STATE OF ISRAEL

Veterinary Services and Animal Health, Beit-Dagan, POB 12, 50250, ISRAEL. FAX: 972-3-968-1753

CA13-007

August 15, 1995

U.S.-Israel Cooperative Development Research Program
Office of Research (R&D/R)
Room 320 SA - 18
Agency for International Development
Washington, D.C. 20523-1818
U.S.A.

Re.: Proposal No. ~~CA 13-007~~ CA13-007, Grant No. TA-MOU-CA 13-007

Dear Sirs,

Enclosed please find one original and 7 copies (1 for Project Office; 4 for the Science Advisor; and 2 for Board on Science and Technology for International Development) of the following:

1) Final Report of the project "Diagnosis and Control of Brucellosis", proposal No. CA 13-007, Grant No. TA-MOU-CA 13-007.
2) A statement of work and budget for cost extension proposal of the current grant from two to three years (Research proposal for 3rd year).

A copy of the original research project is also attached at your convenience.

Because of shortness of time we were not able to obtain signatures from our Kazakhstani counterparts. However, such signatures were obtained a year ago when we submitted a mutual research program (CA 15-027) that unfortunately was declined.

This request is in effort to justify continuation of the project which we feel was a great success and otherwise, if not funded, will loose the momentum and reach a deadend.

We do apologize for not submitting the progress report in due time because of being committed to the national brucellosis control program as well as many other duties.

Thank you for your time and assistance.

Sincerely yours,

Menachem Banai

Menachem Banai, Ph.D.

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DIAGNOSIS AND CONTROL OF BRUCELLOSIS

Proposal No.: CA 13-007

Grant No.: TA-MOU-CA 13-007

Submitted to the Office of the Science Advisor U.S. Agency for International Development

Final Report

November 1993 - July 1995

Submitted by:

Dr. Menachem Banai
The Kimron Veterinary Institute
Bet Dagan
Israel

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1. Executive Summary

Two basic objectives were proposed - e.g., to develop a PCR diagnostic method for *Brucella* and, to develop an ELISA test for mass serological surveillance of the disease. The control of the disease underlies these objectives since although being specific and sensitive the available diagnostic methods are laborious, unefficient and require highly trained personnel to obtain reliable results.

We chose the omp2 and the rplL genes as target DNA sequences for the PCR test. The omp2 gene exists in the *Brucella* chromosome in two copies and therefore expected to increase the test's sensitivity. Moreover, a Pstl restriction site polymorphism within the amplified DNA fragment can distinguish between *B. abortus* and *B. melitensis* - the two important species in Israel and Kazakhstan. Similarly, the rplL gene, shown to be identical in the two species, could be used to facilitate *Brucella* diagnosis.

Using known numbers of bacterial cells (CFUs) the sensitivity of the omp2 and rplL PCR test was similar (about 45%). When 38 field samples of unknown bacteriological status were tested 16 and 17 samples produced PCR fragments with the omp2 and rplL primers, respectively, showing again similar sensitivity of the test between the two gene sequences. The specificty of the test was high as from 23 bacteriologically negative field samples all were also negative in the PCR test. Nevertheless, a DNA fragment similar in size to the *Brucella* rplL gene has been obtained from a field isolate characterized as *Ochrobactrum anthropi*. This fragment differed, however, from the *Brucella* gene by lack of an EcoRI site but it showed DNA homology in hybridization reaction, demonstrating that the established taxonomical relatedness between *Brucella* and *Ochrobactrum anthropi* also occurs in the rplL gene.

Interpretation of the data suggested that inspite the fact that the omp2 gene exists in the *Brucella* chromosome in two copies normal strain's DNAs were only amplified from the omp2b gene sequences. In contrast, the omp2 gene of the vaccine strain *B. melitensis* Rev1 was amplified from the both gene copies. This is first recording of unique characteristics of the vaccine strain on the DNA level (a draft manuscript attached). It could be argued that this phenomenon is associated with attenuation of the vaccine strain. Confirmation of this hypothesis might open new avenues in understanding *Brucella* pathogenicity.

A direct ELISA test was developed and compared with the conventional serological methods. The test is based on coating the wells with brucellin-INRA, the antigen used for the allergic skin test, and analysis of IgG antibody binding to the antigen by using peroxidase conjugate of secondary anti sheep (bovine) antibodies. Sera from either vaccinated (*B. melitensis* Rev1) ewe lambs, vaccinated (*B. abortus* S19) female calves and from infected (*B. melitensis* field strains) sheep and cows were used to demonstrate dissociation by the ELISA test of serological responses due to vaccination and infection (manuscripts in preparation).

As persistence bacteriology and serology have often been recorded after Rev1 vaccination, which hampers surveillance, the

development of the new technologies has significantly improved disease control. The technologies and necessary equipment were transferred to two scientists from Kazakhstan who have stayed with us for over a month and during this period have been trained in all respects (conventional serology, bacteriology, PCR and ELISA).

Section 1

A. Research Objectives

1. To develop a PCR diagnostic test for *Brucella* that will identify the organism and ultimately identify species and biovars.

2. To develop an ELISA test that will replace the conventional serological methods in mass surveillance campaigns.

3. To use the brucellin alergic skin test as a supplementary diagnostic method.

B. Research Accomplishments

1. Development of a PCR diagnostic test for *Brucella*.

PCR amplification of *Brucella* DNA has already been demonstrated by others (1,2,3). The PCR method we have developed is based on using *Brucella* Omp2 gene as target sequences (4). The primers employed are 5' and 3' flanking sequences of a locus in the gene with a Pst 1 restriction fragment endonuclease polymorphism (5). Therefore, the digestion of the PCR fragment product with this restriction enzyme can facilitate biotyping of *B. abortus* and *B. melitensis* species and biotypes.

In addition, we used a second gene for PCR diagnosis. Recently, the *Brucella* rplL gene, encoding the L7/L12 ribosomal protein, has been identified and sequenced (6). Comparison of the *B. abortus* gene with that of *B. melitensis* revealed the two genes are identical (7). This suggested that a PCR diagnosis of *Brucella* is possible by using the gene sequence as target DNA.

A full description of the results is included in the attached draft manuscript (to be submitted to J. Bacteriology, Appendix B).

2. Development of an ELISA test that distinguishes between serological response to vaccination and infection.

An ELISA test in which crude *Brucella* extract enriched with immunodominant proteins (8) is used as the serodiagnostic antigen, has been developed. The antigen is prepared from a rough *B. melitensis* (strain B115) and therefore lacks O-chain antigen of *Brucella*. Usually, this extract is used for allergic skin testing of *Brucella* sensitized animals (9). We used it as coating antigen in the ELISA test to detect antibodies elicited against *Brucella* immunodominat proteins. Therefore, we expected that the immune respone to vaccination, which predominantly involves humoral response against the highly immunogenic O-chain polysaccharide antigen of *Brucella*, will not be detected in the assay. In contrast, we expected that infection would also lead to hyperimmunization against *Brucella* intracellular proteins and the assay will detect these antibodies.

2.1 ELISA tests.

The ELISA test was initially developed in our laboratory for testing sheep sera. For the assay, microtiter plates (flat wells, medium binding, Nunc, USA) were sensitized with Brucellergen (Rhone Merieux, France). This is a commercially available antigen conventionally used in the allergic skin test. It is prepared from a rough *Brucella melitensis*, strain B115, as a protein enriched crude salt extract (9).

Brucellergen was diluted 1:50 in carbonate buffer (pH 9.6) and 100 µl aliguotes were dispensed per well. After 1h incubation at 37°C, the plate was incubated overnight in the cold. The next day, the coating solution was removed and the wells washed five times with phosphate buffered saline (PBS, pH 7.4) containing 0.05% (v/v) Tween-20 (PBST). Then, serum samples diluted 1:100 in the same buffer were added and the plate was incubated for 1h at 37°C.

Following five washes of the wells with PBST 100 µl aliguotes of peroxidase-donkey anti sheep IgG conjugate, diluted 1:8000 in PBST, were added per well and allowed to stand for 1h at 37°C. Then, the wells were washed five times with PBST, and 100µl of o-phenylenediamine dihydrochloride containing 0.05% H_2O_2 were added per well. The absorbance results of the color, developed within 15 minutes, were read at 450 nm using an ELISA reader (Bio-tek EL 340, USA).

