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# Serum PCR Diagnosis of *Brucella melitensis* Infection in Rev. 1 Vaccinated Sheep

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**Abstract:** Serological diagnosis provides a robust and effective approach to monitoring and controlling small ruminant brucellosis. *Brucella melitensis* Rev. 1 is a live vaccine strain used in prophylactic vaccination against small ruminant brucellosis. Because the vaccine strain shares identical serological antigens with the corresponding field strains, differentiating infected from vaccinated animals (DIVA) serological responses hamper surveillance campaigns and interventions that involve vaccination. We have developed a serum PCR-based approach in which we amplify and sequence *Brucella omp2a* as a DIVA solution and *tRNA (uracil-5-)-methyltransferase* as a species marker in the serum samples to determine the etiological agent involved in brucellosis field cases. Using this method, we identified the involvement of both the Rev. 1 vaccine strain and a field strain in an outbreak of brucellosis in a flock. This method represents a novel approach in studying the etiology of brucellosis using serum samples as a source of the pathogen's DNA.

**Keywords:** Brucella melitensis; diagnosis; sheep; serum PCR; omp2a; *tRNA (uracil-5-)-methyltransferase*

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## 1. Introduction

Brucellosis is an important global zoonosis that is caused by Gram-negative bacteria of genus *Brucella* [1]. Historically, *Brucella* species have been classified based upon their growth characteristics on different substrates, sensitivity to brucellaphages and natural affiliation of a strain to a specific mammal host. With the advent of the post-genomic era, genus *Brucella* has been expanded to include atypical isolates that did not fit with the conventional scheme and yet shared genomic similarity with classical type strains. To date, 12 species have been validly published, i.e., *B. melitensis*, *B. abortus*, *B. suis*, *B. neotomae*, *B. ovis*, *B. canis*, *B. pinnipedialis*, *B. ceti. B. microti*, *B. innopinata*, *B. vulpis*, *B. papionis*, among which the first six species are considered "classical" in accordance with their high genomic and metabolic homogeneity [2,3]. However, only B. melitensis and B. abortus have been frequently documented as severe zoonotic species. Furthermore, being naturally associated with small ruminants and bovines, those animals which are in close contact with man, the requirement to eradicate the disease in these farms does not only emerge from the risk to the farms but mostly refers to the risk these organisms pose to human health. As a result, the combined One Health damage is increased in both the livestock industry and the human health component, respectively. B. suis is the third important human pathogen, however, is less frequently reported to-dates, as it is naturally associated with swine which are grown under an intensive reproductive industry [4].

*B. melitensis* strain Rev. 1 and *B. abortus* strain S19 (USA) have been established as live attenuated vaccine strains against brucellosis in their corresponding hosts. These vaccines induce cellular and humoral immune responses in their hosts, the first providing important protection against abortion [5], and the latter involving development of anti-*Brucella* antibodies, including development of a prominent antibody response against

the *Brucella* O-polysaccharide antigen [6]. Because O-polysaccharide of a vaccine strain shares common epitopes with cognate field strains, vaccination with the live vaccine strains hampers serological diagnosis due to the inability to differentiate infected from vaccinated animals (DIVA) [7]. Serum (Tube) Agglutination Test (SAT), which measures agglutinin IgM antibodies elicited during the acute phase of the disease, and Complement Fixation Test (CFT), which measures complement fixing IgG antibodies development during the chronic stage of the disease or alongside persistence of the pathogen in the host, are two common tests used in surveillance programs. The first one is simple and accessible to low-and middle-income countries (LMIC) and the second conceived as a confirmatory test [8,9]. Nevertheless, the serological tests which not always provide a significant predicting value [10] and possibly involving an inaccurate identification of a Rev. 1 adverse performance in the field [11] still constitute paramount tools in achieving a successful national control program.

Ganesh et al. [7] proposed an alternative novel vaccine as a solution to overcoming the DIVA problems associated with the use of live vaccine strains. They assumed that *Brucella* O-polysaccharide could be designed and chemically synthesized, so as to achieve modification of the non-reducing M-epitope of the polysaccharide and binding of an immunodominant protein to the polysaccharide as a means of establishing a potent conjugate vaccine together with a DIVA solution [12,13]. Although based on a sound hypothesis, this approach is just at its beginnings regarding the realm of the brucellosis complexity, while animal vaccination with live attenuated strains still remains the major tool in prophylactic protection against *Brucella* infection. Therefore, finding an alternative DIVA approach that does not rely on serological parameters would establish an important tool in solving the problem of how to integrate vaccination with a test and cull policy.

Serum PCR has been proposed and implemented as a method of indirect diagnosis of brucellosis based upon the presence of *Brucella* DNA residues in the serum samples [14]. Real-Time PCR amplification of *Brucella* protein 31 kDa (BCSP31) has been used in confirming human cases [15,16] and this approach has been similarly adopted in diagnosing animal brucellosis [17,18]. We took this diagnostic approach a step further and developed a "nested" PCR method that increased test sensitivity by establishing a visible DNA band on the gel, sufficing for its extraction and sequencing and establishing SNP differences between DNA samples. We demonstrated feasibility of the use of this method in distinguishing between serological responses due to Rev. 1 vaccine and field infections (a non-serological DIVA test), as well as identifying *B. abortus* and *B. melitensis* species by their *tRNA (uracil-5-)-methyltransferase* SNPs.

Metagenomic next generation sequencing (mNGS) is an emerging novel method which similarly identifies sequences of an etiological agent involved in an infection, however, requiring an in-depth pursuit of the tissue of concern as well as whether an untargeted or targeted sequencing approach should be performed [19]. Here, we suggest that being targeted a priori against a specific disease, and in concern of resolving DIVA cases between filed infection and Rev. 1 vaccine induced antibodies, serum "nested PCR" is superior to mNGS in speed and accuracy of performance, as well as being cheaper and regarded non-invasive. In the long run, we envision that this diagnostic approach would also be applicable in mapping *Brucella* genes of interest based upon testing large numbers of serum samples.

