Brucellosis Reemergence after a Decade of Quiescence in Palestine, 2015-2017: a Seroprevalence and Molecular Characterization Study

Running title: Brucellosis Reemergence in Palestine

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Abstract

Brucellosis is an endemic disease in many developing countries and ranked by the World Health Organization among the top seven "neglected zoonoses". Although a Palestinian brucellosis control program was launched in 1998, the disease reemerged after 2012. Interestingly, a similar reemerging pattern was reported in the neighboring Israeli regions. The aim of this work was to characterize the reemerging strains and delineate their genetic relatedness. During 2015-2017, blood samples from 1324 suspected patients were analyzed using two serological tests. Seropositive samples were cultured, and their DNAs were analyzed by different genetic markers to determine the involved *Brucella* species and rule out any possible involvement of the Rev.1 vaccine strain. The *rpoB* gene was sequenced from 9 isolates to screen for rifampicin-resistance mutations. Multi Locus VNTR Analysis (MLVA-16) was used for genotyping the isolates. The molecular analysis showed that all isolates were *B. melitensis* strains unrelated to the Rev.1 vaccine. The *rpoB* gene sequences showed four single nucleotide variations (SNVs) not associated with rifampicin resistance. MLVA-16 analysis clustered the isolates into 22 unique genotypes that belong to the East Mediterranean lineage. Altogether, our findings show that the reemergence of brucellosis was due to *B. melitensis* strains of local origin, the Palestinian and Israeli control programs' weaknesses could be a major factor behind the reemergence of the disease. However, other socioeconomic and environmental factors must be investigated. Moreover, strengthening brucellosis control programs and enhancing cooperation between all stakeholders is essential to ensure long-term program outcomes to fight brucellosis.

Keywords: Zoonoses; Brucellosis; *Brucella melitensis*; Molecular Typing; Reemerging Infectious Diseases; Rifampicin Resistance; Serologic Tests; Vaccines.

Introduction

Brucellosis is one of the most common bacterial zoonotic diseases worldwide, causing serious health problems to humans and significant economic losses in the livestock industry . The disease is caused by different species of *Brucella* that exhibit different host preferences. The major species associated with human brucellosis are *Brucella melitensis* (*B. melitensis*), *Brucella abortus* (*B. abortus*), and *Brucella suis* (*B. suis*) **.** The infection can pass from animals to humans via ingestion of raw milk and unpasteurized dairy products or through direct contact with contaminated animal tissues . According to the World Health Organization (WHO), the annual global number of notified human cases of brucellosis is around 500,000; however, the true number is believed to be significantly higher . Although a good control of brucellosis has been achieved in most developed countries, in many developing countries brucellosis remains endemic . What is even more serious are the signs of brucellosis reemergence reported in China, Central Asia, and several countries of the Middle East .

Before implementing the Brucellosis Control Program in 1998, the disease incidence rates in Palestine were around 75/100,000 and they reached to 139.9/100,000 in the Hebron Governorate, which was the major affected region . However, the reported incidences of brucellosis decreased significantly after 2000. This reduction was due to implementing a small ruminant vaccination program using the attenuated *B. melitensis* Rev.1 vaccine strain. Despite the efforts made to control brucellosis in Palestine, the disease started to reemerge after 2012 with alarming incidence rates in 2015-2017 . Again, most of the cases were recorded in the Hebron Governorate. Interestingly, after 2012, a significant increase in the incidence rates was also recorded in the Negev “al-Naqab” region by the Israeli Ministry of Health . This region is right

next to, and south of, the Hebron Governorate and has a large semi-nomadic Bedouin population. Although under two different political regimes, the two populations across the border have extended family ties and commercial relationships. In addition, it is relatively difficult to control animal movement across the borders.

The reemergence of brucellosis in Palestine after 2012 stoked a debate in the media and subsequent exchange of blame between concerned authorities and non-governmental organizations. Several questions were raised about the origin of the disease and whether it can be due to *Brucella* species other than *B. melitensis* or might have been imported from abroad or a less plausible possibility that the problem is associated with the Rev.1 vaccine strain in use. In addition, the public health authority had a concern about the misuse of antibiotics and the possibility of emerging antibiotic resistance in the circulating *Brucella* strains. To answer these questions and concerns, we studied a group of collected strains that were isolated between 2015 and 2017. Various molecular techniques were used to determine identity of the isolated samples and to screen for rifampicin resistance associated mutations in the *rpoB* gene. In addition, Multiple Loci VNTR Analysis (MLVA) genotyping method was employed to delineate the genetic relatedness of the isolated samples versus a group of global *B. melitensis* strains. Furthermore, we analyzed the available epidemiological data, both from the Palestinian and Israeli side, in an attempt to understand the possible contribution of different factors in the re-emergence of Brucellosis.

