both were detected in all the sequenced genomes in this study, coding for the homologs of BvrR/BvrS TCS found in *B. abortus*. This system's significance lies in its ability to upregulate the outer membrane proteins needed to penetrate host cells (Omp22 and Omp25), and genes in the *virB* operon (*virB1*, *virB5*, *virB8*, and *virB9*), thus acting as a transcriptional regulator (Lamontagne et al., 2007; Martinez-Nunez et al., 2010). Therefore, a successful intracellular infection in host cells and proper vacuolar maturation is ensured through this two component system and its subsequent interaction with the T4SS components mentioned earlier (Lopez- Goni et al., 2002; Martinez-Nunez et al., 2010).

Moreover, *ureA*, *ureB*, *ureC*, *ureD*, *ureE*, *ureF*, and *ureG* were all found in the sequenced *B. melitensis* in this study, encoding for two urease operons. The *ureD*, *ureE*, *ureF* and *ureG* are accessory genes involved in the assembly of ureases (Paulsen et al., 2002; Sangari el al., 2007). What distinguished *B. melitensis* ureases from ureases in other *Brucella* species, is the fact that they are needed for the successful establishment of an infection in the digestive tract instead of the oral cavity as in *B. abortus* (Paixao et al., 2009), and it does so by allowing *B. melitensis* to withstand the harsh, acidic environment (Seleem et al., 2008).

rsh gene encoding a homologue of *spoT* gene found in *E. coli* was also detected and it encodes a ppGpp (guanosine pentaphosphate) synthetase (Mittenhuber, 2001). The *rsh* is 90% similar to that of *B. suis* and also very closely related (70%) to *spoT* in

Sinorhizobium meliloti. It has two important functions in *Brucella*: cope with nutrient deprivation conditions and regulate the expression of T4SS in *Brucella*, with isolates lacking *rsh* having no functional T4SS (Dozot et al., 2006). Hfq also regulates the expression of T4SS in *B. melitensis* and *B. abortus* and was detected in all the sequenced genomes of this study. Hfq however, has a more prominent role in the virulence of *B. melitensis* than that of *B. abortus*, as it regulates 11% of *Brucella* genes including superoxide dismutase either directly or indirectly and therefore has been considered as an important virulence factor as well (Caswell et al., 2012; Mingquan et al., 2013). Hfq plays a role in causing chronic or persistent infection by overcoming host stresses and by regulating key stress response factors (Kaufmann, 2011).

Moreover, *manA*, *manB* (*pmm*), *manC* genes were all found in our sequenced genomes encoding for phosphomannomutase, mannose-6-phosphate isomerase, and mannose guanylyltransferase respectively, with all being involved in O- antigen biosynthesis. LPS interferes with the host's anti-microbial compounds and renders the organism resistant to defensins and lysozymes (Lapaque et al., 2005; Tumurkhuu et al., 2006). *Brucella* LPS is distinguished from other organisms as its lipid A fatty acid chain is unusually longer, and more virulent than other organisms (Cardoso et al., 2006; Haag et al., 2010), highlighting the importance of the LPS in *Brucella* fitness.

Our findings suggest that despite the absence of classical virulence determinants, *B. melitensis* has an abundant repertoire of virulence factors that contribute to its fitness and pathogenicity.

5.2.2. Resistance patterns

Fluoroquinolone resistance was detected in Bru007, Bru028 and Bru029, while co-trimoxazole resistance in Bru003, Bru014, Bru015, Bru017, Bru019, Bru020, Bru021, Bru022, Bru023, Bru024, Bru028, Bru030, Bru031 and Bru032 (Hamzeh, personal communication). Among those, Bru003, Bru014, Bru015, Bru029 and Bru030 were chosen for whole-genome sequencing and will be thus further discussed. Fluoroquinolones are sometimes administered as therapy for *Brucella* infections because of their low side effects and high affinity for soft tissues and bones. Moreover, fluoroquinolones are able to penetrate cells very efficiently, which made them promising candidates in the treatment of intracellular *Brucella* infections. However, due to high relapse rates, especially with ciprofloxacin, accompanied with cross-resistance to other fluoroquinolones and low bactericidal activity, they are no longer considered as the primary choice for treatment (Al- Sibai, 1992; Rolain et al., 2000). Additionally, and due to the misuse of this antibiotic, increasing microbial resistance has emerged (Valdezate et al., 2010). Alwan et al. (2010) showed that most of the *Brucella* isolated from food products in Lebanon were resistant to ciprofloxacin, which proves resistance was emerging in the country. Fluoroquinolone resistance is attributed to several factors including efflux pumps and mutations in resistance genes (Turkmani et al., 2007). Efflux pumps usually are not specific against one antimicrobial agent and confer multidrug resistance. Among them, the NorMI has a homolog in *B. melitensis* and has been functionally characterized as belonging to the MATE family of toxic compounds and multidrug exclusion. NorMI efflux pumps are linked to norfloxacin and ciprofloxacin

resistance and were detected in the sequenced genomes (Braibant et al., 2002), explaining the resistance against ofloxacin and ciprofloxacin. Another factor could be mutations in the quinolone-resistance determining regions of *gyrA*, *gyrB*, *parC,* and *parE* genes encoding DNA gyrase subunit A, DNA gyrase subunit B, DNA topoisomerase IV subunit A, and DNA topoisomerase IV subunit B respectively (Valdezate et al., 2010). These four genes were detected in the sequenced genomes as well, and we further compared their sequences in our isolates to those of reference strain *B. melitensis* 16M to scan for possible mutations linked to fluoroquinolone resistance. The *B. melitensis gyrA*, *gyrB*, *parC* and *parE* gene sequences were downloaded from the NCBI gene database with the following accession numbers: BME_RS04405, BME_RS09030, BME_RS06035 and BME_RS13510. Bru029, which was the only sequenced isolate resistant to ciprofloxacin, showed no mutations in *parC*, *parE* or *gyrB* with sequences detected being similar to those of *B. melitensis* str. 16M downloaded from NCBI. These results were similar to results reported by Valdezate et al. (2010), where mutations were not detected in any of the above mentioned genes. Based on this, resistance to fluoroquinolones in the sequenced isolates couldn't be attributed to mutations in the *parC*, *parE*, or *gyrB* genes but rather possibly to the overexpression of the NORMI efflux pump.

On the other hand, 42% of isolates in this study were resistant to co-trimoxazole (trimethoprim and sulfonamide) (Hamzeh, personal communication). Co-trimoxazole has offered successful treatment for certain patients and is the drug of choice when tetracyclines cannot be used such as in young children and pregnancy. However,

problems were encountered in therapy in the past such as high relapse rates and resistance reaching 29% among blood samples of *B. melitensis* isolated from a total of 160 patients in Saudi Arabia, which shed light on the emerging resistance against co-trimoxazole in the Middle East (Memish et al., 2000; Roushan et al., 2006). Co-trimoxazole resistance has been linked to the presence of Resistance-Nodulation-Cell division (RND) efflux pumps that extrude trimethoprim, sulfonamides and other drugs as well (Lister et al., 2009), which were detected in all sequenced genomes. In association with the bacterial outer membrane, these pumps extrude drugs to the external membrane instead of the periplasm so that antimicrobial compounds can't re-enter (Nikaido, 1996; Nikaido & Takatsuka, 2009). Co-trimoxazole resistance accordingly could be attributed to the overexpression of RND efflux pumps. RND efflux pumps in *Brucella* conferred multidrug resistance to several antibiotics including co-trimoxazole (Martin et al., 2009). Similar results were reported for other organisms as well, as in *Burkholderia pseudomallei*, where trimethoprim resistance was also linked to efflux systems, whereas in *E. coli* and *Haemophilus influenzae* mutations in the dihydrofolate reductase genes and sulfonamide resistance genes *sul1* and *sul2* caused trimethoprim resistance, respectively (Mohd-Zain et al., 2013; Podnecky et al., 2013).

5.2.3. Detection of prophages

Acquisition of VFs (virulence factors), toxins, genetic diversity and genomic evolution are all the outcome of phage integration into bacterial genomes mediated by horizontal gene transfer (Varani et al., 2013). Enzymes important for bacterial pathogenesis and proteins involved in adhesion and invasion might also be carried by phages (Boyd &

Brussow, 2002). A total of 38 prophage-associated regions were detected in our isolates ranging in size between 6.1 and 60.4kb. Each isolate contained up to three prophages, with two being commonly detected: an intact 13.7kb phage (Paraco_vB_Pmas) and an incomplete 17.4kb phage (Acantho_moumouvirus). The Acantho_moumouvirus phage was similar to that detected in *B. abortus* bv. 1 (Hammerl et al., 2016). However, little is known about prophages and mobile genetic elements in *Brucella* since many prophages were lost during its evolution from a soil to a mammalian pathogen, and therefore, these aspects should be better targeted to understand their role in pathogenesis (Delrue et al., 2004; Hammerl et al., 2016).

5.2.4. Detection of CRISPRs

The CRISPR-Cas system in bacteria is responsible for protection from foreign DNA or RNA that comes from viruses or mobile genetic elements (Barrangou et al., 2007; Hale et al., 2009). Spacers with unique sequences coming from viral origin separate between short repeated sequences and hence act against the viruses (Bolotin et al., 2005; Pourcel et al., 2005). The more the spacers, the more viruses the CRISPRs can recognize and act against (Rath et al., 2015). One possible CRISPR structure was detected in each of the sequenced genomes of length 82bp and having one spacer, and this result indicated that *Brucella* might have acquired resistance against phages and therefore have become more pathogenic in the process. These findings can be correlated with a recent study also reporting the detection of possible CRISPRS in *Brucella* genomes (Chiliveru et al., 2015). Moreover, a diguanylate cyclase (a CRISPR-related protein) was also detected in some *Brucella* genomes and this augments the fact that a CRISPR system might actually

exist (Chiliveru et al., 2015). This finding was consistent with our results as well, with all sequenced isolates having a diguanylate cyclase. This further emphasizes a possible link between CRISPR systems and pathogenicity, which needs to be further investigated in *Brucella* to better understand their role.

5.2.5. Detection of genomic islands

Using Islandviewer3, we identified a number of hypothetical proteins in GIs, in addition to several LPS related genes. Genomic islands (GIs) are clusters of genes acquired by bacteria through horizontal gene transfer and some encode factors such as VFs, antibiotic resistance genes, and metabolic factors that improve the fitness of the corresponding organism. Certain strains of bacteria can be transformed from non-pathogenic to pathogenic through the acquisition of GIs which are also targeted to study polymorphism and evolution among different strains (Dobrindt et al., 2004; Hacker and Kaper, 2000). With respect to *Brucella*, it was thought that the acquisition of GIs helped in its evolution from a soil to an intracellular mammalian bacterium and in withstanding harsh environmental conditions (Rajashekara et al., 2008). Most proteins encoded on *Brucella* GIs are hypothetical, however, some encoded potential virulence factors such as: *virB* locus, genes involved in LPS synthesis, glycosyltransferases, Omp25, exonuclease, and adhesin were also detected on these gene clusters (Delrue et al., 2004; Marchesini et al., 2004; O'Callaghan et al., 1999; Rajashekara et al., 2008). Rajashekara et al. (2004) revealed the presence of nine GIs in *Brucella*. GI-2 was also found to be important for virulence in *B. melitensis* and carrying LPS related genes and *wboA* and *wboB* glycosyltransferases, loss or deletion of which led to a change from smooth to

rough strains and hence attenuation (Mancilla et al., 2011; Rajashekara et al., 2008). However, a deletion of GI-1, 5, or 6 did not cause any change in virulence or growth and hence not involved in pathogenesis (Rajashekara et al., 2008). Among the elements detected in all sequenced genomes in this study were: *per*, *gmd*, *wboA*, *wboB*, *wbkC,* and *wbkA* encoding for perosamine synthetase, GDP-mannose 4-6 dehydratase, glycosyltransferases, formyltransferase, and mannosyltransferase, respectively. Previously it was shown that these genes became part of GIs after the transposases splitting up the original locus of LPS related genes on chromosome I and flanking it with IS*Bm1* insertion sequences, and thus transforming it from a commensal soil bacterium to a virulent intracellular pathogen (Haag et al., 2010; Mancilla, 2011). Moreover, exonuclease involved in base excision repair and protection from oxidative damage, was carried on GIs detected in Bru013, Bru014, Bru015, Bru029 and Bru035, while outer membrane proteins were found on GIs of Bru008, Bru014, Bru015, Bru026, Bru029 which was similar to results reported by Rajashekara et al. (2008) where outer membrane proteins were also detected on GIs. In addition, Bru015 carried components of the *virB* locus, coding for the T4SS, one of the most important VFs in *Brucella*, which was consistent with a previous report which revealed that T4SS components could be acquired by horizontal gene transfer in *Brucella* (Baron et al., 2002). *Brucella* had unique GI regions not harbored by its close ancestor *Ochrobactrum*, which indicated that this acquisition was after diverging from its common ancestor and was mediated by lateral transfer and after interacting with soil or gut bacteria (Wattam et al., 2009). *Brucella* is ingested through contaminated foods and milk and is deposited in fecal

discharges, with the opportunity to interact and acquire virulence determinants from gut bacteria. Although it is an intracellular bacterium, virulence determinants such as LPS, were acquired through horizontal gene transfer, and thus enhancing its survival in macrophages (Pei et al., 2008). Our findings suggest that virulence determinants detected in the studied isolates could be attributed to GI acquisition based on the detection of LPS and T4SS related components, which collectively enhance the organism's fitness and survival, rendering it more pathogenic.