## 2. Materials and Methods

### *2.1. Bacterial DNA Isolation*

*Brucella* strains are maintained at the Kimron National, OIE and FAO Reference Laboratory for Brucellosis as glycerol frozen suspensions. A bacteriological loop was loaded with the frozen culture and transferred to a Tryptic Soy Broth medium, which was then incubated on a rotating platform at 37 °C for 24 h, in the presence of 5% $CO_2$. Genomic DNA was purified using DNeasy blood and tissue kit (Qiagen, Aarhus, Denmark)

according to the manufacturer's recommendations. DNA concentration was measured using microvolume spectrophotometer DS11 (DeNovix Inc, Denver, CO, USA).

### *2.2. Serum Samples and DNA Purification*

Sheep vaccination and blood sampling were conducted as part of the routine activity of the Israeli Veterinary Services in compliance with ISO-17025 (https://www.gov.il/he/departments/general/sheep-health-veterinary-services, Hebrew, accessed on 5 September 2022). Blood samples were collected into vacutainer tubes and sent to the Israeli National Reference Laboratory for Brucellosis to be tested by Complement Fixation (CFT) and Microplate Serum Agglutination (MAT) as described in Baum et al. [8].

### *2.3. DNA Preparation*

An aliquot of 100 µL serum sample was added into 300 µL of 1:10 diluted Edwards' solution [20] in an Eppendorf tube and the mixture was incubated at room temperature for 25–30 min. The reaction mixture was then centrifuged at 15,000 rpm for 5 min, supernatant collected and mixed with 216 µL of ice-cold propanol (kept in a glass bottle overnight at −20 °C) in order to precipitate the DNA. Finally, precipitated DNA was centrifuged at 15,000 rpm for 5 min and supernatant withdrawn. After drying the tube at an ambient temperature, the pellet was dissolved in 70 µL of RNA/DNA-free water and kept in a refrigerator until used.

### *2.4. PCR*

The method comprises two robust techniques, a "nested PCR" amplification of a DNA band first and sequencing of the DNA then after asking for SNP differences between amplicon sequences. Primers were designed according to published target DNA sequences and following the reaction of PCR amplification DNAs were run on agarose gels as a means of developing DNA bands. These DNA bands were then extracted from the gel and DNA contents were sequenced in order to identify SNP differences between analyte genomes.

Primers: For primer design we used chromosomal accession numbers given per a *Brucella* reference and type strain. *B. melitensis* 16M (accession number AE008917.1) has been used as a prototype sequence and locations of the large and short—nested PCR sequences on the chromosome have been depicted in Figures 1A and 2A. The other *Brucella* species included: *B. melitensis* Rev. 1 (accession number CP024715.1), *B. melitensis* 63/9 (accession number CP007789.1), *B. melitensis* Ether (accession number CP007760.1), *B. abortus* S19 (accession number CP000887.1), *B. abortus* 2308 (accession number AM040264.1), *B. suis* 1330 (accession number AE014291.4), *B. microti* (accession number CP001578.1). Primers used in the amplification of the nested PCR reactions were retrieved from the large sequences. Table 1 depicts the primers which were selected. Designated primers were designed and synthesized at Hylabs (Israel) using geneious prime software (Auckland, New Zealand), based on the NCBI and GenBank databases.

**Table 1.** Primers used in the study.

| Target Gene | Intact Gene | | Partial Sequence within the Gene | |
|---|---|---|---|---|
| | Forward (P1) | Reverse (P2) | Forward (P3) | Reverse (P4) |
| *omp2a* BMEI1306 | GGCTATTCAAAATTCTGGCG | ATCGATTCTCACGCTTTCGT | TGGAGGTCAGAAATGAAC | GAGTGCGAAACGAGCGC |
| *tRNA (uracil-5)-methyl transferase* BMEI1496 | ACACTGGCAGGATGAACCTT | GCGTTATAGGGCAGAAGCTC | CGGAAATCATCGCGCGTCT | AGGCACCTTGCCGAAATG |

PCR amplification: Two genes, *omp2a* ((BMEI1306) and *tRNA (uracil-5-)-methyltransferase* (BMEI1496)), were selected as study targets [21]. In an attempt to verify specificity of the method, gene sequences were chosen for amplification after validating their unique specificity to *Brucella*. Reactions were conducted in two steps, the first targeting the entire gene and flanking sequences using primers 1 and 2. Then, in preparation for the second

reaction, amplified DNA from the first reaction was washed and concentrated into a 6 µL solution by a DNA clean and concentrator kit (Zymo Research, Irvine, CA, USA). The second reaction then targeted an internal sequence within the amplified sequence of the first amplification step using primers 3 and 4.

The first reaction mixture composed of 15 µL of HS Red Taq PCR buffer (PCR Biosystem, London, UK), 1.2 µL of primers 1 and 2 (each at a final concentration of 400 nM), 3 µL of bacterial genomic DNA or 10 µL of a serum DNA sample and volume was increased to 30 µL with DNA/RNA-free water. Reaction was initiated by sample heating at 95 °C for 2 min, followed by 40 cycles of 15 s of DNA denaturation at 95 °C, 15 s of DNA annealing at 58 °C (for *omp2a*) or 54.5 °C (for *tRNA [uracil-5-]-methyltransferase*), and ending the reaction with DNA extension at 72 °C for 25 s.

The second amplification cycle was conducted as above, however, using primers P3 and P4 and 6 µL of a concentrated DNA solution obtained at the first reaction and changing the annealing temperature to 50 °C for *omp2a* and 54.5 °C for *tRNA (uracil-5-)-methyltransferase*, respectively.

