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Bioinformatics Analysis Reveals Potential Genetic Markers for *Brucella melitensis* Rev.1 Vaccine Strain: Their Use in Developing a New PCR Approach to Distinguish Rev.1 from Field Strains

By

Mohammad Nouh Suleiman Issa

In Partial Fulfillment of the Requirements for the Degree

Master of Science

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The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in biotechnology

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ABSTRACT

Brucella melitensis is an intracellular bacterium, primarily affecting goats and sheep causing abortion and infertility. In addition, it is considered as one of the zoonotic pathogens that can be transmitted to human causing a depilating disease known as Malta fever.

B. melitensis Rev.1 strain, isolated in 1953, is currently considered as the best available live attenuated vaccine to control *B. melitensis* infection in animals. Unfortunately, Rev.1 vaccination interferes with serological diagnosis of Brucellosis. Rev.1 strain is not attenuated enough; it usually causes abortion in vaccinated animals and can infect human. Since the genetic markers and the molecular evidences for Rev.1 attenuation are not known yet, controlling Rev.1 stability and traceability during production and applying post-vaccination molecular epidemiology tests will remain difficult tasks.

The ancestor of Rev.1 strain acquired a series of in-lab mutations that converted it into an attenuated strain. Discovering the genetic events that lead to Rev.1 attenuation will help in Rev.1 molecular diagnosis, differentiation and quality control of vaccine production.

In this study, Rev.1 genome sequence was compared to the genome sequences of different *B. melitensis* strains that are publically available in various databases. The bioinformatic analysis revealed a set of 24 unique genetic variations (SNPs) that are exclusive to Rev.1 genome. Of these 24 SNPs, ten SNPs were tested in the laboratory and confirmed by PCR. A total of nine SNPs were confirmed by DNA sequencing. A novel bi-directional allele-specific PCR technique was successfully developed to target five of

these SNPs in Rev.1, and shows their presence in Rev.1 and absence in three *B. melitensis* reference strains DNA representing biovar 1, 2 and 3. One SNP were tested with bidirectional allele specific PCR on a collection of *B. melitensis* local isolates.

In conclusion, a group of potential genetic markers were discovered, and are specific for Rev.1 vaccine strain. Selected subsets of the identified markers were successfully used to develop a practical and cost effective PCR assay that can differentiate Rev.1 vaccine strain from other field strains.

Key words: *Brucella melitensis,* Rev.1 strain, genetic markers, bi-directional allele-specific PCR technique, *Brucella* vaccine, SNP.

التحليل الحاسوبي للبيانات الجينية يكشف عن علامات جينية محتملة لعترة اللقاح *Brucella melitensis* Rev.1 واستخدام العلامات المكتشفة في تطوير فحص PCR جديد لتمييز عترة اللقاح عن العترات الحقلية

تسبب جرثومة *Brucella melitensis* مرضا مشتركا بين الانسان و الحيوان. حيث تصيب بشكل اساسي الحيوانات لتسبب اجهاض وقلة خصوبة و تنتقل الى الانسان لتسبب له مرض الحمى المالطية.

تحصين الحيوانات بلقاح حي مضعف عترة *B. melitensis* Rev.1 يعتبر اللقاح الافضل الممكن استخدامه للسيطرة على هذا المرض. و لكن هذا التحصين سيتعارض مع طرق التشخيص المصلي للحيوانات و عترة (Rev.1) ليست مضعفة بما يكفي فهي تسبب الاجهاض في الحيوانات المحصنة و تشكل خطرا بانتقالها للانسان. و بما انه لم تعرف بعد عدد من العلامات الجينية الفارقة لهذه العترة و لم يعرف السبب الجيني لكونها مضعفة ستبقى عملية ضمان جودة اللقاح وسلامته و فاعليته و القدرة على تتبع هذه العترة بعد التحصين امرا صعبا.

لقد تحولت عترة (Rev.1) الى عترة مضعفة عبر اكتسابها مجموعة من الطفرات الجينية المخبرية لعترة كانت ممرضة لسلسا. لذلك فان الكشف عن هذه الطفرات المكتسبة و التي حولت عترة ممرضة الي عترة مضعفة وهي عترة (Rev.1) سيساعد باكتشاف علامات جينية فارقة ممكن ان تستخدم في التشخيص الجزيئي و تساعد في عملية ضمان جودة اللقاح و عملية تتبع العترة بعد التحصين و سيعزز فهمنا لامراضية هذه العترة.

في هذه الدراسة تم مقارنة جينوم (Rev.1) مع ما هو متاح من جينوم عترات اخرى من نفس الجرثومة باستخدام طرق المعلوماتية الحيوية (Bioinformatics) و ادواتها المختلفة. خلصت النتائج الى وجود 24 طفرة نقطية (Point mutation) موجودة حصرا في جينوم (Rev.1), تم تاكيد عشرة طفرات بواسطة تفاعل البلمرة المتسلسل (PCR) و 9طفرات اخرى اكدت بواسطة فحص تحديد التسلسل النيوكلوتيدي للحمض النووي (DNA Sequencing). تم استخدام 5طفرات بنجاح بواسطة فحص جديد يعتمد على تفاعل بلمرة متسلسل ثنائي الاتجاه (bi-directional allele-specific PCR technique) للتمييز بين عترة اللقاح و عترات مرجعية ممثلة للمجموعات المعروفة و هي(Biovar 1,2 and 3). و تم استهداف طفرة واحدة بالفحص الجديد مع مجموعة من العينات و العزلات الحقلية المحلية.

لقد تم اكتشاف مجموعة مقترحة من العلامات الجينية المميزة لعترة (Rev.1) و تم استخدام بعضها بنجاح للتفريق بين هذه العترة و عترات اخرى بواسطة فحص طور خصيصا لهذا الغرض يعتبر قليل التكلفة و عملي و يمكن استخدامه لتسهيل عملية التمييز بين عترة (Rev.1) و باقي العترات الممرضة و كذلك في ضمان جودة االلقاح المستخدم و متابعته في الحقل.

DECLARATION

I declare that the Master Thesis entitled:

Bioinformatics Analysis Reveals Potential Genetic Markers for *Brucella melitensis* Rev.1 Vaccine Strain: Their Use in Developing a New PCR Approach to Distinguish Rev.1 from Field Strains

is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

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DEDICATION

To the memory of my mother

To my dear wife and my lovely daughters

To everyone… who taught me anything

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LIST OF ABBREVIATIONS

a. a	Amino acid
ABC transporters	ATP-binding cassette transporters
ARMS	Amplification Refractory Mutation System
BBP	*Brucella* Bioinformatics Portal
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CFT	Complement Fixation Test
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization of the United Nations
Indels	Nucleotides Insertion or deletions
IS	Insertion Sequence
LPS	lipopolysaccharide
Mbp	Megabase pair (1,000,000) bp
MLVA	Multiple Locus Variable Number Tandem Repeats Analysis
MOA	Ministry of Agriculture
MOH	Ministry of Health
NCBI	National Center for Biotechnology Information
OIE	Office International des Epizooties / World Organization for Animal Health
OMP	Outer membrane proteins
OPS	O-Polysaccharide
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
CVL	Central Veterinary Lab
PHIDIAS	Pathogen Host Interaction Data Integration and Analysis System
RE	Restriction Enzyme
RFLP	Restriction Fragment Length Polymorphism
RPM	Round Per Minute
rpsl	30S ribosomal protein12 gene
SNP	Single Nucleotide Polymorphism
TBE buffer	Tris/Borate/EDTA buffer
TE buffer	Tris/EDTA buffer
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization
Wt	Wild type
µl	Microliter
°C	Degree Celsius

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CHAPTER 1
INTRODUCTION

1.1 Brucellosis

Brucellosis is a serious zoonotic disease caused by *Brucella* species and characterized by infertility and abortion in animals and a debilitating febrile disease in human (Radostitis et al. 2007). It is an ancient disease; scientists have traced Brucellosis back to more than 3700 year ago (Seleem et al. 2010). David Bruce isolated the first *Brucella* in 1887 (known as *Micrococcus melitensis* at that time). He isolated the organism from a dead soldier spleen, during his efforts to discover the causes of Malta fever, a common disease among British soldiers in Malta Island. However, the source of infection was revealed after 1905 when a group of goats imported from Malta by ship, and all the ship crew developed the disease after drinking from the goats' raw milk. As a result, the British Army banned goat milk in their army (Radostitis et al. 2007).

Brucellosis is considered as one of the most important zoonotic diseases, with more than 500,000 human cases reported annually by the World Health Organization (WHO) (Pappas et al. 2006). Also it is considered as one of pathogens that have a bio-weapon potential, as well as the most common lab acquired pathogen (Seleem et al. 2010).

1.1.1 *Brucella* species

Brucella is a facultative intracellular gram negative coccobacillus, non-spore forming and non-capsulated. It belongs to the alpha-2 subdivision of the Proteobacteria. Seven *Brucella* species affect terrestrial animals: *Brucella melitensis*, *B. abortus*, *B. suis*. *B. ovis*, *B. canis*, *B. neotomae* (V. N. Cloeckaert 2005) and new species *B. microti* (Scholz et al. 2008). Two other *Brucella* species *B. pinnipedia* and *B. ceti were* reported in marine mammals (Foster et al. 2007). Each species has a primary host, but can be isolated from other animals (see table 1.1). Human can acquire the disease from domestic animals but are not usually a source of the infection.

Table 1.1 *Brucella* species, hosts and biotypes. Adapted from (Hagan et al. 1988; Winn & Koneman 2006; Seleem et al. 2010)

***Brucella* species**	**Biotypes**	**Principal host**	**Isolated from other hosts**
B. melitensis	Biovar 1,2 and 3	Goat, Sheep	Human, Cattle, Pigs, Dogs
B. abortus	Biovar 1,2 3,4, 5,6, and 9	Cattle	Human, Sheep, Goat, Pigs, Horses, Dogs
B. suis	Biovar 1,2,3,4 and 5.	Pigs	Human, cattle, Horses, Dogs
B. ovis	Nil	Sheep	Nil
B. canis	Nil	Dogs	Human (rare)
B. neotomae	Nil	Wood rat	Nil
B. microti	Nil	Common vole	Nil
B. pinnipedia	Nil	Seals	Nil*
B. ceti	Nil	Cetaceans	Nil*

* Some lab-acquired infections were reported in human (Nymo et al. 2011).

1.1.2 *Brucella* Genome

The different *Brucella* species, which infect terrestrial animals, have a high degree of Deoxyribonucleic acid (DNA) homology, more than 90% in DNA-DNA hybridization assay (V. N. Cloeckaert 2005). Based on DNA homology, some researchers argue that all *Brucella* species should be considered as one species *B. melitensis* and all other species as biovar subspecies. The other two marine *Brucella* species *B. pinnipedia* and *B. cetaceae* have different molecular characteristics (V. N. Cloeckaert 2005). Currently, the taxonomy according to pathogenicity and host preferences is more acceptable than using DNA homology, because extensive analyses for the *Brucella* genomes revealed variations present in species specific genetic markers (Moreno et al. 2002).

Brucella melitensis genome contains 3.29 Megabase pair (Mbp), which has a total of 3,197 predicted Open Reading Frames (ORFs) (DelVecchio et al. 2002). The origin of replication is similar to that found in alpha-proto bacteria (Moreno et al. 2002). The average G/C contents are 57%. *Brucella* is not known as a motile organism, but surprisingly, some genes for construction of flagella are found in *Brucella* genome. Those flagella genes may have relations to the evolution of *Brucella* from motile bacteria (DelVecchio et al. 2002).

There are two replicons found in *Brucella* species chromosome I (2.1 Mbp) and chromosome II (1.17 Mbp). These two replicons are considered two different chromosomes and not as a chromosome and a mega plasmid. Since both replicons contain genes essential for bacterial survival (rRNA operons and other house keeping genes) (DelVecchio et al. 2002). An exception was noticed in *B. suis* biovar 3 which has only one chromosome with 3.1 Mbp size.

Restriction Enzymes (RE) has been used in genotyping of *Brucella* species. The different restriction patterns in *B. melitensis, B. abortus*, *B. ovis* and *B. canis* can be seen by digestion in *Hind*III and by using whole genome restriction fragment and Pulsed Field Gel Electrophoresis (PFGE). while 8 fragments was seen using *Pac*I restriction enzyme, only 5 fragments were see when using *Xba*I restriction enzyme. The differences between species: small insertions and deletions are found more frequently in the small chromosome compared to the other chromosome (V. N. Cloeckaert 2005).

There are repetitive elements known as insertion sequences (IS) that are heterogeneously present along the genomes of the different *Brucella* species. For example, IS 711 varies among species from 5 to 35 copy: in *B. melitensis* 7 to 10 copies of IS711 and 6 to 8 copies in *B. abortus* (V. N. Cloeckaert 2005). These insertion sequences are frequently used for Brucellosis molecular diagnosis and differentiation.

1.1.3 *Brucella* infection and transmission

After ingestion, *Brucella* penetrates the mucosal surface of elementary tract and engulfed by phagocytes where it can survive and even multiply. Outer Membrane Proteins and lipopolysaccharide play a major role in *Brucella's* virulence to resist the digestion inside the macrophages (Hirsh & Zee 1999). The intracellular localization makes the treatment of *Brucella* by antibiotics not fully effective. Localization of *Brucella* occurs initially in the lymph nodes and spreads to other lymphoid tissues, such as spleen, mammary gland, and iliac lymph nodes. Then, *Brucella* tends to localize in the pregnant uterus, testicles and joint capsule, where it causes reproductive failure, abortion in females and orchitis, epididemitis and sterility in males (Radostitis et al. 2007).

Routes of infection are via ingestion, nasal or conjunctival infection, and through skin abrasions (Radostitis et al. 2007), Horizontal transmission between animals usually occurs by direct ingestion of contaminated feed. *Brucella* can persist in grass depending on environmental conditions. It can survive in pasture for 100 days in winter and 30 days in summer (Radostitis et al. 2007). Congenital infection may occur from infected mothers, through in-utero or by consumption of colustrum and milk. The infection persist in young animal with negative serological test and without any clinical signs until sexual maturity and first abortion or parturition occurs (Radostitis et al. 2007). Human infection occurs through consumption of unpasteurized dairy products or by direct contact with infected materials such as: placenta, fetuses and vaginal discharge.

1.1.3.1 Molecular pathogenicity

The pathology of brucellosis reflects the outcome of a battle between the hosts' immunity and the *Brucella* genome. In general the infection cycle of *Brucella* has been partially understood. Many genes were studied to reveal the complex virulence pathways, by studying genes involved in: regulatory proteins, transcriptional regulations, metabolic pathways and factors which organized the quorum sensing (the communication between

bacteria) (Jean-Jaques Letesson & De 2004). Some of the molecular pathogenicity aspects are mentioned below:

Lipopolysaccharides (LPS), which are large molecules composed of lipid and polysaccharides play a major role in *Brucella* virulence by resisting digestion inside the macrophage (Hirsh & Zee 1999). Also, LPS are highly immunogenic molecules and induce a specific antibody response. Generally, glycosyl transferase enzyme and ATP-binding cassette transporters (ABC transporters) play important role in LPS construction. It is known that the *B. abortus* vaccine strain called RB51 has a mutation in WboA gene which codes for glycosyl transferase. This mutation leads to changes in LPS and as a result changes the antibody response in vaccinated animals. Also, this mutation is used in Polymerase Chain Reaction (PCR) assay to differentiate between RB51 vaccine strain and other *Brucella abortus* field strains (Vemulapalli et al. 1999).

Erythritol, a four-carbon atom sugar alcohol, is produced by fetuses and stimulates *Brucella* growth and localization in graved uterus. Increasing Erythritol levels in last third of pregnancy is responsible for *Brucella* abortion in ruminants (Radostitis et al. 2007). The *ery* operon containing four genes (*ery* A, B, C and D) are responsible for Erythritol metabolism. A large deletion in *ery* operon was found in *B. abortus* vaccine strain *S19* (Juan & J.Sangari García 2004). In addition, this deletion considered as one of attenuation causes for vaccine strain *B. abortus S19.*

In macrophages, few bacteria will arrive at endoplasmic reticulum, which is considered as the *Brucella* replicating niche (Moreno & Gorvel 2004). Furthermore, *Brucella* inhibit macrophage apoptosis by increasing cellular cAMP and blocking TNF-α, which keeps macrophages alive as long as possible (Xavier et al. 2004). *Brucella* can extract its entire replication nutritional requirements under harsh condition in macrophage endoplasmic reticulum. These unique properties have been reinforced by the complete genomic analysis of Brucella, which have demonstrated the presence of genes coding for many metabolic alternatives (Xavier et al. 2004). Both the LPS and type-IV secretion apparatus were thought to play role in avoiding phagolysis and in intracellular trafficking. Fretin et al.

reported that *B. melitensis* can construct a functional flagella during intracellular phase, which might be used in intracellular trafficking (Fretin et al. 2005).

1.2 *Brucella melitensis*

Most human Brucellosis cases are caused by *B. melitensis* (Pappas et al. 2006) which is considered as the most invasive and pathogenic *Brucella* species to humans (Radostitis et al. 2007).

1.2.1 *B. melitensis* in the Middle East

Brucellosis caused by *B. melitensis* is a common disease in Middle East; cattle sheep, goat, buffalo, and camel cases were reported. *B. melitensis* biovar 3 has been the most commonly isolated species from animals in Israel, Egypt, Jordan and Turkey (Radostitis et al. 2 007).

The human incidence of Brucellosis in Middle East is relatively high, particularly in Syria (Table 1.2). In Israel, the disease has a high incidence amongst Arabs 70 case/1000 000, compared to 2 case / 1000 000 among Jews (Anis et al. 2011). The highest human incidence has been reported in the southern area among the Arabs in the Naqab regions.

Table 1.2: Brucellosis human annual incidences in Middle East (Source Pappas et al. 2006)

Country	Annual cases /Million
Syria	1603.4
Jordan	23.4
Lebanon	49.5
Egypt	2.95
Iraq	278.4
Saudi Arabia	214.4
Turkey	262.2

1.2.2 *B. melitensis* in Palestine

B. melitensis is the cause of animal and human Brucellosis in Palestine. According to official reports of the Ministry of Agriculture (MOA), 18% of small ruminants (sheep and goat) were serologically positive for *Brucella* in 1998 while 75% of the herds were also affected (Ministry of Agriculture 2009). According to Ministry of Health (MOH), the human cases were 837 in that same year. The human incidence of Brucellosis was the highest in the southern area of West-Bank, particularly in Hebron district (Fig1.1) (Ministry of Agriculture 2009). In 2005, another animal screening for Brucellosis revealed that 50% of the herds of small ruminants were positive while 5% of sheep and goat were positive in the general population (Ministry of Agriculture 2009). The human Brucellosis cases were decreased in the period between 2000 and 2006 and reached less than 200 cases annually. This reduction in human cases after 1999 mainly due to the mass vaccination program to immunize small ruminants using live *B. melitensis* Rev.1 vaccine by conjunctival route (Fig 1.1).

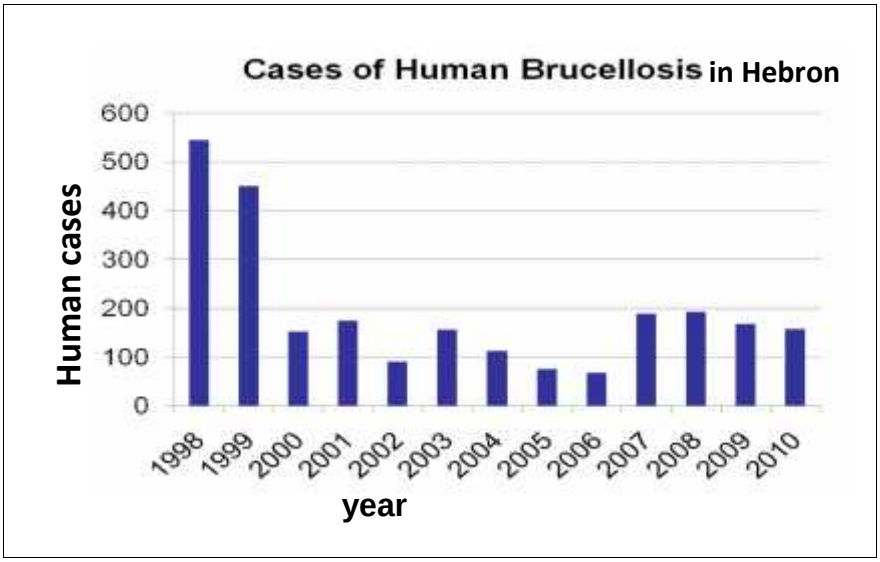

Fig 1.1: Human Brucellosis in Hebron. Show the human cases of Brucellosis reported in Hebron district by the Ministry of Health. Source: Dept. of epidemiology- Hebron (MOH)

In Palestine, the most important risk factor for Brucellosis is the consumption of home-made cheese (70% of cases) (Awad 1998). The adverse effects of being a Brucellosis-endemic country are not limited to losses in livestock health, and in zoonotic risk. It also prevents exporting of dairy products, where many third countries have import-restrictions to the local dairy products due to Brucellosis concerns.

1.2.3 *B. melitensis* Control

The control program for Brucellosis largely depends on controlling the disease in animal population and raising the public awareness about risks of consuming unpasteurized dairy product. In animal farms, control measurements depend on applying high level hygienic conditions at kidding and lambing, correct disposal for infected materials, and vaccination in endemic areas (Radostitis et al. 2007). According to World Organization for Animal Health (OIE), Food and Agriculture Organization (FAO) and WHO recommendations, vaccination with live attenuated *B. melitensis* Rev.1 strain is the best vaccine strain that can be used in small ruminants. In countries with high disease prevalence, vaccination and improvement of the farming management are considered essential practical controlling methods. However, in countries with low incidence rate, a complete eradication program should implemented, depends on a test and slaughter policy, where all *Brucella* serologically positive animals are slaughtered. This control approach is usually prolonged and expensive because of inaccuracy of the serological tests that cannot distinguish whether the positive signal is due to Rev.1 vaccine strain or real field strains (Radostitis et al. 2007).

1.3. B. melitensis Rev.1 Vaccine Strain

1.3.1 Rev.1 History

In 1953 M. Herberg and S Elberg, at the University of California, conducted a series of experiments to attenuate *B. melitensis* to use it as a live attenuated vaccine (Herzberg & Elberg 1953). This work was inspired by similar early works done on *Mycobacterium*

tuberculosis. The efforts of the two scientists began with the *B. melitensis* virulent strain 6056. They tried to get a mutant which has a Streptomycin dependent phenotype. They picked a single mutant *Brucella* colony from a media containing Streptomycin. This mutant *Brucella* was confirmed to be Streptomycin dependent and it was capable to replicate in mice. But when it was inoculated into Guinea pigs, it failed to cause infection. In addition, it did not cause fever when injected into monkeys. Repeated Injection of Streptomycin into tested animals gave no difference in virulence results. It was known at that time that the lack of Streptomycin dependence could regain the virulence to bacteria. The lack of Streptomycin dependence could be regained by further mutational steps, so they grew their avirulent Streptomycin dependent variant in media with low Streptomycin concentration until they got the wanted non-dependent reverts. Fortunately, this Streptomycin non-dependent variant causes infection in Guinea pigs (M Herzberg & Sanford Elberg 1953).

Later on, one clone from 100 reverted clones (reverted from Streptomycin dependent to non dependent variant) was selected and firstly named in 1954 as *B. melitensis* Rev.1 (the name Rev.1 could have come from revert) as non dependent Streptomycin variant from revisions of Streptomycin dependent *B. melitensis* population (Mendel Herzberg & SS Elberg 1955). The *B. melitensis* Rev.1 was grown in Streptomycin free media but remains the Streptomycin resistance. As Elberg stated that Rev.1 “has the characteristic morphological, colonial properties and sensitivity to bacteriostatic dyes typical of *Brucella* melitensis. However, it grows very slowly in broth and on agar and is agglutinated by anti melitensis serum” (Mendel Herzberg & SS Elberg 1955). Rev.1 gave good results when it was tested in goat as a live vaccine (Sanford S Elberg & K Faunce 1957). These early studies revealed the superiority of Rev.1 vaccine compared to killed vaccine (Banai 2002). In 1964, FAO and WHO Experts Committee on Brucellosis recommended Rev.1 as a live vaccine to fight *B. melitensis* in sheep and goat (G. G. Alton et al. 1967) by subcutaneous injection for 3–8 months old animals (WHO 1964). Other brave experiments attempted to use Rev.1 as a human vaccine in 1966, but stopped early because of high virulence of Rev.1 to human (Hoover et al. 2004)**.** From that time until

now, Rev.1 is considered as the best available vaccine for *B. melitensis* controlling in sheep and goats.

1.3.2 Rev.1 performance

Rev.1 strain injection vaccine is not safe to use in all animals. Full vaccine dose of 10^9 cells by subcutaneous injection to pregnant animals causes abortion (G. Alton 1987). Even in vaccination with reduced dose of Rev.1, It causes abortion (Blasco 1997). Adult goat and sheep continue to shed Rev.1 after vaccination with milk and vaginal secretion for various durations (G. Alton 1987). While 4 to 8 months age vaccinated kids and lambs did not excrete any Rev.1 organisms when they reach to the breeding age (G. Alton 1987).

The duration of immunity varies from 2.5 years to lifelong (G. Alton 1987). However, it is generally acceptable as 2.5 years for young vaccinated animals (Banai 2002).
Rev.1 vaccination for adult animals usually interfered with serological tests and made the individual animal diagnosis almost impossible in flocks. The Complement Fixation Test (CFT) needs one year post vaccination to return negative for the majority of animals while the agglutination tests could stay 4 years post vaccination with low titers (G. Alton 1987).

In 1986, WHO/OIE recommended that Rev.1 vaccination should be restricted to lambs and kids of 3 to 8 months old because of safety concerns and the contradiction with serological tests in adult's vaccination. Unfortunately, this age restriction reduced the efficacy of Rev.1 vaccine specially in infected flocks (G. Alton 1987). Rev.1 could reduce abortions, but could not eliminate Brucellosis from infected flocks (Banai, 2002).

Rev.1 vaccine gave good results in many countries, such as Malta and Mongolia where after applying Rev.1 vaccination, a sharp drop in human cases of Brucellosis was reported (G. Alton 1987). Also, some European countries gave the same satisfactory results. While Rev.1 did not give the same good results in other countries such as Israel (G. Alton 1987) and Spain (Blasco 1997). So the classical recommendation for using age

restricted Rev.1 vaccine failed to control Brucellosis in many countries. These limitations of Rev.1 results lead some researchers to look for new techniques to deliver Rev.1. Early studies which described Rev.1 vaccination via conjunctival route showed that Rev.1 could be used for non-pregnant animals with safer results than subcutaneous route. In addition, conjunctival rout can produce good protection without prolonged interference with serological tests in young animals. The seropositive results may stay for about 4 months in young animals while adult might remain seropositive for longer periods (G. Alton 1987; Blasco 1997).

The Spanish Rev.1 experience began with the classical recommendation of subcutaneous vaccination to animals aged 4 to 8 months old. Unfortunately, after 6 years the Brucellosis prevalence in both animals and in human did not drop, so they announce the failure of the program (Blasco 1997). Then they tried another vaccination route, the conjunctival mass vaccination, which gave better results (Blasco 1997)

1.3.3 Rev.1 in Middle East:

In Israel, the peak of human Brucellosis due to *B. melitensis* was recorded in 1988 (Banai, 2002). In 1990, a Rev.1 like strain biovar 1 with same Penicillin and dye sensitive characteristics was isolated from aborted animals and also from human cases (Banai 2002). Banai noted that it was difficult to know if this Rev.1 like strains came from the Rev.1 vaccine or it originated from a field strain because the molecular evidences for Rev.1 attenuation are not known yet neither solid molecular markers were approved (Banai 2002).

Test and slaughter with compensation policy was implemented in Israel beside Rev.1 injection vaccination for young small ruminants. But this program stopped after a year and a half because of budget shortages. Then in 1997 the Veterinary Services moved to another strategy by using Rev.1 conjunctival route (eye drop) using reduced dose to whole flock vaccination. This method has been adapted according to OIE-FAO-WHO recommendation in 1993 for Brucellosis control in Middle East countries (Banai 2002).

Later on, a storm of abortions in vaccinated flocks has occurred as well as isolation of Rev.1 like strain from aborted animals and from human cases. These unexpected events stopped this vaccination program under increasing public concerns about the safety of Rev.1 vaccine. Finally, a complete ban for Rev.1 vaccination for adult animals was implemented and they returned to Rev.1 injection vaccination for young animals (Banai 2002).

Another example about the Rev.1 vaccine safety concerns and manufacturing errors came from South Africa, when a virulent colony was selected as a seed for Rev.1 vaccination stock. This virulent vaccine leads to horizontal spread of the disease in sheep and causes human Brucellosis with Rev.1 like strain infection (Pieterson et al. 1988; Hunter et al. 1989; Banai 2002).

Both Israel and South Africa experience proved the potential hazard of Rev.1 to spread from vaccinated to healthy animals and even to human. Both cases raise the challenge to improve Rev.1 stability, detection and traceability.

In Palestine before 1999, the implemented vaccination program was the Israeli program. Afterward, the Palestinian National Authority through the MOA launched Brucellosis Control Program by mass vaccination with Rev.1 conjunctival route. The mass vaccination repeated every other year until 2008 and the vaccination was limited to small animals from 2000 and then every other year, The mass vaccination for one year and limited vaccination (for young animals) in the next year and so on (FAO 2009). This vaccination program reduces the human cases sharply (see Fig 1.1). The human cases slightly increased in 2006 and became stable in the last four years. Unfortunately, and despite some compliance from farmers who experienced abortion among their animals, especially at the first year of mass conjunctival vaccination and some human Brucellosis cases among the Veterinary Services staff (the author of this thesis is one of them), no serious research has been carried out to assess safety of Rev.1 vaccine in Palestine.

In Jordan, little data has been published about Rev.1 vaccine. In a study to examine Brucellosis in aborted sheep, high percentage of samples were identified as Rev.1 like strain using PCR- Restriction Fragment Length Polymorphism (PCR- RFLP) targeting *omp2* gene, which might indicate an improper use of Rev.1 vaccination.(Samadi et al. 2010).

Abortion due to Rev. 1 was also reported in Turkey, Egypt, and Iran (Aras & Ateş 2011; Hoda et al. 2007; Saeedzadeh et al. 2012).

1.3.4 Rev.1 production quality control:

In its report in 1986, the FAO/WHO Expert Committee on Brucellosis set a group of quality control tests for seed cultures production that should be carried for each Rev.1 vaccine batch (G. Alton 1987) and they are:

(1) Morphology-based identity test and growth characteristics on agar as well as identity test using stained smears.
(2) Test for the presence of contaminating organisms.
(3) Test for dissociation; batches should produce at least 95% smooth colonies.
(4) Test for the number of viable organisms.
(5) Test for reactivity in Guinea pigs (clearance from spleen with 12 weeks of inoculation).
(6) Tests for antigenicity and immunogenicity by challenge of vaccinated Guinea pigs and mouse model with a virulent *Brucella* strain.