For ELISA testing of cows' sera similar procedure was used with slight modifications. These included dilution of the sampled sera 1:250 (compared to sheep sera which were diluted 1:100), and use of peroxidase-rabbit anti bovine immunoglobulin G (IgG) conjugate (Sigma, USA) diluted 1:15,000.

2.2 Sera sampling.

Sheep from brucellosis free flocks were chosen to obtain negative responders, for negative control tests. Ewe-lambs prior to vaccination, or ewes after kidhood vaccination that have completed first gestation (which should not react in the serological tests) were sampled.

Ewe lambs, 20 to 40 days postvaccination, were used in the group of vaccinated animals, to compare ELISA with conventional serology of vaccinated animals.

Sheep from infected flocks were used in the group of infectd animals, to compare ELISA with conventional serology of infected animals.

Cows from brucellosis free dairy cattle herds were sampled for negative control experiments in the ELISA test. Cows from a dairy herd, infected with *B. melitensis* biotype 1 strain, were used to compare ELISA results with the serological results in infected cows. The infected herd consisted of 409 milking cows and all were tested by ELISA, SAT, and CFT.

2.3 Serological tests.

Brucella abortus strain 1119 and *B. melitensis* strain Huddleson were used as antigens to test anti A and anti M serological responses. The antigens were prepared in the laboratory at "Kimron

Veterinary Institute." They were standardized to give 50% agglutination at a serum dilution of 1:650, using national reference serum.

Serum agglutination test (SAT) and complement fixation test (CFT) were carried out as recommended by Alton, G.G., et al. (9). These tests are being routinely conducted in the Israeli laboratory (10).

2.4. Vaccination.

Vaccination of ewe-lambs (3-6 months old) was carried out subcutaneously, using *B. melitensis* Rev1 (commercial vaccine) at a dose of $1\text{-}2 \times 10^9$ colony forming units (CFUs).

Vaccination of calves (3-6 months old) was carried out subcutaneously using *B. abortus* S19 (commercial vaccine) at a dose of 4×10^{10} CFUs. In Kazakhstan, strain *B. abortus* 82 (8×10^{10} CFUs per dose) was also used as a vaccine in several places.

2.5 Preliminary results.

2.5.1. ELISA test in sheep.

Three parameters were studied: a) test's specificity was evaluated by testing sera from serologically negative ewes with, or without, history of kidhood vaccination; b) test's efficacy in distinguishing between serological response to vaccination and infection; and c) tests's efficacy in detecting infected sheep.

The results shown in Table 1 suggest that the ELISA test is specific. From 41 ewes with, or without, history of kidhood vaccination (negative in the conventional serological SAT and CFT tests), none had reacted in the ELISA test. A control of positive reactor's serum was assayed and found positive ($O.D._{450} = 0.26$).

Table 2 depicts a follow up study of the humoral response of vaccinated ewe-lambs (Rev1, normal dose) in conventional SAT and CFT serological tests compared to their results in the ELISA test. As can be seen, vaccination elicited high SAT and CFT titers at the peak of the humoral response (20 and 40 days post-vaccination). In contrast, about 58 to 42% of the ewe-lambs were completely negative in the ELISA test and about 20% more produced suspected results. Sixty days post-vaccination there were still more than 40% negative responders in the ELISA test. Because ewes were negative in the test, irrespective whether or not they had history of kidhood vaccination, we concluded that the ELISA test could, to a certain limit, identify the humoral response which was elicited due to vaccination.

An infected flock, composed of 56 young ewes after kidhood vaccination (age between 12 to 18 months) and 127 ewes from which 22% had been diagnosed positive responders in the CFT, has been chosen for field testing of the ELISA method. As can be seen in Table 3, only partial correlation was obtained between CFT and ELISA. About 7% CFT positive responders were negative in the ELISA test. Similarly, about 11% ELISA positive responders were negative in the CFT. Because ELISA was found specific (Table 1) we concluded that the discrepancy between ELISA and CFT could be explained on the ground of a difference between the methods in their capacity to

diagnose similar antibody isotypes, suggesting that each method may identify different phases of the disease.

The young ewes, which could be true persistent responders to vaccination, were analyzed separately. As depicted in Table 4, the results obtained with this group differed from the results obtained with the whole flock (Table 3). Almost twice as much CFT positive - ELISA negative responders (about 14%) were found in this group while the proportion of ELISA positive - CFT negative responder was similar to the results obtained with the whole flock (about 9% compared to 11%). This suggested that about 7% of the CFT positive responders that were ELISA negative had actually reacted in CFT as a cause of their vaccination.

Although results obtained by ELISA could not be judged conclusively, as some of the vaccinated ewe-lambs could also be responders in the test, the data shown in Table 4 demonstrate the power of the technique in resolving the brucellosis status of problematic sheep flocks. Eventually, identification of the brucellergen proteins which uniquely react with serum from infected animals, but not with serum from vaccinated animals, will facilitate the development of an ultimate ELISA method. We have already identified at least one such protein and in future we will clone the gene and express it in *E. coli* for use as a sole antigen in the ELISA test.

2.5.2. **ELISA test in bovine.**

The ELISA test has also been developed for use in bovine brucellosis. Similar to the organization of the data in Table 2, we have evaluated the ELISA technique in cows. It can be seen in Table 5 that the specificity of the test was very high as no reactor was found among 64 serologically negative heifer calves (first column, day 0). Moreover, no reactor has been found among the vaccinated heifer calves although they were at the peak of their serological (SAT and CFT) response. This proved the efficacy of the test in identification of infected responders among vaccinated animals.

As shown for sheep, we have also evaluated the use of the ELISA test in a dairy herd which was found infected with *B. melitensis* biotype 1 strain. Table 6 depicts the composition of the herd with respect to place of the animals in the premises, their age and their vaccination status. The milking cows composed the largest part of the herd. As can be seen in Table 7, good agreement between ELISA and corresponding reactors in SAT and CFT was shown (39.6%). Similarly, negative reactors in the three tests composed 24.7% of the herd. However, there were deviations that included 8% positive reactors in CFT that were ELISA negative, and about 8.8% with opposite results - e.g., ELISA positive and CFT negative.

In contrast to the results obtained with the milking cows, heifers before and during pregnancy (group 3, Table 8), or heifers after first gestation (group 2, Table 9), have shown higher figures (9 and 14%, respectively) of deviating CFT positive - ELISA negative results suggesting that most of them have elicited the CFT titers due to vaccination. This, again, demonstrated the potence of the ELISA test in distinguishing between serological responders to

vaccination and infection.

We will prepare these data for publication in internationally recognized scientific journals.

3. Research in Kazakhstan.

Brucellosis in Kazakhstan is slightly different than in Israel. Firstly, the overall animal production system is different. Kazakhstan is by far larger than Israel with millions of animals that are at risk of brucellosis. Secondly, while in Israel brucellosis is limited to occurrence of *B. melitensis* only, in Kazakhstan *B. abortus* infection of cattle and camels, and *B. ovis* infection of rams is common (Table 10).

As in Israel, Kazakhstan implemented control measures to reduce the infection rates among the animals. Similar serological surveillance tests were employed, and usually complement fixation test (CFT, Table 11) was used. The allergic skin test was one important element of brucellosis surveys (Tables 10,12).

Until recently, the conventional approach of S19 and Revl vaccination of cattle and sheep and goats, respectively, was common in Kazakhstan. Because this regime of vaccination failed to confer full protection on the animals, other methods were sought. Reimmunization of the animals without hampering the surveillance programs was considered. Therefore, immunization with dead vaccine was a possible solution.

In order to achieve these goals, *B. abortus* strain 82 and *B. melitensis* strain Revl dead vaccines were evaluated using a newly developed adjuvant (Tables 13,14). In general, the vaccine is composed of dead bacterial cells, aluminum hydroxide - as an adjuvant - and thymus extract from neonatal calves as a stimulant of the immune system.