5.2.6. Detection of insertion sequences

Using IS Finder we identified IS3, ISL3, IS481, IS5, IS66, and IS110 in the sequenced isolates. Insertion sequences (ISs) belong to the family of autonomous transposable elements, playing a role in genomic diversity and virulence. This leads to antibiotic resistance in some instances through affecting the gene expression of efflux systems, or attenuating a pathogenic organism as a result of an interruption in a virulence gene (Olliver et al., 2005; Wolter et al., 2004). Moreover, genomic rearrangements caused by IS elements include deletions, inversions and duplications (Iguchi et al., 2006). Using IS IS711 was detected in all sequenced genomes, which is one of the most important ISs in *Brucella* being specific for the genus. Its number also varies between different species and as a result was targeted in molecular typing to differentiate the species. Its variability is based on the number of copies where it varies in different species from seven copies usually found in *B. melitensis*, *B. abortus*, and *B. suis* to more than 30 in *B. ovis* and *B. ceti* (Ocampo-Sosa & Garcia-Lobo, 2008). In *B. abortus* the IS711 is

responsible for attenuating the RB51 strain by interrupting the *wboA* gene involved in LPS O-side chain synthesis (Audic et al., 2011; Vemulapalli et al., 2004).

5.2.7. Phylogenetic analysis

Phylogenetic analysis clustered the sequenced isolates separately from reference strains including strains 16M, ATCC 23457, 63/9, Ether, NI, M28 and M5-90 isolated from USA, India, Italy, Mongolia, and China respectively (Tan et al., 2015). Isolates Bru003, Bru015, Bru035 were clustered together, while the remaining were distributed in four clades and were distantly positioned from Bru003, Bru015 and Bru034 but closer to reference strains 16M and Ether. Since Phylosift is based on concatenated alignment of 37 core genes of a genome, we performed a comparative analysis between the different genomes and the reference strains on RAST to explain this divergence. Recent studies showed that *B. melitensis* strain ATCC23457 and str. 63-9 were closely related to each other and clustered in the same clade, same as str. 16M and Ether based on the concatenated alignment of their core genes and on SNPs (Azam et al., 2016; Kay et al., 2014). When we compared these two pairs (ATCC23457 and 63-9; 16M and Ether) of reference genomes on RAST, the main sequence divergence was due to mobile elements, hypothetical proteins and phage-related proteins. The core genomes were highly conserved, which was consistent with the clustering of both in the same clade. Moreover, a comparative analysis was done between reference strains M28 and M5-90 (Wang et al., 2011), where it was found that M28 is the parent strain of M5-90 with the latter being an attenuated version of M28 strain and both exhibiting little divergence. Comparing those two strains revealed that divergence was linked to virulence

determinants including: cold shock proteins, flagellar genes, respiratory nitrate reductase, ABC transporters and some efflux pumps. This was consistent with previous reports that M28 was more virulent than M5-90 in spite of the close relatedness. This comparative analysis allowed us to better understand the phylogeny of the sequenced isolates. So far, few studies have targeted the whole-genome based phylogenetic relatedness of *B. melitensis* strains especially in the Middle East, due to the limited number of available full genomes deposited in databases (Tan et al., 2015). Therefore, more studies should be done to accurately interpret those findings.

Through whole-genome sequencing we were able to study the pathogenomics characteristics of the clinical *B. melitensis* isolates used in this study, and further explore its pathogenicity and resistance mechanisms in order to identify the emerging cases in Lebanon and importance of controlling it before it becomes a pandemic.

Chapter Six

CONCLUSION

In summary, and to our best knowledge, this is the first study in Lebanon focusing on the whole-genome sequencing of *Brucella* isolated from clinical samples. PCR-RFLP of *omp31* and *omp2a* genes proved useful in detection and identification at the species level. It was also more accurate than the Bruce ladder multiplex PCR. Through whole-genome sequencing we studied antimicrobial resistance genes, VFs, genomic content and structure. Despite the similarities in VFs and gene content, differences were detected in resistance patterns, prophages, ISs, GIs and VFs carried on GIs. Our findings point to the importance of implementing control and prevention measures in order to limit *Brucella* infections in the future and shed light on importance of whole-genome sequencing and its future possibility to become a routine diagnostic tool utilized in labs worldwide. Further studies will be required to understand the pandemic status in Lebanon. In addition, the role of mobile genetic elements in virulence and genetic diversity of *Brucella* in Lebanon should be further investigated.

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ANNEX

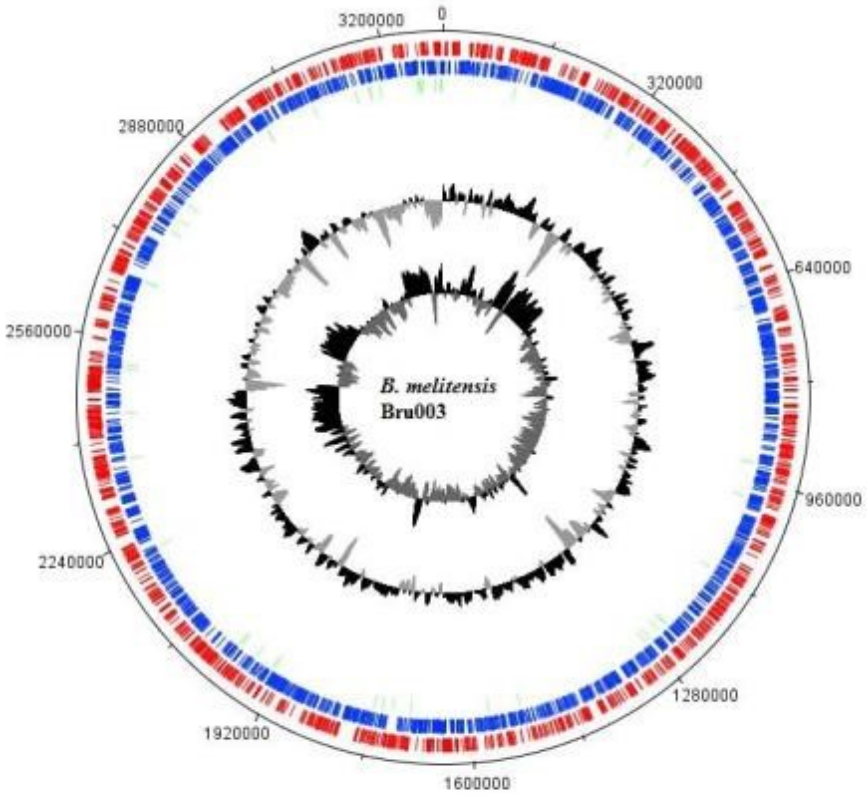

Figure 1. Circular map of Bru003 genome. From outside to inside, the tracks represent: Forward coding sequence (CDS) (red); reverse CDS (blue); tRNAs (green); G+C plot ; G+C skew (black above average and grey below average). DNAPlotter (Carver et al, 2009) was used to generate this image.

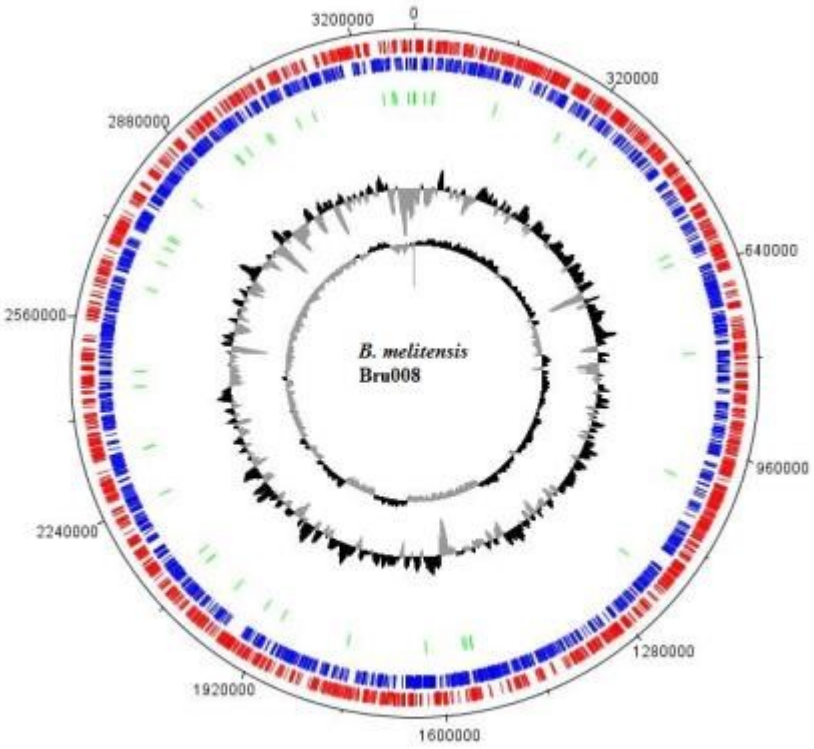

Figure 2. Circular map of Bru008 genome. From outside to inside, the tracks represent: Forward coding sequence (CDS) (red); reverse CDS (blue); tRNAs (green); G+C plot ; G+C skew (black above average and grey below average). DNAPlotter (Carver et al, 2009) was used to generate this image.

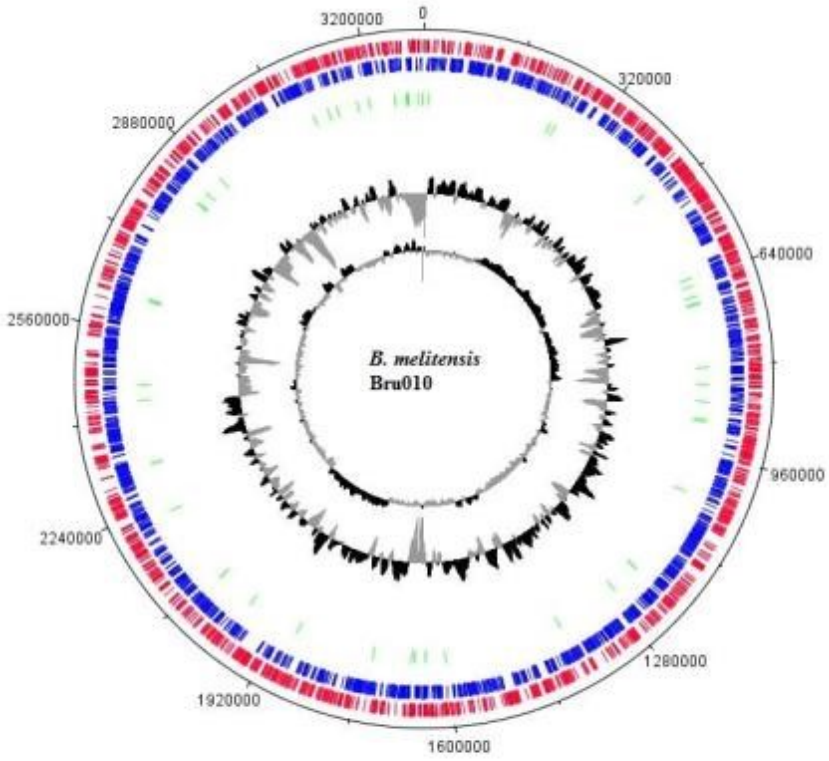

Figure 3. Circular map of Bru010 genome. From outside to inside, the tracks represent: Forward coding sequence (CDS) (red); reverse CDS (blue); tRNAs (green); G+C plot ; G+C skew (black above average and grey below average). DNAPlotter (Carver et al, 2009) was used to generate this image.

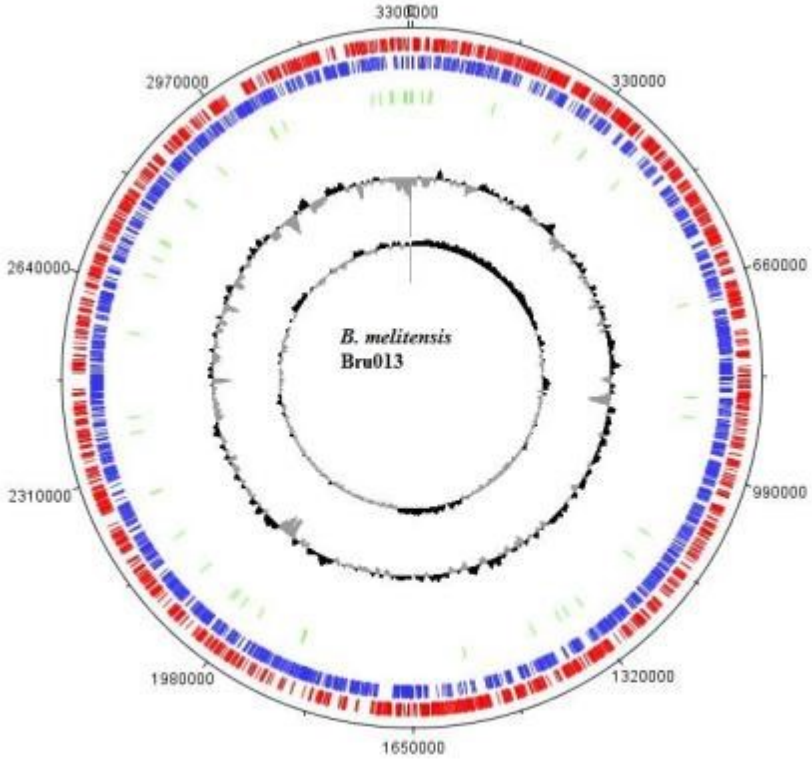

Figure 4. Circular map of Bru013 genome. From outside to inside, the tracks represent: Forward coding sequence (CDS) (red); reverse CDS (blue); tRNAs (green); G+C plot ; G+C skew (black above average and grey below average). DNAPlotter (Carver et al, 2009) was used to generate this image.