1.3.5 Rev.1 stability

In the early studies, there was no evidence that increase Rev.1 passages could lead to increases Rev.1 virulence or to change any of the known Rev.1 characteristics (G. G. Alton et al. 1967). Also, there was no evidence for Rev.1 ability to transmit from vaccinated to non vaccinated animals (G. G. Alton et al. 1967).

The issue of Rev. 1 stability of Rev.1 has later became an interesting question when millions of Rev.1 doses were used worldwide and many laboratories were involved in producing and storing of Rev.1 vaccine (Bosseray 1991). Rev.1 vaccine trails in Spain revealed a different biological pattern among the different commercial batches of Rev.1 vaccine. Some of these vaccine batches caused abortion of pregnant animals while other batches did not. When those different commercial Rev.1 vaccine batches were tested according to stability tests in mice, the results revealed that some of the safe Rev.1 vaccine batch that didn't causes abortion gave poor immunity (Blasco 1997).

Increased evidences showed that Rev.1 batches in different countries are variable from the original Elberg strain (Bosseray 1991). Bosseray made a comparison between the original Elberg strain from batch 101 (Berkeley, California) and other five commercial vaccine batches from different sources. His study revealed that most commercial strains did not alter the antibiotic resistant profile, but there was a variation on their colony morphology and size, and altered phases from smooth and none smooth colonies. Also, most of the examined commercial strains lost their residual virulence (time needed for vaccine strain to recover from mice spleen) compared with the original strain. When immunogenicity test was performed, (by challenge with a virulent strain and spleen count after that) two of the commercial strains were non protective at all. Therefore, Bossaery suggested that since the genetic marker for Rev.1 is not yet identified, the control of vaccine will remain difficult, and the vaccine seeds should come from the original seed strain. Also he recommended to perform the residual virulence immunogenicity, and phase dissociation tests (smooth and non-smooth colonies) so as to ensure good quality of the vaccine. (Bosseray 1991).

1.3.6 Rev.1 diagnosis and differentiation

There are no serological methods to differentiate between Rev.1 vaccine strain and other field strains. Currently, conventional bacteriology tests as well as molecular methods are used for differentiation and detection of Rev.1 strain.

1.3.6.1 Bacteriology method

Rev.1 has many in vivo and in vitro characteristics that are used to differentiate it from other virulent strains such as: colony size, antibiotic resistance, dye susceptibility and persistence of the organism in Guinea pigs spleen (G. Alton 1987). The three main bacteriological characteristics that are acceptable as Rev.1 distinctive features are:

1) Small colony size 1-2 mm.
2) Streptomycin resistant.
3) Penicillin and dyes (Basic Fuchsin & Thionin) sensitivity (Bosseray 1991; G. G. Alton et al. 1967) See table1.3.

Table 1.3 Rev.1 strain bacteriological characteristics: shows the sensitivity to Penicillin and dyes in concentrations which tolerated by virulent strain *B. melitensis* biovar 1 str.640
From (G. G. Alton et al. 1967)

Strain	Colony Size	Dyes				Antibiotics			
		Basic Fuchsin		Thionin		Penicillin		Streptomycin	
		1:50,000	1: 100,000	1:50,000	1: 100,000	5 Unit Per ml	2.5 Unit Per ml	5ug Per ml	2.5ug per ml
Rev.1	**Small**	**-**	**+**	**-**	**++**	**-**	**-**	**-**	**+++**
640*	**Large**	**++++**	**++++**	**++++**	**++++**	**++++**	**++++**	**-**	**-**

+ = 1- 9 colonies, ++ =10-90 colonies, +++ =100-999 colonies, ++++ = >1000 colonies, - = no growth.
* Virulent field *B. melitensis* strain (biovar 1)

1.3.6.2 Molecular methods:

- ***rpsl* gene Single Nucleotide Polymorphism (SNP)**

The Streptomycin resistance in bacteria is usually caused by mutations in *rpsl* gene, which encodes the ribosomal protein S12. Cloeckaert *et al* found a C to T SNP at position 272 of Rev.1 *rpsl* gene (BMEI0752) which leads to amino acids change at the corresponding codon 91 (Pro91Leu) (Axel Cloeckaert et al. 2002). This SNP causes the lack of a *NciI* restriction enzyme site and when it was targeted with PCR-restriction

fragment length polymorphism (PCR –RFLP) it was found exclusive to Rev.1 genome (Axel Cloeckaert et al. 2002). Later on, the OIE recommends the PCR-RFLP genotyping of *rpsl* as a method to differentiate Rev.1 (OIE 2009). This SNP was also targeted with specific primers in multiplex PCR called Bruce-Ladder, a method developed by López-Goñi group. Bruce-Ladder method can simultaneously differentiate many *Brucella* species and vaccine strains (David García-Yoldi et al. 2006; I López-Goñi et al. 2008).

- Multiple Locus Variable Number Tandem Repeats Analysis (MLVA)

The tandem repeats are short tandem repeats, up to 10 base pair (bp) length that have higher mutation rates than the rest of genome (Le Flèche et al. 2006). These mutating series of repeats are known as Variable Number Tandem Repeats (VNTR). Multi locus VNTR Analysis (MLVA) is PCR based molecular approach, targeting those repeats used for DNA finger-printing. MLVA became a powerful tool in differentiation closely related strains, particularly as epidemiological tracing tool. MLVA is applied by targeting 15 loci in *Brucella* genome (MLVA -15) so as to genotype the different strains including Rev.1 (Le Flèche et al. 2006). MLVA-15 assay was also proposed as an indicator for Rev.1 vaccine stability (David García-Yoldi et al. 2007). However, this technique is laborious, expensive, and some MLVA need special software to read the results and a large database for MLVA results to compare with.

- *omp2* gene restriction site

The *omp* genes that encode the Outer Membrane Proteins (OMPs) of *Brucella* are under greater selection pressure compared to other genes. These factors have to deal with environmental changes outside the host and the immunological attack inside the host. At the same time, *omp* genes have to preserve their functions (Bricker 2004). The *omp2* locus contains two genes: *omp2a* and *omp2b that are* arranged head to head on opposite strands. Targeting this region by PCR- RFLP was suggested as method that can distinguish most, but not all, *Brucella* species (Axel Cloeckaert & Verger 1995).

Rev.1 strain was also found to lack the recognition site of *Pst I* restriction enzyme that is naturally present in *omp2a* gene. By using PCR –RFLP specific to *Pst I* site in *omp*2 gene, Rev.1 was distinguished from other *B. melitensis* field strains that were isolated from Israel and other Middle Eastern countries (Bardenstein & Mandelboim 2002). Unfortunately, the same restriction pattern was seen with *B. melitensis 16M* strain (Bardenstein & Mandelboim 2002), which decreases the value of this method in differentiating Rev.1 from other strains.

The molecular genotyping to distinguish Rev.1 vaccine strain from other field strains is still an attractive subject to many researchers. Recently, a SNP based detection with real time PCR technology was proposed (Gopaul et al. 2010). Gopaul targeted a G to A SNP at 933 of the ORF BMEI0208.

Recently, a modified MLVA technique that targets 8 loci of *Brucella* genome combined with Denaturant Gradient Gel Electrophoresis (DGGE) of *omp2a* and *omp2b* was proposed to differentiate Rev.1 from other field strains (Noutsios & Papi 2012). This method looks specific, yet it is quite sophisticated and requires resources that are not frequently available in most laboratories of developing countries.

CHAPTER 2
Problem Statement and Objectives

Brucellosis caused by *B. melitensis* is a serious zoonotic disease*. B. melitensis* Rev.1 strain is an attenuated strain used as a vaccine to control Brucellosis in animals in most countries including Palestine. Unfortunately, Rev.1 vaccination interferes with *Brucella* serological diagnosis. Also, the vaccine strain is not attenuated enough, therefore Rev.1 usually causes abortion in vaccinated animals and can be transmited to infect human. Since both the genetic markers and the molecular evidences for Rev.1 attenuation are not known yet, there is a great need to identify the distinctive genetic profile of this strain to detect its genetic stability, safety to public health, and traceability in the field after animal vaccination. All of that can be improved by identifying and use the Rev.1 distinctive genetic markers.

The specific objectives of this work are:

- To discover distinctive genetic markers of Rev.1 strain using bioinformatics analysis.

- To develop an inexpensive PCR assay to diagnose Brucellosis and differentiate Rev.1 from field strains in one tube PCR reaction.

- To explore the genotype-phenotype association of the founded Rev.1 genetic alterations and their implications in pathogenicity.

CHAPTER 3
Materials and Methods

3.1 Data and Tools

3.1.1 *Brucella* sequence data

The genomic sequence data of various *Brucella* species including *B. melitensis* strains and Rev.1 vaccine strain were retrieved from the following databases:

- National Center for Biotechnology Information database (NCBI)[1].

    1. The full genome sequences *of* chromosome I and chromosome II *of the B. melitensis* (biovar-1 strain 16M ATCC 23456), with the accession numbers NC_003317 and NC_003318, respectively (total size 3294931 bp).
    2. The full genome sequences of chromosome I and chromosome II *of the B. melitensis* (biovar-2strain ATCC 23457/), with the accession numbers NC_012441.1 and NC_012442.1, respectively (total size 3311219 bp).
    3. The genomic sequences of 93 contigs of an unfinished *B. melitensis* Rev.1 genome project (ACEG0100001 to ACEG01000093) with a total size of 3270141 bp (see Appendix.1).

- *Brucella* Group Sequencing Project of Broad Institute[2] :

    1. The DNA sequences of 3221 ORFs of *B. melitensis* Rev.1 (BAMG 00001 to BAMG 03221) were retrieved in FASTA format.

[1] National Center for Biotechnology Information. at <http://www.ncbi.nlm.nih.gov/>
[2] Broad Institute of MIT and Harvard. at <http://www.broadinstitute.org/>

2. Draft genome sequence of 36 *B. melitensis* strains that were available (as accessed on July 2010) for analysis using Broad Institute online tools (For more details see appendix 2).

- *Brucella* Bioinformatics Portal (BBP)[3]. BBP is a gateway to study and compare *Brucella* genomes available at public databases and different analyses from literature (Xiang et al. 2006). A total of 245 virulent genes from different *Brucella* species were used as a set of validated virulence genes for comparison purposes. BBP is considered as a part of the Host Interaction Data Integration and Analysis System (PHIDIAS).

- The Universal Protein Resource (UniProt)[4]. This database was used to retrieve *B. melitensis* protein sequences, for the purpose of comparing and analyzing the mutations' possible effect on protein function.

3.1.2. Bioinformatics tools

The following online tools were used in our study:

1. NCBI\ BLASTn: programs search nucleotide databases using a nucleotide query
2. NCBI\ BLAST (bl2seq): Align two (or more) sequences.
3. NCBI\ Primer-BLAST Finding primers specific to PCR template (using Primer3 and BLAST)
4. NCBI\ BLAST Microbial Genomes: Performs a BLAST search for similar sequences from selected complete eukaryotic and prokaryotic genomes
5. PerlPrimer v1.1.20 software[5]: is free, open-source software that designs primers for PCR.
6. ExPASy Translate Tool[6]: is a tool which allows the translation of a nucleotide sequence to a protein sequence.

[3] PHIDIAS>>Brucella Bioinformatics Portal. at <http://www.phidias.us/bbp/bruvirf/index.php>
[4] UniProt. at <http://www.uniprot.org/>
[5] PerlPrimer. at <http://perlprimer.sourceforge.net/download.html>
[6] ExPASy - Translate tool. at <http://web.expasy.org/translate/>

7. Broad institute\ Browse Polymorphisms tool: which made mutation comparisons for all strains that are available.
8. Broad institute\ Alignment Viewer: a tool to view multiple alignments for selected strains.
9. Broad institute\ View Polymorphism Site tool: displays the mutations' position in the sequenced strains in form of tables.

3.2 Bioinformatics analyses

The primary objective for the sequences similarity analysis was to identify novel genetic alterations that are unique to Rev.1 strain, which could be used as genetic markers for molecular identification purposes.

3.2.1 Searching for large Insertions/deletions (Indels) in Rev.1 Genome

The aim of this approach is to identify relatively large nucleotides insertions or deletions (indels) exclusive to Rev.1 genome that can be used in conventional PCR to distinguish between Rev. 1 and other field strains by amplicon size. Pairwise sequence alignment was performed using Basic Local Alignment Search Tool (BLAST) from NCBI using BLASTn (bl2seq) function to compare all the 93 contigs (contig is from contagious, in shotgun sequencing the short DNA reads are overlapped to form contigs) of *B. melitensis* Rev.1 (biovar-1) against the genome of *B. melitensis* biovar-1 strain 16M. The DNA segments that have indels of 5 bp or more were further compared to *B. melitensis* biovar-2 strain ATCC 23457. The segments with potentially unique indels to Rev.1 genome were further filtered using BLASTn against all available Brucellaceae family genomic sequence in NCBI databases (as accessed on January 2011) (Fig 3.1).

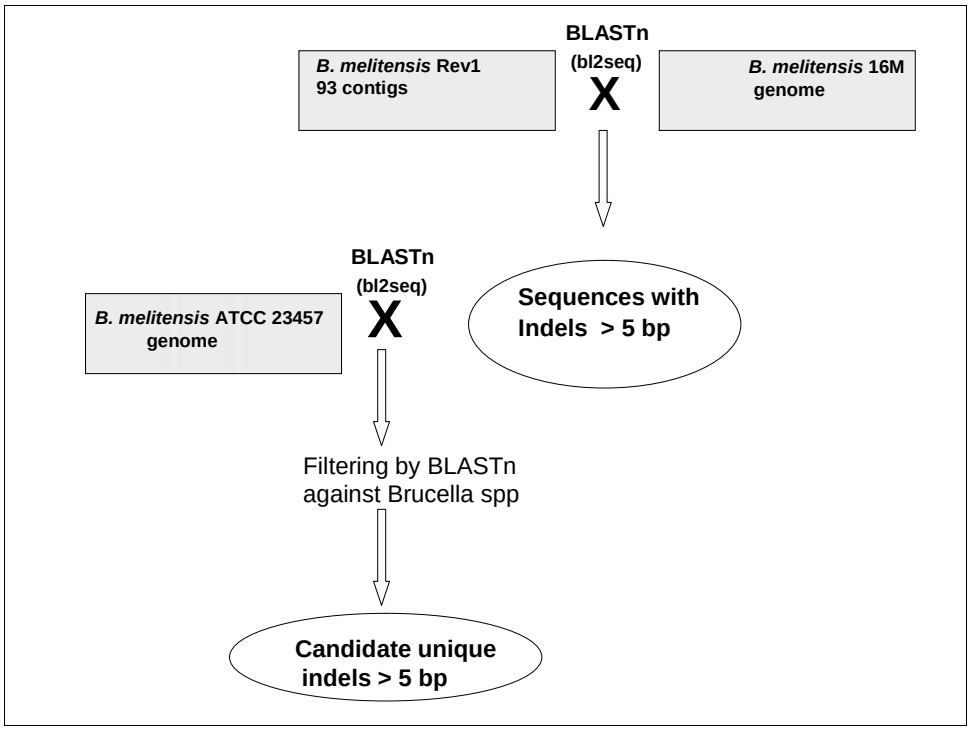

Fig 3.1: The Bioinformatics methods used to find Rev.1 unique large indels (more than 5 bp size). The Rev.1 contigs were analyzed by BLASTn align tool against *B. melitensis* 16M genome. All the resulted indels were analyzed by BlASTn align tool (bl2seq) against *B. melitensis* ATCC 23457 genome. Further BLASTn analysis was preformed to confirm the uniqueness of the indels in Rev.1 by comparing the obtained results to all bacterial genomes available at NCBI.

3.2.2 Searching for Rev.1 point mutations

- **NCBI analyses**

The Broad Institute has designated the Rev. 1 ORFs starting from BAMG 00001 to BAMG 03221. All Broad Institute DNA sequences of Rev.1 were retrieved as FASTA format. All ORFs were compared using BLASTn (bl2seq) tool against *B. melitensis* 16M genome chromosome I and chromosome II.

The BLAST results were filtered using maximum identity value. Any gene which had maximum identity less than 100% was considered as a gene with potential genetic alteration (variant gene). Also, the variant Rev.1 genes were blasted against *B. melitensis* biovar-2 ATCC 23457 chromosome I and chromosome II. The gene locus tag annotations

for *B. melitensis* 16M according to NCBI (BMEI & BMEII format) were obtained by using NCBI BLAST again for the variant Rev.1 genes with BAMG annotation.

- **Broad Institute analysis**

The Broad Institute tool "Polymorphism Predictions tool" was used to search for potential polymorphisms of Rev. 1 genome applying the following parameters:

- Reference genome: *B. melitensis* 16M.
- Mutation in: *B. melitensis* bv. 1, str. Rev.1
- And Mutations Not In: the other 35 strains (repeated many times).

A further confirmation of the uniqueness of the identified Rev.1 mutations was preformed using Broad institute tools such as: View polymorphism tool Multiple Genome Alignments and Alignment Viewer.

The candidate Rev.1 unique mutations that resulted from both NCBI and Broad Institute analyses were filtered by Blasting them using BLASTn to exclude any similar mutation in any orthologous genes of any known bacteria including the Brucellaceae family. The targeted databases in this search were the reference genomes that are provided as a default parameter by BLAST microbial genomes (see fig 3.2).

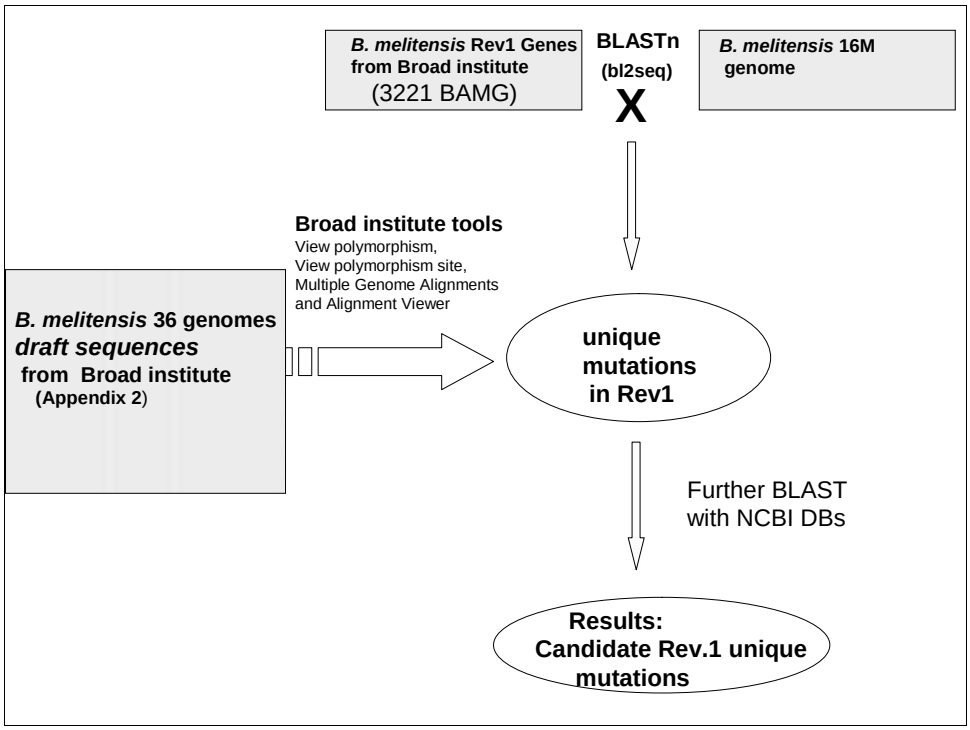

Fig 3.2: Searching for unique Rev.1 mutations in coding sequences. All Rev.1 genes were compared by BLAST with *B. melitensis* 16M genome .The Broad Institute draft sequences of 36 *B. melitensis* strains and tools were used, to find the unique Rev.1 mutations that could be used as a genetic barcode for Rev.1. The final filtration was performed using the genes with potentially unique mutation in Rev. 1 in a BLASTn search to ensure that such mutations are also absent in orthologous genes of any known bacteria.

3.2.3 Analysis of mutations' impact on protein translation

Mutations in coding regions of Rev.1 were tested for their impact on amino acid translation by using ExPASy Translate Tool[7] and using ORFs of *B. melitensis* 16M as wild type references. The mutations were categorized as synonymous or non-synonymous mutations.

All the mutations in coding sequence were tested for association to known virulence factors by using virulent genes list from: Pathogen – Host Interaction Data Integration and Analysis System (PHIDIAS), *Brucella* Bioinformatics Portal (BBP).

[7] ExPASy - Translate tool. at <http://web.expasy.org/translate/>

3.3. DNA samples

3.3.1 DNA Extraction of Rev.1 and Field Isolate

The Rev.1 DNA used in this study is the same Rev.1 vaccine source that has been used by the Palestinian Ministry of Agriculture since 1999. This vaccine is produced by the Laboratory Ovejero SA ® – Spain. The vaccine is lyophilized in smooth phase and sold in vials, each contains 50 doses and each dose contains from $4x10^8 – 5 x 10^9$ bacterial cells. One vaccine vial was dissolved in 5 ml 1X TE buffer. The suspension was boiled for 20 min and then centrifuged for 5000 Round Per Minute (RPM) for 1 min. The supernatant was taken as a Rev.1 DNA template for PCR reactions.

A confirmed non-Rev.1 isolate was obtained from Palestinian Central Veterinary Lab (CVL). This *B. melitensis* filed isolate, which was isolated from sheep milk using conventional bacteriological methods, was named (F3). This filed isolate was used as wild type DNA. One colony was picked from the agar plate, dispensed in 10 ml 1X TE buffer and were boiled for 20 min, then centrifuged at 5000 RPM for 1 min. The supernatant was used as DNA template for PCR.

3.3.2 Brucella reference strains' DNA

DNA samples from reference strains*: B. melitensis* biovar.1 strain 16M (ATCC 23456), *B. melitensis* biovar.2 strain 63/9, and *B. melitensis* biovar.3 strain Ether, which represent the known three *B. melitensis* biovars were generously provided by Dr. Ignacio Lopez Goni, University of Navarra, Pamplona, Spain.

3.3.3 *Brucella* field samples

A group of 36 *B. melitensis* DNA samples were kindly provided by Dr Elina Awad from the Palestinian Central Veterinary Lab (CVL). According to CVL, all the DNA samples were obtained from specimens of vaginal and fetal swabs or milk of aborted sheep or goats.

The samples were collected from different regions of West-Bank, Palestine and they were confirmed as *Brucella* positive samples by the Brucella-specific IS element PCR in Central Veterinary Lab.

3.4 PCR analysis

3.4.1 Bidirectional allele specific PCR technique

Bidirectional allele specific PCR technique is a modification of Amplification Refractory Mutation System (ARMS) (Z. Jiang et al. 2001). This technique can differentiate between two SNP-genotypes in a single tube PCR reaction. This can be achieved by designing two primers in opposite direction: one that targets the wild type and the other targets the mutated genotype. The key point in this technique is the use of two outer primers which produce two different amplicon lengths to distinguish between wild and mutated genotypes (Fig 3.3).

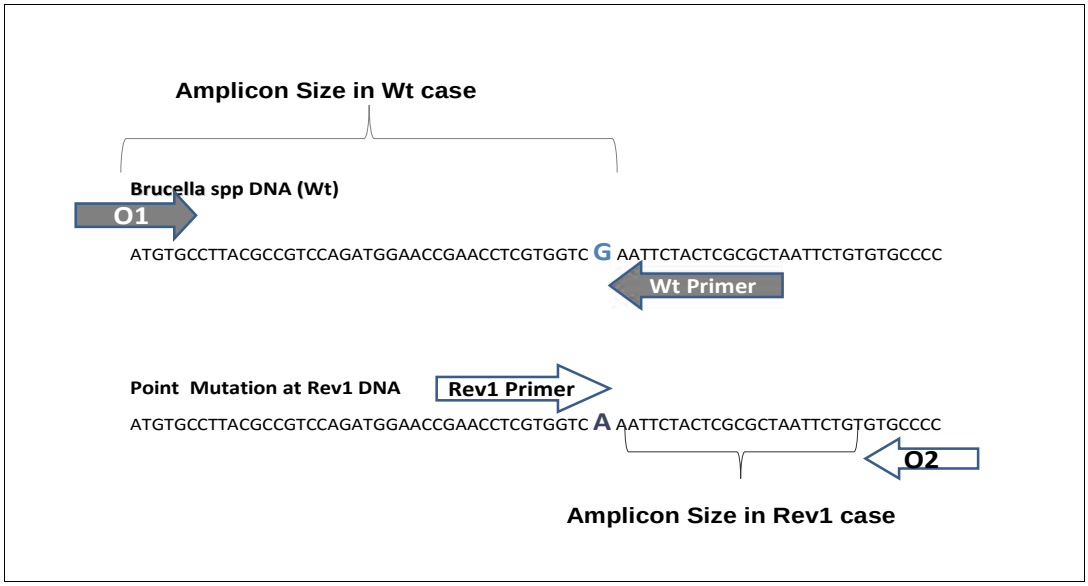

Fig 3.3: Bidirectional allele specific PCR technique. *The DNA sequence represents the Brucella wild type (Wt) DNA sequence, and the point mutation in Rev.1 genome SNP G to A. The arrows represent the primers; O1 and O2 are the outer primer, Wt primer targeted wild type sequence and Rev.1 primer targeted the unique point mutation on Rev.1 genome.*

3.4.2 PCR Primers

All primers used in this study were designed by PerlPrimer v1.1.20 software[8], by using the default parameters. The primers were further tested with Primer-BLAST tool at NCBI[9] to confirm their specificities and matching locations in *Brucella* species.

The primers' full details: sequences, coordination's according to *B. melitensis* 16M genome, and optimal PCR conditions are provided in Appendix 5.

Internal sequencing primers were designed to confirm the presence of the revealed SNPs in Rev.1. The sequencing primers were designed to target regions that included around 50 bp adjacent to the SNPs (see Appendix 6).

The designed primers were delivered by Hy-Labs (Rehovot). The primers were dissolved in 1X TE buffer according to manufacturer instructions to obtain a 100 pmol/µl concentration. Working dilutions have a final concentration of 10 pmol/µl and were stored at -20 °C in aliquot tubes.

[8] PerlPrimer. at <http://perlprimer.sourceforge.net/download.html>
[9] Primer designing tool. at <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>

Table 3.1: The primers used in bi-directional allele specific PCR to test the 11 SNPs; for more details see Appendix 6

SNP code	Primer Name	*Sequence*	TM / °C (pearlprimer)	Size Product (bp)
SNP1	Rev 0006 F	GTTCTGTTTCCATGCAGGTC	59.58	**656**
	Rev 0006 R	TAACTCCAATGGAAGATCGTCG	60,4	
	16M 0006 F	ATCGAAAAAGCAATTGAGGGT	59	**428**
	16M0006 R	CACCACCAATTACAATGACATC	58	
SNP2	Rev 0199F	TTTGAAATATCGGGAGGCAT	57	**1124**
	Rev0199 R	CATGAGGTTCAATATCTGAGT	55	
	16M0199 F	CTGGACCCTTCCATTCAGG	59.8	**662**
	16M0199 R	TATGCCGCCATTATTCGCTA	59.8	
SNP3	Rev 0525 F	CTCCTTGAATTCTCAGTGGT	57	**675**
	Rev0525 R	GTTGGTCGATGTCACCCAG	60	
	16M 0525 F	CTTCGGGATAATGGAAACCATAG	57	**898**
	16M 0525 R	TGACGATAAGGACCCCTTAG	58	
SNP4	Rev 0876 F	GCCAGATGGAGAAGACGCAG	63	**695**
	Rev0876R	CGCGTTCTGTTTCGGGTCT	63	
	16M 0876 F	GCCATGCTGGAGGTGCTG	63.9	**479**
	16M0876 R	GTCACGACACCCACCTGAT	62	
SNP5	Rev 1066 F	AAAGCGGCACCTTTCCTGA	62	**888**
	Rev 1066R	CCTCTCACGTTCGTCGTTCAG	63	
	16M1066F	CAACGCATGACTGCGCCT	64	**334**
	16M1066R	CCGAATATGACCGCCAAGAATGC	64	
SNP6	Rev 1133 F	GAACCGAACCTCGTGGTCA	62	**544**
	Rev 1133R	GGTGCTTCCCGAACAATTGAG	62	
	16M 1133 F	ACCAATGTCAGACAGACCATG	60	**1016**
	16M 1133R	CAGAATTAGCGCGAGTAGAATTC	60	

SNP7	Rev1592 F	GTGGCCTTTCTCGTCCGGT	64	**958**
	Rev1592 R	ACCGATTTCATCTCCAACGAACTG	63	
	16M 1592 F	CAGCCATTCGCACATTCATCG	63	**630**
	16M 1592 R	GAAGACCGGAATGCCGCG	64	
SNP8	Rev 1624F	AAATGCGACGGAAGTGGC	61	**924**
	Rev 1624 R	GCGTTGAACTTCTGAAGAAACTG	61	
	16M1624 F	GCCAATGCAAGCCATGAG	59	**468**
	16M1624 R	GAAATCCTGTATGGAGGCGA	59	
SNP9	Rev1923F	ACTCATTTCCGGTGAGCATGT	62	**468**
	Rev1923R	TTGCGCTCATGCACATAGGA	62	
	16M1923F	TGCCTCGATGTCGTGGTGC	65	**949**
	16M 1923 R	GCCGTGCATGATCCCATGA	63	
SNP10	Rev2044 F	TTCGTGAGGAAAGCCTATGAC	60	**1196**
	Rev2044 R	AAGGAGCAGGGCGGGAT	60	
	16M2044F	AATGGGGCCAGCAAGTG	60	**781**
	16M2044R	CATTGACCGTCTTTCAAATCGATG	61	
SNP 11	16M630F	CCGGTATCAGCGGCG	56	**442**
	16M630R	ACAAGATTGACGAATGTGGTC	56	
	Rev630 F	GTCATTCTTATGCCGGACT	54	**682**
	Rev630 R	GAGATTGCGTTGAGAGGC	55	

3.4.3 PCR Procedure

Each primer pair of the different bidirectional allele specific PCR reactions was initially tested on Rev.1 DNA and on wild type DNA (F3) separately. Both DNA were tested with each primer pair alone in singleplex PCR format.

The bidirectional allele specific PCR reactions were applied using the two pairs of primers for each SNP (Rev.1 primers and wild type primers). Each set of mutation-specific four primers were added in one reaction and were tested on three types of templates: Rev.1 DNA, wild type DNA (F3), and a mixture of DNA from both (Rev.1 and wild type F3). Due to sample amount limitation of the reference strains, only the best working primers were tested on the three reference *B. melitensis* biovars DNA (Biovar-1, Biovar- 2 and Biovar-3).

The PPU-Taq DNA polymerase enzyme and the ready-to-use GoTaq® Green Master Mix (Promega®) were used in PCR reactions according to manufacturers' instructions in a 25 **µl** final volume reaction. All PCR experiments were optimized by changing the annealing temperature, primer concentration, extension time and number of cycles.

The PCR products were analyzed using gel electrophoresis in 1xTBE buffer with 2% agarose gel stained with ethidum bromide dye, and photographed under Ultra Violet (UV) light.