The new vaccine was tested experimentally in heifers and ewe lambs. The results are shown in Tables 13 and 14. As shown in Table 13, certain doses of the dead vaccine provided 100% immunity while reduced doses, and live *B. abortus* strain 82 vaccine, were unsatisfactory. Similarly, the Revl dead vaccine conferred protection on sheep provided that a sufficient dose was used. Live Revl vaccination conferred only 80% immunity on the vaccinated animals (Table 14). Definitely, these experiments provide preliminary evidence as to the efficacy of the newly developed method. Moreover, it has been shown that the serological sensitization due to vaccination disappears within a few months and, therefore, annual revaccination is possible.

Based on these preliminary results a decision was made in Kazakhstan to change the existing vaccination program. The initial vaccination is carried out normally with live vaccine strains. Then after - and prior to insemination of the females - serological CFT test is conducted using ultrasonicated *B. abortus* antigen (see results in Table 11). If proven negative, the animals are revaccinated with the dead vaccine. Males, as well, are being vaccinated annually.

C. Scientific Impact of Collaboration

Since the inception of the collaboration between Israel and Kazakhstan, progress has been made in both countries. Each group undertook an independent course of research leading to the above described results. Moreover, two scientists from Kazakhstan have visited the Israeli laboratory for a period of about two months. During this visit, the techniques developed in Israel were communicated to the Kazakhstanis. Therefore, the scientific level of the both countries has been evened.

D. Description of Project Impact

In April 1995, Israel launched a national brucellosis eradicaiton campaign. It includes serological (CFT) surveillance of the small ruminant population (only ewes and goats that have delivered twice, because their antibody titer to vaccination is expected to drop to nil).

Wherever a serological response is suspected due to vaccination the newly developed ELISA test is being used to confirm the serological result. In addition, dairy cattle is being surveyed by the milk ring test. Suspected herds are being tested by the ELISA test in addition to being tested by CFT. The PCR test is being used at two levels: a) on the experimental level it is being compared routinely with the bacteriological isolation method; b) as a diagnostic test we use it to confirm isolation of *B. melitensis* Rev 1 whenever this is suspected according to bacteriological results. Therefore, the application of the new methods actually improved diagnosis and increased efficacy of the tests.

E. Strengthening of Developing Country Institutions

Prof. Ivanov and Dr. Amireev visited Israel at the beginning of the reported program. At that time, the visit of two Kazakhstani scientists, Dr. Sultanov Akhmedjan and Dr. Victor Ten, was planned. Indeed, these scientists arrived in Israel during the first research year and stayed in our laboratory for almost two months. During this period friendly relationships were established and scientific information was exchanged.

Because the Israeli side controlled the budgetary aspect of the project, the visit of the Kazakhstani scientists to the Israeli laboratory was arranged from Israel. Airline tickets were bought in Israel and sent to Kazakhstan. In addition, funds were allocated by the Israeli administration for accommodation and per diem of the Kazakhstani scientists, with the Israeli administration receiving those expenses from the USA.

Scientific equipment valued at $30,000 e.g., - ELISA Reader, PCR cycler, electrophoresis, micropipetors and disposables - see attached table) was successfully purchased and transferred to the collaborating laboratory in Kazakhstan. The decision for the purchase of the specific items was obtained during the visit of the Kazakhstani scientists in the Israeli laboratory and only after

they were trained in the use of identical equipment.

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Section II

A. Budget

Two changes were made in the Israeli budget. Firtstly, whereas 100% time occupation of technician assistance was approved we only used 75% of it. Secondly, we overcharged the Materials and Supplies, and Equipment sub-budget. A total of $3,000 was allocated whereas we purchased equipment and biological reagents at a sum of $8,705. Expenses were mostly spent on constructing oligonucleotide primers, and purchasing Taq polymerase and ELISA reagents. These are expensive reagents which led to the exceeding of the sub-budget. However, the total budget was kept even.

The Kazakhstani budget was kept promptly (see above).

As the project has so far progressed with great success we request a cost extension of the grant for an additional year (see attached proposal).

B. Collaboration, Travel, Training and Publication

The Israeli P.I. plans to visit Kazakhstan to discuss achievements obtained during the research period. In addition, this visit will be used to prepare a new research proposal.

The results of the current research are being prepared for publication. A draft manuscript on the PCR results is attached. The ELISA results will be prepared very soon.

TABLE 1.

Evaluation of the specificity of the ELISA assay in sheep[a].

Experimental group	No. of sheep	ELISA O.D. 450_{nm}
Kidhood vaccination	19	0.095± 0.038
No kidhood vaccination	22	0.125± 0.037

[a]Forty one ewes, 19 having a history of kidhood vaccination and 22 without it, were tested. All ewes were CFT and SAT negative. A positive responder (control test) produced O.D. 450_{nm}=0.26.

TABLE 2.

Comparison between ELISA and conventional SAT and CFT in evaluation of vaccinated ewe-lambs[a]

Serological category	Percentages of responders (Days after vaccination)			
	0	20	40	60
SAT neg	100	0	2	6
SAT 1:20	0	0	4	16
SAT> 1:40	0	100	94	78
CFT neg	100	0	0	0
CFT 1:5	0	2	0	8
CFT> 1:10	0	98	100	92
ELISA neg[b]	98	58	42	30
ELISA sus	2	20	22	14
ELISA pos	0	22	36	56

[a]Thirty and 20 ewe-lambs, from two flocks, were tested prior to, and following subcutaneous Rev1 vaccination (normal dose).

[b]ELISA negative, suspected and positive results were designated according to $O.D._{450}$ units of 0.15, 0.17 and >0.17, respectively.

TABLE 3.

Comparison between ELISA and conventional CFT in evaluation of infected sheep[a]

CFT	ELISA			Total
	Positive	Suspected	Negative	
Positive	24 (13.1)	3 (1.6)	14 (7.6)	41 (22.4)
Negative	21 (11.5)	18 (9.8)	103 (56.3)	142 (77.6)
Total	45 (24.6)	21 (11.5)	117 (63.9)	183 (100)

[a]A flock consisting of 183 sheep, 56 being in the age between 12 to 18 months, was studied. Numbers in parantheses indicate group's percentages. O.D. limits of 0.15, 0.17 and greater than 0.17 were designated for negative, suspected and positive results, respectively.

TABLE 4.

ELISA evaluation of the infection status of 56 young ewes in the flock (age between 12 to 18 months).

CFT	ELISA			Total
	Positive	Suspected	Negative	
Positive	5 (8.9)	2 (3.6)	8 (14.3)	15 (26.8)
Negative	5 (8.9)	6 (10.7)	30 (53.6)	41 (73.2)
Total	10 (17.8)	8 (14.3)	38 (67.9)	56 (100)

[a]Numbers in parantheses indicate group's percentages, O.D. limits of 0.15, 0.17 and greater than 0.17 were designated for negative, suspected and positive results, respectively.

TABLE 5.

Comparison between ELISA and conventional SAT and CFT in evaluation of bovine sera of different vaccination status

Serological category	Percentages of reactors (time after vaccination)				
	Days[a]			Months[b]	Years[c]
	0	20	40	18	>2
SAT neg	100	11	0	100	89
SAT 1:20	0	6	7	0	10
SAT> 1:40	0	83	93	0	1
CFT neg	100	20	7	100	100
CFT 1:5	0	3	13	0	0
CFT> 1:10	0	77	80	0	0
ELISA neg	100	100	100	100	100
ELISA sus	0	0	0	0	0
ELISA pos	0	0	0	0	0
Number of tested animals	64	35	34	18	136

[a]Heifer calves from 3 different herds in 3 groups of 30, 19 and 15, respectively, were tested. After 20 days, 15 heifer calves from the first group were omitted from the test. After 40 days another heifer calf from the second group was also omitted from the test.

[b]Eighteen heifers in gestation were tested.

[c]One hundred twenty one cows from several herds were tested.

[d]ELISA negative, suspected and positive results were designated according to $O.D._{450}$ units of 0.15, 0.17 and >0.17, respectively.

TABLE 6.

Composition of the dairy herd according to age and vaccination category.