### *2.5. DNA Sequencing*

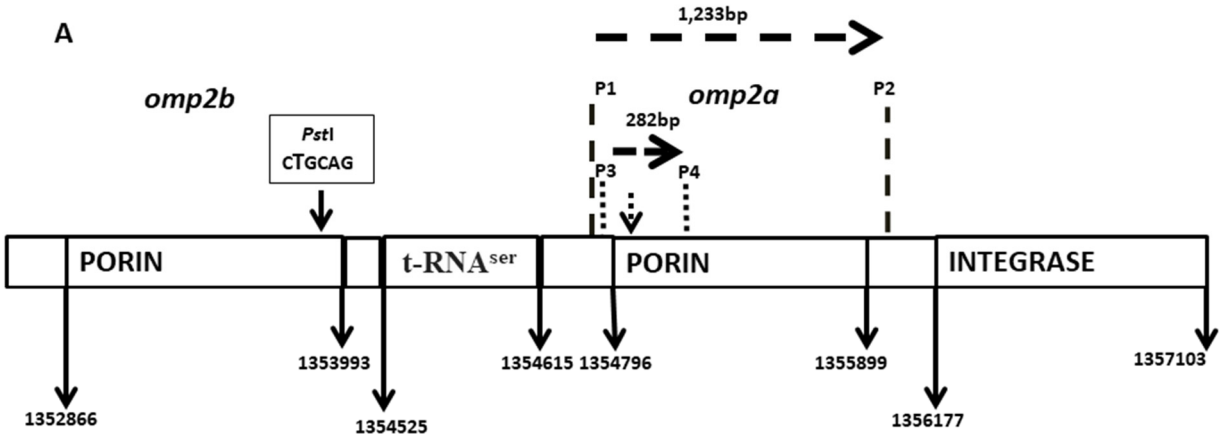
A 25 µL sample of each of the PCR reaction mixture was loaded onto a 1.2% (*w*/*v*) agarose gel (Amresco, Red Bank, NJ, USA), which included Olerup SSP® GelRedTM (Fischer Scientific, Waltham, MA, USA) as a DNA binding dye. Electrophoresis was carried out at 80 V for 45 min and DNA bands detected on a 254 nm UV trans illuminator (Ingenius, Syngene, India) were cut from the agarose using sterile dispensable chirurgic scalpels (Swann Morton, Sheffield, England, UK) and gel pieces were introduced into an Eppendorf tube which was kept under cold until examined. Then, DNA was cleansed using column extraction (DNA Clean up and Concentrater, Zymo, Irvine, CA, USA) before submission to Sanger sequencing at HyLabs, Israel. Finally, sequences were analyzed using geneious prime alignment software (Auckland, New Zealand).

## 3. Results

### *3.1. Design of a DIVA Method*

In a publication by Bardenstein et al., (2002) we identified separated by a *t-RNA*$^{ser}$. Whereas *omp2b* includes a conserved *Pst*I site an *omp2a—Pst*I site SNP difference between Israeli *B. melitensis* field strains and Rev. 1 vaccine strain upon which presence or absence of this SNP distinguishes between the two strains [22]. As shown in Figure 1A, the *omp2* region comprises two closely identical *omp2* gene copies among the strains *omp2a* varies by its *Pst*I SNP according to the studied strain (broken arrow), Primers P1 and P2 identified by their locations on Chromosome I, 1,354,796 (P1) and 1,355,899 (P2), have been designed to amplify a 1233 bp sequence which includes the intact *omp2a* gene and flanking sequence [23]. By delineating into the nested PCR amplicon, primers P3 and P4 within *omp2a* amplify a 282 bp sequence at the 5′ end of the amplicon which may or may not include a *Pst*I site (broken arrow), in accordance with the specific *Brucella* species and strains. Note that some of the genomes of these strains have been annotated at a reverse complementary sequence to that of *B. melitensis* 16M and therefore they are marked by an accompanying star to indicate their opposite alignment. As shown in Figure 1B, a zoom in profile into the *Pst*I site identifies a "C/T" SNP within these sequences which differentiates between *B. melitensis* strain 16M and its cognate Rev. 1 strain and the other *Brucella* species. A *Brucella* BLASTn, taxid:234 analysis against the P3–P4 amplified sequence revealed that *B. melitensis* strains from all around the Eastern Hemisphere of the world including countries from Africa, Western Europe, Eastern Europe, Mediterranean region, and the Gulf [24], as well as China and Mongolia (data not shown) included an intact *Pst*I site in *omp2a*. This indicated that the DIVA method which was primarily validated for *B. melitensis* infections in Israeli strains could be practically employed in most of *B. melitensis* DIVA queries among vaccinated populations worldwide, Unfortunately, the same could not be

indicated in cases of Latin American *B. melitensis* infections as whole genome sequencing (WGS) has not been performed extensively on these strains [24–26].




B

| | | |
|---|---|---|
| *B. melitensis* 16M | AE008917.1 | -C**C**GCAG- |
| **B. melitensis* Rev. 1 | CP024715.1 | -C**C**GCAG- |
| *B. melitensis* 63/9 | CP007789.1 | -C**T**GCAG- |
| *B. melitensis* Ether | CP007760.1 | -C**T**GCAG- |
| **B. abortus* S19 | CP000887.1 | -C**T**GCAG- |
| **B. abortus* 2308 | AM040264.1 | -C**T**GCAG- |
| **B. suis* 1330 | AE014291.4 | -C**T**GCAG- |
| *Brucella* (Taxid:234) | | -C**T**GCAG- |