3.5 DNA Sequencing

The Rev.1 amplicons generated by the outer primer for each tested SNP were sequenced using internal sequencing primers at Hereditary Research Laboratory, Bethlehem University. Using Sanger sequencing method and ABI Genetic analyzer machine from Applied Biosystems®. Each mutation was tested with single direction sequencing primer.

The free software Finch TV version 1.4.0, Geospiza Inc[10] was used to read the sequencing results.

3.6 Protein localization prediction

By using PSORT protein subcellular localization prediction tool[11], for predicting the possible cellular location of the founded mutated proteins.

[10] FinchTV. at <http://www.geospiza.com/Products/finchtv.shtml
[11] http://psort.hgc.jp/form.html

CHAPTER 4
Results

4.1 Bioinformatics Results

4.1.1 Searching for large Indels in Rev.1 genome

In our first attempt to identify unique indels in Rev.1 genome, we used BLASTn (bl2seq) tool to compare the 93 Rev.1 contigs to the *Brucella melitensis* 16M genome (accession numbers NC_003317 and NC_003318). The analysis revealed 6 possible unique indels. Unfortunately, 5 indels were not unique to Rev.1 because they were found in *B. melitensis* strain ATCC 23457. Interestingly, the only indel that was absent in *B. melitensis* strain ATCC 23457 was found in other draft genomic sequence of *B. melitensis* 16 M strain that came from a different sequencing project. In order to resolve this contradiction, we designed a PCR primers surrounding that deletion and the result revealed that the *B. melitensis*16M DNA has indeed the same 17 bp deletion (Table 4.1).

Table 4.1: Large Indels detection results: The second and the third columns show the corresponding coordinates of the each indel in Rev.1 contigs and 16 M genome, respectively. The last column indicates the reason of excluding the indel from being a unique to Rev.1

Indels Size	Corresponding coordinates in Rev.1 contigs	Corresponding coordinates in 16M genome	The reason to exclude the indel
18 bp Insertion	Contig 7 (ACEG0100007) From 154280 to 154262	At Chromosome II (NC_003318) Starting 33326	It was found in *B. melitensis* ATCC 23457
* 17 bp Deletion	Contig 7 (ACEG0100007) Starting at 8202	At Chromosome II (NC_003318) From 179382 to 17939	It was found in *B. melitensis* 16M (newly sequenced contig 22)
25 bp Insertion	Contig 29 (ACEG0100029) From 47623 to 47648	At Chromosome II (NC_003318) Starting at 322918	It was found in *B. melitensis* ATCC 23457
16 bp Insertion	Contig 29 (ACEG0100029) From 30682 to 30698	At Chromosome II (NC_003318) Starting at 339846	It was found in *B. abortus* bv. 3 str. Tulia cont1.55
27 bp Deletion	Contig 40 (ACEG0100040) Starting at 5683	At Chromosome I (NC_003317) From 1704331 to 1704358	It was found in *B. melitensis* ATCC 23457
24 bp Deletion	Contig 89 (ACEG0100089) Starting at 29293	At Chromosome I (NC_003317) From 1940496 to 1940472	It was found in B melitensis ATCC 23457

* This deletion found also in the new sequenced contig 22 for *B. melitensis* 16M was submitted to Genbank at Jun 2009, while the old *B. melitensis* 16M genome was submitted at Dec 2001.

4.1.2 Searching for unique point mutations

4.1.2.1 Results of NCBI BLAST analyses

The BLAST results for all Rev.1 genes against *B. melitensis* 16M genome showed that the Rev.1 genes from BAMG 00001 to BAMG 01277 and genes from BAMG 02392 to BAMG 03221 are located on *B. melitensis 16M* chromosome I. While the rest of Rev.1 genes (BAMG 01278 to BAMG 02391) were located on chromosome II of *B. melitensis* 16M.

In this analysis, a Rev.1 gene is considered a gene with possible variation if the maximum identity using BLASTn analysis with *B. melitensis* biovar.1 (strain 16 M) homologue is less than 100%. Based on this assumption, the Rev.1 genes with possible variations that were found on chromosome I and II were as follows:

- On chromosome I: Out of 2106 tested Rev.1 genes, a total of 178 genes had possible variations compared to *B. melitensis* 16M chromosome I. Of these genes, 128 were also found to be variant when compared with *B. melitensis* biovar.2 *(*strain *ATCC 23457*) (see Appendix 3).

  The short listed genes were further analyzed to examine possible correlation with known virulence genes reported by BBP database. Only 18 mutations were found to be relevant to already known virulence factors (Table 4.2).

- On chromosome II: From 1113 Rev.1 genes, a sum of 109 Rev.1 genes had variations compared to *B. melitensis* 16M chromosome II. Also, 79 of these genes were found to have possible variations when compared with *B. melitensis* (strain ATCC 23457 biovar 2) (see Appendix 4). However, only 14 genes were found to be relevant to known virulence gene according to BBP database - (Table 4.3).

Table 4.2: Rev.1 Variant genes on chromosome 1. This table shows variant gene that are located on chromosome 1 which were also reported as virulence gene in the BBP database.

Rev.1 gene description	**NCBI ORF for *B.melitensis* 16M**	**NCBI Mutations Description**	**Broad Ins. BLAST & Align**
BAMG_00053 | integral membrane sensor hybrid histidine kinase (3516 nt)	BMEI1606	8 bp indels	The gene has 100% similarity with *B. melitensis* 16M newly sequenced
BAMG_00142 | phosphoribosylamine-glycine ligase (1284 nt)	BMEI1519	SNP C to T	SNP C95 T found also in many of 36 strains
BAMG_00163 | 1-deoxyxylulose-5-phosphate synthase (1932 nt)	BMEI1498	SNP G to A	SNP G 1006A and C1179T common in the same 5 strains*
BAMG_00253 | phosphomannomutase (1374 nt)	BMEI1396	SNP C to T	SNP C379T are common in many strains
BAMG_00317 |FeuQ (1389 nt)	BMEI1336	SNP G to A	SNP G424A found in the same 5 strains*
BAMG_00535 | phosphoribosylformylglycinamidine synthase II (2223 nt)	BMEI1127	SNP T to C	has 100% similarity with *B. melitensis* Bv2 and Bv3
BAMG_00817 rotamase (1887 nt)	BMEI0845	SNP C to T	SNP C1128T also found in the same 5 strains*
BAMG_00912 | 30S ribosomal protein S12 (372 nt)	BMEI0752	SNP C to T	known rpsl SNP
BAMG_01119 |. conserved hypothetical protein (1062 nt)	BMEI0545	SNP A to C	has 100% similarity with *B. melitensis* 16M newly sequenced
BAMG_01216 |. 2-isopropylmalate synthase (1728 nt)	BMEI0451	SNP G to T	has 100% similarity with the B. melitensis bv 2 bv3
BAMG_02500 |. diaminopimelate decarboxylase (1266 nt)	BMEI0084	2 bp substitution	has 100% similarity with *B. melitensis* 16M newly sequenced
BAMG_02640 | . hsp70-like protein (1914 nt)	BMEI2002	SNP T to G	has 100% similarity with many *Brucella spp*
BAMG_02755 |. phosphoglucomutase (1632 nt)	BMEI1886	SNP A to G	has 100% similarity with many *Brucella spp*
BAMG_02795 |. dihydroxy-acid dehydratase (1836 nt)	BMEI1848	SNP T to C	SNP T48C also found in the same 5 strains*
BAMG_02807 |. cyclic beta 1-2 glucan synthase (8604 nt)	BMEI1837	8 bp substitution	has 100% similarity with *B. melitensis* 16M newly sequenced
BAMG_02882 |. methionine synthase (3786 nt)	BMEI1759	2 bp substitution	SNP C987T also found in the same 5 strains*
BAMG_03098 | helicase domain-containing protein (3087 nt)	BMEI0275	4 bp substitution	have many SNPs that found in many other strains
BAMG_03108 |. pyruvate carboxylase (3477 nt)	BMEI0266	SNP C to T	C156T found in many strains

(Table 4.3) Rev.1 Variant genes on chromosome 2. This table shows variant gene that are located on chromosome 2 which were also reported as virulence gene in the BBP database.

Rev.1 gene description	NCBI ORF for *B..melitensis 16M*	NCBI Mutations Description	Further analysis by Broad Inst. tools
BAMG_01418 | . type IV secretion system protein virB10 (1161 nt)	BMEII0034	18 BASE DEL OUT BME	has 100% similarity with some *B.melitensis* bv2, bv3 & new 16M
BAMG_01325 |. transcription regulator (1257 nt)	BMEII0128	3 bp indels	has 100% similarity with *B. melitensis* 16M newly sequenced
BAMG_01372 |. AMP-ligase (1764 nt)	BMEII0078	2 bp substitution	has 100% similarity with other *Brucella spp*
BAMG_01419 | . type IV secretion system protein virB9 (870 nt)	BMEII0033	A deletion	has 100% similarity with *B. melitensis* 16M newly sequenced
BAMG_01467 |. 6-phosphogluconate dehydrogenase (1020 nt)	BMEII1124	SNP A to C	SNP T834 G found in many strains
BAMG_01527 |. GntR domain-containing protein (813 nt)	BMEII1066	SNP T to C	in the final unique 24 SNP list
BAMG_01538 |. glucose/galactose transporter (1239 nt)	BMEII1053	SNP C to G	has 100% similarity with *B. melitensis* 16M newly sequenced
BAMG_01822 |. conserved hypothetical protein (1683 nt)	BMEII0761	SNP G to C	has 100% similarity with *B. melitensis* 16M newly sequenced
BAMG_01824 |. cytochrome d oxidase subunit (1155 nt)	BMEII0759	3 bp OUT BME	has 100% similarity with *B. melitensis* bv2 &16M newly sequenced
BAMG_02155 |. erythritol phosphate dehydrogenase (1509 nt)	BMEII0429	SNP G to C	Synonamous / SNP from our candidate SNP list C321G are unique for Rev! in all strains but 1 strain not sure in alignment
BAMG_02282 |. sugar ABC transporter (1533 nt)	BMEII0300	4 bp substitution	C313T found in the same 5 strains *

***** The five *B. melitensis* strains sequenced by Broad institute are genetically similar to Rev.1, many mutations are present just in Rev.1 and those five strains. The strains are: Brucella_melitensis_G6605, Brucella_melitensis_G8755, Brucella_melitensis_H1992, Brucella_melitensis_bv_1_G6191 and Brucella_melitensis_bv_3_G9248.

4.1.2.2 Results of Broad institute tools

In addition to the initial approach that relied heavily on data mining using NCBI database and BLASTn tools, the Broad Institute tools, which were lately exposed to the public, are used.

By using the Broad institute bioinformatics tools the huge numbers of variant genes were minimized. The Rev.1 unique genetic variations that were not shared with any other 36 *B. melitensis* strains were found by using Polymorphism Predictions tools at Broad Institute. In order to confirm the distinctiveness of the genetic variations in Rev.1, other Broad Institute tools were used, such as: View polymorphism, View polymorphism site, Multiple

Genome Alignments and Alignment Viewer. In addition, further NCBI BLAST analyses were conducted to confirm all of those unique mutations against other *Brucella* species and other bacteria. A total of Rev.1 unique 24 point mutations are listed in Table 4.3.

The unique genetic variations of Rev.1 genome which appear in Rev.1 and are absent in all other *B. melitensis* strains and other *Brucella* species are**:**

1. A group of 24 mutations in coding regions (22 /24 are substitution point mutations and 2 /24 are Indel mutations). These 24 mutations are classified as synonymous (Table 4.4) and non-synonymous mutations (Table 4.5).

According to View Polymorphism tool using Alignment viewer, the alignment of the unique 21/24 coding point mutations shows that Rev.1 genes have the point mutation while corresponding gene of all other 35 sequenced genomes of various strains have the wild type sequence in that exact position. The other three mutations were not confirmed to be totally absent in all other 35 genomes because of sequencing-gaps (in Broad institute DB) in the corresponding segments in few strains.

All the 24 point mutations were examined for protein translation using Exapsy Translation Tool. The analysis showed that 6/24 mutations are synonymous (Table 4.4) while 18/24 are non-synonymous mutations (Table 4.5). Two of these non-synonymous mutations were found to be indel mutations causing frame shift of two ORFs (Table 4.5).

2. A group of 8 Non-Coding SNPs were already described by *Brucella* project of Broad Institute as unique mutations to Rev.1 (Table 4.6).

Table 4.4 Synonymous Rev.1 unique point mutations. In this table the: (wt) wild type genotype. The ORF numbering and annotations were based on the genome of *B. melitensis* 16M. The position refers the nucleotide position in the corresponding NCBI ORF.

Nucleotide change wt. to Rev.1	**NCBI/** *B. melitensis* **16M ORFs**	**Position**	**Broad Institute/** *B. melitensis* **Rev. 1 ORFs**	**Our SNP code**
T to C	BMEI0006: tRNA modification : GTPase TrmE	1197	BAMG_02579 : RNA modification GTPase mnmE (1329 nt)	SNP 1
C to T	BMEI0096: electron transfer flavoprotein alpha/beta-subunit	123	BAMG_02489: electron transfer flavoprotein alpha/beta-subunit (747 nt)	
T to G	BMEI0436: peptide ABC transporter permease	51	BAMG_01233: binding-protein-dependent transport system inner membrane component (909 nt)	
G to A	BMEI0876: ATP-dependent protease LA	1398	BAMG_00786 : ATP-dependent protease La (2439 nt)	SNP 4
T to C	BMEII1066 : pyruvate dehydrogenase complex repressor	718	BAMG_01527: GntR domain-containing protein (813 nt)	SNP 5
G to A	BMEI2044: hypothetical protein	444	BAMG_02599: conserved hypothetical protein (510 nt)	SNP 10

Table 4.5: Nonsynonymous Rev.1 unique point mutations. In this table the: (wt) wild type genotype, the ORF numbering and annotations were based on the genome of *B. melitensis* (16M strain). The position refers the nucleotide position in the corresponding NCBI ORF. Mutation effect: the mutation impact on amino acid level.

Nucleotide change wt. to Rev.1	**NCBI/** *B. melitensis* **16M ORFs**	**Position**	**Broad Institute/** *B. melitensis* **Rev. 1 ORFs**	**Mutation effect**	**Our SNP code**
C to G	BMEII0093: replication protein A	61	BAMG_01357: cobyrinic acid ac-diamide Synothase (1194 nt)	R 21 G	
G to A	BMEII0199: oligopeptide transport ATP-binding protein OPPF	688	BAMG_02366: oligopeptide/dipeptide ABC transporter (978 nt)	A230T	SNP 2
C to T	BMEII0525: hypothetical protein	95	BAMG_02057: conserved hypothetical protein	A32V	SNP 3
G to A	BMEII0539: putative cytoplasmic protein	319	BAMG_02043: conserved hypothetical protein (1470 nt)	A107T	
C to T	BMEI0752: 30S ribosomal protein S12	272	BAMG_00912.1: 30S ribosomal protein S12	P91L	
C to T	BMEII0940:maltose/maltodextrin transport ATP-binding protein MALK	65	BAMG_01649: trehalosemaltose transporter ATP-binding protein (846 nt	S 22 L	
A to G	BMEII0971: copper transport ATP-binding protein NOSF	653	BAMG_01620: copper transport ATP-binding protein NOSF	K 218 R	
G to C	BMEI0975: phosphoglycolate phosphatase	83	BAMG_00689: phosphoglycolate phosphatase (705 nt)	S 28T	
C to T	BMEI1068: aspartyl/glutamyl-tRNA amidotransferase subunit B	584	BAMG_00596: aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	T195 I	
C to T	BMEI1120: phosphotyrosyl phosphatase activator (PTPA)	140	BAMG_00542: HpcH/HpaI aldolase (762 nt)	A 47 V	
G to A	BMEI1133: 30S ribosomal protein S4	601	BAMG_00528: SSU ribosomal protein S4P (618 nt)	A 201 K	SNP 6
C to T	BMEI1341: Phage host specificity protein	673	BAMG_00313: conserved hypothetical protein	Q225 stop codon	
C to T	BMEI1592: 3-methyl-2-oxobutanoate hydroxymethyltransferase	403	BAMG_00066: 3-methyl-2-oxobutanoate hydroxymethyltransferase (828 nt)	R 135 C	SNP 7
T to C	BMEI1624: phosphate regulon sensor protein PHOR	986	BAMG_00035 : histidine kinase (1317 nt)	V329A	SNP 8
C to T	BMEI1923: isovaleryl-CoA dehydrogenase	787	BAMG_02717: acyl-CoA dehydrogenase domain-containing protein (1179 nt)	P 263 S	SNP 9
G to A	BMEI2011: 2 hydroxymuconic semialdehyde hydrolase	448	BAMG_02631: conserved hypothetical protein (792 nt)	G150 R	
A insertion	BMEII1129: glycosyl transferase	998	BAMG_01462.1: mannosyl transferase	frame shift	
G deletion	BMEII1129: high-affinity branched-chain amino acid transport ATP-binding protein LIVG	136	BAMG_01950: inner-membrane translocator	frame shift	SNP 11

Table 4.6: Noncoding Rev.1 unique SNPs .

Position	Left Gene	L. gene Annotation	Right Gene	R. gene Annotation	Dist From L Gene	Dist From R Gene
Chr II 52085	BMEII0049	N-formylglutamate deformylase	BMEII0050	sensory transduction histidine kinase	317 (-)	4 (+)
Chr II 555806	BMEII0529	surface protein	BMEII0530	MarR family transcriptional regulator	104 (-)	148 (+)
Chr I 558876	BMEI0536	periplasmic immunogenic protein	BMEI0537	integrase/recombinase	396 (-)	24 (-)
Chr I 634340	BMEI0607	putative cytoplasmic protein	BMEI0608	thymidylate synthase	51 (+)	52 (+)
Chr I 844569	BMEI0820	translation initiation inhibitor	BMEI0821	hypothetical protein	52 (-)	143 (+)
Chr I 878988	BMEI0851	enolase	BMEI0852	methyltransferase	41 (+)	289 (+)
Chr I 1057474	BMEI1017	salicylate hydroxylase	BMEI1018	phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	23 (+)	47 (-)
Chr I 1448000	BMEI1392	ribose ABC transporter ATP-binding protein	BMEI1393	mannosyltransferase C	496 (-)	165 (-)

4.2 PCR Results

In order to validate the bioinformatics analysis, it was decided to examine some of the discovered mutations in the lab. The bidirectional allele specific PCR technique was adopted, as it offers the possibility to distinguish between two genotypes (point mutation) in one reaction. A total of 11 SNPs out of the 24 Rev.1 unique point mutations were selected for the bidirectional allele specific PCR. These 11 SNPs were selected based on the sequence context surrounding the mutation, which enables the designing of two allele-specific primers that fulfill the standard recommended criteria for primer design. The 11 point mutations were given codes SNP 1, SNP 2, etc (See Table 4.5).

The amount of DNA for the three *B. melitensis* reference strains was not sufficient to test the 11 SNPs in singleplex and duplex PCR and to run all the optimization processes. Because of that, the local field isolate DNA (F3) was used as a representative for wild type DNA in the primary PCR tests. The best results of the SNPs primers were later tested with *B. melitensis reference strain* DNA.

Each primers pair was tested separately on Rev.1 DNA and on one the field isolates DNA (F3). The results of singleplex PCR for each primer pair showed that at the same annealing temperature 10 out of 11 SNPs were positive in Rev.1 and negative in F3 field isolate. Although all primer pairs were designed to have very close annealing temperature, some of them showed a slight variation in the annealing efficiency. Therefore, several experiments were carried to find the most optimal annealing temperature for each pair in the singleplex and duplex format. In the preliminary PCR reactions, most primer pairs showed the amplicon of interest with some faint bands representing the other genotype. However, further optimizations of the PCR conditions and primers' concentration produce a better differentiation pattern. The outer product of the bidirectional PCR technique was diminished from some reaction by reducing the extension time. In the following section, the results for each SNP is will describe in details.

4.2.1 PCR results of SNP 1

SNP1 was found at (BMEI0006) [tRNA modification GTPase TrmE] gene, is T to C, substitution at position 1197. In singleplex PCR see (Fig 4.1), it shows that the Rev.1 DNA gives a clear and strong band with Rev.1 primers and no amplicon with wild type (Wt) primers. While, in case of field DNA (F3), it gave faint bands with wild type primers and no bands at Rev.1 primers at same annealing temperature. In duplex PCR, see (Fig 4.2). The Rev.1 DNA gave a clear band and the outer primers product. In field isolate DNA (F3) gave the amplicon size for wild type and faint band for Rev.1 case.
Therefore SNP1 primers worked ideally in Rev.1 DNA and in field DNA it worked partially.

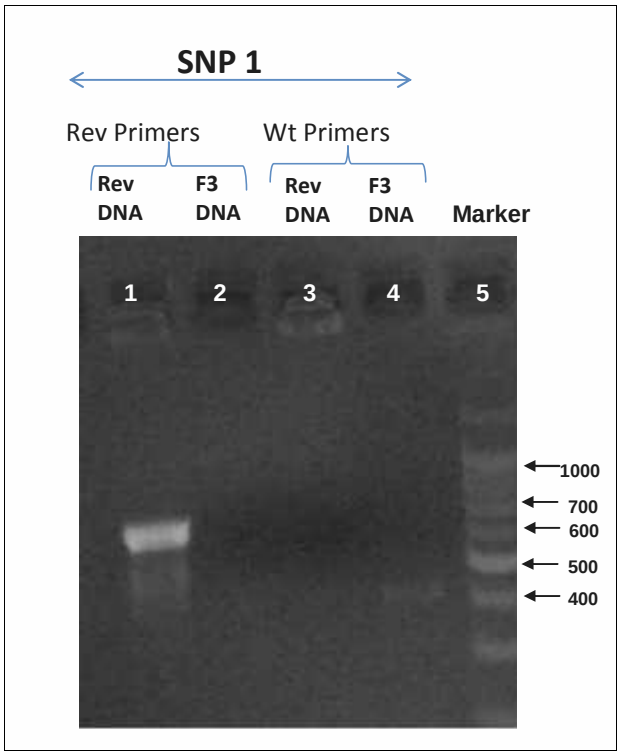

Fig 4.1: Singleplex PCR result for SNP 1. In lane 1 and 2, Rev.1 specific primers were used while in lane 3 and 4 the wild type primers were used. Lane 1: Rev.1 primers with Rev.1 DNA (656 bp product). Lane 2: Rev.1 primers with (F3) *Brucella* field isolate DNA (failed to produce any amplicon). Lane 3: wild type primers with Rev.1 DNA (no amplicon). Lane 4; wild type primers with (F3) the field isolate DNA (428 bp amplicon). (PCR conditions: 95 °C /45 Sec. 61.5 °C /35 Sec.72 °C /01:15 Min. for 27 cycles)

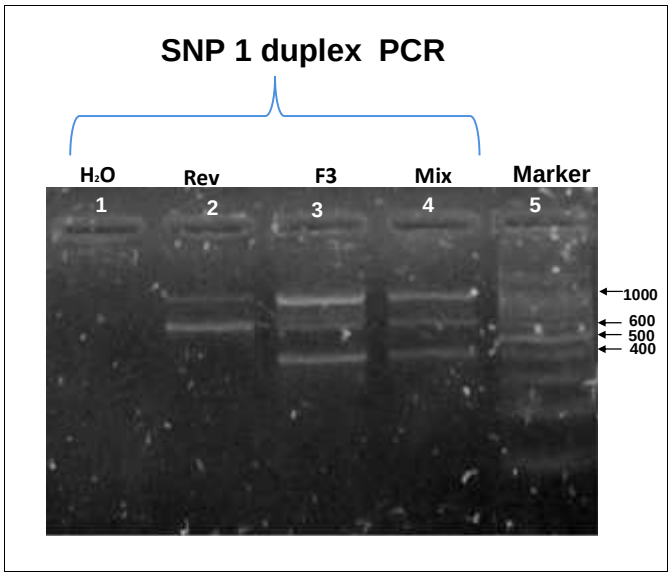

Fig 4.2: Duplex PCR results for SNP 1. Both Rev.1 and wild type primers added to all PCR tubes to test the bidirectional allele specific technique capacity to differentiate Rev.1 DNA from one of *Brucella* field isolate DNA (F3). Lane 1: H2O as a negative control (no amplicon). Lane 2: Rev.1 DNA (656 bp amplicon also there is the outer amplicon between two outer primers~ 1048 bp). Lane 3: (F3) *Brucella* field isolate (428 bp amplicon with faint 656 bp band for Rev.1 and outer primers band 1048 bp). Lane 4: Mix DNA from Rev.1 and *Brucella* field isolate DNA (all bands for Rev.1 and wild type are produced) (PCR conditions: 95°C /2 min, 94 °C /45 Sec. 61.5°C /35 Sec. 72 °C / 2 Min. for 27 cycles)

4.2.2 PCR results of SNP 2 and SNP 3

SNP2 was found at (BMEII0199) [Oligopeptide transport ATP-binding protein OPPF] and it is a G to A substitution at position 688. The result of singleplex PCR shows that SNP 2 primers did not differentiates between the two genotypes as expected even after several modifications of the annealing temperature (Fig 4.3). Therefore, SNP 2 was excluded from further analysis.

SNP 3 was found at (BMEII0525), which is a hypothetical protein and this mutation is a C to T substitution at position 95.

In SNP 3 singleplex PCR (Fig 4.3) the Rev.1 DNA gives a clear and strong band with Rev.1 primers and no amplicon was detected when tested with wild type primers (Wt). On the other hand using field DNA (F3), this mutation it produce faint bands with both wild type primers and Rev.1 primers. In duplex PCR (Fig 4.4), the Rev.1 DNA gave a clear band for Rev.1. In field isolate DNA (F3), the expected amplicon size for wild type appeared also a faint band for Rev.1 case seen. Therefore, we assume that SNP3

primers can be used to distinguish Rev.1 genotype from field isolate in one tube PCR reaction.

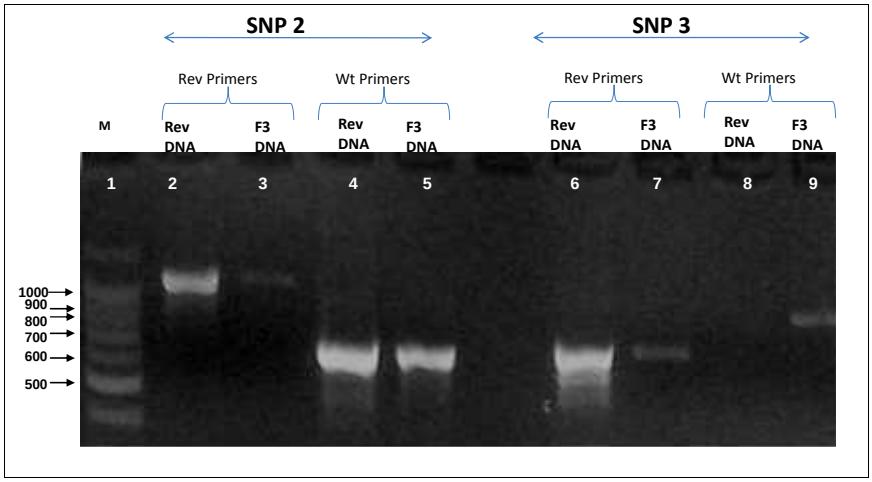

Fig 4.3: Singleplex PCR result for SNP 2 and SNP 3. Lane 1: Marker. Lane 2: SNP 2 Rev.1 primers with Rev.1 DNA (1124 bp amplicon). Lane 3: SNP 2 Rev.1 primers with (F3) *Brucella* field isolate DNA (faint amplicon). Lane 4: SNP 2 wild type primers with Rev.1 DNA (662 bp amplicon). Lane 5: SNP 2 wild type primers with (F3) *Brucella* field isolate (662 bp amplicon). (PCR conditions SNP 2: 95 °C /45 Sec. 59°C /35 Sec.72 °C /01:15 Min. for 27 cycles).
Lane 6: SNP 3 Rev.1 primers with Rev.1 DNA (675 bp amplicon). Lane 7: SNP 3 Rev.1 primers with (F3) *Brucella* field isolate DNA (faint amplicon). Lane 8: SNP 3 wild type primers with Rev.1 DNA (no amplicon). Lane 9: SNP 3 wild type primers with (F3) the field isolate DNA (898 bp amplicon). (PCR conditions SNP 3: 95 °C /45 Sec. 59°C /35 Sec.72 °C /01:15 Min. for 27 cycles).

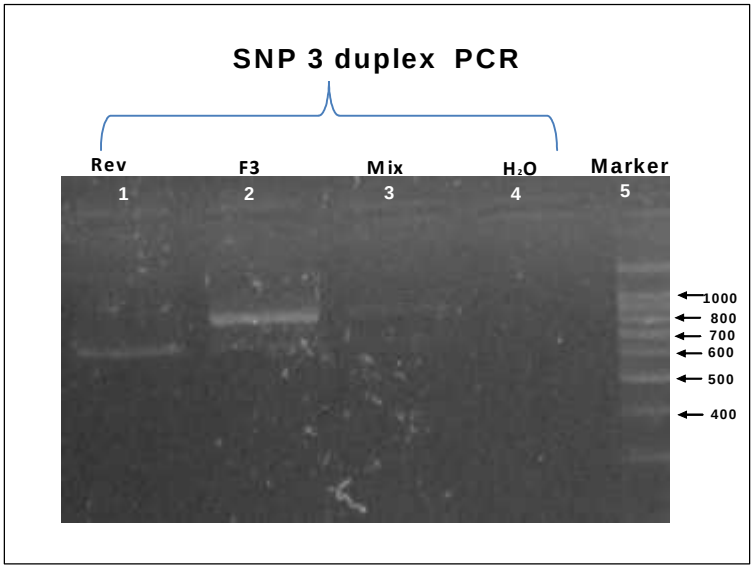

Fig 4.4: Duplex PCR results for SNP 3 Both SNP 3 Rev.1 and wild type primers added to all PCR tubes to test the bidirectional allele specific technique capacity to differentiate Rev.1 DNA from one of *Brucella* field isolate DNA (F3). Lane 1: Rev.1 DNA (675 bp amplicon). Lane 2: (F3) *Brucella* field isolate DNA (898 bp amplicon with very faint 675 bp band for Rev.1). Lane 3: Mix DNA from Rev.1 and *Brucella* field isolates (F3) DNA (faint two bands for Rev.1 and wild type are produced). Lane 4: H2O as a negative control. Lane 5: Marker. (PCR conditions: 95 °C /45 Sec. 57 °C /35 Sec.72 °C / 1:25 Min. for 35 cycles)

4.2.3 PCR results of SNP 4, SNP 5 and SNP 6

SNP 4 was found at (BMEI0876) [ATP-dependent protease LA] gene and it is a G to A substitution at position 1398. In singleplex PCR results, the Rev.1 DNA gave clear strong band with Rev.1 primers and no amplicon with wild type primers. While, in (F3) field DNA gave strong bands with wild type primers and faint band with Rev.1 primers see (Fig 4.5). In duplex PCR the Rev.1 DNA gave a clear band for Rev.1.While, In (F3) field isolate DNA give the amplicon size for wild type. So SNP4 primers worked ideal for both cases in duplex PCR (Fig 4.6).