Group No.	Status of the animals	Age of the animals	No. of animals per group
1	Milking cows	2-10 years	409
2	Heifers after first pregnancy[a]	1.5-2.5 years	80
3	Heifers prior to or in first pregnancy	1-1.5 years	80
4	Female calves 12 days and 2½ months post vaccination	3-6 months	60
5	Unvaccinated young calves	1 week - 2 months	33
Total			861

[a]Twelve to twenty four months after vaccination

TABLE 7

Distribution of the number[a] of nonresponding and responding animals in the SAT, CF and ELISA.

Methods	ELISA			Subtotal
	Positive	Suspect	Negative	
SAT positive CF positive	162 (39.61%)	1 (0.24%)	4 (0.98%)	167 (40.83%)
SAT positive CF negative	1 (0.24%)	1 (0.24%)	1 (0.24%)	3 (0.72%)
SAT negative CF positive	38 (9.29%)	14 (3.42%)	29 (7.09%)	81 (19.8%)
SAT negative CF negative	35 (8.56%)	22 (5.38%)	101 (24.69%)	158 (38.63%)
Subtotal	236 (57.7%)	38 (9.28%)	135 (33%)	409 (100%)

[a]Numbers in parentheses represent percentage of the reactors among total of 409 tested.

TABLE 8

Distribution of the number[a] of nonresponding and responding animals among heifers prior to, or in first pregnancy (group 3).

Methods	ELISA		Subtotal
	Positive	Negative	
SAT positive CF positive	2 (2.5%)	4 (5%)	6 (7.5%)
SAT positive CF negative	1 (1.25%)	2 (2.5%)	3 (3.75%)
SAT negative CF positive	0	3 (3.75%)	3 (3.75%)
SAT negative CF negative	0	68 (85%)	68 (85%)
Subtotal	3 (3.75%)	77 (96.25%)	80 (100%)

[a]Numbers in parentheses represent percentage of the reactors among 80 tested.

TABLE 9

Distribution of the number[a] of nonresponding and responding animals among heifers after first pregnancy (group 2).

Methods	ELISA		Subtotal
	Positive	Negative	
SAT positive CF positive	0	8 (10%)	8 (10%)
SAT positive CF negative	0	3 (3.75%)	3 (3.75%)
SAT negative CF positive	0	3 (3.75%)	3 (3.75%)
SAT negative CF negative	2 (2.5%)	64 (80%)	66 (82.5%)
Subtotal	2 (2.5%)	78 (97.5%)	80 (100%)

[a]Numbers in parentheses represent percentage of the reactors among 80 tested.

TABLE 10.

Skin test and serological surveillance of sheep, cows and camels in infected herds in Kazakhstan.

Animal species	No. of animals tested	Reactor animals			
		A	B	A+B	Total
ewes	19,553	2,260	1,400	1,971	5,631
cows	800	64	34	56	154
camels	300	9	12	5	26

A - Skin test only

B - Serological test only

A+B - Corresponding reactors in the skin test and the serological tests.

TABLE 11.

CFT activity of different *Brucella* abortus antigenic preparations.

Antigen dilution	Methods of preparation[a]			
	A	B	C	D
Abortus 1:10	320	640	320	320
1:20	160	320	160	160
Ovis 1:10	-	-	-	-
Negative	-	-	-	-

[a]Results are presented as reciprocal titer obtained with the same serum. A - Ozone; B - Ultrasonication; C - Commercial antigen (Heat treatment at 80°C); D - Freezing (-20°C) and thawing.

TABLE 12.

The activity of allergenic preparations, prepared from different *Brucella* strains, in guinea pigs sensitized with the same *Brucella*.

Group No.	*Brucella* inoculation	No. of guinea pigs	Skin reaction (mm) by allergens from *Brucella* strains indicated below						
			Rev 1 M±m	3-61 M±m	104M M±m	"B" M±m	19 M±m	82 M±m	Brucellin M±m
1	Rev1 melitensis	10	6.5±0.3	2.7±0.5	5.3±0.3	3.8±0.4	4.4±0.4	1.8±0.2	1.6±0.2
2	3-61 Suis	10	2.5±0.5	6.0±0.2	3.4±0.3	2.3±0.4	1.6±0.4	1.4±0.2	2.7±0.6
3	104M abortus	10	4.3±0.4	2.0±0.2	7.0±0.1	2.4±0.3	4.0±0.3	2.8±0.4	1.4±0.2
4	"B" abortus	10	4.1±0.4	2.9±0.4	5.7±0.3	5.5±0.2	2.4±0.5	1.9±0.4	1.2±0.5
5	19 abortus	10	2.5±0.6	1.7±0.5	4.8±0.6	2.0±0.5	5.8±0.3	1.9±0.2	2.5±0.6
6	82 abortus	10	2.5±0.4	1.5±0.5	3.3±0.1	3.1±0.4	3.2±0.5	5.6±0.3	2.8±0.6
7	Untreated	10	-	-	-	-	-	-	-

TABLE 13.

Immunity conferred on heifers, 12 months after vaccination with a dead strain 82 vaccine, at different doses.

Group No.	No. of animals tested	Dose of vaccine (cm^3)	Immunity %	Index
1	3	4	100	-
2	3	3	100	-
3	3	2	66.6	2.8
4	3	1	33.3	16.7
5[a]	3	$80X10^9$	66.6	11.1
6	3	unvaccinated	-	36.1

[a] Live *B. abortus* strain 82 vaccine.

TABLE 14.

Immunity conferred on sheep, 12 months after vaccination with a dead Rev 1 vaccine, at different doses.

Group No.	No. of animals tested	Dose of vaccine (cm^3)	Immunity %	Index
1	5	2	100	-
2	5	1	100	-
3	5	0.5	80	1.7
4[a]	5	$2X10^9$	80	3.3
5	5	unvaccinated	-	33.8

[a] Live vaccine *B. melitensis* Rev 1.

List of non-expendable equipment and some expendable items (for shipment to Kazakhstan)

Equipment	Ser. No.	Catalog Number	Description	Supplier	Units	Price in U.S. $ Dollars
ELISA	1	EL-340i	Bio-tek automated U.V. kinetic microplate reader	Labotal	1	
	2	307141	Kineti-Calk II kinetics/Endpoint software package for IBM computers	Labotal	1	
Reader and	3	3070186	KC-JR simplified kinetics/Endpoint software package for IBM computers	Labotal	1	15.481
accessories	4	75004	IBM Interface cable for EL-340i	Labotal	1	
	5	9000543	Calibratio plate	Labotal	1	
	6	75006	parallel printer cable	Labotal	1	
PCR	7	5330000.017	Mastercycler 5330 DNA amplification instrument (PCR)	Lumitron	1	6,735
PAGE	8	Mopid-2	Electrophoresis BABY GEL	Tal-Ron	1	710
Digital pipets	9	402-7640	Finnpipette Digital ACL 05-10μl	Labotal	1	
	10	402-7650	Finnpipette Digital ACL 5-40μl	Labotal	1	432
	11	402-7660	Finnpipette Digital ACL 40-200μl	Labotal	1	
	12	402-7670	Finnpipette Digital ACL 200-1000μl	Labotal	1	158
Multi	13	4780000.010	Eppendorf multipette model 4780	Lumitron	1	297
channel	14	4788200.000	8 Channel adapter for multipette	Lumitron	1	
pipette	15	0030058.534	Cartridge 100 μl (20 pcs)	Lumitron	1	887
	16	0030058.518	Cartridge 1.25 μm (20 pcs)	Lumitron	1	
Expendable	17	0030121.023	Micro test tubes 0.5 ml (500 pcs)	Lumitron	2	
items	18	HT-I5-NG	Microcentrifuge tubes 1.5 ml (500 pcs)	Renium	2	84
	19	4844	Natural pipette tips 1-200 μl (1000 pcs)	Costar	2	
	20	000-0000-018	Pipet Tip	EL Kay	1	
	21	462100	Filter 0.45 μm (50 pcs)	Tamar	5	399

TOTAL ± $25,183

Polymerase chain reaction (PCR) based on Brucella *rplL* and *omp*2 gene sequences show distinguished characteristics of the *B. melitensis* vaccine strain Rev1.

