Identification and Molecular Characterization of *Brucella* Strains Isolated from Sheep and Goats in the West Bank, Palestine

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I dedicate this work to my grandfather, my mother and my husband.

All of them are Doctors of Veterinary Medicine.

To my sons Khaled, Salem Rami, Amir and my daughter Aya, Noor. Thanks

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Elena Awwad

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LIST OF ABBREVIATIONS

CFT	Complement Fixation Test
CNS	Central Nervous System
CSF	Cerebrospinal fluid
CVL	Central Veterinary Laboratory
DNA	Deoxyribonucleic Acid
FAO	Food Agriculture Organization
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked immunosorbent assays
HA	Hemagglutinin
IS*711*	Insertion element Sequence no 711
MHD	Minimum hemolytic dose
NC	Negative Control
OIE	Office Infectious Epizooties
OMP	Outer Membrane Protein
OPT	Occupied Palestinian Territories
ORF	Polymerase Chain Reaction
PCBP	Palestinian Brucellosis Control Program
PMN	Polymorphonuclear cells
RBC	Red Blood Cell
RELP	Restriction fragment length polymorphism
RBT	Rose Bengal Test
RES	Reticuloendothelium System
Rev-1	Name of vaccine Brucella melitensis strain
SAT	Saline Agglutination Test

S-LPS Smooth Lipopolysaccharide

UNDP United Nations Development Program

WHO World Health Organization

ABSTRACT

Serum samples were obtained from 350 animals comprising of 302 sheep and 48 goats with signs of brucellosis from different localities in West Bank, Palestine. None of the animals were vaccinated against *Brucella*. These samples were subjected to serological examination for the detection of specific *brucella* antibodies. The percentage of the positive brucellosis sera was found to be as follows: 31% in sheep sera and 52% in goats sera by Rose Bengal test (RBT), giving overall seroprevalence of 34%. The overall seropositivity using Complement Fixation Test (CFT) was 31%; 29% in sheep sera and 44% in goats sera. Eighty milk samples collected from seropositive animals were subjected to bacteriological examination. *Brucella* organisms were detected in 38 (47.5%) of the samples. Testing the milk samples by polymerase chain reaction (PCR), all the 38 positive samples detected by bacterial examination were also detected by PCR. Furthermore, with the PCR, we were able to additionally detect 24 (30%) infected milk samples that were negative by the bacterial isolation method. *B. melitensis* was identified from 42 out of 80 milk samples. The hemagglutinin gene sequence of two of the *B. melitensis* genes was PCR-amplified using the primers ORF and IS*711*, sequenced and subsequently aligned with other GenBank-accessible gene sequences of *B. melitensis* and other *Brucellae* spp. using version 2.0 of BLAST. These sequences were identical to that of the recent sequence of type strain of *B. melitensis* (ATCC 23456). Nucleotide comparison and restriction enzyme analysis of hemagglutinin revealed that the two current isolates were different from vaccine (Rev.1). The results of phylogenetic analysis revealed that they

were genetically close to the isolates from different Mediterranean countries, particularly those from France, Spain and Israel.

الملخص

تشخيص ودراسة جرثومة البروسيلا في الأغنام والمعز المتواجدة في منطقة الضفة الغربية، فلسطين بواسطة تقانات الأحياء الجزيئية

هدف الدراسة هو الكشف عن نسبة تواجد اضداد جرثومة البروسيلا في الأغنام والمعز المتواجدة في منطقة الضفة الغربية، فلسطين التي ظهرت عليها أعراض داء البروسيلا باستخدام اختباري وردية البنكال (RBT) واختبار تثبيت المتمم (CFT). إذ تم جمع 350 عينة مصل 308 من الأغنام و 48 من الماعز. كانت نسبة تواجد أضداد جرثومة البروسيلا باستخدام وردية البنكال في الأغنام والماعز (31%، 52%) على التوالي وبنسبة كلية بلغت (34%) في حين بلغت نسبتها استخدام اختبار تثبيت المتمم في الأغنام والماعز (29%، 44%) وعلى التوالي وبنسبة كلية بلغت (31%). إضافة إلى ذلك جُمعت 80 عينة من الحليب من الأغنام أو المعز التي أظهرت تواجد اضداد جرثومة البروسيلا بغرض زراعة وعزل جرثومة البروسيلا. كانت نسبة الحصول على مزارع لجرثومة البروسيلا 34 (42.5%) من أصل 80 عينة حليب

استعمل فحص تفاعل البلمرة المتسلسل النوعي (PCR) بواسطة بادئات التفاعل النوعية الخاصة بكل نوع من البروسيلا للعنصر الوراثي IS711 لفحص عينات الحليب. تم الكشف عن وجود جرثومة البروسيلا في 62 عينة (77%) من مجموع عينات الحليب (80). وكذلك أشارت نتائج تفاعل البلمرة بواسطة البادئات النوعية الخاصة بجرثومة البروسيلا المالطيه وجود 42 عزلة .

تم دراسة تسلسل الحامض النووي المركب لجين مقترحة هو (Hemagglutinin) لعزلتين من البروسيلا المالطية من العينات الحقلية وتم مقارنتها مع سلالة لقاح البروسيلا المالطية (العترة Rev 1) باستخدام نظام التوافق الموجود في برنامج CLCL حيث كان تشابه بين هاتين العزلتين وسلالة لقاح البروسيلا. بالرغم من هذا التشابه الأ ان هضم الجينن المفترض باستخدام الأنزيم القاطع *Hae* III أظهر اختلاف واضح في طريقة الهضم بين السلأتين التي تم دراستهما و سلأَلة اللقاح .

اظهرت مقارنة تسلسل الحمض النووي للجين المفترض للعزلين السابقين مع جينات مفترضة مشابهة للجينات المشخصة للبروسيلا من حيث التسلسل والتي تم البحث عنها في شبكة المعلومات بواسطة الشجرة الجينية أن هاتين العزلين أقرب لعزلات البروسيلا المالطية المعزولة في فرنسا، إسبانيا وإسرائيل.

أثبتت الدراسة:

1. عدم وجود توافق بين اختياري وردية البنكال (RBT) واختبار تثبيت المتمم (CFT). مما يدل على أنه لا يمكن الاعتماد على اختبار واحد في الكشف عن تواجد اضداد جراثيم البروسيلا كإختبار مسحي وتشخيصي.

2. أن استخدامات الحمض النووي المطور ذات فعالية عالية و سرعة كأداة تشخيصية مميزة لعينات البروسيلأ المعزولة من الحقل مقارنة بطرق التشخيص التقليدية . استخدام هذه التقانات له مستقبل واعد كمصدر فعال في إنتاج القاحات الجزيئية.

3. أظهرت الدراسة اختلاف واضح بين عزلأت البروسيلأ المالطية في فلسطين وسلالة لقاح البروسيلا المالطية (العترة Rev 1)

4. أظهرت الدراسة ان عزلأت البروسيلأ المالطية في فلسطين أقرب لعزلات البروسيلا المالطية المعزولة في فرنسا، إسبانيا وإسرائيل من ناحية وراثية.

توصي الدراسة بالسيطرة على المرض في الحيوان وذلك بإتباع سياسة التطعيم الكلى للقطيع باستخدام سلالة لقاح البروسيلا المالطية ذات العلاقة القريبة للعزلأت في فلسطين أولا ثم تبني سياسة تنفيذ برامج إرشادية للأناس الأكثر عرضة لخطر الإصابة بمرض البروسيلا. كما توصي الدراسة أيضا إجراء مزيد من الدراسات على نطاق واسع تشمل كل القطر لمعرفة لمزيد عن وبائية المرض.

CHAPTER I

INTRODUCTION

Brucellosis has great health significance and economic importance in many countries worldwide. *Brucella melitensis*, a gram negative bacterium, is the leading cause of brucellosis in sheep and goats, and the most important causative agent of brucellosis in humans. Infections in animals caused by *Brucella spp*. frequently result in abortions and diminished levels of milk production. Once the acute period of the disease is over, animals may present little or no disease symptoms, and *Brucella* cells can chronically be located in the supramammary lymphatic nodes and mammary glands of 80% of infected animals; thus, animals continue to secrete the pathogen in their body fluids. Transmission to humans occurs upon consumption of contaminated raw milk and contaminated dairy products. Therefore, there is no doubt that control of this disease in animals will have an immediate impact on the incidence rate of this disease in humans (1, 2). Brucellosis is an ancient disease with a low mortality rate, which causes a substantial residual disability in man. Human brucellosis remains the major bacterial zoonosis in the world with devastating economic effects on the productivity of livestock. Humans, who become incidentally infected due to contact with infected animals or ingestion of dairy products, may develop numerous symptoms. Disease frequently becomes chronic and may relapse, even with treatment (3). In cattle, sheep and goats, the initial phase after infection is often not apparent. In sexually mature animals, the Infection is localized in the reproductive system and typically results in placentitis followed by abortion in pregnant females during the last two months of pregnancy, and epididymitis and orchitis in males (4).

Keeping the mentioned facts about brucellosis in mind, the present study was to examine serum and milk of 350 animals (302 sheep and 48 goats) obtained from different localities in the West Bank, Palestine:

1. To study the seroprevalence of brucellosis in sheep and goats by detecting antibodies in serum employing RBT and comparing it with CFT.
2. Isolation and identification of *Brucella* from milk and confirmation by molecular methods.
3. Genetical characterization of representative isolates of *B. melitensis* related to the vaccine strain used here and some isolates from different Mediterranean countries

1.1 Epidemiology of Brucellosis

Brucellosis is distributed worldwide and is recognized as a zoonosis of great economic importance. In developed countries, certain human infection is associated

with meat-packing and dairy-related occupations. Brucellosis is transmitted among

animals through the gastrointestinal tract, skin and mucus membranes. The organism reaches the lymph nodes and bacteremia occurs. *Brucella* then proliferates in the uterus and in the mammary glands. Growth in the chorionic membranes of the pregnant animal leads to abortion (5).

Brucella spp. are named for their primary host and subdivided into biovars based on serologic agglutination with “smooth lipopolysaccharide (S-LPS) – associated” antigens M and A. *B. melitensis* infect sheep and goats. It may also be found in cattle due to indirect contact with infected sheep and goat flocks sharing contaminated pastures. *B. melitensis* is divided into 3 biovars: biovar 1, 2, 3. *B. abortus* is pathogenic to cattle, but can also infect sheep, goats, canines, horses

and humans. The species comprises 7 biovars (1-6, and 9, biovars 7 and 8 are no longer valid). *B. suis* contains 5 biovars: biovars 1, 2 and 3 are found in swine, while biovar 4 is found in reindeer and caribou in the Arctic regions of North America and Russia, and biovar 5 infects rodents. *B. canis* strains comprise a single biovar that infect dogs in U.S., Mexico, Argentina, Spain, China, Japan and Tunisia. *B.ovis* and *B. neotomae* each contain a single biovar and are found in rams and wood rats. *B. melitensis, B. abortus* and *B. suis* are associated with human disease, with *B. melitensis* the most virulent species. *B. canis* rarely causes infection in humans (6).

Brucellosis is distributed throughout areas of both the eastern and western hemispheres. Indigenous animal and human infections occur in the Mediterranean region, the Arabian gulf, the Indian subcontinent, Latin America, Asia and parts of

Mexico. *B. abortus* distribited worldwide while *B. suis* is endemic in the southern U.S., Southeast Asia and Latin America. Caprine and ovine brucellosis are no longer detected in Northern Europe, Denmark and Norway. This is due to successful control and eradication programs. Southern European regions are still infected with *B. melitensis* in animals and humans. Brucellosis is a big problem in the Balkan region of Central and Southern Europe. *B. abortus* is found in Greece and Macedonia. The countries with the highest incidence of both animals and human brucellosis are

Palestine, Saudi Arabia, Iran, Syria, Jordan and Oman. *B. melitensis* biovar 3 is common in Egypt, Israel, Tunisia, Turkey, and Jordan. Brucella melitesis biovar 2, is common is present in Saudi Arabia, and Turkey while biovar 1 is found in Libya, Oman, and Israel (7).

1.2 Brucellosis in Palestine.

In Palestine, human brucellosis has been reported annually since 1987 with a peak in 1990. In order to encourage people to report this disease, the Ministry of Health offers free treatment to the population. However, under-diagnosis and underreporting of cases is a recognized problem in this country. It is estimated that for each reported case there are at least two additional cases that are not reported and not diagnosed. Therefore, the actual number of cases may be approximately three times as many as the reported number. Irrespective of how one enumerates cases, it is clear that brucellosis has become endemic in the human population in Palestine. Evidence suggests that it is also enzootic in animals, especially sheep and goats, with prevalence in these animals of approximately 5% to 6% in some Palestinian districts.

The environment in the West Bank and Gaza is favorable for the spread of brucellosis,

but incidence rate is less than expected. Reasons for this lower incidence rates can be explained by the underreporting of cases, limited intensive animal breeding and poor economic circumstances resulting in low consumption of milk, milk products and meat. In addition, closure of the border by the Israeli authorities, has resulted in drastically reducing lower the number of infected animals purchased from Bedouins

and farmers residing in Israel. The condition of closed border applies to other neighboring countries such as Jordan, Egypt, Syria and Lebanon (8). The incidence rate of brucellosis in humans is approximately equal in all age groups, suggesting that all age groups are at risk. In Palestine care for animals is carried out mainly by women and this explains higher incidence rates in females. Consumption of milk, dairy products and cheese in particular, constitutes the

major risk factor for transmission among people living in urban areas. Consumption of unpasteurized sour milk is the major risk factor of transmission among people living in refugee camps. Other risk factors for brucellosis may include eating uncooked vegetables contaminated with excreta of infected animals and inhalation of dust contaminated by it. A high proportion of cases occurs during the spring and summer seasons. In spring, the delivery season, transmission occurs by consumption of unpasteurized milk or dairy products as well as direct animal contact during delivery of infected flock. In the summer season, transmission is due to increased consumption of milk and cheese. Consumption of insufficiently cooked or undercooked meat of goats, sheep and cattle with brucellosis in general, and bone marrow, liver and spleen of small ruminants in the bacteremic stage in particular, may also be a source of infection. Brucellosis is a professional hazard where groups of people engaged in handling live infected goats and sheep or their products are continuously at risk of brucellosis. Such groups include shepherds, animal caretakers and handlers, veterinarians, slaughterhouse workers, sheep-shearers and laboratory workers who can become infected while handling materials contaminated with *Brucella spp* (8) or during vaccinating the

animals if the safety rules are not followed correctly(9).

Years	**No of infected people**
1998	837
1999	747
2000	304
2001	273
2002	166
2003	267
2004	153
2005	115

2006	79
2007	206
2008	198

Table 1.2.1: Repeated cases of human Brucellosis from period 1998 to 2008 in the West Bank (Brucellosis project)(10).

The vaccination of sheep and goats with live *Brucella melitensis* Rev1 strain vaccine may cause abortion. But this live vaccine is recommended by international organization - Office International Epizooties (OIE) and directorate of veterinary services and animals health to use for conjunctival vaccination all sheep and goats in Palestine. Instructions dictate that this vaccine should not be applied to pregnant animals. Occasionally, sometimes farmers and veterinarians are unaware of the pregnancies (first or second month of pregnancy) thus animals receiving the vaccine with the consequence of abortion. This holds the Palestinian government responsible. Differentiation in the Central Veterinary Laboratory between circulating wild strain and Rev 1vaccine strain would eliminate ambiguities concerning the real cause of abortion in animals.

1.3 Control of Brucellosis in Palestine

Before 1994 the veterinary service in Occupied Palestinian Territories (OPT) was under authority Israel Civil Administration. Since 1974 were implemented vaccination strategies and in 1981 reports the result of analyses of blood samples

and aborted material in suspected cases of brucellosis. The aim of OPT was to reduce or minimize the incidence of infection in animals and human. Since 1982 have been vaccinated only young female sheep and goats (two-seven month old) with Rev 1 subcutaneous full dose. Vaccination has been compulsory and free charge. In 1992, the OPT veterinary service initiated a partial test and slaughter policy if mean: testing vaccinated animals (those with their ears notched have been vaccinated) after their first delivery (when the animals are 18 month of age and older), testing unvaccinated animals older than 8 month. In June 1992, in the Hebron area, (when one third of the total sheep and goat population of the West Bank is considered), the Israeli Veterinary Department began collecting blood samples and testing them with Rose Bengal and Complement Fixation test. In 1993, the Israel paid compensation for each infected animals and impounded the meat NIS250-300 per animals. Since the initiation of this policy, 200 animals were slaughtered.

In 1997 start Palestinian Brucellosis Control Project. This project was funded by the Spanish Cooperation and implemented by the United Nations Development Program

(UNDP) in Cooperation with the Palestinian Ministry of Agriculture through the Directorate of Veterinary Services and Animal Health. The project aims to contribute to significant reduction of the cases and incidence of Brucellosis in small ruminants, predominantly sheep and goats, as well as humans in the West Bank, Gaza and East Jerusalem. In addition, efforts are being made by the Palestinian Brucellosis Control Program (PCBP) to control Brucellosis as recommended by international organizations (FAO, OIE and WHO). These efforts will result in drastic reduction in the prevalence of Brucellosis in animals to a minimum through conjunctival mass vaccination, which will reflect in lowering the number of human cases. The project also intends to implement new policies to

improve proper diagnosis of brucellosis by veterinarians, develops surveillance and monitoring programs, and to enhances public awareness and eventually eradicates this disease

Within this project public awareness campaign was developed and directed toward educating the public about the menace of brucellosis. This campaign promoted the production and broadcasting of three documentaries about brucellosis, and two TV comedies, with participation and education of students from schools particularly those in hot areas such as Hebron and Dura (10).

1.4 Decrease of animal Brucellosis during Brucellosis project

The effect of the brucellosis project in Palestine in reducing the number of cases in animals and flocks are shown in Tables 2, 3, 4. The drastic reduction in brucellosis in the year 2005 (Table 3) as compared to the year 1998 (Table 2) is clearly evident. The

number of areas and percent positive for both animals and flocks in the year 2009 is shown in Table 4. We can conclude that the effort of this project were fruitful in most cities and areas in the West Bank. Hopefully, with continued effort, we expect to see very low incidence rates (2% international standard)

By animals	**By flocks**
18%	75%

Table 1.4.1: The prevalence of Brucellosis between animals in 1998 in West Bank (10)

District	%+ve by RBT 2005	%+ve by flock 2005
Hebron	5.1	53
Bethlehem	6.9	84
Jerusalem	4.7	47

Jericho	4.9	47
Ramallah	8.2	67
Salfit	7.9	45
Nablus	4.9	35
Tulkarem	3.8	25
Qualquilia	6.8	55
Tubas	3	35
Jenin	5.6	35
Total	**5.8**	**48**

Table 1.4.2: The prevalence of Brucellosis between animals in 2005 by RBT (10)

District	Result by flocks 2009					Result by animals 2009				
	No	No +ve by RBT	% +ve by RBT	No +ve by CFT	% +ve by CFT	No	No +ve by RBT	% +ve by RBT	No +ve by CFT	% +ve by CFT
Dura	14	8	57	6	42	233	18	7.7	12	5.2
Hebron	37	29	78	25	68	824	56	6.8	44	5
Bethlehem	29	7	24	6	21	360	8	2.2	6	1.7
Jerusalem	34	7	21	7	21	1004	8	1	7	0.8
Jericho	49	24	49	21	43	1694	109	6.4	57	3.4
Ramallah	36	15	42	10	28	1117	23	2.1	14	1.3
Salfit	17	4	24	3	18	218	5	2.3	4	1.8
Nablus	57	23	40	20	35	1137	96	8.4	76	6.7
Tulkarem	32	5	16	4	13	451	8	1.8	7	1.6
Qualquilia	5	6	40	4	27	189	10	5.3	7	3.7
Tubas	32	16	50	4	4	829	73	8.8	57	6.9
Jenin	47	14	30	14	30	749	34	4.5	26	3.5
Total	**418**	**159**	**38**	**133**	**32**	**8851**	**483**	**5**	**345**	**3.7**

Table 1.4.3: The prevalence of Brucellosis in 2009 by district in West Bank by RBT and CFT (10).

1.5 Human- to Human Transmission:

Human to human transmission is rare. Infections with *Brucella* can be transmitted via blood transfusion and bone marrow transplantation from infected

donor (11). Neonatal infection can be acquired by the transplacental route, during delivery or via the ingestion of contaminated breast milk during breast feeding (12, 13).

1.6 *Brucella* species

Brucellae are gram-negative coccobacilli (short rods) measuring about 0.6 to 1.5 µm. They are non-spore forming organisms that lack capsule and non flagellated, therefore non-motile. The outer cell membrane closely resembles that of other Gram-negative bacilli with a dominant lipopolysaccharide (LPS) component and three main groups of proteins. The metabolism of the *Brucellae* is mainly oxidative and they show little action on carbohydrates in conventional media. They are aerobes, but some species require an atmosphere with added 5-10% CO_2. Multiplication is slow at the optimum temperature of 37^oC and enriched medium is needed to support adequate growth. *Brucella* colonies become visible on suitable solid media in 2-3 days. The colonies of smooth strains are small, round and convex but dissociation, with loss of the O chains of the LPS, occurs readily to form rough or mucoid variants. Rough or mucoid variants are natural in *B. canis* and *B. ovis* since the LPS of these organisms lack the O chains (14). *Brucella melitensis* is found in sheep, goats and humans, but may be found in cattle sharing contaminated pastures.

1.7 Molecular characteristic of *Brucella* spp

Brucellae spp. are closely related with percent relatedness of 96 ± 4% between species. An insertion element IS711 is common in all species, but may be located on different sites on the bacterial chromosome depending on the species. Variations among different *Brucella species* are found on the lipopolysaccharide (LPS) and outer membrane proteins (OMP) (16). A recent study (17) proposed

that all *Brucellae* species belong to a single species, *Brucella melitensis,* and all other species should be regarded as biovars of *Brucella melitensis (Brucella melitensis* biovar *melitensis, abortus, suis, canis*). Molecular genetic studies and restriction endonuclease mapping revealed gene polymorphism that can differentiate between *B.abortus, B.melitensis, B.suis,* and *B.canis* (17). One of the polymorphic genes that help differentiate the various *Brucella species* is *omp 2 porin gene* which encodes for a 36-kDa OMP which is responsible for susceptibility to the dyes used for conventional species identification (12). *Brucella* species have two chromosomes except *B.suis* biovar which has a single chromosome. The genome of *Brucella melitensis* strain 16M was sequenced and found to contain 3,294,931 base pairs distributed over two circular chromosomes of 2,117,144 bp and 1,177,787 bps (12). Each chromosome encodes functions that are essential for replication and survival of the organism. Endogenous plasmids, transformation and conjugation have not been described in *Brucella* species (17).