SNP 5 found at (BMEII1066) [Pyruvate dehydrogenase complex repressor] gene, substitution T to C at position 718. In singleplex PCR (Fig 4.5) the Rev.1 DNA give clear strong band with Rev.1 primers and no amplicon with wild type primers. While, in field DNA (F3) gave faint bands with wild type primers and faint band in Rev.1 primers. In duplex PCR (Fig 4.6) the Rev.1 DNA did not work. While, (F3) DNA gave a faint band 888 bp for Rev.1 case beside the strong band for wild type case 334 bp. SNP5 primers failed to genotype Rev.1 in duplex PCR and the SNP was confirmed with singleplex PCR.

SNP 6 found at (BMEI 1133) [30S ribosomal protein S4] gene, substitution G to A at position 601. Singleplex PCR results, see (Fig 4.5), the Rev.1 DNA gave clear strong band with Rev.1 primers and no amplicon with wild type primers. While, in field DNA (F3) it gave expected band with wild type primers and no band in Rev.1 primers. In duplex PCR (Fig 4.7), Rev.1 DNA gave a clear band for with expected length for Rev.1. And in field isolate DNA (F3) the amplicon size for wild type. So SNP6 primers worked ideal for both cases in duplex PCR.

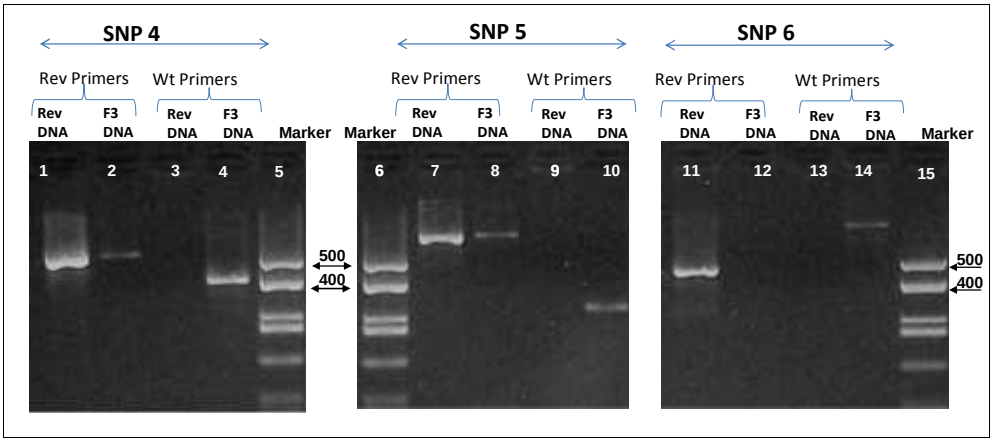

Fig 4.5: Singleplex PCR results for SNP 4, SNP 5 and SNP 6. Lane1: SNP 4 Rev.1 primers with Rev.1 DNA (695 bp amplicon). Lane 2: SNP 4 Rev.1 primers with (F3) field isolate DNA (faint amplicon). Lane 3: SNP 4 wild type primers with Rev.1 DNA (no product). Lane 4: SNP 4 wild type primers with (F3) the field isolate DNA (amplicon 479 bp). (PCR conditions SNP 4: 95 °C /45 Sec. 62°C /35 Sec.72 °C /01:00 Min. for 28 cycles) Lane 5& 6: Marker. Lane7: SNP 5 Rev.1 primers with Rev.1 DNA (888 bp product). Lane 8: SNP 5 Rev.1 primers with (F3) *Brucella* field isolate DNA (faint amplicon). Lane 9: SNP 5 wild type primers with Rev.1 DNA (no product). Lane 10: SNP 5 wild type primers with (F3) *Brucella* field isolate DNA (amplicon 334 bp). (PCR conditions SNP 5: 95 °C /45 Sec. 63°C /35 Sec.72 °C /01:15 Min. for 26 cycles) Lane11: SNP 6 Rev.1 primers with Rev.1 DNA (544 bp amplicon). Lane 12: SNP 6 Rev.1 primers with (F3) *Brucella* field isolate DNA (no amplicon). Lane 13: SNP 6 wild type primers with Rev.1 DNA (no product). Lane 14: SNP 5 wild type primers with (F3) *Brucella* field isolate DNA (amplicon 1016 bp). (PCR conditions SNP 6: 95 °C /45 Sec. 62°C /35 Sec.72 °C /01:15 Min. for 27 cycles)

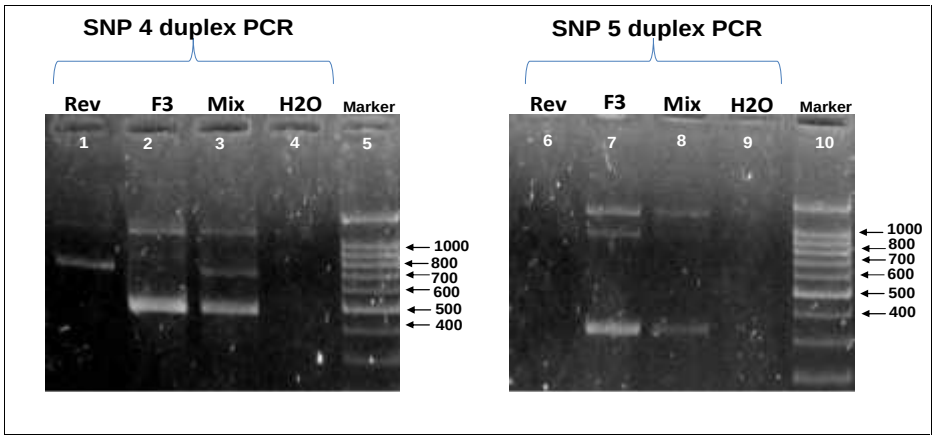

Fig 4.6: Duplex PCR results for SNP 4 and SNP 5. SNP 4 and SNP 5 duplex PCR tested by add both Rev.1 and wild type primers to all PCR tubes to test the bidirectional allele specific technique capacity to differentiate Rev.1 DNA from one of *Brucella* field isolate DNA (F3). The first 4 lanes were for SNP 4 primers. Lane 1: Rev.1 DNA (695 bp amplicon). Lane 2: (F3) *Brucella* field isolate DNA (479 bp amplicon and the outer primers product 1120 bp). Lane 3: Mix DNA from Rev.1 and (F3) *Brucella* field isolate DNA (faint two bands for Rev.1 and wild type are produced). Lane 4: H2O as a negative control. Lane 5: Marker. Lane 6, 7, 8 and 9 for SNP 5 primers. Lane 6: Rev.1 DNA (no amplicon). Lane 7: (F3) *Brucella* field isolate DNA (amplicon 334 bp and faint band for Rev.1 888 bp and the outer primers product ~1150 bp). Lane 8: Mix DNA from Rev.1 and (F3) *Brucella* field isolate DNA (faint two bands for wild type and outer primers are produced). Lane 9: H2O as a negative control. Lane 10: Marker. (PCR conditions: 95 °C /45 Sec. 62.5°C /35 Sec.72 °C / 1:00 Min. for 28 cycles)

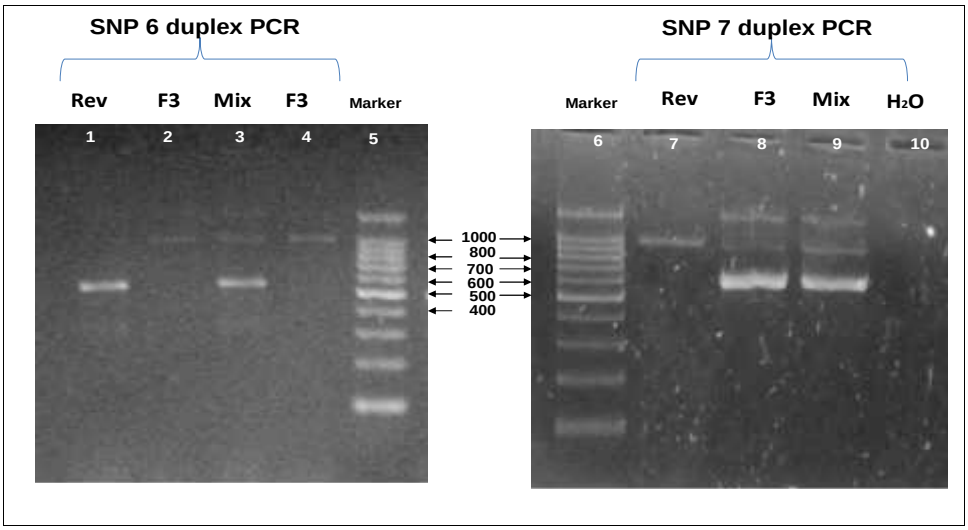

Fig 4.7: Duplex PCR results SNP 6 and SNP 7. The SNP 6 and SNP 7 duplex PCR tested by add both Rev.1 and wild type primers to all PCR tubes to test the bidirectional allele specific technique capacity to differentiate Rev.1 DNA from one of *Brucella* field isolate DNA (F3). The first 4 lanes were for SNP 6 primers. Lane 1: Rev.1 DNA (544 bp amplicon). Lane 2: (F3) *Brucella* field isolate DNA (amplicon 1016 bp). Lane 3: Mix DNA from Rev.1 and (F3) *Brucella* field isolate DNA (two bands for Rev.1 and wild type are produced). Lane 4: (F3) *Brucella* field isolate DNA (amplicon1016 bp) more (F3) DNA than lane 2. Lane 5: Marker. Lane 6,7,8,9 and 10 are for SNP 7 primers. . Lane 6: Marker Lane 7: Rev.1 DNA (958 bp amplicon). Lane 8: (F3) *Brucella* field isolate DNA (630 bp amplicon and faint Rev.1 band and the outer primers product ~1550 bp). Lane 9: Mix DNA from Rev.1 and (F3) field isolate DNA (two bands for wild type Rev.1 and outer primers are produced). Lane 10: H2O as a negative control.
(SNP 7 PCR conditions were: 95 °C /45 Sec. 62.5°C /35 Sec.72 °C / 1:00 Min. for 28 cycles. SNP 6 PCR conditions: 95 °C /45 Sec. 59°C /35 Sec.72 °C / 1:40 Min. for 30 cycles)

4.2.4 PCR results of SNP 7 and SNP 11

SNP 7 found in (BMEI1592) [3-methyl-2-oxobutanoate hydroxymethyltransferase] gene, substitution C to T at position 403. Singleplex PCR results (Fig 4.8) showed that the Rev.1 DNA gave clear strong band with Rev.1 primers and faint amplicon with wild type primers. While, in field DNA (F3) gave no bands with Rev.1 primers and clear band in wild type primers. In duplex PCR (Fig 4.7) the Rev.1 DNA gave the expected band only, while the (F3) DNA gave band for wild type and faint band for Rev.1 case. SNP 7 primers can genotype Rev.1.

SNP11 found in (BMEII1129) [High-affinity branched-chain amino acid transport ATP-binding protein LIVG] gene, G deletion, location at the gene 136. Singleplex PCR results

in (Fig 4.8) the Rev.1 DNA gave clear strong band with Rev.1 primers and no amplicon with wild type primers. While, field DNA (F3) gave expected band with wild type primers and no band in Rev.1 primers. In duplex PCR (Fig 4.9) the Rev.1 DNA gave a clear band with expected length and field isolate DNA (F3) the amplicon size for wild type appears. So SNP11 primers worked ideal for both cases in duplex PCR.

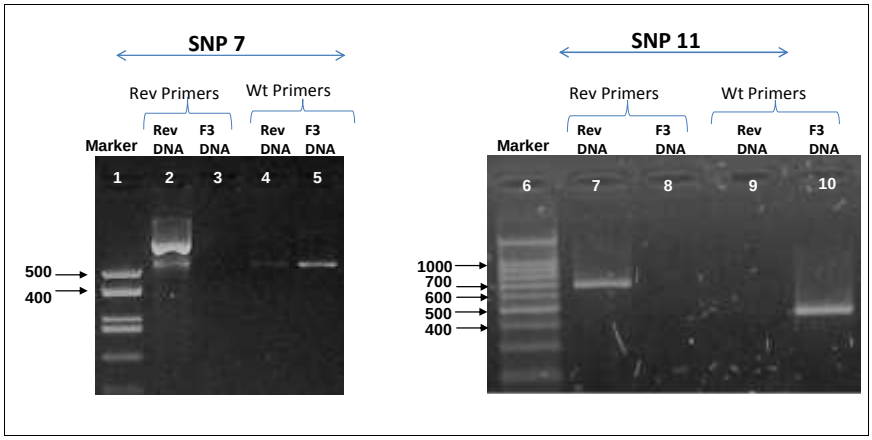

Fig 4.8: Singleplex PCR result for SNP 7 and SNP 11. Lane 1: Marker. Lane 2: SNP 7 Rev.1 primers with Rev.1 DNA (958 bp amplicon). Lane 3: SNP 7 Rev.1 primers with (F3) *Brucella* field isolate DNA (no amplicon). Lane 4: SNP 7 wild type primers with Rev.1 DNA (faint 630 bp amplicon). Lane 5: SNP 7 wild type primers with (F3) *Brucella* field isolate (630 bp amplicon). (PCR conditions SNP 7: 95 °C /45 Sec. 63°C /35 Sec.72 °C /01:15 Min. for 26 cycles) Lane 6: Marker. Lane 7: SNP 11 Rev.1 primers with Rev.1 DNA (682 bp amplicon). Lane 8: SNP 11 Rev.1 primers with (F3) *Brucella* field isolate DNA (no amplicon). Lane 9: SNP 11 wild type primers with Rev.1 DNA (no amplicon).Lane 10: SNP 11 wild type primers with (F3) the field isolate DNA (442 bp amplicon). (PCR conditions SNP 11: 95 °C /45 Sec. 58°C /35 Sec.72 °C /01:15 Min. for 30 cycles)

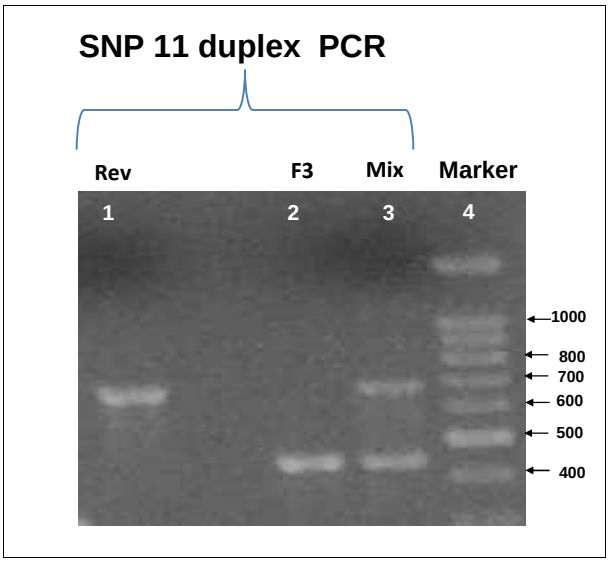

Fig 4.9: Duplex PCR results for SNP 11. Duplex PCR tested by add both Rev.1 and wild type primers to all PCR tubes to test the bidirectional allele specific technique capacity to differentiate Rev.1 DNA from one of *Brucella* field isolate DNA (F3). Lane 1: Rev.1 DNA (682 bp amplicon). Lane 2: (F3) *Brucella* field isolate DNA (442 bp amplicon). Lane 3: Mix DNA from Rev.1 and *Brucella* field isolate DNA (two bands for Rev.1 and wild type are produced). Lane 4: Marker. (PCR conditions: 94 °C/ 2 Min 95 °C /35 Sec. 58°C /35 Sec.72 °C / 45 Sec. for 30 cycles)

4.2.5 PCR results of SNP 8 SNP 9 and SNP 10

SNP 8 found in (BMEI1624) [Phosphate regulon sensor protein PHOR] gene, the substitution T to C, at position 986.

Singleplex PCR results (Fig 4.10) the Rev.1 DNA gave clear strong band with Rev.1 primers and no amplicon with wild type primers. While, in field DNA (F3) gave strong bands with wild type primers and faint band in Rev.1 primers. In duplex PCR the Rev.1 DNA gave expected band length for Rev.1 and the field isolate DNA (F3) gave the amplicon size for wild type (data not shown). So SNP 8 primers worked ideal for both cases in duplex PCR.

SNP 9 found in (BMEI1923) [Isovaleryl-CoA dehydrogenase] gene, substitution C to T, at position 787. Singleplex PCR results (Fig 4.10) the Rev.1 DNA give clear strong band with Rev.1 primers and no amplicon with wild type primers. While in field DNA (F3) gave expected band with wild type primers and no band in Rev.1 primers. In duplex PCR (Fig

4.11) the Rev.1 DNA gave a clear band with expected length beside faint band for wild type, and in field isolates DNA (F3) the amplicon size for wild type appears. SNP 9 primers worked ideal for both cases in duplex PCR.

SNP 10 found in (BMEI2044) hypothetical protein gene, substitution G to A at position 444. Singleplex PCR results (Fig 4.10) the Rev.1 DNA give clear strong band with Rev.1 primers and no band with wild type primers. While, the field DNA (F3) gave expected band with wild type primers and no band in Rev.1 primers. In duplex PCR (Fig 4.11) the Rev.1 DNA gave a clear band with expected length, and the fields isolate DNA (F3) the amplicon size for wild type appears. So SNP10 primers worked ideal for both cases in duplex PCR.

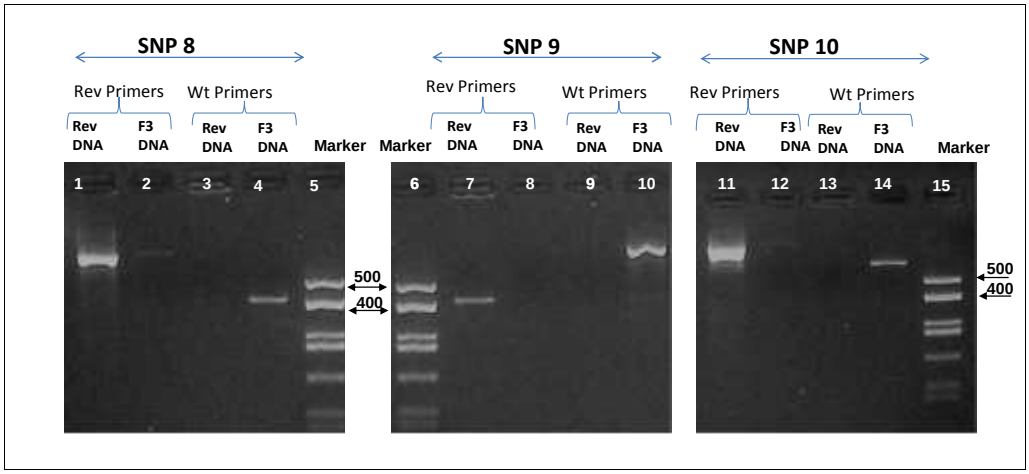

Fig 4.10: Singleplex PCR result for SNP 8, SNP 9 and SNP 10. Lane1: SNP 8 Rev.1 primers with Rev.1 DNA (924 bp amplicon). Lane 2: SNP 8 Rev.1 primers with (F3) *Brucella* field isolate DNA (very faint amplicon). Lane 3: SNP 8 wild type primers with Rev.1 DNA (no product). Lane 4: SNP 8 wild type primers with (F3) the *Brucella* field isolate DNA (468 bp amplicon). (PCR conditions SNP 8: 95 °C /45 Sec. 59°C /35 Sec.72 °C /01:15 Min. for 26 cycles) Lane 5& 6: Marker. Lane7: SNP 9 Rev.1 primers with Rev.1 DNA (468 bp amplicon). Lane 8: SNP 9 Rev.1 primers with (F3) *Brucella* field isolate DNA (no amplicon). Lane 9: SNP 9 wild type primers with Rev.1 DNA (no amplicon). Lane 10: SNP 9 wild type primers with (F3) *Brucella* field isolate DNA (949 bp amplicon). (PCR conditions SNP 9: 95 °C /45 Sec. 63°C /35 Sec.72 °C /01:15 Min. for 26 cycles). Lane11: SNP 10 Rev.1 primers with Rev.1 DNA (1196 bp amplicon). Lane 12: SNP 10 Rev.1 primers with (F3) *Brucella* field isolate DNA (no amplicon). Lane 13: SNP 10 wild type primers with Rev.1 DNA (no product). Lane 14: SNP 10 wild type primers with (F3) *Brucella* field isolate DNA (781 bp amplicon). (PCR conditions SNP10: 95 °C /45 Sec. 59°C /35 Sec.72 °C /01:15 Min. for 29 cycles).

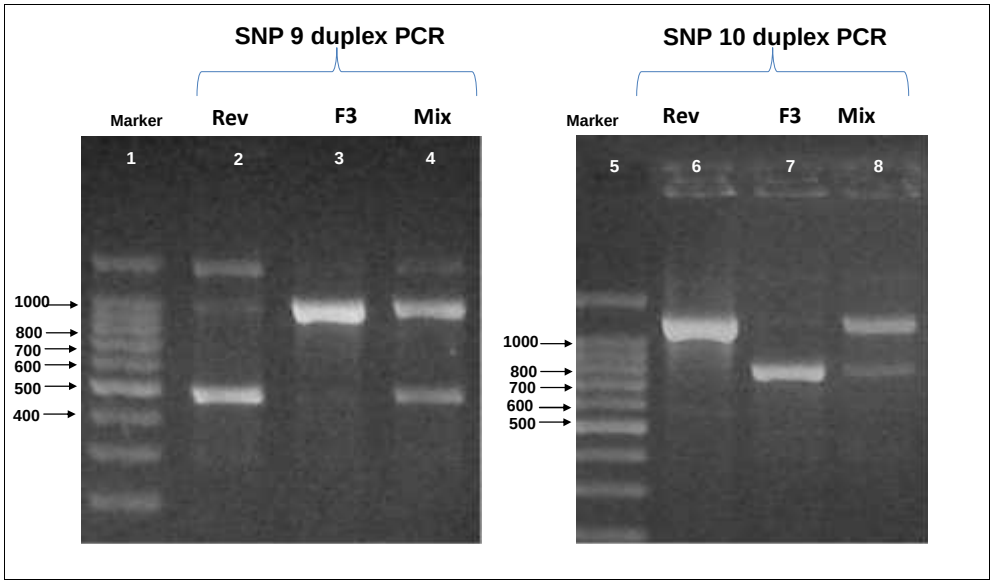

Fig 4.11: Duplex PCR results for SNP 9 and SNP 10. The SNP 9 and SNP 10 duplex PCR tested by add both Rev.1 and wild type primers to all PCR tubes to test the bidirectional allele specific technique capacity to differentiate Rev.1 DNA from one of *Brucella* field isolate DNA (F3). The first 4 lanes were for SNP 9 primers. Lane 1: Marker. Lane 2: Rev.1 DNA (468 bp amplicon and band for outer primer ~1350 bp). Lane 3: (F3) *Brucella* field isolate DNA (949 bp amplicon). Lane 4: Mix DNA from Rev.1 and *Brucella* field isolate DNA (two bands for Rev.1 and wild type are produced). Lane 5, 6, 7, and8 for SNP 10 primers. Lane 5: Marker. Lane 6: Rev.1 DNA (1196 bp amplicon). Lane 7: (F3) *Brucella* field isolate DNA (781 bp amplicon). Lane 8: Mix DNA from Rev.1 and *Brucella* field isolate DNA (two bands for wild type Rev.1). (PCR conditions SNP 10: 94 °C/ 2 Min 95 °C /35 Sec. 57.5°C /35 Sec.72 °C / 1:45 Sec. for 35 cycles) (PCR conditions SNP 9: 94 °C/ 2 Min 95 °C /35 Sec. 61°C /35 Sec.72 °C / 1:10 Sec. for 30 cycles)

In summary, the primer pairs that showed good differentiation capacity (primer pairs for SNP 4, 6, 8, 9, 10 and 11) were used to examine the three *B. melitensis* reference biovars: biovar 1, biovar 2 and biovar 3.

4.2.6 PCR results of the three *B. melitensis* reference strains

The SNP 4, 6, 8, 9, 10 and 11 were tested using the three known *B. melitensis* reference biovars DNA: *B. melitensis* 16M biovar-1 (ATCC 23456), *B. melitensis* biovar.2 str. 63/9, and *B. melitensis* biovar 3 strain Ether. SNP 11, 10, 4 and SNP 8 showed the right amplicon size for Rev.1, which was different from the amplicon sizes produced by the three reference strains DNA with minimum PCR artifacts. (See fig 4.12, 4.13, 4.14 & 4.15).

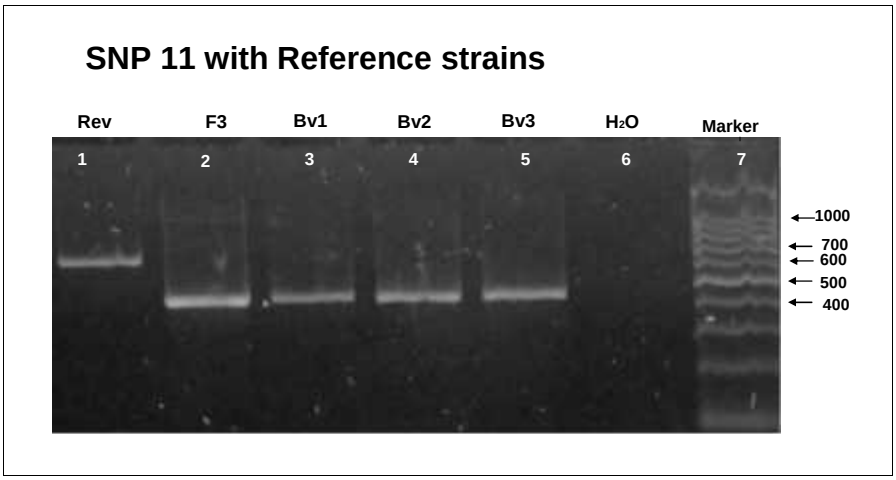

Fig 4.12: Bidirectional PCR result for SNP 11 primers with the *B. melitensis* three reference biovars. Lane 1: *B. melitensis* Rev.1 DNA (682 bp amplicon). Lane 2: (F3) field isolate from *B. melitensis* (442 bp amplicon). Lane 3: *B. melitensis* biovar 1 16M DNA (442 bp amplicon). Lane 4: *B. melitensis* biovar 2 str. 63/9 DNA (442 bp amplicon). Lane 5: *B. melitensis* biovar 3 str. Ether DNA. (442 bp amplicon).Lane 6: H2O as a negative control. Lane 7: Marker. Rev.1 DNA gave amplicon size different than other strains, so we can genotype Rev.1 by SNP 11 primer with bidirectional PCR assay. (PCR conditions: 94 °C/ 2 Min 95 °C /35 Sec. 58°C /35 Sec.72 °C / 0:45 Min. for 35 cycles)

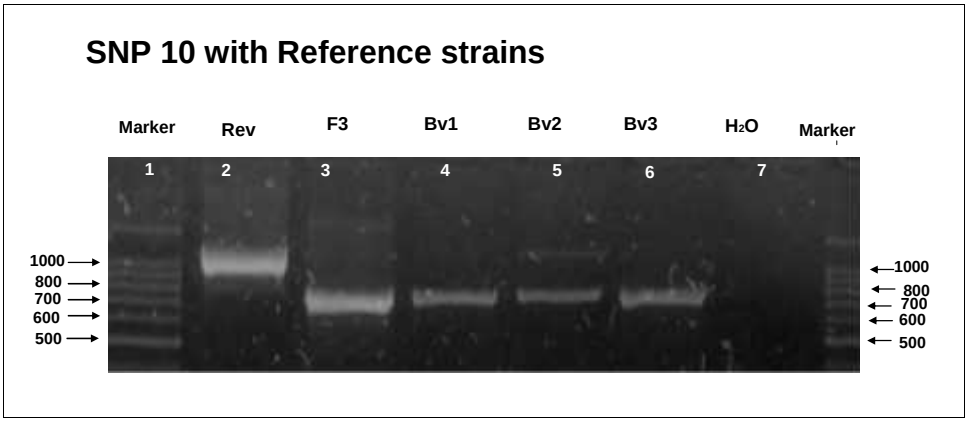

Fig 4.13: Bidirectional PCR result for SNP 10 primers with the *B. melitensis* three reference biovars. Lane 1: Marker. Lane 2: *B. melitensis* Rev.1 DNA (1196 bp amplicon). Lane 3: (F3) field isolate from *B. melitensis* (781 bp amplicon). Lane 4: *B. melitensis* biovar 1 16M DNA (781 bp amplicon). Lane 5: *B. melitensis* biovar 2 str. 63/9 DNA (781 bp amplicon) and faint band 1196 bp. Lane 6: *B. melitensis* biovar 3 str. Ether DNA. (781 bp amplicon).). Lane 7: H2O as a negative control. Lane 8: Marker Rev.1 DNA gave amplicon size different than other strains, so we can genotype Rev.1 by SNP 10 primers with bidirectional PCR assay. (PCR conditions: 94 °C/ 2 Min 95 °C /35 Sec. 58°C /35 Sec.72 °C / 1:15 Min. for 35 cycles)

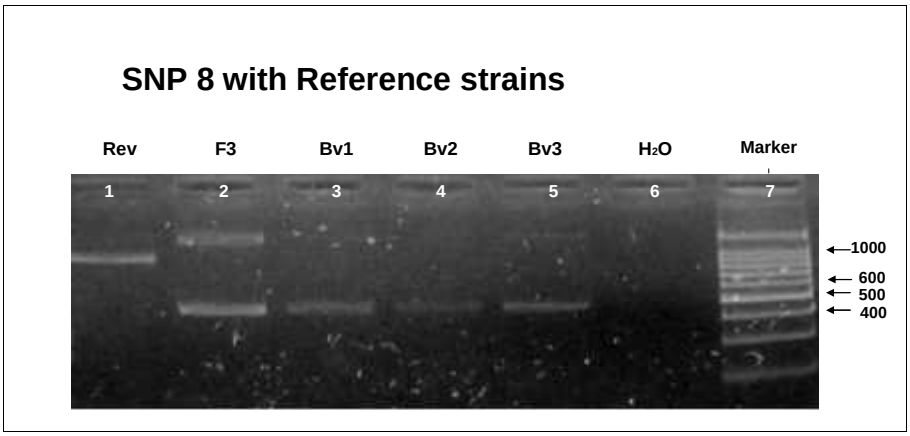

Fig 4.14: Bidirectional PCR result for SNP 8 primers with the *B. melitensis* three reference biovars. Lane 1: *B. melitensis* Rev.1 DNA (924 bp amplicon). Lane 2: (F3) field isolate from *B. melitensis* (468 bp amplicon) and the outer primers amplicon (~1350 bp). Lane 3: *B. melitensis* biovar 1 16M DNA (468 bp amplicon). Lane 4: *B. melitensis* biovar 2 str. 63/9 DNA (468 bp amplicon). Lane 5: *B. melitensis* biovar 3 str. Ether DNA. (468 bp amplicon).). Lane 6: H2O as a negative control. Lane 7: Marker. Rev.1 DNA gave amplicon size different than other strains, so we can genotype Rev.1 by SNP 8 primers with bidirectional PCR assay.
(PCR conditions: 94 °C/ 2 Min 95 °C /35 Sec. 59°C /35 Sec.72 °C /1:20 Min. for 30 cycles)

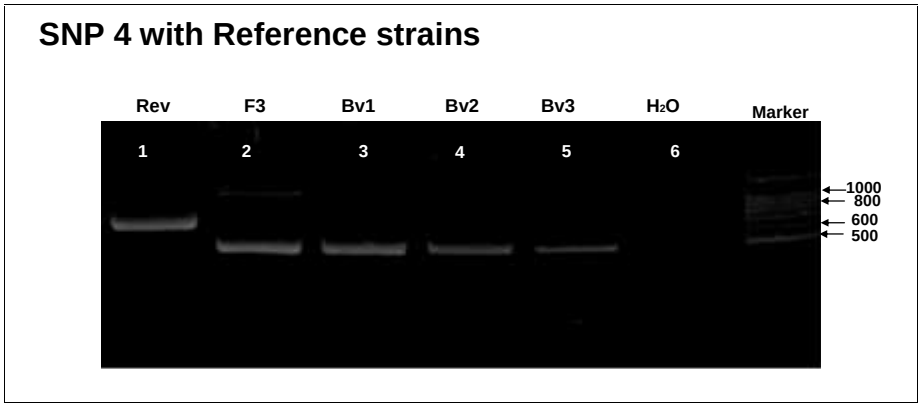

Fig 4.15: Bidirectional PCR result for SNP 4 primers with the *B. melitensis* three reference biovars. Lane 1: *B. melitensis* Rev.1 DNA (695 bp amplicon). Lane 2: (F3) field isolate from *B. melitensis* (479 bp amplicon). Lane 3: *B. melitensis* biovar 1 16M DNA (479 bp amplicon). Lane 4: *B. melitensis* biovar 2 str. 63/9 DNA (479 bp amplicon). Lane 5: *B. melitensis* biovar 3 str. Ether DNA. (479 bp amplicon).Lane 6: H2O as a negative control. Lane 7: Marker. Rev.1 DNA gave amplicon size different than other strains, so we can genotype Rev.1 by SNP 4 primer with bidirectional PCR assay from the three *B. melitensis reference* biovars DNA
(PCR conditions: 94 °C/ 2 Min 95 °C /35 Sec. 62°C /35 Sec.72 °C /1:20 Min. for 30 cycles)

4.2.7 A preliminary screening of some field isolates and samples

SNP 11 primers, which gave a strong differentiation results, were tested with bidirectional PCR approach on 36 field isolates and clinical samples of *B. melitensis* that were collected by CVL. The PCR result shows that most of the field isolates and clinical samples are most likely not Rev.1 (Fig 4.16). The results showed that the majority of isolates gave just the wild type band, while isolates in lanes; 10, 13, 14, 18, 26 and 36 gave bands for wild type and faint band for Rev.1. Some of samples DNA didn't gave any product. In this PCR, the cycles were elevated to 50 to enhance the detection sensitivity since some of the samples are diluted old samples. Unfortunately, the complete data about these *Brucella* samples were not available.