Mandelboim, M.,[1] Banai, M.,[1]* Keren, G.,[2] Ficht, T.,[3] Bercovier, H.,[4] and Bachrach, G.[4]

Department of Bacteriology, The Kimron Veterinary Institute, Bet Dagan 50250,[1] Infectious Disease Unit, Sheba Medical Hospital, Sackler School of Medicine, Tel Aviv University,[2] Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77845,[3] and Department of Clinical Microbiology, The Hebrew University-Hadassah Medical School, Jerusalem,[4] Israel

Abstract

Polymerase chain reaction (PCR) based on *Brucella omp*2 and *rplL* genes has been evaluated as a means of *Brucella* diagnosis. The *omp*2 gene exists in the *Brucella* chromosome in two copies suggesting that the efficacy of its amplification might be doubled in comparison to amplification of a single copy gene. Moreover, within the amplified DNA sequence a *Pst*1 restriction site polymorphism exists that enables to distinguish between *B. abortus* and *B. melitensis*, the two most disseminated *Brucella* nomenspecies over the world. The *rplL* gene has been shown to be identical in these nomenspecies suggesting that it might be useful as a diagnostic tool. It was demonstrated that non-*Brucella* DNA, including closely

Polymerase chain reaction (PCR) based on Brucella *rplL* and *omp2* gene sequences show distinguished characteristics of the *B. melitensis* vaccine strain Rev1.

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Abstract

Polymerase chain reaction (PCR) based on *Brucella omp2* and *rplL* genes has been evaluated as a means of *Brucella* diagnosis. The *omp2* gene exists in the *Brucella* chromosome in two copies suggesting that the efficacy of its amplification might be doubled in comparison to amplification of a single copy gene. Moreover, within the amplified DNA sequence a *Pst1* restriction site polymorphism exists that enables to distinguish between *B. abortus* and *B. melitensis*, the two most disseminated *Brucella* nomenspecies over the world. The *rplL* gene has been shown to be identical in these nomenspecies suggesting that it might be useful as a diagnostic tool. It was demonstrated that non-*Brucella* DNA, including closely

related genera such as *Agrobacterium* and *Rhizobium*, did not produce amplified fragments corresponding with the *Brucella* amplified DNA fragments. This suggested that the both tests are specific to the *Brucella* genus. However, this conclusion was biased by identification of a corresponding *rplL* PCR product from a bacterial isolate from a cow, identified as *Ochrobactrum anthropi*, which was homologous to the *brucella* gene by DNA hybridization. This fragment, however, differed from the *Brucella* gene by lack of a known *Eco*RI restriction site. Biological samples of unknown bacteriological status, mainly composed of milk samples from sheep and cows, were diagnosed by either gene with similar efficacy (around 45% of the samples). Direct testing of *Brucella* cultures suggested that the threshold number for *Brucella* identification with the both genes was similar, being around 50 to 100 CFUs. *Pst*1 digestion analysis of the *omp*2 PCR amplification products revealed that only the *omp*2b gene has been amplified when normal *B. melitensis* strains were tested. However, when the *B. melitensis* vaccine strain Revl was tested the both genes were amplified. This unique result was also obtained with Revl field isolates, demonstrating the importance of the technique in diagnosis (identification of vaccine strain secretion in animals after kidhood vaccination), and as a taxonomical tool in establishing evolutionary changes in *B. melitensis*. To the best of our knowledge, this is first recording of unique genotypic characterization of the Revl vaccine strain which might give a clue to understanding the attenuation of the strain.

Introduction

Brucellosis is a worldwide distributed zoonotic disease. The causative agent of brucellosis is a small gram negative coccobacillus usually persisting as an intracellular parasite in the host's macrophage and monocyte cells. It is the causative agent of abortion in the natural host species while it can infect other animal species. The genus is divided into six species corresponding to preferred host, phage susceptibility and unique oxidative metabolism profile.

Live attenuated vaccine strains were developed to control the disease (8,11,25). *Brucella melitensis* strain Rev1 is currently the vaccine of choice for small ruminant vaccination (1) but its use has also been suggested to vaccinate cattle. The Rev1 was shown superior in conferring protection when compared to *B. abortus* S19 and *B. suis* strain 2 (6).

In the recent years, Israel has conducted an intensive control campaign during which most of the female ewe-lambs and goat kids at the age between 3 to 6 months were vaccinated with a single, full dose vaccine. Nevertheless, there were occassional outbreaks of the disease among vaccinated animals. In addition, the vaccine strain was isolated frequently from vaccinated ewes and rarely from unvaccinated animals. In a single incident of a sheep flock infection with Rev1 the owner contracted the disease and the vaccine straine was isolated from his blood (4). On top of these, atypical *B. melitensis* biovar 1 strains were identified and one of them has been found endemic in the country (7).

The complications in bacteriological characterization of the field isolates and the need to continuously survey diary cattle herds, vaccinated with *B. abortus* S19, for *B. melitensis* infection constituted a demand for improved diagnosis. The polymerase chain reaction (PCR) method has been used in the recent years as a powerful diagnostic tool for both identification of *Brucella* and for biotyping the strains (7,12,13). We sought use of this method as a supplementary test to diagnose *Brucella* spp. as well as for biotyping the isolates. The *Brucella rplL* and *omp*2 genes were selected as target sequences.

The *rplL* gene was recently identified, cloned and sequenced from both *B. melitensis* strain 16M (3) and *B. abortus* strain 2038 (20). Sequence analysis revealed the gene encodes the L7/L12 ribosomal protein and it is identical in the two species. Moreover, it has been demonstrated that the first 14 amino acid residues in the gene's N terminus are unique to *Brucella* spp. despite the fact that downstream it is highly conserved in comparison with other microrganisms (3). Therefore, the oligonucleotide primers were designated for the PCR test according to these sequences.

The *omp*2 gene encodes for the *brucella* 36 kDa outer membrane protein (14). There are two gene copies in the *brucella* chromosome (*omp*2a and *omp*2b) but only one (*omp*2b) is supposedly expressed (16). The gene duplication was thought to improve sensitivity of the PCR test. Moreover, the presence of a *Pst*1 restriction enzyme site polymorphism in the two genes and their flanking sequences (15) provided an opportunity to distinguish between *Brucella*

species according to the size of the fragments that a PCR product will form after digestion with different restriction endonucleases.

In this article we show that the *rplL* gene was usually amplified at higher efficacies than the *omp*2 gene. In addition, *omp*2 gene amplification of normal *B. melitensis* strains resulted in a different pattern than that produced by amplification of the *B. melitensis* Rev1 DNA. It is suggested that in contrast to our expectations when normal *B. melitensis* strains were tested amplification occurred from only the *omp*2b gene whereas the two gene copies were amplified from the vaccine starin's chromosomal DNA. The possibility that this observation is associated with the *Brucella* melitensis virulence is indicated.

Materials and Methods

Bacterial strains: The bacterial strains used in this study are listed in Table 1. *Brucella* reference strains and field isolates were from our national collection after many passages in the laboratory. *Escherichia coli* strain NCTC 9001 was used to obtain non *Brucella* DNA as control. *Rhizobium meliloti* strains 158M and 161M were obtained from Dr. Dov Kishinevsky and *Agrobacterium radiobacter* strains At 5/re and At 96216 were obtained from Dr. Dan Zutra, The Volcani Center, Beit Dagan, Israel. Other bacterial strains were obtained either from the Bacteriology Department, The Kimron Veterinary Institute, Beit Dagan, or from The Clinical Microbiology Department, The Hadassah Medical School, Jerusalem. Bacteria were grown using standard conditions for each strain.

DNA preparation: Rapid PCR analysis was conducted on bacterial cells suspended in double distilled water after boiling the sample for 20 min. Alternatively, biological samples - e. g., milk, placenta and lymphatic glands, were smeared and heat fixed on glass slides and then, a sample was collected from the slide directly into the PCR test tube by a needle scratching of the smear.

To prepare chromosomal DNA, bacterial cells were harvested in saline and incubated for 20 min at 4°C with 4 mg/ml lysozyme. Then, SDS (0.5%) and proteinase K (200ug/ml) were added and incubation was continued at 37°C for 1 hr. The cell lysate was first extracted with phenol-chloroform-isoamylalcohol (1:1:24) and then with chloroform-isoamylalcohol (1:24). The purified DNA was alcohol precipitated and resuspended in TE (50 mM Tris-HCl, 1 mM EDTA, pH 8.0) until used.

