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Chapter 8

LABORATORY DIAGNOSIS OF BRUCELLOSIS

Maria José Saavedra[1,*], PhD,
Conceição Fernandes[2], PhD
and Cristina Queiroga[3], DVM, PhD

[1]Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB) and Animal and Veterinary Research Centre (CECAV), Department of Veterinary Science, Medical Microbiology Laboratory, University of Trás-os-Montes e Alto Douro, Vila Real, Portugal
[2]Mountain Research Centre (CIMO), ESA-Polytechnic Institute of Bragança, Bragança, Portugal
[3]Department of Veterinary Medicine, School of Sciences and Technology and Institute of Mediterranean Agricultural and Environmental Sciences (ICAAM), University of Évora, Portugal

[*] Corresponding Author's Email: saavedra@utad.pt.

ABSTRACT

This chapter addresses the different methods used to diagnose brucellosis. Rapid diagnosis of the disease is essential for its control and to protect public health. Basically, there are two types of tests, the direct tests, which detect the presence of *Brucella* and are used in clinical situations where the animals are affected and show clinical signs, and indirect tests that are mainly used for screening to detect subclinical conditions. These are widely used as part of control and eradication programs. Main aspects of direct tests are described, namely bacterial isolation and identification and molecular methods: conventional and real-time PCR, multiple locus variable-number tandem repeat analysis, multi-locus sequence typing and luminex xMAP technology. Regarding indirect tests, serological tests are prefered: milk ring test, buffered *Brucella* antigen tests, namely rose Bengal test and buffered plate agglutination test, serum agglutination test, complement fixation test, indirect and competitive enzyme-linked immunosorbent assays, fluorescence polarization assay, immunoprecipitation tests and lateral flow immunochromatography. Another indirect test, interferon-gamma release assay, performed on whole blood and brucellin skin tests are discussed. Concerning sensitivity (DSe) and specificity (DSp) of the methods, none of the tests are *Brucella* species-specific, but some of those, specifically the buffered agglutination tests, set a high standard with regards to the DSe/DSp. Although no current serological test provides enough DSe for the 100% DSp required, some test combinations can be of great help.

Keywords: bacterial methods, direct tests, indirect tests, molecular methods, serological methods

INTRODUCTION

To control and eradicate brucellosis is extremely important to diagnose it promptly and accurately. Brucellosis diagnostic tests fall into two categories: those that demonstrate the presence of organisms and those that detect an immune response to their antigens.

In ruminants, flock identification of the disease depends on the presence of clinical manifestations such as reproductive failure, i.e.,

abortion and birth of weak offspring in females, and orchitis and epididymitis in males. Brucellosis identification, in one or more infected animals, is sufficient evidence that the infection is present and, therefore, other animals may be incubating the disease and present a risk (Garin-Bastuji et al., 1998; Corbel, 2006).

The laboratory diagnosis of brucellosis can be based on direct and indirect methods and should be performed whenever clinical signs or epidemiological evidences suggestive of the disease are observed. Accurate diagnostic and standard procedures are critical for the success of the brucellosis control and eradication. Furthermore, the identification of the different species is of great epidemiological importance. In the European Union, according to Directive 2003/99/EC, brucellosis and its agents are included within the list of zoonosis that requires surveillance.

Several biological samples can be used for monitoring and laboratory confirmation of the *Brucella* spp. infection. The collected samples can be examined fresh or frozen and transported to the laboratory. Samples of milk, vaginal swabs, blood, aborted materials (aborted fetus, fetus membranes) and carcasses may be used (Alton et al., 1975).

The differentiation between *Brucella* species and their different biovars has been based on serotyping, phage typing, sensitivity to dyes, CO_2 requirement, H_2S production, and metabolic properties. However, the variability of some phenotypic characteristics in different *Brucella* strains impairs the identification of species and biovars. Therefore, the design of stable markers based on DNA is presently considered essential for the detection and identification of *Brucella*.