1.8 Virulence Factors and Pathogenecity of *Brucella melitensis*

Brucella melitensis is an intracellular pathogen that can survive and multiply within phagocytic cells of the host. The mechanism by which *B. melitensis* evades intracellular killing is not fully elucidated. Nevertheless, *Brucella* organisms ultimately become sequestered within monocytes and macrophages of the reticuloendothelial system (RES) in lymph nodes, liver, spleen and bone marrow (16). Although humoral antibodies appear to play some role in resistance to infection, the principal mechanism of recovery from brucellosis is cell-mediated. Cellular immunity involves the development of specific cytotoxic T lymphocytes and activation of macrophages, enhancing their bactericidal activity, through the release

of cytokines (e.g. gamma interferon and tumor necrosis factor) from specifically committed helper T lymphocytes. Coincident with the development of cell-mediated immunity, the host usually demonstrates delayed type hypersensitivity to *Brucella* antigens (12, 16).

At the molecular level, antigenic variation is the result of decreased expression of genes encoding the additional glycosylation of the polysaccharide moieties of the cell wall lipopolysaccharide (LPS). Organisms that are in the smooth phase posses a smooth-type LPS (S-LPS) and are resistant to intracellular killing by polymorphonuclear cells (PMNs), presumably by inhibiting lysosomal degranulation and the respiratory burst associated with PMN activation. After its entrance in the host, the monocytic-macrophagic system is the target for the pathogen, where it is able not only to survive, but also replicate. The pathogen evades host defence by inhibiting endsome fusion with lysome and may reach the endoplasmic reticulum (18). The markers for biovar determination, also have a role in organism virulence, as monoclonal antibodies directed against the S-LPS are protective in animal challenge models, and smooth isolates that have lost S-LPS by transposon mutagenesis have attenuated pathogenicity for mice. The S-LPS O (somatic antigen) chain from smooth *B. melitensis* and smooth *B. abortus* strain are both composed of polymers of 4,6-dideoxy-4-formamido-D-mannose (N-formyl-D-perosamine). In *B. melitensis* O chain, α 1,2 and α 1,3 linkages occur in a 4:1 ratio (M determinants). The serodominant A antigen tends to be rod-shaped, the shape being determined by

the five consecutive α 1,2-linked residues, whereas the serodominant M antigen is

“kinked” in shape because the fourth residue is linked to the fifth by an α 1,3 linkage. The common expression of nonterminal α 1,2-linked N-formyl-D-perosamine is responsible for the cross-reactivity see between S-LPS of smooth

B. abortus and smooth *B. melitensis* strains and the cross-reactivity that is seen with other species (16).

1.9 The characteristics of a variant strain of *Brucella melitensis Rev 1*.

The *Rev.1* vaccine was developed in the 1950s by a two-step selection involving firstly streptomycin resistance and dependence and secondly reversion of dependence but keeping streptomycin resistance. The evidence is presented for the

occurrence of a variant of a vaccine strain of *B. melitensis Rev 1,* designated "FSA" (foreign South African). FSA resembles *Rev 1* in its reactions to penicillin and streptomycin but reacts closer to a field strain of *B. melitensis* with regards to the dyes (thionine and basic fuchsin) sensitivity and colony size. Colonies of *Rev 1* were consistently smaller than other *B. melitensis* strains, their size 0,75 mm as opposed to the 1-2 mm, while *B. melitensis* 16M colonies were 1,25-1,5 mm. *Rev 1* was found to be urease positive, unless a test of low sensitivity was applied.(19, 20). The live attenuated strain *B. melitensis* Rev.1 is considered the best vaccine available for the prophylaxis of brucellosis in sheep and goats. (20). Chromosomally acquired streptomycin resistance is frequently due to mutations in the gene encoding the ribosomal protein S12, *rpsL*. Nucleotide sequencing revealed one mutation in the *rpsL* gene of vaccine strain *Rev.1* compared to that of reference strain 16 M leading

to an amino acid Pro-to-Leu change at codon position 91 (Pro91Leu). This mutation resulted also in the lack of a *Nci*I restriction site in the gene. PCR-restriction fragment length polymorphism (PCR-RFLP) using *Nci*I applied to a large number of *Brucella* reference and field strains showed that the mutation detected was specific of vaccine strain Rev.1 (21).

1.10 Laboratory Diagnosis of *Brucella melitensis*

It is extremely important to follow safety rules when handling specimens for cultivation of *Brucella* which poses significant hazard to clinical laboratory personnel. The diagnosis of brucellosis in the laboratory is primarily based on serology. Rose Bengal test is commonly performed for screening. If positive, subsequent Complement fixation test (CFT) and saline agglutination test (SAT) are performed. Culture for brucellosis is rarely needed to diagnose human infections (22, 16).

Culture for *B. melitensis* is usually done for blood and body fluid samples. Blood cultures should be incubated for total of 21 days. Blind subcultures should be made every 7 days before reporting the culture as negative (23). Rapid detection methods of *Brucella* in clinical samples are considered to be optimal for the identification of infections caused by this organism. Polymerase chain reaction (PCR), enzyme-linked immunosorbent assays (ELISA) and immunofluorescent staining are usually used for rapid detection. PCR is primarily used to detect *B. melitensis* directly in the milk of suspected animals. In addition, this highly sensitive and specific technique can be performed on vaginal swabs obtained from animals who ercentlyhad abortion (24,25,26,27).

1.11 Clinical manifestations of Brucellosis in humans.

1.11.1 Osteoarticular complications

Bone and joint involvement are the most frequent complications of brucellosis, occurring in up to 40% of cases. Patients usually present with fever and back pain, often radiating down the legs (sciatica). Children may refuse to walk. Vertebral osteomyelitis is readily apparent through radionucleide scans. A post-

infectious spondyloarthropathy involving multiple joints has been described, and is believed to be caused by circulating immune complexes (3).

1.11.2 Gastrointestinal complications

B. melitensis is primarily foodborne and transmitted to humans via the consumption of unpasteurized milk and dairy products (as cheese). Foodborne brucellosis resembles typhoid fever. Some patients with the disease experience nausea, vomiting, and abdominal discomfort (2).

1.11.3 Hepatobiliary complications

The liver is commonly involved in brucellosis. Liver function tests can be normal or only mildly elevated. A spectrum of hepatic lesions has been described in cases due

to *B. melitensis*, including scattered small foci of inflammation resembling viral hepatitis. Occasionally larger aggregates of inflammatory cells are found within the liver parenchyma with areas of hepatocellular necrosis. In other cases, small, loosely formed epitheloid granulomas with giant cells can be found. Acute and chronic cholecystitis has been reported in association with brucellosis (2).

1.11.4 Respiratory tract complications

Aerosol inhalation is a recognized route of transmission of brucellosis, especially in abattoirs where infected animals are slaughtered. A variety of pulmonary complications have been reported, including hilar and paratracheal lymphadenopathy, interstitial pneumonitis, bronchopneumonia, lung nodules, pleural effusions, and emphysema (3).

1.11.5 Genitourinary complications

Orchitis and epididymitis are the most frequent genitourinary complications of brucellosis in men. Usually unilateral, *Brucell*a orchitis can mimic testicular cancer or tuberculosis. Although *Brucella* organisms have been recovered from banked human spermatozoa, there have been a few reports implicating sexual transmission. Renal involvement in brucellosis is rare, but it too can resemble renal tuberculosis. In women, rare cases of pelvic abscesses and salpingitis have been reported (12).

1.11.6 Pregnancy and breastfeeding

Brucellosis during the course of pregnancy carries the risk of spontaneous abortion or intrauterine transmission to the infant. Abortion is a frequent complication of brucellosis in animals, where placental localization is believed to be associated with erythritol, a growth stimulant for *B. abortus*. Although erythritol is not present in human placental tissue, *Brucella* bacteremia can result in abortion, especially during the early trimesters (12,11).

1.11.7 Cardiovascular complications

Infective endocarditis is the most common cardiovascular manifestation and most common cause of death from brucellosis. The aortic valve is involved more often than the mitral valve. Mycotic aneurysms, usually involving the middle cerebral artery, can be a neurological complication of infective endocarditis (16).

Neurobrucellosis refers to a variety of neurological complications associated with brucellosis. Direct invasion of the central nervous system occurs in about 5% of cases of *B. melitensis* infection, and meningitis or meningoencephalitis are the most common manifestations. *Brucella* meningitis can be acute or chronic.

Analysis of cerebrospinal fluid (CSF) usually reveals elevated protein content, normal or low glucose concentration, and a lymphocytic pleocytosis. *Brucella* organisms are rarely isolated from CSF, but specific antibodies can be demonstrated in the CSF and serum. Other CNS manifestations of brucellosis include cerebral vasculitis, mycotic aneurysms, brain and epidural abscesses, infarcts, haemorrhage, and cerebellar ataxia. Peripheral nerve complications include neuropathy/radiculopathy, Guillain-Barré syndrome, and a poliomyelitis-like syndrome (12).

1.11.8 Cutaneous complications

A variety of skin lesions have been reported in patients with brucellosis, including

rash, nodules, papules, erythema nodosum, petechiae, and purpura. Occasionally, epistaxis, gingivorrhea, haematuria, and cutaneous purpura occur in association with severe thrombocytopenia, which has been ascribed to hypersplenism, bone marrow

haemaphagocytosis, and/or anti-platelet antibodies (16).

1.11.9 Opthalmic complications

Although uncommon, a variety of ocular lesions have been reported in patients with

brucellosis. Uveitis is the most frequent manifestation, and can present as chronic iridocyclitis, nummular keratitis, multifocal choroiditis or optic neuritis. Since Brucella organisms have not been isolated from the structures of the eye in humans, many of these lesions are considered to be late complications, possibly immunologically mediated (16).

1.11.10 Chronic brucellosis

Chronic brucellosis should be reserved for patients whose clinical symptoms persist for 12 months or more from the time of diagnosis. Using this criterion, patients fall into three categories: (1) relapse, (2) chronic localized infection, and (3) delayed

convalescence. Relapse is defined as the recurrence of characteristic signs and symptoms occurring at some time after the completion of a course of treatment. Patients with relapse characteristically have objective signs of infection, such as fever, and persistently elevated titers of IgG antibodies in their serum. Most relapses occur within six months after therapy, and relapse is not usually due to the emergence of antibiotic resistant strains (12).

1.12 Treatment

Brucellae are sensitive to a number of oral antibiotics and to aminoglycosides in vitro. Therapy with a single drug has resulted in a high relapse rate. Therefore,

treatment with multiple antibiotics is encouraged. Combinations of doxycycline and streptomycin, rifampin and doxycycline, rifampin, streptomycin, and doxycycline, and rifampin with trimethoprim/sulfamethoxazole are commonly used for effective treatment of brucellosis and its manifestations. World Health Organization Expert Committee recommends the treatment of pregnant women with rifampin (12).

1.13 Vaccination

The Rev.1 live *Brucella melitensis* vaccine is the most widely used vaccine in control programs against brucellosis in small ruminants. When properly used, the Rev.1 vaccine confers a long lasting protection against field infections in a high proportion of animals. This vaccine however shows a considerable degree of

virulence and induces abortions when the first vaccine dose is administered during

pregnancy (28, 29).

The antibody response to vaccination cannot be differentiated from the one observed after field infection, and this therefore impedes control programs. Attempts have been made to develop new vaccines based on “rough” (R) strains or genetically modified strains of the *Brucella* species. Those vaccines await further evaluation in

field experiments. Control programs focus on the proper application of vaccination as an indispensable step to eradicate *Brucella melitensis* Control programs require a well functioning surveillance system, the co-operation with livestock owners and considerable financial support. The appropriate application of vaccination will result in suppression of the infection pressure and has been shown to reduce the zoonotic spread of the disease (29).

1.14 Outline of the study

Reported cases of brucellosis in Palestine are mainly diagnosed by serology tests and culture followed by identification by of phenotypic tests. However, up to now there is no data about the local circulating *Brucella* strains in comparison with the strains used in vaccine production. Hence, it is of epidemiological interest to see whether the allelic frequency in Palestinian *Brucella* circulating strains is similar to the one reported in other Mediterranean countries and to determine if vaccination selects for strains that have different genotypes from those used to make the vaccine, allowing the spread of escape mutants to the vaccine strain. Therefore, the current study was conducted,

1. To study the seroprevalence of brucellosis in sheep and goats suspected to have infected with Brucellosis by detecting antibodies in serum employing RBT and comparing it with CFT.

2. To isolate and identify *Brucella* from milk with confirmation by molecular methods.

3. To genetically characterize representative isolates of *B. melitensis* related to the Rev 1 vaccine strain used here and isolates from different Mediterranean countries.

CHAPTER II

MATERIALS AND METHODS

2.1. Area of study

The West Bank is a landlocked territory and the eastern part of the Palestinian territories was chosen for the study (Figure 2.1). The geographical location of the West Bank between the 31°21` and 32°33` latitude and between 34°52` and 35°32` longitude, makes the area highly influenced by the Mediterranean climate. The Mediterranean climate is characterized by a long, hot, dry summer and short, cool, rainy winter. Rainfall is limited to the winter and spring months. It usually starts in the middle of October and continues up to the end of April. Snow and hail, although uncommon, may occur anywhere in the area especially to the west of and over the highlands. The total animal population in this area according to last statistic Ministry of Agriculture and directorate veterinary services and animals health 2009 are 800.000 sheep and goats and 4886 bovines.

Figure2.1 Map of West Bank, Palestine

2.2. Subjects and study

This study on serological, cultural and molecular detection of *Brucella* infection. It was performed at the Master program in Clinical Laboratory Science, Birzeit University, Palestine between January and December, 2008. A total of 350 sera samples comprising of 302 from sheep, 48 from goats were collected from five different districts in the West Bank, Palestine. Eighty pooled milk samples were

also included in this study (Table 2.1). None of the animals were vaccinated against *Brucella*. Also, these samples were also collected from the flocks when owners were infected by Brucellosis according to report by Ministry of Health.

District	Samples					
	Serum			Milk		
	Sheep	Goat	Total	Sheep	Goat	Total
Hebron	180	22	202	29	6	35
Jericho	47	4	51	25	10	35
Ramallah	35	7	42	2	1	3
Nablus	20	5	25	2	0	2
Jenin	20	10	30	3	2	5
Total	302	48	350	61	19	80

Table 2.1: Distribution of the serum and milk samples collected from sheep and goats flocks in different districts of the West Bank.

2.3. Collection of samples

2.3.1. Animal sera

About 5 to 10 ml of blood was collected aseptically from each animal from the jugular vein in a plain tube without EDTA. It was essential to avoid the shaking of the tubes (which contain blood) during transport to prevent hemolysis of the RBCs. The serum was screened immediately for *Brucella melitensis* by the Rose Bengal

Plate Test (RBPT) as a screening test, and Complement Fixation Test (CFT) as the confirmatory test. If either of these tests were positive, the animal was considered infected with brucellosis.

2.3.2. Milk samples

Sheep and goat milk samples were collected in a sterile plastic vial in the morning. The milk samples were immediately cultured on *Brucella* agar medium (Oxoid).

2.4. Laboratory Procedure

2.4.1. Rose Bengal Plate Test (RBPT)

The Rose Bengal Plate Test antigen for *Brucella* was obtained from Jordan Bio-Industries Center (JOVAC) Jordan. The test was performed by a rapid slide screening method, according to manufacturer's instruction. Before performing the test, antigen and sera were brought to room temperature and aliquots of 30 µl of the serum were placed on a glass slide by micropipette. The antigen bottle was shaken well to ensure homogenous suspension and then one drop (30 µl) of Rose Bengal antigen was added. The antigen and serum were mixed thoroughly with the spreader and then the slide was rotated for four minutes. Definite clumping (agglutination) was considered as positive reaction (Figure 2.2). Positive and negative controls were used with each test plate (30, 31).

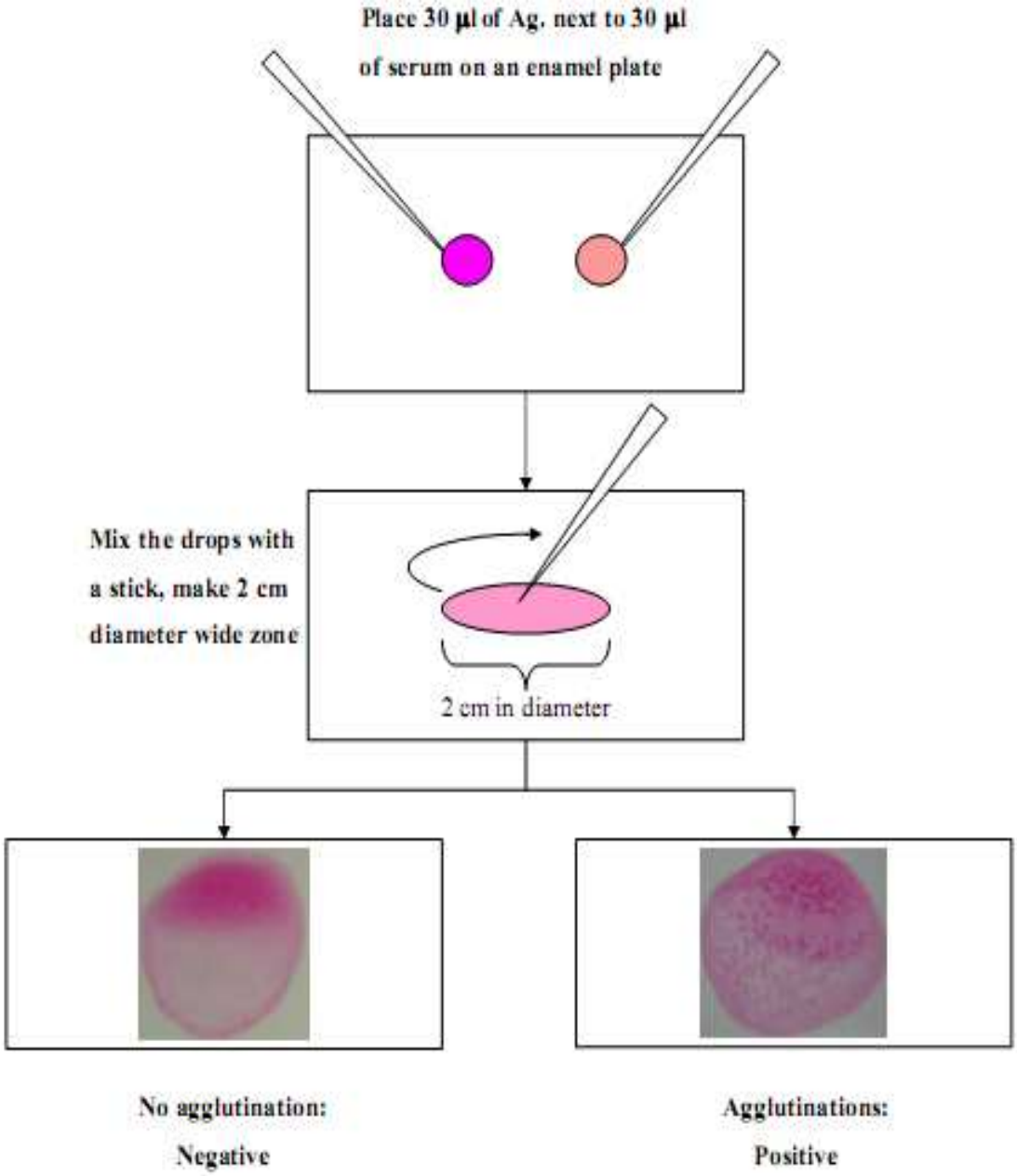


Figure 2.2 Rose Bengal Plate Test (RBPT)

2.4.2 Complement Fixation Test (CFT)

The principle of in the complement fixation antigen-antibody reaction involved binding of the complement. Absence of antibody in animal's serum leaves the added complement unfixed. The addition of an indicator, consisting of sheep red

blood cells sensitized with hemolysin (specific antibody to sheep red blood cells), to the reaction well enables any residual complement to be detected and visualized by the lysis of sheep red blood. Absence of complement and therefore presence of antibody in the animal's serum is visualized by the sheep red blood cells remaining contact (Figure 2.3).

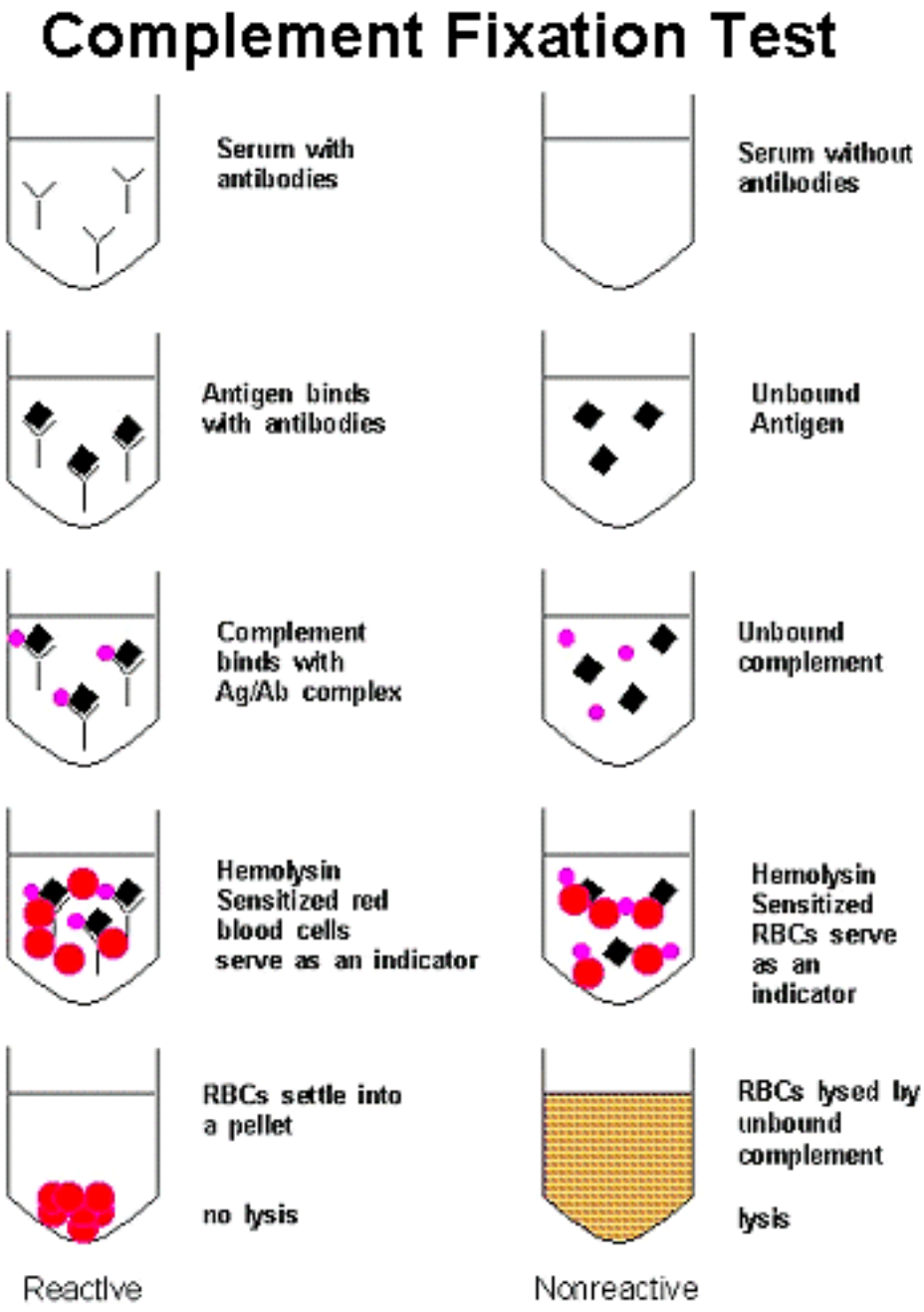


Figure 2.3: The principle of the Complement Fixation Test

2.4.2.1 Hemolysin titration

To determine the minimum concentration required for 100% lysis of the sensitized sheep red blood cells, the Minimum Hemolytic Dose (MHD), the following procedure is used: An aliquot of 25 µl of veronal buffer saline (Biomerieux) was placed in each well of columns 2 -12 of the 96-well U-bottom microtitre plates (Figure 2.4). An aliquot of 50 µl of diluted hemolysin (Biomerieux) solution in veronal buffer saline in a ratio 1:250 was placed in each well of the first column, followed by twofold dilution. Then 25 µl of complement (Biomerieux) was added to each well in the microtitre plate followed by the addition of 25 µl of 2% suspension of red blood cells (RBC).