DNA digestion: Reaction was carried out by incubation of target DNA with a restriction endonuclease and buffer according to the manufacturer instructions (Boehringer Mannheim, Gmbh, Germany). The digested DNA was separated by electrophoresis on either 1. 5% agarose gels (Tris-Acetate buffer) or, 10% polyacrylamide gels (Tris-Borate buffer). DNA fragments were visualized by staining the gel with 1. 5 ug/ml ethidium bromide.

DNA hybridization: DNA fragments were transferred to nitrocellulose filter by Southern blotting (21). The NEBlot Phototope™ Chemiluminescent Kit (New England Biolabs, Inc., U.S.A.) was used, according to manufacturer instructions. Hybridization was detected with the Lumigen-PPD reagent.

Oligonucleotide primers: The *Brucella omp*2 and *rplL* genes were used as target DNAs. The forwards 5' primer (p1) and reverse 3'primer (p2) of the *omp*2 gene were obtained from National Biosciences, Inc., U.S.A.

p1 - TGGAGGTCAGAAATGAAC

p2 - GAGTGCGAAACGAGCGC

The forwards 5' primer (p3) and reverse 3' primer (p4) of the *rplL* gene were obtained from General Biotechnologies, Inc., U.S.A.

p3 - GCTGATCTCGCAAAGATCGTTGAA

p4 - TCCAAACTTACTTGAGTTCAACCT

Amplification reactions: PCR amplification of the DNAs was carried out using the method of Mullis and Faloona (18). A typical reaction mixture included 50mM KCl, 1. 5mM MgCl2, 0. 1% Triton X-100, 0. 2mg/ml BSA (fraction IV, Sigma, U. S. A.) and 10mM Tris-HCl, pH8. 5. The four nucleotides were added at a final concentration of 1mM, and the template DNA and the oligonucleotide primers were each added at a final amount of 100ng. Finally, the reaction was initiated by adding 1. 25U *Taq* polymerase (Boehringer Mannheim Gmbh, Germany). The reaction mixture was covered with 15ul mineral oil (Sigma, U. S. A.) to prevent evaporation.

Thirty cycles were employed using Eppendorf Thermocycler (Eppendorf, Germany), as follows: 20sec at 95°C for DNA denaturation, 1min at 50°C or 68°C for DNA annealing of the *omp*2 and *rplL* gene fragments, respectively, and 1min at 72°C for polymerase mediated primer extension. Reaction was terminated by additional incubation of the sample at 72°C for 7min followed by

cooling at 4°C. Ten microliter of the amplified product were analysed by electrophoresis in 1. 5% agarose gels in TAc buffer.

Results

Bacteriological diagnosis of brucellosis has been complicated in Israel, in the last years, due to isolation of atypical *Brucella* strains that resemble the vaccine strain in their susceptibility to penicillin (5) in addition to isolation of normal strains. Moreover, in several cases the vaccine strain, in normal and atypical forms, was isolated from vaccinated and unvaccinated ewes in flocks identified by the serological tests as infected. Therefore, a supplementary diagnostic method was necessary to confirm the bacteriological results. A PCR method was sought that will be sensitive enough to identify even a small number of *Brucella* cells in a biological sample coventionally used for *Brucella* isolation - e. g. , milk samples, lymphatic nodes such as the retropharyngeal glands and supramammary glands, and aborted material such as the placentum and the embryonal tissues. In addition, the PCR test was aimed at improving biotyping techniques of *Brucella* by using unique DNA sequences that will distinguish between biovars and species. Specifically, *B. melitensis* is predominant in Israel in the both small ruminants and cattle populations. *B. abortus*, on the other hand, has been completely eradicated but the cattle population is still being vaccinated with the *B. abortus* S19 vaccine strain.

The *rplL* gene has recently been cloned and sequenced in B.

melitensis and *B. abortus* and the both genes were found identical (3,20). Taken that DNA homology between the *Brucella* species is very high (17,23) we made the hypothesis that this conserved gene can be used as a possible candidate for PCR diagnosis of *Brucella*. Although being conserved between different microorganisms at the gene's 3' sequences a significant degeneration was found in the 5' terminus (3) allowing the use of the two gene's termini as targets for primer amplification by the PCR method. Accordingly, primers p3 and p4 were synthesized (Materials & Methods).

The *omp*2 gene was found in two copies in all *Brucella* spp. (16) suggesting that PCR amplification will be twice as efficient as compared to amplification of single copy genes. Moreover, the DNA sequences include a *Pst*1 restriction endonuclease site polymorphism within the gene and its flanking ends (15). Therefore, it could be predicted that a PCR test based on these sequences will be sensitive and further allow differentiation between strains and species of *Brucella*. Accordingly, primers p1 and p2 were selected (Materials & Methods).

The results depicted in Figure 1 confirm the specificity of the PCR test with the both *rplL* and *omp*2 gene primers. As can be seen in Figure 1B, the amplification product of the *rplL* gene (375bp) was obtained only when *Brucella* DNA was used as a substrate. Blank sample or bacterial DNAs obtained from bacteria other than *Brucella*, did not produce a DNA fragment with a similar size. Dot blot hybridization of the PCR products, using the *rplL* gene as a probe, confirmed the specificity of the PCR analysis. No

amplification product besides the one produced from *Brucella* strains hybridized with the probe (data not shown). As shown in Fig. 1A similar specificity was obtained with the PCR amplification of the *omp*2 gene. Only *Brucella* DNA produced a DNA fragment (282bp) which hybridized specifically with the gene's probe (data not shown).

The possibility of a false positive result was raised only once using the *rplL* gene primers but not the *omp*2 gene primers. In this case a bacterial isolate obtained from the uterin of a serologically positive responder cow, produced a PCR fragment identical in size to the *rplL* gene fragment while not reacting in the PCR test with the *omp*2 gene primers. The *rplL* fragment was further analysed for the presence of an *Eco*RI site established in the *Brucella* gene (3) but no such digest products were found (data not shown). Preliminary results from our laboratory suggested the isolate was *Ochrobactrum anthropi*, a bacterium closely related phylogeneticly to *Brucella* spp. (7,22).

The sensitivity of the test was evaluated by two criteria. Firstly, *Brucella* cells counted as colony forming units (CFU) were consecutively diluted from 10^7 to less than 10 in the test tube and analysed by the PCR reaction for the two gene sequences. Whereas the *rplL* gene product was identified in samples with less than 100 cells (one experiment) the *omp*2 gene product was usually obtained only if more than 100 cells were used (out of 11 experiments the *omp*2 gene product was identified only once when less than 100 cells

were sampled, data not shown). In general, it was concluded that the reaction, with the two genes, produced PCR products at similar sensitivities, requiring about 100 cells in the sample to obtain a positive signal. Secondly, the biological samples sent to our laboratory for *Brucella* diagnosis were tested simultaneously for the *rplL* and the *omp*2 gene products. As can be seen in Table 2, the percentages of successful PCR amplification reactions obtained from a total of 37 field samples, composed of 33 milk samples and 4 fetuses, was similar for the both *rplL* and the *omp*2 genes.

The data shown in Figure 2 suggested that field samples could be directly diagnosed from the slides. Samples confirmed positive for *Brucella* by bacteriological isolation of the strain were tested simultaneously in the PCR test. With almost no exception the amplification products obtained with the both gene primers corresponded with the isolation of the strain.

The possibility of biotyping *brucella* strains according to the *Pst*1 restriction fragment polymorphism of the *omp*2 gene (15) was evaluated using different *Brucella* species. As can be seen in Figure 3 the PCR products obtained from normal *B. melitensis* strains, atypical strains, and *B. abortus* strain, were completely digested with *Pst*1 showing one fragment of 238bp and a smaller one of 44bp. In contrast, the PCR amplification product of the *B. melitensis* vaccine strain was digested only partially showing an uncut fragment, comigrating with the PCR uncut product, and two digest products similar in size to the digested fragments obtained from the other *Brucella* DNAs. Similar results were obtained with

all Rev1 field isolates and with the human isolate.