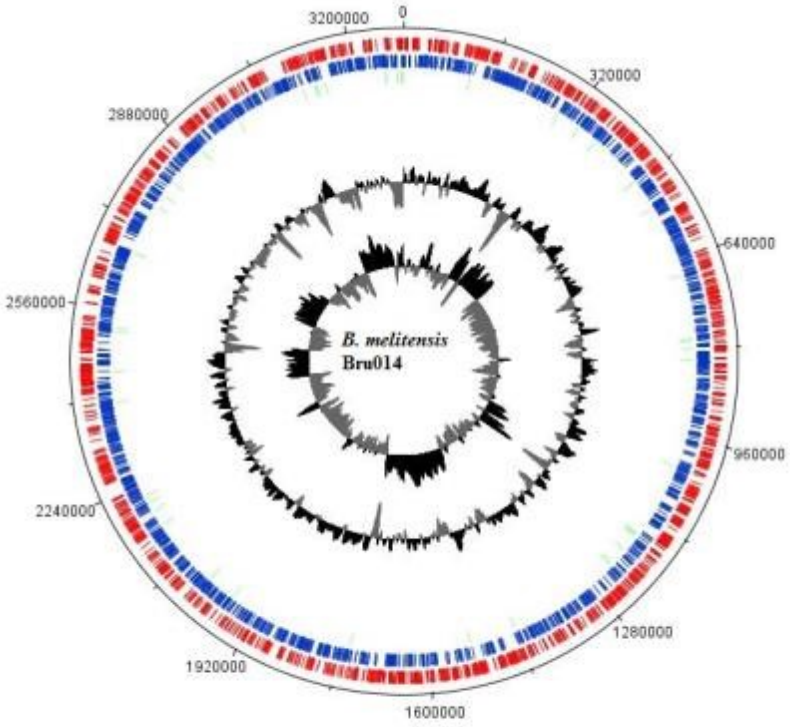

Figure 5. Circular map of Bru014 genome. From outside to inside, the tracks represent: Forward coding sequence (CDS) (red); reverse CDS (blue); tRNAs (green); G+C plot ; G+C skew (black above average and grey below average). DNAPlotter (Carver et al, 2009) was used to generate this image.

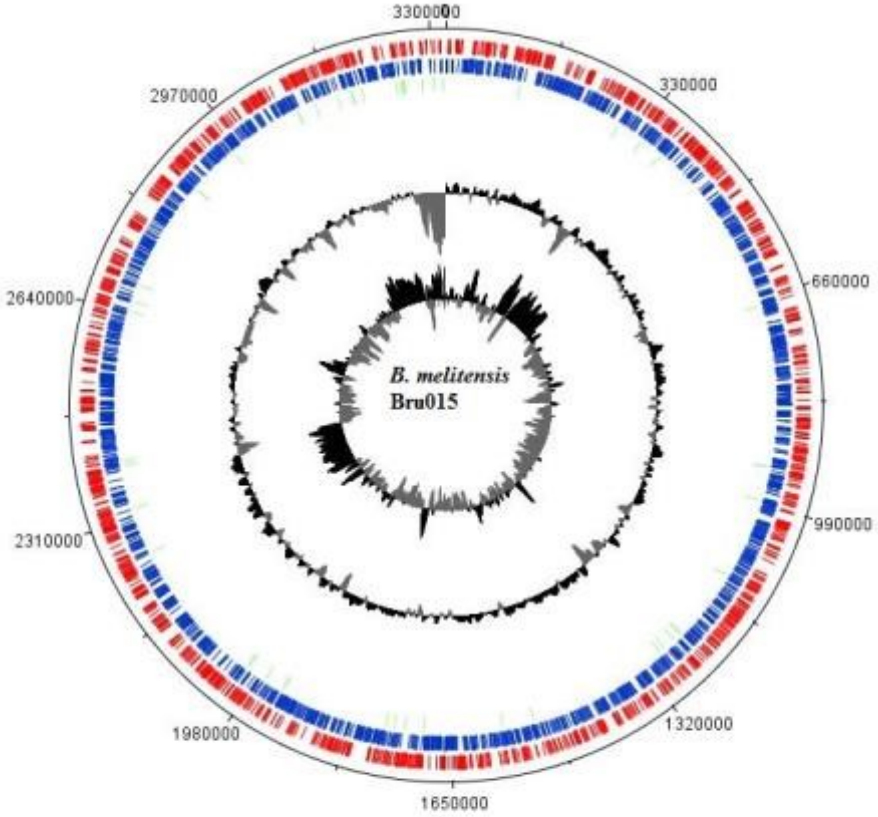

Figure 6. Circular map of Bru015 genome. From outside to inside, the tracks represent: Forward coding sequence (CDS) (red); reverse CDS (blue); tRNAs (green); G+C plot ; G+C skew (black above average and grey below average). DNAPlotter (Carver et al, 2009) was used to generate this image.

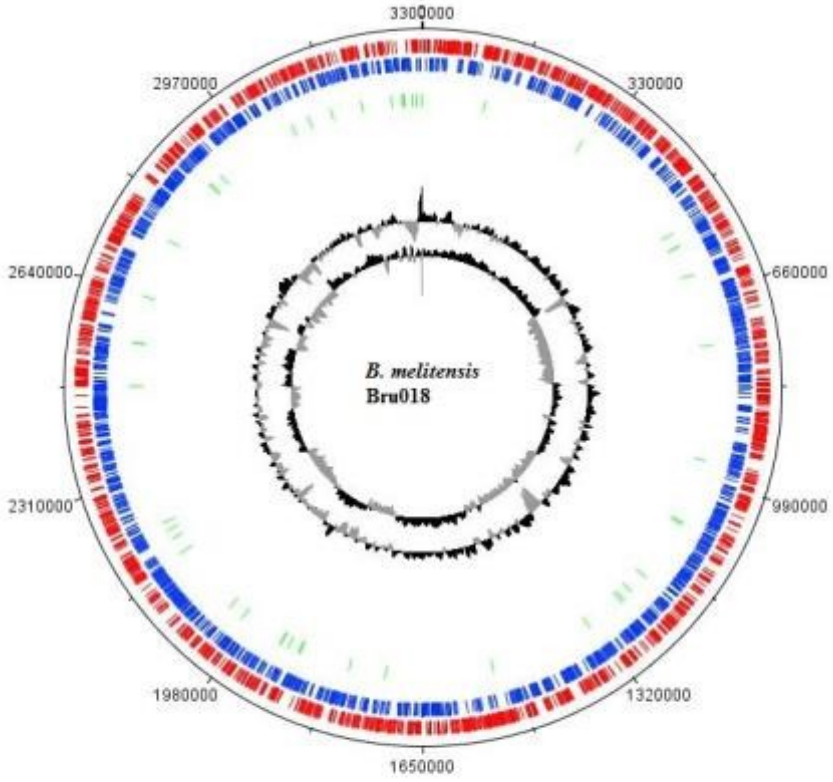

Figure 7. Circular map of Bru018 genome. From outside to inside, the tracks represent: Forward coding sequence (CDS) (red); reverse CDS (blue); tRNAs (green); G+C plot ; G+C skew (black above average and grey below average). DNAPlotter (Carver et al, 2009) was used to generate this image.

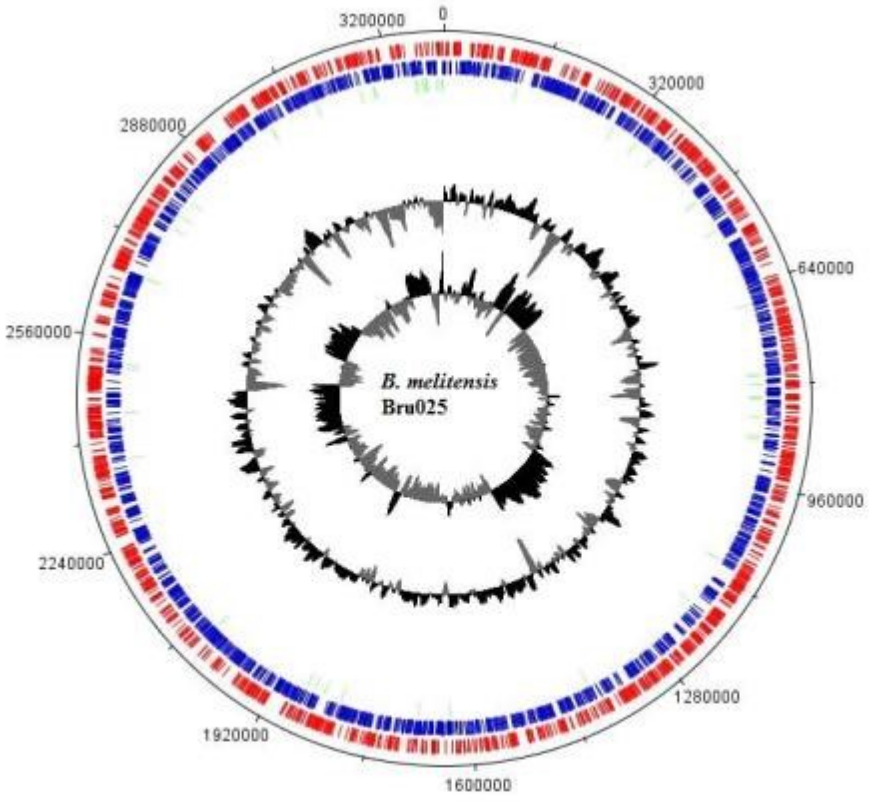

Figure 8. Circular map of Bru025 genome. From outside to inside, the tracks represent: Forward coding sequence (CDS) (red); reverse CDS (blue); tRNAs (green); G+C plot ; G+C skew (black above average and grey below average). DNAPlotter (Carver et al, 2009) was used to generate this image.

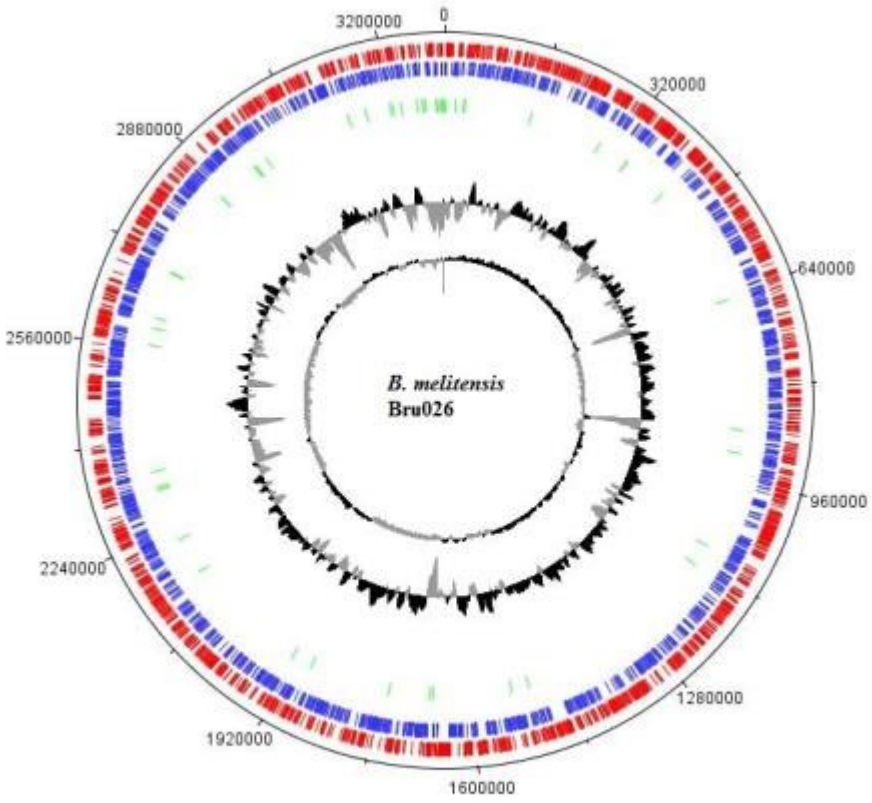

Figure 9. Circular map of Bru026 genome. From outside to inside, the tracks represent: Forward coding sequence (CDS) (red); reverse CDS (blue); tRNAs (green); G+C plot ; G+C skew (black above average and grey below average). DNAPlotter (Carver et al, 2009) was used to generate this image.

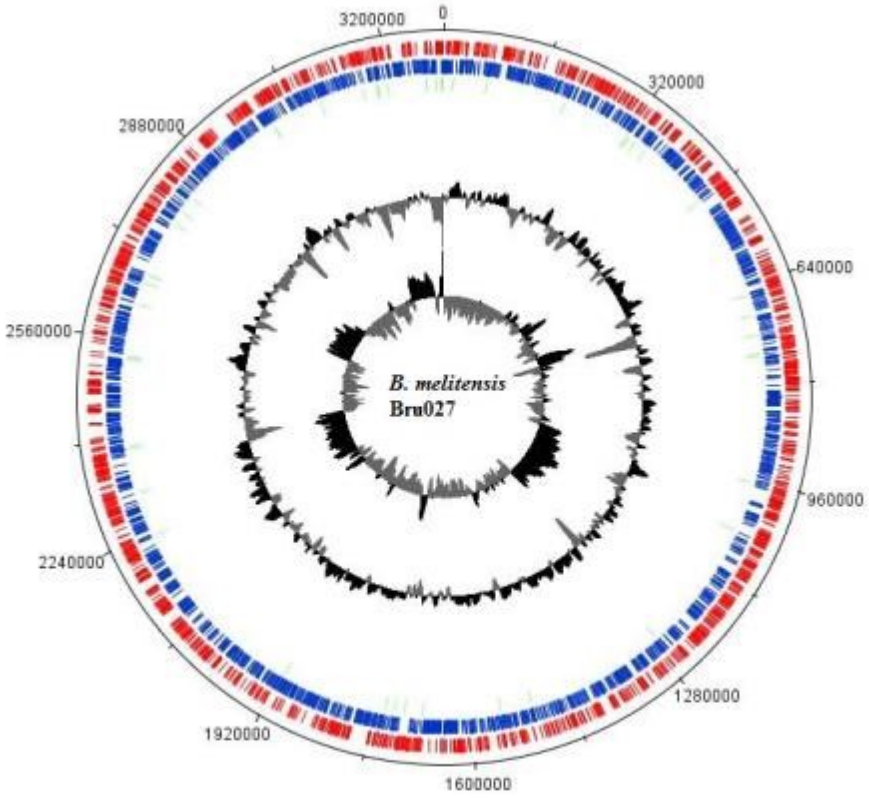

Figure 10. Circular map of Bru027 genome. From outside to inside, the tracks represent: Forward coding sequence (CDS) (red); reverse CDS (blue); tRNAs (green); G+C plot ; G+C skew (black above average and grey below average). DNAPlotter (Carver et al, 2009) was used to generate this image.