The plate was then incubated at 37°C for 30 minutes and subsequently checked for agglutination. The working solution of the hemolysin is considered as two folds higher than the observed agglutination.

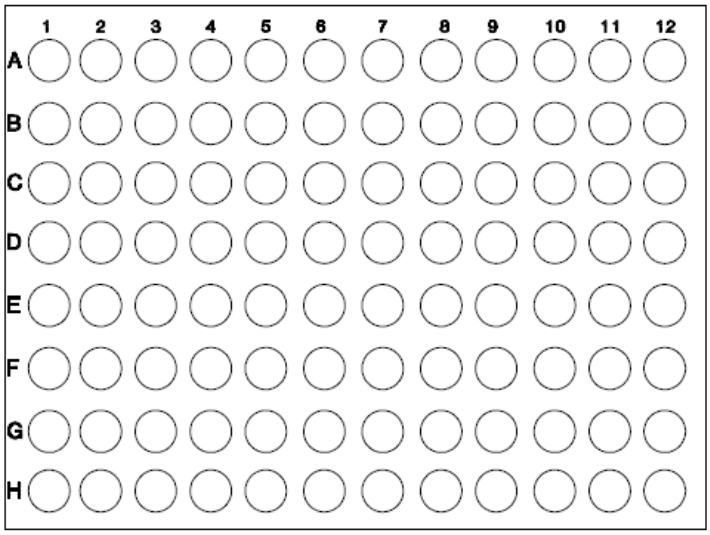


Figure 2.4: Orientation of the microtitre plate

2.4.2.2 Complement Fixation Test Method

Serum samples, positive and negative standards were diluted 1:5 in Veronal buffer saline and placed in a water bath at 56°C for 30 minutes. An aliquot of 25 µl of Veronal buffer was added to all wells of the 96-well U-bottom microtitre plates. In the first column, 25 µl of the inactivated samples was added to wells of the first column, followed by a 2 fold dilution. An aliquot of 25 µl antigens (1:500 dilutions) and complement (1:30 dilution) was added to all wells of plate and incubated for 30 minutes at 37°C. A mixture of hemolysin working solution and 2% RBC solution was mixed in equal amounts and prepared 15 minutes before the end of the incubation of the previous step. An aliquot of 25µl of this mixture was added to all wells of the plate followed by an incubation period of 30 minutes at 37°C. The plate was shaken three times during this incubation period. The plate was then removed from the incubator and placed at room temperature for a total of two hours before interpretation. Wells that show complete lysis of the sensitized red blood cells were considered negative while wells with sedimentation of the sensitized red blood cells were considered positive (Figure 2.5) (29)

	1	2	3	4	5	6	7	8	9	10	11	12
A	+ ve Control											
B	- ve Control											
C	Sample 1											
D	Sample 2											
E	Sample 3											
F	Sample 4											
G	Sample 5											
H	Sample 6											
	(1:5)	(1:10)	(1:20)	(1:40)	(1:80)	(1:160)	(1:320)	(1:640)	(1:1280)	(1:2560)	(1:5)	(1:10)

50 µl Sample(S) 25 µl VBD 50µl S 25µl S

Transfer 25 µl of sample from col. 1 – 10 & discard 25 µl from col. 10

Transfer 25µl of sample from col. 11–12 & discard 25µl from col. 12

25 µl of diluted antigen 25 µl VBD

50 µl of diluted complement

50 µl of hemolytic system

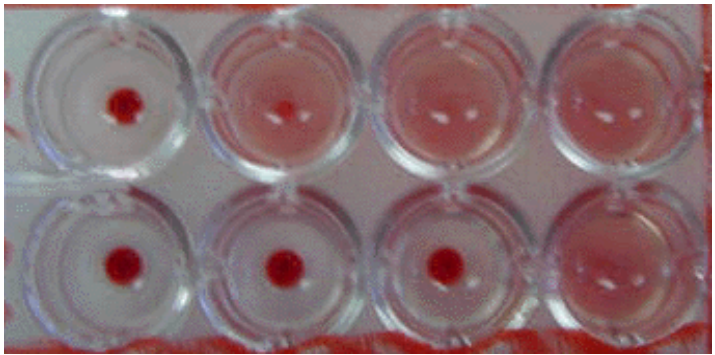


Figure 2.5 Complement Fixation Test in Microtiter Plate

.4.3. Isolation and Identification of *Brucella* Culture from Milk samples

The milk collected from seropositive flock and positive control, Rev 1 vaccine strain, were cultured on *Brucella* agar (Oxoid) supplemented with *Brucella* supplement (Oxoid) and 7% sterile horse serum (Oxoid). Growth appeared after 24 hours for the control strain while it took 3 to 7 days for the cultured milk. The isolates were identified as *Brucella* according to colonial and microscopic morphology, oxidase (Oxoid) positive and urease (Oxoid) positive.

2.4.4. Genomic DNA Preparation

DNA was extracted from both positive milk cultures and collected milk samples from seropositive animals using DNA extraction Nucleospin kit (Promega, Germany) according the protocol suggested by the manufacturer. An aliquot of 200 µl of milk or a suspension of 200 µl of bacteria was mixed with 180 µl pre-lysis buffer (T-1) and 50 µl proteinase K, vortexed and placed in a dry bath at 56ºC for 3 hours, then 200 µl of lysis buffer (B-3) was added, vortexed and incubated at 72ºC for 10 min in a dry bath as before. Subsequently, 200 µl of ethanol was added, to make the DNA float. The DNA sample was placed in the tube within the column where the DNA will be bound. The tube was then centrifuged for 1 min at 8000 rpm, washed twice with 500 µl of washing buffer (B5). The membrane in the tube was dried by centrifugation for 1 min at 14 000 rpm and then the DNA sample was eluted by adding 50 µl elution buffer (BE buffer) followed by placing the filter in a sterile collection tube and centrifuged for 1 min at 8000 rpm where the DNA was

collected in the sterile collection tube.

2.4.5. Primers

A set of the primers used in the PCR reactions is summarized in Table 2.2. Sequences for primers IS711G, IS711, BM and BMR7521 were obtained from the literature (37, 27, 28). DNA sequences of primers for *Brucella* used in DNA sequencing were constructed via Search for open reading frames (ORF 1) and putative gene used in the DNA sequence of *Brucella melitensis* was performed with a computer-assisted program (Primer3 Output; http://frodo.wi.mit.edu/primer3). The primer sequence was selected with the following properties: 20-25 nucleotides starting with at least one G or C at the 5', terminated with GC at 3'end and the GC content of about 50%.

2.4.6 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) amplification was performed using a MasterCycler (BioRad Laboratories, Inc., Hercules, CA). The DNA was amplified using the primers listed in Table 2 below. Each reaction mixture was prepared by placing 12.5 µl master mix with loading dye obtained from Promega, Germany (0.5 U Taq polymerase, 10 mMTris – HCl, 3 mM MgCl2, 50 mMKCl, 0.1% Triton, 200 mM and the four nucleotides), 6.5 µl nuclease free PCR water (Qiagen, Germany), 0.5 µl 20 nmol forward primer and 0.5 µl 20 nmol reverse primer (Syntezza, Israel) and 5 µl DNA template. PCR amplification was performed as summarized in Table

2.3.

Primer name	Oligonucleotide sequences (5'-3')	Amplified product (bp)	Target species

IS711G	F- GGT TGT TAA AGG AGA ACA GC	600 bp	*Brucella* spp.
	R – GAC GAT AGC GTT TCA ACT TG		
IS711B	F – TGC CGA TCA CTT AAG GGC CTT CAT	731 bp	*B.melitensis*
M			
	R – AAA TCG CGT CCT TGC TGG TCT GA		
BMR752	F - CAG GCA AAC CCT CAG AAG C	752 bp	*B.melitensis*
	R - GAT GTG GTA ACG CAC ACC AA		*Rev.1*
ORF 1	F – GAA CCA GAA TAC GGC AAA A	2 kb	DNA sequence
	R – CTC ACG GCT GTT CTC CTT TAA CA		

Table 2.2: The sequences of primers for *Brucella* used in this study

	Cycling conditions			
Primers	**Initial denaturation**	**Denaturation**	**Annealing**	**Extension**
IS711G	95ºC, 120 s	95ºC, 30 s	51ºC, 45 s	72ºC, 30 s
IS711BM	95ºC, 120 s	95ºC, 30 s	51ºC, 45 s	72ºC, 30 s
		Repeated for 35 cycles		
ORF 1	95ºC, 120 s	95ºC, 30 s	51ºC, 45 s	72ºC, 30 s
BMR752	95ºC, 120 s	95ºC, 30 s	51ºC, 45 s	72ºC, 30 s
		Repeated for 35 cycles		

Table 2.3 Conditions of thermal cycling for different primer pairs in PCR

2.4.7. Agarose Gel Electrophoresis

DNA products generated via PCR were mixed with 2 µl of agarose gel loading dye and separated on a 2% agarose (Promega, Germany), run at 100 V in a gel electrophoresis unit, BioRad, Germany until the dye front was approximately 1 inch from the bottom of the gel. A 100-bp DNA ladder molecular weight standard was visualized on gels to determine the size of linearized DNA samples

being tested. Gels were incubated in 1.0 µg/ml ethidium bromide for 5-8 minutes and destained in dH_2O for 30 minutes. Gels were photographed on a 392-nm wavelength transilluminator **(**BioRad, Germany) and the resultant bands were recovered using a Polaroid Gel Documentation System.

2.4.8. PCR Purification procedure for sequencing

This step was essential to eliminate undesirable components that may be present and to obtain a clean concentrated product adequate for sequencing. Purification of the positive PCR products was conducted by using the MinElute PCR purification kit (Qiagen, Germany). The purified PCR products were then re-amplified in a total volume of 50 µl instead of the 25 µl previously used to get reasonable quantities adequate for sequencing.

In brief, 250 µl of sodium iodide was added to 50 µl of the positive PCR product. The mixture was then gently vortexed and added to the DNA binding column, centrifuged to elute unbounded components and washed twice with the washing buffer included in the kit. Bound DNA (PCR product) was eluted with 10 µl ddH2O. Agarose gel electrophoresis was then carried out using 2 µl products to ensure purity and concentration prior to sequencing.

2.4.9. DNA sequencing

Sequencing of the purified PCR products was performed at Bethlehem University using the sequencer ABI PRISM 301 Genetic analyzer. Sequencing was performed for both the reverse and forward primer in each reaction with a mix of labeled nucleotides (Big Dye Terminator V 1.1).

2.4.10. DNA analysis.

The open reading frames (ORFs) sequences of the two representative *B. melitensis* isolates and the vaccine strain (*B.melitensis* Rev.1*)* were compared to the Genbank sequences of reference *Brucella* with gaps treated as "missing", as per the PAUP rule. Sequence comparisons to reference *Brucella* were conducted using CLC Main Workbench software; (Website, www.clcbio.com, version 5.6.1, 2009) Alignments of individual *Brucella* sequences were created using the ClustalX program (BLAST). The phylogenetic relationships between the *Brucella* isolate and selected reference *Brucella* were estimated from their nucleotide sequences by employing the neighbour-joining algorithm in the program NEIGHBOR in the Phylip program package version 3.52. Bootstrap analysis on 1000 replicas was performed using the SEQBOOT and CONSENSE programs in the Phylip package**.**

2.5 Statistical analysis

To compare the sensitivity and specificity between the tests used in our study, the statistical formula given by Samad *et al.* (33) was used as described below.

Test		**Gold standard test**		**Total**
		Positive	**Negative**	
The test Positive to be compared	Positive	A	B	A+B
	Negative	C	D	B+D
	Total	A+C	B+D	A+B+C+D

The notations used above are defined as.

A: Number of samples positive to both conventional and the gold standard tests

B: Number of samples positive to conventional but negative to the gold standard test

C: Number of samples negative to conventional but positive to the gold standard test

D: Number of samples negative to both conventional and the gold standard tests

A+B+C+D = Total number of samples

Sensitivity: It is the capacity of the test to detect diseased animals, when compared with the gold standard test (A/A+C x 100).

Specificity: It is the capacity of the test to detect non-diseased animals, when compared with the gold standard test (D/B+D x 100)

CHAPTER III

RESULTS

3.1. Serological Results

Brucellosis is a zoonotic infection with a variety of clinical pictures, and may be confused with a number of other illnesses in diagnosis. Thus, various serological tests are employed with varying degree of sensitivity and specificity. Isolation of organisms is tedious, cumbersome and time consuming thus it is generally not being followed in routine diagnostic laboratories. In the present study, the sera of 350 animals with clinical suspicion of Brucellosis comprising of 302 from sheep and 48 from goat collected from five different districts in the West Bank, Palestine were analyzed by Rose Bengal Plate Test (RBPT) and Complement Fixation Test (CFT). Culture and PCR techniques were used for detection of *Brucella* organisms and *Brucella* DNA, respectively, in the milk samples. The percentage of the positive brucellosis sera was found to be as follows: 31 % in

sheep sera and 52% in goats sera by RBPT, giving overall seroprevalence of 34 %. Whereas the overall seropositivity using CFT was 31%, 29% in sheep sera and 44% in goats sera (Table 3.1)

Animal sp.	Total samples	RBPT		CFT	
		Positive samples	(%)	Positive samples	(%)
Sheep	302	94	31	87	29
Goats	48	25	52	21	44
Overall	350	119	34	108	31

Table 3.1 The percentage of the positive brucellosis in serum samples from different animals by RBPT and CFT

To find out relative sensitivity and specificity of RBPT and CFT, cross tabulation of results of RBPT with that of CFT, considering CFT as a gold standard test, are shown in Table 3.2.

Test		**CFT**		**Total**	**Sensitivity (%)**	**Specificity (%)**
		Positive	**Negative**			
RBPT	Positive	102	27	129	90.3	89
	Negative	11	210	221		
	Total	113	237	350		

Table 3.2 Evaluation of RBT in comparison to CFT

3.2. Isolation and Identification of *Brucella*

Milk samples collected from seropositive animals were subjected to bacteriological examination. *Brucella* organisms were detected in milk samples collected from 38 (47.5%) 80 seropositive animals only. All cultures were typical of isolates of

Brucella in morphology, colonial appearance and growth characteristic on *Brucella* agar medium. The round, glistening and smooth or mucoid colonies on plates of BAM were suspected to be of *Brucella* (Figure 4.1). All the isolates were oxidase positive and urease positive.

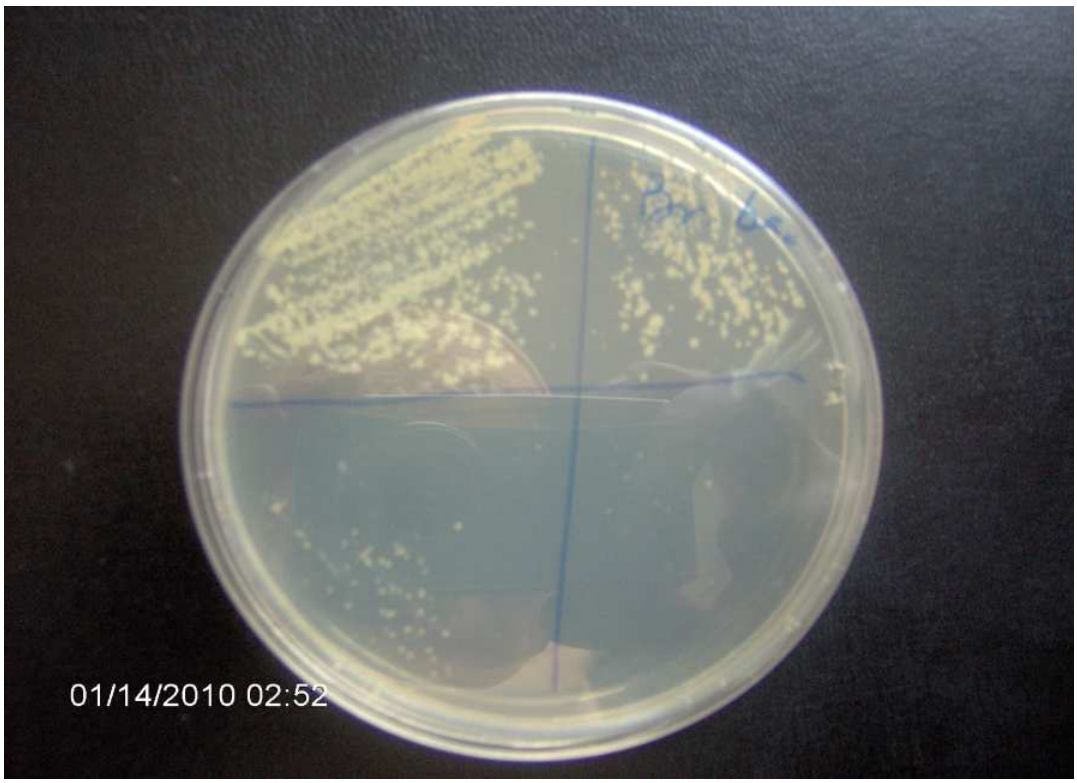


Figure 3.1 *Brucella* agar plate growing *Brucella*

3.3. Testing of milk samples with *IS*711 PCR

IS*711* elements, also known as IS*6501*, have been described as useful targets for molecular characterization of *Brucella* species and biovars. All species of the

Brucella genus contain several copies (between 10 and 40) of an insertion sequence,

IS711. The position of copies of this insertion sequence appears to differ in each species and this can be used to discriminate between them. A new polymerase chain reaction test, called IS711 PCR was developed. It was based on a combination of a primer bound on the sequence of I IS711 with a second primer chosen arbitrarily. The patterns obtained reflect the position of the insertion sequence in the genome. By testing the milk samples collected in our study, all 34 positive (42.5%) milk samples detected by bacterial isolation were also detected by IS*711* PCR. Furthermore, with the IS*711* PCR, we were able to additionally detect 24 (30%) of the samples that were negative by the bacterial isolation method. Table 3.3 shows the number of positive milk samples detected by bacterial isolation and IS*711* PCR. *Brucella* species-specific DNA fragments with 600 bp were amplified from all isolates and no DNA was observed in negative control samples (Figure 3.2)

Method	Total milk samples	Positive samples detected

		No.	%
Bacterial isolation	80	38	47.5
IS*711* PCR	80	62	77.5

Culture		**PCR from cultured bacteria**				**PCR from milk**					
		Brucella group		***B. melitensis***		***B. group***		***B.melitensis***		***B.abortus***	
No	%	No	%	No	%	No	%	No	%	No	%
38/80	47.5	38/38	100%	38/38	100%	62/80	77.5	42/80	52.5	29/80	36.3

Table 3.3. Detection of *Brucella* spp. in milk samples by bacterial isolation and IS*711* PCR

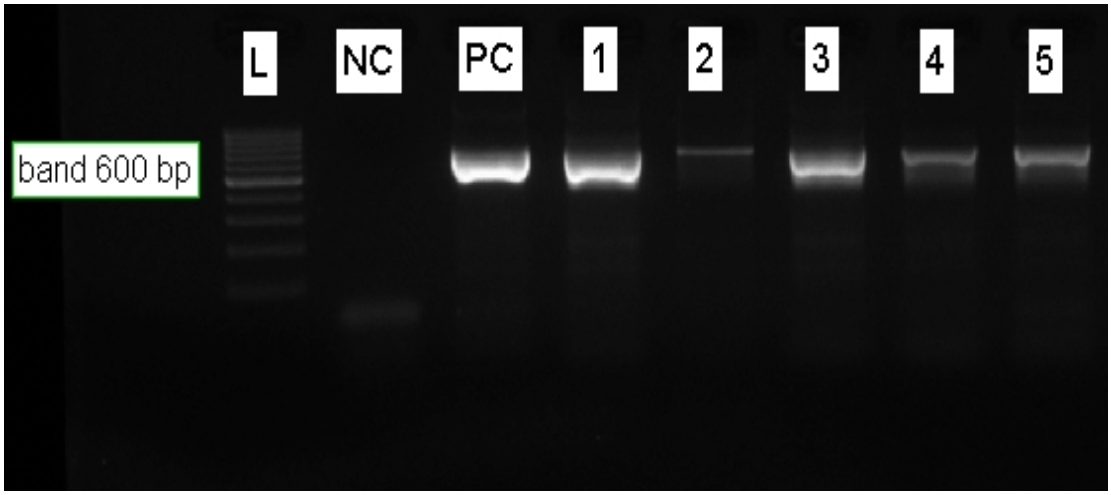


Figure 3.2 Representative IS*711* PCR profiles of DNA from 5 *Brucella* isolates. Lane L, molecular sizes marker (100-bp ladder DNA); NC, Negative control contained no template; PC, Positive control; Lane 1-5, *Brucella* species

To find out relative sensitivity and specificity of *Brucella* culture and PCR, cross tabulation of results of culture with that of PCR, considering PCR as a gold standard test, are shown in Table 3.4. Sensitivity of culture method was found to be of 61.3%, with considering IS711 PCR as a gold standard test while specificity was found to be of 100.0%.

Test	**PCR**		**Sensitivity**	**Specificity**

		Positive	Negative	Total	(%)	(%)
Cult ure	Positive	38	0	38	61.3	100
	Negative	24	18	42		
	Total	62	18	80		

Table 3.4: Evaluation of RBT in comparison to CFT.

