

A Review on Diagnostic Methods of Brucellosis

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Abstract

We reviewed different diagnostic methods of brucellosis in different livestock and humans. Bacteriological diagnosis, immunohistochemistry (an alternative technique for direct diagnosis) and different molecular methods for *Brucella* species genotyping are among the direct methods for diagnosis of brucellosis discussed in this review. The well-established indirect methods for diagnosis of brucellosis, serological and brucellin allergic skin tests were also critically conferred. Finally, for effective control and prevention of brucellosis around the world, the direct diagnostic methods are advised in order to develop vaccine against the circulating *Brucella* strain in the specific country.

Keywords: Allergic skin test; Brucellosis; Immunohistochemistry; Molecular tool; Serological tests

Introduction

Brucellosis is an ancient disease that can possibly be traced back to the 5th plague of Egypt around 1600 BC. Recent examination of the ancient Egyptian bones, dating to around 750 BC, showed evidence of sacroilitis and other osteoarticular lesions, common complications of brucellosis [1]. David Bruce isolated *Brucella melitensis* (*B. melitensis*) (*Micrococcus melitensis* at that time) in 1887 from the spleen of a British soldier who died from a febrile illness (Malta fever) common among military personnel stationed on Malta. For almost 20 years after isolation of *M. melitensis*, Malta fever remained a mystery and was thought to be a vector-borne disease until Themistocles Zammit accidentally demonstrated the zoonotic nature of the disease in 1905 by isolating *B. melitensis* from goat's milk. It was believed that goats were not the source of infection since they did not become ill when inoculated with *Brucella* cultures. The discovery that healthy goats could be carriers of the disease has been termed one of the greatest advances ever made in the study of epidemiology [2,3].

Brucellosis is caused by Gram-negative coccobacilli of the genus *Brucella* [4,5]. In livestock, the disease results in significant economic losses due to reproductive impairment caused by abortion, stillbirth or weak calves and neonatal mortality, infertility [6]. In humans, *Brucella* spp. infection causes a febrile disease that may be associated with a broad spectrum of symptoms, and it may be fatal in some cases [5,7]. Currently, there are ten spp. described in the genus *Brucella*. Each one may infect different host spp., but each *Brucella* spp. has a preference for its host spp., *B. melitensis* (sheep and goats), *B. abortus* (cattle), *B. suis* (pigs), *B. ovis* (rams), *B. canis* (dogs), *B. microti* (rodents-*Microtus arvalis*), *B. neotomae* (rodents - *Neotoma lepida*), *B. pinnipedialis* (pinnipeds), *B. ceti* (cetacea), and *B. inopinata* (originally isolated from a human patient, but its preferential host is not known) [8,9]. Three of this *Brucella* spp. can be subdivided in biotypes [10,11].

Therefore, three biotypes (1-3) have been identified in *B. melitensis*; eight biotypes (1-7,9) in *B. abortus*; and five biotypes (1-5) in *B. suis* [12]. All *Brucella* spp. are considered potentially pathogenic for

humans, with the exceptions of *B. neotomae*, *B. microti*, and *B. ovis* [6,9].

A precise diagnosis of *Brucella* spp. infection is important for the control of the disease in animals and consequently in man. Clinical diagnosis is based usually on the history of reproductive failures in livestock, but it is a presumptive diagnosis [13] that must be confirmed by laboratory methods [13,14]. The "gold standard" in the diagnosis of brucellosis is bacterial isolation from blood or bone marrow specimens that requires long cultivation periods (4 to 7 days up to 40 days) and often the blood cultures are unsuccessful [15]. Serological tests, such as serum agglutination test (SAT), rose Bengal plate test (RBPT), complement fixation test (CFT), and enzyme-linked immunosorbent assay (ELISA) are still frequently used [5,16]. Since the routine identification and differentiation of brucellosis suspected specimens, based on culture isolation and phenotypic characterization, requires biosafety level-3 (BSL-3) protocols for the high risk of laboratory-acquired infections [17], molecular methods have been explored in order to overcome these difficulties. Furthermore, the polymerase chain reaction (PCR)-based assays have shown a higher sensitivity with respect to the standard microbiological assay for the diagnosis of brucellosis [18].

Therefore, the objectives of this paper are to review a diagnostic methods that are used for isolation, screening, monitoring or epidemiological surveillance and complementary or confirmatory for brucellosis in livestock and humans.