Materials and Methods

Ethics statement

The serological and blood culture examinations followed by molecular confirmation of brucellosis by genus specific PCR were performed as part of the standard diagnosis and follow-

up protocols offered to the patients referred to the department of preventive medicine, Hebron health directorate, Ministry of Health (MOH). The molecular analysis of stored DNA from confirmed blood culture isolates was made retrospectively. All the serological protocols and the molecular tests used in this study were approved by MOH according to the principles of the Declaration of Helsinki.

Serological and culture tests

The commercial agglutination Rose Bengal test (RBT) (Atlas Medical) was used to screen for the presence of anti-*Brucella* antibodies in patients' sera. Standard Tube Agglutination Test (STAT) was used to confirm the RBT positive samples. To improve sensitivity, sera with a titer of ≥ 1:80 were considered seropositive for brucellosis. Blood samples from seropositive patients that are not under antibiotic treatment were cultured up to 15 days using BACTEC 9240 (Becton Dickinson).

Bacterial isolates and DNA preparation

The positive blood culture samples were autoclaved and bacterial DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN). The same kit was used to extract DNA from two different commercial *B. melitensis* Rev.1 vaccine strains used in the Palestinian territories for the vaccination program. The first, from Laboratorios Ovejero (Spain), used in 1998-2008; and the second, from Jordan Bio-Industries Center JOVAC (Jordan), used in 2009-2015. DNA samples from *B. melitensis* strain 16M and *B. abortus* strain 544 were used as positive controls.

Genetic identification tests

Each DNA sample from positive cultures was tested by PCR using B4/B5 primers (Appendix Table 1), which targets a *Brucella* genus-specific bcsp31 gene **.** The *Brucella* positive samples

were further analyzed to identify the associated *Brucella* species based on Probert *et. al* method . The method was modified by designing PCR primers (Appendix Table 1) that can differentiate *B. abortus* from *B. melitensis* by multiplex PCR on agarose gel instead of real time PCR. The samples were also examined to rule out infection by the live attenuated vaccine strain Rev.1 using two PCR techniques; the PCR restriction fragment length polymorphism (PCR-RFLP) of rpsL gene and the bidirectional allele specific PCR (BiDAS-PCR) for SNP11 of BMEII0630 gene .

3 Screening for variations in *rpoB* Gene

According to the routine clinical follow-up performed by the department of preventive medicine, 9 samples were selected as they manifested signs of poor response to Rifampicin. The DNA from these isolates was analyzed using Next Generation Sequencing (NGS) to screen for Rifampicin resistance-associated mutations in *rpoB* gene. A targeted NGS was performed to sequence the amplified full sequence of *rpoB* gene by Hylabs (Rehovot, Israel). The *rpoB* sequences from 13 strains representing different global regions were downloaded from NCBI database and compared with the sequences obtained in this study using MUSCLE software to perform multiple sequence alignment (MSA).

4 MLVA-16 genotyping

MLVA- 16 analysis was used to genotype *B. melitensis* samples as previously described . The primers used for the VNTRs amplification are presented in Appendix Table 1. BioNumerics version 7.6 software (Applied Maths, Belgium) was used to normalize agarose gel images and to estimate amplicon lengths. Amplicon lengths were converted into numbers of tandem repeat units according to *Brucella* data available at the MLVAbank .

Clustering analysis was performed using BioNumerics V.7.6 software based on the categorical coefficient (also called Hamming's distance) and Unweighted Pair Group Method with Arithmetic mean (UPGMA). The use of categorical parameters implies that the character states are considered unordered. The same weight is given to a large or a small number of differences in the number of repeats at each locus. Polymorphisms at each locus were measured using Hunter and Gaston diversity index (HGDI) via the online tool V-DICE available at the HPA website .