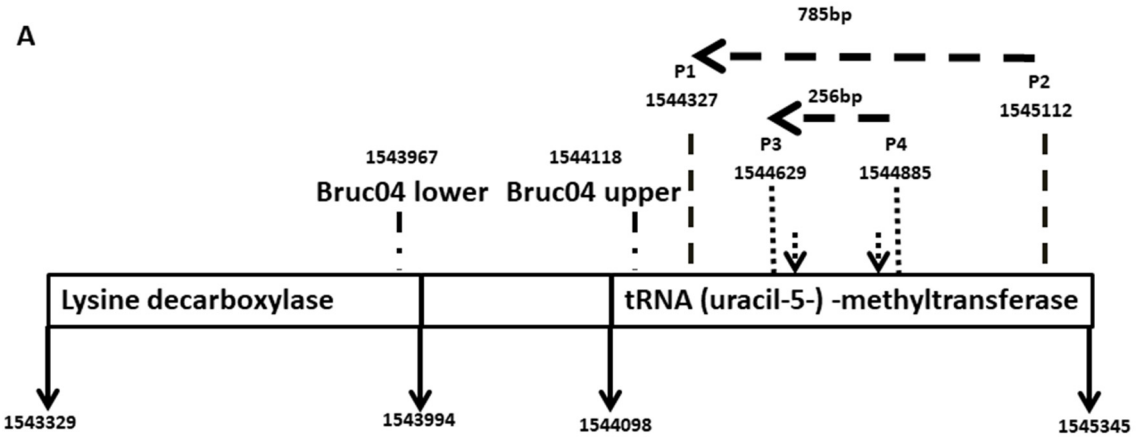
**Figure 1.** (**A**) Schematic presentation of the *B. melitensis* strain 16M *omp2* region (arrows indicate locations in the chromosome of ORFs and primers). P stands for a primer. The size of the first amplicon (1233 bp) and nested PCR product (282 bp) as well as direction of gene transcription are indicated by broken arrows. Vertical broken short arrows stand for an apparent *Pst*I site. (**B**) The specific *omp2a* sequence which aligns with the corresponding *Pst*I site and accession numbers are given. Note that the *Pst*I site is lacking exclusively from strains 16M and Rev. 1. Star (*) stands for a reverse complementary sequence in comparison to that of *B. melitensis* 16M. SNPs are depicted by letters with an increased font size.

Figure 2A shows a schematic genetic map of the *tRNA (uracil-5-)-methyltransferase* gene and flanking sequences in chromosome I of *B. melitensis* strain 16M. Figure 2B focuses on two SNPs differences between *Brucella* species. Although this is not applied for *B. microti* which shares identical SNPs with *B. suis*, one can rule out involvement of the two species in most if not all brucellosis cases among small ruminants and cattle. Intriguingly, as can be seen in Figure 2A, these sequences are linked to a hypervariable Bruc04 sequence of the MLVA technique [27], possibly implying that this region is a hotspot of mutational events.

### *3.2. Preliminary Analyses of the Specificity and Reproducibility of the Technique*

Two sets of experiments were examined. In the first set, the sensitivity of the amplification reaction of the final *omp2a* 282 bp product was studied using a series of 10 folds dilutions of genomic DNA in the first cycle of the reaction mixture and detection of a DNA band at the expected size in the "nested" amplification of the smaller sequence. DNA bands have been then sliced out from the gels and the samples sent for DNA sequencing as a validation of the specific sequence. In comparison, it was important to confirm that negative

control samples do not produce false positive DNA bands on the gel. Thus, we also sent suspected false bands for sequencing and confirmed that they were non-sense DNA.




B

| | | |
|---|---|---|
| *B. melitensis* 16M | AE008917.1 | -GCCGCC- -TGAGT- |
| **B. melitensis* Rev. 1 | CP024715.1 | -GCCGCC- -TGAGT- |
| *B. melitensis* 63/9 | CP007789.1 | -GCCGCC- -TGAGT- |
| *B. melitensis* Ether | CP007760.1 | -GCCGCC- -TGAGT- |
| **B. abortus* S19 | CP000887.1 | -GCCTCC- -TGAGC- |
| **B. abortus* 2308 | AM040264.1 | -GCCTCC- -TGAGC- |
| **B. suis* 1330 | AE014291.4 | -GCCGCC- -TGAGC- |
| **B. microti* | CP001578.1 | -GCCGCC- -TGAGC- |

**Figure 2.** (**A**) Schematic presentation of the *B. melitensis* strain 16M *tRNA (uracil-5) methyltransferase* region (arrows indicate locations in the chromosome of ORFs and primers). The size of the first amplicon (785 bp) and nested PCR product (256 bp) as well as direction of gene transcription are indicated by broken arrows. Note, *tRNA (uracil-5) methyltransferase* is linked to an MLVA Bruc04 sequence. P stands for a primer. SNP locations on the nested PCR amplicon are indicated by short vertical broken arrows. (**B**) The specific *tRNA (uracil-5) methyltransferase* sequence which aligns with the corresponding SNPs and accession numbers are given. Star (*) stands for a reverse complementary sequence in comparison to that of *B. melitensis* 16M. SNPs are depicted by letters with an increased font size.

After approval of the test sensitivity, we then tested field serum samples from sheep which reacted positive by the CFT tests. In these experiments we validated the established conditions using field serum samples and tried to find how many of the tested serum samples yielded true bands (according to sequencing results). In these studies, we again confirmed our negative control results, using serum samples from brucellosis free animals before vaccination.

### *3.3. Determining Analytical Sensitivity*

As can be seen in the left-hand side of Figure 3, lanes 1 to 8, none of the *B. melitensis* 16M genomic DNA solutions sufficed to produce an intact gene amplicon of approximately 1200 bp in the first amplification cycle. In contrast, as shown at the right-hand side of Figure 3, 4.5 ng (Lane 1) and 0.45ng (Lane 2) of the original DNA amounts used in the first reaction yielded a visible DNA band of an expected 282 bp in the second "nested" PCR reaction.