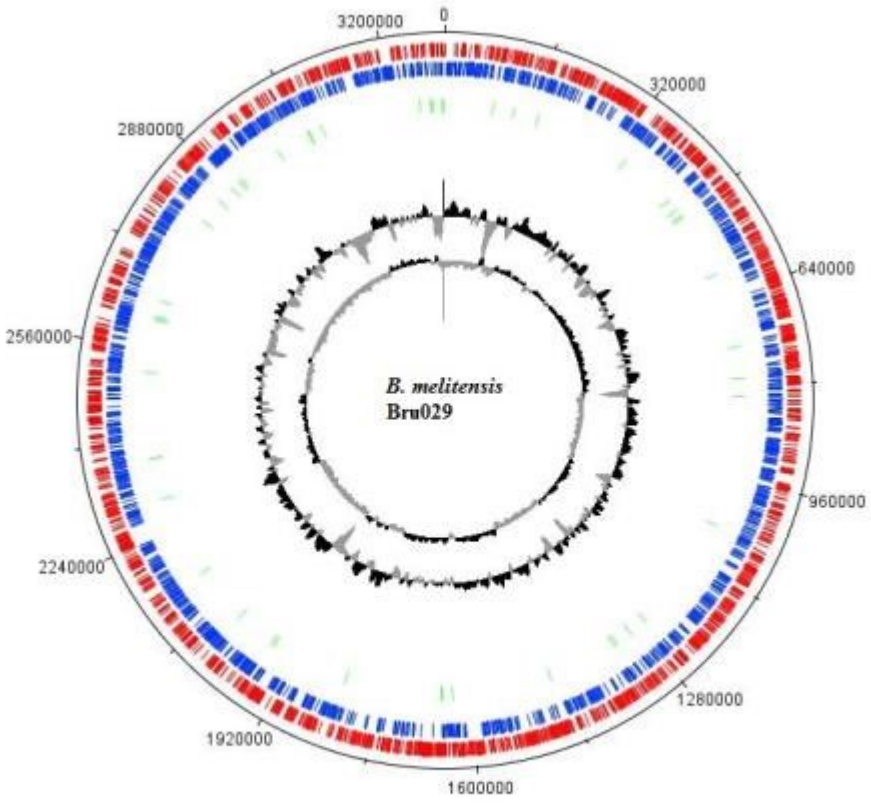

Figure 11. Circular map of Bru029 genome. From outside to inside, the tracks represent: Forward coding sequence (CDS) (red); reverse CDS (blue); tRNAs (green); G+C plot ; G+C skew (black above average and grey below average). DNAPlotter (Carver et al, 2009) was used to generate this image.

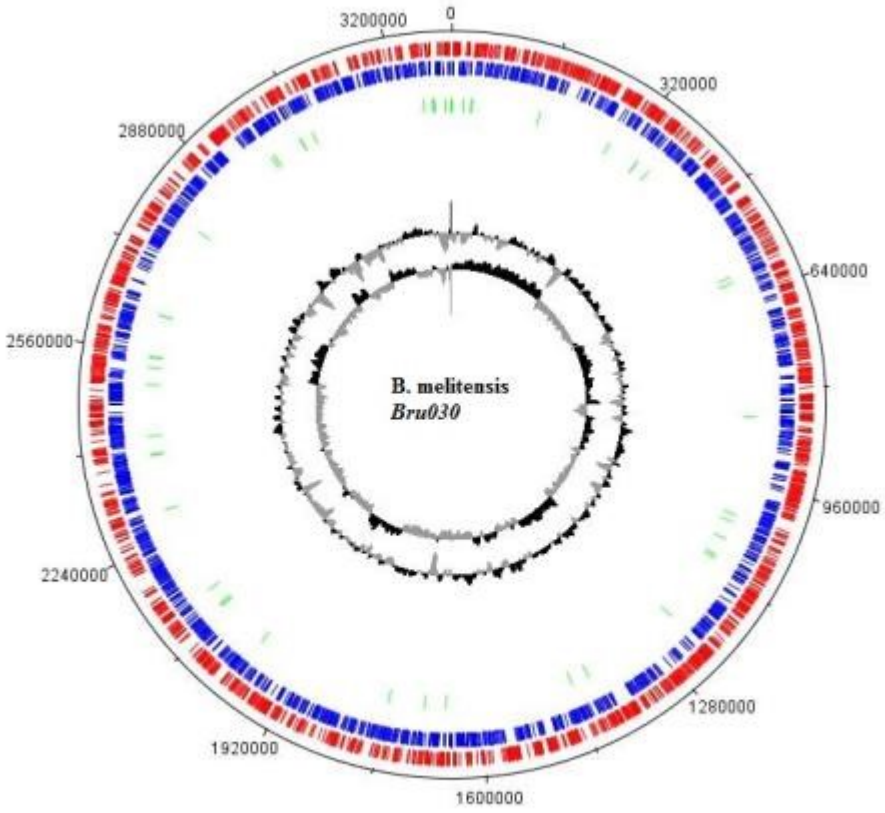

Figure 12. Circular map of Bru030 genome. From outside to inside, the tracks represent: Forward coding sequence (CDS) (red); reverse CDS (blue); tRNAs (green); G+C plot ; G+C skew (black above average and grey below average). DNAPlotter (Carver et al, 2009) was used to generate this image.

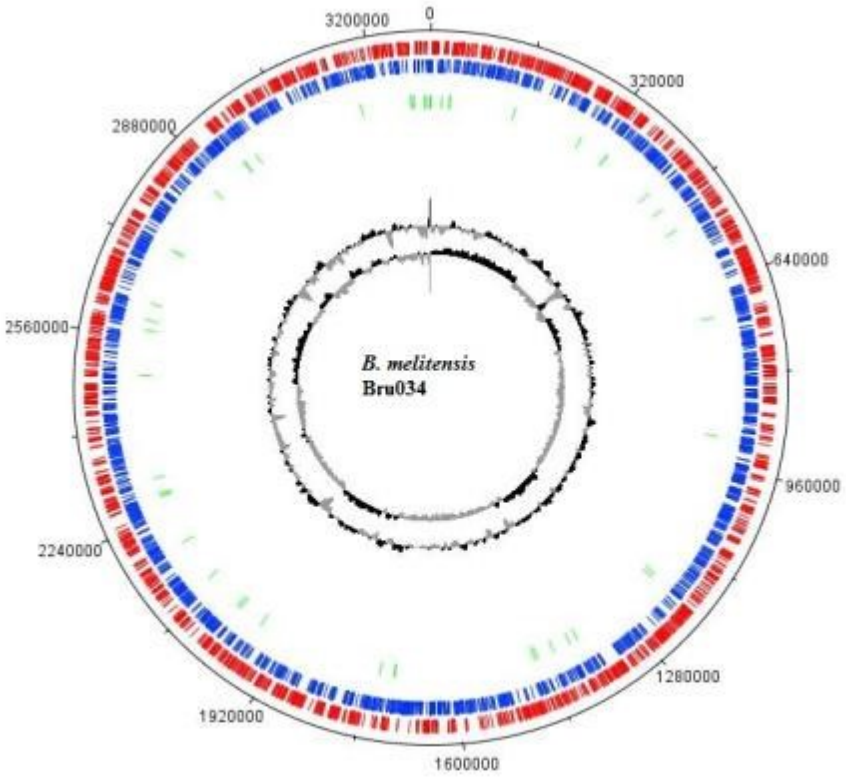

Figure 13. Circular map of Bru034 genome. From outside to inside, the tracks represent: Forward coding sequence (CDS) (red); reverse CDS (blue); tRNAs (green); G+C plot ; G+C skew (black above average and grey below average). DNAPlotter (Carver et al, 2009) was used to generate this image.

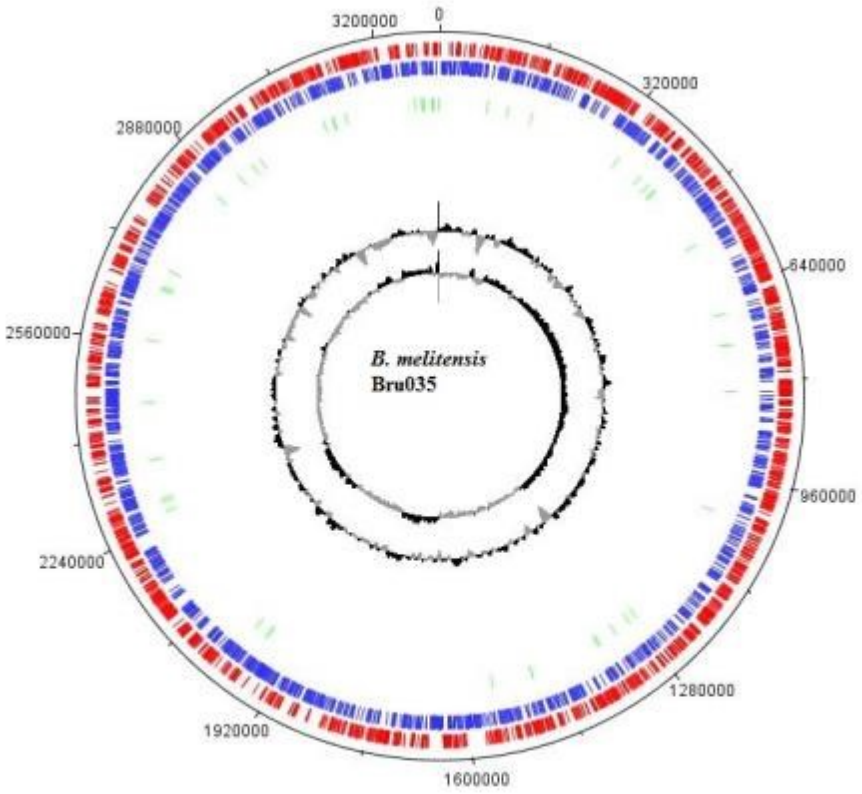

Figure 14. Circular map of Bru035 genome. From outside to inside, the tracks represent: Forward coding sequence (CDS) (red); reverse CDS (blue); tRNAs (green); G+C plot ; G+C skew (black above average and grey below average). DNAPlotter (Carver et al, 2009) was used to generate this image.

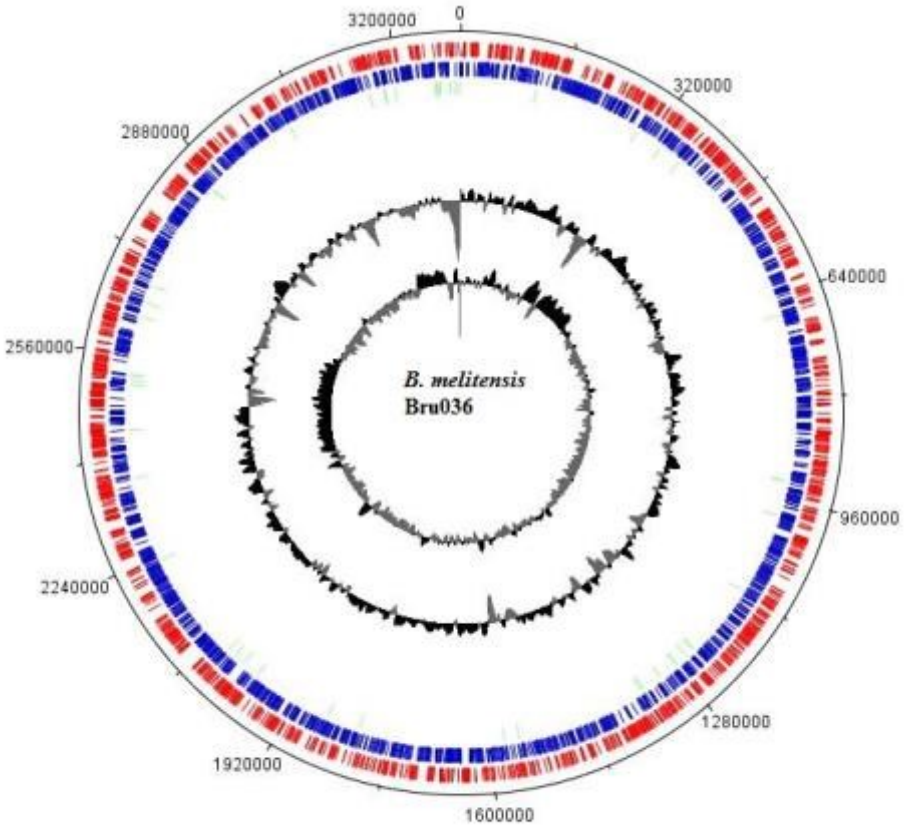

Figure 15. Circular map of Bru036 genome. From outside to inside, the tracks represent: Forward coding sequence (CDS) (red); reverse CDS (blue); tRNAs (green); G+C plot ; G+C skew (black above average and grey below average). DNAPlotter (Carver et al, 2009) was used to generate this image.

