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NEW TRENDS IN THE LABORATORY DIAGNOSIS OF BRUCELLOSIS IN PALESTINE: A SHORT REVIEW

TENDINȚE NOI IN DIAGNOSTICUL DE LABORATOR AL BRUCELOZEI IN PALESTINA: O SCURTĂ ANALIZĂ

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ABSTRACT | REZUMAT

In Palestine, brucellosis is an endemic and zoonotic disease reported mainly in small ruminant flocks. In endemic areas, rapid detection of *Brucella melitensis* strains, and the information on prevalence, may shed new light on the epidemiology of brucellosis and for the efficacy of control policy. Laboratory diagnostic is an important tool of the brucellosis control program.

The aim of this review is the evaluation of the Palestinian diagnostic capacity of brucellosis. Principal trends of laboratory practice in Palestine are focused on the increasing of the epidemiological investigation accuracy by using various diagnostic methods of active surveillance. Currently, four directions are involved: 1) rapid laboratory diagnosis of brucellosis during outbreaks, especially when there are reported infected people and use RBT on serum samples followed by RT-qPCR on milk samples of all seropositive animals; 2) routine diagnosis of brucellosis performed with RBT, CFT, ELISA, isolation of bacteria and molecular techniques; 3) differentiation between *B melitensis* field strains and vaccine Rev 1 strain by applying detection of polymorphism mutation in *rpl*L and *omp*2 genes; and 4) epidemiological investigation through sequencing of *lps*B gene, of which variability from one isolated to another allows the construction of phylogenetic trees. All this are necessary to discover sources of infection and transmission pathways in Palestine.

Keywords: *Brucella melitensis*, small ruminants, infectious diseases, zoonosis

În Palestina, bruceloza este o boală endemică și zoonotică raportată în principal în turmele de rumegătoare mici. În zonele endemice, detectarea rapidă a tulpinilor de *Brucella melitensis* și informațiile privind prevalența acestora pot aduce o nouă lumină asupra epidemiologiei brucelozei și eficacității politicilor de control. Diagnosticul de laborator este un instrument important al programului de control al brucelozei. Scopul acestei scurte analize este evaluarea capacității palestiniene de diagnosticare a brucelozei. Tendințele principale ale practicii de laborator din Palestina se concentrează pe creșterea preciziei anchetei epidemiologice prin utilizarea diverselor metode de diagnosticare a supravegherii active. În prezent sunt implicate patru direcții: 1) diagnosticul rapid de laborator al brucelozei în focare, în special atunci când sunt raportate persoane infectate și se utilizează RBT pe probe de ser urmat de RT-qPCR pe probele de lapte la toate animalele seropozitive; 2) diagnosticul de rutină al brucelozei efectuat cu RBT, CFT, ELISA, izolare bacteriană și tehnici de biologie moleculară; 3) diferențierea tulpinilor *B melitensis* sălbatice și Rev 1 vaccinală prin detectarea mutațiilor de la nivelul genelor *rpl*L și *omp* 2; și 4) analiză epidemiologică prin secvențiere a genei *lps*B, a cărei variabilitate de la un izolat la altul permite construirea de arbori filogenetici. Toate acestea sunt necesare să se descopere sursele de infecție și căile de transmitere din Palestina.

Cuvinte cheie: *Brucella melitensis*, rumegătoare mici, boli infecțioase, zoonoză

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Brucellosis is a worldwide zoonosis with a public health impact, mainly in Mediterranean countries (17, 23, 33). The genus *Brucella* includes 11 species (31, 35), the most important one is *Brucella melitensis* (16). The human infection is frequently produced by consumption of contaminated dairy products or as professional exposure to infected livestock and manifested in most cases as flu and if is not diagnosed and

treated properly, it can become chronic and affect multiple body systems (17, 24, 34, 36, 39, 40). In animals, the disease causes significant economic losses by decreasing milk and offspring productions, and loss of trade opportunities (18). The essential virulence factor of pathogenesis are the ability of survive, replicate and avoid apoptosis into phagocytes (9, 14, 15, 22, 25, 26, 29, 35).