Direct Methods for Diagnosis of Brucellosis

Bacteriological diagnosis

Isolation of the organism is considered the gold standard diagnostic method for brucellosis since it is specific and allows biotyping of the isolate, which is relevant under an epidemiological point of view [10,19]. However, in spite of its high specificity, culture of *Brucella* spp. is challenging. *Brucella* spp. is a fastidious bacterium and requires rich media for primary cultures. Furthermore, its isolation requires a large

number of viable bacteria in clinical samples, proper storage and quick delivery to the diagnostic laboratory [9,20].

Contamination of clinical samples is a complicating factor for *Brucella* spp. isolation. Therefore, the use of nutrient-rich media supplemented with antibiotics (Polymixin B 5,000 UI/L; bacitracin 25,000 UI/L; cyclohexamide 100 mg/L; nalidixic acid 5 mg/L; nystatin 100,000 UI/L and vancomycin 20 mg/L) is used to inhibit growth of contaminants that may prevent isolation of *Brucella* spp. [21].

Another limiting factor for culturing *Brucella* spp. is the requirement for appropriate laboratory conditions and personnel training so the procedure can be performed safely [22]. *Brucella* spp. is classified as a Biosafety level 3 organism, whose manipulation should be performed in biosafety level-3 laboratories [23]. Importantly, brucellosis is one of the most common accidental laboratory infections, particularly in research laboratories [24,25].

Samples for *Brucella* spp. isolation from cattle include fetal membranes, particularly the placental cotyledons where the number of organisms tends to be very high. In addition, fetal organs such as the lungs, bronchial lymph nodes, spleen and liver, as well as fetal gastric contents, milk, vaginal secretions and semen are samples of choice for isolation [23,26]. Vaginal secretions should be sampled after abortion or parturition, preferably using a swab with transporter medium, allowing isolation of the organism up to six weeks post parturition or abortion [13]. Milk samples should be a pool from all four mammary glands. Non- pasteurized dairy products can also be sampled for isolation [13,23].

Samples of choice in slaughterhouses include mammary, iliac, pharyngeal, parotids and cervical lymph nodes, and spleen. Samples must be immediately sent to the laboratory, preferentially frozen at -20°C, and they must be identified as suspect of *Brucella* spp. infection [13]. Vaginal swabs, semen and seminal fluid have low numbers of viable organisms, and therefore isolation is more difficult, often resulting in false negative results. Enrichment media containing selected antibiotics can improve the sensitivity in these cases [21,27].

Brucella spp. colonies are elevated, transparent, convex, with intact borders, smooth, and a brilliant surface. The colonies have a honey color under transmitted light. Optimal temperature for culture is 37°C, but the organism can grow under temperatures ranging from 20°C to 40°C, whereas optimal pH ranges from 6.6 to 7.4. Some *Brucella* spp. requires CO₂ for growth. Typical colonies appears after 2 to 30 days of incubation, but a culture can only be considered negative when there are no colonies after 2 to 3 weeks of incubation [28]. False negative results should be considered in the absence of bacterial growth since the sensitivity of culture is low [13].

Usually, solid media such as dextrose agar, tryptose agar, and trypticase soy agar, are recommended for primary isolation of *Brucella*, but some species, i.e., *B. ovis* and *B. canis* require addition of 5-10% of sterile bovine or equine serum to the culture media. In the case of blood or milk, biphasic media such as Castañeda's medium is recommended for improving sensitivity [13].

Immunohistochemistry

Immunohistochemistry is an alternative technique for direct diagnosis of *Brucella* spp. infection. It has been extensively used in studies of pathogenesis and diagnosis of brucellosis, allowing in situ localization of the organisms within *Brucella* induced lesions [29]. An advantage of this technique is that it does not require viable bacteria

and allow retrospective studies [30]. Although immunohistochemistry is simple, several factors may affect the result, including the fixation protocol and selection of the primary antibody [31].

Molecular methods for *Brucella* species genotyping

Molecular techniques are important tools for diagnosis and epidemiologic studies, providing relevant information for identification of species and biotypes of *Brucella* spp., allowing differentiation between virulent and vaccine strains [32,33]. Molecular detection of *Brucella* spp. can be done directly on clinical samples without previous isolation of the organism. In addition, these techniques can be used to complement results obtained from phenotypic tests [34].