B. melitensis was isolated from 42 out of 80 (52.5%) milk samples examined in this study. The identification was performed by PCR utilizing primers specific to IS*711* gene of *B. melitensis. B. melitensis*-specific DNA fragments with 731 bp were amplified from all isolates and no DNA was observed in negative control s

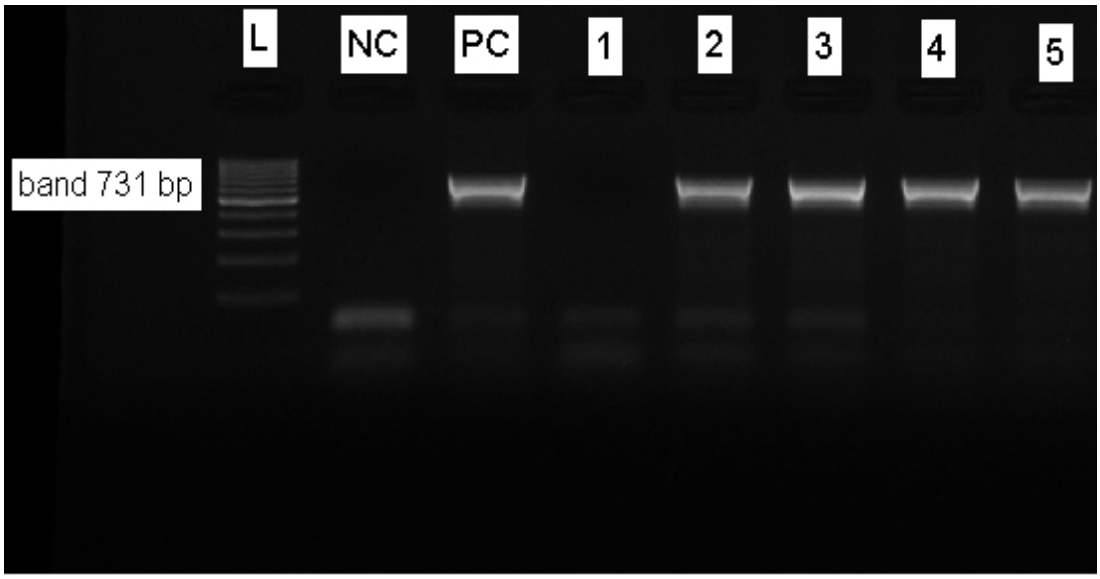
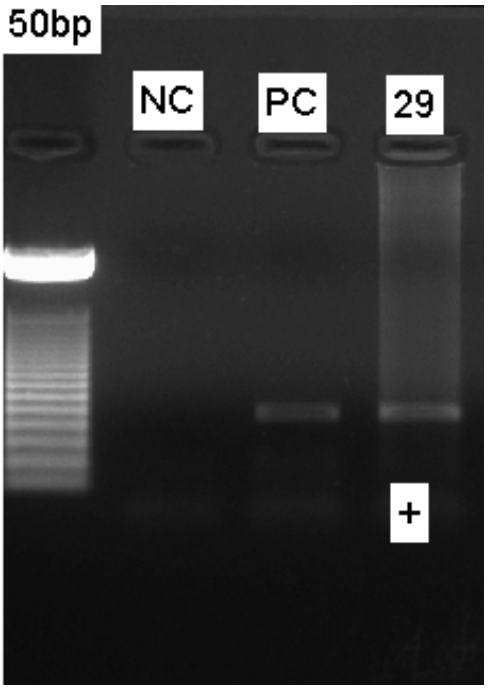


Figure 3.3).

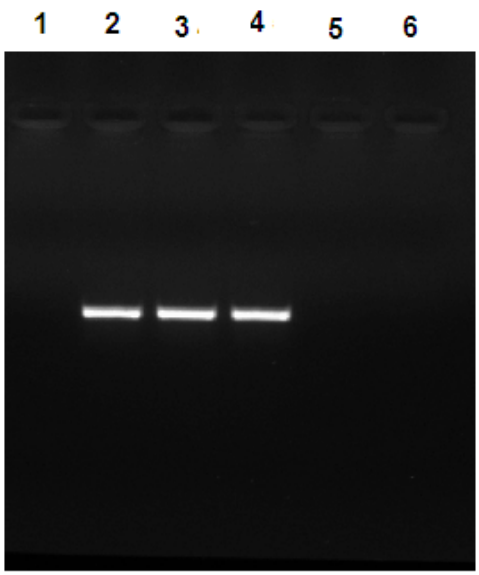
Figure 3.3 Representative PCR profiles of DNA from 5 *Brucella melitensis* isolates using primer of *B. melitensis*. Lane L, molecular sizes marker (100-bp ladder DNA); NC, Negative control contained no template; PC, Positive control; Lane 1-5, *Brucella* species



B. abortus was distinguished by amplification of a specific 240-bp fragment in 36% (29/80) of the milk samples. A representative example of the PCR with *B. abortus* DNA results is presented in Figure 3.4.

Figure 3.4 PCR profile of DNA from a representative *B. abortus* isolate using primer of *B. abortus*. Lane L, molecular sizes marker (100-bp ladder DNA); NC, Negative control contained no template; PC, Positive control; Lane 29, *Brucella* species

Distinguishing vaccine strains from strains that cause infections among vaccinated herds in the field is essential. To accomplish this, our PCR-based, species-specific assay was used to identify *Brucella melitensis* vaccine strains Rev 1 using primers specific for Rev1. These primers were selected on the basis of polymorphism which occurs at a locus containing the genetic element IS*711* arising. This new primer was designed to differentiate Rev 1 from other *B.*



melitensis strains by PCR amplification of an additional 752-bp product. Our data confirmed the expected paradigm for *B. melitensis* strains and the vaccine strain Rev.1. All our *B. melitensis* field isolates were different from the vaccine strain Rev.1 by the absence of the 752-bp fragment (Figure 3.5).

Figure 3.5 Identification and differentiation of all *B. melitensis* and *B. melitensis* Rev1 vaccine strains by PCR. Lane 1, Negative control; Lane 2-4, *B. melitensis* Rev1; Lane 5-6, *B. melitensis* field strain

3.4. Genetic analysis

3.4.1. *B. melitensis* field isolates and commercial vaccine strain *Rev.1* alignment.

Two of the *B. melitensis* isolates and the vaccine strain *B. melitensis* Rev1 were used for genetic strain phylogeny. For this purpose, Hemagglutunin (HA) gene was PCR-amplified from two field isolates and the vaccine strain *B. melitensis* Rev1 using the primers ORF-1F and ORF-1R. The amplified PCR products were subsequently sequenced (Figure 3.6,a b, c) at Bethlehem University using the Genetic analyzer (sequencer) ABI PRISM 301. The primers used for PCR are indicated in Table 2.2.

Figure 3.6, a. DNA sequence of *B. melitensis* isolate number 1

Figure 3.6, b. DNA sequence of *B. melitensis* isolate number 2

Figure 3.6, c. DNA sequence of *B. melitensis* Rev1

The ORFs sequences of the two field isolates were aligned and compared with other GenBank-accessible gene sequences of *B. melitensis* and other *Brucellae* spp. using version 2.0 of BLAST. These sequences were identical to that of the recent sequence of type strain of *B. melitensis* (ATCC 23456, GenBank access no: CP001489.1 and AE008918.1),confirming that the isolate belonged to the genus

Brucella and were also corresponding to the *Brucella* hemagglutinin or Glycoprotein X precursor gene sequences. Several other GenBank-deposited sequences of *B. melitensis* biovar/strain, *ovis*, *suis*, and *canis* were also found to be identical to isolate sequences of the current study. (Table 3.5).

Accession	Description	E value*	Identity (%)
CP001489.1	*Brucella melitensis* ATCC 23457 chromosome II, complete sequence	0.0	95%
AE008918.1	*Brucella melitensis* 16M chromosome II, complete sequence	0.0	95%
CP000709.1	*Brucella ovis* ATCC 25840 chromosome II, complete sequence	0.0	95%
CP000912.1	*Brucella suis* ATCC 23445 chromosome II, complete sequence	0.0	95%
CP000873.1	*Brucella canis* ATCC 23365 chromosome II, complete sequence	0.0	95%
AE014292.2	*Brucella suis* 1330 chromosome II, complete sequence	0.0	95%

*Expect value; The lower the E-value, or the closer it is to zero, the more "significant" the match is.

Table 3.5 Genetic homology of the two *B. melitensis* isolated in Palestine with related sequences available in GenBank.

In Palestine, whole cell *Brucella* vaccine is still the only kind of *Brucella* vaccine being used. Up to now there is no data about the local circulating *Brucella* strains in comparison with the strains used in vaccine production. Hence, it is of epidemiological interest to see whether the allelic frequency in Palestinian *Brucella* circulating strains is similar or not to the one reported in the other Mediterranean countries and to determine if vaccination selects for strains that have different genotypes from those used to make the vaccine, allowing the spread of escape mutants to the vaccine strain. Therefore, the ORFs sequences of hemagglutinin gene of two *B. melitensis* field isolates isolates were Clustal

aligned and compared with the vaccine strain Rev.1 using version 5.6.1 of CLCL Main Workbench. A good alignment was observed between the two *B. melitensis* isolates and the vaccine strain (Figure 3.7).

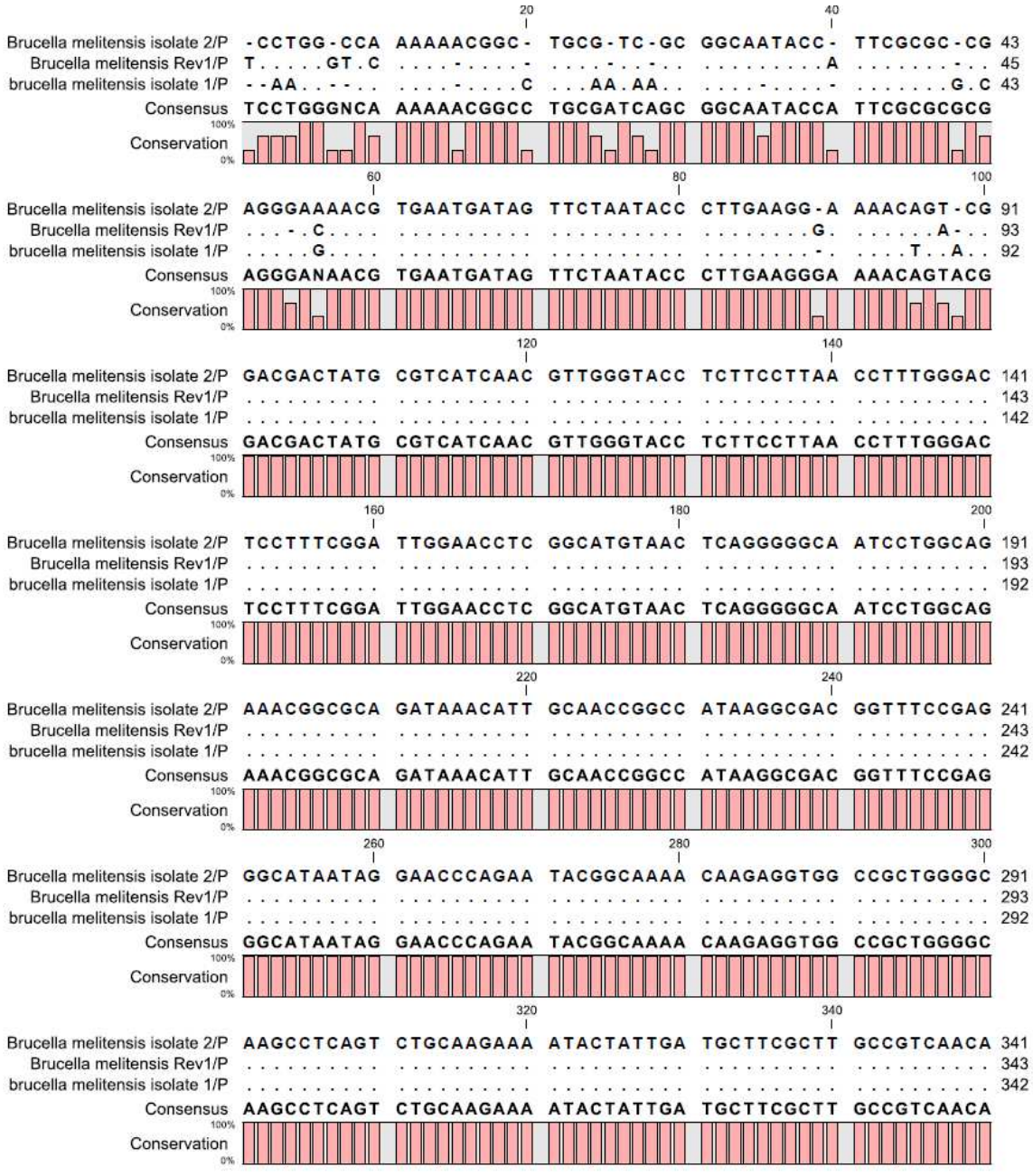


Figure 3.7. Nucleotide comparison between ORFs of the homology gene of *B. melitensis* field isolates and the vaccine strains. Dots represent nucleotides similar to the consensus.

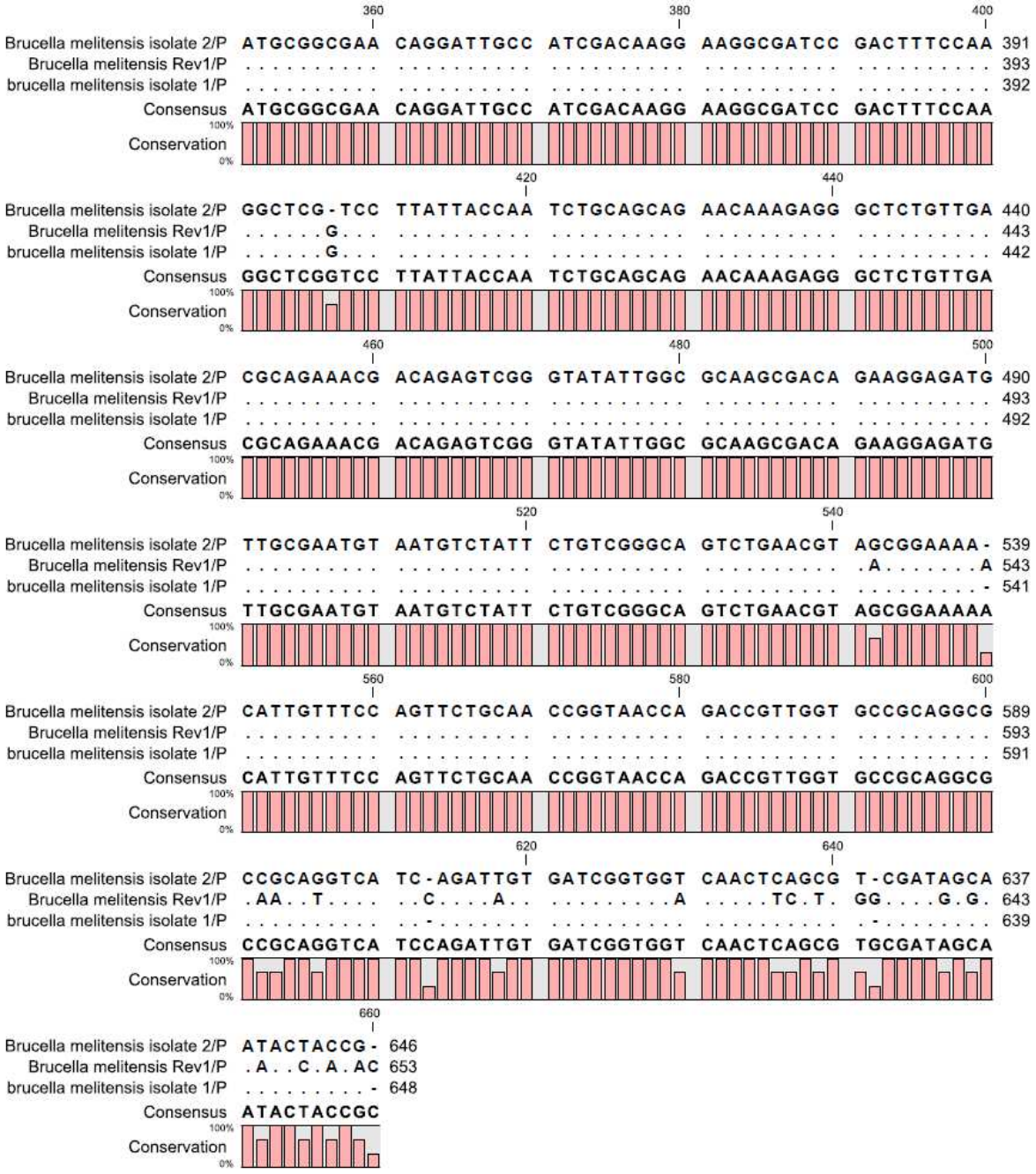


Figure 3.7. (Continued).

3.4.2. Restriction analysis

We analyzed the *Pst*I digestion profiles of the ORFs sequenced fragment of hemagglutinin gene obtained from the field isolates and that obtained from vaccine strain using version 5.6.1 of CLCL Main Workbench. As can be seen in

Figure 3.8 and Table 3.6, *Pst*I digestion of the field isolates (lines 2 and 3) produced a uniform identical to that obtained for vaccine strain Rev.1, however, The *Hae*III digestions of the amplified fragments from the vaccine strain Rev.1 yielded a different pattern (line 2).

Sequence	Name	Pattern	Length	Overhang	Number of cut sites	Cut position(s)
Brucella melitensis isolate 1/P	PstI	ctgcag	6	3'	2	418, 910
Brucella melitensis isolate 2/P	PstI	ctgcag	6	3'	1	416
Brucella melitensis Rev1/P	HaeIII	ggcc	4	Blunt	2	221, 283
Brucella melitensis Rev1/P	PstI	ctgcag	6	3'	1	419

Table 3.6. Restriction site analysis of the ORFs of *B. melitensis* field isolates and the vaccine strains.

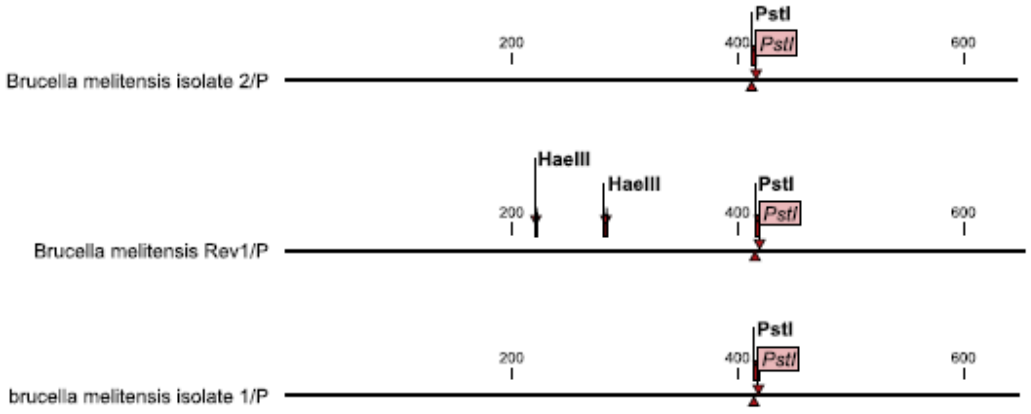


Figure 3.8. Restriction map of the ORFs of *B. melitensis* field isolates and the vaccine strains.

3.4.3. Phylogenetic analysis

The results of a maximum likelihood phylogenetic analysis by creating a Clustal tree using version 5.6.1 of CLCL Main Workbench of the two *B.melitensis* field isolates with other GenBank-accessible gene sequences of *B. melitensis* isolated from several Mediterranean countries are shown in Figure 3.8. This tree sorts the *Brucella* sequences studied here into three lineages (1-3). Each node received 100% bootstrap. Although the tree is technically branching, the shortness and suboptimal support of those branches in the lineage number 1 suggest a higher average of similarity between Palestine/Israel/France/Spain *Brucella* strains; they appear to have radiated explosively. The tree generated (Figure 3.9) also shows, as expected, that strain with an accession number AY518304 which belongs to Israeli strains is the closest relative to our *Brucella* field isolates.

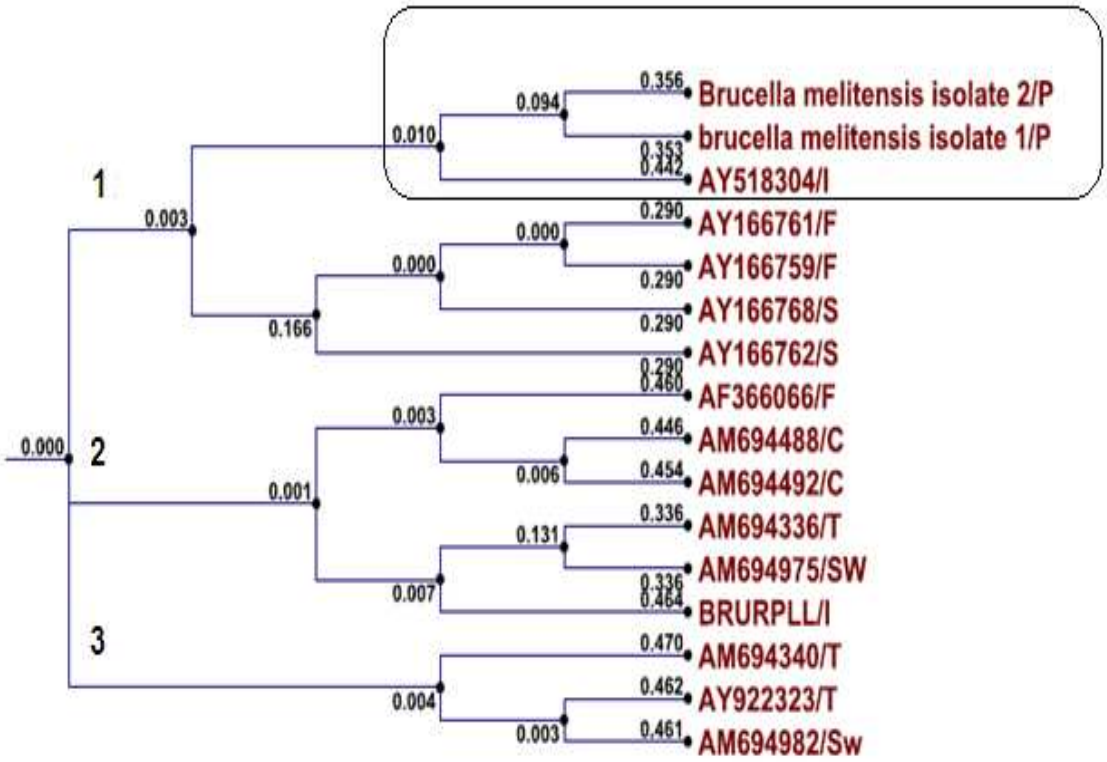


Figure 3.8. A distance phylogenetic tree based on the partial nucleotide sequences of the ORFs sequences of two selected *B. melitensis* field isolates and GenBank-accessible gene sequences of *B. melitensis* isolated from several Mediterranean countries. Horizontal distances are proportional to genetic distance. P; Palestine; I, Israel; F, France; S, Spain; C,Cyprus, T, Turkey; SW, Switezerland. The tree is rooted to version 5.6.1 of CLCL Main Workbench.