Discussion

The taxonomical division of the genus *Brucella* is currently under debate. The genus is conventionally divided into six recognized species, each having unique characteristics - e.g., affiliation to a specific host, distinguishable susceptibility to certain phages and demonstration of a specific oxidative metabolism profile. However, on the level of their nucleic acid composition (17), chromosomal DNA digestion profile with restriction endonucleases (19) and identity of genes (3,20) and ribosomal DNA (23) it was proposed that the *Brucella* should be considered one species (23,24). The results reported in this manuscript provide additional dimension on the taxonomy of *Brucella*.

The analysis of the *rplL* gene sequences revealed high conservation among eubacteria at the 3' terminus of the gene but very degenerate sequences in the first 14 amino acid residues of the 5' terminus of the gene (3). Therefore, we chose this gene for PCR diagnosis of *Brucella* spp. using primers deduced from the two termini of the gene. The PCR analysis of bacterial DNAs from different genera and species by these primers actually confirmed our expectation that the PCR test will be highly specific. Even when taxonomically related bacteria, such as *Agrobacterium* and *Rhizobium* (7,10,22), were tested no false positive results were obtained (Figure 1).

However, while testing a bacterial isolate, from the uterus of

a serologically positive responder cow, a PCR product comigrating with the *Brucella rplL* gene fragment was obtained (Figure 1B). Although this fragment lacked an *Eco*RI site, which exists in the *Brucella rplL* gene, it did hybridize with the gene probe (data not shown), suggesting a certain degree of homology exists between the two genes. Therefore, the strain was also tested for the amplification of the *omp*2 gene sequences by the PCR reaction but in this test the result was negative (Figure 1A). Since the bacteriological tests identified the strain as *Ochrobactrum anthropi*, which is also related taxonomically to *Brucella* (7,22), these results could suggest that the two genera share a common *rplL* gene but they are distant when compared on the *omp*2 gene level.

The *omp*2 gene was chosen as an alternative target DNA for PCR diagnosis because it exists in two copies in the *Brucella* chromosome, suggesting a possible increased sensitivity of the test. Another important criterium was the fact that a *Pst*1 restriction polymorphism between *B. melitensis* and *B. abortus* exists in the gene sequences flanked with the primers. This should have allowed to distinguish between *B. abortus* and *B. melitensis* field isolates obtained from infected dairy cows (all vaccinated with *B. abortus* S19). In the last decade Israel has been free of *B. abortus*, but *B. melitensis* constituted a major problem in dairy cows (9).

The results were surprising at two levels. Firstly, unlike we expected, the PCR test with the *rplL* gene primers was usually more sensitive than the test with the *omp*2 primers (Table 2). These data

could not be explained on the basis of a simple principle. However, the observation that the PCR product of the *B. melitensis* 16M DNA was completely digested with *Pst*1 (Figure 3) suggested that although the *omp*2 gene exists in two copies in the *Brucella* chromosom only the *omp*2b gene was amplified. Similarly, the PCR products obtained from all other field isolates, characterized in our laboratory as *B. melitensis* virulent biovars, were also digested completely by *Pst*1 (Figure 3).

The reason for this selected amplification of the *omp*2b gene has not been clarified. It could be suggested that the *omp*2a gene sequences occure in normal *B. melitensis* strains in a conformation that inhibits its amplification by the polymerase chain reaction. In contrast, this phenomenon was not shown in the vaccine strain *B. melitensis* Rev1. As could be judged from the *Pst*1 digest of the amplification product (Figure 3) the two gene copies were actually produced, one being cut by the *Pst*1 and the second being resistant to this digestion. Therefore, the PCR test could specifically distinguish Rev1 DNA from the rest of the *B. melitensis* virulent strains' DNAs. To the best of our knowledge, this is the first recording of Rev1 unique characteristics based on DNA analysis.

Whether this phenomenon is unique to *B. melitensis* or it occures in all other *Brucella* species is difficult to evaluate because the *Pst*1 site polymorphism characterizes only *B. melitensis*. So far, however, all the *B. melitensis* isolates biotyped in our laboratory as Rev1 strains (Table 1) have shown this unique characteristics compared to normal *B. melitensis*

isolates (Figure 3). Moreover, the atypical strains (9413, 68875/3, Table 1) produced each a *Pst*1 digest profile which corresponded with the *B. melitensis* normal strain's profile. This provided evidence, in the first time, that the atypical strains were originated from a field strain that had mutated rather than from a Rev1 mutant. Therefore, besides the taxonomical importance of this observation it could be hypothesized that the attenuation of the vaccine strain could be associated with the structure of the *omp*2a gene or, with its expression.

It has been suggesteded that only *omp*2b is actively expressed in the *Brucellae* whereas the other gene is remaining silent (14). It will be interesting to test whether DNA modification affects expression of either gene and to what extent this is correlated with the virulence of the *Brucella* strain? We aim our future studies to test this hypothesis. In this context it is important to note that the two *omp*2 genes of *B. melitensis* strain 16M were amplified by the PCR method once a genomic library, constructed in *E. coli* (3, data not shown), was used as the template DNA. This indicates that the proposed modification of the DNA in *Brucella* did not occur in *E. coli* while replication of the phage genome occurred. Additional studies are required to elaborate on this observations.

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This research was initiated under Israel Health Ministry Grant 1963, "Rapid diagnosis of Human Brucellosis by the polymerase chain reaction (PCR)", with the participation of Michael Dan and Gad Frenkel. Dr. Frankel (current address - Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY) has designed the *omp*2 primers. The research was fully supported under Grant No.TA MOU CA 13-007, U.S.-Israel Cooperative Development Research Program, Office of the Science Advisor, U.S. Agency for International Development.

Legends to Figures

Figure 1. PCR analysis of known strains with the *omp*2 gene primers (Panel A) and *rplL* gene primers (Panel B).Lanes from right to left depict the following samples: Molecular weight markers (1857,1058, 929,383,121,13, bp, respectively); *B.melitensis* Ether; *B.melitensis* 16M; *B.melitensis* Rev1; *B.melitensis* 9413; *B.abortus* 544; *B.abortus* S19; *B.abortus* Tulya; *B.suis* strain 2; Field isolate of *Ochrobactrum anthropi*; *Agrobacterium radiobacter*, pv.tumefacient, biovar 1, strain At 96216; *Agrobacterium radiobacter*, pv. tumefacient, biovar 1, strain At 5/re; *Rhizobium meliloti* 158M; *Rhizobium meliloti* 161M; *Escherichia coli* NCTC 9001; Blank sample;

The bacteria are listed in Table 1.The non *Brucella* strains, listed also in Table 1, were tested and found negative in the both PCR tests.

Figure 2. PCR analysis of field isolates with the *omp*2 (Panel A) and *rplL* (Panel B) gene primers.Lanes from right to left depict the following samples: Molecular weight markers (1857,1058,929, 383,121,13, bp, respectively); *B.melitensis* 16M (reference); sheep milk samples, smeared on glass slides, contaminated with isolates 70335-3, 75153-6 and 83911-20, respectively; sheep aborted placenta, smeared on glass slides, with isolates 69160, 68875/3, and 69194, respectively; *Brucella* cultures, smeared on glass slides, isolated from cow's milk samples: 68176, 73318, 13, and 744; *Brucella* Rev1 cultures, smeared on glass slides, isolated from sheep's milk samples: 64945, and 51971; *Brucella* culture from human isolate 85035 smeared on glass slide; *B.abortus* 544 smeared on glass slide; The bacterial samples are listed in Table 1.

Figure 3.Comparative *Pst*1 restriction endonuclease digestion analysis of the *omp*2 PCR gene amplification products of normal *B. melitensis* and *B.abortus* strains and of *B.melitensis* original and rield Rev1 isolates.Lanes from right to left depict the following samples: Molecular weight markers (1857,1058,929, 383,121,13, bp, respectively); uncut DNA (S19); *B.melitensis* Ether; *B.melitensis* 9413; *B.melitensis* Rev1 field isolate 75153-6; *B.melitensis* Rev1; *B.melitensis* 16M; *B.melitensis* atypical biotype 1 sheep isolate

68875/3; *B.melitensis* Rev1 human isolate 5000; *B.melitensis* rough Rev1 sheep isolate 43094-38; *B.melitensis* normal human isolate 85035; *B.melitensis* normal sheep isolate 69160; *B.abortus* 544; *B.abortus* Tulya.The bacterial samples are listed in Table1.