The understanding of the *Brucella* epidemiology is critical for refining the control of brucellosis, mainly in countries with limited resources, who can't implement the same policy as the high-income countries (13, 23, 24, 30). Therefore, early, rapid and accurate detection by valid diagnostic tools it is the main goal during an outbreak in order to prevent spreading of the disease, to manage and eradicate the brucellosis (1).

Culture methods have low sensitivity, are time-consuming and hazardous, but remain necessary in epidemiological investigations and in research (1, 33). Serological methods are rapid, but not conclusive because not all infected animals produce detectable levels of antibodies and cross-reactivity with other antigens can give false-positive results (2). Molecular diagnostic techniques represent an important breakthrough in the diagnostic practice due to their high sensitivity and specificity in detection of the all *Brucella* species, in various samples (20, 29). Consequently, the combination of laboratory tests it is a basis for successful implementation of diagnostic goals.

General aims of this short review were to point out our success experiment of laboratory diagnostic through a combination of different techniques which allows to control of brucellosis in Palestine and could guide the decision makers to select the suitable alternative for future control strategies and eradication of animal and human brucellosis.

BRUCELLA DIAGNOSIS IN PALESTINIAN CENTRAL VETERINARY LABORATORY

New trends begin with the implementation of the molecular technique in *Brucella melitensis* diagnosis. The brucellosis reports are mainly based on serological tests and the isolation of the organism followed by identification with a set of molecular tests. The plan of the *Brucella* laboratory diagnosis is moving in four main directions. The first direction is focused to a rapid detection of the infected animals during the outbreaks through the implementation of faster agglutination tests as Rose Bengal Test (RBT) of serum samples followed by real-time quantitative polymerase chain reaction (RT-qPCR) of milk samples from all seropositive animals. The second direction involve the routine laboratory diagnostic of all suspected animals with various serological test as RBT, Complement Fixation Test (CFT) and ELISA, bacteriological methods as *Brucella* isolation and molecular biology methods as classical PCR and RT-qPCR. There were performed molecular biology methods able to distinguish between *Brucella melitensis* field and vaccine Rev 1 strains by detection of *rps*L gene mutation with classical PCR and identification of genetic polymorphism in *omp*2 gene with RFLP-PCR. In addition, phylogenetic tree analysis through sequencing of *Brucella* isolates aid in disease epidemiological investigations (5).

SAMPLES COLLECTION AND PREPARATION

Blood samples are collected from sheep and goats flocks with a history of abortion or when human infection occurs. All information about infected animals (e.g. age, breed, origin, and owner) were recorded at the time of the farm visit. The samples are kept in the ice box at about 4°C and transported immediately to the Laboratory. The sera are extracted by centrifugation at 3000 rpm for 3 min and tested immediately or kept at –20°C until required. The milk samples are obtained from animals during their routine milking time. The cream and sediment mixtures of milk assess after centrifugation of samples (10 mL) at 2000g, 4°C for 20 minutes and the sediment will be used for isolation and extraction purposes. Other samples (stomach content, vaginal discharge, cotyledons, organs and semen fluid) are used in their original form for isolation purpose and 10 g of samples are grinded with phosphate buffer saline (PBS) for extraction and further PCR identification.

SEROLOGICAL EXAMINATION OF SERUM SAMPLES

Rose Bengal test (RBT) is highly sensitive and is mostly used as a screening test (8). RBT uses antigen prepared from a concentrated suspension of *B abortus*. The test is positive if agglutination appears within 4 minutes. False positive results can appear due to cross-reaction with other bacteria as *Yersinia enterocolitica*, but also prozone effect may occur due to a high concentration of antibodies, then samples dilution are necessary. RBT will be confirmed with CFT and the positive animal will be retest after 2 weeks. RBT in

combination with RT-qPCR is a very successful tool to manage outbreaks (5, 6, 7).

COMPLEMENT FIXATION TEST

The principle of the complement fixation test (CFT) is based on the ability of some antigens (bacterial or viral) to use the complement in the recognition process by their specific antibodies. Usually, in early stages of the disease, CFT can be negative due to the low level of specific antibodies. In Palestinian laboratory, CFT is using diluted sheep and goat sera (from 1/5 to 1/2560), whole bacterial cells as antigen (Jovac, Jordan) and a pretitrated amount of complement guinea pigs serum (BioMeriux, France).

