Jundishapur J Microbiol. 2019 May; 12(5):e91200. doi: 10.5812/jjm.91200.

Published online 2019 May 20. **Review Article**

Laboratory Diagnostic Procedures for Human Brucellosis: An Overview of Existing Approaches

Afshar Etemadi [1], Rezvan Moniri [2, *], Heinrich Neubauer [3], Yasaman Dasteh Goli [4] and Saeed Alamian [5]

[1] Kashan University of Medical Sciences, Kashan, Iran
[2] Department of Microbiology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran
[3] Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Bacterial Infections and Zoonoses, Jena, Germany
[4] University of Maryland College Park, Maryland, United States
[5] Brucellosis Department, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization, Karaj, Iran

[*] *Corresponding author*: Anatomical Sciences Research Center, Kashan University of Medical Sciences, Kashan, Iran. Email: moniri@kaums.ac.ir

Received 2019 March 03; **Revised** 2019 April 04; **Accepted** 2019 April 16.

Abstract

Context: Diagnosis of human brucellosis still challenges clinicians and scientists with several considerable aspects, particularly in endemic countries. The current study aimed at reviewing laboratory tests in the diagnosis of human brucellosis.
Evidence Acquisition: A literature search was conducted in PubMed, Scopus, Thompson Reuters, and Mesh databases using keywords for articles published until December 2018. Seventy studies were selected for data collection.
Results: The current inclusive review included information about the currently used advanced diagnostic tests to confirm the detection of human brucellosis.
Conclusions: The article reviewed the methods for the diagnosis of human brucellosis and summarized developments for the future.

Keywords: Brucellosis, Diagnosis, Molecular Techniques

1. Context

Brucellosis is a zoonotic disease, endemic in many parts of the world especially the Middle East, South and Central Asia, Mediterranean region, Europe, North and East Africa, and Latin America with over half a million new cases annually (1-3). Clinical management of brucellosis is one of the most challenging obstacles due to a high rate of failure in treatment and subsequent relapse (4, 5). Definitive diagnosis of brucellosis needs comprehensive evaluation of the living conditions of the patient, medical history, clinical examinations, and careful interpretation of laboratory test results and radiologic findings (6, 7). Indeed, diagnosis of brucellosis is frequently delayed and often missed especially in the developing countries (8). The gold standard for diagnosis still is bacterial culture, which often fails. Thus, diagnosis relies on the combination of several methods (9).The present study aimed at reviewing laboratory tests in the diagnosis of brucellosis.

2. Evidence Acquisition

In the current review, data were retrieved by search in MEDLINE (via PubMed), Web of Science, Embase, and Cochrane databases, as well as references of the related articles. The following search keywords were used with the help of Boolean operators (AND or OR): *Brucella*, brucellosis, human, and diagnosis. Articles published from 1953 to December 2018 were included. Inclusion criterion was articles using the following techniques and/or methods: direct isolation and identification, conventional cultural examinations, lysis-centrifugation, blood clot culture, automated and semi-automated techniques, serological diagnostic tests, and molecular assays. After screening the abstracts in terms of applied techniques and methods, information was extracted from selected articles in terms of microbiological, serological, and molecular techniques.

3. Results

Diagnostic approaches for human brucellosis are presented in Figure 1. Comparison of different diagnostic

methods for human brucellosis is described in Table 1.

3.1. Direct Isolation and Identification

The isolation of *Brucella* spp. is considered as the gold standard technique for the diagnosis of brucellosis. The culture of *Brucella* is specific and allows definitive identification and typing of the isolates of *Brucella* spp. that is particularly valuable for epidemiological investigations (10). Sensitivity of the *Brucella* spp. isolation is variable depending on the culture method, type of clinical sample, stage of the disease, and history of antibiotic use (11, 12). The risk of acquiring an infection from laboratory ranges 40% to 100% and depends on various factors; e. g., exposures due to laboratory accidents and aerosolization of microorganisms during routine identification activities (13). The ability to direct isolation and culture of *Brucella* spp. can vary between acute and chronic manifestations. Although 50% - 80% of acute cases yield positive blood cultures, only 5% of chronic cases are culture-positive (12). In order to increase the sensitivity, multiple blood sampling should be conducted in the acute phase of brucellosis (14). The frequency of bacteremia episodes is another factor, which should be considered in terms of the time, frequency, and volume of blood collected for culturing. Use of bone marrow aspirate is more sensitive in patients who underwent antibiotic therapy, as well as the ones with a chronic form of brucellosis (15).

3.2. Conventional Culture Examinations

There are numerous available culture media in solid, broth, or biphasic forms for growing *Brucella* spp. isolated. Biphasic media such as the Castaneda blood culture bottles, SEPTI-CHEK™ blood culture (BD BBL®), and Hemoline performance diphasic medium (bioMerieux®) can be used to avoid subculture (15, 16). Commercially available biochemical tests such as API 20 NE® (bioMérieux®) are particularly useful for the rapid and easy identification.

3.3. Lysis-Centrifugation

LC technique is used to concentrate intracellular *Brucella* spp. in blood samples and consequently, increase the test sensitivity (17). The sensitivity, specificity, and positive and negative predictive values of the LC method are 100%, 87.8%, 81.6%, and 100%, respectively compared with those of the Castañeda method (17).

3.4. Blood Clot Culture

Clot culture is a more suitable choice when a second blood sample is not available. Since clot culture techniques are sensitive, simple, and inexpensive and yield earlier results, they can be settled in the areas where automated systems are far from reach. The overall mean time-to-detection of clot culture technique is approximately four days less than that of the conventional methods (18).

3.5. Automated and Semi-Automated Techniques

BACTEC™ (Becton Dickinson Diagnostic Systems®), and BacT/ALERT™ (bioMérieux®) are two frequently used systems in many laboratories that continuously monitor the growth of microorganisms by labeled CO_2. The BACTEC™ Myco/F-Lytic system (Becton Dickinson Diagnostic Systems®) is also developed to improve the recovery rate of intracellular pathogens such as *Brucella* spp. by combined lytic activity and automation (19). Recently, the Micronaut™ semi-automated biotyping System (Merlin Diagnostika®), which facilitates the metabolization of various substrates by bacterial cells, was used for the identification of *Brucella* species and biovars (20).