Polymerase chain reaction (PCR) and its variants, based on amplification of specific genomic sequences of the genus, species or even biotypes of *Brucella* spp., are the most broadly used molecular technique for brucellosis diagnosis [10]. The technique is chosen based on the type of biological sample and the goal, i.e., diagnosis or molecular characterization or epidemiological survey. Most of the molecular diagnostic methods for brucellosis have sensitivity ranging from 50% to 100% and specificity between 60% and 98%. The DNA extraction protocol, type of clinical sample, and detection limits of each protocol, are factors that can influence the efficiency of the technique [35].

Since the routine identification and differentiation of brucellosis suspected specimens, based on culture isolation and phenotypic characterization, requires Biosafety level-3 (BSL-3) protocols for the high risk of laboratory-acquired infections [17], molecular methods have been explored in order to overcome these difficulties. Furthermore, the PCR-based assays have shown a higher sensitivity with respect to the standard microbiological assay for the diagnosis of brucellosis [18].

Multiplex polymerase chain reaction typing

Several multiplex PCRs which identify the genus *Brucella* at the species level and partly at the biovar level using different primer combinations have been reported. The first multiplex PCR, called AMOS PCR assay (AMOS is an acronym from "abortus-melitensis-ovis-suis"), comprised five oligonucleotide primers for the identification of selected biovars of four species of *Brucella*. The assay exploited the polymorphism arising from species-specific localization of the genetic element IS711 in the *Brucella* chromosome. Identity was determined by the size of the product amplified from primers hybridizing at various distances from the element.

This method could identify three biovars (1, 2, and 4) of *B. abortus*, all three biovars of *B. melitensis*, all *B. ovis* biovars and biovar 1 of *B. suis*. An abbreviated multiplex AMOS PCR assay based on three additional primers was developed to differentiate *B. abortus* vaccine strains S19 and RB51 from field strains [36]. In 2005 the finding of a deletion next to one of the IS711 copies in *B. abortus* biovars 5, 6, 9 and in some field strains of biovars 3 of *B. abortus* has allowed to design and add a specific primer to the eight primer mixtures of AMOS PCR, allowing enhancing the discrimination power of this assay.

A random amplified polymorphic DNA (RAPD-PCR) was used in order to develop a multiplex PCR that uses the AMOS primers, additional specific loci of the insertion element IS711, and other unique insertions and deletions. This novel PCR assay differentiates

between all presently recognized *Brucella* species, including the recently described species *B. ceti* (formerly named '*B. maris*' or '*B. cetaceae*'), *B. pinnipedialis* (formerly named '*B. maris*' or '*B. pinnipediae*'), and *B. microti*, including some more recently described strains of the latter species [37-39], and also allows accurate differentiation of certain biovars of *B. abortus* and *B. suis* [40].

Real-time PCR

Real-time PCR is more rapid and more sensitive than conventional PCR. It does not require post amplification handling of PCR products, thereby reducing the risk of laboratory contamination and false-positive results. Real-time PCR assays have been recently described in order to test *Brucella* cells [41], urine [42], blood, and paraffin-embedded tissues [43].

Three separate real-time PCRs were developed to specifically identify seven biovars of *B. abortus*, three biovars of *B. melitensis* and biovar one of *B. suis* using fluorescence resonance energy transfer. The upstream primers used in these real-time PCRs derived from the insertion element, IS711 whereas the reverse primer and FRET probes are selected from unique species or biovar-specific chromosomal loci. Sensitivity of *B. abortus*-specific assay was as low as 0.25 pg DNA corresponding to 16-25 genome copies and similar detection levels were also observed for *B. melitensis* and *B. suis*-specific assays [41].

High resolution melt

The development of a molecular technique which utilizes real-time PCR followed by high-resolution melt (HRM) curve analysis to reliably type members of this genus has been described by Winchell et al. [44]. The assay targeted discriminating loci within the genomes of *Brucella* spp. and through the dissociation curve analysis allowing the accurate identification of *Brucella* isolates at the species level and of unusual *Brucella* isolates such as BO1 and BO2. This assay also proved successful for discriminating *B. suis* from *B. canis*, but was unable to accurately differentiate a *B. suis* biovar 4 from *B. canis*. However, this particular *B. suis* biovar has previously been reported to exhibit a genotypic pattern identical to *B. canis*, and it is still debated as to whether this is truly a unique biovar of *B. suis* [45,46].