Inactivation of the serum samples complement is done by heating 30 min at 56°C. The incubation temperature is 37 °C for 1 hour for warm method and 4°C for 18-24 hours for cold method. The test will be considerate negative if 100% haemolysis appears in 1/5 diluted sera (Fig. 1).

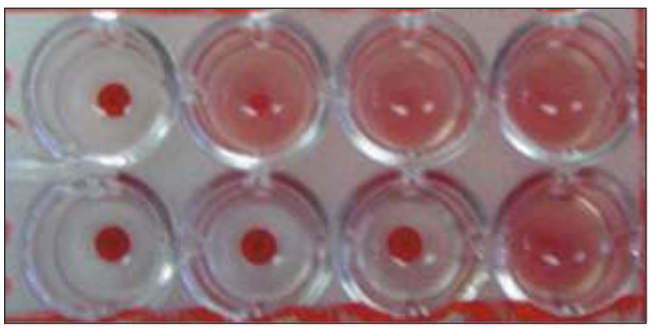

Fig. 1. Complement Fixation Test in *Brucella* diagnostic. Negative: 100% haemolysis in 1/5 diluted sera.

INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY

The indirect enzyme-linked immunosorbent assay (iELISA) is an important tool for the diagnosis of brucellosis on early stage, able to distinguish and detect different classes of antibodies (IgM, IgG, and IgA). Palestinian laboratory uses a commercial iELISA kit (IDEXX CHEKIT Brucellose Serum ELISA Test Kit - IDEXX Laboratories, ME, USA).

BRUCELLA ISOLATION AND IDENTIFICATION

Palestinian laboratory is using *Brucella* agar plates (Oxoid, UK) with *Brucella* supplement (Oxoid, UK) and 7% calf serum. The plates are incubated at 37°C for 2 to 7 days and daily examined. In positive samples, after 48-72h of incubation, characteristic *Brucella* colonies (0.5-1.0 mm, convex and circular outline) are present. Smooth strains are transparent and pale yellow, resembling droplets of honey, with a shiny surface (3). Rough colonies are more opaque with a granular surface. In Palestinian laboratory, dissociation of smooth and rough strains is performed by the emulsification of a colony in 0.1% w/v aqueous acriflavine. Smooth colonies produce a yellow uniform suspension while rough colonies produce granular agglutinates (3, 38). *Brucellae* are very small, faintly stained Gram-negative coccobacillus, resembling with "fine sand" (3).

Identification and typing of *Brucella* isolates is performed in Palestinian laboratory by using following tests: CO_2 requirement, H_2S production, urease and oxidase activity, agglutination with monospecific sera (A and M), selective inhibition of growth on *Brucella* dye tolerance media with basic fuchsin or with thionin, and phage typing. Sometimes, commercially available biochemical identification systems (e.g. API20NE bio Mérieux, Nürtingen, Germany) are used.

MOLECULAR DIAGNOSTIC OF BRUCELLOSIS

Singleplex and multiplex PCR

The design of primers was made with Primer3 software (http://frodo.wi.mit.edu/primer3) or were chosen from scientific articles, and the extraction was made with commercial kit.

The positive control is obtaining from *Brucella melitensis* Rev.1 vaccine (Ovejero, Spain).

Extraction is made with QIAamp RNA Mini Kit (Qiagen, Germany), without adding polyA, in accordance with the procedure of the commercial kit.