3.6. Serological Diagnostic Tests

The serological diagnosis of brucellosis commonly relies on the confirmation of the rising titters of *Brucella*-specific antibodies. This is the indirect proof of infection. Serological assays are used for the primary diagnosis of infection, as well as treatment follow-up (21) (Table 1). Immunoglobulin (Ig) M isotype antibodies against the lipopolysaccharide (LPS) of *Brucella* spp. are the first immunoglobulins emerge after infection and are the predominant antibodies during the acute phase of the disease (22). The presence of specific IgM is considered suggestive of acute or recent infection. But, IgM antibody detection in the absence of IgG may lead to a misdiagnosis of acute brucellosis and may be a source of controversy (23, 24). However, the early IgM response might not be seen in patients infected with slow-onset strains, as well as in those appeared late in the course of the disease, or in those with relapses.

The titer of antibody should decline after an effective treatment. Otherwise, the patient should be examined for the possibility of chronic focal disease or relapse. Furthermore, the significant titers of antibody may persist for several months or even years in patients with the history of brucellosis. False positives in the determination of anti-*Brucella* IgM may be due to the presence of cross-reactions and rheumatoid factor. It may be difficult to distinguish

Table 1. Comparison of Different Diagnostic Methods for Human Brucellosis

Method	Advantage	Disadvantage
Conventional culture	Gold standard and specificity	Time consuming, insensitive or low sensitive, and posing a risk for laboratory staff
BACTEC™ and BacT/ALERT™	Rapid, sensitive, and limiting exposure to infectious agents	Costly, and need of subsequent identification
Serum agglutination test	Safe, inexpensive, and appropriate for primary screening	Cross-reactivity with other microorganisms, false-negative results in the early stages of infection, and prozone phenomenon
2-Mercaptoethano	A confirmatory test that allows selective quantification of IgG anti-*Brucella*	Toxicity of mercaptoethanol, the possibility of IgG degradation by the 2-ME, which may lead to false negative results
Coombs antiglobulin agglutination test	Sensitive for relapsing and chronic brucellosis	Labor-intensive and time consuming
Rose Bengal plate agglutination test	Rapid for primary screening, simple, and inexpensive	Cross-reactivity with the antibodies of other microorganisms, false-negative results in the early stages of infection, and prozone phenomenon
Complement fixation test	Sensitive and specific	Complexity, high prices of reagents, need of trained laboratory technicians, and expensive equipment
ELISA	Highly sensitive and specific, rapid, simple, and capable of distinguishing between acute and chronic stages	Cross-reactivity
Fluorescence polarization immunoassay	Highly sensitive and specific, and capable of distinguishing between acute and chronic stages	Costly, need of trained laboratory technicians, and expensive equipment
Lateral flow assay	Easy, rapid, sensitive, and specific	Expensive and possibility of cross-reactivity
PCR	Rapid and accurate; can be performed on blood, serum, CSF, and other clinical samples; can yield positive results as early as 10 days after inoculation	Expensive equipment, genus specific Brucladder has low detection limit, and works only on pure cultures
Real-time PCR	Highly sensitive, specific, and rapid; can be performed on blood, serum, CSF and other clinical samples	Expensive equipment
MALDI-TOF MS	Highly sensitive and specific; can be performed on blood, serum, CSF, and other clinical samples	Expensive equipment
Immunoblot	Sensitive and specific	Cross-reactivity
NGS	Specific technique	Expensive equipment; need of software and complicated analysis

between active infection and simply exposure to the bacteria without clinical relevance in endemic regions by serological methods (25, 26).

3.7. Fluorescence Polarization Immunoassay

Fluorescence polarization immunoassay utilizes molecular rotation, measuring antigen-antibody binding without the need for separation procedures. It requires one-step serum dilution, assessment of background fluorescence, addition of the labelled antigen, and finally measurement of antigen-antibody interaction (27). The accuracy of the FPA is equal or superior to other serological assays such as the complement fixation test (CFT) or the enzyme-linked immunosorbent assay (ELISA). The specificity and sensitivity of FPA for culture-confirmed human brucellosis is 98% and 96%, respectively (12, 28).

3.8. Immunochromatographic Lateral Flow Assay

Immunochromatographic lateral flow assay is a simplified version of the ELISA for the detection of *Brucella*-specific IgM and IgG antibodies (22). Immunochromatographic lateral flow assay is capable of identifying acute, persistent, and relapsing infections. It can also be used to monitor treatment. The sensitivity and specificity of ILFA to detect *Brucella* IgM and IgG in comparison with ELISA or CFT reported 96% and 99%, respectively (22). Therefore, ILFA for both *Brucella* IGM and IgG antibodies is a suitable method for endemic areas with limited resources (29).

3.9. Molecular Assays

Molecular methods become valuable tools for clinical diagnosis and public health surveillance purposes, as well as identification of species and subspecies (30). These techniques can be more sensitive than blood culture and more specific than serologic tests. Molecular assays can be