Restriction fragment length polymorphism based approaches

Polymerase chain reaction (PCR)-restriction fragment length polymorphism (PCR-RFLP) is a common approach for typing of *Brucella* spp., providing a good tool for taxonomic, epidemiological, evolutionary and diagnostic studies. The method has especially been utilized in studies of various outer membrane protein (omp) genes [47].

Single nucleotide polymorphisms typing

Single nucleotide polymorphisms (SNPs) represent powerful markers that allow accurately describing the phylogenetic framework of a species, particularly in a genetically conserved group as *Brucella*. The approach is based on a series of discrimination assays interrogating SNPs that shown to be specific to a particular *Brucella* spp. Scott et al. [48] described the use of SNPs in order to develop a multiplex SNP detection assay, based on primer extension technology that can rapidly and unambiguously identify an isolate as a member of

one of the six classical *Brucella* spp. or as a member of the recently identified marine mammal group.

An alternative approach based on minor groove binding protein (MGB) probes applied on a real-time PCR platform was described [49,50].

The assay distinguishes all members of the classical species, but the differentiation of *B. suis* and *B. canis* was difficult as no *B. suis* specific SNP has been identified. However, as a specific *B. canis* SNP has been identified [45], it is possible a discrimination with *B. suis*/*B. canis* specific SNP and the *B. canis* specific SNP [12].

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Bacterial identification based on peptidic spectra obtained by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was proposed 30 years ago. This method represents a new diagnostic tool in established microbiological laboratories [51]. Databases have been developed that include the main pathogenic microorganisms, thus allowing the use of this method in routine bacterial identification from plate culture. Recently, to identify *Brucella* species a reference library was constructed using 12 *Brucella* strains. With this '*Brucella* library' discrimination was not possible to the species level [52].

Tandem repeat based typing

In the last years the availability of microbial genome sequences has facilitated the development of multilocus sequence-based typing approaches such as multiple locus variable number of tandem repeats (VNTR) analysis (MLVA). The VNTR, allelic hyper variability related to variation in the number of tandemly repeated sequences observed at several genomic loci in the *Brucella* genomes, were used for the discrimination of bacterial species that display very little genomic diversity.

The first application of VNTR based typing to *Brucella* was the HOOOF-Prints scheme (Hyper Variable Octomeric Oligonucleotide Finger-Prints) published by Bricker et al. [53]. The approach was based on a comparison of the newly completed genome sequences of *B. suis* and *B. melitensis* along with a draft *B. abortus* sequence which identified an eight base pair tandem repeat sequence at nine distinct genomic loci [12].

Indirect Methods for Diagnosis of Brucellosis

Serological tests

Serological tests are crucial for laboratory diagnosis of brucellosis since most of control and eradication programs rely on these methods. Inactivated whole bacteria or purified fractions (i.e., lipopolysaccharide or membrane proteins) are used as antigens for detecting antibodies generated by the host during the infection. Antibodies against smooth *Brucella* species (e.g., *B. abortus*, *B. melitensis*, and *B. suis*) cross react with antigen preparations from *B. abortus*, whereas antibodies against rough *Brucella* species (e.g., *B. ovis* and *B. canis*) cross react with antigen preparations from *B. ovis* [14].

Although several serological methods are currently available, these tests can be classified as screening tests (e.g., buffered antigen plate agglutination-BPAT), monitoring or epidemiological surveillance tests

(e.g., milk ring test), and complementary or confirmatory tests (e.g., 2-mercaptoethanol, complement fixation, ELISAs, and fluorescence polarization assay). Selection of a given test should take into account the species affected as well as local regulations [13,14].

Standard slow agglutination tube test

Standard slow agglutination tube test (SAT), which was the first developed serological test for diagnosis of brucellosis, is based on bacterial antigen agglutination, particularly by IgM under neutral pH. This test has low specificity, and therefore it is not recommended [13,14,53].

Milk ring test

The milk ring test is based on agglutination of antibodies secreted into the milk. This test allows screening of large number of cattle by using milk samples from tanks or pools from several cows. This test is useful for monitoring cattle herds or areas free of brucellosis so it is classified as surveillance or monitoring test [53]. Importantly, the number of false positive results is proportional to the number of cows secreting acidic milk due to colostrums or mastitis [53]. A positive result indicates the presence of infected cattle in the herd so the test should be followed by individual serological test in the entire herd.