Indirect diagnosis methods are widely routinely used in control and eradication and surveillance programs for ruminants. Immunological tests are required at each step of intervention against brucellosis: the evaluation of the prevalence, the assessment of the efficacy of control and elimination measures, and the confirmation of erradication of disease through surveillance (Ducrotoy et al., 2018). For human brucellosis diagnosis, as the *Brucella* organism grows very slowly *in vitro*, serological tests are used as screening tests for preliminary diagnosis of brucellosis (Khan et al., 2017).

DIRECT DIAGNOSIS

As mentioned before, brucellosis direct diagnostic tests are based on bacterial isolation and identification, and molecular methods. Cultural methods are time-consuming and costly. Molecular methods, on the other hand, have been increasingly applied for the diagnosis of infection in human and in veterinary medicine. In particular, Polymerase Chain Reaction (PCR)-based methods, have been used successfully for this purpose. When compared to bacteriological isolation those methods are advantageous for its speed, sensitivity and safety. In fact, molecular methods allow rapid diagnosis and differentiation of various bacterial species, especially slow-growing ones.

According to OIE Terrestrial Manual (OIE, 2016), there is no single test by which a bacterium can be identified unequivocally as *Brucella*. A combination of growth characteristics, serological, bacteriological or molecular methods is required for a definitive identification.

Bacteriological Methods

Isolation and identification of the etiologic agent is an unequivocal method of diagnosis of brucellosis. It is relatively sensitive when performed in skilled and experienced laboratories.

The classical microbiological identification of brucellae strains is based on colonial morphology, microscopic appearance and biochemical properties, such as CO_2 requirement, H_2S production, urea hydrolysis, sensitivity to basic fuchsin and thionin, and also agglutination with monospecific sera, and phagetyping (Alton et al., 1988). For bacterial culture, samples from uterine discharges, aborted fetuses, udder secretions or selected tissues, such as lymph nodes and male and female reproductive organs, should be aseptically taken and immediatly cooled or frozen if they are to spend more than 12 hours before being cultured.

Before culturing, smears of organs or biological fluids may be performed and stained after fixed with heat or ethanol. Brucellae are Gram

negative coccobacilli that usually do not show bipolar staining and stain red by the Stamp's modification of the Ziehl–Neelsen's method (OIE, 2016).

Direct isolation and culture of *Brucella* are usually performed on solid media. Different basal media supplemented with 2–5% bovine or equine serum, with or without appropriate antibiotics to suppress the growth of contaminant organisms, may be used. After a 4-day incubation at 37°C ± 2°C in air supplemented with 5–10% (v/v) CO_2, brucellae colonies appear as round, 1–2 mm in diameter, with smooth margins, translucent and pale honey coloured when observed through a transparent medium and convex and pearly white when viewed from above. These colonies may undergo variation during growth and develop rough forms.

For identification, characteristic colonies (both smooth and rough colonies) should be examined using a Gram or Stamp stained-smear, and urease and oxidase tests, the slide agglutination test with a polyclonal anti-*Brucella* serum. Species and biovar identification relies on the CO_2 requirement for growth, production of H_2S, growth in the presence of basic fuchsin and thionin, phage lysis and agglutination with monospecific sera. As dyes and phage sensitivity are usually altered in the non-smooth phases, attention to the colonial morphology is essential for the correct interpretation of typing tests (OIE, 2016).

PCR Assay

PCR amplification of specific DNA regions have been successfully used for *Brucella* identification and typing.

The first PCR-based method has been directed toward detection of a single unique genetic *locus* that is common among all species of *Brucella*, such as 16S and 23S rRNA operon, IS*711* insertion sequence or *bcsp31* gene. PCR techniques have been developed directed toward detection of gene loci, that are variable among the species/biovars (Çiftci et al., 2017). PCR primers could be used to screen the *Brucella* spp., but the sensitivity of the test for bacterial detection in blood and milk is low, mainly due to

the difficulty of lysing the microorganisms. In *B. abortus* two copies of DNA sequence of the gene coding for omp2 were described. Furthermore, studies have shown that for the detection *B. abortus*, in blood and milk samples, primers for both copies are required (Ficht et al., 1989; Leal-Klevezas et al., 1995).