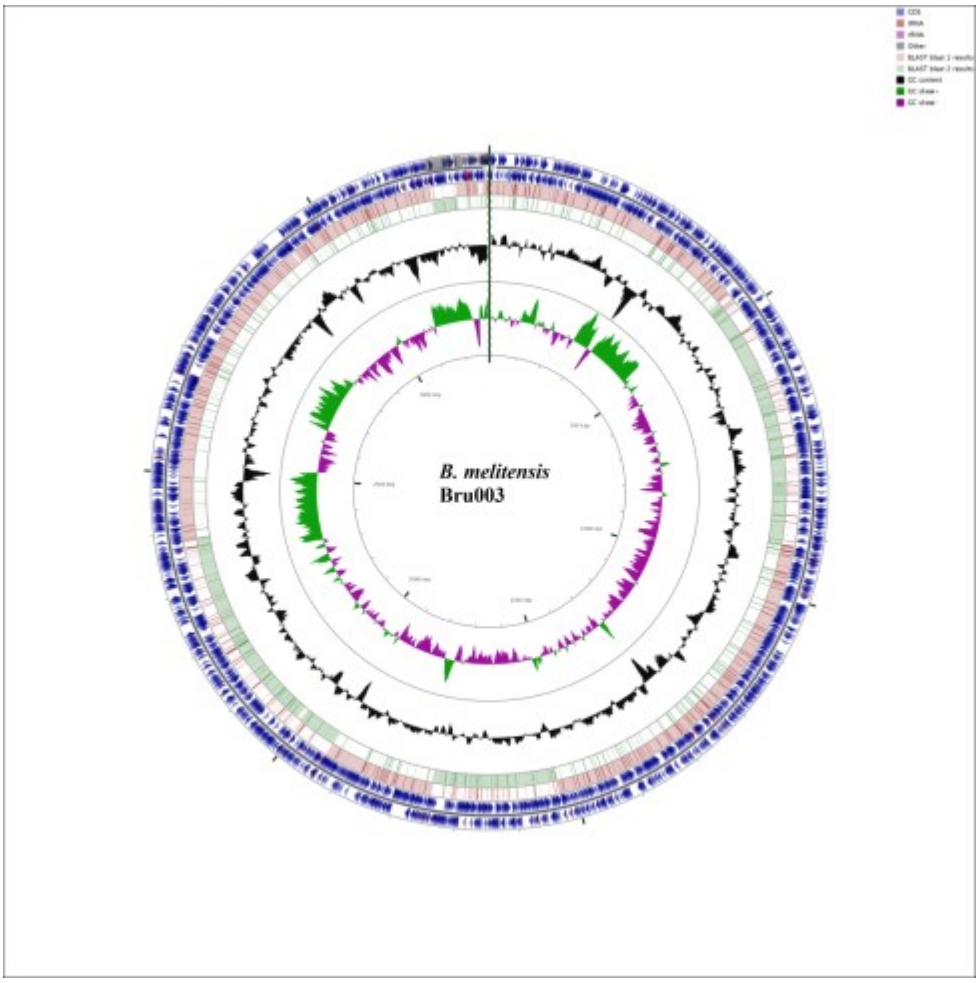

Figure 16. Circular map of Bru003 compared with *B. melitensis* str. 16M reference genome. From outside to inside the rings represent: Bru003 coding DNA sequence (CDS) on the forward and reverse strand (blue), *B. melitensis* 16M chromosome 1 and 2 Blast results (light pink and light green) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green) and G+C negative skew (purple). CGview Server V 1.0 (Grant & Stothard, 2008) was used to construct this image.

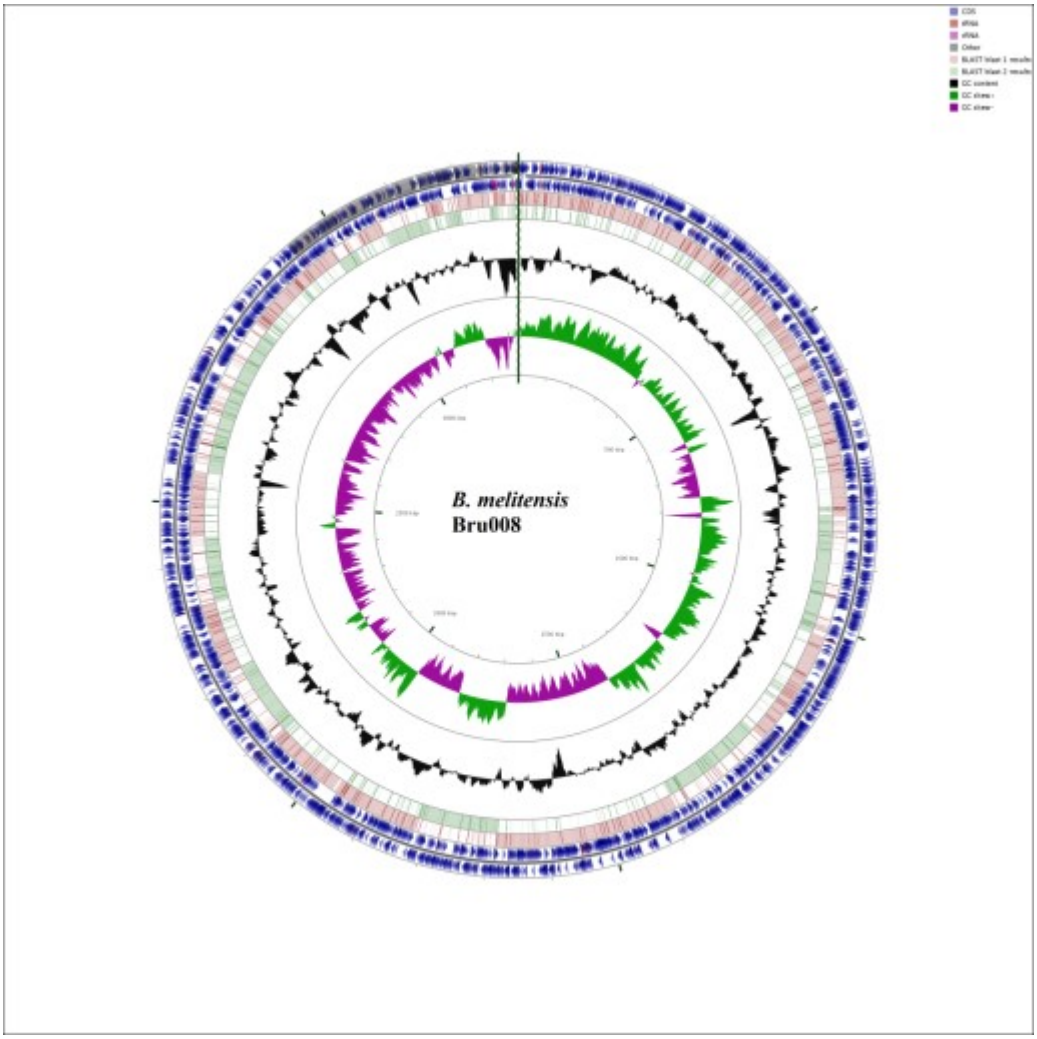

Figure 17. Circular map of Bru008 compared with *B. melitensis* str. 16M reference genome. From outside to inside the rings represent: Bru008 coding DNA sequence (CDS) on the forward and reverse strand (blue), *B. melitensis* 16M chromosome 1 and 2 Blast results (light pink and light green) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green) and G+C negative skew (purple). CGview Server V 1.0 (Grant & Stothard, 2008) was used to construct this image.

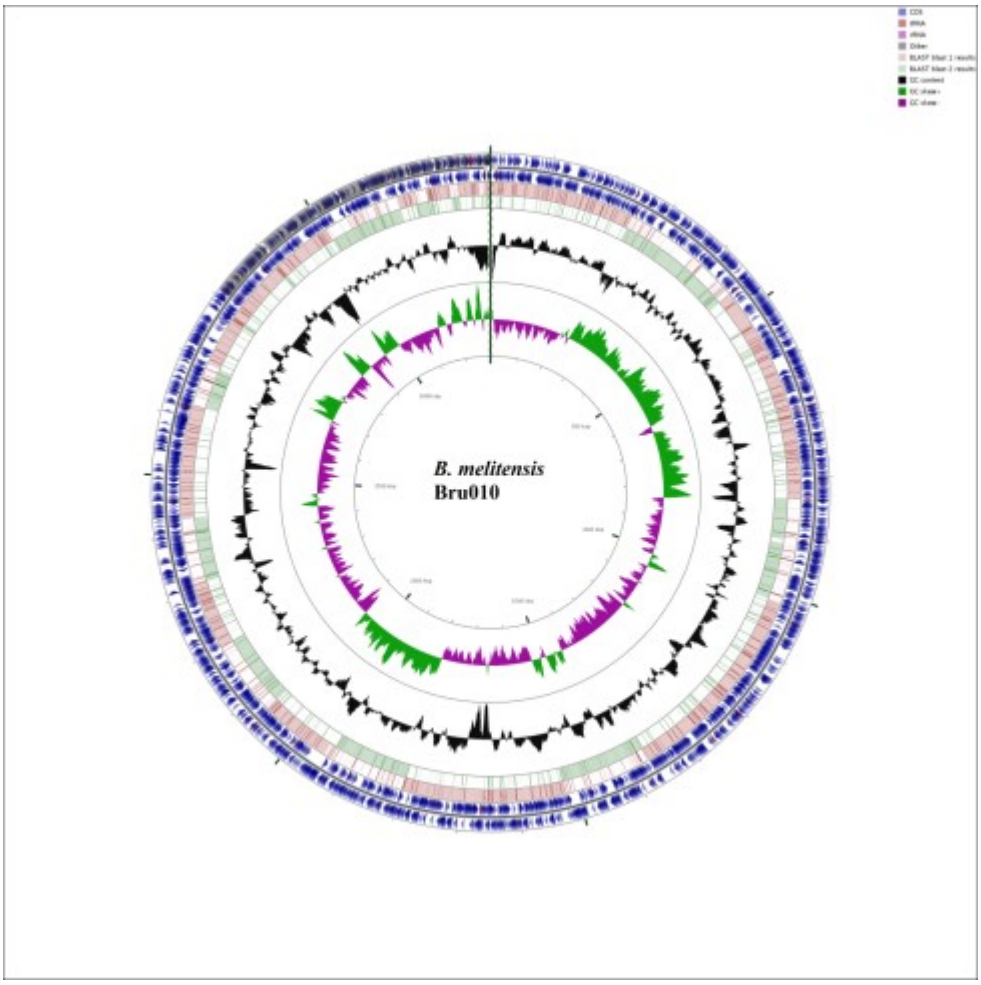

Figure 18. Circular map of Bru010 compared with *B. melitensis* str. 16M reference genome. From outside to inside the rings represent: Bru010 coding DNA sequence (CDS) on the forward and reverse strand (blue), *B. melitensis* 16M chromosome 1 and 2 Blast results (light pink and light green) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green) and G+C negative skew (purple). CGview Server V 1.0 (Grant & Stothard, 2008) was used to construct this image.

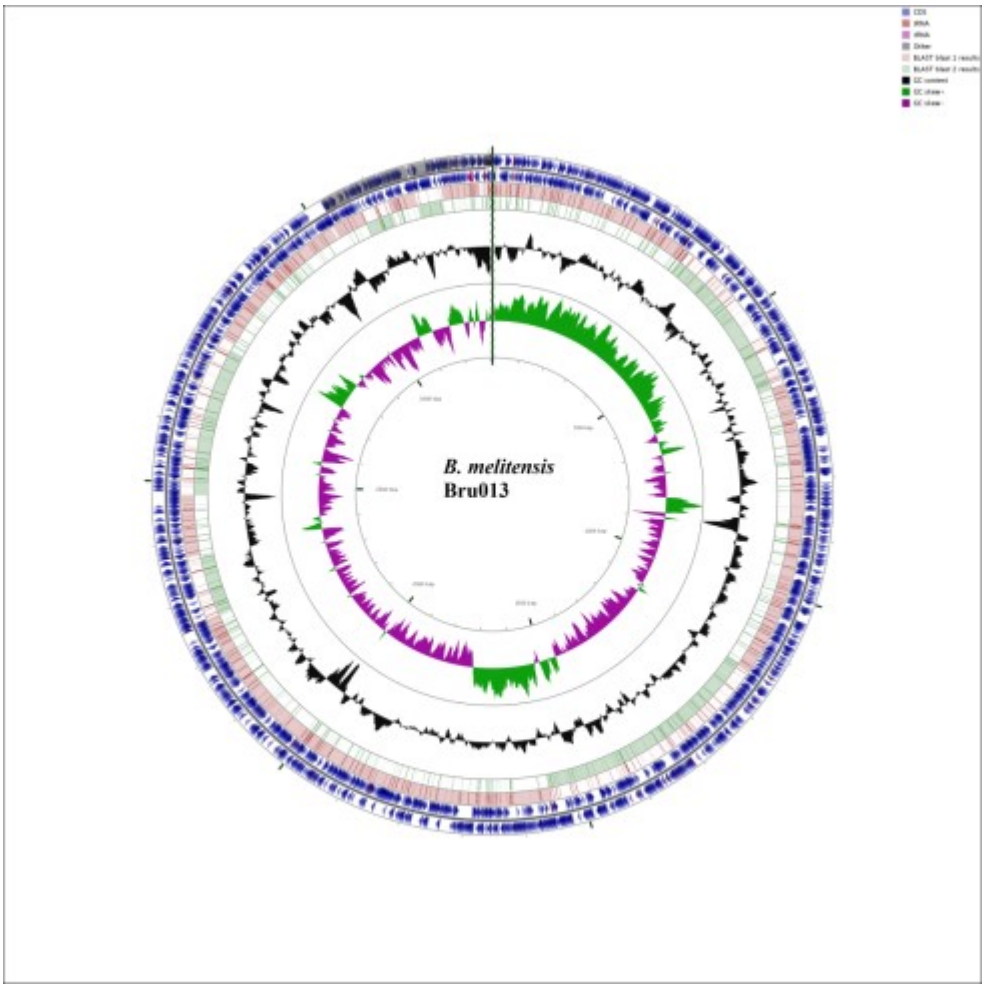

Figure 19. Circular map of Bru013 compared with *B. melitensis* str. 16M reference genome. From outside to inside the rings represent: Bru013 coding DNA sequence (CDS) on the forward and reverse strand (blue), *B. melitensis* 16M chromosome 1 and 2 Blast results (light pink and light green) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green) and G+C negative skew (purple). CGview Server V 1.0 (Grant & Stothard, 2008) was used to construct this image.