The MLVA genotypes of *B. melitensis* strains from this study were compared with 60 MLVA genotypes from different international clades representing 3 main geographical groups (East Mediterranean, West Mediterranean, and Americas), which were retrieved from MLVABank database (Appendix Table 2). Each isolate was given a unique key based on the MLVA strain name available on MLVABank. The isolates of this work were given a unique key (Pal_Bm_Hu) followed by a serial number of each isolate. The DNA of two reference strains, namely *B. abortus* strain 544 and *B. melitensis* strain Rev.1 was used as MLVA validation controls. In addition to UPGMA-based dendrogram analysis to generate hierarchical clustering, a minimum spanning tree (MST) clustering was constructed using BioNumerics V.7.6 software. Each isolate was given a unique code according to the international standard three-letter country codes.

Epidemiological data

The annual incidence rates in Palestine was obtained from the annual health report 2019 for Palestine, issued by MOH . The annual incidence rates in Israel was obtained from the published data .

Results

Serological and culture results

RBT was performed to detect anti *Brucella* antibodies in 1324 suspected human serum samples collected during 2015-2017. According to the collected records by the department of preventive medicine, the cases were suspected because of their possible consumption of unpasteurized dairy products and none of them was associated with brucellosis occupational exposure. RBT was positive in 704 (53.2%) samples (Appendix Table 3). The sex and age distribution of the 704 RBT positive patients were 50% females (2 to 74 years, M= 28.4, SD= 16.5) and 50% males (1 to 78 years, M=25.2, SD= 17.5). Of the 704 RBT positive samples, 559 (79.4%) were considered positive for brucellosis as their STAT test showed antibody titer ≥ 1:80, which is a cutoff value to confirm the diagnosis. The antibody titer of 376/704 (53.4%) of the samples were within 1:160 to 1:640 IU/ml (Figure 1). Interestingly, 91 (12.9%) of the cases had antibody titers ≥ 1280 IU/ml. Unfortunately, only 111 out of the 559 seropositive cases were not on antibiotic treatment at least one week before sample collection. The blood culture results of these 111 samples showed a positive correlation between successful blood culture and STAT titer (Figure 1).

Genus and species identification

Blood culture was performed for RBT positive samples with a STAT titer ≥ 1:80 providing that patients are not on antibiotic treatment. Only 111 of the 559 brucellosis patients fulfilled the above criteria (Figure 1). In addition, three blood samples for patients referred from local hospitals were included in the group for blood culture tests although their STAT titer was not available. Of the 114 blood cultures, only 85 showed positive growth signals within 15 days of incubation. The 85 positive-cultures were tested to verify the presence of *Brucella* using the genus specific bcsp31 marker (Appendix Figure 1). The results confirm the presence of *Brucella*

in 72 out of 85 samples and 13 positive-cultures were negative. Using a PCR that distinguishes *B. melitensis* from *B. abortus*, it was found that all of the 72 *Brucella* samples are *B. melitensis* species (Appendix Figure 2).

Testing for the Rev.1 genetic markers

To rule out the possibility that some of the cases could be due to *B. melitensis* Rev.1 vaccine strains, the 72 samples were screened for two unique Rev.1 genetic markers located in the rpsL gene and the BMEII0630 gene. The results revealed that none of the 72 samples have any of these Rev.1 genetic markers (Appendix Figure 3A and 3B).

Screening for variations of *rpoB* Gene

NGS analysis was used to test for possible variations in the *rpoB* gene. Using *B. melitensis* 16 M *rpoB* gene as a reference, the 9 sequenced samples showed 4 identical single nucleotide variations (SNVs) namely; 1886 T>C, 2954 T>C, 3747 G>A, and 3927 A>G (Figure 2). However, none of these SNVs is located within the rifampicin resistance determining regions (RRDRs). The *rpoB* consensus sequence of the local strains (designated Pal_Bm_Hu) was aligned with r*poB* sequences from 13 different *B. melitensis* strains that represent the three main clades (East Mediterranean, West Mediterranean, and Americas). The multiple sequence alignments adjacent to the 4 SNVs demonstrate that the isolated strains in this study belong to the East Mediterranean clade. The results also prove that the strains are not related to the Rev.1 vaccine strain.