The IS*711* insertion sequence is unique to *Brucella* species, and its copy number in the genome varies between species and biovars. Thus, the amplification of this mobile element is used to differentiate between species, producing band sizes with 731 bp (*B. melitensis*), 498 bp (*B. abortus)*, 285bp (*B. suis)*, 976 (*B. ovis*) (Çiftci et al., 2017). Based on the observation that this genetic element occurs at several species-specific or biovar-specific chromosomal loci, Brickera and Halling (1994) developed a PCR protocol, AMOS (Abortus-Melitensis-Ovis-Suis)-PCR, designed to amplify species-specific-sized products by using five primers, one of which hybridizes to the IS*711* element and the others hybridize to one of four species-specific regions adjacent to the element. Identification was based on the products' size of resulting amplicons. The performance of the assay, for field isolates, was highly effective, allowing the differentiation of *B. abortus* (1, 2, and 4), *B. melitensis* (biovars 1, 2 and 3), *B. suis* biovar 1, and *B. ovis*. However, this AMOS -PCR was not able to differentiate all subspecies. Later, new oligonucleotide primers have been added to the AMOS-PCR multiplex allowing the discrimination between *B. abortus* vaccine strains (S19 and RB51) and wild-type isolates (Bricker and Halling, 1995). An improvement of this AMOS-PCR format was later performed by Ocampo-Sosa and colleagues (2005) in order to discriminate *B. abortus* biovars 3b, 5, 6 and 9 (Bricker and Halling, 1995; Ocampo-Sosa et al., 2005).

Also using as target the multiple insertion element IS*711*, which is stable in both number and position in the *Brucella* chromosomes, Hinić et al. (2008) designed seven primer pairs for individual reactions for the rapid detection of the *Brucella* genus, and the differentiation between *B. melitensis, B. abortus, B. suis, B. ovis, B. canis*, and *B. neotomae*. Lysates from 18 reference and 47 *Brucella* field strains were analyzed and each of the PCR reactions generated a specific PCR product, which correlated in

all cases to the corresponding *Brucella* species, while non-*Brucella* species showed no amplification with any of the primers.

To increase the sensitivity of the conventional PCR methods for tissues, it is necessary to optimize PCR and DNA extraction protocols. In a recent study on aborted sheep and bovine fetuses, Çiftci et al. (2017), using different target genes for *B. melitensis* (Ba148/928, 31ter/sd, IS*711*, JPF/JPR, and F4-R2) and for *B. abortus* (Eri1-Eri2 genes), showed higher sensitivity in tissues, blood, milk and semen, when compared with conventional bacteriological isolation, using different conditions of PCR protocols optimization.

Currently, the Bruce-ladder multiplex PCR is the most commonly used method for the differentiation of the majority of *Brucella* species and S19, RB51, and Rev.1 vaccine strains and the identification is based on the numbers and sizes of seven products amplified by the PCR. In a study including 625 *Brucella* isolates from different geographic origins and different animal species, including humans, Bruce-ladder showed to be species-specific. Nevertheless, this PCR assay cannot differentiate among biovars from the same species (García-Yoldi et al., 2006; López-Goñi et al., 2008). Lopez-Goñi et al. (2011) developed a multiplex PCR (Suis-ladder) that, besides the differentiation of *B. suis*, *B. canis* and *B. microti*, is able to differentiate the five biovars of *B. suis*.

Real-Time PCR Assay

The Real-Time PCRs for species differentiation are based on unique genetic *loci* and provides a means of detecting and quantifying DNA targets by monitoring PCR product accumulation during cycling by increased fluorescence. Different PCR protocols were optimized for *Brucella* spp. detection. Newby et al. (2003) designed a pair of primers and respective hybridization probes for *B. abortus* to produce a 156-bp amplicon spanning a region of the genome that includes portions of the *alkB* gene and the IS*711* insertion element, which is highly specific to

detect this species, using a particular protocol, but not aplicable for other *Brucella* spp.