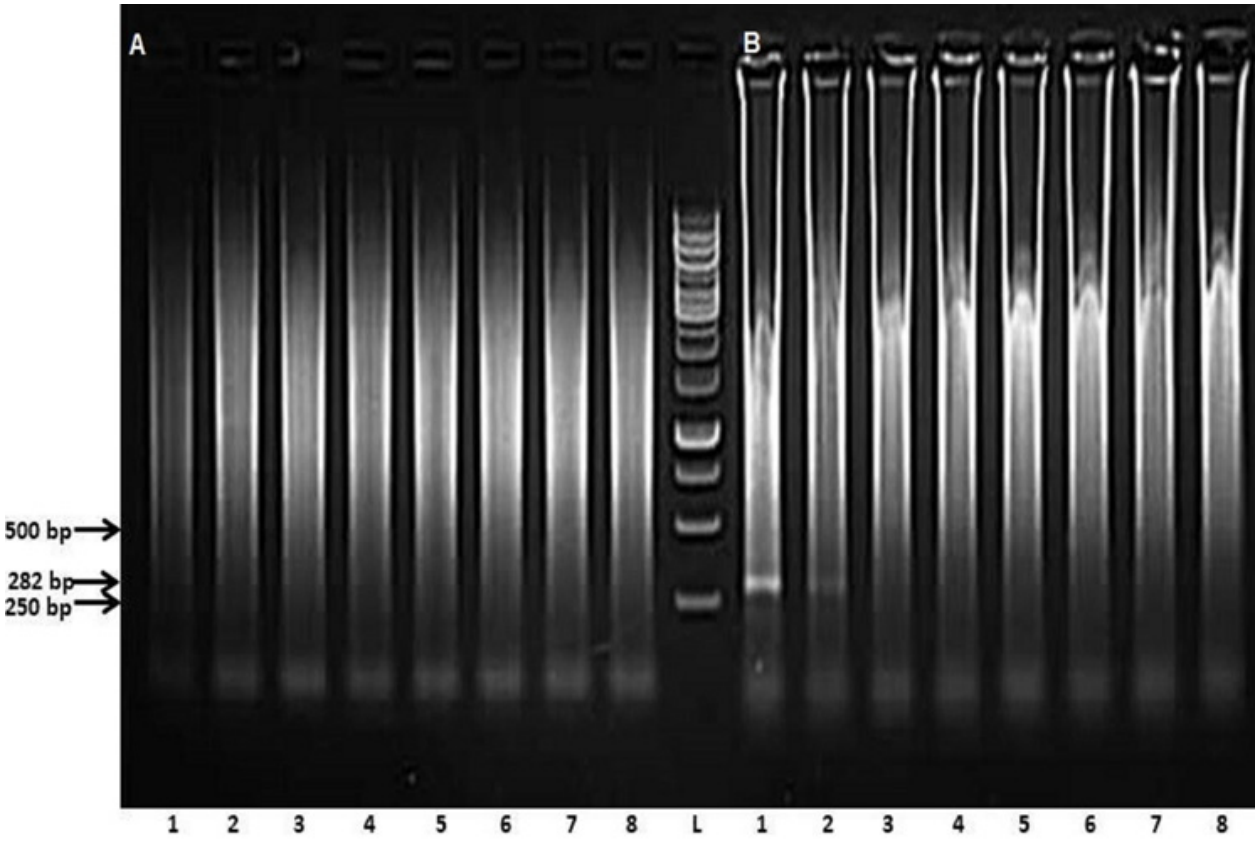



**Figure 3.** Calibration of the nested PCR reaction using *B. melitensis* 16M genomic DNA. Left-hand side, DNA amplification of the intact *omp2a* gene of a genomic DNA source diluted by a ten-fold factor. An expected band at 1200 bp is not depicted in any of the reactions, Lanes 1–8. Right-hand side, a "nested PCR" DNA amplification of a 282 bp size partial sequence within the intact *omp2a* gene is seen in Lanes 1 and 2, to the right side of a GeneRuler 1 kb DNA ladder, corresponding to 4.5 and 0.45 ng of the original genomic DNA concentrations in the first amplification reactions (Left hand side, Lanes 1 and 2), respectively.

Importantly, these DNA amounts yielded a visible band on the gel which could be extracted and sent for DNA sequencing. Further DNA dilutions thereof in the first reaction also produced negative PCR bands in the second reaction, implying that a minimal content of 0.45 ng or more of the genomic DNA in the serum sample should suffice to establish a positive "nested" band for sequencing.

### *3.4. Validating Serum PCR*

In the transition between laboratory validation of the method and conducting field diagnosis, during running preliminary analyses in order to improve the test's performance, we often encountered a problem of non-reproducibility of results due to quality variations between commercial molecular kits. Hence, conditions described in M&M are advised as being superior to alternative combinations. Further, we only used serum samples which were close to time of collection and confirmed serologically positive by CFT at a titer of 1:20 or higher. CFT is a highly reproducible, specific and sensitive method which stands alone in confirming herds' exposure to the disease (used as an OIE recommended method in both sheep and goats and in cattle brucellosis). By obtaining a positive CFT result animal infection is confirmed even without bacteriological isolation of the strain (a gold test), which justify animal culling against compensation to the owner. While only addressing CFT positive responders as target animals, aiming at increasing test's sensitivity, Batrinou et al., (2022) [11] have justifiably indicated that a minor number of undiagnosed responders may also be positive in the test.

In order to validate the versatility of the nested PCR method to fit with several diagnostic purposes, we investigated two genes: omp2a, which is intended to be implemented as a DIVA test, and *tRNA (uracil-5-)-methyltransferase*, intended to be used as a method that identifies Brucella species. omp2a is common among core Brucella species establishing an identical DNA band of 282 bp. Submission of the DNA for sequencing reveals a C/T SNP difference between vaccine strain Rev. 1 (as well as its counter type strain 16M) and B. melitensis field strains (Figure 1). Brucella *tRNA (uracil-5) methyltransferase* is found in the three zoonotic Brucella species and therefore all three genomes yield a DNA band of 256 bp. Submission of the DNA for sequencing reveals a difference in a SNP combination between two nucleotides within the sequence which identifies the Brucella species (Figure 2).

The fraction of field serum samples which contain a minimal *Brucella* genomic DNA amount required to produce a DNA band of expected size on the agarose gel is not known a priori. To address this question, we performed serum PCR analyses in three infected farms, testing 9 (first farm, Figure 4A) and 6 (second farm, Figure 4B) CFT positive serum samples questioning *omp2a* amplification and 3 (third farm, Figure 5) CFT positive serum samples asking amplification of *tRNA (uracil-5-)-methyltransferase*, respectively. As can be seen, the *omp2a* study resulted with four serum samples out of 9 (Lanes 2, 4, 7, 8; 44% sensitivity, Figure 4A) and six out of 6 serum samples (100% sensitivity, Figure 4B) which yielded a positive DNA band of 282 bp. In the *tRNA (uracil-5-)-methyltransferase* study, two out of 3 (66% sensitivity, Figure 5) serum samples yielded a DNA band of 256 bp. This established a test's sensitivity which could vary among farms and animals between 44% to 100% with expectation to yield DNA bands of sufficient amount for sequencing. However, the test's performance per serum sample did not always match between the two genes of study, *omp2a* and *tRNA (uracil-5-)-methyltransferase*, indicating that differences between PCR conditions may play a role in the statistics of successful amplification results.