MLVA-16 genotyping

The full set of MLVA-16 markers (Panel I, Panel IIA and IIB) was used for strain-level genotyping . In the first experiment, the two commercial Rev.1 vaccine strains from Ovejero

194Labs and JOVAC, which were used in the Palestinian Brucellosis Control Program, were 195analyzed to validate their genetic similarity. The result showed that the two strains have identical 196MLVA-16 patterns that match the expected pattern derived theoretically from the genomic DNA 197sequences (CP024715 and CP024716) of the original *B. melitensis* strain Rev.1 Elberg (passage 198101) (Appendix Figure 4).

199In the second experiment, the MLVA-16 assay was used to genotype the 72 isolates. The MLVA 200scheme grouped the 72 isolates into 22 unique genotypes not reported in the MLVABank 201database. The genotypes were designated genotype 1 to genotype 22 as shown in Figure 3. 202Interestingly, most of the isolated strains tend to cluster in some genotypes including genotype 6 203(strains=13), genotype 3 (strains=11), genotype 10 (strains=9), genotype 11 (strains=8), and 204genotype 4 (strains=5). The 22 genotypes showed no variability in Panel I markers, while 205different markers of Panel II showed significant variability (Bruce16 with 7 alleles, Bruce04 with 2064 alleles, Bruce19 with 3 alleles, and Bruce30 with 3 alleles) (Appendix Table 4A).

207The diversity index was calculated for each marker using the HGDI method with 95% 208confidence interval (CI). While no diversity was detected using Panel I markers, Panel IIA 209markers showed diversity in Bruce19 marker and Panel IIB markers showed diversity in 3 210markers, namely Bruce04, Bruce16, and Bruce30 (Appendix Table, 4B).

211The second part of clustering analysis aimed to determine the genetic relatedness between the 72 212isolates and 60 different global isolates of *B. melitensis* representing the clades of East 213Mediterranean, West Mediterranean, and Americas, which are the major clades in the 214MLVABank database. Both hierarchical clustering and MST analyses showed that the 215Palestinian isolates belong to the East Mediterranean clade (Figure 4) (Appendix Figure 5).

Despite their clustering within the East Mediterranean clade, only one of our isolates, namely Pal_Bm_Hu33, showed a close similarity with strains from some neighboring countries.

Discussion

Although a good control of brucellosis has been achieved in most developed countries, in many developing countries brucellosis remains endemic . Despite the implementation of the Palestinian Brucellosis Control Program since 1998, the disease reemerged after 2012. In this work, we examined 1324 suspected cases associated with this reemergence. Various genotyping techniques were employed to elucidate identities and characterize the isolated strains in order to resolve the debate about the origin of the disease. This work reveals the indigenous origin of the isolated *B. melitensis* strains, which supports the postulate that the reemergence of brucellosis in Palestine is due to persistent field strains from local nomadic herds. Most likely, these strains have been circulating silently and they exploited the accumulated deteriorations in brucellosis control programs to resurface after 2012, causing the reemergence of brucellosis in Palestine, Israel, and possibility in other neighboring countries.

In this work, the RBT test was used for initial screening followed by STAT as a confirmatory test. Even though the STAT test cutoff value was set at ≥ 1:80, a significant number of RBT results were false positives (145/704). This high ratio of RBT false positive results can be attributed to the well-recognized low specificity of RBT in endemic regions where the chance to have a history of brucellosis or a previous exposure to *Brucella* organism is high . Although cutoff values of ≥ 1:160 or even ≥ 1:320 have been used in endemic regions to improve specificity of the STAT test , we decided to set the cutoff value at ≥ 1:80 in order to increase the sensitivity at the cost of specificity. This decision did actually increase the sensitivity and 7 out of the 16 samples with 1:80 STAT titer showed true positive results.

In this work, the majority of seropositive patients were on blind antibiotic treatment. This irrational use of antibiotics tended to delay the diagnosis and this is reflected in the high percentage of patients who were diagnosed when having high antibody titer. This delay is usually associated with more patients suffering and may increase the risk for developing chronic brucellosis leading to severe complications. The misuse of antibiotics is also associated with drug resistance which has a serious public health impact. Unfortunately, and due to limited biosafety conditions, we were unable to perform antimicrobial susceptibility testing for our isolates. Nevertheless, and as rifampicin was the drug of choice for treating the patients of this study, *rpoB* gene from nine cases with suspected rifampicin resistance were sequenced. The cases were selected based on their poor clinical response. Sequencing of the *rpoB* gene did not reveal any mutations related to rifampicin resistance. It is interesting to note a country-to-country variation in detecting *rpoB* mutations related to rifampicin resistance. While reports from endemic countries like Spain, Turkey, and Qatar did not detect such mutations , reports from other countries like Egypt demonstrate the occurrence of these mutations in various isolates of *Brucella spp.*. This variation may be attributed to an uncontrolled use of rifampicin for veterinary purposes despite the fact that it is a disapproved antibiotic for animal treatment. This study was limited to screening for *rpoB* gene mutations because of its essential role in response to rifampicin. Nevertheless, the role of other genes cannot be underestimated and a whole-genome screening to identify antimicrobial resistance determinants worth further investigation.