Amplification of the specific fragment is performed in a 25 µL volume master mix (Promega, Madison, WI, USA) containing 10 mM tris-HCl (pH 8.4), 50 mM KCl, 1 mM MgCl2, 200 µM each deoxyribonucleotide triphosphate (dATP, dGTP, dTTP, dCTP) and 0.5 U of Taq polymerase, and 10 nM of each primer (Syntezza, Israel) (Table 1). Thermocycling conditions are detailed in Table 1. The PCR products are visualized in agarose (2.0%) gel electrophoresis with ethidium bromide (0.5 mg/mL). The use of more than one marker-based PCR gave increased sensitivity and higher specificity, providing a better diagnostic approach for screening of field animals. Molecular assays targeting the IS711 insertion element improve analytical sensitivity, be-

Table 1

Singleplex and multiplex PCR for *Brucella melitensis* diagnostic

Primer types (code)	Sequence (5'-3')	Amplified product (bp)	PCR protocol*	No of cycles	Reference
Multiplex PCR					
***Brucella* group (*BG*) specific primer**			ID-2min at 95C D-20sec at 95C A-45sec at 51C E-30sec at 72C FE-6min at 72C	35	Garcia-Yoldi, 2006 (20)
BG-forward	GGTTGTTAAAGGAGAACAGC	600			
BG-reverse	GACGATAGCGTTTCAACTTG				
***B. melitensis* (BM-IS711) specific primer**					
BM-IS711 - forward	AAATCGCGTCCTTGCTGGTCTGA	731			
BM-IS711 – reverse	TGCCGATCACTTAAGGGCCTTCAT				
***B. melitensis* Rev 1specific primer**					
*rps*L Rev1-forward	CAGGCAAACCCTCAGAAGC	752			
*rps*L Rev1-reverse	GATGTGGTAACGCACACCAA				
Singleplex PCR					
Hemagglutinin gene (*HA*)					
HA-forward	GAACCAGAATACGGCAAAA	944	ID-2min at 95C D-45sec at 95C A-45sec at 45C E-30sec at 72C FE-2min at 72C	30	Awwad, 2015 (4)
HA-reverse	GTCGTTTCTGCGTCAACAGA				
***lps*B gene**					
*lps*B- forward	TTCAGCTCATTCCATTGCAG	978	D-30sec at 94C A-30sec at 65C E-30sec at 72C FE-5min at 72C	30	Awwad, 2015 (4)
*lps*B-reverse	CGGATTATCTTCCTGCGAAA				

*ID-initial denaturation, D-denaturation, A-annealing, E-extension, FE-final extension

cause is found in multiple copies of *Brucella* chromosomes (21). *B melitensis* Rev 1 strain is used in vaccines for sheep and goat. Therefore, it is very important to be able to identify and differentiate vaccine and pathogenic *B melitensis* strains.

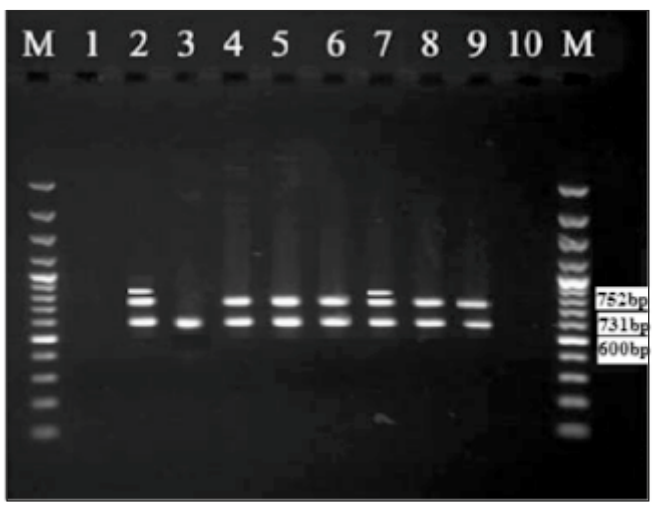

Fig. 2. Agarose gel electrophoresis of multiplex PCR product for *B melitensis*. Line M – ladder; line 1 – negative control; line 2 – positive control (*B melitensis* Rev1 strain); line 3 – field sample positive only for *Brucella* group primers (600 bp); lines 4, 5, 6, 8, and 9 – field samples positive for *B melitensis* IS711 specific primers (731 bp); line 7 – field sample positive for *B melitensis* Rev1 *rps*L specific primers (752 bp); line 10 – negative sample.

One pair of primers based on sequences of the *rps*L locus was used to identify Rev 1, while in field strains of *Brucella melitensis* this locus is absent due to *rps*L gene mutations.