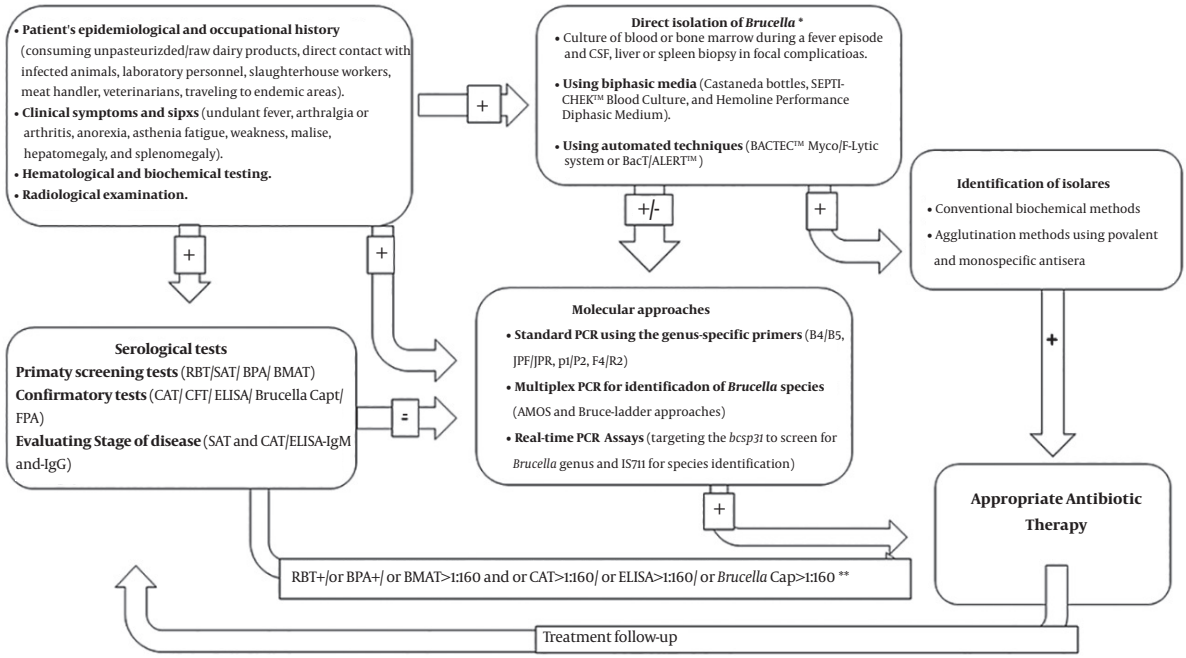

Figure 1. Diagnostic approaches for human brucellosis. * Direct isolation of *Brucella* needs BSL-3 laboratory capability. ** In endemic areas, high titers (cutoff points) may be considered as a positive reaction. BMAT, *Brucella* microagglutination test; BPA, buffered plate antigen test; CAT, Coombs antiglobulin agglutination test; CFT, complement fixation test; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; FPA, fluorescence polarization immunoassay; SAT, serum agglutination tube test.

performed on various clinical samples including serum, whole blood, cerebrospinal fluid (CSF), synovial or pleural fluid, urine, and even tissue specimens. Furthermore, they can supplement phenotypic tests (31, 32). However, direct detection of *Brucella* DNA in patients suspected of brucellosis may be a challenge due to the small number of circulating bacteria in the blood, especially in chronic courses or after antibiotic therapy. Moreover, the detection of *Brucella* DNA cannot demonstrate an active infection with viable pathogens, and thus, may not efficiently support the therapeutic decision making. The type of clinical sample, the DNA extraction method, the specific gene that is tracked, and the employed technique are factors that can influence the efficiency of molecular assays (33).

3.10. Standard Polymerase Chain Reaction

PCR can be performed to amplify and detect *Brucella* DNA in clinical samples or pure cultures. Previously, Navarro et al. described several advantages of using serum samples for nucleic acid amplification (34). Several single-step PCR assays are developed to amplify and detect specific genomic sequences of the genus, species, or even biotypes of *Brucella*. Primer pairs used to detect *Brucella* at the genus-specific level include the primers for sequences encoding BCSP31 (B4/B5), 16SrRNA (F4/R2), 16S - 23S intergenic transcribed spacers (16S - 23S ITS) (Bru ITS-S/Bru ITS-A), 16S - 23S rDNA interspace (ITS66/ITS279), IS711 (IS313/IS639), outer membrane proteins (*omp2b*, *omp2a* and *omp31*), *per* (*bruc1*/*bruc5*), and proteins of the *omp25*/*omp31* family of *Brucella* (35-38). Specificity and sensitivity of these techniques vary depending on the sets of primers, type of clinical sample, and presence of human genomic DNA (Table 2).

B4/B5 primers targeting *bcsp31* are often used for the detection of eukaryotic brucellosis in clinical settings. This primer pair has the highest sensitivity (> 98%) in testing buffy coat or whole blood samples (40). Four primer pairs including B4/B5, JPF/JPR, P1/P2, 26A/26B, and F4/R2 can be applied in four distinct PCR assays to detect *B. abortus*, *B. melitensis*, *B. suis*, and *B. canis* at the genus level. These assays are ideal for rapid confirmation of human brucellosis (41). Two multiplex PCR assays, called AMOS and Bruce-ladder, are standardized and used to detect *Brucella* strains of animal or human origin (42). A multiplex PCR assay was described by Kumar et al. for the simultaneous detection of *B. melitensis*, *B. abortus*, and *B. suis* (43). Researchers re-

Table 2. Comparison of Different PCR Techniques for the Identification of Human Brucellosis

PCR Technique	Primer Name	Primer Sequence	Amplicon Size, bp	Annealing Temp, °C	No. of cycles	Specificity, %	Sensitivity, %	PPN, %	NPN, %	Detection Limit, fg	Reference
bcsp31[a]	B4	TGGCTCGGTTGCCAATATCAA	223	60	40	Bc: 100; Wb: 100; S: 100	Bc: 100; Wb: 100; S: 97	Bc: 100; Wb: 100; S: 100	Bc: 100; Wb: 98; S: 94.3	10 - 100	(39)
	B5	CGCGCTTGCCTTTCAGGTCTG									
omp2[b]	JPF	GCGCTCAGGCTGCCGACGCAA	193	58	35	Bc: 100; Wb: 100; S: 100	Bc: 100; Wb: 98; S: 95.5	Bc: 100; Wb: 100; S: 100	Bc: 100; Wb: 96.1; S: 91.7	25 - 250	(39)
	JPR	ACCAGCCATTGCGGTCGGTA									
omp2	P1	TGGAGGTCAGAAATGAAC	282	50	30	Bc: 100; Wb: 100; S: 100	Bc: 100; Wb: 99; S: 97	Bc: 100; Wb: 100; S: 100	Bc: 100; Wb: 98; S: 94.3	12.5 - 125	(39)
	P2	GAGTGCGAAACGAGCGC									
bp26[c]	26A	GCCCCTGACATAACCCGCTT	1029	58	30	Bc: 100; Wb: 100; S: 100	Bc: 100; Wb: 98.5; S: 96.5	Bc: 100; Wb: 100; S: 100	Bc: 100; Wb: 97.1; S: 93.5	20 - 200	(39)
	26B	GAGCGTGACATTTGCCGATA									
16srRNA gene	F4	TCGAGCGCCCGCAAGGGG	905	54	35	Bc: -[d]; Wb: 100; S: -	Bc: -; Wb: 53.1; S: -	Bc: -; Wb: 53.1; S: -	Bc: -; Wb: 100; S: -	210000	(38)
	R2	AACCATAGTGTCTCCACTAA									

Abbreviations: Bc, buffy coat; NPN, negative predictive number; PPN, positive predictive number; S, serum; Wb, whole blood.
[a] Encoding an immunogenic 31-kDa outer membrane protein, which is highly conserved with each known *Brucella* species and biovar (except *B. ovis*).
[b] Encoding a 26-kDa outer membrane protein of *Brucella* spp.
[c] Encoding a *Brucella* immunodominant antigen, named BP26, CP28, or Omp28 protein.
[d] Undetermined.

ported various procedures that can detect and distinguish *Brucella* spp. in human serum and blood samples via a simple and robust multiplex PCR approach (44-46).