A protocol, named TaqMan® real-time PCR assay, was designed, optimized and evaluated for the detection of *Brucella* at genus level by targeting a conserved region of three specific genes: (i) the insertion sequence IS*711*, (ii) *bcsp31* and (iii) *per* genes. It presented several advantages over conventional PCR when used for *B. ovis*, *B. melitensis* bv. 1, *B. abortus* bv. 1 and *B. canis* reference strains. This protocol showed to be less labourious, faster and uses a closed system with no need of post-PCR handling, preventing DNA contamination (Bounaadja et al., 2009).

Doosti and Moshkelani (2011) developed a real time PCR assay for identification and species differentiation of *B. melitensis* and *B. abortus* from mice tissue, targeting *B. melitensis* BMEII0466 gene and B. abortus BruAb2_0168 gene. Real time PCR showed higher specificity over gel electrophoresis. Similar results were obtained, with the IS*711* gene, using different primers, for simultaneous detection and differentiation of the species of *B. abortus* and *B. melitensis* (Mirnejad et al., 2012). Primers targeting the multiple insertion element IS*711*, may be used with corresponding TaqMan® probes, for real-time PCR assay for the identification of the *Brucella* genus, as well as the differentiation between *B. melitensis, B. abortus, B. suis, B. ovis, B. canis*, and *B. neotomae* (Hinić et al., 2008).

To improve the specificity of the analysis methodologies, real time PCR followed by High-Resolution Melt (HRM) was developed. This molecular technique utilizes curve analysis to reliably type members of the *Brucella* genus, using a panel with seven primer sets to identify species and respective biovars. Gene target and corresponding *Brucella* species were *vdcc* (*Brucella* spp.), *int-hyp* (*B. canis*, *B. suis* bv3 and bv4), *BP26/IS711* (marine species), *int-hyp* (*B. melitensis*), *glk* (*B. neotomae*), Transposase gene (*B. suis*) and *glk* (*B. ovis* and *B. abortus*). Results showed >99% accuracy compared to traditional techniques, based on 153 *Brucella* spp. isolates (Winchell et al., 2010).

The advantages of real-time PCR are speed (since there is no need to analyze the PCR products by agarose gel electrophoresis), higher

sensitivity, and specificity for the detection of the *Brucella* species in clinical samples when compared with conventional PCR. However, protocols should be carefully validated on a representative numbers of *Brucella* infected samples and *Brucella* free controls before being implemented in routine diagnosis for animal and human brucellosis (Bounaadja et al., 2009).

Multiple Locus Variable-Number Tandem Repeat Analysis (MLVA) and Multi Locus Sequence Typing (MLST)

MLVA is based on the analysis of variable number-tandem repeats (VNTR), which exists in bacterial genomes and seems to be highly discriminatory markers, even when the pathogens investigated belong to monomorphic species with high similarity, such as *Brucella* spp. (DNA–DNA homology >90%) (AI Dahouk et al., 2007; Kiliç et al., 2011). Tandem repeats are copies of an elementary unit into the genome and can be observed in different bacterial strains. Tandem repeats are classified in satellites (megabases of DNA) present in many eukaryotic genomes, minisatellites (spanning hundreds of base pairs with a repeat unit size of at least 9 bp), and microsatellites (spanning a few tens of nucleotides with a repeat unit size 2-6 bp) (Denœud and Vergnaud, 2004; Le Flèche et al., 2006; Sweet et al., 2012).