Though those results are not deterministic, yet they provide an initial confirmation about the validity of our Rev.1 genotyping approach. It is important to assert that further analysis of other field strains and different vaccine batches are needed to support our new PCR assay sensitivity and specificity.

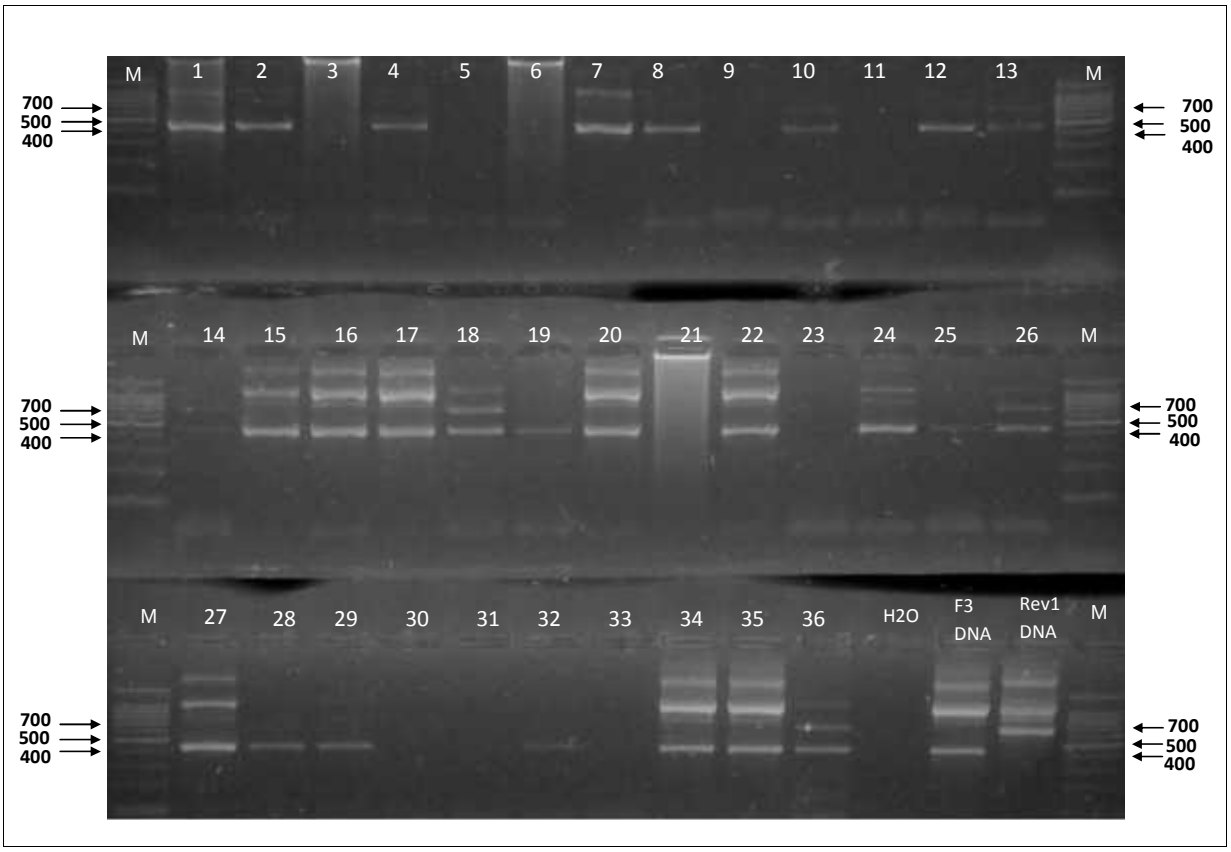

Fig 4.16: Bidirectional PCR result for SNP 11 primers with 36 local field isolates. Lane 1 to lane 36 different samples and isolates (for details see table 4.6):Lane 37: H2O Lane 38:(F3)field DNA Lane 39: Rev.1 DNA (PCR conditions: 94 °C/ 2: 00 Min, 95 °C /45 Sec. 57 °C /40 Sec.72 °C /02:30 Min. for 50 cycles)

4.3 - Sequencing Results

The SNPs 1, 3, 4, 5, 6, 7, 8, 9 &10 were confirmed by sequencing of Rev.1 DNA (see Fig 4.17 to Fig 4.25), while SNP 2 and SNP 11 are still under investigation.

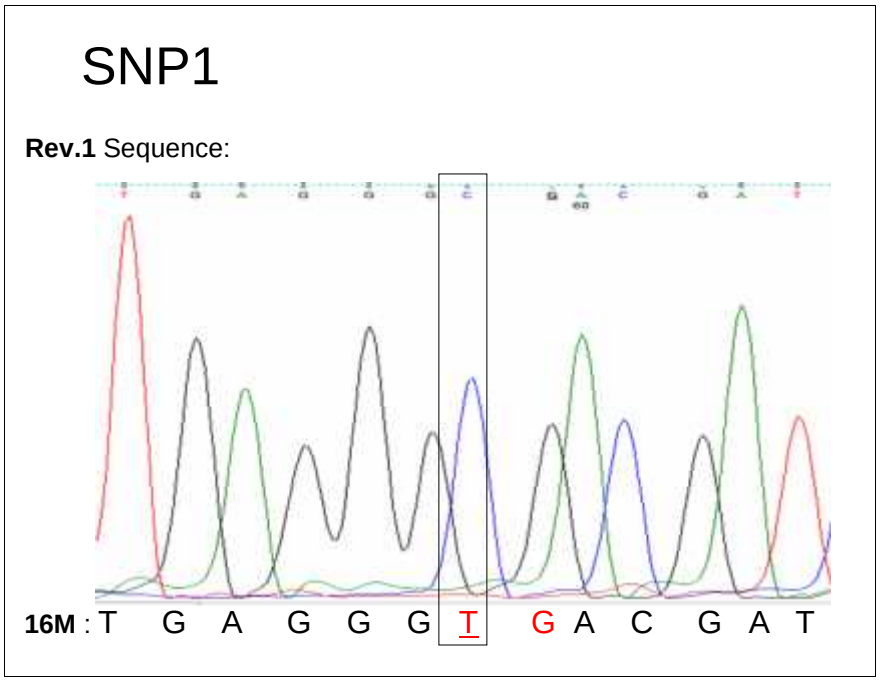

Fig 4.17: SNP 1 chromatogram sequencing results for Rev.1 compared to the *B. melitensis* 16M strain (Wt) sequence.

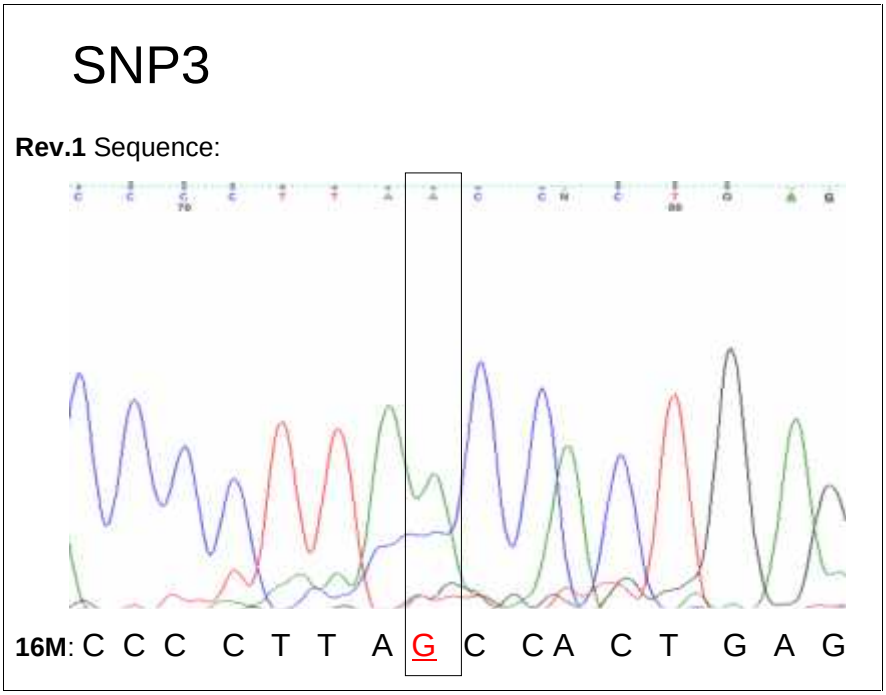

Fig 4.18: SNP 3 chromatogram sequencing results for Rev.1 compared to the *B. melitensis* 16M strain (Wt) sequence.

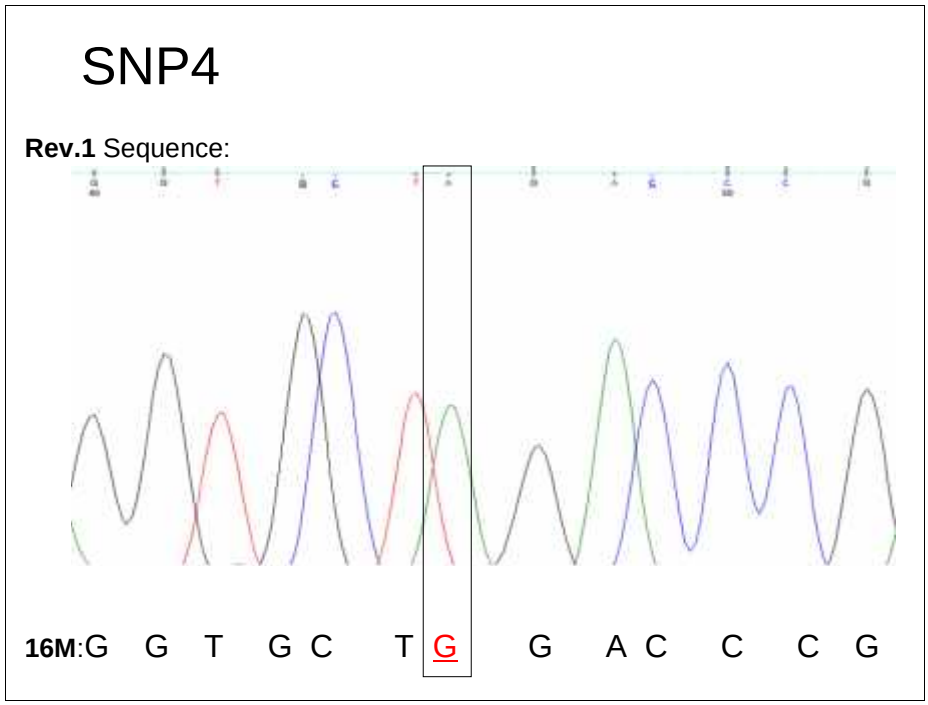

Fig 4.19: SNP 4 chromatogram sequencing results for Rev.1 compared to the *B. melitensis* 16M strain (Wt) sequence.

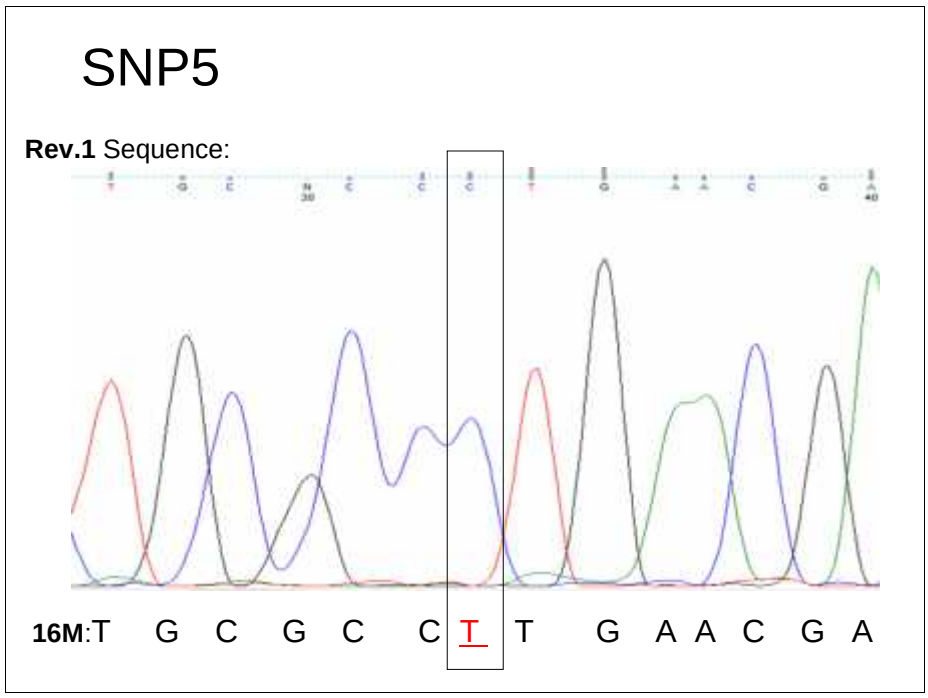

Fig 4.20: SNP 5 chromatogram sequencing results for Rev.1 compared to the *B. melitensis* 16M strain (Wt) sequence.

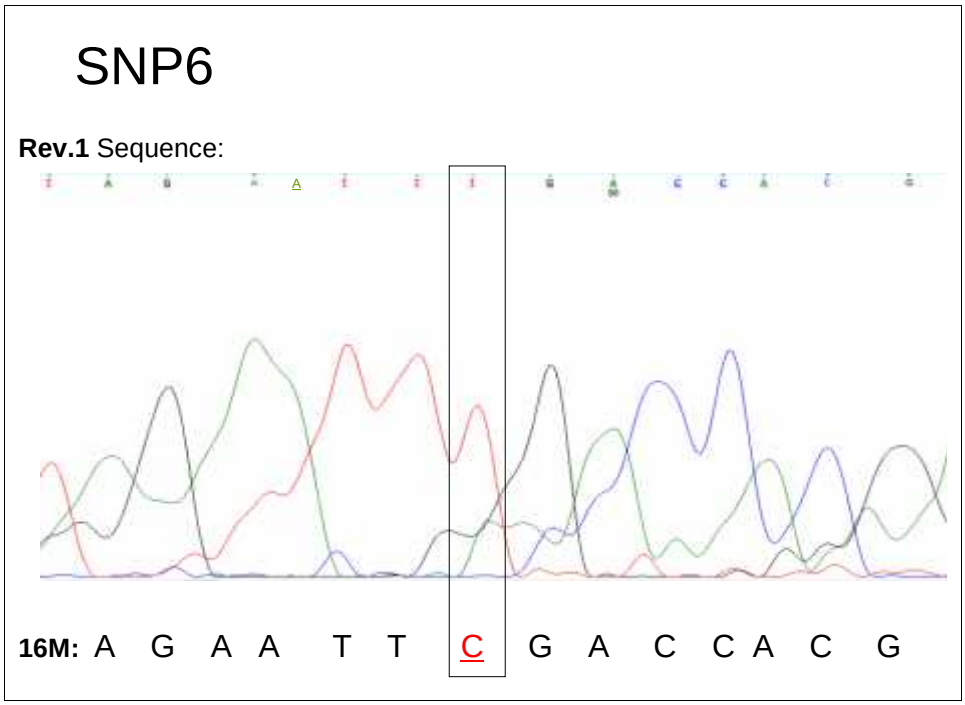

Fig 4.21: SNP 6 chromatogram sequencing results for Rev.1 compared to the *B. melitensis* 16M strain (Wt) sequence

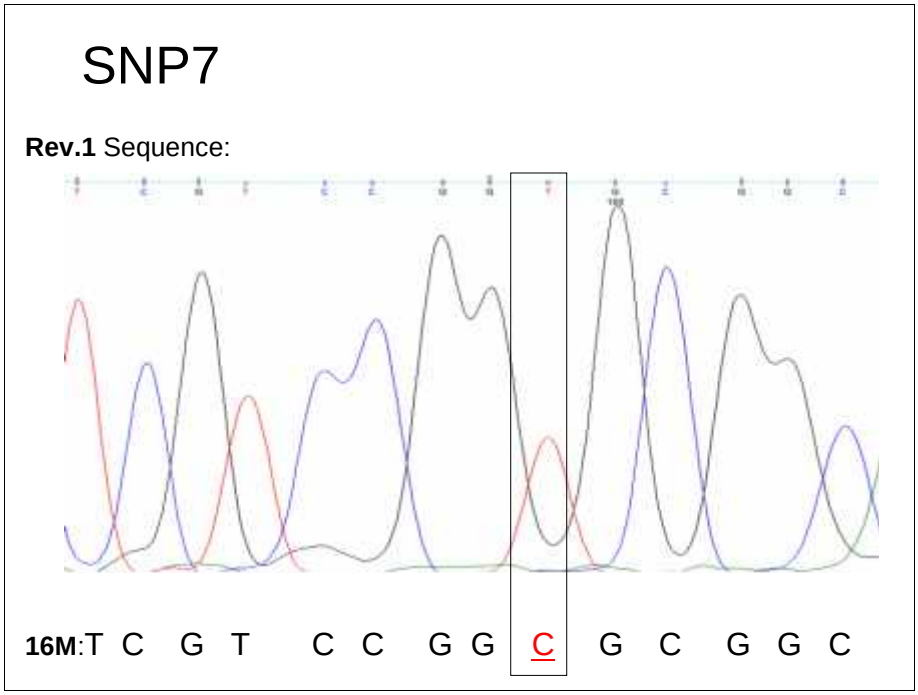

Fig 4.22: SNP 7 chromatogram sequencing results for Rev.1 compared to the *B. melitensis* 16M strain (Wt) sequence.

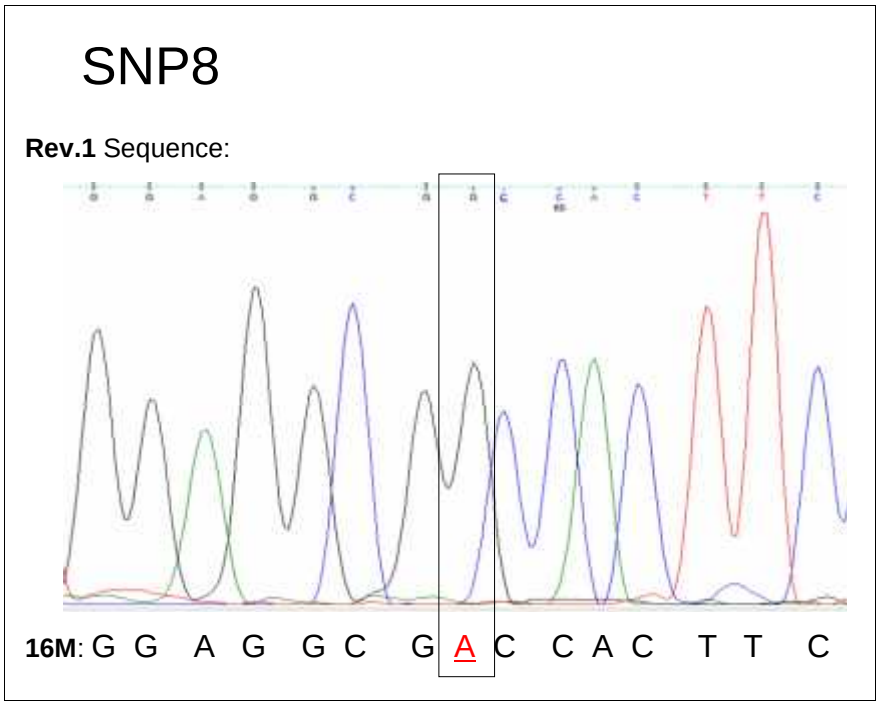

Fig 4.23: SNP 8 chromatogram sequencing results for Rev.1 compared to the *B. melitensis* 16M strain (Wt) sequence.

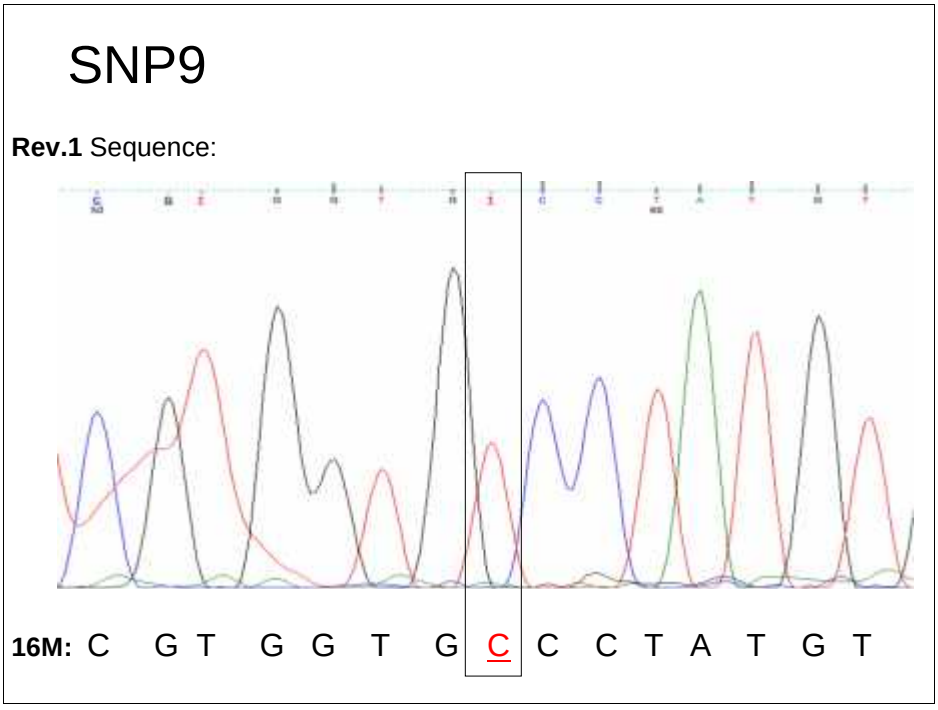

Fig 4.24: SNP 9 chromatogram sequencing results for Rev.1 compared to the *B. melitensis* 16M strain (Wt) sequence.

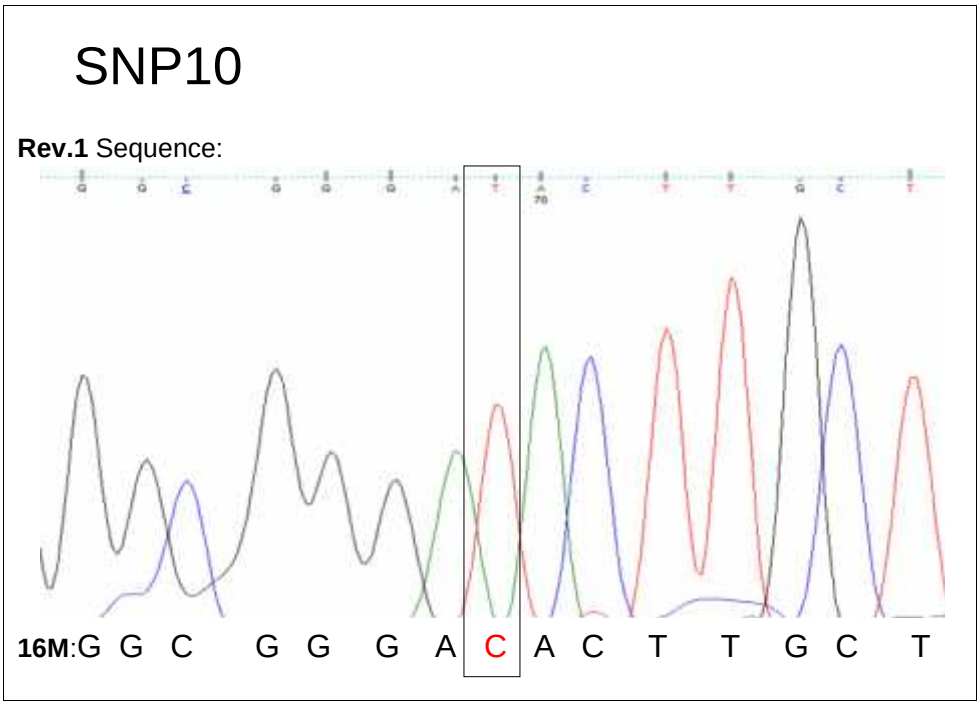

Fig 4.25: SNP 10 chromatogram sequencing results for Rev.1 compared to the *B. melitensis* 16M strain (Wt) sequence.

CHAPTER 5
Discussion and Conclusions

5.1 Rev.1 genetic markers

Brucellosis is a world-wide disease that dose not only causes huge losses to the livestock sector but also has a serious zoonotic risk. Countries are spending millions of US Dollars annually to fight this disease to reduce its effect on public health. *Brucella melitensis* Rev.1 is currently the best used vaccine to control Brucellosis caused by *B. melitensis*. Despite the fact that Rev.1 vaccine has been used since 1960s, the distinctive genetic markers for Rev.1 are not yet identified (Bosseray 1991). Moreover, the molecular evidences for its attenuation are not yet known. Consequently, the control of Rev.1 vaccine production and its post-vaccination traceability will remain difficult and challenging tasks.

Discovering the genetic mutations which took place in Elberg lab and made one of his virulent strains an attenuated strain “Rev.1” that has later became the most important vaccine for *B. melitensis*, will facilitate Rev.1 molecular differentiation and improve the quality control of the vaccine production. In addition, revealing the molecular events that caused Rev. 1 attenuation will help in developing this vaccine and to reducing its side effects. For that goal, many previous attempts were directed to discover the unique genetic alteration that can be used for genotyping this vaccine. These efforts resulted in the discovery of few individual SNPs: one confirmed in *rpsl* gene and another potential one in *omp2* gene. This work is unique and different from the previous attempts because a novel and massive comparative genomic approach were used to identify all possible genetic alternations that are unique to Rev.1. This methodology has the advantage to benefit from recent huge increase in DNA sequence data that are available for analysis. It is worth mentioning that unlike many other bacterial genomes, *Brucella* bioinformatics databases are scarce and dispersed. Also, Rev.1 genome was available in the form of contigs and scaffolds (scaffold is a portion of genome sequence, constructed from contigs and gabs) and not as one reference genome sequence. These limitations made this task even harder.

In this study, the first goal was to reveal the mutations which converted the virulent strain to the attenuated Rev.1 vaccine strain by using a bioinformatics approach. Then to suggest some or all of the distinctive mutations as genetic markers that can be considered as a reliable genetic barcode for Rev.1 strain. The bioinformatics analysis resulted in identification of 24 candidate genetic markers exclusive to Rev.1 strain. Of these 24 genetic variations (SNPs), 11 were selected for further experimental validations. Ten out of these 11 variations were tested and confirmed by PCR and nine out of these ten SNPs were confirmed by sequencing. A total of 5/9 of these SNPs were found to be appropriate for straightforward genotyping using bidirectional PCR technique. Indeed, these 5 SNPs prove to be specific when tested on three *B. melitensis* biovars (biovar 1, 2 and 3).

The proposed Rev.1 genetic markers need to be confirmed in different Rev.1 vaccine batches and against other *B. melitensis* isolates to confirm the uniqueness and stability of these mutations. Unfortunately, in Palestine, we do not have *Brucella* reference strains or bank of isolates. Because of that, our research team has ongoing co-operation with Dr. Ignacio Lopez-Goni from Navarra University -Spain to confirm the founded SNPs on other isolates and to develop a solid Rev.1 genetic barcode.

The discovered genetic markers could be used in vaccine quality control production and in traceability of Rev.1 in the field after vaccination. Also, Rev.1 genetic markers can be used to develop new diagnostic tools, such as: multiplex PCR assay or microarray chips. In addition, understanding the molecular attenuation evidences can help to develop new *B. melitensis* vaccine that is safer and more stable with fewer side effects than the current Rev.1 vaccine.

5.2 Bidirectional PCR assay

The second goal of this study was to experimentally confirm some of the found mutations by bioinformatics methods as well as to develop an accurate and inexpensive PCR assay to diagnose *Brucella* and to differentiate Rev.1 from other field strains.

Five SNPs gave a clear differentiation pattern when tested in bidirectional PCR technique against the three known *B. melitensis* biovars. One SNP, "SNP11" was tested against a panel of *Brucella* clinical samples and isolates and holds a potential to be used in large-scale screening for the diagnosis of Brucellosis and differentiation of Rev.1 in one tube PCR assay. Differentiating between vaccinated and infected animals is a critical step in Brucellosis eradication program. While it is almost impossible to differentiate between Rev.1 and field strains with serological methods, several other methods were used with limited success. These methods depend mainly on post culture identification of *Brucella* species. Though the proposed bidirectional PCR had a potential to work with clinical samples directly, yet this method needs further optimization.