3.11. Other PCR-Based Approaches

Several nested and semi-nested PCR assays were developed to detect *Brucella* spp. in human blood samples (47). A nested-PCR assay was described for the diagnosis of relapse or chronic brucellosis in clinical practice (48). Both sensitivity and specificity was 100%. A semi-nested PCR assay for *bcsp31* and IS6501 was evaluated on whole blood samples (49). However, these assays may increase the probability for primer-dimer formation and/or nonspecific amplification products. Moreover, the reported nested-PCRs can only detect a set of *Brucella* strains, but not single species. A novel loop-mediated isothermal amplification assay (LAMP) was developed to detect *Brucella* spp. DNA in human blood samples. The LAMP assay, based on the sequence of the highly repetitive *omp25* gene, can detect 9 femtogram (fg)/μL of *Brucella* DNA with a sensitivity of 10 times higher than that of the nested-PCR (50).

Considering its advantages as simple operation, rapid amplification, and easy detection, the LAMP has potential applications for clinical diagnosis besides surveillance of human brucellosis in the developing countries without requiring sophisticated equipment or skilled personnel. An inexpensive and simple device such as a water bath or a heat block that can provide a constant temperature of 63°C is sufficient and, unlike conventional PCR result, it can be readout by the naked eye without the need for electrophoretic analysis (29). An arbitrarily primed-PCR (AP-PCR) to detect and identify 25 different *Brucella* strains is also introduced (51). Some PCR-restriction fragment length polymorphism (PCR-RFLP) techniques are successfully used to distinguish *Brucella* species and various biovars.

Restriction maps of *omp2a* and *omp2b* genes showed a greater diversity among and within *Brucella* spp. than other genes investigated so far. PCR-RFLP assays may serve as tools for diagnostic and epidemiological surveillance purposes (52). Furthermore, a PCR-enzyme immunoassay (EIA) was used by Vrioni for the diagnosis of human brucellosis directly from peripheral blood. Following the amplification of the *bcsp31* target sequence, the amplified product was detected in a hybrid well-microtiter plate by hybridization analysis. The diagnostic specificity of the PCR-EIA for both whole blood and serum specimens was 100%, whereas the sensitivity was 81.5% for whole blood specimens and 79% for serum specimens. Vrioni et al. recommend that the detection of *Brucella* DNA in whole blood and serum specimens by PCR-EIA, as a sensitive and specific method, can help the rapid and accurate diagnosis of acute brucellosis (53)

3.12. Real-Time PCR Assay

The real-time PCR technique is more sensitive, specific, reproducible, and rapid than the conventional PCR. The quantitative real-time (qRT)-PCR allows both detection and quantification of the PCR product in real-time, while it is synthesized (54). Real-time PCR can be used for the rapid diagnosis of chronic, but serologically positive, brucellosis and acute brucellosis when serum and blood samples of known clinical presentations are investigated (40). These assays are developed targeting the 16S - 23S ITS region, IS711 element, and *omp25*, *omp31*, and *bcsp31* genes (55-58). The *bcsp31* gene target can be recommended for the detection

of bacteria at the genus level. Species-specific identification confirming the primary diagnosis by a second gene target such as IS711 can be done (59, 60). Several multiplex real-time PCR approaches are developed for the simultaneous detection of *Brucella* spp. and *Mycobacterium tuberculosis* complex (MTC). These techniques amplify the *bcsp31*, IS711, and *omp2a* genes for the detection of *Brucella* spp. and target the *senX3-regX3*, IS6110, and *cfp31* genes for the identification of the MTC (31, 32). Sanjuan-Jimenez et al. evaluated three molecular targets (IS711, *bcsp31*, and *omp2a*) of *Brucella* and three targets of MTC (IS6110, *cfp31*, and *senX3-regX3*) for their simultaneous detection by a multiplex real-time PCR (61).

3.13. Single Nucleotide Polymorphisms Typing

Some investigators previously described unique real-time PCR assays that can characterize *Brucella* isolates to the species level. They used single nucleotide polymorphisms (SNPs) multilocus sequencing (62). Foster et al. applied SNPs to housekeeping genes and introduced gene sequences that can identify the seven main *Brucella* species using the TaqMan assays with contained probes specific to each allele. The assays can detect DNA concentrations of less than 10 fg/mL that is their detection limit (63). However, finding SNPs that can separate *B. canis* from *B. suis* is challenging due to a high degree of sequence homology that indicates a recent split between these species (63).

3.14. Multilocus Variable Number of Tandem Repeats Analysis

PCR methods can detect *Brucella* spp. based on the finding of specific sequences, but limits of these techniques, for example, failure to differentiate among biovars within a species, encouraged the development of other molecular typing methods such as multilocus variable number of tandem repeats analysis (MLVA). Multilocus variable number of tandem repeats analysis measures the number of tandem repeats at a specified locus and can discriminate between isolates within a certain *Brucella* biovar. The MLVA is a quick and efficient method for typing and clustering *Brucella* strains. Moreover, multilocus sequence typing (MLST), sequencing of multiple genetic loci in bacteria, is increasingly accepted as a mean for the classification of microbial populations (64).

3.15. Matrix-Assisted Laser Desorption Ionization

The time of flight mass spectrometry (MALDI-TOF MS) is used as a fast and reliable technique for bacterial identification based on protein profile characteristics of microorganisms (65). MALDI-TOF MS is a reliable test for direct detection of *Brucella* to the genus level from blood culture bottles and culture plates. However, *Brucella* has not been yet incorporated into some of the main available databases due to its potential bioterrorism application (66). Another limitation of MALDI-TOF MS to detect *Brucella* is the need for pure cultures, which pose health hazards to laboratory personnel. Mesureur et al. described a simple and safe method for inactivation of *Brucella* isolates prior to their analysis by MALDI-TOF MS (67).