In Palestinian laboratory primer pairs used to identify *Brucella spp.* at the genus-specific level include the primers for sequences encoding *Brucella* group and *B melitensis* IS711, *rps*L gene, hemagglutinin gene, and *lps*B genes (Table 1).

Real-time PCR

The analytical sensitivity will be increased by using real-time PCR assays, which can detect at least five bacteria per reaction. Moreover, real-time PCR enables high-throughput screening of clinical samples and delivers results in a few hours. Genus-specific PCR assays are generally adequate for the molecular diagnosis of brucellosis (1, 2). The *bcsp*31 gene, coding for a 31-kDa immunogenic outer membrane protein conserved among all *Brucella* spp., is the most common molecular target in clinical applications (10, 32). DNA amplification mix consist in 7.12 µl of RNase-free water,5.0µl QIAGEN OneStep RT-PCR Buffer, 5x (Qiagen, UK), 0.8 µl dNTP Mix, 10 mM each (200 µM) (Qiagen, UK), 0.5 µl forward and reverse primers (20 µM/µl),

Table 2

Real Time qPCR used for *Brucella melitensis* diagnostic

Primer/probe code	Sequence (5'-3')	PCR protocol*	No of cycles	Reference
Brucella goup specific *bcsp*31 gene				
*bcsp*31-forward	GCTCGGTTGCCAATATCAATGC	ID-3min at 95C D-15sec at 95C A-35sec at 60C E-35sec at 60C	50	Probert, 2004 (32)
*bcsp*31-reverse	GGGTAAAGCGTCGCCAGAAG			
*bcsp*31-probe	6FAM-AAATCTTCCACC-TGCCCT-GCCATCA-BHQ1			
*ID-initial denaturation, D-denaturation, A-annealing, E-extension, FE-final extension				

0.5 µl (5 µM/µl) probe (Syntheza, Israel), 1 µl Taq polymerase (Promega, Germany), and 5.0 µl of DNA product. Smart Cycler (Cepheid, France) thermocycling conditions are detailed in Table 2.

Detection of genetic polymorphism by DNA restriction fragment length polymorphism analysis

Gene's *omp*2a and *omp*2b as a locus of two nearly homologous repeated copies indicate a greater degree of diversity in all species of *Brucella. Omp*2a does not have the restriction site of *Pst*I and, therefore, is not a good target for differentiation between vaccine strains and field strain in restriction fragment length polymorphism analysis of PCR-amplified fragments (PCR-RFLP). *Omp*2b has the mentioned site for the *Pst*I enzyme and can be used successfully for differentiation between *Brucella* vaccine and field strains (11, 19). PCR-amplified fragments of *omp*2 gene (282 bp) are digested with restriction enzymes *Pst*I according to the manufacturer's instructions (Thermo Fisher Scientific, USA) and separated in 12% SDS-PAGE polyacrylamide gel. Palestinian laboratory prepare polyacrylamide gel as follow: 37.5 ml 40% acrylamide/bis, 4.8 ml H_2O, 2.4 ml of 5xTBE buffer, 200 µl 10% ADS and 20µl of TEMED. Polyacrylamide gels run at 90V, 1.2W, in 0.05% TBE buffer, and stained with ethidium bromide.

Sequencing and phylogenetic analysis

The PCR products are purified with MinElute PCR Purification Kit (Qiagen, Germany) according to manufacturer recommendation. Sequencing includes 3 steps: 1) Big Dye-PCR running (BigDye Terminator v3.1 Kit, ABI, USA); 2) Purification of the sequencing products using DyeEx 2.0 Spin Kit (Qiagen, Germany); 3) sequencing in ABI PRISM 3500xL 8-channel Genetic Analyser (ABI, USA).