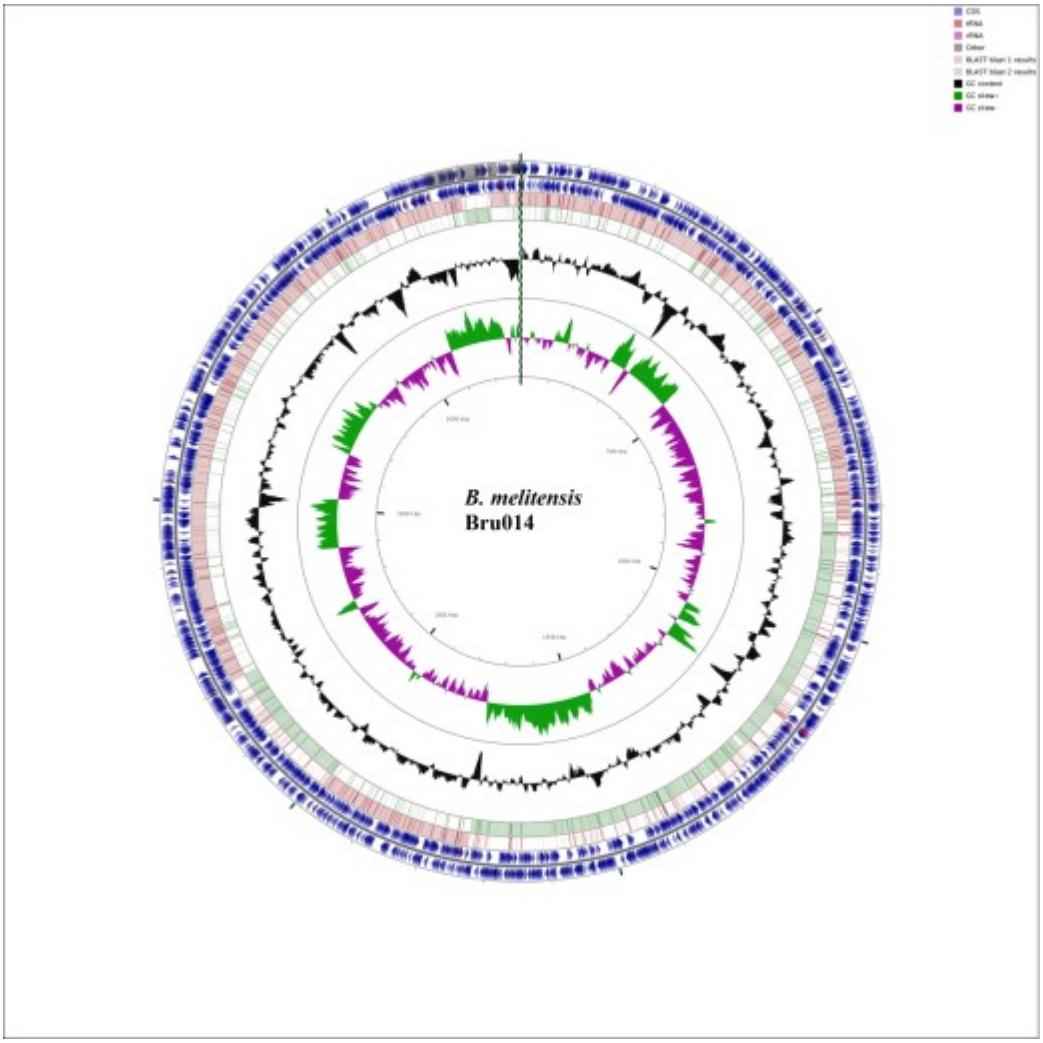

Figure 20. Circular map of Bru014 compared with *B. melitensis* str. 16M reference genome. From outside to inside the rings represent: Bru014 coding DNA sequence (CDS) on the forward and reverse strand (blue), *B. melitensis* 16M chromosome 1 and 2 Blast results (light pink and light green) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green) and G+C negative skew (purple). CGview Server V 1.0 (Grant & Stothard, 2008) was used to construct this image.

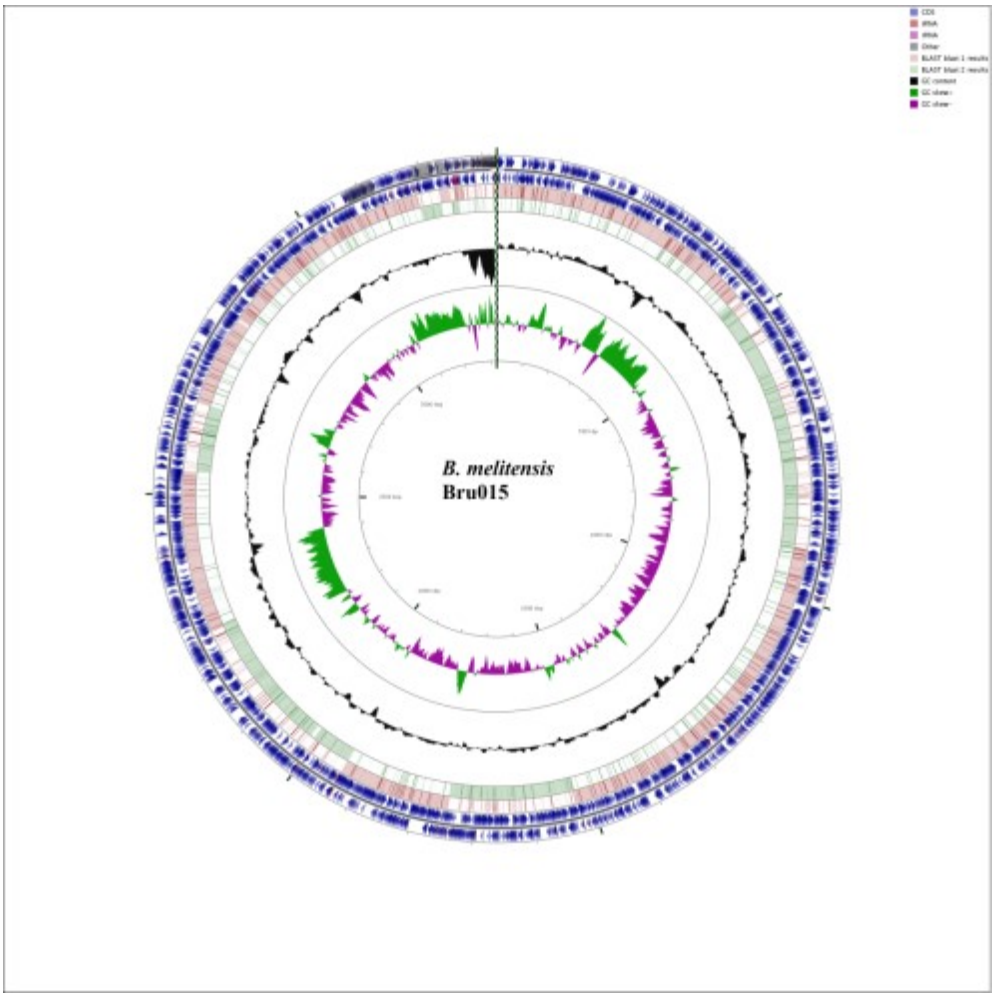

Figure 21. Circular map of Bru015 compared with *B. melitensis* str. 16M reference genome. From outside to inside the rings represent: Bru015 coding DNA sequence (CDS) on the forward and reverse strand (blue), *B. melitensis* 16M chromosome 1 and 2 Blast results (light pink and light green) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green) and G+C negative skew (purple). CGview Server V 1.0 (Grant & Stothard, 2008) was used to construct this image.

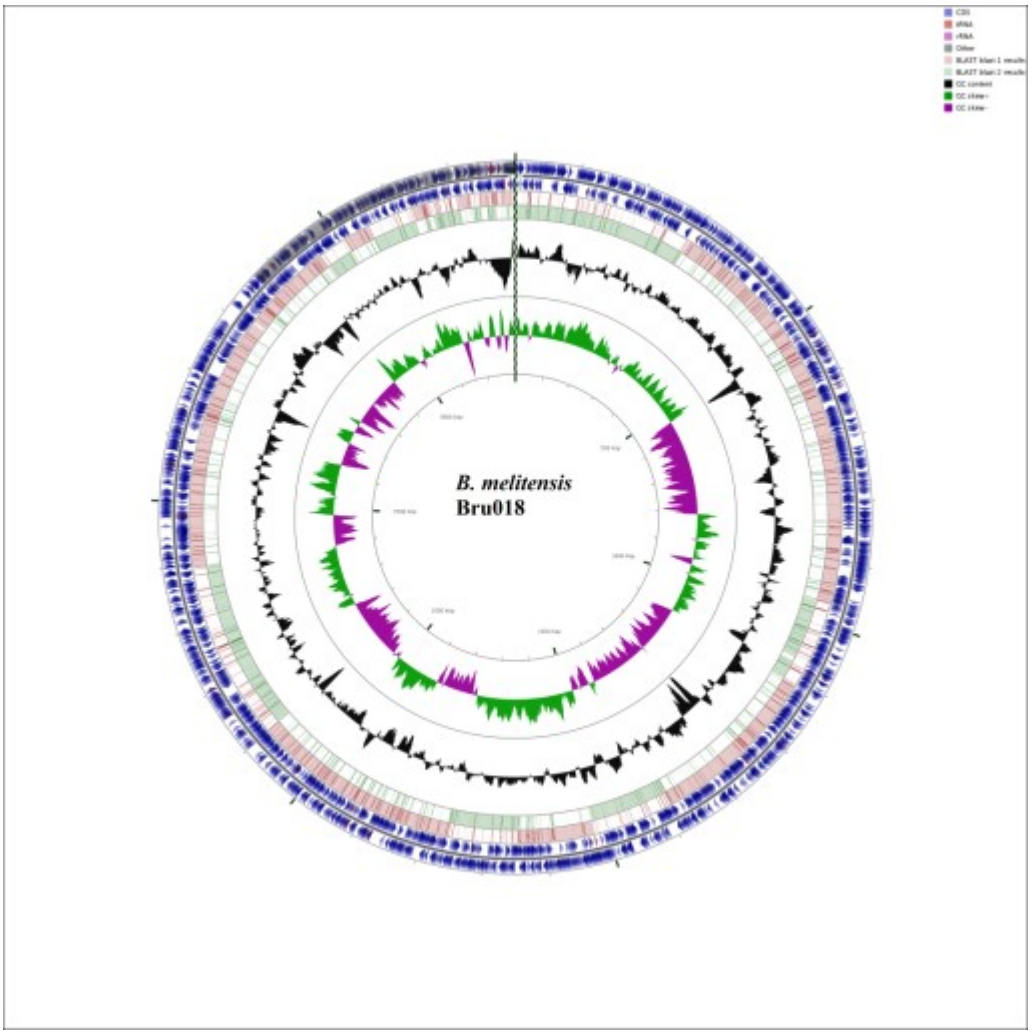

Figure 22. Circular map of Bru018 compared with *B. melitensis* str. 16M reference genome. From outside to inside the rings represent: Bru018 coding DNA sequence (CDS) on the forward and reverse strand (blue), *B. melitensis* 16M chromosome 1 and 2 Blast results (light pink and light green) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green) and G+C negative skew (purple). CGview Server V 1.0 (Grant & Stothard, 2008) was used to construct this image.

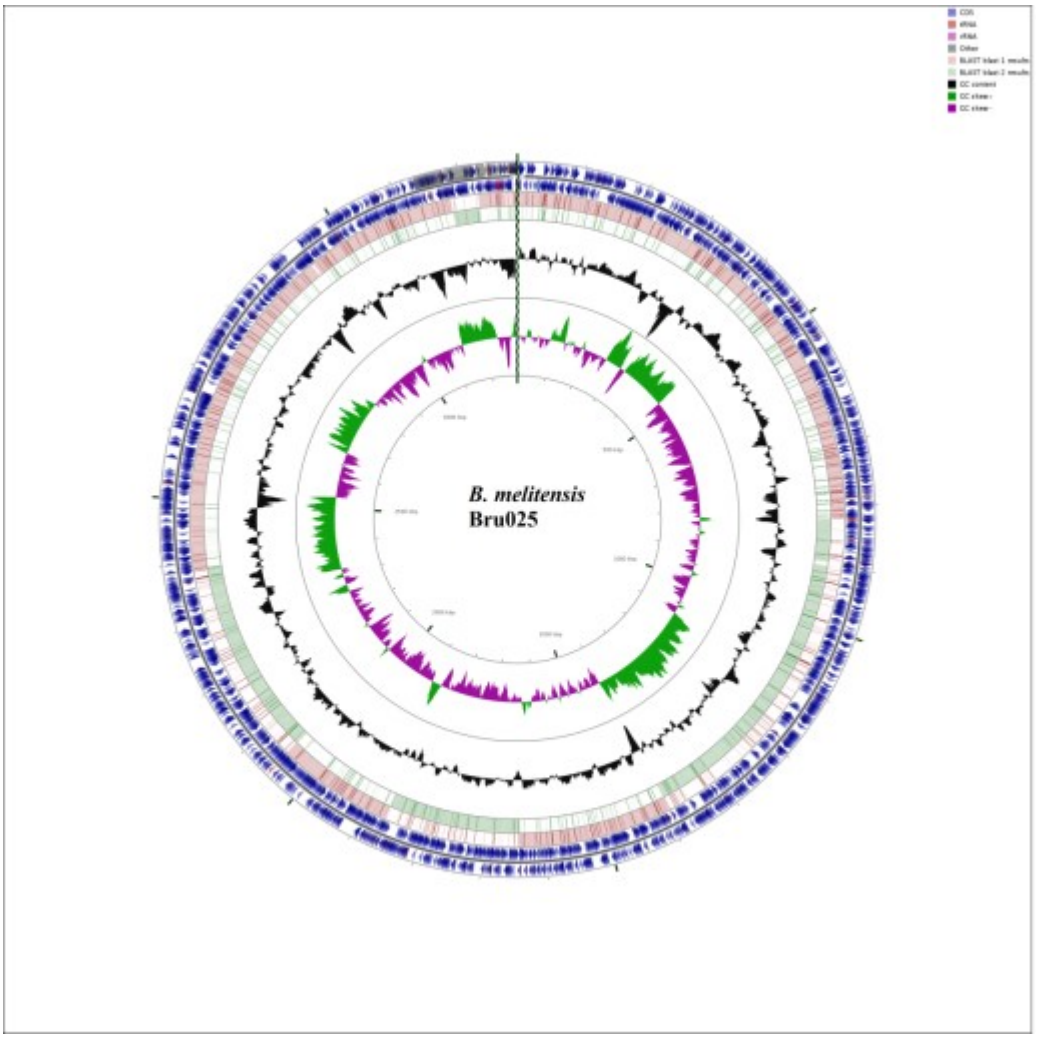

Figure 23. Circular map of Bru025 compared with *B. melitensis* str. 16M reference genome. From outside to inside the rings represent: Bru025 coding DNA sequence (CDS) on the forward and reverse strand (blue), *B. melitensis* 16M chromosome 1 and 2 Blast results (light pink and light green) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green) and G+C negative skew (purple). CGview Server V 1.0 (Grant & Stothard, 2008) was used to construct this image.