CHAPTER IV

DISSCUSSION

Brucellosis is a major bacterial zoonosis of global importance (3). The disease is manifested by abortion and infertility and caused by *Brucella* species Gram-negative facultative intracellular bacteria. Six species in the genus of *Brucella* are currently recognized on the basis of the phenotypic characteristics, antigenic properties and host distribution (6). *Brucella melitensis* is the main etiological agent of brucellosis and the most important pathogenic *Brucella* species for humans causing clinically apparent human brucellosis. Brucellosis is geographically distributed in Mediterranean region, Middle East, parts of Africa, Western Asia and Latin America (7). *B. melitensis* is a big problem in Palestine and is the main cause of abortion in sheep (7).

In Palestine, diagnosis of brucellosis in animals is based mainly on clinical signs, serological and bacteriological investigations. However, *B. melitensis* strains isolated or detected in Palestine have not been genetically characterized. Since isolation and genotyping of the infectious agents are vital for management

practices of the farms and for the ability of the country to implement and finance prevention and control program, we isolated *B. melitensis* from sheep and goats in the West Bank, Palestine and characterized it phenotypically, serologically and genetically.

The application of multiple serological assays currently available for the detection of *Brucella* antibodies in various species of animals indicates that no single test can detect all infected animals and therefore, combination of serological tests should include more sensitive tests designed to reduce the number of false negative reactions which contribute to the persistence of infection as a herd problem.

In the present study, we comprehensively compared the RBT and CFT methods in animals (sheep and goats) in order to evaluate their applicability as alternative methods in surveillance programs. The obtained results revealed that in RBPT and CFT, 119 (34%) and 108 (31%) were positive respectively (Table 3.1). When CFT was used as the reference, the sensitivity and specificity of RBT were being in the range of 90.3% and 88.9%, respectively. The obtained results do not mention the prevalence of Brucellosis in Palestine, since our samples have been taken from animals with signs of Brucellosis. This research indicates that brucellosis is still representing a big problem that affects the domestic animals in this part of Palestine, that agree with the previous seroepidemiologic studies (34).

The higher number of RBT 119 (34%) reactors compared to CFT 108 (31%) is the result of high number of false positive results of RBT. This result from the examiner recording any slight agglutination which may neither be neither accurate nor recommended by the manufacturers as positive. Keeping in mind the well documented problem of extensive serological cross-reactions of the RBT with other bacteria including *Yersinia enterocolitica* O:9, *Campylobacter fetus, Vibrio cholera, Bordetella bronchiseptica* and *Salmonella* species which are commonly

found in sheep and goats, it is highly likely that a great number of these samples were indeed false positives (35).

From the results obtained by RBT and CFT, we concluded that it was impossible to

detect all infected animals using a single test. The combination of RBT with CFT is recommended since the CFT method is one of the superior serological tests as reported (34).

Bacteriological examination of milk samples obtained from 80 serologically positive animals revealed the recovery of *Brucella* isolates from only 38 (47.5%) samples. This indicated that the sensitivity of serological test was higher than that of the culture method. The same conclusion was reached by Hamdy and Amin (36) who suggested that the most specific diagnostic test involves isolation of the causative organism, but this suffers from the drawback of requiring a long incubation period and low sensitivity especially in the chronic stage of the disease. Moreover, the culture material must be handled carefully as the *Brucella* organism is a class III pathogen.

Although bacteriological isolation and identification of *Brucella* from culture has been considered to be the gold-standard, PCR is reliable in diagnosis of brucellosis. Taking into consideration the ability of PCR to be applied directly on milk specimens, it is more advantageous to use for diagnosing brucellosis than culture (27). In this study, we report the performance of PCR assay for the detection of *Brucella* species in milk samples. PCR was able to increase the number of positive milk samples which were negative by bacterial isolation. When PCR was used as the reference, the sensitivity and specificity of isolation of *Brucella* were being in the range of 61.3% and 100%, respectively.

Even though PCR is more sensitive, more rapid and less biohazardous than cultural

techniques, the isolation of the organism is still accepted as gold standard. The culture

isolation followed by the confirmation by PCR in this study is an another approach of diagnosis since PCR confirmation can fasten the identification at species level while remaining isolates can provide material for further biologic, phenotypic and antigenic studies.

Historically, *Brucella* cell components specific for cell adhesion and invasion have not been characterized, and attempts to detect invasin genes have failed. With the completion of entire *Brucella* species genomes, specifically *B. melitensis* 16M, studies have been and are currently being done to detect and characterize novel genes that may be involved in *Brucella* pathogenicity. Of particular note is a putative hemagglutinin gene found within the *B. melitensis* genome that is absent in *B. abortus* (37). The gene is present in *B. suis* and *B. canis* with minor nucleotide substitutions. There are two copies of the gene in *B. ovis*. The study utilized the hemagglutination test (HA), which has been extensively used for molecular detection or taxonomic analyses of many different bacterial species. This putative gene was PCR-amplified from two *B. melitensis* field isolates using the primers ORF-1F and ORF-1R. The PCR fragments sequenced and aligned with other GenBank-accessible gene sequences of *B. melitensis* and other *Brucellae* spp. The finding of identical sequence from *Brucella* in Palestine with the hemagglutinin gene consensus gene sequences from divergent GenBank-accessible gene sequences also confirms that this gene sequence among *Brucella* spp. is extremely conserved. Since then, this method was extensively used to genotype *Brucella* isolates. This method, therefore, was carried

out to compare two of the *B. melitensis* isolate in the current study with Rev.1 strain

of *B. melitensis.*

The nucleotide alignment of hemagglutinin ORFs results showed close similarities of the two field isolates to the current vaccine strains. However, the results of restriction analysis presented in this study have highlighted some of the potential hazards associated with use of the Rev.1 vaccine in Palestine control programs. Restriction analyses showed that the *B. melitensis* described here demonstrated restriction profile different from Rev.1 strain suggesting that field strain of *B. melitensis* in Palestine may be genetically different from the vaccine strain of the same organism. Although there are no studies on the capacity or efficiency of this vaccine for protection of brucellosis in this region, the field strains need to be also antigenically characterized and compared with the vaccine strain in order to better evaluate potential of Rev.1 vaccine. This antigenic determination should be a subject for a further study to establish a base for vaccine developments if necessary. Phylogenetic analysis provides a method of assigning strains to groups on the basis of similarities. The phylogenetic analysis strongly suggested that the two Palestinian *Brucella* field isolates and the *Brucella* isolates from Israel, France, and Spain are closely related. The phylogenetic tree presented in Figure 3.9 also shows, as expected, that strain with an accession number AY518304 which belongs to Israeli strains is the closest relative to our *Brucella* field isolates. These results demonstrated conclusively that an Israeli *Brucella* has been transmitted to sheep and goats in Palestine. As show study (38) that's atypical strain characterized in Israel raised the possibility that this strain

originated from a mutation of the vaccine stain.

In conclusion, further studies are needed to identify the route of transmission of *Brucella* into sheep and goats in Palestine.

Conclusion and Recommendations

1. This study has shown that the zoonotic brucellosis (*Brucella abortus*) is still acting threat as a public health problem in Palestine.

2. No single serological test can detect all infected animals by Brucella and therefore, combination of serological tests should include more sensitive tests designed to reduce the number of false negative reactions which contribute to the persistence of infection as a herd problem.

3. Comparatively PCR was found more suitable method for detection *Brucella* in milk as compared to cultural methods because more numbers of milk samples were found positive by this method as well as all culturally positive bovines also found positive in PCR.

4. Nucleotide alignment and Phylogenetic tree analysis of hemagglutinie gene revealed that the current representative isolate of *B. melitensis* obtained from our collection was genetically close to the isolates from different Mediterranean countries, particularly those from France, Spain and Israel, however, different from vaccine (Rev.1)

Base on the outcome results, the following recommendations should be made to improve the farm management practices and to reduce the risk of brucellosis in the whole country:

1. For the continued rise of public awareness of brucellosis, it is necessary to raise the level of health knowledge and farm management practices among the public and the farmers.

2. Adequate epidemiological studies are is necessary to implement correct country vaccination strategy should be discussed.

3. In the future, further study is necessary to be done about in investigations the risk status on the public health issues related to brucellosis. e.g. cohort study are important in investigations to identify risk factors associated with brucellosis in

4. The field strains need to be antigenically characterized and compared with the vaccine strain in order to better evaluate potential of Rev.1 vaccine. This antigenic determination should be a subject for a further study to establish a base for vaccine developments if necessary.

5. Development more safety vaccine against Brucellosis of animals.

6. Further studies are needed to identify the route of transmission of *Brucella* into sheep and goats in Palestine.

REFERENCE

1. **Pappas G**, Akritidis N, Bosilkovski M. Mile. Brucellosis. The New England Journal of Medicine; 352:2325-36, 2005

2. **Corbel M.** Brucellosis in human and animals. World Health Organization, 2006

3. Cutler S and Cutler R. Brucellosis remains endemic in the Mediterranean basin, Middle East, Western Asia, Africa and South America. The biomedical scientist, April 2006

4. European commission health and consumer protection directorate general Brucellosis in sheep and goat, 2001

5. **Young E.** An overview of human brucellosis. Clinical Infectious Diseases 21:283-290, 1995

6. **Shimshony A**. Epidemiology of emerging zooneses in Israel. Emerg Infect Dis; 3: 229-238, 8. 1997

7. **Refai M**. Incidence and control brucellosis in the Near East region. Vet Microbiol; 90: 81-110, 2002

8. **Awad R**. Human brucellosis in the Gaza Strip, Palestine Eastern Mediterranean Health Journal Volume 4, Issue 2, 225-233, 1998

9. **Pishva E**, Salehi M. First report of isolation of *Brucella melitensis*, vaccine strain Rev 1 as a source of cattle infection in Iran. Journal of Science Islamic Republic of Iran 19 (1), 19-23, 2008

10. Directorate of veterinary services and animal health and UNDP archive documentation. Ministry of Agriculture, 2009

11. Alton G, J. Forsyth. *Brucella*., 2008

12. Food and Agriculture Organization of the United Nations, World Organization for Animal Health , Word health organization Brucellosis in humans and animals, 2008

13. Al-Kharfy T. Neonatal brucellosis and blood transfusion: case report and review of the literature. Ann Trop Paediatr, 21:349-362, 2001

14. Allardet-Servent A, Bourg G, Ramuz M et al. DNA polymorphism in strains of the genus *Brucella*. J. Bateriol, ; 170:4603-4607, 1998

15. Muler W, Adams L, Ficht T et al. *Brucell*a induced abortion and infection in bottlenose dolphins (*Tursiops truncates*). Zoo wield Med; 30:100-110, 1999

16. Koneman's, Color atlas and Textbook of diagnostic microbiology, 2006

17. Gandara B, Merino A, Rogel M et al. Limited genetic diversity of *Brucella* species. J. Clin microbiology; 39:235-240, 2001

18. Lecaroz C, Blasco-Prieto M, Burrell M. Intracellular killing of *Brucella melitensis* in human macrophages with microsphere-endocapsulated gentamycin. Journal of Antimicrobial Chemotherapy, 58, 549-556, 2006

19. Dieterson P, Gummon et al. The characteristic of a variant strain of *Brucella melitensis*. Onderstepoort J. Vet Res, 55(1), 15-7, March 1981

20. Banai M, Mayer I and Cohen A. Isolation, Identification and Characterization in Israel of Brucella melitensis Biovar 1 Atypical Strains Susceptible to Dyes and

Penicillin, Indicating the Evolution of New Variant. Journal of Clinical Microbiology,
p. 1057-1059, May 1990

21. **Cloeckaert A**, Gragon M et al. Identification of *Brucella melitensis* vaccine strain by PCR-RFLP based on a mutation in the rpsL gene; Vaccine 20, Issue 19-20, p.2546-2550, June 2002

22. Chachra D, Saxena H, Kaur G et al. Comparative efficiency of Rose Bengal plate test, standard tube agglutination test and Dot ELISA in immunological detection of antibodies to *Brucella abortus* in sera; Journal of Bacteriology Research Vol. 1, p 030-033, June, 2009

23. Unver A, Erdogan H. Isolation, identification and molecular characterization of *Brucella melitensis* from aborted sheep fetuses in Kars, Turkey; Revue Med. Vet., 157, 1, 42-46, 2006

24. Vemulapalli R, McQuiston J, et al. Identification of an IS711 element interrupting the wboA gene of *B. abortus* vaccine strain RB51 and PCR assay to distinguish strain RB51 from other *Brucella spp* and strains; Clinical and Diagnostic Laboratory Immunology, p. 760-764, 1999

25. Ilhan Z, Solmaz H, et al. Detection of *Brucella melitensis* DNA in the milk of sheep after abortion by PCR; Arch. Med. Vet. 40, N2, p. 141-146, 2008

26. Leal-Klevezas D, Martinez-Vazquez I, et al. Single – step PCR for detection of *Brucella spp* from blood and milk of infected animals; Journal of Clinical Microbiology, p. 3087-3090, 1995

27. **Lopez-Goni I**, Garcia-Yoldi D, et al. Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all *Brucella* species, including the vaccine strains; Journal of Clinical Microbiology, p. 3484-3487, 2008

28. Perry Q, Xavier B, et al. *Brucella melitensis*: the evaluation of a putative hemagglutinin gene's effect on virulence in the caprine model; 2007

29. Berhe G, Belihu K, Asfaw Y. Seroepidemiological investigation of bovine brucellosis in the extensive cattle production system of Tigray region of Ethiopia; Intern J Appl Res Vet Med, vol. 5, No 2, 2007

30. Gomez M, Nieto J, Rosa G et al. Evaluation of serum test for diagnosis of human brucellosis in an area where the disease is endemic. Clinical and vaccine Immunology, p. 1031-1033, June 2008

31. Chachra D, Saxena H, Kaur G et al. Comparative efficacy of Rose Bengal plate test, standard tube agglutination test and dot ELISA in immunological detection of Ab to *Brucella abortus* in sera. Journal of Bacteriology research, Vol. 1(3), p. 030-033, June, 2009

32. Gerrit J, Viljoen, Lous H et al. Molecular diagnostic PCR handbook, 2006

33. Samad A, Awaz K and Sarkate L. Diagnosis of bovine traumatic reticuloperitonitis I: strength of clinical signs in predicting correct diagnosis. J. of Appli. Anim.Res. **6:** 13-18, 1994

34. Ilhan Z, Solmaz H, et al. Detection of *Brucella melitensis* DNA in the milk of sheep after abortion by PCR; Arch. Med. Vet. 40, N2, p. 141-146, 2008

35. Mainar-Jaime R, Munoz P. Specificity dependence between serological tests for diagnostic bovine brucellosis in *Brucella*-free farms showing false positive serological reaction due to *Yesinia enterocolitica* O:9. Can Vet J, 46(10), 913-916, October 2005

36. Ocampo-Sosa A, Garcia-Lobo J. Demonstration of IS711 transposition in *Brucella ovis and Brucella pinnipedialis*; BMC Microbiology, 8:17, 2008.

37. Del Vecchio V, Wagner M, Eschenbrenner M, Horn T, Kraycer J, Estock F, Elzer P, and Mujer C. *Brucella* Proteomes--A Review. Vet. Microbiol. 90: 592-603, 2002c

38. Bardenstein S, Mandelboim M, Ficht T et al. Identification of the *Brucella melitensis* vaccine strain Rev 1 in animals and humans in Israel by PCR analysis of the *PstI* site polymorphism of its *omp2* gene. Journal of clinical microbiology, p. 1475-1480, April 2002.

APPENDICES

Appendix 1: Positive result of the serum sample by RBT and CFT

Number	Sample	RBT	CFT	Culture
1	8	++++	1:360	**Positive**

2	10	+++	1:40	**Positive**
3	11	++	1:10	Negative
4	13	++++	1:360	**Positive**
5	15	**+++**	**Negative**	**Positive**
6	20	**+++**	**Negative**	Negative
7	22	++++	1:160	**Positive**
8	24	++	1:20	Negative
9	26	++	1:10	Negative
10	28	++++	1:80	**Positive**
11	29	+++	1:40	Negative
12	30	**Negative**	**1:360**	**Positive**
13	34	++++	1:360	**Positive**
14	37	**Negative**	**1:360**	**Positive**
15	41	+++	1:40	Negative
16	43	+++	1:40	Negative
17	45	++++	1:320	**Positive**
18	47	++++	1:180	**Positive**
19	50	++++	1:20	Negative
20	51	++++	1:360	**Positive**
21	53	++++	1:160	**Positive**
22	54	**++++**	**Negative**	
23	55	**Negative**	**1:80**	Negative
24	59	++++	1:40	Negative
25	63	+++	1:20	Negative
26	64	++++	1:80	Negative
27	72	+++	1:40	Negative
28	74	**Negative**	**1:360**	**Positive**
29	80	++++	1:180	**Positive**
30	82	+++	1:40	Negative
31	83	**++++**	**Negative**	Negative
32	84	++++	1:360	**Positive**
33	88	++	1:20	Negative
34	90	+++	1:40	Negative
35	91	+++	1:40	Negative

36	92	+++	1:40	Negative
37	93	++++	1:360	**Positive**
38	94	++++	1:180	**Positive**
39	96	+++	1:40	
40	98	+++	1:40	
41	99	+++	1:40	
42	105	++++	1:80	**Positive**
43	107	**+++**	**Negative**	
44	108	+++	1:40	Negative
45	112	**+++**	**Negative**	Negative
46	114	+++	1:40	Negative
47	116	**++++**	**Negative**	
48	120	+++	1:40	
49	126	++++	1:80	**Positive**
50	128	**++++**	**Negative**	
51	132	**++++**	**Negative**	
52	134	**Negative**	**1:80**	
53	137	++++	1:80	**Positive**
54	138	**++++**	**Negative**	
55	140	++++	1:40	Negative
56	141	++++	1:80	**Positive**
57	143	**++++**	**Negative**	
58	147	**++++**	**Negative**	
59	149	**Negative**	**1:160**	Negative
60	151	+++	1:40	Negative
61	152	+++	1:40	Negative
62	153	++++	1:80	**Positive**
63	154	++	1:20	Negative
64	155	**+++**	**Negative**	
65	156	+++	1:40	
66	160	**+++**	**Negative**	
67	161	++	1:40	
68	162	++++	1:360	**Positive**
69	163	++++	1:360	**Positive**

70	165	**Negative**	**1:40**	
71	173	+++	1:40	
72	174	+++	1:20	
73	177	++	1:20	**Positive**
74	178	**+++**	**Negative**	
75	179	+++	1:40	Negative
76	180	++++	1:80	
77	189	++++	1:360	Negative
78	190	**++++**	**Negative**	
79	192	++++	1:320	**Positive**
80	196	++++	1:180	**Positive**
81	189	++++	1:360	**Positive**
82	190	**++++**	**Negative**	
83	192	++++	1:320	**Positive**
84	196	++++	1:180	**Positive**
85	200	**Negative**	**1:80**	
86	203	++++	1:160	**Positive**
87	207	**++++**	**Negative**	
88	210	**++++**	**Negative**	
89	213	++++	1:20	Negative
90	215	++	1:10	
91	219	++++	1:320	**Positive**
92	220	++++	1:40	Negative
93	226	++++	1:40	
94	228	++++	1:360	**Positive**
95	230	+++	1:40	Negative
96	231	++++	1:320	**Positive**
97	232	++++	1:40	
98	234	**Negative**	**1:80**	
99	235	++++	1:80	
100	237	++	1:20	Negative
101	238	++++	1:80	
102	240	**+++**	**Negative**	
103	241	++++	1:40	

104	242	++++	1:20	
105	244	++++	1:40	
106	263	++++	1:40	
107	270	++	**Negative**	
108	279	++	1:40	Negative
109	280	++++	1:80	
110	282	**Negative**	**1:180**	Negative
112	285	++++	1:360	
113	287	++	1:20	
114	290	+++	1:40	
115	295	+++	1:40	Negative
116	297	++++	1:360	
117	301	++++	1:40	Negative
118	302	++++	1:40	Negative
119	304	++++	1:80	**Positive**
120	305	++++	1:320	**Positive**
121	306	++++	1:360	**Positive**
122	307	++	**Negative**	
123	308	++++	1:40	Negative
12	311	++++	1:80	Negative
125	312	+++	**Negative**	
126	314	++	**Negative**	
127	315	++++	1:20	
128	316	+++	**Negative**	
129	317	++++	1:40	Negative
130	324	++++	1:160	**Positive**
131	325	++++	**Negative**	

Appendix 2: The result o milk sample from seropositive animals by culture (*Brucella melitensis* agar) and PCR.