The PCR–RFLP technique targeting the Rev.1 specific SNP in *rpsl* gene is generally an accepted Rev.1 differentiation method. This SNP is also included in the Bruce-Ladder assay (I López-Goñi et al. 2008). We believe that our approach, which aims toward targeting various Rev.1-distinctive SNPs, may be more reliable and accurate than targeting the single SNP of *rpsl* gene for several reasons. Firstly, *rpsl* SNP is a single marker that is linked to streptomycin resistant phenotype with no relation to Rev.1 attenuation and therefore it is better to target a wider range of attenuation-associated markers to ensure detection specificity and to track attenuation. Secondly, *rpsl* detection by direct PCR cannot rule out possible contamination of Rev. 1 by virulent strains. However, the bidirectional PCR method can detect mixed infections by Rev. 1 as well as other virulent strains. Finally, the *rpsl* SNP detection with PCR-RFLP method is an expensive technique compared to the used bidirectional PCR approach.

Another accepted molecular approach to differentiate Rev.1 from wild type is the Multiple Locus Variable Number Tandem Repeats Analysis (MLVA). MLVA can identify the genotype of closely related bacterial strains by revealing variations in the number of tandem repeated DNA sequences that occur naturally in many different loci in bacterial genomes . However, MLVA assay is considered an expensive, laborious and dependent on high level labs facilities such as automated genetic analyzer. It is important to recall that most laboratories of the developing countries, which suffer from Brucellosis, do not usually have the optimal setup to perform MLVA technique. On the other hand, the bidirectional PCR is practical, inexpensive, and can be run in molecular labs of most developing countries.

The *omp2* gene differentiation depends on PCR - RFLP method, which is considered as unspecific method, since the *B. melitensis 16M* gives the same restriction pattern as Rev.1. A recent report by *Gopaul et al*., suggested a real-time PCR assay to distinguish Rev. 1 by targeting a G to A SNP at position 933 of *proA* gene BMEI0208 (Gopaul et al. 2010). However, our bioinformatics analysis showed that this SNP is also found in other five *B. melitensis* strains. Gopaul et al. were keen to examen our crtique and they indeed found that SNP in other *B. melitensis* strains (personal communication with Dr. Whatmore)

The 11 tested SNPs with bidirectional PCR were chosen according to appropriateness of DNA sequence flanking the SNPs for designing bidirectional primers. One out of the 11 SNPs (SNP 2) was not capable to different Rev.1 in singleplex PCR. Whereas, the other 10 SNPs were capable to differentiate Rev.1 in singleplex PCR. Some of these SNP primers worked partially, that means the Rev.1 primers gave PCR product in Rev.1 DNA and no bands in wild type DNA, but the wild type primers gave a product in both wild type and Rev.1 DNA, or vice versa. These unusual results could be due to using the same PCR conditions for both primer pairs which were tested in the same annealing temperature. Further optimization for each primer set, which may include redesign the primers, could enhance detection sensitivity.

It is worth mentioning that, except SNP 11, all targeted SNPs were single nucleotide substitutions. The fact that some nucleotide differences at the 3' end of primers are more promiscuous such as G and T, had contributed in lowering the differentiation power of some of bidirectional PCRs. As mentioned earlier, SNP 11 bidirectional PCR was the only one that targeted a G nucleotide deletion. Therefore, the difference between Rev.1 and wild type in this PCR was two nucleotides at the 3' end of the internal primers. This is the reason why SNP 11 primers gave the best discrimination capability.

The experimental PCR work was made based on three *B. melitensis* references biovar DNA (biovar 1, biovar 2 and biovar 3), kindly supplied as a gift from Dr. Ignacio Lopez Goni. Since the amount of biovars DNA was not sufficient to test the 11 different SNPs and to optimize PCR conditions for a lot of primers, one of *B. melitensis* field isolate (F3) was used, for optimization purposes, in our initial PCR tests.

Our preliminary trail to test a group of field samples using SNP 11 bidirectional PCR can be considered promising though we faced several constrains. The DNA samples that were supplied kindly by Central Veterinary lab, were not fully labeled (the date, location, Isolate or clinical samples etc). In addition, few of them were from field strains that were isolated on agar, while the majority was just DNA extracted from clinical samples such as milk and aborted materials. Therefore the DNA template concentration was significantly different from sample to sample. Some of these samples gave faint band for Rev.1 and other band for wild type. These unexpected results could be due to cross contamination with Rev.1 DNA, or due to a PCR artifact due to increase in cycle numbers to 50 cycles.

5.3 *B. melitensis Rev.1* virulence

In this section, the biological roles of Rev.1 genes that were discovered to have unique SNPs will be discussed. In addition, their possible implication in virulence and the likelihood that the revealed SNPs are molecular evidence of Rev.1 attenuation.

5.3.1 ATP-binding cassette (ABC) transporters

ABC transporters are transmembrane proteins, which require energy to transport various substances across membranes. ABC transporters also play a role in DNA repair and RNA translation (Garmory & Titball 2004). In bacteria, the efflux system use ABC transporters to excrete harmful substances (Antibiotics) and excrete toxins. Also, ABC transporters have a role in building the membrane lipo-polysaccharides. Bacteria which lives in complex environment usually requires numerous ABC systems (Garmory & Titball 2004). *Brucella* has relatively high percentage of ABC system genes about 8.8% of its genome (Jenner et al. 2009) compared with *E. coli* genome which has 5% of its genome coding for ABC systems (Garmory & Titball 2004).

ABC transporters play an obvious role in bacterial virulence by affecting the nutrient and ions uptake, antibiotic resistance and cell attachment (Garmory & Titball 2004). Manipulation of ABC transporters can generate attenuated bacteria, e.g. a deletion in one of ABC transporter genes in *Brucella abortus* attenuated this mutant and when tested it gives better immunogenicity than *B. abortus* vaccine S19 strain (Garmory & Titball 2004). Jenner et al (2009) noticed that *Brucella ovis*, which cannot infect human, contained less ABC transporters than other aggressive *Brucella* which can infect human (Jenner et al. 2009).

This work revealed that the Rev.1 strain has many ABC transporters genes which suffer from unique mutations. Because of that, it is suggest that the reduced virulence of Rev.1 could be partially due to shortage in some of its ABC transporters' functions. Also, the susceptibility to Penicillin and dyes in Rev.1 (G. G. Alton et al. 1967) could be due to shortages in efflux system as a result of ABC transporters mutations. Iron acquisition through siderophores is very important for full virulence of *B. melitensis, the* ABC transporters have a role in transporting siderophore which carry Fe^{3+} ions (Danese et al. 2004) Its known that Rev.1 suffering from a miss regulation of iron (DelVecchio & C. Mujer 2004), this could due to ABC transporters defects in Rev.1. Some of ABC transporter system genes which could affect the Rev.1 virulence are:

- High-affinity branched-chain amino acid transport ATP-binding protein livg (BMEII0630)

The SNP11 is a “G” deletion at 136 in this gene (BMEII0630) that causes a frame shift and premature stop codon. In *E. coli*, this gene is a part of ABC transporter and its deletion was associated to failure in leucine transport (Nazos et al. 1985; Adams et al. 1990). Leucine is an essential amino acid and a main compound of ferritin, which store iron and release it based on bacterial needs. In proteomic analysis of Rev.1, an over expression of some proteins which are needed for iron regulations are reported. Also, bacterioferritin was accumulated in Rev.1 more than it was in the virulent strain 16M. Additionally, there are over expression of iron-regulated outer membrane protein and an iron (III)-binding periplasmic protein (Eschenbrenner et al. 2002). Altogether, these findings indicates that Rev.1 suffers from a miss regulation of iron homeostasis (DelVecchio & C. Mujer 2004).

- **Oligopeptide transport ATP-binding protein OPPF (BMEII0199)**

A Nonsynonymous mutation was found in this gene that leads to an amino acid substitution (A230T). According to Uniprot database, this gene’s function is “Part of the binding-protein-dependent transport system for oligopeptides. Probably, responsible for energy coupling to the transport system”.

- **Maltose/maltodextrin transport ATP-binding protein MALK (BMEII0940)**

Nonsynonymous mutation leads to change amino acid (S22L). This gene is involved in maltose/maltodextrin importation.

- Copper transport ATP-binding protein NOSF (BMEII0971)

Nonsynonymous mutation leads to change amino acid (K218R). This gene’s function is to transport Copper

It is assumed that a mutation of one of the above ABC transporter or a combination of more than one mutation in different genes might be the reason behind the unique susceptibility of Rev1 to Penicillin and Basic Fuchsine and Thionin dyes, unlike other *B. melitensis* strains which can tolerate these agents. A possible proof of this assumption is the accumulated evidence which showed that over expression of ATP transporter increases the antibiotic resistance. While blocking ABC transporter increase the sensitivity to antibiotic (Garmory & Titball 2004).

5.3.2. Glycosyl Transferase

The function of Glycosyl transferase enzyme group is to transfer glycosyl group from one compound (donor) to another one (acceptor). *B. abortus* strain RB51, is an attenuated vaccine. The RB51 strain has a mutation in wboA gene, which encodes a glycosyl transferase. This enzyme is required for the synthesis of O-Polysaccharide (OPS), which are part of the outer membrane lipopolysaccharide (LPS). In fact, this difference makes *B. abortus RB51* distinguishable from other virulent strains using serological methods. Also, this RB51 mutation has been used in molecular differentiation of this strain by PCR (Hoover et al. 2004). The outer membrane OPS is important in Antibody (Ab) opsonization; the rendering of bacteria for phagocytes. In Brucella, OPS are resistant to complement mediated lysis inside phagosomes (Moreno & Gorvel 2004)

- **Glycosyl transferase (BMEII1129) gene.**

One of the unique Rev.1 mutations that was found in this study is an “A” insertion at position 998 in the glycosyl transferase gene (BMEII 1129). This is a frame shift mutation that leads to a premature stop codon. According to the Broad Institute’s annotation, this gene is called mannosyl transferase which is glycosyl transferase. According to the general annotation of the Uniprot database, this protein is essential for lipopolysaccharide synthesis.

In general LPS is important in Penicillin resistance mechanism. Also, *Brucella* LPS is known as a virulence factor and induces immune response. Our findings agree with the

proteomic-based study of Eschenbrenner et al (2002) which showed that Rev.1 had an alteration in immunogenic OMP (Eschenbrenner et al. 2002). Another study of Zygmunt et al., shows that Rev.1 vaccinated sheep have less antibody response against O-polysaccharide (O-PS), compared with infected sheep with virulent strains (M S Zygmunt et al. 1994).

5.3.3 Ribosomal protein

A unique nonsynonymous mutation was found in the *30S ribosomal protein S4* gene (BMEI1133). This mutation causes an amino acid substitution (A201K). According to Uniprot database, mutations in the *30S ribosomal protein S4* have correlation with suppression of streptomycin dependence in *E. coli*. The Rev.1 strain was obtained by a two-step selection involving: Firstly streptomycin resistance and dependence (Sanford S Elberg & K Faunce 1957). Secondly, from those Streptomycin dependents a nondependent mutant was selected, tested as a vaccine, and then named Rev.1 strain (Sanford S Elberg & K Faunce 1957). Therefore, if this mutation in 30S ribosomal protein S4 (BMEI1133) altered the protein function, then this mutation will be the real mutation which made Rev.1 as it is. This mutation (SNP 6) was confirmed by bidirectional PCR against the three *B. melitensis* biovars, and in Rev.1 by sequencing.

5.3.4 Other genes with unique nonsynonymous mutation

- Phage host specificity protein (BMEI1341)

A unique nonsynonamous SNP leads to premature stop codon (Q 225 stop codon) in this gene. This agrees with the work of Hernández et al 2004, who noticed change in phago typing pattern in Rev.1 compared to other *Brucella* species (Hernández et al. 2004).

- Isovaleryl-coa dehydrogenas (BMEI1923)

A unique non-synonamous point mutation which leads to (P263S). Using proteomic analysis, D'Haeze found that this enzyme was under-expressed in Rev.1 (D'Haeze 2002)

- Replication protein A (BMEII0093)

A nonsynonymous SNP leads to (R21G). According to Uniprot, this gene plays an important role in DNA repair mechanisms, initiation of replication and in gene regulation.

- Phosphoglycolate phosphatase (BMEI0975)

A nonsynonymous SNP leads to (S28T). This hydrolase enzyme plays a key role in DNA repair.

- Aspartyl/glutamyl-tRNA amidotransferase subunit B (BMEI1068)

A non-synonymous SNP leads to (T195I). According to Uniprot this protein allows the formation of correctly charged Asn-tRNA or Gln-tRNA.

5.3.5 Subcellular localization of mutated proteins

Using PSORT protein subcellular localization prediction tool[12], to predict the possible cellular location of the abovementioned affected proteins. The analysis of the prediction results showed that none of the affected proteins is located in the bacterial outer membrane. Interestingly, this finding indicates that the key attenuation changes seem to affect intracellular or inner membrane factors that might be important for internal homeostasis of the organism. The observation that none of the affected proteins is an outer membrane protein supports the fact that Rev.1 is still capable to induce a relatively light infection that can stimulate protective immunity but it persists for a shorter time in the host compared to the other virulent strains (Bosseray 1991).

[12] http://psort.hgc.jp/form.html

APPENDICES

Appendix 1: The accession numbers for the 93 Rev.1 contigs from NCBI. (Accessed, April 2010).

B. melitensis Rev.1 contigs	Size bp	B. melitensis Rev.1 contigs	Size bp	B. melitensis Rev.1 contigs	Size bp
ACEG0100001	1180	ACEG01000034	43696	ACEG01000067	931
ACEG0100002	671	ACEG01000035	1654	ACEG01000068	149469
ACEG0100003	1024	ACEG01000036	3246	ACEG01000069	35463
ACEG0100004	1687	ACEG01000037	1183	ACEG01000070	3872
ACEG0100005	1239	ACEG01000038	1235	ACEG01000071	3144
ACEG0100006	1566	ACEG01000039	631	ACEG01000072	2988
ACEG0100007	204775	ACEG01000040	130872	ACEG01000073	741
ACEG0100008	27920	ACEG01000041	118724	ACEG01000074	807
ACEG0100009	31459	ACEG01000042	3198	ACEG01000075	6271
ACEG01000010	12333	ACEG01000043	103606	ACEG01000076	1209
ACEG01000011	32075	ACEG01000044	5972	ACEG01000077	1353
ACEG01000012	31030	ACEG01000045	23632	ACEG01000078	806
ACEG01000013	2377	ACEG01000046	102826	ACEG01000079	2071
ACEG01000014	19999	ACEG01000047	6361	ACEG01000080	678
ACEG01000015	17442	ACEG01000048	33124	ACEG01000081	4984
ACEG01000016	9587	ACEG01000049	13779	ACEG01000082	2062
ACEG01000017	42527	ACEG01000050	37608	ACEG01000083	79680
ACEG01000018	87047	ACEG01000051	31450	ACEG01000084	2653
ACEG01000019	84825	ACEG01000052	27175	ACEG01000085	4261
ACEG01000020	2005	ACEG01000053	20987	ACEG01000086	6828
ACEG01000021	29755	ACEG01000054	62174	ACEG01000087	27978
ACEG01000022	166568	ACEG01000055	42301	ACEG01000088	208750
ACEG01000023	2602	ACEG01000056	12296	ACEG01000089	30163
ACEG01000024	57684	ACEG01000057	135150	ACEG01000090	15963
ACEG01000025	11553	ACEG01000058	16773	ACEG01000091	70135
ACEG01000026	20743	ACEG01000059	20120	ACEG01000092	139042
ACEG01000027	9490	ACEG01000060	93718	ACEG01000093	819
ACEG01000028	85005	ACEG01000061	131087	**Total Size BP**	**3270141**
ACEG01000029	49170	ACEG01000062	5034		
ACEG01000030	41899	ACEG01000063	67400		
ACEG01000031	8692	ACEG01000064	20415		
ACEG01000032	25404	ACEG01000065	25143		
ACEG01000033	1941	ACEG01000066	1176		

Appendix 2: The *B. melitensis* 36 strains draft sequences available at Broad Institute site

BruMel_16M_GB1 *
B. melitensis 16M
Brucella_melitensis_E8127
Brucella_melitensis_G6605
Brucella_melitensis_G8755
Brucella_melitensis_H1992
Brucella_melitensis_bv_1_G5013
Brucella_melitensis_bv_1_G6191
Brucella_melitensis_bv_1_H0866
Brucella_melitensis_bv_1_H0877
Brucella_melitensis_bv_1_str_63_143
Brucella_melitensis_bv_1_str_63_149
Brucella_melitensis_bv_1_str_63_33
Brucella_melitensis_bv_1_str_63_43
Brucella_melitensis_bv_1_str_63_61
Brucella_melitensis_bv_1_str_65_112
Brucella_melitensis_bv_1_str_65_16
Brucella_melitensis_bv_1_str_65_32
Brucella_melitensis_bv_1_str_65_56
Brucella_melitensis_bv_1_str_65_57
Brucella_melitensis_bv_1_str_65_61
Brucella_melitensis_bv_1_str_80_1
Brucella_melitensis_bv_1_str_80_4
Brucella_melitensis_bv_1_str_85_20
Brucella_melitensis_bv_1_str_90_1
Brucella_melitensis_bv_1_str_F1_01
Brucella_melitensis_bv_1_str_F3_05-371
Brucella_melitensis_bv_1_str_F3_05-372
Brucella_melitensis_bv_1_str_F3_99-548
Brucella_melitensis_bv_1_str_R36_03-60
Brucella_melitensis_bv_1_str_Rev.1
Brucella_melitensis_bv_3_G9248
Brucella_melitensis_bv_3_G9319
Brucella_melitensis_bv_3_G9481
Brucella_melitensis_bv_3_K26
Brucella_melitensis_bv_3_str _Ether
Brucella. melitensis bv. 2 str. 63/9

*This is the old sequence for *B. melitensis* 16M from Gene Bank (NCBI)

Appendix 3 and 4 The Results NCBI BLAST analyses of all Rev.1 genes against 16M to find all the variant genes (less than 100% in max identity) as follow:

Appendix 3: Rev.1 variant genes compared with B*. melitensis 16M* strain found in chromosome I (NC_003317) found by NCBI BLAST tool and analysis if the gene is from PHIDIAS- BBP virulent genes.

	Rev.1 Broad institute Gene Description	BLAST max identity	16M strain NCBI gene descreption	Virulent genes from PHDEAS	Mutations Description
1	BAMG_00069 |- conserved hypothetical protein (330 nt)	97%	Paert of BMEI1590		7 base deletion (ccccccc) INSIDE BME
2	BAMG_01200 |- conserved hypothetical protein (261 nt)	97%	BMEI0468		6 base subestitution ..far from each
3	BAMG_01024 |- cobalt transporter CbiM (636 nt)	98%	BMEI0639		8 base / del ins sub…OUT BME
4	BAMG_00005 |- urease subunit gamma (303 nt)	99%	BMEI1654		SNP del c after bmi
5	BAMG_00008 |- urease accessory protein ureE (489 nt)	99%	BMEI1651		SNP sub T to C BME
6	BAMG_00028 |- efflux transporter (1194 nt)	99%	BMEI1630		SNP G to A
7	BAMG_00029 |- hydrophobe/amphiphile efflux-1 family protein (3156 nt)	99%	BMEI1629		
8	BAMG_00035 |- histidine kinase (1317 nt)	99%	BMEI1624		SNP G del
9	BAMG_00040 |- 33K chaperonin protein (1023 nt)	99%	BMEI1619		SNP A to T
10	BAMG_00053 |- integral membrane sensor hybrid histidine kinase (3516 nt)	99%	BMEI1606	YES	8 base del sub ins
11	BAMG_00066 |- 3-methyl-2-oxobutanoate hydroxymethyltransferase (828 nt)	99%	BMEI1592		SNP G to A
12	BAMG_00079 |- TRAP dicarboxylate transporter (1575 nt)	99%	BMEI1580		SNP ins G
13	BAMG_00084 |- xanthine dehydrogenase small subunit (1557 nt)	99%	BMEI1576		3 base del GCG
14	BAMG_00096 |- Cbb3-type cytochrome oxidase component (159 nt)	99%	BMEI1566		
15	BAMG_00111 |- predicted protein (1317 nt)	99%	BMEI1549		SNP A to G
16	BAMG_00132 |- glycyl-tRNA synthetase subunit beta (2286 nt)	99%	BMEI1529		SNP C to T
17	BAMG_00142 |- phosphoribosylamine-glycine ligase (1284 nt)	99%	BMEI1519	YES	SNP G to A
18	BAMG_00157 |- histone deacetylase superfamily protein (951 nt)	99%	BMEI1504		SNP C to T
19	BAMG_00163 |- 1-deoxyxylulose-5-phosphate synthase (1932 nt)	99%	BMEI1498	YES	SNP G to A
20	BAMG_00168 |- peptidoglycan-binding LysM (1299 nt)	99%	BMEI1493		SNP A \to G
21	BAMG_00227 |- pyruvate phosphate dikinase (2682 nt)	99%	BMEI1436		SNP G to C

22	BAMG_00253 |- phosphomannomutase (1374 nt)	99%	BMEI1396	YES	SNP T to C
23	BAMG_00258 |- D-ribose transport system ATP-binding protein (447 nt)	99%	BMEI1392		2 base del 1447070
24	BAMG_00259 |- ABC transporter (1200 nt)	99%	BMEI1392		2 base del G C 1447077
25	BAMG_00272 |- predicted protein (291 nt)	99%	bart of BMEI1379		SNP T to C
26	BAMG_00313 |- conserved hypothetical protein (3864 nt)	99%	between BMEI1340 and BMEI1341		del g and sub (2 bases diff)
27	BAMG_00317 |- FeuQ (1389 nt)	99%	BMEI1336	YES	SNP C to T
28	BAMG_00318 |- LipA (408 nt)	99%	BMEI1335		SNP A to G
29	BAMG_00334 |- endonuclease/exonuclease/phosphatase (1077 nt)	99%	BMEI1319"		7 insert befor BME
30	BAMG_00338 |- glutathione S-transferase domain-containing protein (711 nt)	99%	BMEI1316		SNP C to T
31	BAMG_00346 |- phage integrase (927 nt)	99%	BMEI1307		SNP A to G
32	BAMG_00354 |- lytic transglycosylase catalytic (1629 nt)	99%	BMEI1302		SNP G to A
33	BAMG_00378 |- conserved hypothetical protein (765 nt)	99%	BMEI1280		SNP G to A
34	BAMG_00389 |- transporter DMT superfamily protein (948 nt)	99%	BMEI1269		3 base del CCC
35	BAMG_00398 |- leucyl aminopeptidase (1494 nt)	99%	BMEI1261"		2 sub 2 inser
36	BAMG_00429 |- L-lactate permease (1695 nt)	99%	BMEI1232		2 Base sub
37	BAMG_00436 |- predicted protein (420 nt)	99%	BMEI1225		SNP T to C
38	BAMG_00444 |- apeA (1374 nt)	99%	BMEI1217		SNP T to C
39	BAMG_00469 |- peptidoglycan binding domain-containing protein (1341 nt)	99%	BMEI1193		SNP OUT BME
40	BAMG_00470 |- serine hydroxymethyltransferase (1317 nt)	99%	BMEI1191		SNP ins G
41	BAMG_00476 |- membrane-bound proton-translocating pyrophosphatase (2157 nt)	99%	BMEI1185		SNP A to G
42	BAMG_00491 |- agmatinase (951 nt)	99%	BMEI1170		SNP A to G
43	BAMG_00509 |- NADH-quinone oxidoreductase (2085 nt)	99%	BMEI1152		flep CG toGC & sub c to G
44	BAMG_00510 |- NADH-quinone oxidoreductase subunit H (1044 nt)	99%	BMEI1151		2 base del inser
45	BAMG_00528 |- SSU ribosomal protein S4P (618 nt)	99%	BMEI1133		SNP C to T
46	BAMG_00535 |- phosphoribosylformylglycinamidine synthase II (2223 nt)	99%	BMEI1127	YES	SNP T to C
47	BAMG_00542 |- HpcH/HpaI aldolase (762 nt)	99%	BMEI1120		SNP G to A
48	BAMG_00564 |- nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase (1020 nt)	99%	BMEI1099		SNP A tto T

49	BAMG_00596 |- aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B (1503 nt)	99%	BMEI1068"		SNP G to A
50	BAMG_00604 |- DSBA oxidoreductase (798 nt)	99%	BMEI1060		SNP G to A
51	BAMG_00614 |- alkyl hydroperoxide reductase (465 nt)	99%	BMEI1049		SNP C to A
52	BAMG_00617 |- anhydro-N-acetylmuramic acid kinase (1122 nt)	99%	BMEI1046		SNP C to G
53	BAMG_00637 |- valyl-tRNA synthetase (1425 nt)	99%	BMEI1027		SNP T to C
54	BAMG_00654 |- conserved hypothetical protein (1026 nt)	99%	BMEI1012		SNP T to C
55	BAMG_00661 |- predicted protein (171 nt)	99%	BMEI1006		SNP C to A
56	BAMG_00665 |- conserved hypothetical protein (1452 nt)	99%	BMEI0999		SNP G to A
57	BAMG_00672 |- arsenate reductase (480 nt)	99%	BMEI0992		SNP A to C
58	BAMG_00689 |- phosphoglycolate phosphatase (705 nt)	99%	BMEI0975		SNP G to C
59	BAMG_00697 |- praline-rich extensin (753 nt)	99%	NO BME in this locas The Beginning of BMEI0968		4 base del
60	BAMG_00698 |- malic enzyme (2325 nt)	99%	BMEI0967		SNP
61	BAMG_00708 |- conserved hypothetical protein (621 nt)	99%	BMEI0957		SNP C to A
62	BAMG_00718 |- multi-sensor hybrid histidine kinase (2676 nt)	99%			SNP G to C and ins T after BME
63	BAMG_00746 |- nucleoside triphosphate pyrophosphohydrolase (825 nt)	99%	BMEI0920		SNP C to G
64	BAMG_00757 |- site-specific recombinase (1308 nt)	99%	in the end there is BMEI0907		SNP out BME
65	BAMG_00768 |- efflux transporter (1182 nt)	99%	BMEI0892		2 inse & 2 del
66	BAMG_00786 |- ATP-dependent protease La (2439 nt)	99%	BMEI0876		SNP G to A
67	BAMG_00817 |- rotamase (1887 nt)	99%	BMEI0845	YES	SNP G to A
68	BAMG_00819 |- indole-3-glycerol phosphate synthase (807 nt)	99%	BMEI0843		SNP C to T
69	BAMG_00839 |- 30S ribosomal protein S2 (771 nt)	99%	BMEI0823		2 Base sub
70	BAMG_00893 |- 50S ribosomal protein L6 (534 nt)	99%	BMEI0772		SNP G to A
71	BAMG_00894 |- 30S ribosomal protein S8 (399 nt)	99%	BMEI0771		SNP T to C
72	BAMG_00897 |- 50S ribosomal protein L24 (312 nt)	99%	BMEI0768		SNP T to C
73	BAMG_00907 |- ribosomal protein L4 (621 nt)	99%	BMEI0758		SNP C del
74	BAMG_00911 |- 30S ribosomal protein S7 (471 nt)	99%	BMEI0753		SNP C to A
75	BAMG_00912 |- 30S ribosomal protein S12 (372 nt)	99%	BMEI0752	YES	SNP C to T

76	BAMG_00927 |- transglutaminase cysteine peptidase BTLCP (606 nt)	99%	BMEI0737"		SNP T to C
77	BAMG_00939 |- fructose-1,6-bisphosphatase (987 nt)	99%	BMEI0726		SNP A to G
78	BAMG_00953 |- precorrin-3B C17-methyltransferase (1707 nt)	99%	BMEI0712		SNP G to C
79	BAMG_00958 |- cobalamin biosynthesis protein cobD (984 nt)	99%	BMEI0707		SNP T to C
80	BAMG_00982 |- transcriptional regulator (999 nt)	99%	BMEI0685		2 base G to c &G to A
81	BAMG_00983 |- FAD dependent oxidoreductase (1509 nt)	99%	part of BMEI0684		SNP A del
82	BAMG_00984 |- FAD dependent oxidoreductase (1158 nt)	99%	BMEI0684		SNP A ins
83	BAMG_01011 |- ABC transporter ATP binding/permease (1995 nt)	99%	BMEI0654		SNP G del
84	BAMG_01021 |- urease accessory protein UreH (909 nt)	99%	BMEI0643		SNP T- G
85	BAMG_01030 |- conserved hypothetical protein (1027 nt)	99%	BMEI0632		2 BASE T ins & T to C
86	BAMG_01033 |- NUDIX hydrolase (732 nt)	99%	BMEI0629		SNP C del
87	BAMG_01066 |- conserved hypothetical protein (510 nt)	99%	BMEI0598		SNP G to A
88	BAMG_01073 |- peptidase M24 (1827 nt)	99%	BMEI0591		SNP G to T
89	BAMG_01080 |- cell division protein FtsZ (1701 nt)	99%	BMEI0585		2 del 1 inser
90	BAMG_01091 |- UDP-N-acetylmuramoyl-L-alanyl-D-glutamate-2,6-diaminopimelate ligase (1473 nt)	99%	BMEI0574		SNP G to A
91	BAMG_01103 |- lytic Murein transglycosylase (1233 nt)	99%	BMEI0561		SNP G del
92	BAMG_01105 |- 5,10-methylenetetrahydrofolate reductase (912 nt)	99%	BMEI0559		SNP OUT BME
93	BAMG_01115 |- Rieske domain-containing protein (1248 nt)	99%	BMEI0549		2 base c to g & T to C
94	BAMG_01119 |- conserved hypothetical protein (1062 nt)	99%	BMEI0545	YES	SNP A to C
95	BAMG_01129 |- integrase/recombinase (758 nt)	99%	BMEI0537 & BMEIt15		two BME OUT SNP T to C between
96	BAMG_01136 |- Na+ cotransporter (1677 nt)	99%	BMEI0528		SNP OUT BME
97	BAMG_01210 |- conserved hypothetical protein (2070 nt)	99%	BMEI0457		SNP C del
98	BAMG_01214 |- NUDIX hydrolase (483 nt)	99%	BMEI0453		SNP C to T
99	BAMG_01216 |- 2-isopropylmalate synthase (1728 nt)	99%	BMEI0451	YES	SNP G to T
100	BAMG_01224 |- major facilitator transporter (1293 nt)	99%	BMEI0445		C del
101	BAMG_01227 |- predicted protein (237 nt)	99%	BMEI0442		SNP G to C
102	BAMG_01233 |- binding-protein-dependent transport system inner membrane component (909 nt)	99%	BMEI0436		SNP T to G
103	BAMG_02404 |- methyltransferase (894 nt)	99%	BMEI0182		SNP T to C