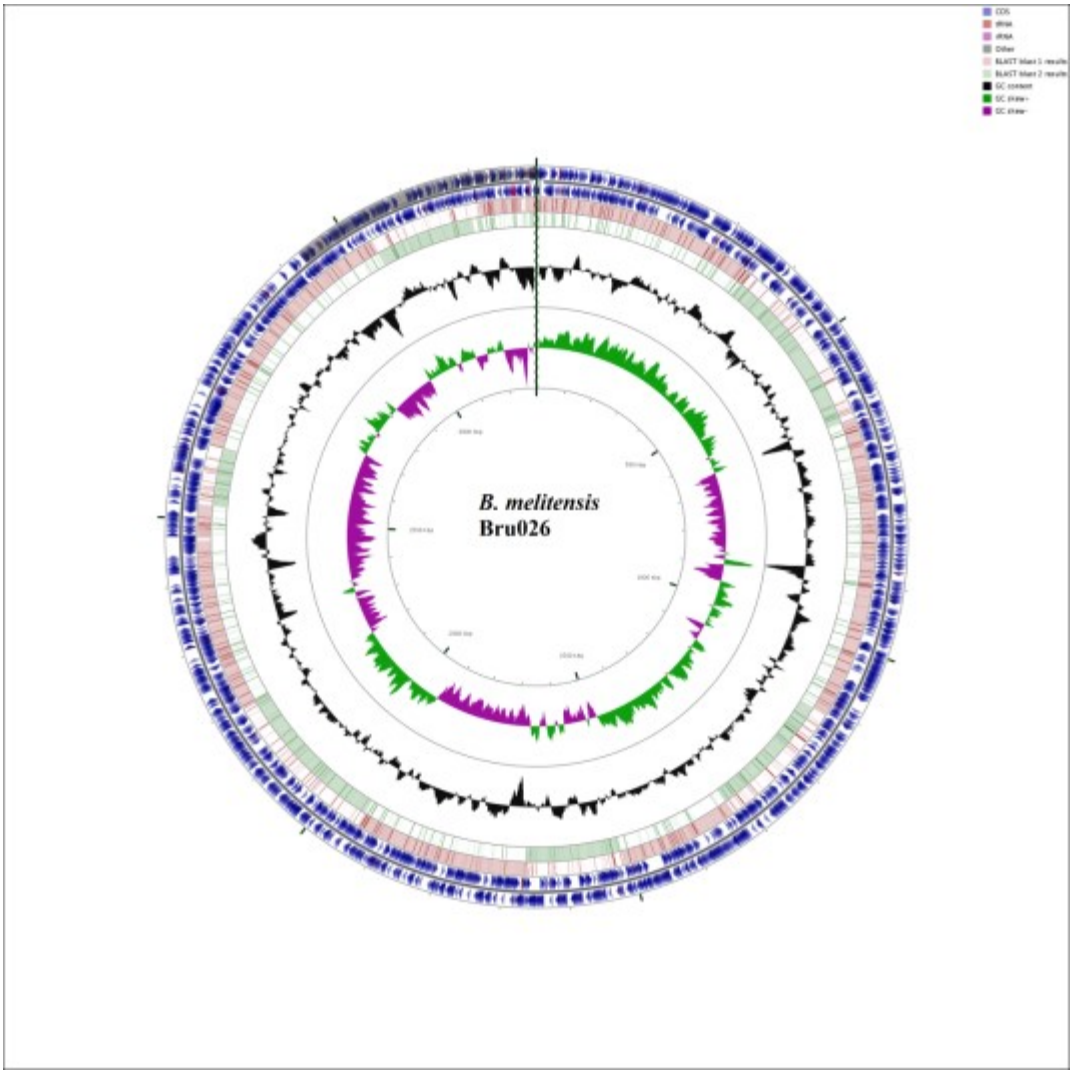

Figure 24. Circular map of Bru026 compared with *B. melitensis* str. 16M reference genome. From outside to inside the rings represent: Bru026 coding DNA sequence (CDS) on the forward and reverse strand (blue), *B. melitensis* 16M chromosome 1 and 2 Blast results (light pink and light green) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green) and G+C negative skew (purple). CGview Server V 1.0 (Grant & Stothard, 2008) was used to construct this image.

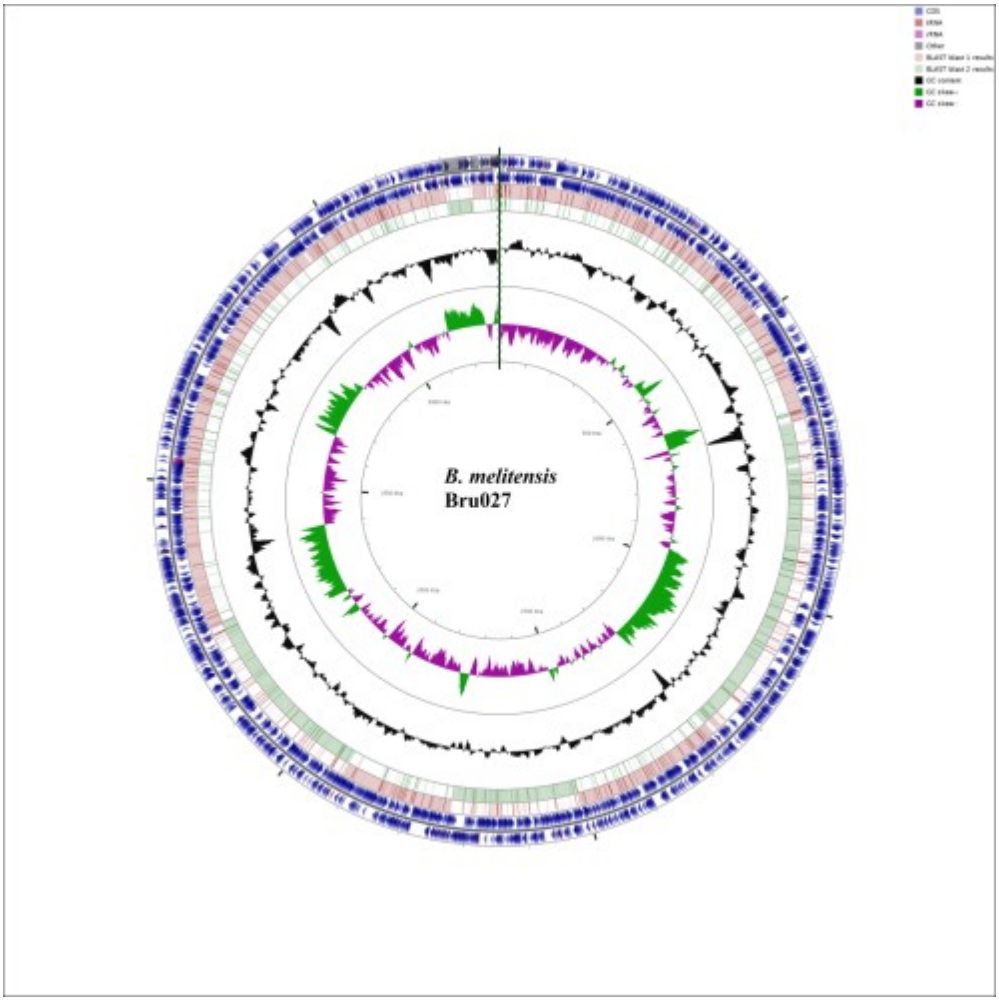

Figure 25. Circular map of Bru027 compared with *B. melitensis* str. 16M reference genome. From outside to inside the rings represent: Bru027 coding DNA sequence (CDS) on the forward and reverse strand (blue), *B. melitensis* 16M chromosome 1 and 2 Blast results (light pink and light green) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green) and G+C negative skew (purple). CGview Server V 1.0 (Grant & Stothard, 2008) was used to construct this image.

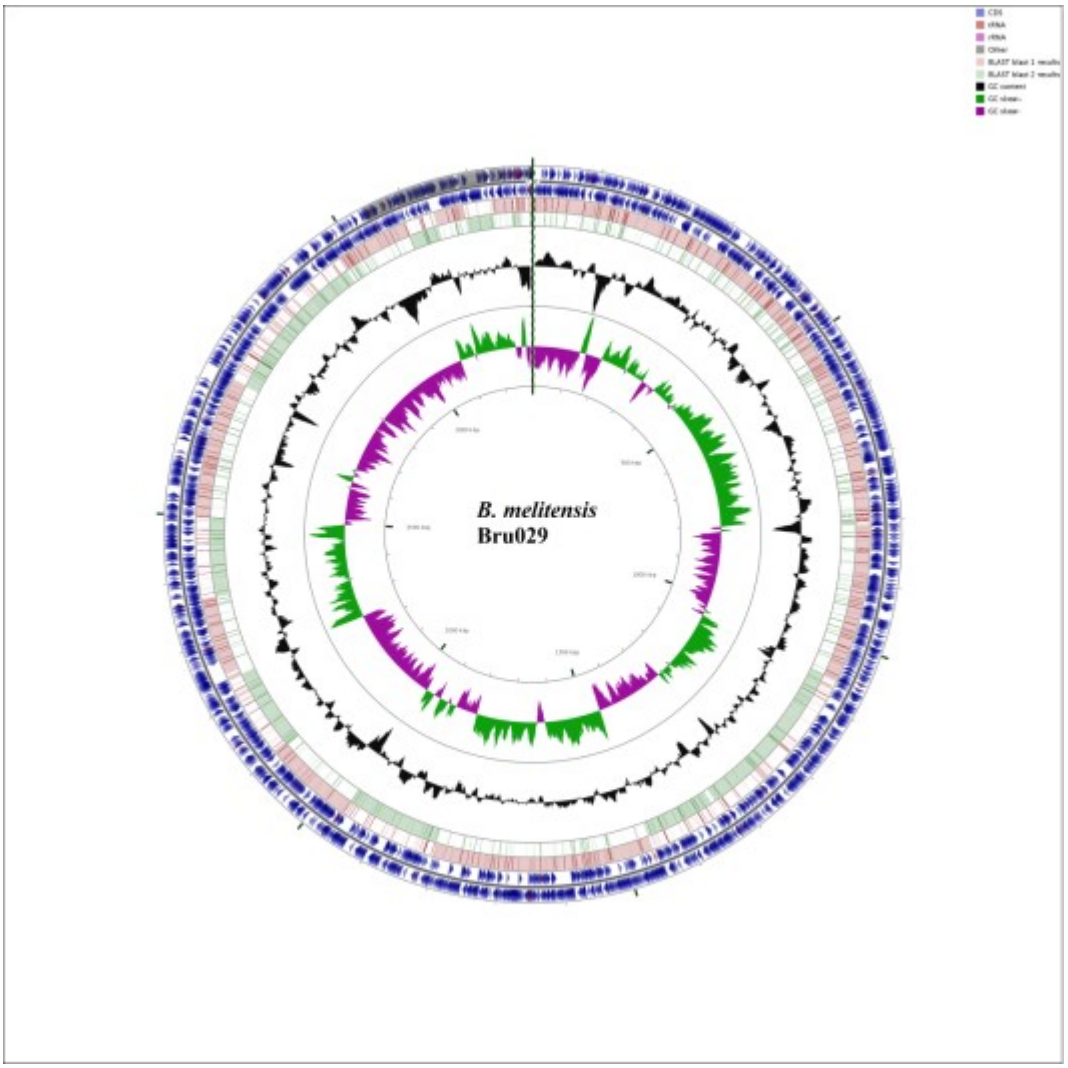

Figure 26. Circular map of Bru029 compared with *B. melitensis* str. 16M reference genome. From outside to inside the rings represent: Bru029 coding DNA sequence (CDS) on the forward and reverse strand (blue), *B. melitensis* 16M chromosome 1 and 2 Blast results (light pink and light green) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green) and G+C negative skew (purple). CGview Server V 1.0 (Grant & Stothard, 2008) was used to construct this image.