Sample	**Culture**	**PCR from Culture**		**PCR from Milk**		
		***Brucella* group**	***B. melitensis***	***Brucella* group**	***B. melitensis***	***B. abortus***
1	Positive	Positive	Positive	Positive	Positive	Negative
2	Positive	Positive	Positive	Positive	Positive	Positive
3	Negative			Positive	Positive	Positive

4	Positive	Positive	Positive	Positive	Positive	Negative
5	Positive	Positive	Positive	Positive	Positive	Negative
6	Negative			Positive	Negative	Positive
7	Positive	Positive	Positive	Positive	Positive	Negative
8	Negative			Positive	Positive	Negative
9	Negative			Positive	Positive	Negative
10	Positive	Positive	Positive	Positive	Positive	Negative
11	Negative			Positive	Positive	Negative
12	Positive	Positive	Positive	Positive	Positive	Negative
13	Positive	Positive	Positive	Positive	Positive	Negative
14	Positive	Positive	Positive	Positive	Positive	Negative
15	Negative			Negative	Negative	Negative
16	Negative			Negative	Negative	Negative
17	Positive	Positive	Positive	Positive	Positive	Negative
18	Positive	Positive	Positive	Positive	Positive	Negative
19	Negative			Negative	Negative	Negative
20	Positive	Positive	Positive	Positive	Positive	Negative
21	Positive	Positive	Positive	Positive	Positive	Negative
22	Negative			Negative	Negative	Negative
23	Negative			Positive	Negative	Positive
24	Negative			Positive	Negative	Positive
26	Negative			Positive	Negative	Positive
27	Positive	Positive	Positive	Positive	Positive	Negative
28	Positive	Positive	Positive	Positive	Positive	Negative
29	Negative			Positive	Negative	Positive
30	Negative			Negative	Negative	Negative
31	Positive	Positive	Positive	Positive	Positive	Negative
32	Negative			Positive	Negative	Positive
33	Negative			Negative	Negative	Negative
34	Negative			Negative	Negative	Negative
35	Negative			Negative	Negative	Negative
36	Positive	Positive	Positive	Positive	Positive	Negative
37	Positive	Positive	Positive	Positive	Positive	Negative
38	Positive	Positive	Positive	Positive	Positive	Positive
39	Negative			Negative	Negative	Negative
40	Negative			Positive	Negative	Positive
41	Negative			Negative	Negative	Positive
42	Positive	Positive	Positive	Positive	Positive	Negative
43	Positive	Positive	Positive	Positive	Positive	Positive
44	Negative			Negative	Negative	Negative
45	Negative			Positive	Negative	Positive
46	Negative			Negative	Negative	Negative
47	Positive	Positive	Positive	Positive	Positive	Positive
48	Positive	Positive	Positive	Positive	Positive	Negative
49	Positive	Positive	Positive	Positive	Positive	Positive
50	Negative			Negative	Negative	Negative
51	Positive	Positive	Positive	Positive	Positive	Negative
52	Positive	Positive	Positive	Positive	Positive	Negative
53	Positive	Positive	Positive	Positive	Positive	Negative
54	Negative			Positive	Negative	Positive
55	Negative			Positive	Negative	Positive

56	Positive	Positive	Positive	Positive	Positive	Negative
57	Positive	Positive	Positive	Positive	Positive	Negative
58	Positive	Positive	Positive	Positive	Positive	Negative
59	Negative			Positive	Negative	Positive
60	Positive	Positive	Positive	Positive	Positive	Negative
61	Negative			Negative	Negative	Negative
62	Positive	Positive	Positive	Positive	Positive	Positive
63	Negative			Positive	Negative	Positive
64	Positive	Positive	Positive	Positive	Positive	Negative
65	Negative			Negative	Negative	Negative
66	Positive	Positive	Positive	Positive	Positive	Negative
67	Negative			Positive	Negative	Positive
68	Negative			Positive	Negative	Positive
69	Negative			Negative	Negative	Negative
70	Negative			Negative	Negative	Negative
71	Positive	Positive	Positive	Positive	Positive	Negative
72	Positive	Positive	Positive	Positive	Positive	Negative
73	Negative			Positive	Negative	Positive
74	Negative			Positive	Negative	Positive
75	Negative			Positive	Negative	Positive
75	Negative			Positive	Negative	Positive
76	Positive	Positive	Positive	Positive	Positive	Negative
77	Positive	Positive	Positive	Positive	Positive	Negative
78	Negative			Positive	Negative	Positive
79	Negative			Positive	Negative	Positive
80	Negative			Positive	Negative	Positive

Appendix 3: Primer design

For design of the internal primer pair, the Open read frame Brucella melitensis gene was downloaded Primer3 Output (http://frodo.wi.mit.edu/primer3). The primer sequence was selected with the following properties: 20-25 nucleotides starting with at least one G or C nucleotides at the 5', terminated with GC at 3'end. The GC content of ~ 50%.

```
No mispriming library specified
Using 1-based sequence positions
OLIGO                    start  len    tm    gc%   any   3'  seq
LEFT PRIMER          253    20    59.94    45.00   2.00   0.00
GAACCCAGAATACGGCAAAA
RIGHT PRIMER         454    20    60.03    50.00   4.00   3.00
GTCGTTTCTGCGTCAACAGA
SEQUENCE SIZE: 1025
INCLUDED REGION SIZE: 1025
PRODUCT SIZE: 202, PAIR ANY COMPL: 7.00, PAIR 3' COMPL:
1.00
```

```
    1
AAGGCAAAAACGGCCTGCAATAAGCGGCATACCTTCGCGCGCCAGGGAGAACGTGAATG
A

   61
TAGTTCTAATACCCTTGAAGGAAAACTGTACGGACGACTATGCGTCATCAACGTTGGGT
A

  121
CCTCTTCCTTAACCTTTGGGACTCCTTTCGGATTGGAACCTCGGCATGTAACTCAGGGG
G

  181
CAATCCTGGCAGAAACGGCGCAGATAAACATTGCAACCGGCCATAAGGCGACGGTTTCC
G

  241
AGGGCATAATAGGAACCCAGAATACGGCAAAACAAGAGGTGGCCGCTGGGGCAAGCCTC
A
          >>>>>>>>>>>>>>>>>>>

  301
GTCTGCAAGAAAATACTATTGATGCTTCGCTTGCCGTCAACAATGCGGCGAACAGGATT
G

  361
CCATCGACAAGGAAGGCGATCCGACTTTCCAAGGCTCGGTCCTTATTACCAATCTGCAG
C

  421
AGAACAAAGAGGGCTCTGTTGACGCAGAAACGACAGAGTCGGGTATATTGGCGCAAGCG
A
                    <<<<<<<<<<<<<<<<<<<

  481
CAGAAGGAGATGTTGCGAATGTAATGTCTATTCTGTCGGGCAGTCTGAACGTAGCGGAA
A

  541
ACATTGTTTCCAGTTCTGCAACCGGTAACCAGACCGTTGGTGCCGCAGGCGCCGCAGGT
C

  601
ATCAGATTGTGATCGGTGGTCAACTCAGCGTCGATAGCAATACTACCGGAAACGGCAGT
T

  661
CAACGATATCGCATGACGGAGGCTCTGCATTTGCCGAAACCGCCGCTGACTTTGTTATT
G
```

721
CCAATAACCAGGCAACATCGTGACAGATGCGGCTGATCATTTGACAATCTCCAGCGCGT
C

781
TATTGTGTGGAGGGTTACAGCTGACTGATTGTGCAGGTGATGCATGCCGACGAAAGCAT
G

841
TTCCCGATTAGTCCTAGTCGGAATAATGCCTGTGACCTCTAAACATCGATCAGTACACC
A

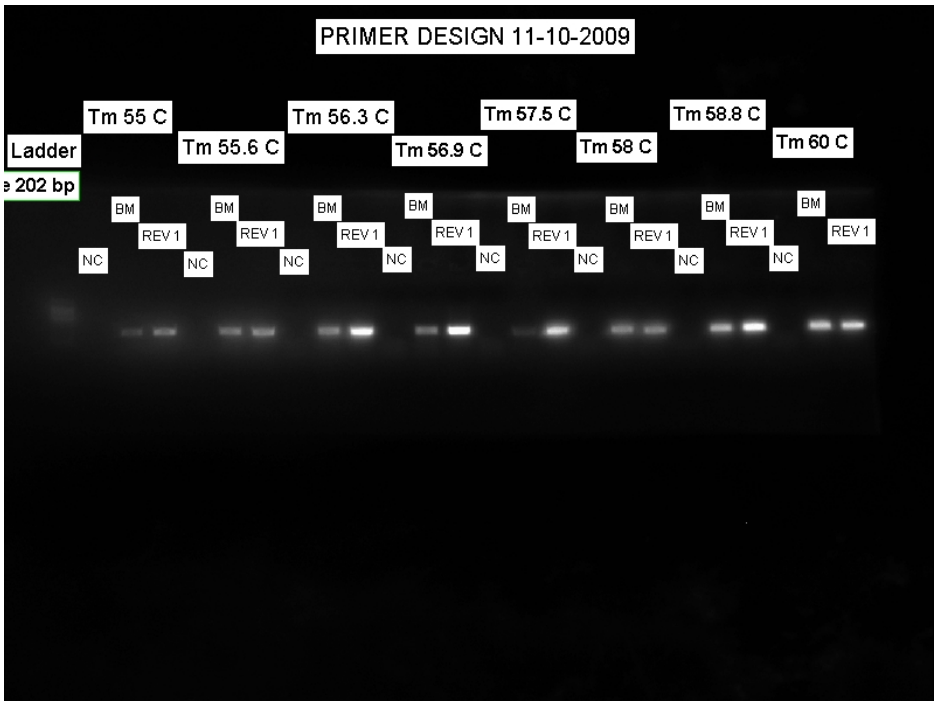
901
TCCATCTGCAGCAACAGTCCTTCTACGGTATGACTGAAACTCACGGCCATACGGACTTG
G

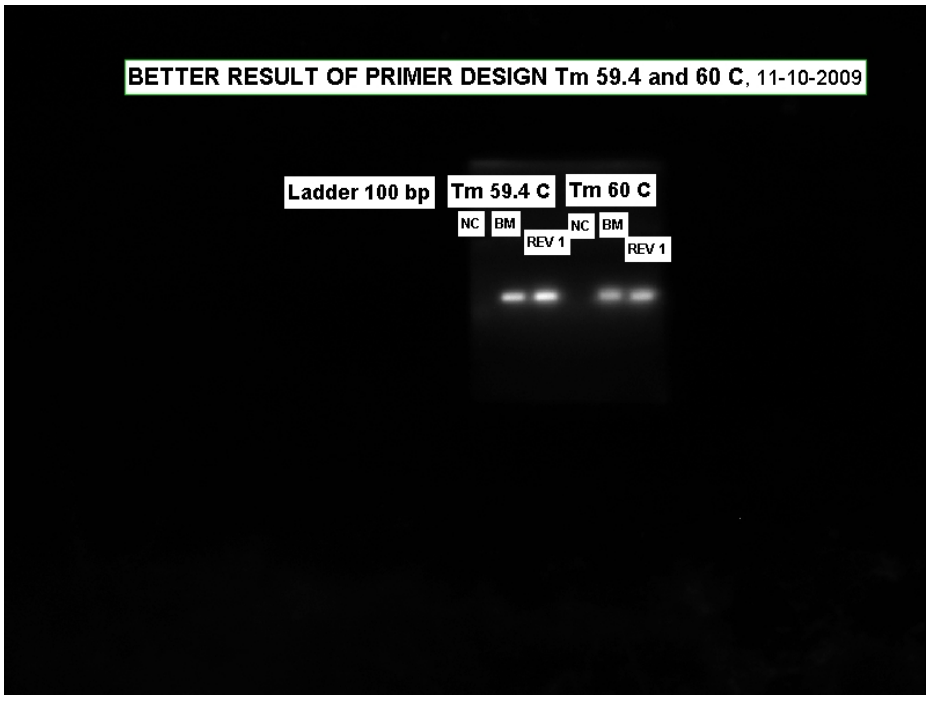
961
AACGCATTTAGCCTGGCCTCGTGGCTAACCATCCAGAATAACTCCATGTTATATCTGAA
A

1021 TGTCG

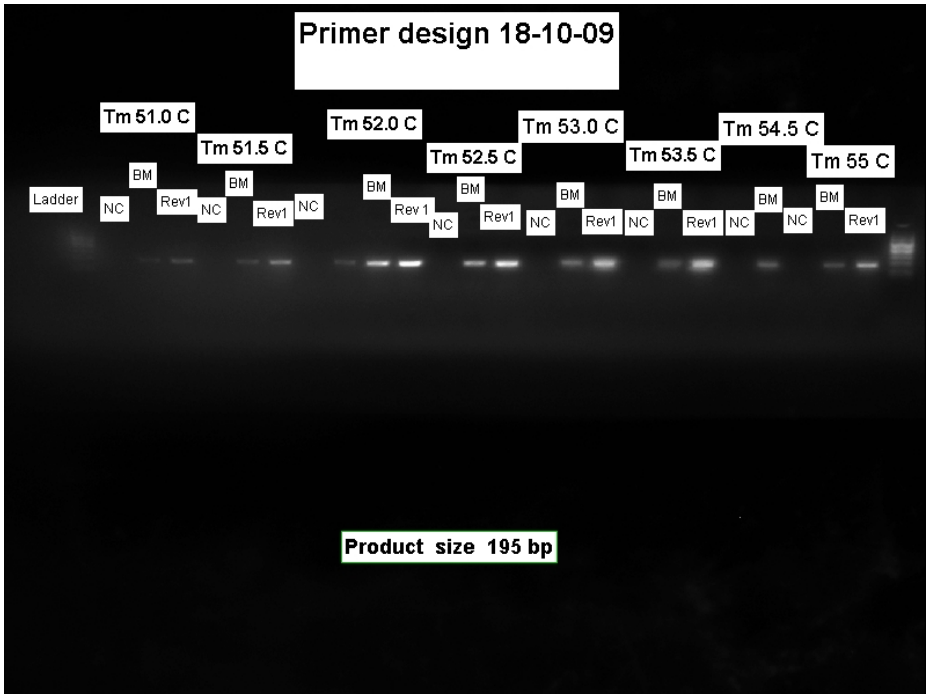
Sequence choose for primer design, red color primer 1, and blue – primer 2

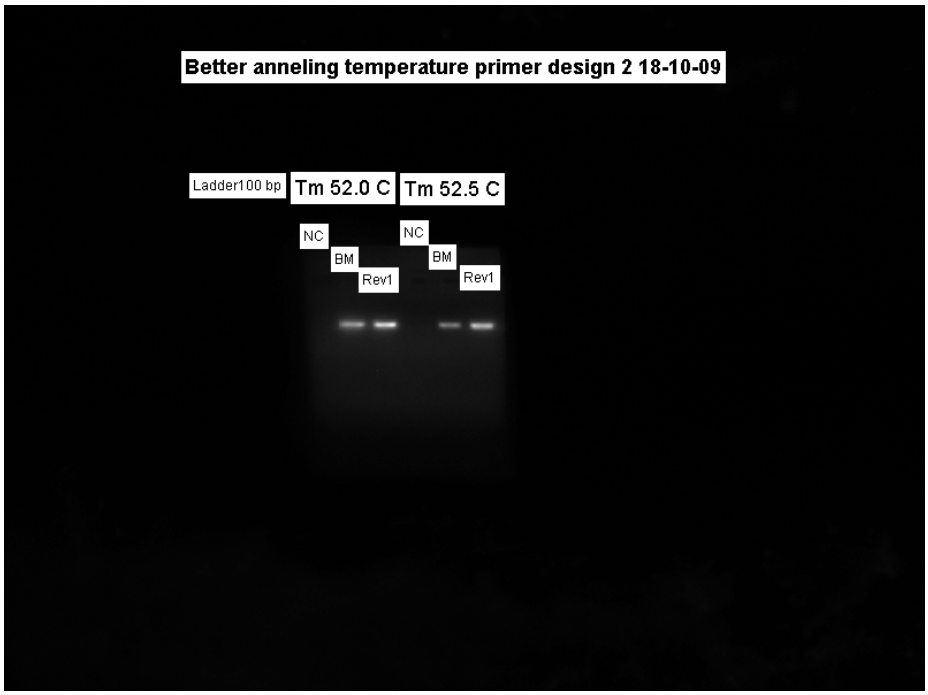
Name of primer	**Sequence**	**Annealing temperature**	**Size**	**Reference**
BM-ORF1-forward	5’-GAACCAGAATACGGCAAAA-3’	52ºC	202 bp	Our design
BM-ORF1-reverse	5’-GTCGTTTCTGCGTCAACAGA-3’			
BM-ORF2-forwadr	5’-AGGGAGAACGTGAATGAT-3’	59.5ºC	195 bp	Our design
BM-ORF2-reverse	5’-GCCGGTTGCAATGTTTATCT-3’			





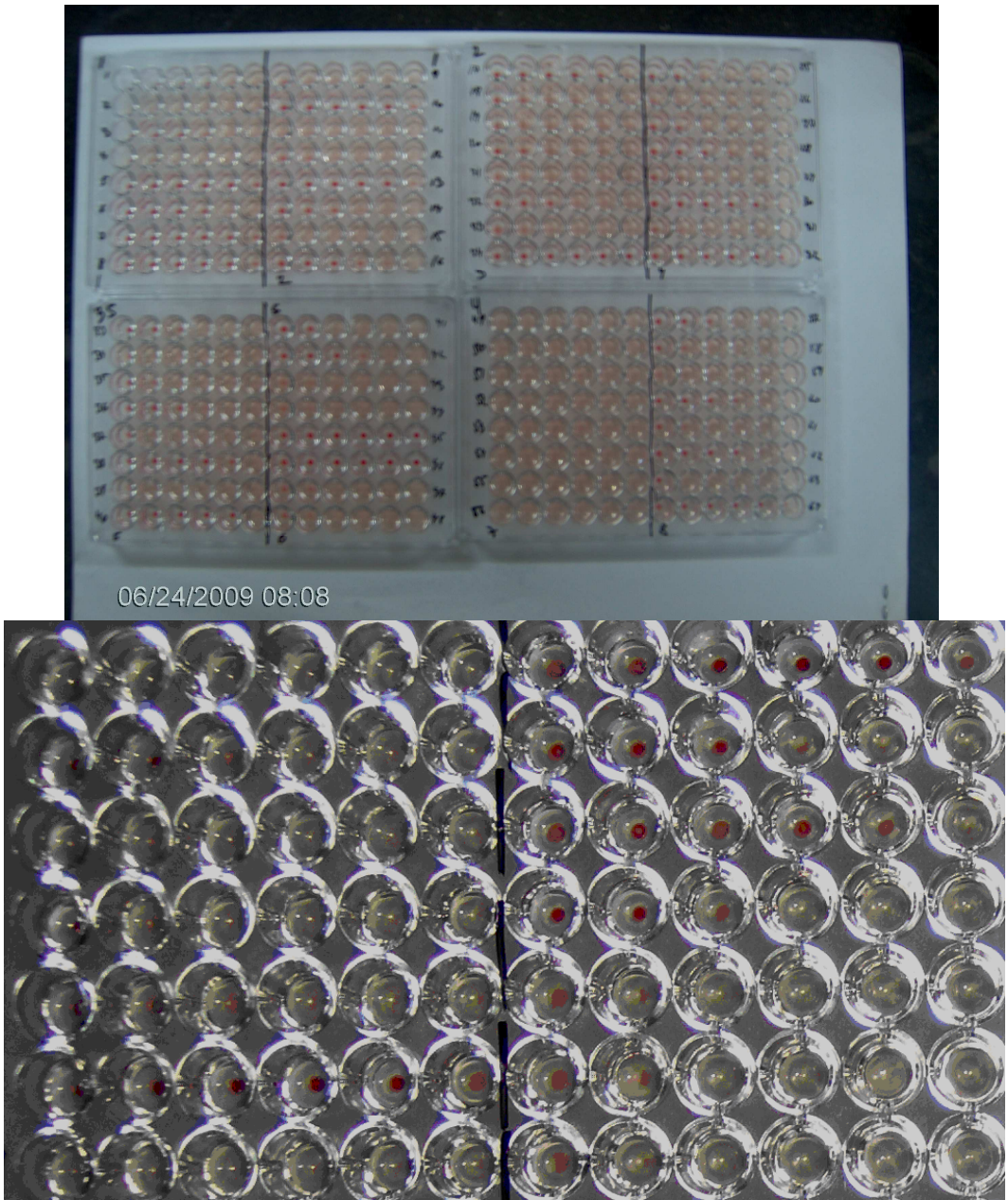
Primer design 1: product size 202 bp



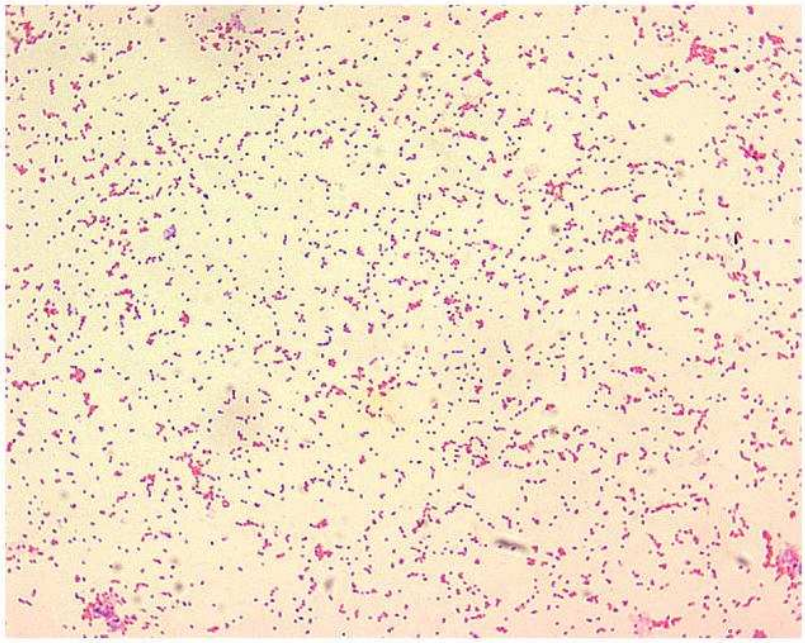


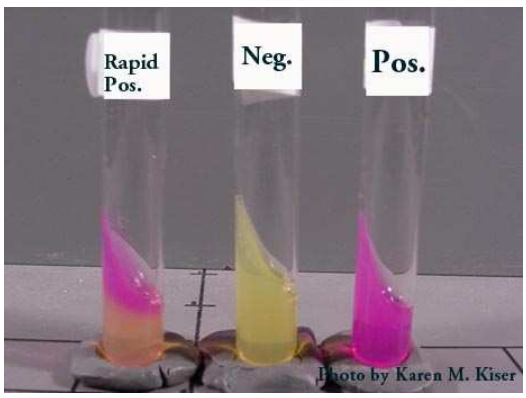
Primer design 2, product size 195 bp

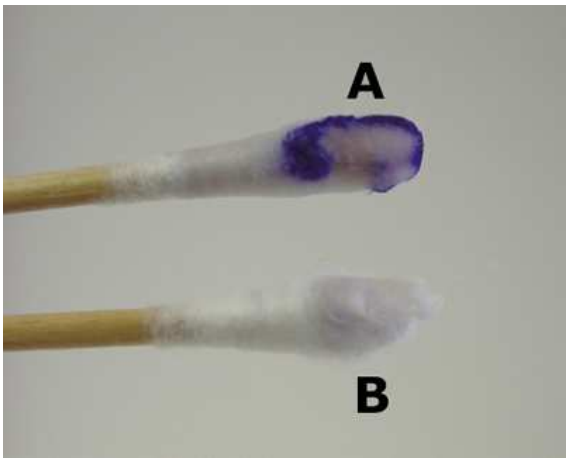
Appendix 5: Complement Fixation Test picture



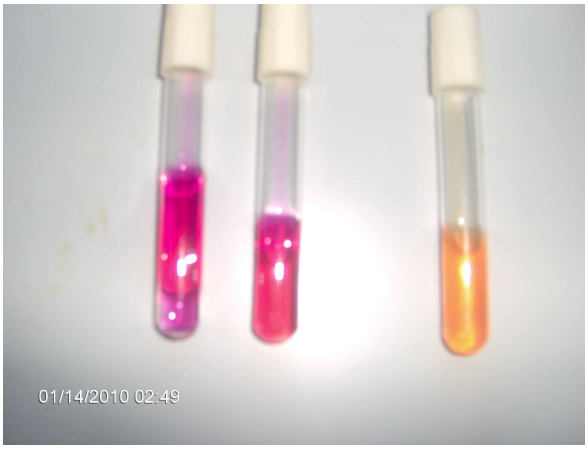
Appendix 5: Brucella melitensis Biochemical test