The most used MLVA genotyping system for brucellosis is MLVA-16, originally developed by Le Flèche et al. (2006) and modified by Al Dahouk et al. (2007), which consists of 16 genetic markers (MLVA-16) comprising eight minisatellite markers most appropriate for species-level identification (panel 1-Bruce06, Bruce08, Bruce11, Bruce12, Bruce42, Bruce43, Bruce45, and Bruce55), and eight microsatellite markers with higher discriminatory power (panel 2A-Bruce18, Bruce19, and Bruce21; panel 2B-Bruce04, Bruce07, Bruce09, Bruce16, and Bruce30). Based on simple PCR reactions, MLVA-16 is accessible and the number of alleles (PCR amplicons) can be analyzed by simple agarose gel electrophoresis or automatic high-throughput procedures. Even tandem repeats differing by a

single repeat unit can be distinguished by these methods. MLVA genotypes can correlate with the geographic origin of the strains, comprising a tool for molecular epidemiological studies of brucellosis.

MLVA is a reliable method of monitoring phylogeny of the lineage and the regional and temporal distribution of the disease. Results are strengthened when a larger number of strains are included in the analysis (AI Dahouk et al., 2007; Ficht, 2010; Ferreira et al., 2017). In this way, a collaborative public online database, based on a MLVA-16 scheme, has been built up with the aim of promoting the creation of a global epidemiological map of *Brucella* spp. (*Brucella* MLVA database at http://mlva.u-psud.fr/), where genotypes can be submitted and compared to other published results.

According to required test specificity, MLVA can be performed using the 16 *loci* (MLVA-16; panels 1 and 2 markers), using 11 *loci* (MLVA-11; panels 1 and 2A markers) or using eight *loci* (MLVA-8; panel 1 markers) (Le Flèche et al., 2006; AI Dahouk et al., 2007; Kiliç et al., 2011; Ferreira et al., 2017).

The MLVA-16 and MLVA-11 were used for investigating the epidemiological relationship and genetic diversity of human *B. melitensis* isolates, collected in Turkey regions. Results showed that the most prevalent MLVA genotype is typically *B. melitensis* biovar 3, frequently isolated in humans and common in the East Mediterranean region (Kiliç et al., 2011). The same biovar 3 *B. melitensis* was also found in a human epidemiological study in China (Xiao et al., 2015), with genotypic profiles from different countries, such as Israel, Irac, Lebanon and Syria. These results highlight the importance of quarantine rules, suggesting that poor importation quarantine policies may account for a set of *B. melitensis* infections.

This method has been widely used to study genotype distribution of *Brucella* isolates, such as *B. canis* (Di et al., 2014), marine mammal isolates, *B. ceti* and *B. pinnipedialis* (Maquart et al., 2009), *B. suis* (Ferreira et al., 2017), *B. melitensis* in Italy (De Massis et al., 2015) and in Mongolia (Kang et al., 2017). Recently a MLVA-13Bc assay was developed and validated using a combination of 13 VNTRs specifically

designed for genotyping *B. canis* strains, with high discriminatory power (Yang et al., 2017).

The MLVA assay is rapid, highly discriminatory, and reproducible within human *Brucella* isolates (Al Dahouk et al., 2007). This technique is useful for analysis of *Brucella* spp., in especially *B. melitensis,* the most pathogenic for humans. Molecular typing methods improve epidemiological surveillance efficiency, determine pathogenic relationships and trace-back brucellosis for potential risk factors (season, rearing system, product, and environment condition) of outbreak regions (Kang et al., 2017). Studies based on MLVA are helpful to understand the dynamic distribution of brucellosis in the world and can improve the prevention, surveillance, and management of brucellosis in neighbouring countries, and countries involved in trade and distribution of animal species at risk of brucellosis.