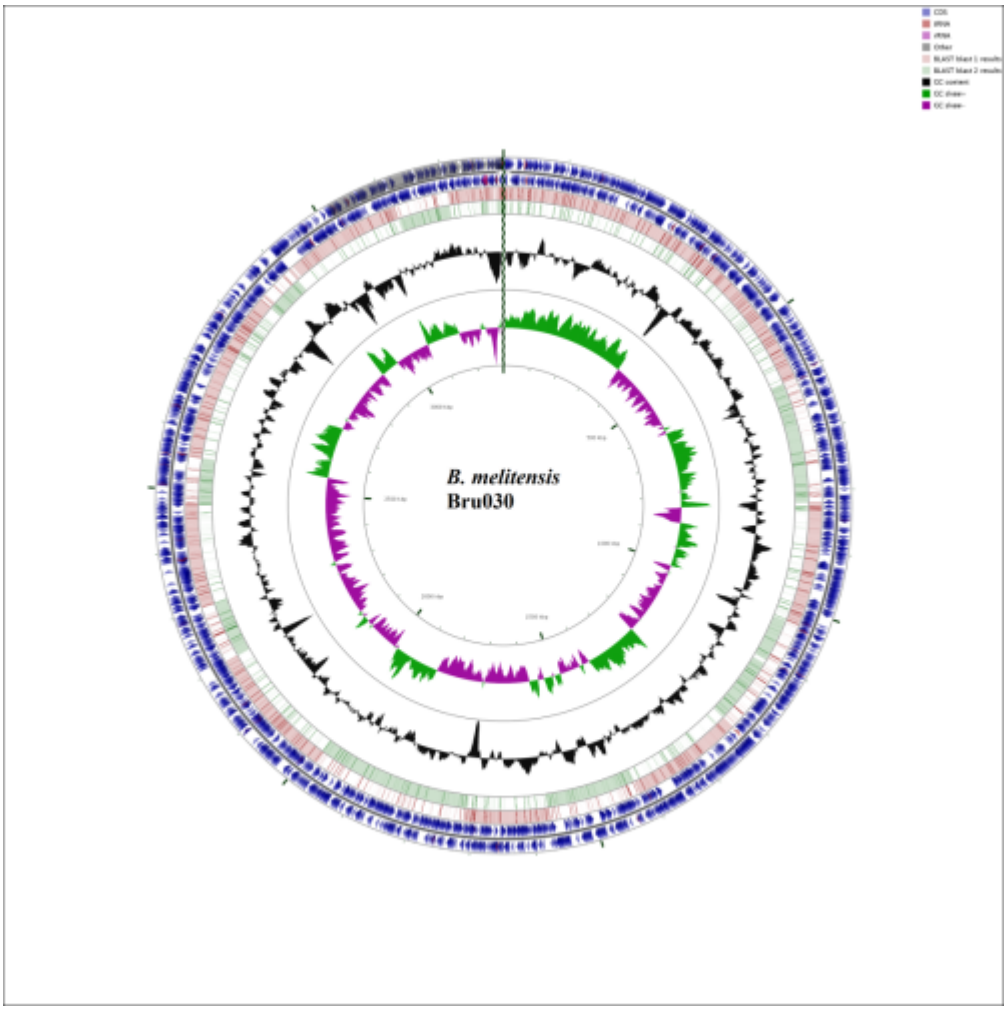

Figure 27. Circular map of Bru030 compared with *B. melitensis* str. 16M reference genome. From outside to inside the rings represent: Bru030 coding DNA sequence (CDS) on the forward and reverse strand (blue), *B. melitensis* 16M chromosome 1 and 2 Blast results (light pink and light green) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green) and G+C negative skew (purple). CGview Server V 1.0 (Grant & Stothard, 2008) was used to construct this image.

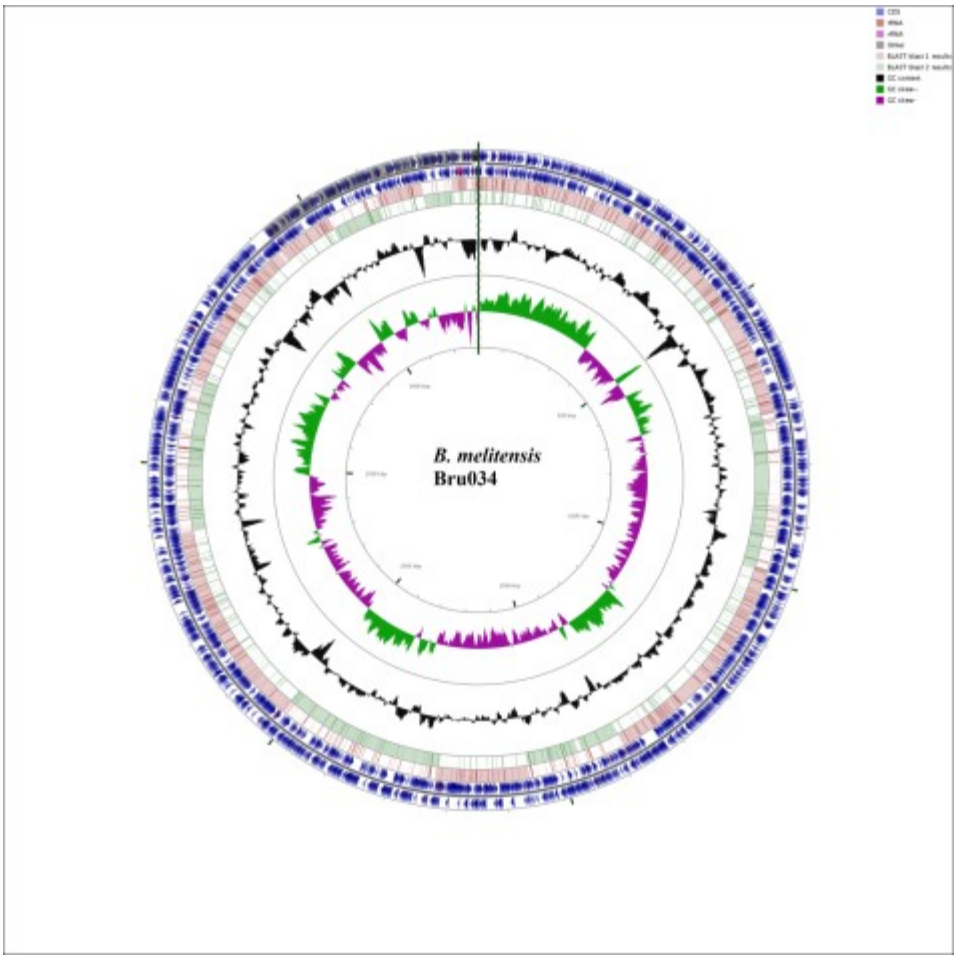

Figure 28. Circular map of Bru034 compared with *B. melitensis* str. 16M reference genome. From outside to inside the rings represent: Bru034 coding DNA sequence (CDS) on the forward and reverse strand (blue), *B. melitensis* 16M chromosome 1 and 2 Blast results (light pink and light green) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green) and G+C negative skew (purple). CGview Server V 1.0 (Grant & Stothard, 2008) was used to construct this image.

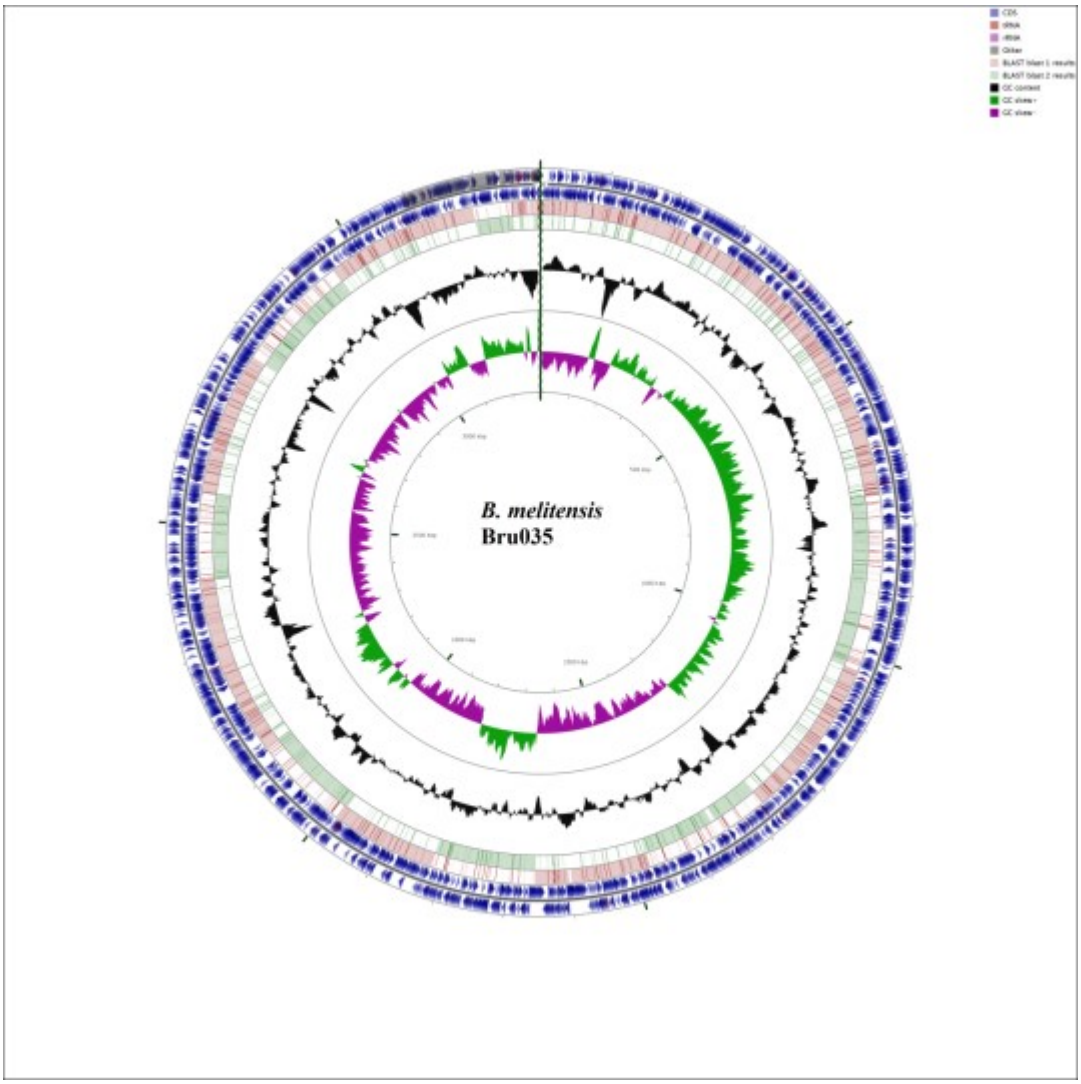

Figure 29. Circular map of Bru035 compared with *B. melitensis* str. 16M reference genome. From outside to inside the rings represent: Bru035 coding DNA sequence (CDS) on the forward and reverse strand (blue), *B. melitensis* 16M chromosome 1 and 2 Blast results (light pink and light green) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green) and G+C negative skew (purple). CGview Server V 1.0 (Grant & Stothard, 2008) was used to construct this image.

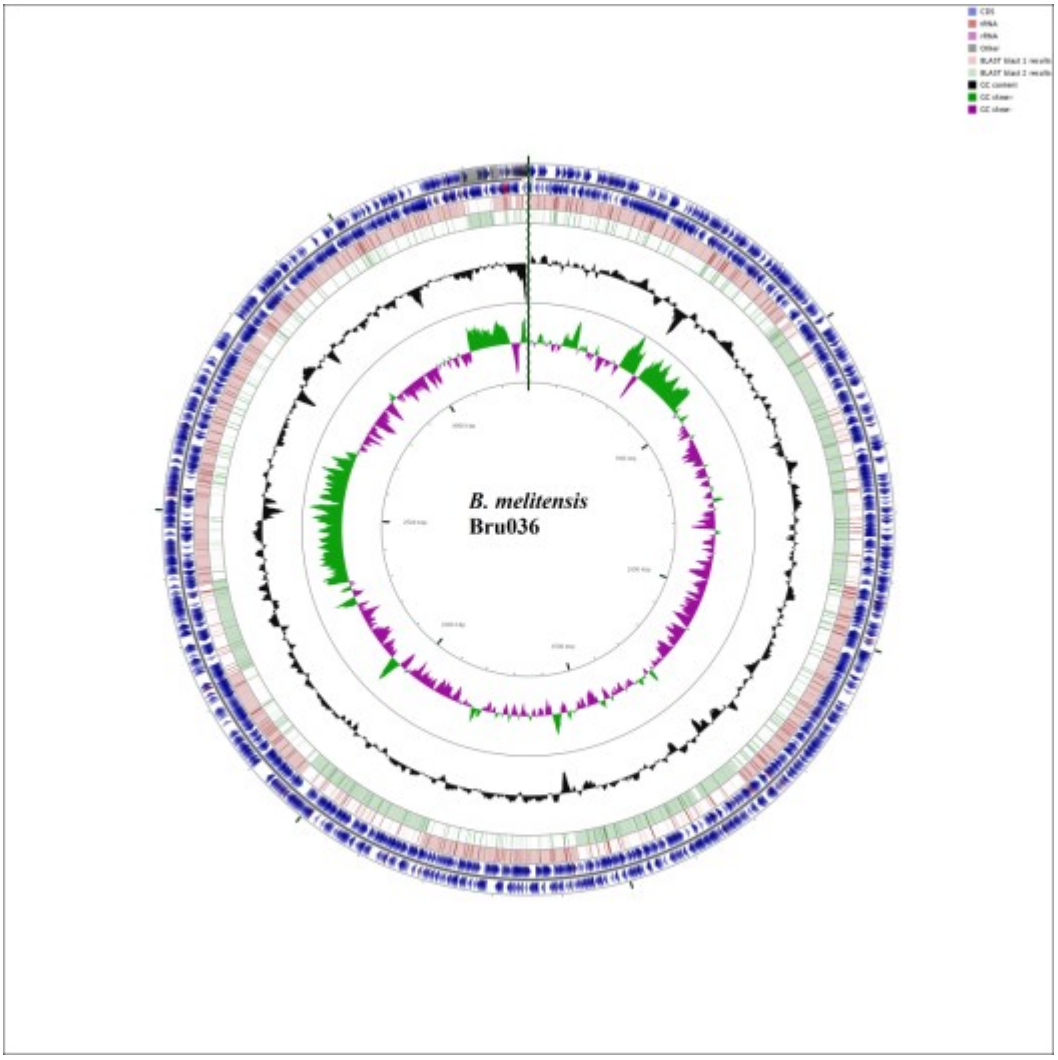

Figure 30. Circular map of Bru036 compared with *B. melitensis* str. 16M reference genome. From outside to inside the rings represent: Bru036 coding DNA sequence (CDS) on the forward and reverse strand (blue), *B. melitensis* 16M chromosome 1 and 2 Blast results (light pink and light green) representing the positions covered by the BLASTN alignment, G+C content (black), G+C positive skew (green) and G+C negative skew (purple). CGview Server V 1.0 (Grant & Stothard, 2008) was used to construct this image.