3.16. Novel Technologies for the Serologic Diagnosis of Brucellosis

The immunoblot-based assay showed several immunodominant proteins of *B. abortus* and *B. melitensis* in a previous study; this technique can be used to identify new candidate antigens for the serologic detection of brucellosis (68). Immunoproteomics of *B. abortus* RB51 (a mutant strain lacking the LPS portion) revealed several candidate antigens. The highly immunogenic proteins may be useful as alternative antigens to avoid cross-reactivity (69). Immunoproteomics of *B. abortus* also showed differential antibody profiles for *B. abortus* strain, S19-vaccinated and naturally infected cattle, and differentiation between vaccinated cattle and those animals infected with field strains (70).

4. Conclusions

Laboratory diagnosis of brucellosis still relies upon culture of bacteria followed by various biochemical and serological test results. Nucleic acid tests such as PCR are the novel-generation technologies that have higher sensitivity than blood cultures and better specificity than serologic tests. Molecular techniques such as PCR facilitate rapid, sensitive, and specific detection. MLVA is helpful for following an infection. Finally, it should be emphasized that novel technologies such as microfluidic lab-on-chip and next-generation sequencing (NGS) can provide a rapid, accurate, and safe diagnosis of brucellosis, especially in endemic countries. Future research on immunoproteomics and the selection of highly immunogenic protein spots can be useful as alternative antigens for the diagnosis of brucellosis.

Footnotes

Authors' Contribution: Study concept and design: Rezvan Moniri and Afshar Etemadi. Search in literature: Rezvan Moniri, Afshar Etemadi, and Saeed Alamian. Drafting of the manuscript: Rezvan Moniri and Afshar Etemadi.

Critical revision of the manuscript for important intellectual content: Heinrich Neubauer. English editing of the manuscript: Yasaman Dasteh Goli.

Conflict of Interests: The authors declared no conflict of interest.

Ethical Considerations: None required.

Financial Disclosure: It is not declared by the authors.

Funding/Support: This research supported by the Kashan University of Medical Sciences (grant No.: 95152).