The results of species determination demonstrated that all of the isolated strains belonged to *B. melitensis* species and none of them was *B. abortus*. The endemicity of *B. melitensis* in our region can be related to the livestock breeding patterns. In contrast to the tightly controlled intensive farming systems in the dairy cattle, sheep, and goats, rearing of these animals in

Palestine and Israel follow traditional farming systems which rely on small farmers and nomadic herders. It is also noteworthy that *B. abortus* has never been reported since 1985 after the successful eradication of this species both from Israel and the West-Bank by the Israeli agricultural authorities .

Despite the weak possibility that shedding of the *B. melitensis* Rev.1 vaccine strain in milk could be a potential cause of human infection, we decided to examine this possibility. The Rev.1 genetic markers analysis along with the MLVA-16 genotyping and *rpoB* sequencing results demonstrate that none of the isolated strains was related to the Rev.1 vaccine strain. This result was expected as most of the previously reported human brucellosis caused by the Rev.1 strain were associated with accidental occupational exposure to the vaccine and only a few rare cases contracted brucellosis following infection by the Rev.1 strain through vaccinated animals . In addition, and to rule out the possibility that the used Rev.1 vaccines are not related to the original Elberg vaccine strain, the two commercial Rev.1 vaccines used in Palestine between 1998 to 2015 were analyzed using MLVA-16 assay. The two vaccines showed MLVA-16 profiles identical to the profile produced by the original Elberg (passage 101) *B. melitensis* strain Rev.1, which disproved the allegations, circulated on social media, that the vaccine used could be contaminated with a pathogenic strain. Nevertheless, it is important to highlight that genetic similarity does not imply equivalent efficacy of the two vaccines and, since no quality control test was performed to compare the two vaccines, the possibility that part of the problem could be associated with suboptimal vaccine efficacy cannot be excluded.

In this study, MLVA-16 assay was used to delineate the genetic relationships between the 72 strains and to compare them with a group of global *B. melitensis* strains from MLVABank. Because of its power to determine the different *Brucella* species and to differentiate isolates

within the same species, MLVA-16 has been widely used to assess genetic diversity to uncover epidemiological relationships and to trace back the source of outbreaks among *Brucella* strains . MLVA assay revealed 22 unique genotypes among the investigated 72 strains. Some of these genotypes such as 6, 3, 10, 11, and 4 include several strains, which may represent focal outbreaks associated with common sources of infection. According to patients' data maintained by the department of preventive medicine, some of these genotypes have actually more than one isolate from different members of the same family. Nevertheless, a thorough analysis showed that some of the strains with identical MLVA profiles were isolated from different years and different geographical points. This strongly suggests the presence of few persistent genotypes that have certain evolutionary advantages. Unfortunately, the studied samples were limited to human cases collected in the period 2015-2017. Although this period represents the peak of the disease reemergence, we believe that analyzing isolates from nomadic herds of sheep, goats and camels with a wider spatial and temporal representation could provide more insight into the evolutionary aspects and socioeconomic factors related to brucellosis.

Clustering analysis based on MLVA-16 assay results strongly pointed out the Eastern Mediterranean origin of the studied 72 strains. Analysis of the hierarchical clustering results and the *rpoB* MSA suggest that the Eastern Mediterranean clade might be divided into subclades that represent two geographical areas. Our samples are clustered within a subclade that represents the historical Fertile Crescent region including Palestine, Jordan, Lebanon, Syria, Iraq, and Southern regions of Turkey. Until the 20th century, these countries had very strong political, cultural and social connections. A few isolates suggest a second possible subclade including samples from Central Asia and the Iberian Peninsula. Although the connection between Central Asia and the Iberian Peninsula is unclear, previous reports also showed a similarity of *B. melitensis* isolates

from the two regions . To obtain an accurate delineation of *B. melitensis* clades and to reconstruct a clear phylogeographic map of this species, a large number of global isolates should be analyzed using the whole-genome SNP genotyping approach , which is a more reliable phylogenetic method.