104	BAMG_02421 |- acyl-CoA thioesterase II (903 nt)	99%	BMEI0166		SNP C to T
105	BAMG_02426 |- succinate dehydrogenase hydrophobic membrane anchor (396 nt)	99%	BMEI0160		SNP T to C
106	BAMG_02436 |- transcriptional regulator (666 nt)	99%	BMEI0150		
107	BAMG_02441 |- dihydrolipoamide dehydrogenase (1404 nt)	99%	BMEI0150		
108	BAMG_02443 |- threonine efflux protein (639 nt)	99%	BMEI0143		SNP A -T
109	BAMG_02445 |- dihydrolipoamide succinyltransferase (1227 nt)	99%	BMEI0141		SNP C-T
110	BAMG_02449 |- malate dehydrogenase (963 nt)	99%	BMEI0137		SNP G –C
111	BAMG_02457 |- intracellular septation protein A (663 nt)	99%	BMEI0130		3 base sub
112	BAMG_02465 |- PpiC-type peptidyl-prolyl cis-trans isomerase (996 nt)	99%	BMEI0123		SNP C –G
113	BAMG_02466 |- protein translocase subunit secA (2721 nt)	99%	BMEI0121		SNP C-T
114	BAMG_02475 |- aspartate ammonia-lyase (864 nt)	99%	BMEI0109		SNP G- A
115	BAMG_02480 |- major facilitator family transporter (1338 nt)	99%	BMEI0104		del T
116	BAMG_02489 |- electron transfer flavoprotein alpha/beta-subunit (747 nt)	99%	BMEI0096		SNP C – T
117	BAMG_02490 |- 6-pyruvoyl tetrahydrobiopterin synthase (312 nt)	99%	inside this BMEI0094		G del
118	BAMG_02491 |- radical SAM domain-containing protein (756 nt)	99%	BMEI0093		SNP T – C
119	BAMG_02492 |- ATP-cobalamin adenosyltransferase (588 nt)	99%	in side BMEI0092		2 base sub & del
120	BAMG_02500 |- diaminopimelate decarboxylase (1266 nt)	99%	BMEI0084	YES	2 base
121	BAMG_02503 |- methyltransferase (780 nt)	99%	BMEI0081		ins C
122	BAMG_02527 |- predicted protein (381 nt)	99%	bart of BMEI0061		SNP t to c
123	BAMG_02553 |- amidohydrolase (1164 nt)	99%	BMEI0033		SNP G –A
124	BAMG_02574 |- chromosome segregation DNA-binding protein (882 nt)	99%	BMEI0010		SNP A to G
125	BAMG_02576 |- ribosomal RNA small subunit methyltransferase G (642 nt)	99%	BMEI0008		SNP C –G
126	BAMG_02579 |- tRNA modification GTPase mnmE (1329 nt)	99%	BMEI0006		SNP T – C
127	BAMG_02593 |- smr family protein (603 nt)	99%	BMEI2051		OUT of bme
128	BAMG_02597 |- ATP-dependent protease hslV (555 nt)	99%	BMEI2047		SNP A to G
129	BAMG_02599 |- conserved hypothetical protein (510 nt)	99%	BMEI2044		SNP C to T
130	BAMG_02619 |- double-strand break repair helicase AddA (3543 nt)	99%	BMEI2023		3 base
131	BAMG_02625 |- tryptophan synthase subunit beta (1269 nt)	99%	BMEI2018		SNP T –G

132	BAMG_02626 |- N-(5'-phosphoribosyl)anthranilate isomerase (669 nt)	99%	BMEI2017		SNP T – C
133	BAMG_02630 |- benzoate transporter (1152 nt)	99%	BMEI2012		SNP G to A
134	BAMG_02631 |- conserved hypothetical protein (792 nt)	99%	BMEI2011		SNP G to A
135	BAMG_02638 |- phenylalanyl-tRNA synthetase subunit beta (2415 nt)	99%	BMEI2004		3 base
136	BAMG_02639 |- oxidoreductase domain-containing protein (987 nt)	99%	BMEI2003		SNP t to c
137	BAMG_02640 |- hsp70-like protein (1914 nt)	99%	BMEI2002	YES	SNP T to G
138	BAMG_02654 |- phosphate ABC transporter (1311 nt)	99%	BMEI1987		SNP C –A
139	BAMG_02661 |- peptidase M29 aminopeptidase II (1254 nt)	99%	BMEI1981		3 BASE
140	BAMG_02667 |- PhoH family protein (1062 nt)	99%	BMEI1975		DEL C
141	BAMG_02670 |- apolipoprotein N-acyltransferase (1599 nt)	99%	BMEI1972		SNP T– C
142	BAMG_02672 |- methionine adenosyltransferase (1266 nt)	99%	BMEI1970		4 BASE SUB
143	BAMG_02684 |- beta-ketoacyl synthase (1224 nt)	99%	BMEI1957		SNP G – C
144	BAMG_02696 |- enoyl-CoA hydratase/isomerase (774 nt)	99%	BMEI1945		SNP A –G
145	BAMG_02711 |- extracellular ligand-binding receptor (1107 nt)	99%	BMEI1930		SNP C – T
146	BAMG_02717 |- acyl-CoA dehydrogenase domain-containing protein (1179 nt)	99%	BMEI1923		SNP C – T
147	BAMG_02732 |- NUDIX hydrolase (948 nt)	99%	Inside BME		3 Base
148	BAMG_02755 |- phosphoglucomutase (1632 nt)	99%	BMEI1886	YES	SNP A – G
149	BAMG_02764 |- lytic transglycosylase catalytic (864 nt)	99%	BMEI1878		C del
150	BAMG_02784 |- conserved hypothetical protein (2550 nt)	99%	BMEI1860		5 Base: 3 del &2 sub
151	BAMG_02795 |- dihydroxy-acid dehydratase (1836 nt)	99%	BMEI1848	YES	SNP T – C
152	BAMG_02796 |- conserved hypothetical protein (459 nt)	99%	BMEI1847		SNP A – G
153	BAMG_02807 |- cyclic beta 1-2 glucan synthase (8604 nt)	99%	BMEI1837	YES	8 Base
154	BAMG_02824 |- conserved hypothetical protein (657 nt)	99%	BMEI1821		SNP T – C
155	BAMG_02828 |- ATP-dependent helicase HrpB (2497 nt)	99%	BMEI1817		SNP T – C
156	BAMG_02829 |- integral membrane sensor signal transduction histidine kinase (1287 nt)	99%	IN SIDE THIS BMEI1816		SNP C –G
157	BAMG_02853 |- predicted protein (369 nt)	99%	BMEI1788		SNP A - G
158	BAMG_02882 |- methionine synthase (3786 nt)	99%	BMEI1759	YES	2 BASE
159	BAMG_02904 |- thiamine-phosphate pyrophosphorylase (612 nt)	99%	BMEI1736		SNP T - C

160	BAMG_02926 |- binding-protein-dependent transport system inner membrane component (924 nt)	99%	BMEI1715		SNP T - A
161	BAMG_02933 |- fumarylacetoacetate hydrolase (846 nt)	99%	BMEI1708		SNP G - C
162	BAMG_02934 |- mandelate racemase/muconate lactonizing protein (1278 nt)	99%	BMEI1707		SNP A – G
163	BAMG_02944 |- conserved hypothetical protein (1530 nt)	99%	BMEI1696		SNP A-G
164	BAMG_02967 |- TIR protein (828 nt)	99%	BMEI1674		SNP A - G
165	BAMG_02989 |- N-acetyltransferase (495 nt)	99%	BMEI0379		SNP T - C
166	BAMG_03006 |- ExbB (960 nt)	99%	BMEI0365		SNP G - C
167	BAMG_03033 |- TolR protein (453 nt)	99%	BMEI0337		SNP G - T
168	BAMG_03082 |- ABC transporter (1818 nt)	99%	BMEI0288		SNP A- C
169	BAMG_03098 |- helicase domain-containing protein (3087 nt)	99%	BMEI0275	YES	4 BASE
170	BAMG_03099 |- acetyl-CoA acetyltransferase (1185 nt)	99%	INSIDE BMEI0274		2 BASES
171	BAMG_03108 |- pyruvate carboxylase (3477 nt)	99%	BMEI0266	YES	SNP C - T
172	BAMG_03115 |- inner-membrane translocator (1380 nt)	99%	BMEI0259		2 BASE T-C \ G-C
173	BAMG_03128 |- primosome assembly protein PriA (2235 nt)	99%	BMEI0245		C DEL
174	BAMG_03147 |- conserved hypothetical protein (837 nt)	99%	BMEI0229		SNP T-C
175	BAMG_03151 |- chorismate mutase (315 nt)	99%	BMEI0226		C DEL
176	BAMG_03170 |- gamma-glutamyl phosphate reductase (1281 nt)	99%	BMEI0208		SNP G - A
177	BAMG_03189 |- glutamine ABC transporter (459 nt)	99%	BMEI0111		SNP T - C
178	BAMG_03198 |- modification methylase (888 nt)	99%	BMEI0192		SNP A -C

Appendix 4: Rev.1 variant genes compared with B*. melitensis 16M* genome found in chromosome II (NC_003318) using NCBI BLAST tool and analysis if the gene is from PHIDIAS-BBP virulent genes.

	Rev.1 Broad institute Gene Description	BLAST max identity	16M strain NCBI gene descreption	Virulent genes from PHDEAS	Mutations Description
1	BAMG_01378 | RNA polymerase (525 nt)	97%	inside BMEII0072		13 Base
2	BAMG_01288 |flagellar biosynthesis protein FliR (751 nt)	97%	BMEII0168		17 BUTIFUL BASES :)
3	BAMG_01418 | type IV secretion system protein virB10 (1161 nt)	98%	BMEII0034	Yes	18 BASE DEL OUT BME
4	BAMG_02049 | HlyD family secretion protein (867 nt)	98%	BMEII0533		9 BASE DEL
5	BAMG_01306 | predicted protein (8277 nt)	99%	BMEII0148		T- C

6	BAMG_01318 | 5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase (1515 nt)	99%	BMEII0135		A-C
7	BAMG_01323 |aminotransferase (1359 nt)	99%	BMEII0130		A-C
8	BAMG_01325 | transcription regulator (1257 nt)	99%	BMEII0128	Yes	3 BASE INS DEL
9	BAMG_01327 | predicted protein (1494 nt)	99%	BMEII0126		7 BASE …
10	BAMG_01328 | aldehyde dehydrogenase (1479 nt)	99%	BMEII0124		5 BASE INSID BME AND 10 AFER
11	BAMG_01340 | conserved hypothetical protein (1173 nt)	99%	BMEII0112		C - T
12	BAMG_01354 | heavy metal translocating P-type ATPase (1941 nt)	99%	BMEII0097		T - C
13	BAMG_01355 |- coproporphyrinogen III oxidase (1353 nt)	99%	BMEII0096		T -C
14	BAMG_01357 |- cobyrinic acid ac-diamide synthase (1194 nt)	99%	BMEII0093		G -C
15	BAMG_01358 |- conserved hypothetical protein (987 nt)	99%	BMEII0092		2 BASE DEL INS
16	BAMG_01359 |- replication protein C (1194 nt)	99%	BMEII0091		T DEL
17	BAMG_01372 |- AMP-ligase (1764 nt)	99%	BMEII0078	YES	2 BASES
18	BAMG_01379 |- conserved hypothetical protein (651 nt)	99%	BMEII0072		A -C
19	BAMG_01419 |- type IV secretion system protein virB9 (870 nt)	99%	BMEII0033	YES	C - G & A DEL OUT BME
20	BAMG_01436 |- homospermidine synthase (1446 nt)	99%	BMEII0015		A del
21	BAMG_01440 |- sigma-54 dependent DNA-binding response regulator (1491 nt)	99%	BMEII0011		2 base ins ^del
22	BAMG_01462 |- mannosyl transferase (1192 nt)	99%	BMEII1129		A ins
23	BAMG_01467 |- 6-phosphogluconate dehydrogenase (1020 nt)	99%	BMEII1124	YES	A - C
24	BAMG_01486 |- flagellum-specific ATP synthase (1362 nt)	99%	BMEII1105		A INS OUT OF BME
25	BAMG_01527 |- GntR domain-containing protein (813 nt)	99%	BMEII1066	YES	T -C
26	BAMG_01537 |- ATP phosphoribosyltransferase (696 nt)	99%	BMEII1054		2 BASE G-C\ A-C
27	BAMG_01538 |- glucose/galactose transporter (1239 nt)	99%	BMEII1053	YES	C - G
28	BAMG_01543 |- hsp60-like protein (1641 nt)	99%	BMEII1048		2 BASE G -C\ T- C
29	BAMG_01548 |- riboflavin biosynthesis protein RibF (873 nt)	99%	BMEII1044		G - A
30	BAMG_01559 |- PmbA (1371 nt)	99%	BMEII1033		2 BASE FLIP GC -CG
31	BAMG_01560 |- inositol monophosphatase (810 nt)	99%	BMEII1032		T -C
32	BAMG_01567 |- major facilitator superfamily transporter cis,cis-muconate transporter (1239 nt)	99%	BMEII1024		3 BASE SUB

33	BAMG_01579 |- oxidoreductase FAD/NAD(P)-binding subunit (2205 nt)	99%	BMEII1011		3 BASE INS
34	BAMG_01597 |- conserved hypothetical protein (1368 nt)	99%	BMEII0994		C - T
35	BAMG_01611 |- FGGY-family pentulose kinase (1341 nt)	99%	BMEII0979		A - G
36	BAMG_01627 |- ABC transporter periplasmic binding protein (1041 nt)	99%	BMEII0963		C DEL
37	BAMG_01641 |- nitrate reductase (3765 nt)	99%	BMEII0949		2 DEL
38	BAMG_01643 |- transcriptional activator FtrB (711 nt)	99%	BMEII0947		A - T
39	BAMG_01644 |- alanine racemase (789 nt)	99%	BMEII0946		2 BASE
40	BAMG_01649 |- trehalosemaltose transporter ATP-binding protein (846 nt)	99%	BMEII0940		G - A
41	BAMG_01666 |- binding-protein-dependent transport system inner membrane component (1137 nt)	99%	inside BMEII0921		C DEL
42	BAMG_01672 |- hydrophobe/amphiphile efflux-1 family protein (3225 nt)	99%	BMEII0915		2 BASE IN & ONE OUT BME
43	BAMG_01681 |- EAL domain-containing protein (1785 nt)	99%	BMEII0904		A DEL
44	BAMG_01697 |- GMP synthase (1563 nt)	99%	BMEII0887		C DEL
45	BAMG_01701 |- conserved hypothetical protein (1434 nt)	99%	BMEII0882		6 BASES
46	BAMG_01705 |- cytochrome P450 (1164 nt)	99%	BMEII0879		C -T
47	BAMG_01707 |- zinc-containing alcohol dehydrogenase superfamily protein (1008 nt)	99%	BMEII0876		C - T
48	BAMG_01709 |- branched-chain amino acid ABC transporter (2724 nt)	99%	BMEII0874		T - C
49	BAMG_01714 |- succinate-semialdehyde dehydrogenase (1452 nt)	99%	BMEII0869		A - C
50	BAMG_01722 |- oligopeptide ABC transporter (873 nt)	99%	BMEII0861		T -C
51	BAMG_01737 |- Bme6 (1125 nt)	99%	BMEII0845		A - C
52	BAMG_01743 | Bme31 (1122 nt)	99%	BMEII0839		A - C
53	BAMG_01753 | Bme21 (1302 nt)	99%	BMEII0828		C INS OUT
54	BAMG_01765 |- acetyl-coenzyme A synthetase (1670 nt)	99%	BMEII0815		2 BASE C INS
55	BAMG_01778 |- secretion protein HlyD family protein (945 nt)	99%	BMEII0803		T - C
56	BAMG_01800 |- glycoside hydrolase (888 nt)	99%	BMEII0782		G - A
57	BAMG_01806 |- 8-amino-7-oxononanoate synthase (1137 nt)	99%	BMEII0776		C -A
58	BAMG_01815 |- NADH-ubiquinone/plastoquinone oxidoreductase (1629 nt)	99%	BMEII0768		2 BASE
59	BAMG_01816 |- cation antiporter (516 nt)	99%	BMEII0767		T - G

60	BAMG_01822 |- conserved hypothetical protein (1683 nt)	99%	BMEII0761	YES	G - C
61	BAMG_01824 |- cytochrome d oxidase subunit (1155 nt)	99%	BMEII0759	YES	3 BASE OUT BME
62	BAMG_01832 |- oxidoreductase domain-containing protein (1071 nt)	99%	BMEII0751		C - G
63	BAMG_01842 |- cell divisionFtsK/SpoIIIE (2466 nt)	99%	BMEII0742		G -C
64	BAMG_01865 |- transposase (1119 nt)	99%	BMEII0713		2 BASE
65	BAMG_01874 |- bacterioferritin (486 nt)	99%	BMEII0704		2 BASE
66	BAMG_01881 |- phosphatidylcholine synthase (801 nt)	99%	BMEII0695		C - T
67	BAMG_01888 |- transcription-repair coupling factor (3513 nt)	99%	BMEII0688		G - A
68	BAMG_01916 |- virulence-associated protein (2352 nt)	99%	BMEII0665		C -G
69	BAMG_01932 |- DNA polymerase III (702 nt)	99%	BMEII0650		C - T
70	BAMG_01950 |- inner-membrane translocator (987 nt)	99%	BMEII0631		G INS & 2 BASE OUT BME
71	BAMG_01957 |- sn-glycerol-3-phosphate transport system permease ugpE (848 nt)	99%	BMEII0622		T - G
72	BAMG_01961 |- xanthine/uracil/vitamin C permease (1296 nt)	99%	BMEII0618		2 BASE IN & 3 OUT BME
73	BAMG_01969 |- conserved hypothetical protein (1656 nt)	99%	BMEII0610		A - T
74	BAMG_01981 |- ppx/GppA phosphatase (1497 nt)	99%	BMEII0598		4 BASE
75	BAMG_01994 |- binding-protein-dependent transport system inner membrane component (1719 nt)	99%	BMEII0585		C DEL
76	BAMG_02009 |- thiamine pyrophosphate protein (1824 nt)	99%	BMEII0571		G - C
77	BAMG_02014 |- binding-protein-dependent transport system inner membrane component (2226 nt)	99%	BMEII0566		2 BASE IN &3 OUT BME
78	BAMG_02039 |- short-chain dehydrogenase/reductase SDR (774 nt)	99%	BMEII0543		T - C
79	BAMG_02042 |- translation initiation inhibitor (372 nt)	99%	BMEII0540		A - T
80	BAMG_02043 |- conserved hypothetical protein (1470 nt)	99%	BMEII0539		C - T
81	BAMG_02066 |- branched-chain amino acid transport (300 nt)	99%	BMEII0516		A - G
82	BAMG_02082 |- lysyl-tRNA synthetase (1656 nt)	99%	BMEII0500		2 BASE
83	BAMG_02085 |- 3-hydroxyacyl-CoA dehydrogenase (2217 nt)	99%	BMEII0497		G - A
84	BAMG_02094 |- nickel transporter permease NikC (855 nt)	99%	BMEII0489		3 BASE
85	BAMG_02095 |- nickel transporter permease NikB (945 nt)	99%	BMEII0488		3 BASE OUT BME
86	BAMG_02102 |- ABC transporter (1182 nt)	99%	BMEII0479		C - G

87	BAMG_02133 |- type I restriction modification enzyme (2970 nt)	99%	BMEII0449		C DEL
88	BAMG_02144 |- UbiA prenyltransferase (1440 nt)	99%	BMEII0437		2 BASE
89	BAMG_02147 |- periplasmic binding protein/LacI transcriptional regulator (948 nt)	99%	BMEII0435		G INS
90	BAMG_02150 |- ribose ABC transporter (1545 nt)	99%	BMEII0432		A - G
91	BAMG_02155 |- erythritol phosphate dehydrogenase (1509 nt)	99%	BMEII0429	YES	G - C
92	BAMG_02161 |- fructose-bisphosphate aldolase (1065 nt)	99%	BMEII0423		C =G
93	BAMG_02193 |- RNA-binding S1 domain-containing protein (2142 nt)	99%	BMEII0392		A - G
94	BAMG_02203 |- acriflavin resistance protein (3075 nt)	99%	BMEII0382		A \-C
95	BAMG_02239 |- aminotransferase class-III (3074 nt)	99%	BMEII0348		C - T
96	BAMG_02247 |- inner-membrane translocator (996 nt)	99%	BMEII0340		3 BASE
97	BAMG_02256 |- conserved hypothetical protein (1196 nt)	99%	BMEII0329		ONE BASE OUT
98	BAMG_02274 |- WD-repeat family protein (975 nt)	99%	BMEII0307		T - G
99	BAMG_02282 |- sugar ABC transporter (1533 nt)	99%	BMEII0300	YES	4 BASE
100	BAMG_02283 |- sugar ABC transporter (1131 nt)	99%	Bart of BMEII0300		7 BASE
101	BAMG_02290 |- conserved hypothetical protein (1302 nt)	99%	BMEII0293		T - C & 3 BASE OUT BME
102	BAMG_02294 |- glutamyl-tRNA amidotransferase subunit A (1390 nt)	99%	BMEII0289		G INS
103	BAMG_02296 |- oligopeptide/dipeptide ABC transporter (1026 nt)	99%	BMEII0287		T - C
104	BAMG_02299 |- extracellular solute-binding protein (1479 nt)	99%	BMEII0284		2 bases
105	BAMG_02310 |- inner membrane protein oxaA (1833 nt)	99%	BMEII0275		A - C
106	BAMG_02317 |- succinyl-diaminopimelate desuccinylase (1188 nt)	99%	BMEII0268		C - T
107	BAMG_02366 |- oligopeptide/dipeptide ABC transporter (978 nt)	99%	BMEII0199		C - T
108	BAMG_02391 |- transcriptional regulatory protein (732 nt)	99%	BMEII0226		C - T
109	BAMG_01298 |- lytic transglycosylase catalytic (573 nt)	99%	BMEII0157		A - T

Appendix 5: unique mutations full details and primers for Rev.1

NOTES: - All Gene descriptions and Sequences acquired from NCBI for the [*Brucella* melitensis bv. 1 str. 16M] as the Wild type (Wt).

- The PCR conditions are for singleplex PCR.

SNP 1

Gene description	trmE tRNA modification GTPase TrmE			
Gene locus_tag	BMEI0006	**Gene ID**	1195717	
NCBI Accession	NC_003317.1	**Gene location**	5526..6854	
SNP location	6722	**SNP location at gene (Nucleotide coordinate)**	1197	
Mutation Type	Synonymous	**SNP sequence**	**Rev.1**	C
			Wt	T

SNP 1 Primers

Name	**Sequence**	**TM** °C	**Primer coordinates on Gene Bank Acc AE008917.1**	**Product Size bp**
Rev 0006 F	GTTCTGTTTCCATGCAGGTC	51.3	6088 6107	656
Rev 0006 R	TAACTCCAATGGAAGATCGTCG	52.1	6743 6722	
16M 0006 F	ATCGAAAAAGCAATTGAGGGT	50.5	6702 6722	428
16M0006 R	CACCACCAATTACAATGACATC	49.9	7129 7108	

Gene Sequence SNP 1

> gi|17986284:5526-6854 *Brucella* melitensis bv. 1 str. 16M chromosome I, complete sequence

ATGAGCGAGATCGGTTCTTATCACGATACGATTTTTGCATTATCGAGCGGACGCTTGCCCTCAGGGGTCG
CGGTCATTCGCATTTCCGGCCCAAAAACTCGATTCGTTTACGAAACAATCTGCCAAGCAATTCCGGAACC
GCGCCATGCCGCATTGCTTACATTCCGTAGCCGAAATGGTGACGCCATTGATCGCGGCCTCACTCTGTTT
TTCCCTGCTCCTCATAGTTTTACTGGGGAAGACTGCGCCGAATTTCATTTGCACGGCGGTAAGGCCGTTG
TGGAAAAGATGCTTGCGGTGTTGGGCGAACTGCCGGGATGCCGAATTGCGGAGGCTGGTGAATTTACGCG
ACGTGCTTTTGCCAATGGCAAGATGGACTTGACGATTGCAGAAGGTCTGGCCGACCTCATTGCTGCGGAA
ACCGAGGGGCAGCGACGGCTTGCAATGCAAGTGGCTTCCGGTAATCAGCGCAAACTATATAGTGAGTGGC
GCCAGCGACTTATCAATGCGCGGGCTTTCATTGAAGCTGAACTGGATTTTGCCGATGAAAGCGACGTACC
CGGTTCTGTTTCCATGCAGGTCTGGCAACAGCTTTCGGCGCTCAAACATGAAATCGAGCACCATATCGCA
AGCGGTAAGCGCGCTGCGATGCTGCGGGATGGATTGCATGTTGTTATTGTCGGCGCGCCAAATGCCGGTA
AATCCAGCTTGCTTAATTTTCTAGCCGGGCGCGATGTTGCGATCATTTCCGAAGAAGCTGGTACGACGCG
CGATCTTCTGGAAGTGAAACTCGATCTCGGCGGTATCCCGGTTTATGTCACCGATACGGCGGGTCTTCGC
GAAACGGATAGTGTCGTTGAGAAGATCGGCATTGAGCGTGCGCGTGCCCGGATGGCCGAAGCCGATCTGG
TGCTCTCGCTGGAAGATATGAGCGGACCTGTATCTGTTACCGTTGAGAAAATCGAAGCAGAAACCTGGCT
GATCGGTACCAAGGCCGACCTCGGTGGAAGTGCTTCGGGCTTGTGGAAATATCACATTTCCACCATGACT
GGCAGTGGTCTGGAACAATTGCTGGACGCTCTACAGGCTTTTGCTGAAGCGAAGATCGGCCAGATTGAAG
ATGCTGTTCCTACCCGGCAGCGCCATATCAACCTGTTGCGGGCAACGATTGAAGAAATCGAAAAAGCAAT
TGAGGG **T** GACGATCTTCCATTGGAGTTAAGAGCCGAGAATATGCGGCTTGCTTCGCAATTCCTTGGACGG
ATCACGGGCGATGTGGATGTCGAGGAAATTCTGGACGTTATTTTCTCGCAATTCTGCATCGGGAAGTGA

PCR SNP 1 conditions and notes			
Primer concentration 5 picomole/ µl we add 1 µl to 25 µl reaction			
PCR Program	Tem °C	Time/ min	
Initial Denaturation	94	04:00	
Cycles	95	00:45	
	61.5	00:35	
	72	01:15	
Number of cycles	27 X		
Final extension	72	04:00	

SNP 2

Gene description	oligopeptide transport ATP-binding protein OPPF			
Gene locus_tag	BMEII0199	**Gene ID**	1197970	
NCBI Accession	NC_003318.1	**Gene location**	210614..211720	
SNP location	211033	**SNP location at gene (Nucleotide coordinate)**	688	
Mutation type	Nonsynonymous	**SNP sequence**	**Rev.1**	A
			Wt	G

SNP 2 Primers

Primer Name	**Sequence**	**TM** °C	**Primer coordinates on Gene Bank Acc AE008918.1**	**Size bp**
Rev 0199F	TTTGAAATATCGGGAGGCAT	48.9	212136 212117	
Rev0199 R	CATGAGGTTCAATATCTGAGT	46.9	211013 211033	1124
16M0199 F	CTGGACCCTTCCATTCAGG	51.2	211051 211033	
16M0199 R	TATGCCGCCATTATTCGCTA	51.4	210390 210409	662

The gene sequence : SNP 2

>gi|17988344:c211720-210614 *Brucella* melitensis bv. 1 str. 16M chromosome II, complete sequence

TTGCCCATCCTCGGGCGCCCTGTGTCCGACCCATTCACCGGATTTCATCCAACTTTTCTCGTCGGGACAG
GCCACCGCTTGTCATTACCCAGCAATCTCTGCGGACAGATTGCAACAGGAGCAGCTTTTATGACAGTCTC
GCTATTACAGGTTCAGGACCTGGTCAAAAGCTTTCCCTCTCGCCACGGGACGCATCCCGTGCGTGCTGTT
GACGGTGTATCGTTCACTCTTTCGCGGGGAGAAACACTCGGTCTCGTCGGTGAATCCGGGTGTGGAAAAT
CCACTTTGGCAAGGCTGGTGCTGAAGCTCATGCCGGTTTCCGATGGAAGCATTTCGGTCGAAGGGCACGA
TGTGACGTCCATGCCCGATTCAATTTTCCGAGACTGGCGGAAACGTATCCAGATGATTTTCCAGGATCCC
TATTCCACACTCAACCCGCGTCATTCGCTTCGGCGGATATTCCAGGAAGTCTTCGAAACACATTGCATTC
CGACCCCGGAAGGTATCGAGAACCGCATCATGAAGCTCCTGGCAGATGTGGGTCTGGGAGAAGTGGATTT
GTCCAAATTGCCACATGAATTTTCTGGCGGCCAACTTCAACGCATCGGGATTGCCAAGACACTCGCCCTC
GATCCCGATCTTATCGTTGCAGACGAACCCACTTCTGCGCTGGACCCTTCCATTCAGGCTCAGATATTGA
ACCTCATGCTTAAAATCAGGCGCGAGCGTGGAGTGGCTTTTCTGCTAATTTCACACGATTTGGAAGTAGT

C GGCCATCTGGCGGACCGGATTGCCGTCATGTATCTCGGCCGGATCGTGGAGGAGGGTCCTGCAAGCGAA
ATTCTCACTTCCCCGGCGCATCCCTATACAATGGCGCTGCTGTCCGCTTCACCAACGCTCGCGGATATTC
GTATGGGTCGGGATCAGCGTATTCGGCTGCGAGGCGACCCACCCAATCCCGCGAATGTGCCGGATGGCTG
CCGTTTTCATCCGCGCTGCCAACACGCCATGGATATCTGCAGGCGCCAGGACCCGCAATTTACATCAATC
GCGGAAGGGCGCAGATGCGCCTGTCATTTTTGGGCTGAAGGAGGCAAGGCCGCATAA

PCR conditions and notes

Primer concentration 5 picomole/µl we add 1 µl to 25 µl reaction

PCR Program	Tem °C	Time/ min	
Initial Denaturation	94	04:00	
Cycles	95	00:45	
	59	00:35	
	72	01:15	
Number of cycles	27 X		
Final extension	72	04:00	

SNP 3

Gene description	hypothetical protein			
Gene locus_tag	BMEII0525	**Gene ID**	1198297	
NCBI Accession	NC_003318.1	**Gene location**	550612..550764	
SNP location	550706	**SNP location at gene (Nucleotide coordinate)**	95	
Mutation Type	Nonsynonymous	**SNP sequence**	**Rev.1**	T
			Wt	C

SNP 3 Primers

Name	**Sequence**	**TM** °C	**Primer coordinates on Gene Bank Acc AE008918.1**	**Product Size bp**
Rev 0525 F	CTCCTTGAATTCTCAGTGGT	48.8	550687 550706	675
Rev0525 R	GTTGGTCGATGTCACCCAG	52.4	551361 551343	
16M 0525 F	CTTCGGGATAATGGAAACCATAG	50.9	549828 549850	898
16M 0525 R	TGACGATAAGGACCCCTTAG	49.9	550725 550706	

The gene sequence :SNP 3

>gi|17988344:550612-550764 *Brucella* melitensis bv. 1 str. 16M chromosome II, complete sequence