MLST has been used to identify the species and genotypes of these *Brucella* isolates (Ma et al., 2016). MLST is a DNA sequence-based typing method for many different bacterial species to differentiate strains and identify clonal lineages. The procedure characterizes isolates using the DNA sequences of multiple genetic loci, usually but not exclusively housekeeping genes, which are then accurately sequenced on both strands using an automated DNA sequencer. For each gene, the different sequences are assigned as alleles and the alleles at the loci provide an allelic profile or sequence type (ST). A series of profiles can then be the identification marker for strain typing and characterizes strains by their unique allelic profiles. Whatmore et al. (2007) amplified by PCR nine distinct genome fragments. Products were separated by agarose gel electrophoresis; PCR products were then purified and sequenced. Each unique allelic pattern over all nine loci was identified as a ST. Sequences of the nine loci were concatenated to produce a 4,396 bp sequence for each genotype. Phylogenetic analysis was performed with software, and neighbour joining trees were constructed. These authors stated that the sequencing of these nine fragments is a potentially valuable tool for the identification of *Brucella*.

To improve the resolution of MLST for *Brucella*, an extended MLST (EMLST) by increasing the sequencing length was described by Chen et al. (2011). Through analysis of a large number of sequence data was possible to improve resolution of MLST. These authors found that the EMLST method could increase the length by about 50%. With the increased sequences, more alleles and STs were identified, and the genotyping resolution of the MLST was greatly improved.

Luminex xMAP Technology

Luminex xMAP system is a multiplexed microsphere-based suspension array platform capable of analyzing and reporting up to 100 different reactions in a single reaction vessel by performing discrete assays on the surface of colour-coded beads known as microspheres, which are then read in a compact analyzer (Dunbar, 2006). This technology may be used for high-throughput nucleic acid detection methods. For that purpose, DNA probes to detect PCR amplicons are covalently coupled to the microspheres (Dunbar et al., 2003). The Luminex® xMAP™ System has the advantage that they allow for simultaneous detection of multiple nucleic acid sequences in a single reaction vessel which reduces time, labour and cost as compared to single-reaction-based detection methods.

Pfefer et al. (2018) developed a multiplexed assay called Luminex bead-based suspension array for detection and identification of the most common *Brucella* species (*B. abortus, B. melitensis, B. suis, B. suis bv5, B. canis, B. ovis, B. pinnipedialis,* and *B. neotomae*) as well as the *Brucella* genus level. The work demonstrated overall excellent accuracy for all strains tested and the platform also allows for flexibility in assay design to easily add more *Brucella* species and to attain excellent target accuracy due to the customization of both primers and probe.

INDIRECT DIAGNOSIS

Indirect methods, or immunological methods, detect an immune response to *Brucella* antigens. They are mostly used for simplicity of execution and interpretation and are based on antibody detection. The detection of these *Brucella*-specific antibodies in milk or serum samples may be performed through numerous immunological diagnostic tests, including milk ring test (MRT), buffered *Brucella* agglutination tests (i.e., Rose Bengal test (RBT); Card Test (CT), and buffered plate aglutination test (BPAT), complement fixation test (CFT), enzyme-linked immune-sorbent assay (ELISA) and fluorescence polarisation assay (FPA). Most of them do not have high sensitivity and specificity and it is usually necessary to associate several techniques to increase the level of detection (Garin-Bastuji et al., 2006).

Indirect tests are used worldwide for screening of herds/flocks and individual small ruminants, camelids and bovines, to contribute to eradication policies and to study herd/flock prevalence of infection and surveillance (OIE, 2016). The World Organisation for Animal Health (OIE) emphasizes that no single serological test is appropriate in all epidemiological situations, since all have limitations, especially when it comes to screening individual animals or humans.

B. abortus strain 99 (Weybridge) (S99), *B. abortus* strain 1119-3 (USDA) (S1119-3) or *B. melitensis* strain 16M are used for the production of antigens for different serological tests. These bacterial cells may be used either as all cell antigen or as a source of soluble antigen extracts as smooth lipopolysaccharide (S-LPS) or O-polysaccharide (OPS). The *Brucella* OPS represents the most immunogenic bacterial portion (Olsen and Palmer, 2014; OIE, 2016).