In the last two decades, reemergence of brucellosis has been reported in several countries with distinctive circumstances for each one. For example, in Bulgaria, the reemergence of brucellosis during 2005-2007 was most likely imported from neighboring countries as the Balkan region at that period witnessed socioeconomic changes with a significant increase in animal trade, animal movement, and occupational migration . In 2012, a brucellosis outbreak caused by *B. melitensis* was reported in the French Alp region . The analysis indicated the alpine ibex as a silent wildlife reservoir of the disease. In our opinion, the reemergence of brucellosis in Palestine after 2012 must be studied considering the situation of the disease in the adjoining Israeli region of Negev. Remarkably, plotting the disease incidence rates, reported in the two regions in the last two decades, showed a strong similarity in the infection pattern (Figure 5). After 2009, the Palestinian brucellosis control program suffered from scarcity of funding, which was reflected on a very sharp decline in vaccine coverage . Moreover, the limited resources to perform efficient surveillance and to control animal movement across the borders with the Israeli regions were additional weaknesses of this program. On the Israeli side, the cessation of *B. melitensis* control campaign due to budgetary constraints and movement of infected animals across the border with the Palestinian regions were the major weaknesses . Therefore, the most reasonable postulate for the reemergence of brucellosis with a concordant incidence patterns in the two adjacent regions is due to some quiescent strains that have been lurking in nomadic herds of sheep and goats or, maybe, camels. Due to vulnerabilities in the brucellosis control and surveillance programs, these

strains presumably started to spread slowly to more herds and finally to humans through dairy products.

Conclusion

The present study demonstrates that the reemergence of brucellosis in Palestine after 2012 was due to *B. melitensis* strains of Eastern Mediterranean origin that are not associated with Rev.1 vaccine strain. The results prove that MLVA-16 is an expensive and useful tool for investigating brucellosis in countries with limited resources. It has become evident that brucellosis does not give up easily and that surveillance and control programs with short-term objectives cannot be relied upon to control this disease. The weaknesses in the control programs in Palestine and the neighboring countries could be one of the critical reasons for the reemergence of this disease. However, it is necessary to take into account several other factors that may have a role in this problem, such as socioeconomic factors, climate changes, political conflicts and wars, and the weak regional cooperation. Therefore, strengthening brucellosis control programs and enhancing cooperation between all national and regional stakeholders are critical factors to ensure good outcomes of the long-term program to fight brucellosis.

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Author contributions

Design and supervision of the project, Y.A.; Methodology and lab work, B.A., K.Z., A.T., and W.H.; Sample collection, M.H.A. and K.A.; Data Analysis, B.A., A.T., and Y. A.; Manuscript writing, Y.A.; Review manuscript, B.A., A.T., and Y.A.

Conflict of interest

The authors declare that they have no conflict of interest.

Figure legends

Figure 1. Antibody titer by STAT. The X-Axis represents the antibody titer by STAT and the Y-Axis represents the number of cases; the number above the bars are the number of cases for patients on antibiotics (grey bars), patients without antibiotic (Red bars), and for positive *Brucella* culture samples (green bars).

Figure 2. Variations within the *rpoB* gene using NGS. RRDRs refer to the rifampicin resistance determining regions within the *rpoB* gene. The 4 SNVs are shown and their positions are indicated above the *rpoB* gene. The 13 *rpoB* sequences from different global regions plus one Palestinian sample are indicated by their strain names and origin. The variations are detected on the sequence by blue and red colors. The texts (in red) under the boxes refer to the amino acid change and position.

Figure 3. Dendrogram of the clustered 72 MLVA-16 genotypes of the isolates. The 22 genotypes are indicated on the tree by different colors (Genotype1- Genotype 22) in addition to B. a_strain 544 and B. m_strain Rev.1. In the columns, the following data are presented: strain name, Host, Year, Species, and Region.

Figure 4. MST clustering for the field isolates and 60 samples from different global origins. The samples were clustered into four different groups according to region. The circles outline the genetic profiles of strains. The connecting lines represent the difference between samples in the number of markers. The size of the circles is proportional to the number of strains (1 or 2) bearing the same genetic profile.