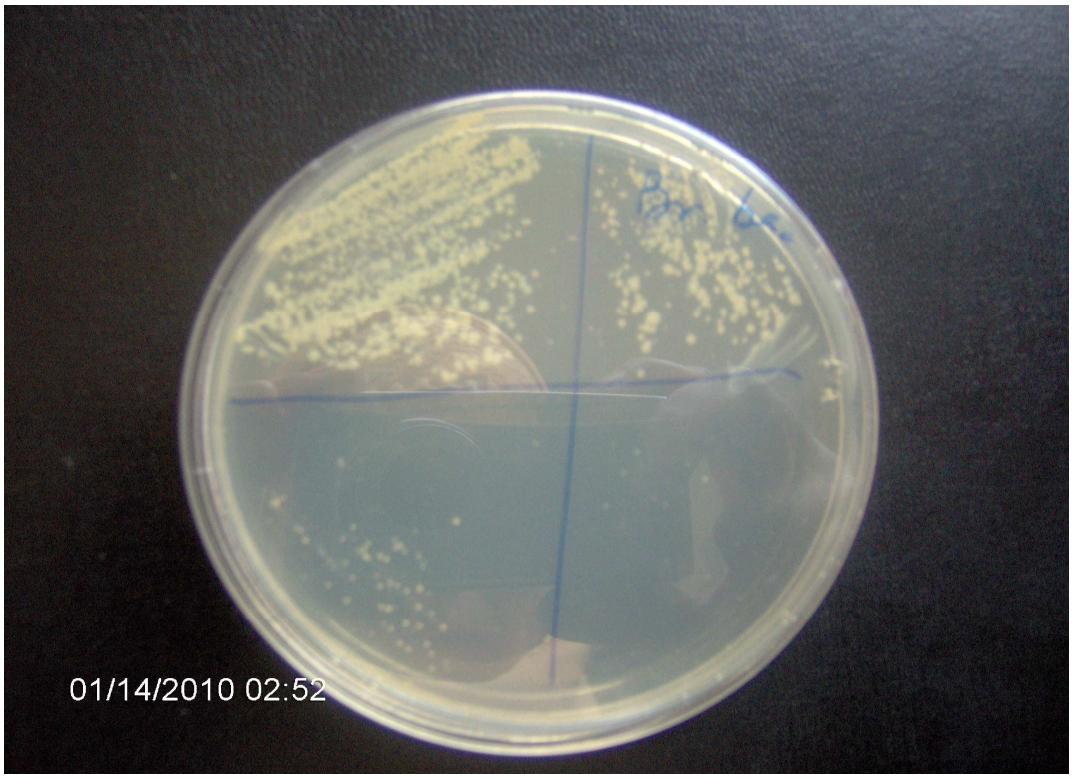


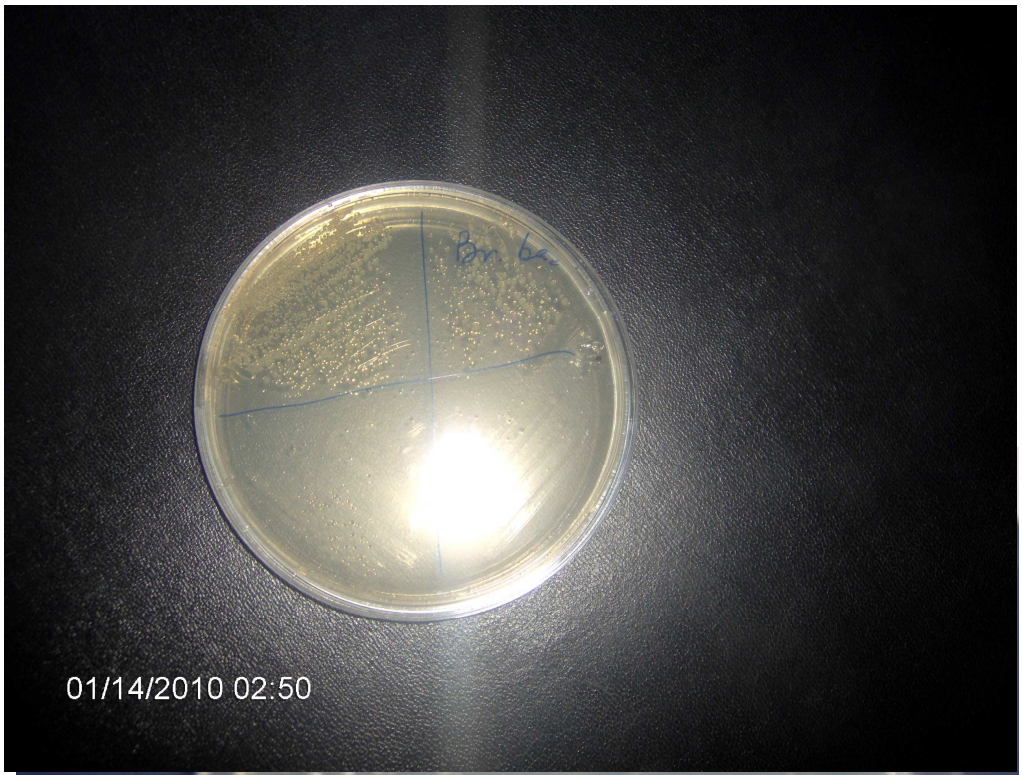




The stain, urea and oxidase test




01/14/2010 02:52


01/14/2010 02:50


01/13/2010 04:01

Appendix 6: Alignment

DNA analysis

Sequenced Open read frame further were compare by BLAST with reference gene library strain of Brucella melitensis. In figure 4 shown that's our isolate alignment with hemagglutinin and glycoprotein X precursor sequence *Brucella melitensis* of reference strain. In addition, our isolate was alignment with hypothetical protein for *B. suis*, putative cell wall surface protein of *B. ovis*, cell wall protein AWA1 precursor of *B. canis*

gb|AE008918.1| Brucella melitensis 16M chromosome II, complete sequence
Length=1177787

Features in this part of subject sequence:
hemagglutinin

Score = 1404 bits (760), Expect = 0.0
Identities = 846/883 (95%), Gaps = 24/883 (2%)
Strand=Plus/Plus

```
Query     5                CAAAAACGGCCTGCAATAAGCGGC-ATACC-TTCGCGCGCCAGGGA-
GAACGTGAATGAT  61
                |||||  ||||  |||||  |  ||||||  |||||  |||||||  |||    ||  ||
||||||||||
Sbjct          755420          CAAATACGG-CTGCAGTCAGCGGCAATACCATTCGCGC-CCA--
GACGATCGTGAATGAT  755475

Query                                                                    62
AGTTCTAATACCCTTGAAGGAAAACTGTACGGACGACTATGCGTCATCAACGTTGGGTAC  121
                |||||||||||||||||||||||||||||
||||||||||||||||||||||||||||||
Sbjct                  755476                AGTTCTAATACCCTTGAAGGAAAACTGT-
CGGACGACTATGCGTCATCAACGTTGGGTAC  755534

Query                                                                   122
CTCTTCCTTAACCTTTGGGACTCCTTTCGGATTGGAACCTCGGCATGTAACTCAGGGGGC  181

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                             755535
CTCTTCCTTAACCTTTGGGACTCCTTTCGGATTGGAACCTCGGCATGTAACTCAGGGGGC  755594

Query                                                                   182
AATCCTGGCAGAAACGGCGCAGATAAACATTGCAACCGGCCATAAGGCGACGGTTTCCGA  241

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                             755595
AATCCTGGCAGAAACGGCGCAGATAAACATTGCAACCGGCCATAAGGCGACGGTTTCCGA  755654

Query                                                                   242
GGGCATAATAGGAACCCAGAATACGGCAAAACAAGAGGTGGCCGCTGGGGCAAGCCTCAG  301

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                             755655
GGGCATAATAGGAACCCAGAATACGGCAAAACAAGAGGTGGCCGCTGGGGCAAGCCTCAG  755714

Query                                                                   302
TCTGCAAGAAAATACTATTGATGCTTCGCTTGCCGTCAACAATGCGGCGAACAGGATTGC  361

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                             755715
TCTGCAAGAAAATACTATTGATGCTTCGCTTGCCGTCAACAATGCGGCGAACAGGATTGC  755774

Query                                                                   362
CATCGACAAGGAAGGCGATCCGACTTTCCAAGGCTCGGTCCTTATTACCAATCTGCAGCA  421

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                             755775
CATCGACAAGGAAGGCGATCCGACTTTCCAAGGCTCGGTCCTTATTACCAATCTGCAGCA  755834

Query                                                                   422
GAACAAAGAGGGCTCTGTTGACGCAGAAACGACAGAGTCGGGTATATTGGCGCAAGCGAC  481
```

```
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                 755835
GAACAAAGAGGGCTCTGTTGACGCAGAAACGACAGAGTCGGGTATATTGGCGCAAGCGAC  755894

Query                                                                    482
AGAAGGAGATGTTGCGAATGTAATGTCTATTCTGTCGGGCAGTCTGAACGTAGCGGAAAA  541

|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                 755895
AGAAGGAGATGTTGCGAATGTAATGTCTATTCTGTCGGGCAGTCTGAACGTAGCGGAAAA  755954

Query                                                                    542
CATTGTTTCCAGTTCTGCAACCGGTAACCAGACCGTTGGTGCCGCAGGCGCCGCAGGTCA  601

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Sbjct                                                                 755955
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Query                                                                    602
TCAGATTGTGATCGGTGGTCAACTCAGCGTCGATAGCAATACTACCGGAAACGGCAGTTC  661

|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                 756015
TCAGATTGTGATCGGTGGTCAACTCAGCGTCGATAGCAATACTACCGGAAACGGCAGTTC  756074

Query                                                                    662
AACGATATCGCATGACGGAGGCTCTGCATTTGCCGAAACCGCCGCTGACTTTGTTATTGC  721

|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                 756075
AACGATATCGCATGACGGAGGCTCTGCATTTGCCGAAACCGCCGCTGACTTTGTTATTGC  756134

Query              722                                        CAATAACCAGGCAA-
CATCGTGACAGATGCGGCTGATCATTTGACAATCTCCAGCGCGTC  780
                   ||||||||||||||                                        ||||||
|||||||||||||||||||||||||||||||||||||
Sbjct                                                                 756135
CAATAACCAGGCAAACATCGTTACAGATGCGGCTGATCATTTGACAATCTCCAGCGCGTC  756194

Query       781                  TATTG-TGTGGAGGGTTACAGCTGACTGATTG-TGCAGGT-
GATGCATGCCGACGAAAGC  837
                   |||||  ||||||||||  |||  |  |||  |||||  |||  |||  |  ||  ||||||  ||||
|
Sbjct         756195         TATTGGTGTGGAGGGT-ACACC-GAC-GATTGGTGC-GGTTGTTG-
ATGCCGTCGAAGG-   756248

Query  838     ATGTTCCCGATTAGTCCTAGTCGGAATAATGCCTGTGACCTCT  880
               |  ||||   |  ||  |||||  |  ||||  |||||||  |||||  |||
Sbjct  756249  AGGTTCG-G-TT-GTCCTCG-CGGA-TAATGCC-GTGACGTCT  756285
```

Alignment of the flock 1 Palestinian hemagglutinin gene 16M Brucella melitensis (Query), Sbjct (ATCC)

gb|CP001489.1| Brucella melitensis ATCC 23457 chromosome II, complete sequence
Length=1185518

Features in this part of subject sequence:
Glycoprotein X precursor

Score = 1404 bits (760), Expect = 0.0

Identities = 846/883 (95%), Gaps = 24/883 (2%)
Strand=Plus/Minus

```
Query     5                CAAAAACGGCCTGCAATAAGCGGC-ATACC-TTCGCGCGCCAGGGA-
GAACGTGAATGAT  61
                 ||||  ||||  |||||  |  ||||||  |||||  |||||||  |||    ||  ||
||||||||||
Sbjct          514620          CAAATACGG-CTGCAGTCAGCGGCAATACCATTCGCGC-CCA--
GACGATCGTGAATGAT  514565

Query                                                                    62
AGTTCTAATACCCTTGAAGGAAAACTGTACGGACGACTATGCGTCATCAACGTTGGGTAC  121
                 ||||||||||||||||||||||||||||
||||||||||||||||||||||||||||||
Sbjct                  514564                 AGTTCTAATACCCTTGAAGGAAAACTGT-
CGGACGACTATGCGTCATCAACGTTGGGTAC  514506

Query                                                                   122
CTCTTCCTTAACCTTTGGGACTCCTTTCGGATTGGAACCTCGGCATGTAACTCAGGGGGC  181

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                514505
CTCTTCCTTAACCTTTGGGACTCCTTTCGGATTGGAACCTCGGCATGTAACTCAGGGGGC  514446

Query                                                                   182
AATCCTGGCAGAAACGGCGCAGATAAACATTGCAACCGGCCATAAGGCGACGGTTTCCGA  241

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                514445
AATCCTGGCAGAAACGGCGCAGATAAACATTGCAACCGGCCATAAGGCGACGGTTTCCGA  514386

Query                                                                   242
GGGCATAATAGGAACCCAGAATACGGCAAAACAAGAGGTGGCCGCTGGGGCAAGCCTCAG  301

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                514385
GGGCATAATAGGAACCCAGAATACGGCAAAACAAGAGGTGGCCGCTGGGGCAAGCCTCAG  514326

Query                                                                   302
TCTGCAAGAAAATACTATTGATGCTTCGCTTGCCGTCAACAATGCGGCGAACAGGATTGC  361

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                514325
TCTGCAAGAAAATACTATTGATGCTTCGCTTGCCGTCAACAATGCGGCGAACAGGATTGC  514266

Query                                                                   362
CATCGACAAGGAAGGCGATCCGACTTTCCAAGGCTCGGTCCTTATTACCAATCTGCAGCA  421

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                514265
CATCGACAAGGAAGGCGATCCGACTTTCCAAGGCTCGGTCCTTATTACCAATCTGCAGCA  514206

Query                                                                   422
GAACAAAGAGGGCTCTGTTGACGCAGAAACGACAGAGTCGGGTATATTGGCGCAAGCGAC  481

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                514205
GAACAAAGAGGGCTCTGTTGACGCAGAAACGACAGAGTCGGGTATATTGGCGCAAGCGAC  514146

Query                                                                   482
AGAAGGAGATGTTGCGAATGTAATGTCTATTCTGTCGGGCAGTCTGAACGTAGCGGAAAA  541

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Sbjct                                                                514145
AGAAGGAGATGTTGCGAATGTAATGTCTATTCTGTCGGGCAGTCTGAACGTAGCGGAAAA  514086

Query                                                                  542
CATTGTTTCCAGTTCTGCAACCGGTAACCAGACCGTTGGTGCCGCAGGCGCCGCAGGTCA  601

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                514085
CATTGTTTCCAGTTCTGCAACCGGTAACCAGACCGTTGGTGCCGCAGGCGCCGCAGGTCA  514026

Query                                                                  602
TCAGATTGTGATCGGTGGTCAACTCAGCGTCGATAGCAATACTACCGGAAACGGCAGTTC  661

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                514025
TCAGATTGTGATCGGTGGTCAACTCAGCGTCGATAGCAATACTACCGGAAACGGCAGTTC  513966

Query                                                                  662
AACGATATCGCATGACGGAGGCTCTGCATTTGCCGAAACCGCCGCTGACTTTGTTATTGC  721

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                513965
AACGATATCGCATGACGGAGGCTCTGCATTTGCCGAAACCGCCGCTGACTTTGTTATTGC  513906

Query              722                                       CAATAACCAGGCAA-
CATCGTGACAGATGCGGCTGATCATTTGACAATCTCCAGCGCGTC  780
                   ||||||||||||||                                    ||||||
||||||||||||||||||||||||||||||||||
Sbjct                                                                513905
CAATAACCAGGCAAACATCGTTACAGATGCGGCTGATCATTTGACAATCTCCAGCGCGTC  513846

Query       781                  TATTG-TGTGGAGGGTTACAGCTGACTGATTG-TGCAGGT-
GATGCATGCCGACGAAAGC  837
                ||||| |||||||||| ||| | ||| ||||| ||| ||| | || |||||| ||||
|
Sbjct         513845         TATTGGTGTGGAGGGT-ACACC-GAC-GATTGGTGC-GGTTGTTG-
ATGCCGTCGAAGG-  513792

Query  838     ATGTTCCCGATTAGTCCTAGTCGGAATAATGCCTGTGACCTCT  880
                | ||||  | || ||||| | |||| ||||||| ||||| |||
Sbjct  513791  AGGTTCG-G-TT-GTCCTCG-CGGA-TAATGCC-GTGACGTCT  513755
```

Alignment Palestinian glycoprotein x precursor 16M *Brucella melitensis* (Query), Sbjct (ATCC)

gb|CP000912.1| Brucella suis ATCC 23445 chromosome II, complete sequence
Length=1400844

Features in this part of subject sequence:
Hypothetical protein, conserved

Score = 1393 bits (754), Expect = 0.0
Identities = 845/884 (95%), Gaps = 26/884 (2%)
Strand=Plus/Minus

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Query    5                CAAAAACGGCCTGCAATAAGCGGC-ATACC-TTCGCGCGCCAGGGA-
GAACGTGAATGAT  61
                ||||  ||||  |||||  |  ||||||  |||||  |||||||  |||    ||  ||
||||||||||
Sbjct         535145          CAAATACGG-CTGCAGTCAGCGGCAATACCATTCGCGC-CCA--
GACGATCGTGAATGAT  535090
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Query                                                                  62
AGTTCTAATACCCTTGAAGGAAAACTGTACGGACGACTATGCGTCATCAACGTTGGGTAC  121
                ||||||||||||||||||||||||||||
||||||||||||||||||||||||||||||
Sbjct                  535089                AGTTCTAATACCCTTGAAGGAAAACTGT-
CGGACGACTATGCGTCATCAACGTTGGGTAC  535031

Query                                                                 122
CTCTTCCTTAACCTTTGGGACTCCTTTCGGATTGGAACCTCGGCATGTAACTCAGGGGGC  181

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          535030
CTCTTCCTTAACCTTTGGGACTCCTTTCGGATTGGAACCTCGGCATGTAACTCAGGGGGC  534971

Query                                                                 182
AATCCTGGCAGAAACGGCGCAGATAAACATTGCAACCGGCCATAAGGCGACGGTTTCCGA  241

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          534970
AATCCTGGCAGAAACGGCGCAGATAAACATTGCAACCGGCCATAAGGCGACGGTTTCCGA  534911

Query                                                                 242
GGGCATAATAGGAACCCAGAATACGGCAAAACAAGAGGTGGCCGCTGGGGCAAGCCTCAG  301

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          534910
GGGCATAATAGGAACCCAGAATACGGCAAAACAAGAGGTGGCCGCTGGGGCAAGCCTCAG  534851

Query                                                                 302
TCTGCAAGAAAATACTATTGATGCTTCGCTTGCCGTCAACAATGCGGCGAACAGGATTGC  361

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          534850
TCTGCAAGAAAATACTATTGATGCTTCGCTTGCCGTCAACAATGCGGCGAACAGGATTGC  534791

Query                                                                 362
CATCGACAAGGAAGGCGATCCGACTTTCCAAGGCTCGGTCCTTATTACCAATCTGCAGCA  421

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          534790
CATCGACAAGGAAGGCGATCCGACTTTCCAAGGCTCGGTCCTTATTACCAATCTGCAGCA  534731

Query                                                                 422
GAACAAAGAGGGCTCTGTTGACGCAGAAACGACAGAGTCGGGTATATTGGCGCAAGCGAC  481

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          534730
GAACAAAGAGGGCTCTGTTGACGCAGAAACGACAGAGTCGGGTATATTGGCGCAAGCGAC  534671

Query                                                                 482
AGAAGGAGATGTTGCGAATGTAATGTCTATTCTGTCGGGCAGTCTGAACGTAGCGGAAAA  541
                ||||||||||||||||||||||||||||||||||||||||
||||||||||||||||
Sbjct                                                          534670
AGAAGGAGATGTTGCGAATGTAATGTCTATTCTGTCGGGCAATCTGAACGTAGCGGAAAA  534611

Query                                                                 542
CATTGTTTCCAGTTCTGCAACCGGTAACCAGACCGTTGGTGCCGCAGGCGCCGCAGGTCA  601

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          534610
CATTGTTTCCAGTTCTGCAACCGGTAACCAGACCGTTGGTGCCGCAGGCGCCGCAGGTCA  534551

Query                                                                 602
TCAGATTGTGATCGGTGGTCAACTCAGCGTCGATAGCAATACTACCGGAAACGGCAGTTC  661
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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                534550
TCAGATTGTGATCGGTGGTCAACTCAGCGTCGATAGCAATACTACCGGAAACGGCAGTTC  534491

Query                                                                  662
AACGATATCGCATGACGGAGGCTCTGCATTTGCCGAAACCGCCGCTGACTTTGTTATTGC  721

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                534490
AACGATATCGCATGACGGAGGCTCTGCATTTGCCGAAACCGCCGCTGACTTTGTTATTGC  534431

Query            722                                 CAATAACCAGGCAA-CATCG-
TGACAGATGCGGCTGATCATTTGACAATCTCCAGCGCGT  779
                 |||||||||||||                     |||||                  |
||||||||||||||||||||||||||||||||||||
Sbjct                     534430                    CAATAACCAGGCAAACATCGGT-
ACAGATGCGGCTGATCATTTGACAATCTCCAGCGCGT  534372

Query       780                 CTATTG-TGTGGAGGGTTACAGCTGACTGATTG-TGCAGGT-
GATGCATGCCGACGAAAG  836
                |||||| |||||||||| ||| | ||| ||||| ||| ||| | || |||||| ||||
|
Sbjct        534371        CTATTGGTGTGGAGGGT-ACACC-GAC-GATTGGTGC-GGTTGTTG-
ATGCCGTCGAAGG  534317

Query  837     CATGTTCCCGATTAGTCCTAGTCGGAATAATGCCTGTGACCTCT  880
                 | ||||  | || ||||| | |||| ||||||| ||||| |||
Sbjct  534316  -AGGTTCG-G-TT-GTCCTTG-CGGA-TAATGCC-GTGACGTCT  534280
```

Alignment our isolate with hypothetical protein *Brucella sius*

gb|CP000709.1| Brucella ovis ATCC 25840 chromosome II, complete sequence
Length=1164220

Features in this part of subject sequence:
putative cell wall surface protein

Score = 1399 bits (757), Expect = 0.0
Identities = 846/884 (95%), Gaps = 26/884 (2%)
Strand=Plus/Minus