References

1. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The new global map of human brucellosis. *Lancet Infect Dis*. 2006;**6**(2):91–9. doi: 10.1016/S1473-3099(06)70382-6. [PubMed: 16439329].
2. Lucero NE, Ayala SM, Escobar GI, Jacob NR. Brucella isolated in humans and animals in Latin America from 1968 to 2006. *Epidemiol Infect*. 2008;**136**(4):496–503. doi: 10.1017/S0950268807008795. [PubMed: 17559694]. [PubMed Central: PMC2870831].
3. Wolfram JH, Butaev MK, Duysheev A, Gabbasova AR, Khasanov OS, Kulakov YK, et al. Epidemiology chapter. *Vaccine*. 2010;**28 Suppl 5**:F77–84. doi: 10.1016/j.vaccine.2010.04.050. [PubMed: 20850689].
4. Alavi SM, Alavi L. Treatment of brucellosis: A systematic review of studies in recent twenty years. *Caspian J Intern Med*. 2013;**4**(2):636–41. [PubMed: 24009951]. [PubMed Central: PMC3755828].
5. Demirturk N, Demirdal T, Erben N, Demir S, Asci Z, Kilit TP, et al. Brucellosis: A retrospective evaluation of 99 cases and review of brucellosis treatment. *Trop Doct*. 2008;**38**(1):59–62. doi: 10.1258/td.2006.006266. [PubMed: 18302876].
6. Buzgan T, Karahocagil MK, Irmak H, Baran AI, Karsen H, Evirgen O, et al. Clinical manifestations and complications in 1028 cases of brucellosis: A retrospective evaluation and review of the literature. *Int J Infect Dis*. 2010;**14**(6):e469–78. doi: 10.1016/j.ijid.2009.06.031. [PubMed: 19910232].
7. Ariza J, Pellicer T, Pallares R, Foz A, Gudiol F. Specific antibody profile in human brucellosis. *Clin Infect Dis*. 1992;**14**(1):131–40. doi: 10.1093/clinids/14.1.131. [PubMed: 1571417].
8. Franc KA, Krecek RC, Hasler BN, Arenas-Gamboa AM. Brucellosis remains a neglected disease in the developing world: A call for interdisciplinary action. *BMC Public Health*. 2018;**18**(1):125. doi: 10.1186/s12889-017-5016-y. [PubMed: 29325516]. [PubMed Central: PMC5765637].
9. Gupte S, Kaur T. Diagnostic approach to brucellosis. *J Trop Dis*. 2016;**4**(1). doi: 10.4172/2329-891X.1000e109.
10. Al Dahouk S, Tomaso H, Nockler K, Neubauer H, Frangoulidis D. Laboratory-based diagnosis of brucellosis–A review of the literature. Part I: Techniques for direct detection and identification of Brucella spp. *Clin Lab*. 2003;**49**(9-10):487–505. [PubMed: 14572205].
11. Hadush A, Pal M. Brucellosis - An infectious re-emerging bacterial zoonosis of global importance. *Int J Livestock Res*. 2013;**3**(1):28. doi: 10.5455/ijlr.20130305064802.
12. Christopher S, Umapathy BL, Ravikumar KL. Brucellosis: Review on the recent trends in pathogenicity and laboratory diagnosis. *J Lab Physicians*. 2010;**2**(2):55–60. doi: 10.4103/0974-2727.72149. [PubMed: 21346896]. [PubMed Central: PMC3040083].
13. Singh K. Laboratory-acquired infections. *Clin Infect Dis*. 2009;**49**(1):142–7. doi: 10.1086/599104. [PubMed: 19480580].
14. Erdem H, Kilic S, Sener B, Acikel C, Alp E, Karahocagil M, et al. Diagnosis of chronic brucellar meningitis and meningoencephalitis: The results of the Istanbul-2 study. *Clin Microbiol Infect*. 2013;**19**(2):E80–6. doi: 10.1111/1469-0691.12092. [PubMed: 23210984].
15. Yagupsky P. Detection of Brucellae in blood cultures. *J Clin Microbiol*. 1999;**37**(11):3437–42. [PubMed: 10523530]. [PubMed Central: PMC85661].
16. Ruiz J, Lorente I, Perez J, Simarro E, Martinez-Campos L. Diagnosis of brucellosis by using blood cultures. *J Clin Microbiol*. 1997;**35**(9):2417–8. [PubMed: 9276429]. [PubMed Central: PMC229981].
17. Espinosa BJ, Chacaltana J, Mulder M, Franco MP, Blazes DL, Gilman RH, et al. Comparison of culture techniques at different stages of brucellosis. *Am J Trop Med Hyg*. 2009;**80**(4):625–7. doi: 10.4269/ajtmh.2009.80.625. [PubMed: 19346389].
18. Mangalgi S, Sajjan A. Comparison of three blood culture techniques in the diagnosis of human brucellosis. *J Lab Physicians*. 2014;**6**(1):14–7. doi: 10.4103/0974-2727.129084. [PubMed: 24696554]. [PubMed Central: PMC3969635].
19. Akpinar O. Historical perspective of brucellosis: A microbiological and epidemiological overview. *Infez Med*. 2016;**24**(1):77–86. [PubMed: 27031903].
20. Al Dahouk S, Scholz HC, Tomaso H, Bahn P, Gollner C, Karges W, et al. Differential phenotyping of Brucella species using a newly developed semi-automated metabolic system. *BMC Microbiol*. 2010;**10**:269. doi: 10.1186/1471-2180-10-269. [PubMed: 20969797]. [PubMed Central: PMC2984481].
21. Ciocchini AE, Rey Serantes DA, Melli LJ, Iwashkiw JA, Deodato B, Wallach J, et al. Development and validation of a novel diagnostic test for human brucellosis using a glyco-engineered antigen coupled to magnetic beads. *PLoS Negl Trop Dis*. 2013;**7**(2). e2048. doi: 10.1371/journal.pntd.0002048. [PubMed: 23459192]. [PubMed Central: PMC3573069].
22. Smits HL, Abdoel TH, Solera J, Clavijo E, Diaz R. Immunochromatographic Brucella-specific immunoglobulin M and G lateral flow assays for rapid serodiagnosis of human brucellosis. *Clin Diagn Lab Immunol*. 2003;**10**(6):1141–6. doi: 10.1128/CDLI.10.6.1141-1146.2003. [PubMed: 14607880]. [PubMed Central: PMC262433].
23. Fadeel MA, Hoffmaster AR, Shi J, Pimentel G, Stoddard RA. Comparison of four commercial IgM and IgG ELISA kits for diagnosing brucellosis. *J Med Microbiol*. 2011;**60**(Pt 12):1767–73. doi: 10.1099/jmm.0.033381-0. [PubMed: 21835974].
24. Solis Garcia Del Pozo J, Lorente Ortuno S, Navarro E, Solera J. Detection of IgM antibrucella antibody in the absence of IgGs: A challenge for the clinical interpretation of brucella serology. *PLoS Negl Trop Dis*. 2014;**8**(12). e3390. doi: 10.1371/journal.pntd.0003390. [PubMed: 25474572]. [PubMed Central: PMC4256177].
25. Casanova A, Ariza J, Rubio M, Masuet C, Diaz R. BrucellaCapt versus classical tests in the serological diagnosis and management of human brucellosis. *Clin Vaccine Immunol*. 2009;**16**(6):844–51. doi: 10.1128/CVI.00348-08. [PubMed: 19369480]. [PubMed Central: PMC2691052].
26. Xu J, Qiu Y, Cui M, Ke Y, Zhen Q, Yuan X, et al. Sustained and differential antibody responses to virulence proteins of Brucella melitensis during acute and chronic infections in human brucellosis. *Eur J Clin Microbiol Infect Dis*. 2013;**32**(3):437–47. doi: 10.1007/s10096-012-1767-7. [PubMed: 23224716].
27. Lucero NE, Escobar GI, Ayala SM, Silva Paulo P, Nielsen K. Fluorescence polarization assay for diagnosis of human brucellosis. *J Med Microbiol*. 2003;**52**(Pt 10):883–7. doi: 10.1099/jmm.0.05217-0. [PubMed: 12972582].
28. Nielsen K, Gall D. Fluorescence polarization assay for the diagnosis of brucellosis: A review. *J Immunoassay Immunochem*. 2001;**22**(3):183–201. doi: 10.1081/IAS-100104705. [PubMed: 11506271].
29. Gupte S, Kaur T. Diagnosis of human Brucellosis. *J Trop Dis*. 2016;**4**(1). doi: 10.4172/2329-891x.1000185.