Figure 5. Brucellosis incidence rate in Palestine and among Arabs in Israel. **A.** A plotted diagram indicating the incidence rate of brucellosis among Palestinian (in blue) and the incidence rate among Arabs in Israel (in red) from 1998 to 2018. **B.** Palestine territory map showing the highest *Brucella* cases among Palestinian people in Hebron, Bethlehem, and Jericho (indicated in blue color) compared to highest cases among Arabs in Israel in Negev region and Jerusalem (indicated in red color).

Appendix legends

Appendix Table 1. Primers sequences for the different molecular techniques used in the study, including *Brucella* genus and species identification, Rev.1 strains identification, and the 16 primer pairs for MLVA-16 genotyping assay.

Appendix Table 2. The 60 isolates MLVA genotypes collected from MLVABank database and the 72 local isolates MLVA genotypes in addition to the two reference strains *B. abortus* strain 544 and *B. melitensis* strain Rev.1.

Appendix Table 3. Anonymized patient data collected from the serological, culture and molecular methods in the study, Palestine, 2015-2017.

Appendix Table 4. Diversity of MLVA-16 markers. **A.** The 22 unique genotypes among local samples obtained from MLVA-16 analysis. **B.** Diversity indices obtained by Hunter Gaston Diversity index (HGDI) method using V-Dice online tool.

Appendix Figure 1. Genus identification assay: A representative gel electrophoresis image for PCR using B4, B5 primers on 2% Agarose. Lanes 1, 2, 3: samples, Lane 4: 16M *B. melitensis* as a positive control, and lane 5: No Template Control (NTC), lane M: 100 bp DNA ladder (GeneDireX).

Appendix Figure 2. *B. melitensis* and *B. abortus* discrimination assay: A representative gel electrophoresis picture for PCR products on 2% Agarose that aim at discriminating *B. melitensis* and *B. abortus*. Lanes 1, 2: samples, lane 3: Rev.1 vaccine, lane 4: 16M strain of *B. melitensis* as positive control, lane 5: *B. abortus* 544, lane 6: No Template Control (NTC), lane M: 100 bp DNA ladder (GeneDireX).

Appendix Figure 3. Identification of the Rev.1 strain using *rpsl* gene and BiDAS-PCR. **A.** A representative gel electrophoresis picture for identification of Rev.1 Associated Strains on 2% Agarose gel, bands tagged with (-) represent PCR products before cutting with NciI restriction enzyme, bands tagged with (+) represent PCR product of rpsl gene after targeting it with NciI restriction enzyme. Lanes 1, 2, 3, 4: samples, lane 5: Rev.1 vaccine, lane 6: 16M *B. melitensis*, lane N: No template control of Rpsl PCR, lane M: 100 bp DNA ladder (GeneDireX). **B.** A representative gel electrophoresis picture for identification of Rev.1 associated strains using BiDAS-PCR technique to differentiate the Rev.1 DNA from *Brucella* field isolates on 1.5 % Agarose gel. Lanes 1, 2, 3, 4: fields samples, lane 5: Rev.1 vaccine, lane 6: 16M *B. melitensis*, lane NTC: No template control of PCR, lane M: 100 bp DNA ladder (GeneDireX).

Appendix Figure 4. Gel electrophoresis results of MLVA-16 assay comparing the two commercial Rev.1 vaccines. The 16 MLVA markers were resolved on 2% agarose gel. For each marker, lane A represents *B. melitensis* Rev.1 vaccine from Ovejero Lab and lane B represents *B. melitensis* Rev.1 vaccine from JOVAC Co. Lane M for Panel I was the 20bp ladder (Sigma-Aldrich) and for panel II was the 100bp DNA ladder (GeneDirex),

Appendix Figure 5. Dendrogram of clustered MLVA-16 genotypes for local isolates and MLVABank isolates. The 72 human *B. melitensis* isolates and the 60 different geographical samples were clustered into three different groups (East Mediterranean, West Mediterranean, and Americas) using similarity-based clustering (UPGMA). In the columns the following data are presented: Strain names (According to MLVABank), year, group, region, and key (full names and data are available in Appendix Table 2. The different colors of the branches related to the different groups plus the local isolates.

References