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Table 1. Bacterial strains used in the study

Organism	Strain designation	Source	Organism	Strain designation	Source
Culture Collection			Isolates		KVI
Brucella abortus bv. 3	544	Ref. 2	*B. melitensis* bv. 1	5000	Human, Rev1 from blood
Brucella abortus bv. 1	Tulya	Ref. 2	*B. melitensis* bv. 1	95/75153-6	Vaccinated sheep, Rev1 from milk
Brucella abortus bv. 1	Strain 19 vaccine	Ref. 2	*B. melitensis* bv. 1	51971	Vaccinated sheep, Rev1 from milk
Brucella suis	Strain 2 vaccine	Ref. 2	*B. melitensis* bv. 1	94/64945	Vaccinated sheep, Rev1 from milk
Brucella melitensis bv. 1	16 M	Ref. 2	*B. melitensis* bv. 1 rough	94/43094-38	unvaccinated sheep, Rev 1 from milk
Brucella melitensis bv. 1	Strain Rev1 vaccine	Ref. 2	*B. melitensis* bv. 1	95/85035	Human, from blood
Brucella melitensis bv. 3	Ether	Ref. 2	*B. melitensis* bv. 1	94/68176	cow, from milk
Agrobacterium radiobacter pv. tumefaciens	At 5/re peach isolate	ARO	*B. melitensis* bv. 1	94/73318	cow, from milk
Agrobacterium radiobacter pv. tumefaciens	At 96216	ARO	*B. melitensis* bv. 1	13	cow, from milk
Rhizobium meliloti	Strain 158M	ARO VCRC	*B. melitensis* bv. 3	744	cow, from milk
Rhizobium meliloti	Strain 161M	ARO VCRC	*B. melitensis* bv. 1 atypical	9413	sheep, from placenta. Ref.
Escherichia coli	NCTC 9001		*B. melitensis* bv. 1 atypical	68875/3	Sheep, from mik
Salmonella typhimurium	ATCC 15277		*B. melitensis* bv. 2	95/83911-20	sheep, from milk
Klebsiella pneumoniae	ATCC 8044		*B. melitensis* bv. 3	94/69160	Sheep, from aborted fetus
Proteus vulgaris	NCTC 4175		*B. melitensis* bv. 3	94/69194	sheep, from aborted fetus
Streptococcus mutans	NCTC 104		*B. melitensis* bv. 3	94/70335-3	as 94/69194
Mycobacterium smegmatis	ATCC 607				
Mycobacterium bovis	ATCC 19210		*Ochrobactrum anthropi*		cow, from uterus

ARO - Agricultural Research Organization
VCRC - Volcani Center Rhizobium Collection
KVI - Kimron Veterinary Institute

Table 2. Percentages of positive PCR results of *omp*2 and *rplL* gene sequence amplification of several biological samples[a]

Gene primers	Biological samples on glass slide (% positive identification)		
	Milk	Tissues from aborted fetuses	
		lung[b]	Abomasum[c]
*omp*2	39	75	50
rplL	42	75	75

[a]Samples of unknown bacteriological status of brucellosis were each smeared and heat fixed on a glass slide prior to testing in the PCR reaction. Thirty three milk samples (from sheep and from cows) and tissues from 4 aborted fetuses were tested.

[b]Two samples were identified positive by Stamp staining. These also produced positive PCR results with the both *omp*2 and *rplL* gene primers. An additional PCR product has been obtained with the both gene primers from a Stamp negative slides.

[c]Two positive Stamp samples were identified. These also produced positive PCR results with the both *omp*2 and *rplL* gene primers. An additional *rplL* PCR amplification product has been obtained with the *rplL* primers and it was negative with the *omp*2 PCR test.

A
B
H_2O
E. coli-NCTC9001
R. meliloti-161M
R. meliloti-158M
A.radiobacter-At 5/re
A.radiobacter-At 96216
O. anthropi
B.suis-strain 2
B.abortus-Tulya
B.abortus - S19
B.abortus - 544
B.melitensis-9413
B.melitensis-Rev1
B.melitensis-16M
B.melitensis-Ether
Marker

FIG. 1

FIG. 2

B.abortus-544

B.melitensis-85035

B.melitensis-51971

B.melitensis-64945

B.melitensis-744

B.melitensis-13

B.melitensis-73318

B.melitensis-68176

B.melitensis-69194

B.melitensis-68875/3

B.melitensis-69160

B.melitensis-83911-20

B.melitensis-75153-6

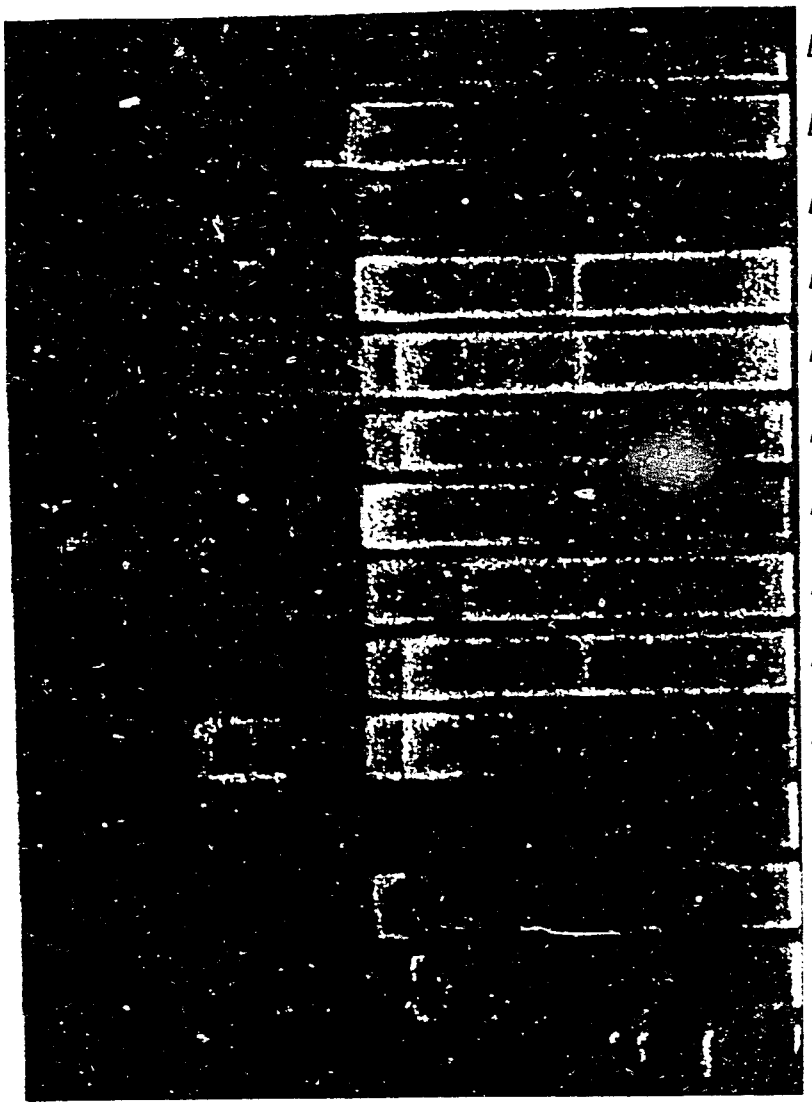
B.melitensis-70335-3

B.melitensis-16M

Marker

52

FIG. 3



B.abortus-Tulya

B.abortus-544

B.melitensis-69160

B.melitensis-85035

B.melitensis-43094-38

B.melitensis-5000

B.melitensis-63875/3

B.melitensis-16M

B.melitensis-Rev1

B.melitensis-75153-6

B.melitensis-9413

B.melitensis-Ether

B.melitensis-Rev1(uncut)

Marker