We then asked if vaccination by strain Rev. 1 elicits residual DNA in the serum sample as infection does. To test this hypothesis, we followed development of an *omp2a* band in ewe-lambs which have been vaccinated by the Rev. 1 vaccine. Serum samples were taken prior to vaccination as a proof of lack of infection and 15 and 25 days postvaccination to determine DNA analyte. CFT and MAT were negative before vaccination and turned positive postvaccination, according to the individual response, indicating that vaccination was achieved successfully (data not shown), We then conducted the serum PCR test twice, obtaining inconclusive DNA bands in 2 out of 2 ewe-lambs in the first experiment and as a result, we caried out a second experiment in which 10 ewe-lambs were tested. In this test, we also included a serum sample from an infected ewe as an internal positive control. After 15 days postvaccination only faded DNA bands were shown in 2 out of 10 serum samples which were not depicted after 25 days postvaccination. In this test, all other serum samples were also negative, yet, the internal control of a serum sample of an infected sheep provided a clear positive DNA band at the expected size, validating the test performance. This ruled out persistence of Rev. 1 DNA in serum samples due to prompt ocular vaccination per se (Data not shown).

### *3.5. A Case Study*

At the beginning of June 2019, a person was diagnosed infected with *B. melitensis* bv. 1 as confirmed by strain isolation.

An epidemiological investigation identified an unregistered sheep flock composed of around 100 heads as a suspected source of this infection. Some of the animals were at late gestation and others had already lambed, most being untagged. After completion of a renewed animal tagging and registration, all adult animals were tested by CFT, revealing 2 males and 7 females positive for the disease. On a second follow up visit, a month later, 3 males and 20 females were tested and found positive, indicating an ongoing spread of the disease in the flock. Because some of the animals were tagged with the original vaccine yellow color ear tags, we were intrigued to find out how many of the animals positive by CFT at $\geq$1:20 titers responded to Rev. 1 vaccination, or were infected by a

field strain. We then implemented an *omp2a* serum PCR as a DIVA method and *tRNA (uracil-5-)-methyltransferase* gene analysis as a means of identifying which *Brucella* species were involved in this event.

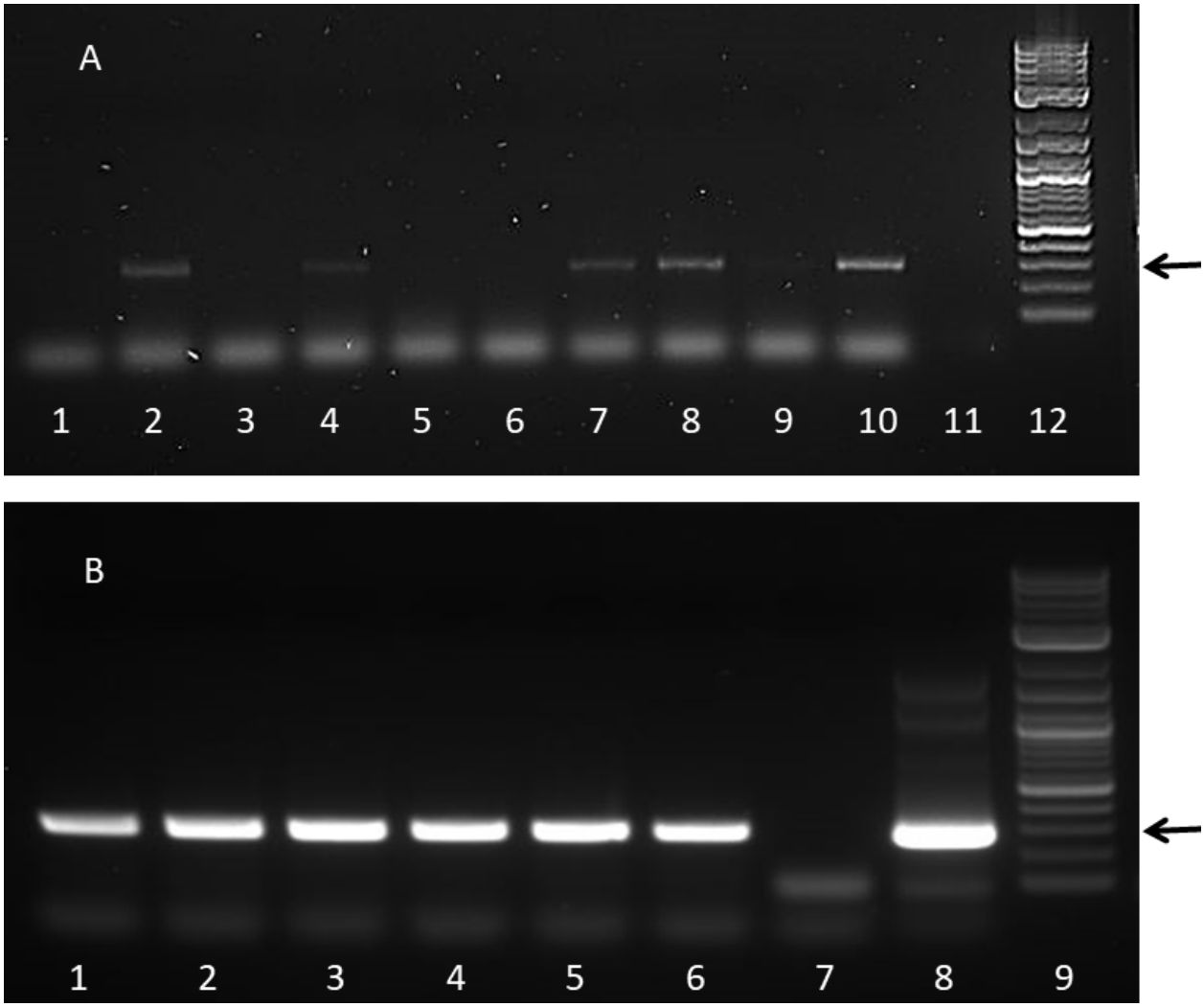



**Figure 4.** An *omp2a* test analysis of field serum samples confirmed positive by CFT serology, obtained from two infected sheep farms, (**A**,**B**). (**A**)—Experiment 1—Serum samples number 2, 4, 7 and 8 out of nine as well as a control sample (lane 10) depict the correct 282 bp DNA band (black arrow). Lane 11 depicts a negative control sample. (**B**) Experiment 2—six out of six serum samples as well as a control sample (lane 8) depict the correct 282 bp DNA band (black arrow). Lane 7 depicts a negative control sample.