30. Lin F, Xu Y, Chang Y, Liu C, Jia X, Ling B. Molecular characterization of reduced susceptibility to biocides in clinical isolates of Acinetobacter baumannii. *Front Microbiol*. 2017;**8**:1836. doi: 10.3389/fmicb.2017.01836. [PubMed: 29018420]. [PubMed Central: PMC5622949].
31. Colmenero JD, Morata P, Ruiz-Mesa JD, Bautista D, Bermudez P, Bravo MJ, et al. Multiplex real-time polymerase chain reaction: A practical approach for rapid diagnosis of tuberculous and brucellar vertebral osteomyelitis. *Spine (Phila Pa 1976)*. 2010;**35**(24):E1392–6. doi: 10.1097/BRS.0b013e3181e8eeaf. [PubMed: 21030888].
32. Queipo-Ortuno MI, Colmenero JD, Bermudez P, Bravo MJ, Morata P. Rapid differential diagnosis between extrapulmonary tuberculosis and focal complications of brucellosis using a multiplex real-time PCR assay. *PLoS One*. 2009;**4**(2). e4526. doi: 10.1371/journal.pone.0004526. [PubMed: 19225565]. [PubMed Central: PMC2639699].
33. Al Dahouk S, Nockler K. Implications of laboratory diagnosis on brucellosis therapy. *Expert Rev Anti Infect Ther*. 2011;**9**(7):833–45. doi: 10.1586/eri.11.55. [PubMed: 21810055].
34. Navarro E, Escribano J, Fernandez J, Solera J. Comparison of three different PCR methods for detection of Brucella spp in human blood samples. *FEMS Immunol Med Microbiol*. 2002;**34**(2):147–51. doi: 10.1111/j.1574-695X.2002.tb00616.x. [PubMed: 12381466].
35. Alamian S, Esmaelizad M, Zahraei T, Etemadi A, Mohammadi M, Afshar D, et al. A novel PCR assay for detecting Brucella abortus and Brucella melitensis. *Osong Public Health Res Perspect*. 2017;**8**(1):65–70. doi: 10.24171/j.phrp.2017.8.1.09. [PubMed: 28443226]. [PubMed Central: PMC5402848].
36. Etemady A, Mohammdi M, Esmaelizad M, Alamian S, Vahedi F, Aghaeipour K, et al. Genetic characterization of the wboA gene from the predominant biovars of Brucella isolates in Iran. *Electron Physician*. 2015;**7**(6):1381–6. doi: 10.14661/1381. [PubMed: 26516446]. [PubMed Central: PMC4623799].
37. Yu WL, Nielsen K. Review of detection of Brucella spp. by polymerase chain reaction. *Croat Med J*. 2010;**51**(4):306–13. doi: 10.3325/cmj.2010.51.306. [PubMed: 20718083]. [PubMed Central: PMC2931435].
38. Baddour MM, Alkhalifa DH. Evaluation of three polymerase chain reaction techniques for detection of Brucella DNA in peripheral human blood. *Can J Microbiol*. 2008;**54**(5):352–7. doi: 10.1139/w08-017. [PubMed: 18449219].
39. Mitka S, Anetakis C, Souliou E, Diza E, Kansouzidou A. Evaluation of different PCR assays for early detection of acute and relapsing brucellosis in humans in comparison with conventional methods. *J Clin Microbiol*. 2007;**45**(4):1211–8. doi: 10.1128/JCM.00010-06. [PubMed: 17267626]. [PubMed Central: PMC1865811].
40. Wang Y, Wang Z, Zhang Y, Bai L, Zhao Y, Liu C, et al. Polymerase chain reaction-based assays for the diagnosis of human brucellosis. *Ann Clin Microbiol Antimicrob*. 2014;**13**:31. doi: 10.1186/s12941-014-0031-7. [PubMed: 25082566]. [PubMed Central: PMC4236518].
41. Imaoka K, Kimura M, Suzuki M, Kamiyama T, Yamada A. Simultaneous detection of the genus Brucella by combinatorial PCR. *Jpn J Infect Dis*. 2007;**60**(2-3):137–9. [PubMed: 17515651].
42. Mayer-Scholl A, Draeger A, Gollner C, Scholz HC, Nockler K. Advancement of a multiplex PCR for the differentiation of all currently described Brucella species. *J Microbiol Methods*. 2010;**80**(1):112–4. doi: 10.1016/j.mimet.2009.10.015. [PubMed: 19887090].
43. Kumar S, Tuteja U, Sarika K, Singh D, Kumar A, Kumar O. Rapid multiplex PCR assay for the simultaneous detection of the Brucella Genus, B. abortus, B. melitensis, and B. suis. *J Microbiol Biotechnol*. 2011;**21**(1):89–92. doi: 10.4014/jmb.1007.07051. [PubMed: 21301197].
44. Kamal IH, Al Gashgari B, Moselhy SS, Kumosani TA, Abulnaja KO. Two-stage PCR assay for detection of human brucellosis in endemic areas. *BMC Infect Dis*. 2013;**13**:145. doi: 10.1186/1471-2334-13-145. [PubMed: 23517532]. [PubMed Central: PMC3614503].
45. Garofolo G, Ancora M, Di Giannatale E. MLVA-16 loci panel on Brucella spp. using multiplex PCR and multicolor capillary electrophoresis. *J Microbiol Methods*. 2013;**92**(2):103–7. doi: 10.1016/j.mimet.2012.11.007. [PubMed: 23174277].
46. Mirnejad R, Mohamadi M, Piranfar V, Mortazavi SM, Kachuei R. A duplex PCR for rapid and simultaneous detection of Brucella spp. in human blood samples. *Asian Pac J Trop Med*. 2013;**6**(6):453–6. doi: 10.1016/S1995-7645(13)60073-5. [PubMed: 23711705].
47. Al-Nakkas A, Mustafa AS, Wright SG. Large-scale evaluation of a single-tube nested PCR for the laboratory diagnosis of human brucellosis in Kuwait. *J Med Microbiol*. 2005;**54**(Pt 8):727–30. doi: 10.1099/jmm.0.45772-0. [PubMed: 16014425].
48. Hasanjani Roushan MR, Marashi SM, Moulana Z. Polymerase chain reaction-based assays for the diagnosis of active and relapsed cases of human brucellosis. *Am J Trop Med Hyg*. 2016;**95**(6):1272–6. doi: 10.4269/ajtmh.16-0344. [PubMed: 27928078]. [PubMed Central: PMC5154438].
49. Cirak MY, Hizel K. [The value of polymerase chain reaction methods targeting two different gene regions for the diagnosis of brucellosis]. *Mikrobiyol Bul*. 2002;**36**(3-4):271–6. Turkish. [PubMed: 12838660].
50. Lin GZ, Zheng FY, Zhou JZ, Gong XW, Wang GH, Cao XA, et al. Loop-mediated isothermal amplification assay targeting the omp25 gene for rapid detection of Brucella spp. *Mol Cell Probes*. 2011;**25**(2-3):126–9. doi: 10.1016/j.mcp.2011.01.001. [PubMed: 21232598].
51. Fekete A, Bantle JA, Halling SM, Stich RW. Amplification fragment length polymorphism in Brucella strains by use of polymerase chain reaction with arbitrary primers. *J Bacteriol*. 1992;**174**(23):7778–83. doi: 10.1128/jb.174.23.7778-7783.1992. [PubMed: 1360006]. [PubMed Central: PMC207493].
52. Al Dahouk S, Tomaso H, Prenger-Berninghoff E, Splettstoesser WD, Scholz HC, Neubauer H. Identification of brucella species and biotypes using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). *Crit Rev Microbiol*. 2005;**31**(4):191–6. doi: 10.1080/10408410500304041. [PubMed: 16417200].
53. Vrioni G, Gartzonika C, Kostoula A, Boboyianni C, Papadopoulou C, Levidiotou S. Application of a polymerase chain reaction enzyme immunoassay in peripheral whole blood and serum specimens for diagnosis of acute human brucellosis. *Eur J Clin Microbiol Infect Dis*. 2004;**23**(3):194–9. doi: 10.1007/s10096-003-1082-4. [PubMed: 14986157].
54. Garibyan L, Avashia N. Polymerase chain reaction. *J Invest Dermatol*. 2013;**133**(3):1–4. doi: 10.1038/jid.2013.1. [PubMed: 23399825]. [PubMed Central: PMC4102308].
55. Kattar MM, Zalloua PA, Araj GF, Samaha-Kfoury J, Shbaklo H, Kanj SS, et al. Development and evaluation of real-time polymerase chain reaction assays on whole blood and paraffin-embedded tissues for rapid diagnosis of human brucellosis. *Diagn Microbiol Infect Dis*. 2007;**59**(1):23–32. doi: 10.1016/j.diagmicrobio.2007.04.002. [PubMed: 17532591].
56. Debeaumont C, Falconnet PA, Maurin M. Real-time PCR for detection of Brucella spp. DNA in human serum samples. *Eur J Clin Microbiol Infect Dis*. 2005;**24**(12):842–5. doi: 10.1007/s10096-005-0064-0. [PubMed: 16341519].
57. Queipo-Ortuno MI, Colmenero JD, Bravo MJ, Garcia-Ordonez MA, Morata P. Usefulness of a quantitative real-time PCR assay using serum samples to discriminate between inactive, serologically positive and active human brucellosis. *Clin Microbiol Infect*. 2008;**14**(12):1128–34. doi: 10.1111/j.1469-0691.2008.02095.x. [PubMed: 19046166].
58. Zhang B, Wear DJ, Stojadinovic A, Izadjoo M. Sequential real-time PCR assays applied to identification of genomic signatures in