2-Mercaptoethanol

The 2-mercaptoethanol is a confirmatory test that allows selective quantification of IgG anti-*Brucella* due to inactivation of IgM in the test sample. Production of IgG is usually associated with chronic infection, and therefore, a positive result with this test is a strong indicator of brucellosis. However, this test has some drawbacks including the toxicity of mercaptoethanol, which requires a fume hood for its manipulation, and the possibility of IgG degradation caused by the 2-mercaptoethanol, which may result in false negative results [13]. Sensitivity of the 2-mercaptoethanol test varies from 88.4 and 99.6%, and its specificity from 91.5 and 99.8% [54].

Complement fixation test

Due to its high accuracy, complement fixation is used as confirmatory test for *B. abortus*, *B. melitensis*, and *B. ovis* infections and it is the reference test recommended by the OIE for international transit of animals [55,56]. However, this method has some disadvantages such as high cost, complexity for execution, and requirement for special equipment and trained laboratory personnel. In addition, the test presents limitations with hemolysed serum samples or serum with anti-complement activity of some sera, and the occurrence of prozone phenomena [54]. Sensitivity of complement fixation ranges from 77.1 to 100% and its specificity from 65 to 100% [56,57].

Rose bengal plate test

The rose bengal test (RBT) is a rapid, slide-type agglutination assay performed with a stained *B. abortus* suspension at pH of 3.6-3.7 and plain serum. Its simplicity made it an ideal screening test for small laboratories with limited resources. The drawbacks of RBT include: low sensitivity particularly in chronic cases, relatively low specificity in endemic areas and prozones make strongly positive sera appear negative in RBT [58]. The overall sensitivity is 92.9%, so the use of RBT should be considered carefully in endemic areas, particularly in

individuals exposed to brucellosis and those having history of *Brucella* infection [59]. Rose Bengal plate test [RBT] is an agglutination test that is based on reactivity of antibodies against smooth lipopolysaccharide (LPS). As sensitivity is high, false negative results are rarely encountered. To increase specificity, the test may be applied to a serial dilution (1:2 through 1:64) of the serum samples [60]. The present World Health Organization (WHO) guidelines recommend the confirmation of the RBT by other assays such as serum agglutination tests [60,61].

Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assay (ELISA) has become popular as a standard assay for the diagnosis of brucellosis, serologically. It measures IgG, IgA and IgM antibodies and this allows a better interpretation of the clinical situation. The diagnosis of brucellosis is based on the detection of antibodies against the smooth LPS. Detection of IgG antibodies is more sensitive than detection of IgM antibodies for diagnosing cases of brucellosis but specificity is comparable [61-63]. Compared to the conventional agglutination methods, ELISA is more sensitive in acute and chronic cases of brucellosis and it offers a significant diagnostic advantage in the diagnosis of brucellosis in endemic areas. For case detection and an accurate diagnosis of suspected cases, the combination of ELISA IgM and IgG tests should be used as this combination of laboratory tests has been shown to be the most efficient technique in the detection and diagnosis of brucellosis.

For follow-up and monitoring of prognosis, ELISA Ig M and 2-mercapto ethanol (2-MET) are more promising [64,65].

Enzyme linked immunosorbent assay (ELISA) is an excellent method for screening large populations for *Brucella* antibodies and for differentiation between acute and chronic phases of the disease [66]. It is the test of choice for complicated, local or chronic cases particularly when other tests are negative while the case is under high clinical suspicion. It can reveal total and individual specific immunoglobulins (IgG, IgA and IgM) within 4-6 hours with high sensitivity and specificity. In addition to the detection of immunoglobulin classes, ELISA can also detect *Brucella*-specific IgG subclasses and other *Brucella* immunoglobulins such as IgE [61].

The indirect ELISA (i-ELISA) has been used for serologic diagnosis of brucellosis in sheep, goats and pigs. It has also been used for diagnosis using serum or milk from cattle [66,67]. O ELISA-i has been usually used for smooth LPS *Brucella* spp., and it is sensitive and specific for *B. abortus* or *B. melitensis*, but it is not capable of differentiating antibodies induced by the vaccine strains S19 or Rev1 [68,69]. Sensitivity of i-ELISA varies from 96 to 100% and its specificity from 93.8% and 100% [56,70].