As can be seen in Table 2, six out of 10 serum samples yielded a positive *omp2a* band of which three animals, numbers 4002118, 4898827 and 4898865, produced an amplicon with a DNA sequence that correlated with a Rev. 1 profile, and other 3, numbers 4898849, 3673916 and 4505924, produced a DNA band with a sequence that correlated with a field strain infection. Two of these serum samples, numbers 3673916 and 4002118, also yielded a *tRNA (uracil-5-)-methyltransferase* band that confirmed the species as *B. melitensis*. Two additional serum samples, numbers 4813646 and 3991347, which did not amplify an *omp2a*, still yielded a *B. melitensis tRNA (uracil-5-)-methyltransferase* positive sequence. The fact that some serum samples yielded amplicons of the 2 genes in parallel, in comparison to others that only yielded a single gene amplicon, highlights on the importance of the double test approach as a means of increasing test sensitivity.

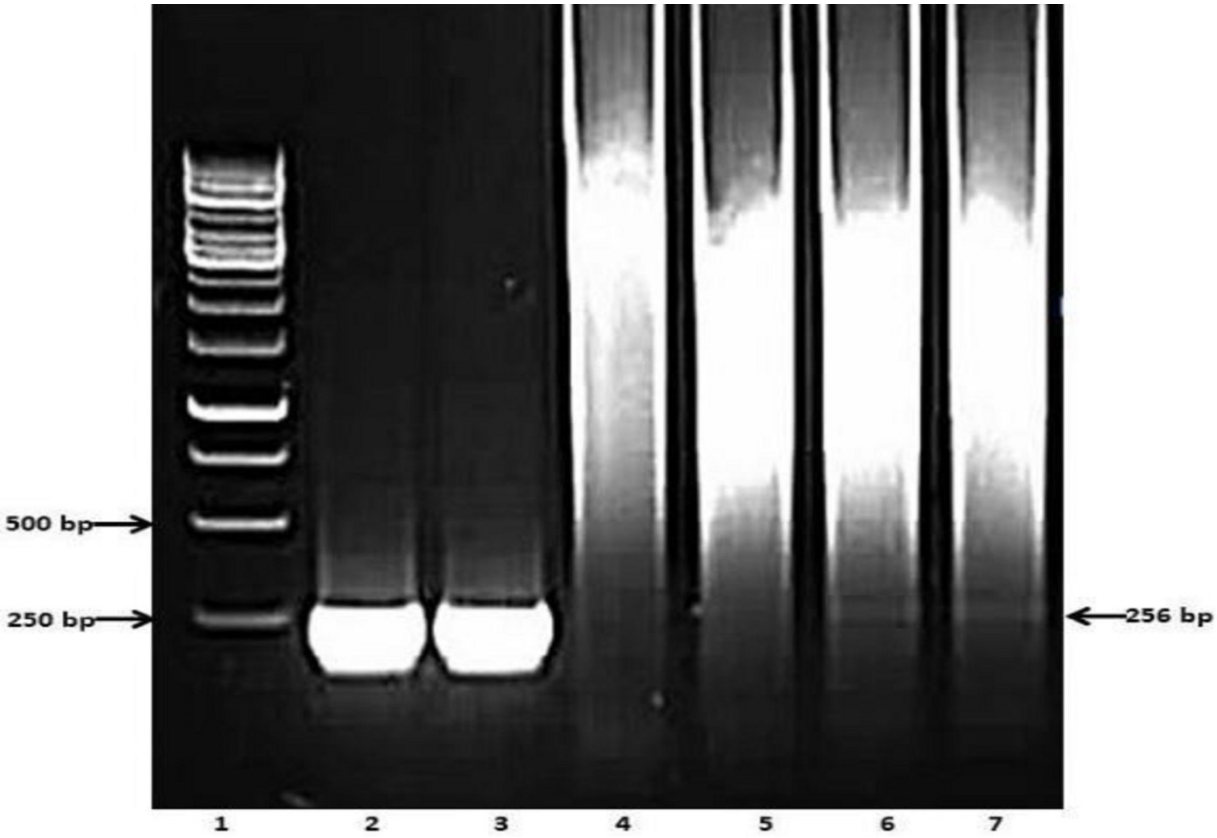



**Figure 5.** Serum PCR amplification of *tRNA (uracil-5-)-methyltransferase* yields a common DNA band among core *Brucella* species. Differences between species is acknowledged only after submission of the DNA band for sequencing and correlating a SNP combination of two nucleotides within this sequence as fingerprints of the *Brucella* agent (Figure 2). Lane 1—GeneRuler 1kb DNA ladder. Lane 2—A positive control, genomic DNA from an Israeli field strain (DNA sequence proven to be *B. melitensis*). Lane 3—A positive control, genomic DNA from *B. abortus* strain 19 (DNA sequence proven to be *B. abortus*). Lane 4—A negative control sample. Lane 5—Serum 4002118, negative. Lane 6—Serum 4813646, Lane 7—Serum 3991347. Positive DNA bands confirmed as *B. melitensis* are indicated by an arrow.