formalin-fixed paraffin-embedded tissues: A case report about brucella-induced osteomyelitis. *Mil Med*. 2013;**178**(1):88–94. doi: 10.7205/MILMED-D-12-00274. [PubMed: 23356125].

59. Colmenero JD, Clavijo E, Morata P, Bravo MJ, Queipo-Ortuno MI. Quantitative real-time polymerase chain reaction improves conventional microbiological diagnosis in an outbreak of brucellosis due to ingestion of unpasteurized goat cheese. *Diagn Microbiol Infect Dis*. 2011;**71**(3):294–6. doi: 10.1016/j.diagmicrobio.2011.06.016. [PubMed: 21855249].
60. Bounaadja L, Albert D, Chenais B, Henault S, Zygmunt MS, Poliak S, et al. Real-time PCR for identification of Brucella spp.: A comparative study of IS711, bcsp31 and per target genes. *Vet Microbiol*. 2009;**137**(1-2):156–64. doi: 10.1016/j.vetmic.2008.12.023. [PubMed: 19200666].
61. Sanjuan-Jimenez R, Colmenero JD, Bermudez P, Alonso A, Morata P. Amplicon DNA melting analysis for the simultaneous detection of Brucella spp and Mycobacterium tuberculosis complex. Potential use in rapid differential diagnosis between extrapulmonary tuberculosis and focal complications of brucellosis. *PLoS One*. 2013;**8**(3). e58353. doi: 10.1371/journal.pone.0058353. [PubMed: 23520501]. [PubMed Central: PMC3592798].
62. Gopaul KK, Sells J, Bricker BJ, Crasta OR, Whatmore AM. Rapid and reliable single nucleotide polymorphism-based differentiation of Brucella live vaccine strains from field strains. *J Clin Microbiol*. 2010;**48**(4):1461–4. doi: 10.1128/JCM.02193-09. [PubMed: 20181906]. [PubMed Central: PMC2849582].
63. Foster JT, Okinaka RT, Svensson R, Shaw K, De BK, Robison RA, et al. Real-time PCR assays of single-nucleotide polymorphisms defining the major Brucella clades. *J Clin Microbiol*. 2008;**46**(1):296–301. doi: 10.1128/JCM.01496-07. [PubMed: 18032628]. [PubMed Central: PMC2224295].
64. Maio E, Begeman L, Bisselink Y, van Tulden P, Wiersma L, Hiemstra S, et al. Identification and typing of Brucella spp. in stranded harbour porpoises (Phocoena phocoena) on the Dutch coast. *Vet Microbiol*. 2014;**173**(1-2):118–24. doi: 10.1016/j.vetmic.2014.07.010. [PubMed: 25115787].
65. Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, Rolain JM, et al. Ongoing revolution in bacteriology: Routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin Infect Dis*. 2009;**49**(4):543–51. doi: 10.1086/600885. [PubMed: 19583519].
66. Ferreira L, Vega Castano S, Sanchez-Juanes F, Gonzalez-Cabrero S, Menegotto F, Orduna-Domingo A, et al. Identification of Brucella by MALDI-TOF mass spectrometry. Fast and reliable identification from agar plates and blood cultures. *PLoS One*. 2010;**5**(12). e14235. doi: 10.1371/journal.pone.0014235. [PubMed: 21151913]. [PubMed Central: PMC2997794].
67. Mesureur J, Ranaldi S, Monnin V, Girard V, Arend S, Welker M, et al. A simple and safe protocol for preparing brucella samples for matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis. *J Clin Microbiol*. 2016;**54**(2):449–52. doi: 10.1128/JCM.02730-15. [PubMed: 26582837]. [PubMed Central: PMC4733191].
68. Wareth G, Eravci M, Weise C, Roesler U, Melzer F, Sprague LD, et al. Comprehensive identification of immunodominant proteins of Brucella abortus and Brucella melitensis using antibodies in the sera from naturally infected hosts. *Int J Mol Sci*. 2016;**17**(5). doi: 10.3390/ijms17050659. [PubMed: 27144565]. [PubMed Central: PMC4881485].
69. Kim JY, Sung SR, Lee K, Lee HK, Kang SI, Lee JJ, et al. Immunoproteomics of Brucella abortus RB51 as candidate antigens in serological diagnosis of brucellosis. *Vet Immunol Immunopathol*. 2014;**160**(3-4):218–24. doi: 10.1016/j.vetimm.2014.05.009. [PubMed: 24908638].
70. Pajuaba AC, Silva DA, Almeida KC, Cunha-Junior JP, Pirovani CP, Camillo LR, et al. Immunoproteomics of Brucella abortus reveals differential antibody profiles between S19-vaccinated and naturally infected cattle. *Proteomics*. 2012;**12**(6):820–31. doi: 10.1002/pmic.201100185. [PubMed: 22539433].