The competitive ELISA (c-ELISA) with smooth *Brucella* LPS as antigen is used for detection of anti-*Brucella* in serum samples from cattle, sheep, goats, and pigs. This test is capable of differentiating vaccine antibody response from actual infections, and its sensitivity varies from 92 to 100%, whereas the specificity ranges from 90 and 99% [57,71].

Fluorescence polarization assay

The fluorescence polarization assay (FPA) was initially developed for testing serum. However, the technology has been extended to testing whole blood and milk samples from individual animals.

Fluorescence polarization assay (FPA) is based on the rotational differences between a small soluble antigen molecule in solution and the antigen molecule complex with its antibody. It measures the size of a fluorescent tagged molecule such as an antigen. The utilization of the O-side chain of LPS from *Brucella* species has shown encouraging results. The test is a valuable alternative to conventional serological tests.

Sensitivity of FPA is 96% for culture-confirmed human brucellosis and specificity is about 98% [60,72]. The fluorescence polarization assay has been used for the diagnosis of *Brucella* spp. infection in man [73] and several animal species, using serum, milk or whole blood in EDTA. This test can be performed under field conditions [14]. Sensitivity of the fluorescence polarization assay varies from 87.5 and 100%, and specificity from 84 to 100% [71], which is similar to the levels obtained with c-ELISA [56].

Species	Screening tests	Confirmatory test	Reference
<i>B. abortus</i>	BPAT,MRT	2ME,CF,ELISAc	OIE [53]
<i>B. melitensis</i>	BPAT	BPAT,CF	OIE [55]
<i>B. suis</i>	BPAT	2ME,CF,AGIT,ELISAc	Di Febo et al. [67]
<i>B. canis</i>	–	2ME,AGIT,ELISAi	Ebani et al. [76]
<i>B. ovis</i>	–	CF, AGIT, i-ELISA	Gall et al. [66]; OIE [55]

Table 1: Screening and confirmatory tests commonly used in the serological diagnosis of *Brucella* spp. infection. 2ME-2-mercaptoethanol, AGIT-Agar gel immunodiffusion test, BPAT-Buffered antigen plate agglutination, CF-Complement fixation, ELISAi-Indirect ELISA, ELISAc-Competitive ELISA, FPA-Fluorescence polarization assay, MRT-Milk ring test.

Coombs test

This is the most suitable and sensitive test for confirmation of relapsing patients with persistent disease [60]. It is an extension of the SAT test i.e., if the SAT test yields negative results due to the presence of blocking antibodies, Coombs test may be used instead. Agglutination can be determined visually, as for SAT, by using an agglutinoscope or a drop on a slide examined under the microscope [61]. Coombs test is used for detection of incomplete, blocking or non-agglutinating IgG. It is time consuming, technically difficult, requires skilled personnel and not routinely performed in clinical laboratories. It is good for complicated and chronic cases but misses about 7% of cases compared with ELISA [76,77].

Dipstick assay

The IgM dipstick assay is one of the tests that have been adapted to detect IgM antibodies to the smooth LPS. The assay has shown high sensitivity for patients with disease lasting less than 3 months [78,79]. IgM dipstick assay offers higher sensitivity and easier manipulation than IgM ELISA to detect IgM antibodies to *Brucella* species and improves the interpretation of results thus establishing cut-off points. IgM dipstick assay could be used as a rapid and simple alternative to the ELISA IgM for the serodiagnosis of patients with acute brucellosis.

The combined results of SAT and IgM dipstick assays can provide an indication of the stage of disease for those patients in whom the onset of clinical manifestations is not known [80].

Agar gel immunodiffusion test

The agar gel immunodiffusion test is based on precipitation of the antigen-antibody complex. This method is often used for the diagnosis of *B. ovis* infection. This test has a low cost, it is easily performed and it has sensitivity levels that are comparable to complement fixation. However, it has some disadvantages such as a marked decrease in sensitivity in chronic infections and high variability of the quality of commercially available antigens. Therefore, it is highly advisable to perform complementary diagnostic techniques such as PCR [74]. Sensitivity of the agar gel immunodiffusion test varies from 50 to 92.7% and the specificity from 94.3 and 100% [70,75] (Table 1).