```
Query    5                CAAAAACGGCCTGCAATAAGCGGC-ATACC-TTCGCGCGCCAGGGA-
GAACGTGAATGAT  61
                |||| ||||||  |||  |  ||||||  |||||  |||||||  |||    ||  ||
||||||||||
Sbjct         536302          CAAATACGGCC-GCAGTCAGCGGCAATACCATTCGCGC-CCA--
GACGATCGTGAATGAT  536247

Query                                                                   62
AGTTCTAATACCCTTGAAGGAAAACTGTACGGACGACTATGCGTCATCAACGTTGGGTAC  121
                |||||||||||||||||||||||||||
||||||||||||||||||||||||||||||
Sbjct                  536246                 AGTTCTAATACCCTTGAAGGAAAACTGT-
CGGACGACTATGCGTCATCAACGTTGGGTAC  536188

Query                                                                  122
CTCTTCCTTAACCTTTGGGACTCCTTTCGGATTGGAACCTCGGCATGTAACTCAGGGGGC  181

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Sbjct                                                                536187
CTCTTCCTTAACCTTTGGGACTCCTTTCGGATTGGAACCTCGGCATGTAACTCAGGGGGC  536128
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Query                                                                 182
AATCCTGGCAGAAACGGCGCAGATAAACATTGCAACCGGCCATAAGGCGACGGTTTCCGA  241

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          536127
AATCCTGGCAGAAACGGCGCAGATAAACATTGCAACCGGCCATAAGGCGACGGTTTCCGA  536068

Query                                                                 242
GGGCATAATAGGAACCCAGAATACGGCAAAACAAGAGGTGGCCGCTGGGGCAAGCCTCAG  301

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          536067
GGGCATAATAGGAACCCAGAATACGGCAAAACAAGAGGTGGCCGCTGGGGCAAGCCTCAG  536008
Query                                                                 302
TCTGCAAGAAAATACTATTGATGCTTCGCTTGCCGTCAACAATGCGGCGAACAGGATTGC  361

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          536007
TCTGCAAGAAAATACTATTGATGCTTCGCTTGCCGTCAACAATGCGGCGAACAGGATTGC  535948

Query                                                                 362
CATCGACAAGGAAGGCGATCCGACTTTCCAAGGCTCGGTCCTTATTACCAATCTGCAGCA  421

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          535947
CATCGACAAGGAAGGCGATCCGACTTTCCAAGGCTCGGTCCTTATTACCAATCTGCAGCA  535888

Query                                                                 422
GAACAAAGAGGGCTCTGTTGACGCAGAAACGACAGAGTCGGGTATATTGGCGCAAGCGAC  481

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          535887
GAACAAAGAGGGCTCTGTTGACGCAGAAACGACAGAGTCGGGTATATTGGCGCAAGCGAC  535828

Query                                                                 482
AGAAGGAGATGTTGCGAATGTAATGTCTATTCTGTCGGGCAGTCTGAACGTAGCGGAAAA  541

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          535827
AGAAGGAGATGTTGCGAATGTAATGTCTATTCTGTCGGGCAGTCTGAACGTAGCGGAAAA  535768

Query                                                                 542
CATTGTTTCCAGTTCTGCAACCGGTAACCAGACCGTTGGTGCCGCAGGCGCCGCAGGTCA  601

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          535767
CATTGTTTCCAGTTCTGCAACCGGTAACCAGACCGTTGGTGCCGCAGGCGCCGCAGGTCA  535708

Query                                                                 602
TCAGATTGTGATCGGTGGTCAACTCAGCGTCGATAGCAATACTACCGGAAACGGCAGTTC  661

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Sbjct                                                          535707
TCAGATTGTGATCGGTGGTCAACTCAGCGTCGATAGCAATACTACCGGAAACGGCAGTTC  535648

Query                                                                 662
AACGATATCGCATGACGGAGGCTCTGCATTTGCCGAAACCGCCGCTGACTTTGTTATTGC  721

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          535647
AACGATATCGCATGACGGAGGCTCTGCATTTGCCGAAACCGCCGCTGACTTTGTTATTGC  535588

Query             722                                 CAATAACCAGGCAA-CATCG-
TGACAGATGCGGCTGATCATTTGACAATCTCCAGCGCGT  779
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               |||||||||||||                    |||||                    |
||||||||||||||||||||||||||||||||||||
Sbjct                     535587                      CAATAACCAGGCAAACATCGGT-
ACAGATGCGGCTGATCATTTGACAATCTCCAGCGCGT  535529

Query       780                 CTATTG-TGTGGAGGGTTACAGCTGACTGATTG-TGCAGGT-
GATGCATGCCGACGAAAG  836
               |||||| |||||||||| ||| | ||| ||||| ||| ||| | || |||||| ||||
|
Sbjct        535528        CTATTGGTGTGGAGGGT-ACACC-GAC-GATTGGTGC-GGTTGTTG-
ATGCCGTCGAAGG  535474

Query  837     CATGTTCCCGATTAGTCCTAGTCGGAATAATGCCTGTGACCTCT  880
                | ||||  | || ||||| | |||| ||||||| ||||| |||
Sbjct  535473  -AGGTTCG-G-TT-GTCCTTG-CGGA-TAATGCC-GTGACGTCT  535437
```

Alignment our isolate with putative cell wall surface protein protein Brucella ovis

gb|CP000873.1| Brucella canis ATCC 23365 chromosome II, complete sequence
Length=1206800

Features in this part of subject sequence:
Cell wall protein AWA1 precursor

Score = 1038 bits (562), Expect = 0.0
Identities = 604/623 (96%), Gaps = 8/623 (1%)
Strand=Plus/Minus

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Query     10                CAAA-ACGGCTGC-GTC-GCGGCAATACCATTCGCGCCGAGGAC-
AACGTGAATGATAGT  65
               ||||   ||||||||   |||   ||||||||||||||||||||   |   |||   |
||||||||||||
Sbjct              534533       CAAATACGGCTGCAGTCAGCGGCAATACCATTCGCGCCCA-
GACGATCGTGAATGATAGT  534475

Query                                                                  66
TCTAATACCCTTGAAGGGAAAACAGACGGACGACTATGCGTCATCAACGTTGGGTACCTC  125
               |||||||||||||||                  |||||||                |
||||||||||||||||||||||||||||||||
Sbjct  534474  TCTAATACCCTTGAA-GGAAAACTGTCGGACGACTATGCGTCATCAACGTTGGGTACCTC
534416

Query                                                                 126
TTCCTTAACCTTTGGGACTCCTTTCGGATTGGAACCTCGGCATGTAACTCAGGGGGCAAT  185

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          534415
TTCCTTAACCTTTGGGACTCCTTTCGGATTGGAACCTCGGCATGTAACTCAGGGGGCAAT  534356

Query                                                                 186
CCTGGCAGAAACGGCGCAGATAAACATTGCAACCGGCCATAAGGCGACGGTTTCCGAGGG  245

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                          534355
CCTGGCAGAAACGGCGCAGATAAACATTGCAACCGGCCATAAGGCGACGGTTTCCGAGGG  534296

Query                                                                 246
CATAATAGGAACCCAGAATACGGCAAAACAAGAGGTGGCCGCTGGGGCAAGCCTCAGTCT  305

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Sbjct                                                                534295
CATAATAGGAACCCAGAATACGGCAAAACAAGAGGTGGCCGCTGGGGCAAGCCTCAGTCT  534236

Query                                                                  306
GCAAGAAAATACTATTGATGCTTCGCTTGCCGTCAACAATGCGGCGAACAGGATTGCCAT  365

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                534235
GCAAGAAAATACTATTGATGCTTCGCTTGCCGTCAACAATGCGGCGAACAGGATTGCCAT  534176

Query                                                                  366
CGACAAGGAAGGCGATCCGACTTTCCAAGGCTCGGTCCTTATTACCAATCTGCAGCAGAA  425
                |||||||||||
||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                534175
CGACAAGGAAAGCGATCCGACTTTCCAAGGCTCGGTCCTTATTACCAATCTGCAGCAGAA  534116

Query                                                                  426
CAAAGAGGGCTCTGTTGACGCAGAAACGACAGAGTCGGGTATATTGGCGCAAGCGACAGA  485

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                                                                534115
CAAAGAGGGCTCTGTTGACGCAGAAACGACAGAGTCGGGTATATTGGCGCAAGCGACAGA  534056

Query                                                                  486
AGGAGATGTTGCGAATGTAATGTCTATTCTGTCGGGCAGTCTGAACGTAACGGAAAAACA  545
                |||||||||||||||||||||||||||||||||||||||||||||||||||   |||||||
||
Sbjct  534055  AGGAGATGTTGCGAATGTAATGTCTATTCTGTCGGGCAGTCTGAACGTAGCGGAAAA-
CA  533997

Query                                                                  546
TTGTTTCCAGTTCTGCAACCGGTAACCAGACCGTTGGTGCCGCAGGCGCAACATGTCATC  605
                |||||||||||||||||||||||||||||||||||||||||||||||||||||        ||
||||||
Sbjct                                                                533996
TTGTTTCCAGTTCTGCAACCGGTAACCAGACCGTTGGTGCCGCAGGCGCCGCAGGTCATC  533937

Query  606     CAGATAGTGATCGGTGGACAACT  628
                ||||  ||||||||||| |||||
Sbjct  533936  -AGATTGTGATCGGTGGTCAACT  533915
```

Alignment our isolate with cell wall protein AWA1 precursor protein *Brucella canis*

Alignment 7: DNA sequencing

Sequence Brucella melitensis from farm 1 - forward

ORF F3-1 F

AAGGCAAAAACGGCCTGCAATAAGCGGCATACCTTCGCGCGCCAGGG
AGAACGTGAATGATAGTTCTAATACCCTTGAAGGAAAACTGTACGGAC
GACTATGCGTCATCAACGTTGGGTACCTCTTCCTTAACCTTTGGGACTC
CTTTCGGATTGGAACCTCGGCATGTAACTCAGGGGGCAATCCTGGCAG
AAACGGCGCAGATAAACATTGCAACCGGCCATAAGGCGACGGTTTCC
GAGGGCATAATAGGAACCCAGAATACGGCAAAACAAGAGGTGGCCGC
TGGGGCAAGCCTCAGTCTGCAAGAAAATACTATTGATGCTTCGCTTGC
CGTCAACAATGCGGCGAACAGGATTGCCATCGACAAGGAAGGCGATC
CGACTTTCCAAGGCTCGGTCCTTATTACCAATCTGCAGCAGAACAAAG
AGGGCTCTGTTGACGCAGAAACGACAGAGTCGGGTATATTGGCGCAA
GCGACAGAAGGAGATGTTGCGAATGTAATGTCTATTCTGTCGGGCAGT
CTGAACGTAGCGGAAAACATTGTTTCCAGTTCTGCAACCGGTAACCAG
ACCGTTGGTGCCGCAGGCGCCGCAGGTCATCAGATTGTGATCGGTGGT
CAACTCAGCGTCGATAGCAATACTACCGGAAACGGCAGTTCAACGATA
TCGCATGACGGAGGCTCTGCATTTGCCGAAACCGCCGCTGACTTTGTT
ATTGCCAATAACCAGGCAACATCGTGACAGATGCGGCTGATCATTTGA
CAATCTCCAGCGCGTCTATTGTGTGGAGGGTTACAGCTGACTGATTGT
GCAGGTGATGCATGCCGACGAAAGCATGTTCCCGATTAGTCCTAGTCG
GAATAATGCCTGTGACCTCTAAACATCGATCAGTACACCATCCATCTG
CAGCAACAGTCCTTCTACGGTATGACTGAAACTCACGGCCATACGGAC
TTGGAACGCATTTAGCCTGGCCTCGTGGCTAACCATCCAGAATAACTC
CATGTTATATCTGAAATGTCG

Sequence Brucella melitensis from farm 2 - forward

ORF F3 – F

CCTGGCCAAAAAACGGCTGCGTCGCGGCAATACCTTCGCGCCGAGGG
AAAACGTGAATGATAGTTCTAATACCCTTGAAGGAAAACAGTCGGAC
GACTATGCGTCATCAACGTTGGGTACCTCTTCCTTAACCTTTGGGACTC
CTTTCGGATTGGAACCTCGGCATGTAACTCAGGGGGCAATCCTGGCAG
AAACGGCGCAGATAAACATTGCAACCGGCCATAAGGCGACGGTTTCC
GAGGGCATAATAGGAACCCAGAATACGGCAAAACAAGAGGTGGCCGC
TGGGGCAAGCCTCAGTCTGCAAGAAAATACTATTGATGCTTCGCTTGC
CGTCAACAATGCGGCGAACAGGATTGCCATCGACAAGGAAGGCGATC
CGACTTTCCAAGGCTCGTCCTTATTACCAATCTGCAGCAGAACAAAGA
GGGCTCTGTTGACGCAGAAACGACAGAGTCGGGTATATTGGCGCAAG
CGACAGAAGGAGATGTTGCGAATGTAATGTCTATTCTGTCGGGCAGTC
TGAACGTAGCGGAAAACATTGTTTCCAGTTCTGCAACCGGTAACCAGA
CCGTTGGTGCCGCAGGCGCCGCAGGTCATCAGATTGTGATCGGTGGTC
AACTCAGCGTCGATAGCAATACTACCGGAAACGGCAGTTCAACGATAT
CGCATGACGGAGGCTCTGCATTTGCCGAAACCGCCGCTGACTTTGTTA
TTGCCAATAACCAGGCAAACATCGTTACAGATGCGGCTTGATCATTTG
ACAATCTCCAGCGCGTCTATTTGGTGTGGAGGGTACACCGACGATTGG
TGCGGTTGTTGATGCCGTCGAAGGATGTTTCGGTGTCCCTCGCCGGAT
AATGCCCGGTGACGTCCTCAGGCCGGTAAGGTAACATCCACATCCCGC
ATGCAAGTCCTGAAGGATTGATCGACCTCCGCAAATCGGCTTGATGCA
TCAGCCTGGCCCCCTGGCTACCATTCAGATTAAACCTGATTCTGAATAT
TTGCGGCATCGATGCT

Sequence Brucella melitensis from vaccine strain REV 1

ORF REV 1 – 1 F

TCCTGGGTCCAAAACGGCTGCGTCGCGGCAATACCATTCGCGCCGAGG
ACAACGTGAATGATAGTTCTAATACCCTTGAAGGGAAAACAGACGGA
CGACTATGCGTCATCAACGTTGGGTACCTCTTCCTTAACCTTTGGGACT
CCTTTCGGATTGGAACCTCGGCATGTAACTCAGGGGGCAATCCTGGCA
GAAACGGCGCAGATAAACATTGCAACCGGCCATAAGGCGACGGTTTC
CGAGGGCATAATAGGAACCCAGAATACGGCAAAACAAGAGGTGGCCG
CTGGGGCAAGCCTCAGTCTGCAAGAAAATACTATTGATGCTTCGCTTG
CCGTCAACAATGCGGCGAACAGGATTGCCATCGACAAGGAAGGCGAT
CCGACTTTCCAAGGCTCGGTCCTTATTACCAATCTGCAGCAGAACAAA
GAGGGCTCTGTTGACGCAGAAACGACAGAGTCGGGTATATTGGCGCA
AGCGACAGAAGGAGATGTTGCGAATGTAATGTCTATTCTGTCGGGCAG
TCTGAACGTAACGGAAAAACATTGTTTCCAGTTCTGCAACCGGTAACC
AGACCGTTGGTGCCGCAGGCGCAACATGTCATCCAGATAGTGATCGGT
GGACAACTTCGTGGGCGATGGGAAAACCAACACAAAACGCGCGGTTT
AGTGGTTTG

Sequence Brucella melitensis from farm 1 - reverse

ORF F3 – 1R

ATCTTGGTACGGAATTTGTTACAAAAGTGTAATCCTTGCCACTGCAGG
AGAGGGGCTAGGGCATTGAGTGTCCCCCCAATCGACTGATGGACGAA
AGAGCCATAGGCCGCCATGATCATAGGCTGATGAATGGTATGGAAACT
ATCTTCGTTATCGCGTACATACGCTCCGTCAAGACCGGTATTTGATGAA
GTGGGATGCTCTTGAGCACCAGTTCCAATATTGGCAGTGACGGAATTG

CTCTCGACCACCAGTTCATTGCGGCTCTGGGTGCCGCTGGCTGAGGCG
CTCTGCACATTGTCGGTTACCGAAAAGCTGGATTCCTCCTGTTTAACGT
TGGCATTAAAAAACGGTAGCGTTATGGGAGCCCTACTGCTGATTCCTG
GGAATCCTGCGACCGCATCGGTCAACGAAGCAATGACCGACGATCCA
GCATCACCCACCTGAACGTTGGCAATACCGGCCGAACCGGCGACACTG
TTTCCGCCAAGTCCGGCTACATGATCCGCCTTGCCGTCCTCATAATGCA
CTTGTCCAAGCAGATTAGTAGCGTCGCTACCAATCGCAGTCGCCTGCT
GCGTGTTGTTCTCGACGGTTAATTTGGCTCCAGATATGTCGCTACCAAC
CTTGTTGTCGCCGTAGACACCAACCACCGACGCCGCATTGTTGGCGGG
AAATGTCACTGCTATAGTTTTGCCTGGTAGGCTGGGTAAGCATATTAC
CATGCCTTAACCCCGAAAGGCCGACTATCATGGGGTAGTCCTGGTCAG
TTCGGTCCCGACCTGTCCGCACACCCTAGTGCTGATCCCCAGCGGTTTA
GGGTTATTGGGCGTTCCAGGGGCCACCTGGCTGGGCTGGAGGCTGTTG
CCCAACGCCCAGTGCGGAATATACTTGGTTCCCCCAGAAAGCACAAGG
GGGTCCCCTATACTCGAAGATCACCCTGTTCCTTTCGGCTTTTACCGGC
ACAGTGGCGAACCCACCGTGGACCCACTATTTTGTCCGGGTCTGCCCC
CCCGCCATCTATTCCAGAAAAAAAACAACCAGTATATTAATCTCATGA
GTGATGGGT

Sequence Brucella melitensis from farm 2 - reverse

ORF F3 – 2 R

ACATTCTGGTACGGATTCCGTACCATTTTGGTAATCCTTGCATGCAGGC
AGGAAAAAGAGGGCATTGACTCTCCCCCCAATCGACTGATCGAAAGA
TAAGGCCATAGGCCGCCATGATCATAGGCTGATGAATGGTATGGAAAC
TATCTTCGTTATCGCGTACATACGCTCCGTCAAGACCGGTATTTGATGA
AGTGGGATGCTCTTGAGCACCAGTTCCAATATTGGCAGTGACGGAATT
GCTCTCGACCACCAGTTCATTGCGGCTCTGGGTGCCGCTGGCTGAGGC
GCTCTGCACATTGTCGGTTACCGAAAAGCTGGATTCCTCCTGTTTAACG
TTGGCATTAAAAAACGGTAGCGTTATGGGAGCCCTACTGCTGATTCCT
GGGAATCCTGCGACCGCATCGGTCAACGAAGCAATGACCGACGATCC

AGCATCACCCACCTGAACGTTGGCAATACCGGCCGAACCGGCGACACT
GTTTCCGCCAAGTCCGGCTACATGATCCGCCTTGCCGTCCTCATAATGC
ACTTGTCCAAGCAGATTAGTAGCGTCGCTACCAATCGCAGTCGCCTGC
TGCGTGTTGTTCTCGACGGTTAATTTGGCTCCAGATATGTCGCTACCAA
CCTTGTTGTCGCCGTAGACACCAACCACCGACGCCGCATTGTTGGCGG
AAATATCACTGCTATAGTTTGCCTGTAAGCTGGTAAGCATAGCACCTG
CCTTAGCCCGAAGGCCGCTATCATGGGTAGTCTCGTCGTTCGGCCCGC
CCGTCAGCAACCCAGTGCTATCCCCAGCGGTTTAGGTTATTGGCGTCC
AGGGCCCAACTGCTGGCTGGCGCTGTTGCCAACGCCAGTGCGTAATCT
TGTTCCCCGAAACATCAACGGTACCATTCATCGAGGATACTGTCTCTC
AGCTTACGGACATGGCACCACTGAACATTTAGCGTGGCTCGCCGCATA
TTCCGAAACAGTTAATTCGATGTTAGCCAGGGCAGCTGGTAGGACTAT
CCAAGGCCGCGA

Sequence Brucella melitensis REV 1 strain - reverse

ORF REV1 – 1R

AGTGTTCGCCGGTGACGCATTGGGTAAATGAGTTATCCTTGCCATGCA
GGGAGGGAGCAGGGCATTGATTGTTCCCCCAATCGACTGAGCGCCAA
AAAGGCCATAGGCCGCCATGATCATAGGCTGATGAATGGTATGGAAA
CTATCTTCGTTATCGCGTACATACGCTCCGTCAAGACCGGTATTTGATG
AAGTGGGATGCTCTTGAGCACAGTTCCAATATTGGCAGTGACGGAATT
GCTCTCGACCACCAGTTCATTGCGGCTCTGGGTGCCGCTGGCTGAGGC
GCTCTGCACATTGTCGGTTACCGAAAAGCTGGATTCCTCCTGTTTAACG
TTGGCATTAAAAAACGGTAGCGTTATGGGAGCCCTACTGCTGATTCCT
GGGAATCCTGCGACCGCATCGGTCAACGAAGCAATGACCGACGATCC
AGCATCACCCACCTGAACGTTGGCAATACCGGCCGAACCGGCGACACT
GTTTCCGCCAAGTCCGGCTACATGATCCGCCTTGCCGTCCTCATAATGC
ACTTGTCCAAGCAGATTAGTAGCGTCGCTACCAATCGCAGTCGCCTGC
TGCGTGTTGTTCTCGACGGTTAATTTGGCTCCAGATATGTCGCTACCAA

CCTTGTTGTCGCCGTAGACACCAACCACCGACGCCGCATTGTTGGCGG
AAATATCACTGCTATAGTTTGCCTGTAAGCTGGTAAGCATAGCACCTG
CCTTAGCCCCGAAGGCCGCTATCATGGGTAGTCTCGTCGTTCGGCCCG
CCCGTCAGCAACCCAGTGCTATCCCAACGGTAAGGTTATTGGCGTCCA
GGTCAACTGCTGGCTGGCGCTGTTGCCAAACGCCAGTGCGAAAATCCT
TGGTACCCCGAAACATCAACGTACAATCAATCGCACGATACATGTTCT
TTTCGCGTTTGACTGAACAATGCAAGCGACTTGAGCCAATTACCTTGC
CGGCGCAACTTCAGGATACCAGTATCTGATGGTTAAGCCAAGGGCCAG
CCGTATGAGCTGATCCATAAG