**Table 2.** Serum PCR analysis of sheep from a flock partially vaccinated with Rev. 1 *.

| Serial No. | Ear Tag No. | File No. | Amplified Sequences | | |
|---|---|---|---|---|---|
| | | | *omp2a* | | *tRNA (uracil-5-)-methyltransferase* Sequence |
| | | | Rev. 1 Sequence | Field Strain Sequence | |
| 201 | 4898849 R | E238264 | | + | Negative |
| 202 | 3673916 Y 2014 | E238264 | | + | + |
| 203 | 4505960 R | E238264 | Negative | Negative | Negative |
| 204 | 4505924 R | E238264 | | + | Negative |
| 205 | 4813646 Y 2020 | E238264 | Negative | Negative | + |
| 206 | 3991347 Y 2016 | E238264 | Negative | Negative | + |
| 207 | 4002118 Y 2017 | E238264 | + | | + |
| 208 | 4898827 R | E238264 | + | | Negative |
| 209 | 4898865 R | E238264 | + | | Negative |
| 210 | 3651960 R | E238264 | Negative | Negative | Negative |

* *omp2a* and *tRNA (uracil-5-)-methyltransferase* serum PCR analyses of serum samples from ≥1:20 CFT positive sheep. The sequential order of the ear tag numbers indicates different times of visits of the flock (R—red; Y—yellow). Whereas Yellow labels indicate a true time of animal vaccination as young ewe-lambs, the red tag labeled animals indicate at least four visits of the flock, with some animals being retagged due to loss of the original label.

Four serum samples were derived from sheep labeled by the original yellow ear tag, however they differed as to their time of vaccination, e.g., sheep number 3,673,916 was vaccinated in 2014, 3,991,347 in 2016, 4,002,118 in 2017, while 4,813,646 had been vaccinated only recently. This indicates that the animals varied in age, the oldest being at least 6 years old and the youngest being possibly closer to one year old. A closer inspection revealed that animal number 4,002,118 had been vaccinated while in quarantine, three years prior to being tested, while two others that carried the recently labeled red ear tags 4,898,827 and 4,898,865, and therefore could not be directly associated with past vaccinations, were also diagnosed as Rev. 1 carriers of a persisting strain. In contrast, the older animal from the vaccinated group, number 3,673,916, and two others, numbered 4,505,924 and 4,898,849, wearing red ear tags and of undetermined vaccination status, were conclusively infected by a field strain. As this study started with the diagnosis of a patient infected with *B. melitensis* identification of three animals infected with a non-vaccine *B. melitensis* strain allowed the conclusion that *B. melitensis* was the causative agent of this infection.

## 4. Discussion

Detection of *Brucella* agents has been historically based upon strain isolation and phenotype characterization according to their growth patterns in presence of dyes, agglutination by A or M monospecific serum and susceptibility to brucellaphages, respectively. Yet, although contributing to our understanding of *Brucella* epidemiology and taxonomy, these methods, which relied on strain isolation and bacteriological typing beforehand, have become obsolete in the presence of the newly evolving high throughput alternative molecular typing approaches. In contrast, serum PCR, first introduced as a means of diagnosing human brucellosis [15,16] and further used to diagnose camel brucellosis—based upon a consensus protein BCSP31 sequence—has opened a window to profiling *Brucella* infections in real time without necessitating *Brucella* isolation [17,18].

Here, granted that a vast number of serum samples are sent to a diagnostic laboratory for serological investigation on a daily basis, we exploited these serum samples in diagnosing *Brucella* infections according to DNA fingerprints determined by "nested" PCR. In order to show the versatile applicability of the method, we studied two genes: one that identifies strain Rev. 1 DNA based upon an *omp2a Pst*I DIVA site proven relevant to most *Brucella* genomes by a BLASTn, taxid:234 search (Figure 1B, [24–26]), and the second, based upon SNPs within a *tRNA (uracil-5-)-methyltransferase* gene, which defines the difference between *B. abortus* and *B. melitensis*. Our data confirmed the applicability of the method with both genes depicting the corresponding DNA bands in serum samples obtained from *B. melitensis* field strain infected sheep (Figures 4 and 5).

Analytical sensitivity of the method was highly significant, as it successfully identified between 4.5 ng to 0.45 ng of *Brucella* genomic DNA in the sample (Figure 3). Thus, achieving a sensitivity which varied between 44% (lowest) and 100% (highest) among infected flocks (Figure 4A,B) indicates that failing in showing DNA bands in a field study most likely correlates with lack of *Brucella* genomic DNA in the negative serum samples. In other words, confirmation of a positive band, even if obtained from a single sheep, suffices to judge the flock as infected, and as a consequence put it in quarantine and enforce an immediate brucellosis control program in the farm [28].

In a case study, a sheep flock consisting of about 100 heads had been linked to a human illness. Because some animals bore yellow ear tags indicating their vaccination status, serology could not differentiate infected from vaccinated animals (DIVA). We therefore considered our newly developed serum PCR as a means of distinguishing between these possibilities.

From 10 serum samples, the nested PCR approach identified 3 field strain cases which confirmed the infectious status of these animals. This supported the decision to quarantine the flock and test and cull infected animals according to our national policy. However, additional 3 sera samples depicted the presence of Rev. 1 DNA (Table 2) in this flock, suggesting on an ongoing mixed infection between a field strain and the Rev. 1 vaccine

strain. Moreover, this revealed that a Rev. 1 infection persisted in a sheep 3 years after vaccination, also accompanied by a serological titer, reopening concerns regarding the safety of Rev. 1 vaccine lots [28,29]. In light of this finding, we renew our call for using a safer Rev. 1 vaccine based upon Elberg strain, passage 101, 1970, which has been validated and standardized as the only safe seed stock [30]. Taken that serum—PCR relies on existence of nucleic acid components of pathognomonic infections in the serum samples, one can design an effective campaign to monitor small ruminant brucellosis among vaccinated populations based upon serum PCR tests.

Together, despite not knowing which would be the conditions that drive *Brucella* DNA to persist in a serum sample and for how long, our data open up a novel approach in studying the etiology of *Brucella* infections by serum PCR and facilitate development of a pan-genomic map of prevailing *Brucella* strains in an epidemiological unit.

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