GTGAATGCGGAAACCCGTCCGCTCCCTGCCGGTAAATCTGTCGCCGTAAAATTGCTGGTCGCTATCATGT
CCATTCTCCTTGAATTCTCAGTGG **C** TAAGGGGTCCTTATCGTCAGGGTCATTCGCTTACGCGGCTCCTTT
TCCTTTACGATGA

PCR conditions and notes

Primer concentration 5 picomole/µl we add 1 µl to 25 µl reaction

PCR Program	Tem °C	Time/ min	
Initial Denaturation	94	04:00	
Cycles	95	00:45	
	59	00:35	
	72	01:15	
Number of cycles	26 X		
Final extension	72	04:00	

SNP 4

Gene description	ATP-dependent protease LA			
Gene locus_tag	BMEI0876	Gene ID	1196587	
NCBI Accession	NC_003317	Gene location	905053..907524)	
SNP location	906450	SNP location at gene (Nucleotide coordinate)	1398	
Mutation type	Synonymous	SNP sequence	Rev.1	A
			Wt	G

SNP 4 Primers

Name	Sequence	TM °C	Primer coordinates on Gene Bank Acc AE008917.1	Product Size bp
Rev 0876 F	GCCAGATGGAGAAGACGCAG	55.0	905774 905793	695
Rev0876R	CGCGTTCTGTTTCGGGTCT	55.0	906468 906450	
16M 0876 F	GCCATGCTGGAGGTGCTG	55.4	906433 906450	479
16M0876 R	GTCACGACACCCACCTGAT	53.5	906911 906893	

The gene sequence :SNP 4

>gi|17986284:905053-907524 *Brucella* melitensis bv. 1 str. 16M chromosome I, complete sequence

TTGGGTCGGAAGGCCCGAGAAAGGACTAGTGTTATGACGGGTATTGAACAGAAAACACCGGTTGGTGGTT
CTGAAACGGGTGGTGCGGACGGGCTCTATGCTGTCTTGCCGCTGCGTGACATCGTCGTCTTTCCCCATAT
GATCGTCCCGCTTTTTGTGGGCCGCGAAAAATCCATTCGCGCGCTTGAAGAAGTGATGGGCGTTGACAAG
CAGATATTGCTTGCCACCCAGAAGAACGCTGCCGATGACGATCCGGCGCCGGACGCGATCTATGAGATTG
GTACGATCGCCAATGTGTTGCAGCTTCTGAAACTGCCGGACGGCACCGTCAAGGTGCTGGTCGAAGGCAC
GGCACGCGCCAAAATTTCCAAGTTTACCGATCGTGAAGATTATCACGAGGCTTATGCTGCGGCTCTGCAG
GAGCCGGAGGAAGATGCTGTCGAGATCGAGGCACTGGCCCGCTCGGTGGTTTCCGACTTTGAAAATTACG
TCAAGCTGAACAAGAAGATTTCGCCGGAAGTGGTTGGCACGGCCAGCCAGATCGACGATTATTCCAAGCT
TGCCGATACGGTTGCCTCGCACCTTGCGATCAAAATCCCTGAAAAGCAGGAAATGCTGTCGGTTCTTTCG
GTGCGCGAGCGCCTTGAGAAAGCCCTTTCCTTCATGGAAGCCGAAATTTCTGTTCTACAGGTTGAGAAGC
GTATTCGCAGCCGCGTCAAGCGCCAGATGGAGAAGACGCAGCGCGAATATTATCTCAATGAGCAGATGAA
GGCGATCCAGAAGGAGCTTGGCGACAGTGAGGACGGCCGTGATGAAGTGGCCGAAATCGAAGAGCGCATC
ACCAAGACCAAGCTCAGCAAGGAAGCGCGCGAAAAAGCTCTGGCCGAGCTGAAGAAACTGCGCAGCATGA
GCCCGATGTCCGCTGAAGCGACGGTGGTTCGCAATTATCTCGACTGGTTGCTCTCCATTCCATGGGGCAA
GAAGTCGAAGGTGAAGCAGGATCTGAACTTTGCGCAGGAAGTGCTGGATGCGGAGCATTTCGGCCTTGGC
AAGGTCAAGGAACGCATCGTCGAATATCTGGCGGTGCAGGCCCGTTCGACCAAGATCAAGGGTCCGATCC
TCTGCCTCGTTGGCCCTCCCGGCGTCGGCAAGACCTCGCTTGCGCGCTCGATTGCCAAGGCAACGGGCCG
CGAATATGTCCGCATGTCGCTTGGCGGCGTACGCGACGAGGCTGAAATCCGCGGTCATCGCCGCACCTAT
ATCGGCTCGATGCCCGGCAAGGTCATCCAGTCGATGAAGAAGGCGAAGAAGTCCAATCCGCTTTTCCTGC
TCGATGAAATCGACAAGATGGGCCAGGATTTCCGCGGCGATCCGTCTTCGGCCATGCTGGAGGTGCT **G** GA
CCCGAAACAGAACGCGACCTTCATGGATCACTACCTTGAAGTTGAGTATGATCTATCGAACGTCATGTTC
GTGACGACCGCCAATACGATGAATATTCCCGGTCCACTTCTGGATCGTATGGAGATCATCCGTATCGCCG
GTTACACGGAAGACGAAAAGCTGGAGATCGCCAAGCGGCACCTGTTGCCGAAGGCCATCAAGGACCATGC
CCTGCAACCGAAGGAGTTTTCGGTTACGGAAGATGCGCTGCGCAACGTTATCCGCCATTATACGCGGGAA
GCGGGCGTGCGTAGCCTTGAACGCGAGGTGATGACCCTTGCGCGCAAGGCCGTGACGGAAATCCTGAAGA
CGAAGAAGAAGTCGGTAAAGATTACCGACAAGAACCTCTCCGATTACCTTGGTGTGGAGAAGTTCCGCTT
CGGTCAGATCGACGGTGAAGATCAGGTGGGTGTCGTGACTGGCCTTGCCTGGACCGAAGTCGGCGGTGAG
CTTTTGACCATCGAAGGCGTCATGATGCCGGGTAAGGGCCGCATGACGGTTACGGGTAATCTCCGTGACG
TGATGAAGGAATCGATTTCGGCCGCGGCATCCTATGTCCGCTCGCGCGCGATCGATTTCGGCATCGAGCC
TCCGCTGTTCGACAAGCGCGATATCCACGTGCACGTGCCGGAAGGCGCGACGCCGAAGGATGGTCCTTCT
GCCGGTATTGCCATGGTTACGGCCATCGTCTCCGTGCTGACGGGTATTCCCGTTCGCAAGGACATCGCCA
TGACGGGTGAAGTCACGTTGCGCGGTCGGGTTCTGCCAATCGGCGGGTTGAAGGAAAAGCTGCTTGCGAC
CTTGCGCGGCGGTATCAAGAAGGTTCTGATCCCGGAAGAGAACGCCAAGGATCTGGCGGAAATCCCGGAC
AATGTGAAGAACAATCTTGAGATCGTTCCGGTATCCCGCGTCGGTGAAGTGCTGAAGCACGCGCTCGTGC
GCCAGCCTGAACCGATTGAATGGACCGAGCAGGAGAATCCCACTGCCGTGCCTCCGGTGGAGGATGAAGC
AGGGGCTTCGCTGGCCCATTAA

PCR conditions and notes

Primer concentration 5 picomole/µl we add 1 µl to 25 µl reaction

PCR Program	Tem °C	Time/ min	
Initial Denaturation	94	04:00	
Cycles	95	00:45	
	62	00:35	
	72	01:15	

Number of cycles	26 X		
Final extension	72	04:00	

SNP 5

Gene description	pyruvate dehydrogenase complex repressor			
Gene locus_tag	BMEII1066	**Gene ID**	1198838	
NCBI Accession	NC_003318	**Gene location**	1108409..1109221	
SNP location	1109126	**SNP location at gene (Nucleotide coordinate)**	718	
Mutation type	Synonymous	**SNP sequence**	**Rev.1**	C
			Wt	T

SNP 5 Primers

Name	**Sequence**	**TM** °C	**Primer coordinates on Gene Bank Acc AE008918.1**	**Product Size bp**
Rev 1066 F	AAAGCGGCACCTTTCCTGA	54.0	1108259 1108277	
Rev 1066R	CCTCTCACGTTCGTCGTTCAG	55.3	1109146 1109126	888
16M1066F	CAACGCATGACTGCGCCT	55.8	1109109 1109126	
16M1066R	CCGAATATGACCGCCAAGAATGC	56.6	1109442 1109420	334

The gene sequence :SNP 5

>gi|17988344:1108409-1109221 *Brucella* melitensis bv. 1 str. 16M chromosome II, complete sequence

ATGGCCGACACAGTTTTTTCTCGTATACAAGCAAGCCGCACGGCGGACGACGTGGTTCACCAGATCGAAT
CGCTCATCCTTGAGGGGGTTCTGCGCGGCGGTGATCGCCTTCCCGGCGAACGCGAGCTTGCGCGCCAGTT
CGACATTTCCCGCCCCATCCTGCGCGATGCGCTGAAGCGCCTTGAAACGGCAGGCCTGCTCACATCACGC
CATGGCGGCGGCACCTTCGTTGCCGATGTCATCGGACAGGTTTTCAGCGCGCCTGTGGTGAAGCTCTTTG
CCGATCATCACAAGGCCACCGCCGATTATCTCGAATATCGCCGGGAGATTGAAAGCGTAGCCGCCGAATA
TGCCGCGCTGCGCGCCACGCCCGCCGACCGCGCGCTCCTGACCGAGATCATGAACGCGATGGAAAAGGCC
CACGGCGCTGACGATTTCGCCACCGAGGCACGTCTCGACGTGGAACTGCACAATGCCATTGGTGAGGCGG
CGCATAATATTGTGCTGCTGCACACCTTGCGCTCCTGCTACCAGCTTTTGAAGGACGGTGTATTCTACAA
TCGCAGCGTGATCTATAATCACCCGGGCGTGGCCGATGTGTTCCTTTCCCAGCACCGCGCCATCTACGAT
GCGGTGATGGCTGGAAACCCACAGGCCGCGCGCGAAGCAGTGCAGAAACATATCCGCTTCGTCGAACAGG
CAACGCATGACTGCGCC **T** TGAACGACGAACGTGAGAGGGTGGCGCGCCTTCGCTATCGCCAGCGCGCCGG
CTCCAAGGAACTTAAAGAAATCAGGGAAGACGAGAGCGAATGA

PCR conditions and notes			
Primer concentration: 5 picomole/ul. we add 1.25 µl to 25 µl reaction			
PCR Program	Tem °C	Time/ min	
Initial Denaturation	94	04:00	
Cycles	95	00:45	
	63	00:35	
	72	01:15	
Number of cycles	26 X		
Final extension	72	04:00	

SNP 6

Gene description	30S ribosomal protein S4			
Gene locus_tag	BMEI1133	**Gene ID**	1196844	
NCBI Accession	NC_003317	**Gene location**	1176916..1177533	
SNP location	1176933	**SNP location at gene (Nucleotide coordinate)**	601	
Mutation type	Nonsynonymous	**SNP sequence**	**Rev.1**	A
			Wt	G

SNP 6 Primers

Name	**Sequence**	**TM** °C	**Primer coordinates on Gene Bank Acc AE008917.1**	**Product Size bp**
Rev 1133 F	GAACCGAACCTCGTGGTCA	53.9	1176951 1176933	
Rev 1133R	GGTGCTTCCCGAACAATTGAG	54.0	1176387 1176407	544
16M 1133 F	ACCAATGTCAGACAGACCATG	52.0	1177926 1177906	
16M 1133R	CAGAATTAGCGCGAGTAGAATTC	52.0	1176911 1176933	1016

The gene sequence SNP 6

: >gi|17986284:c1177533-1176916 *Brucella* melitensis bv. 1 str. 16M chromosome I, complete sequence

ATGAGCAAGCGCGAATCCGCAAAGTATAAGATTGACCGCCGTCTTGGCGAAAACATCTGGGGCCGTCCGA
AGTCCCCGGTCAACCGTCGTGAATATGGCCCGGGCCAGCACGGCCAGCGCCGCAAGGGCAAGCTGTCGGA
CTTCGGTGTGCAGCTGCGCGCCAAGCAGAAGCTTAAGGGTTTCTACGGCGATATCTCGGAAAAGCAGTTC
CGCAAGACCTATGAAGAAGCCGCTCGCCGCAAGGGCGATACGGGTGAAAACCTGATCGGTCTTCTCGAAT
CGCGTCTGGATGCTGTTGTGTATCGCGCAAAGTTCGTTCCGACGATTTTCGCCGCACGCCAGTTCATCAA
CCACGGCCACGTGAACGTGAACGGCCGCCGCGTCAACATTCAGTCCTACCGCCTCAAGGTCGGTGACGTT
GTTGAAGTCCGCGAAAAGTCGAAGCAGCTCGCCATCGTTCTCGAAGCCGTTCAGCTCGCAGAACGCGATG
TGCCGGACTACATCGATGTCGATCACAACAAGATGGTCGCGACCTACAACCGTGTTCCGGGTCTTTCGGA
TGTGCCTTACGCCGTCCAGATGGAACCGAACCTCGTGGTC **G** AATTCTACTCGCGCTAA

PCR conditions and notes

Primer concentration 5 picomole/µl we add 1 µl to 25 µl reaction

PCR Program	Tem °C	Time/ min	
Initial Denaturation	94	04:00	
Cycles	95	00:45	
	62	00:35	
	72	01:15	
Number of cycles	27 X		
Final extension	72	04:00	

SNP 7

Gene description	3-methyl-2-oxobutanoate hydroxymethyltransferase			
Gene locus_tag	BMEI1592	**Gene ID**	1197303	
NCBI Accession	NC_003317	**Gene location**	1640909..1641736	
SNP location	1641334	**SNP location at gene (Nucleotide coordinate)**	403	
Mutation type	Nonsynonymous	**SNP sequence**	**Rev.1**	T
			Wt	C

SNP 7 Primers

Name	**Sequence**	**TM** °C	**Primer coordinates on Gene Bank Acc AE008917.1**	**Product Size bp**
Rev.1592 F	GTGGCCTTTCTCGTCCGGT	56.4	1641352 1641334	958
Rev.1592 R	ACCGATTTCATCTCCAACGAACTG	55.5	1640395 1640418	
16M 1592 F	CAGCCATTCGCACATTCATCG	54.9	1641946 1641926	630
16M 1592 R	GAAGACCGGAATGCCGCG	56.0	1641317 1641334	

The gene sequence : SNP 7

>gi|17986284:c1641736-1640909 *Brucella* melitensis bv. 1 str. 16M chromosome I, complete sequence

ATGAGTGCACCCGTAACCCGCAAACGGCTGACGCCGAAGGTTATTCAGGCGATGAAGGGCGAGTGTCCCA
TCGTCAGCCTCACCGCTTATACGACGCCGGTTGCGCGTCTGCTCGACCCGCATTGCGACCTGCTTCTGGT
GGGGGATTCGCTTGGCATGGTACTTTACGGCATGGAGTCCACCCTTGCCGTCACGCTCGACATGATGATC
ATGCATGGGCAGGCGGTCATGCGCGGTACAAGCCATGCCTGCGTGATCGTGGATATGCCTTTCGGCTCCT
ATCAGGAATCGAAGGAGCAGGCCTTCCGCAATGCCGCCCGTGTGATGCAGGAAACGGGCTGTGACGGCGT
GAAGCTGGAAGGCGGCGAGGAAATGGCCGAAACGGTGGCCTTTCTCGTCCGG **C** GCGGCATTCCGGTCTTC
GGCCATGTCGGGCTGATGCCGCAGCAGGTGAATACGGTCGGCGGGTTTCGTTCGCTGGGGCGCGGCGACG
ATGAAGCCGGCAAGATACGCCGCGACGCGCAAGCCATTGCCCAAGCGGGTGCTTTCGCGGTGGTCATCGA
AGGCACGGTCGAGCCGCTTGCGCGCGAAATAACGGCCTTGATCGATATTCCGACCGTCGGTATCGGCGCT
TCATCCGCCTGCGACGGGCAGGTTCTGGTTTCGGATGACATGCTGGGCCTGTTCCAGGATTTCACGCCGC
GTTTCGTCAAGCGTTTTGCGCATCTGGCGCCGCAGGTGTCGCAAGCGGCCGAAGCCTATGCCGAAGAGGT
GCGCGCGCGCAGATTTCCCGGCCCGGAACATGTTTTCGGGGCCAAGCCCGGAGCGTGA

PCR conditions and notes

Primer concentration 5 picomole/µl we add 1.25 µl to 25 µl reaction

PCR Program	Tem °C	Time/ min	
Initial Denaturation	94	04:00	
Cycles	95	00:45	
	63	00:35	
	72	01:15	
Number of cycles	26 X		
Final extension	72	04:00	

SNP 8

Gene description	phosphate regulon sensor protein PHOR			
Gene locus_tag	BMEI1624	**Gene ID**	1197335	
NCBI Accession	NC_003317	**Gene location**	1670762..1672000	
SNP location	1671015	**SNP location at gene (Nucleotide coordinate)**	986	
Mutation type	Nonsynonymous	**SNP sequence**	**Rev.1**	C
			Wt	T

SNP 8 Primers

Name	**Sequence**	**TM** °C	**Primer coordinates on Gene Bank Acc AE008917.1**	**Product Size bp**
Rev 1624F	AAATGCGACGGAAGTGGC	53.1	1671032 - 1671015	924
Rev 1624 R	GCGTTGAACTTCTGAAGAAACTG	53.0	1670109 - 1670131	
16M1624 F	GCCAATGCAAGCCATGAG	51.5	1671463 - 1671446	468
16M1624 R	GAAATCCTGTATGGAGGCGA	51.2	1670996 - 1671015	

The gene sequence :SNP 8

>gi|17986284:c1672000-1670762 *Brucella* melitensis bv. 1 str. 16M chromosome I, complete sequence

GTGTGCTTTTCGTCAGCATGGTGCTGGCGATCTGTGTCATGGCGCTCGACCTTCCTTTCTGGTTCCGCGC
CATATTGCTGGCGCTCGTTGTGCTGGGCGCGGTCTGGGCGGCGGCGGGCATTGGCAATGGGGAGGATGAA
AAGGTTCCCTTGCGGGAAGTGGCCAGCCCGTGAAATGGCGGCTGTCTCGGGCGAGCGGCTGGCCGATCTT
CTGACCGATCCGATGATCGTCTTCGATCACACCGCAACGGTGCGTTTTGCCAACGTGGCGGCGCTTGAAG
CGTTCCAGTCGCTGCAAAACGGCACCGCGCTTTATCTGCGCTTTCGCGCCCCGGAAATGCTCGCGCTGAT
CCAGGGCGTGATTGCCGACGGCGAACCGCGCAATATCGAATATTTCGAGCGCGTTCCCATCGATCGCTGG
TACAAGGCCATGGTCAAGGCGCTGCGTGATGCGGAAGGAAAGCCGGAACTCTTCGTGCTTATTTTCCGCG
ACCAGAGCGAAACACGGCGTATCGACCGTATGCGTTCCGACTTTATCGCCAATGCAAGCCATGAGCTGCG
CACCCCGCTTGCCTCGCTGCGCGGCTTCATCGAAACGCTGCAAGGCCCGGCGCGCAACGATGCCGCCGCC
CGTGACCGCTTTCTCGACATCATGCAGAAACAGGCCGAGCGCATGTCGCGTTTGATCGACGATCTCCTGT
CGCTGTCGCGGCTTGAAATGCGTGCTCATCTGGCGGTCAATGAAAGCGTGGATGTGGCGGCCACGCTGGC
CCATGTGGCCGATACGCTGACGCCACTTGCCGCCGGTCTCGGCGTGACCATCGAGCGCCACCTGCCGGAA
CATCCGGTTCATGTGATGGGCGCGCGTGATGAGCTGATTCAGGTCTTCCAGAATCTTGTCGAAAATGCCT
GCAAATACGGGCAGGAGGGCAAGCGCGTCATCATAAGATTAAGCGAGGAAGACACGGGAAATGCGACGGA
AGTGG **T** CGCCTCCATACAGGATTTCGGGCCGGGCATTGCTGCCGAACACCTTCCGCGCCTCACAGAGCGT
TTCTATCGCATCGACGTGGAGACAAGCCGCGCGCACAAGGGCACAGGTCTTGGCCTTGCCATCGTCAAGC
ATATTCTGGCCCGCCATCGCGGCCGGCTTGTGGTGCGTTCGCAATTGGGGGAAGGCTCTACTTTCATGGT
GCGCCTGCCGGGGCAGAACGCGCGCTCCGCCGAGGCTGCAACAATATGA

PCR conditions and notes			
Primer concentration 5 picomole/µl we add 1 µl to 25 µl reaction			
PCR Program	Tem °C	Time/ min	
Initial Denaturation	94	04:00	
Cycles	95	00:45	
	59	00:35	
	72	01:15	
Number of cycles	28 X		
Final extension	72	04:00	

SNP 9

Gene description	isovaleryl-CoA dehydrogenase			
Gene locus_tag	BMEI1923	**Gene ID**	1197634	
NCBI Accession	NC_003317	**Gene location**	1979349..1980497	
SNP location	1980135	**SNP location at gene (Nucleotide coordinate)**	787	
Mutation Type	Nonsynonymous	**SNP sequence**	**Rev.1**	T
			Wt	C

SNP 9 Primers

Name	**Sequence**	**TM** °C	**Primer coordinates on Gene Bank Acc AE008917.1**	**Product Size bp**
Rev.1923F	ACTCATTTCCGGTGAGCATGT	54.1	1979687 - 1979707	468
Rev.1923R	TTGCGCTCATGCACATAGGA	54.3	1980154 - 1980135	
16M1923F	TGCCTCGATGTCGTGGTGC	57.2	1980117 - 1980135	949
16M 1923 R	GCCGTGCATGATCCCATGA	54.7	1981065 - 1981047	

The gene sequence SNP 9 :

>gi|17986284:1979349-1980497 *Brucella* melitensis bv. 1 str. 16M chromosome I, complete sequence

ATGAACTTCGGTCTTGGCGAGGAGATCGAAGCCCTTCGTGACACTGTGCGCCGTTTCGCCGAAAGCCGCA
TTGCGCCGCTTGCCGCTGAAACCGACCGCAATAATCAATTTCCCATGCATCTTTGGCGTGAATTTGGTGA
GCTTGGCGTACTCGGCATTACGGCGCCGGAAGACTATGGTGGCGCAGGCATGGGCTATCTCGCCCATTGC
ATCGCCATGGAGGAGATCAGCCGTGCTTCCGCCTCCATCGGCCTTAGCTACGGCGCCCATTCCAATCTCT
GCGTCAACCAGATCACCCGCAACGGTTCGCCCGAACAGCGCGCGAAATATCTGCCGAAACTCATTTCCGG
TGAGCATGTCGGCGCGCTGGCCATGTCTGAGCCGGGCGCGGGGTCCGATGTTGTTTCCATGAAGCTTGCC
GCCGAAAAGCGCGGGGACCGTTACGTCCTCAACGGCAACAAGATGTGGATCACCAATGGCCCGGATGCCG
ATGTGCTGGTGGTCTATGCCAAGACAGACCTGTCGGCTGGCCCGCGCGGGATCAGCGCCTTTATCATTGA
AAAAGGGTTCAAGGGTTTTTCGACCGCGCAAAAGCTCGACAAACTCGGTATGCGCGGTTCCAACACCTGC
GAACTGGTGTTCGAGGATTGCGAAGTGCCAGCCGAAAACCTGCTCGGAACCGAGGGCAAGGGCGTCAATG
TGCTGATGTCCGGCCTCGATTATGAGCGCGTGGTGCTGGCGGGTGGGCCGCTCGGCATCATGGCGGCCTG

CCTCGATGTCGTGGTG **C** CCTATGTGCATGAGCGCAAGCAGTTCGACCAGCCAATCGGTGAATTCCAGCTC
ATGCAGTGCAAGCTTGCCGACATGTATGTGACCTTCAACGCTTCACGCGCTTATGTCTATGCCATGGCCG
CAGCCTGCGACCGGGGTGAGACGACGCGCAAGGATGCGGCGGGCTGCATTCTTTACTCTGCGGAAAATGC
CACGCAGATGGCCTTGCAGGCGATCCAGTCTCTCGGCGGCAACGGCTATATCAACGATTATCCGACGGGC
CGCCTTTTGCGCGATGCCAAGCTTTATGAAATCGGCGCGGGCACGTCGGAAATCCGGCGTATGCTGATCG
GACGGGAACTCTTTCAGGAGACCCGTTGA

PCR conditions and notes			
Primer concentration 5 picomole/µl we add 1.25 µl to 25 µl reaction			
PCR Program	**Tem °C**	**Time/ min**	
Initial Denaturation	94	04:00	
Cycles	95	00:45	
	63	00:35	
	72	01:15	
Number of cycles	26 X		
Final extension	72	02:00	

SNP 10

Gene description	hypothetical protein			
Gene locus_tag	BMEI2044	**Gene ID**	1197755	
NCBI Accession	NC_003317.1	**Gene location**	2104370..2104879	
SNP location	2104436	**SNP location at gene (Nucleotide coordinate)**	444	
Mutation type	Synonymous	**SNP sequence**	**Rev.1**	A
			Wt	G

SNP 10 Primers

Name	**Sequence**	**TM °C**	**Primer coordinates on Gene Bank Acc AE008917.1**	**Product Size bp**
Rev2044 F	TTCGTGAGGAAAGCCTATGAC	51.9	2105615 2105595	1196
Rev 2044 R	AAGGAGCAGGGCGGGAT	54.4	2104420 - 2104436	
16M2044F	AATGGGGCCAGCAAGTG	51.6	2104452 - 2104436	781
16M2044R	CATTGACCGTCTTTCAAATCGATG	53.2	2103672 - 2103695	

The gene sequence SNP 10 :

>gi|17986284:c2104879-2104370 *Brucella* melitensis bv. 1 str. 16M chromosome I, complete sequence

ATGGCGCAATTCGTCGTTCTTGAACCGACCGGAGCCGAGGCATTTTCAGGAGAGGCGGGAACCGGCTCTC
CGTCCGAAAAGGCCGTGTTCGTGCGCGACGGATTTTCTATACTGGCGCTGGTGTTTCCTTTCGTCTGGCT
TTTGATGCAGCGGCTCTGGTTCGAGGCATTCGCCGTTCTGGGCATAACCATCCTGCTTGGACTTGCCGGG
ACCAGCCTGGGTATCGACGACGCGGTTCCGCTGCTTGCCATTCTGGTCTCGCTGTTCGTGGCTCTTGAGG
GCGCCGGCTGGAAGATCGCGAAGCTTGAACGGCAGGGTTATGTCCAGCGCGCAGTCATTGAGGCAGCCGA
TCTGGATGAGGCGGAAATCCGCTATTTCATCGGGCGCGACGGGGTTGAAACCGCGTCTGCGGATATCCCC
GTTCCGCAATGGGGCCAGCAAGT **G** TCCCGCCCTGCTCCTTCCACCTTTGGCGCGACTATAGGATTTGTCG
GCCATCGCGGAGAAAACTGA

PCR conditions and notes

Primer concentration 5 picomole/µl we add 1.5 µl to 25 µl reaction

PCR Program	Tem C	Time/ min	
Initial Denaturation	94	04:00	
Cycles	95	00:45	
	59	00:35	
	72	01:15	
Number of cycles	29 X		
Final extension	72	04:00	

SNP 11

Gene description	high-affinity branched-chain amino acid transport ATP-binding protein LIVG			
Gene locus_tag	BMEII0630	**Gene ID**	1198402	
NCBI Accession	NC_003318.1	**Gene location**	665880..666314	
SNP location	666179	**SNP location at gene (Nucleotide coordinate)**	136	
Mutation type	Nonsynonymous	**SNP sequence**	**Rev.1**	- (deletion)
			Wt	G

SNP 11 Primers

Name	Sequence	TM °C	Primer coordinates on Gene Bank Acc AE008918.1	Product Size bp
16M630F	CCGGTATCAGCGGCG	56.8	666193 - 666179	
16M630R	ACAAGATTGACGAATGTGGTC	56.5	665748 - 665768	442
Rev630 F	GTCATTCTTATGCCGGACT	54.6	666828 - 666846	
Rev630 R	GAGATTGCGTTGAGAGGC	55.9	666163 - 666179	682

The gene sequence SNP 11:

>gi|17988344:c666314-665880 *Brucella* melitensis bv. 1 str. 16M chromosome II, complete sequence

GTGCGCTCCCCCTTTGGCATGTTGTGCCGGGGCGTGAAGGAAGACCCGATCCGCATCCACGCCATGGGCG
CTTCGGTGCAAGGCGCGCAACTTCGCATGTATGTGATTTCCGGCGCGGTTGCCGGTATCAGCGGC **G** CTCT
CAACGCAATCTCCACACAGGTCGTCGGCCTCGACAGTCTCAGCTTCACCATGTCGGCGGAAGCCCTTGTC
ATGCTGGTGCTGGGGGGGGGGCCGGATCTCTTTTCGGCGGGCTGGTTGGCACGGTCGTCTTCATGTGGTT
TGAGGACGTGGTATCCACCGCCAATCCGTTCCATTGGCTCACCATGGTCGGCGCGCTCCTCATTGCCGTG
GTGCTCTTCGCCCCGCGCGGCCTTTACGGAACCCTCGCCTATTATTTTGAAAAGAAGGCGCGCCGCAAAT
GAGCACCGTGTTTGA

PCR conditions and notes

Primer concentration 5 picomole/µl we add 1 µl to 25 µl reaction

PCR Program	Tem C	Time/ min	

Initial Denaturation	94	04:00	
Cycles	95	00:45	
	58	00:35	
	72	01:15	
Number of cycles	30 X		
Final extension	72	04:00	

Appendix 6: **Sequencing primer list for the tested 11 Rev.1 unique SNPs**

Primer Name	***Sequences from 5' to 3'***	***TM °C***	**Primer coordinates on Gene Bank Acc AE008917 (Chromosome I)**	**Primer coordinates on Gene Bank Acc AE008918 (Chromosome II)**
Seq- BMEII0630	**GACATGGTGAAGCTGAGA**	56		666121 - 666138
Seq- BMEI0006	**GATTGAAGATGCTGTTCCTAC**	56	6638 - 6658	
Seq- BMEII0199	**GAGATACATGACGGCAATC**	56		210914 - 210932
Seq-BMEII0525	**CGTTGGAACAATCAATTCGA**	57		550800 - 550781
Seq-BMEI0876	**CTCGATGAAATCGACAAGA**	55	906382 - 906400	
Seq- BMEII1066	**GAAGCAGTGCAGAAACATA**	55		1109072 -1109090
Seq-BMEI1133	**GTTCCAGGATAACGCATAA**	55	1176821 -1176839	
Seq-BMEI1592	**ATCAGGAATCGAAGGAGCA**	58	1641456 -1641438	
Seq-BMEI1624	**CACGTCGATGCGATAGAA**	57	1670933 -1670950	
Seq-BMEI1923	**GTCCGGCCTCGATTATGA**	58	1980056 -1980073	
Seq-BMEI2044	**GTCAATAATAGCAACACGCA**	57	2104347 - 2104366	

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