Immunocapture agglutination test; *Brucella Capt*

Recently, new immunocapture agglutination for anti-*Brucella* (BCAP) assay has been developed to detect agglutinating and non-agglutinating antibodies with high sensitivity [81].

It is based on the sandwich ELISA system, where a microwell is covered with Coombs antibodies against human origin IgG, IgA and IgM antibodies. This *Brucella* agglutination test occurs in a microwell and is performed with Coombs antibodies and determines the 3 antibodies that form against brucellosis. It has been suggested as a possible substitute for Coombs test and a better marker for disease activity [81].

Compared to Coombs test, it has similar sensitivity and specificity but both can remain positive for long time after treatment in cured patients. BCAP determines blocking antibodies at diagnosis and during follow up for patients having brucellosis. It is easier to carry out in 24 hours without a second step necessary as in Coombs test [82]. In comparison with other tests: it is more complex, expensive and slow. It can hardly replace rapid screening tests such as RBT and dipstick as a screening or first diagnostic test. However, it could help to diagnose disease in patients with longstanding evolution of brucellosis that is not detected by SAT. So, like Coombs test, *Brucella Capt* which is based on the immunocapture-agglutination of the total anti-*Brucella* antibodies, could be a second level serological test [60,79].

Lateral flow assay

An immunochromatographic *Brucella* IgM / IgG lateral flow assay is a simplified version of the ELISA test and has a great potential as a

rapid point-of-care assay. The test has high sensitivity and specificity for *Brucella* IgM and IgG. It uses a drop of blood obtained by finger prick. It can be done as a bedside procedure. So it is a rapid and a simple diagnostic test that is also easy to interpret [60].

Rapid slide agglutination test

Since routine brucellosis diagnosis does not include *B. canis* investigation, infection with this species may be more widespread than is currently suspected. The rapid slide agglutination assay test (RSAT) could be a suitable screening test for the diagnosis of human brucellosis and a supplementary technique, such as ELISA, performed on all positive RSAT samples that were negative by *B. abortus* antigen could ensure diagnostic specificity and confirm the diagnosis. It is recommended to use MAT and 2-ME/RSAT to check sera of all patients, who have symptoms of brucellosis but are negative for brucellosis using a smooth *Brucella* antigen [83,84].

Brucellin allergic skin test

The skin test is an allergic test that detects the specific cellular immune response induced by *Brucella* spp. infection. The injection of brucellergene, a protein extract of a rough strain of *Brucella* spp., is followed by a local inflammatory response in a sensitized animal. This delayed type hypersensitivity reaction is measured by the increase in skin thickness at the site of inoculation. This test is highly efficient in discriminating between true brucellosis cases and false positive serological reactions. The skin test is highly specific but its weak sensitivity makes it a good test for herds but not for individual certification. It cannot discriminate between infection and vaccination [85]. Pouillot et al. [86] made an assessment of the diagnostic value of the Brucellin allergic skin test (AST) in a brucellosis false positive serological reaction and reported that allergenic skin test is to be more specific than RBT and CFT. Therefore, this test could be used as a confirmatory test on cattle non-vaccinated against brucellosis. This test is prescribed as an alternative test by the OIE [53].

Conclusion

A precise diagnosis of *Brucella* spp. infection is, important for the control of the disease in animals and consequently in man. Clinical diagnosis is based usually on the history of reproductive failures in livestock, but it is a presumptive diagnosis that must be confirmed by laboratory methods. Direct diagnosis of brucellosis involves bacteriological, immunohistochemistry and molecular methods. Under an epidemiological point of view, bacterial isolation from different specimen is more relevant, since it is more specific and allows biotyping of the isolate but it requires BSL-3 protocols for the high risk of laboratory-acquired infections. For retrospective studies, immunohistochemistry allows in situ localization of the organisms within *Brucella* induced lesion since it does not require viable bacteria. Molecular techniques are important tools for diagnosis and epidemiologic studies, providing relevant information for identification of species and biotypes of *Brucella* spp., allowing differentiation between virulent and vaccine strains. Serological methods are among the well-established indirect laboratory diagnosis of brucellosis since most of control and eradication programs rely on these methods while brucellin allergic skin test could be used as a confirmatory test on animal non-vaccinated against brucellosis and more specific than RBT and CFT.

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