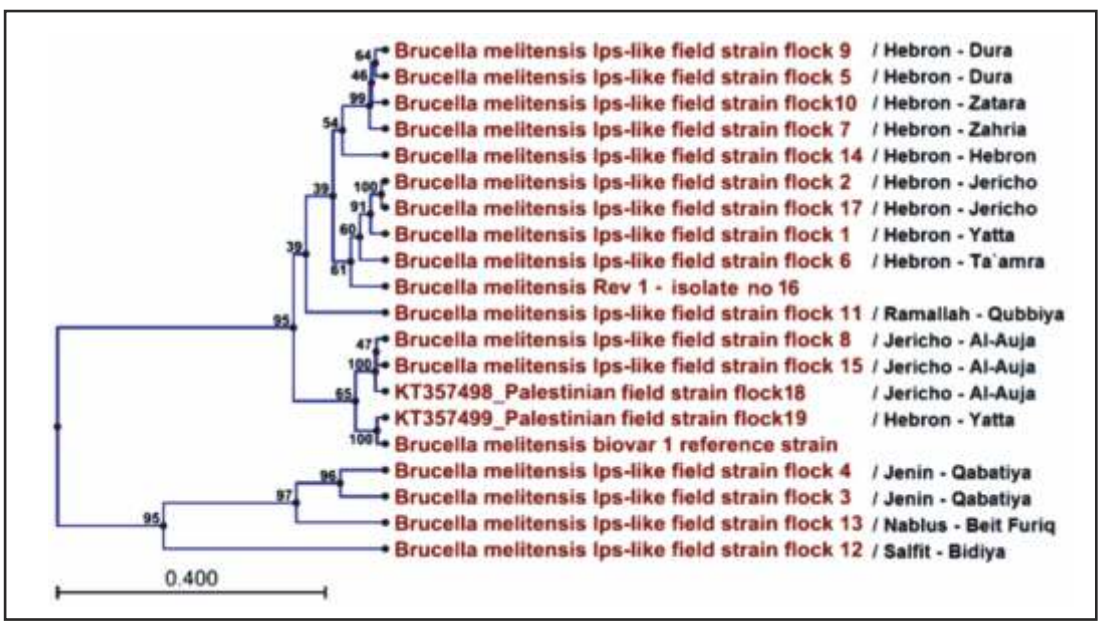

Fig. 3. Phylogenetic relationship of 18 *Brucella melitensis* field strains with *B. melitensis* biovar 1 reference strain and *B. melitensis* Rev1 strain

The alignment of the DNA sequences and the phylogenetic relationships of the *Brucella* field isolates, compared with the reference strain and the vaccine strain Rev.1, are made with CLC Main Workbench software (version 5.6.1, 2009), through UPGMA model of the phylogenetic tree that reflects the structure present in a pair-wise similarity matrix and based on the assumption that all nucleotide substitutions are equally likely and use to determine genetic distances (Fig.3).

DISCUSSION

Brucellosis is still a public health problem in Palestine. The accurate diagnosis and the epidemiological surveillance have clearly benefited from the appearance of molecular techniques, but no single laboratory test can detect all infected animals.

Combination of serological, bacteriological and molecular analysis made more accurate laboratory diagnosis. The rapid serological tests should always be followed by at least one confirmatory test, more specific and sensitive, designed to reduce the number of false negative and positive reactions (1).

Molecular techniques provide several epidemiological data as:

1) genotype of *Brucella* strains;
2) differentiation between field and vaccine strains;
3) similarity of the local circulating strains with other Mediterranean strains;
4) detection of new field strains (20, 21, 27).

Ability to design phylogenetic tree among field isolates is necessary to discover origin and source of the infection, transmission pathways and can be done during an outbreak. In addition, the possibility of data utilization at the global level and comparing with neighbouring regions can increase the rate of policy control success (5). Also, it is predicting the relationships between isolates from neighbouring regions (12, 13, 23, 28, 29, 37).

In order to reduce the prevalence of human infections in the endemic areas it is extremely important to control and remove infected animals from suspected flocks and carry out awareness campaigns to emphasize farmers to boil the milk for all purposes.

In addition, preventive measures should be adopted to deal with animal health in general and with infected animals in particular.

Eradication of brucellosis can only be achieved by test-and-slaughter policy combined with animal movement control.

CONCLUSION

The protocol of diagnostics that include serological, bacteriological and molecular techniques that allows to obtain more accurate result and it is a highly useful tool for the monitoring of brucellosis and for the assessment of the policy control.

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