The list of available tests for the diagnosis of infection with smooth *Brucella* species, in particular *B. abortus, B. melitensis* and *B. suis,* are available on the OIE Web site (http://www.oie.int), in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Table 8.1). The most used tests are the buffered *Brucella* agglutination tests (BBAT; i.e., RBT, BPAT) CFT, FPA and indirect or competitive enzyme-linked

immunosorbent assays (I-ELISA and C-ELISA, respectively) (CFSPH, 2009; OIE, 2016).

Table 8.1. List of brucellosis diagnostic tests: the "prescribed tests" are required by the OIE Terrestrial Animal Health Code for the international movement of animals and animal products; and "alternative tests" are suitable for the diagnosis of disease within a local setting and can also be used in the import/export of animals after bilateral agreement

Disease name	Prescribed tests	Alternative tests
Bovine brucellosis (*B. abortus and B. melitensis*)	BBAT, CFT, ELISA, FPA, SAT	BBAT+NH, MRT, IFN-γ
Caprine and ovine brucellosis (excluding *B. ovis*)	BBAT, CFT	Brucellin test, FPA, NH
Ovine brucellosis (*B. ovis*)	CFT	ELISA
Swine brucellosis (*B. suis*)	ELISA	BBAT, FPA

Abbreviations: buffered *Brucella* antigen test (BBAT); complement fixation test (CFT); enzyme-linked immunosorbent assay (ELISA); fluorescence polarisation assay (FPA); serum agglutination test (SAT); native hapten test (NH); milk ring test (MRT), interferon gama (IFN-γ).

Adapted from OIE (2008, 2018)

Milk Ring Test (MRT)

In lactating cattle, the Milk ring test (MRT) can be used for screening herds for brucellosis. However, the MRT is not suitable in milk from small ruminants (OIE, 2016).

The test consists of mixing coloured *Brucella* haematoxylin-staining whole-cell antigen with fresh bulk/tank milk. In the presence of anti-*Brucella* antibodies, antigen-antibody complexes form and migrate to the cream layer, forming a dark blue ring on the surface, as fat globules adsorb the immunoglobulins by their Fc fractions and act as passive carriers promoting an effective clumping of brucellae. In the absence of antigen-antibody complexes, the suspension remains uniform and the cream remains colorless. This test is not considered sensitive but this lack of

sensitivity is compensated by the fact that the test can be repeated, usually monthly, due to its very low cost (OIE, 2009).

Buffered *Brucella* Antigen Tests (BBAT)

Buffered *Brucella* antigen tests are simple spot agglutination tests using stained antigen. These include the Rose Bengal test (RBT) and the buffered plate agglutination test (BPAT).

Agglutination tests are the most widely used serological tests, for screening purposes as they give specific and reliable preliminary diagnosis, and are cost effective (Naik et al., 2017**)**.

Rose Bengal Agglutination Test (RBT)

The RBT is a simple method of brucellosis diagnostics and is the most widely used for the serological diagnosis of sheep and goat brucellosis. RBT is performed with a stained *B. abortus* suspension at pH of 3.6-3.7. RBT consists of a simple spot agglutination test where drops of rose Bengal-stained antigen and serum are mixed on a plate and any resulting agglutination signifies a positive reaction (Alton et al., 1988). The RBT is an affordable, quick, simple and efficient screening test and is used as a diagnostic test for screening individual animals and herds, as well as in humans. This test was found efficient in diagnosis of the acute human brucellosis and still is used in the diagnosis of chronic cases. Normally, results are obtained in few minutes (Khan et al., 2017; Teng et al., 2017).

Although rapid and excellent for screening, this test is not reliable for vaccinated animals, because it can generate false positives due to its high sensitivity (Smirnova et al., 2013). It is conventional that RBT have little specificity in animals and humans that are already immunized with strain 19. Therefore, a positive blood sample should be confirmed by definitive test. In fact, in most countries, the RBT is mostly used as a screening test,

followed by the CFT as a confirmatory test for diagnosis of brucellosis (Khan et al., 2017).

Buffered Plate Agglutination Test (BPAT)

The BPAT is also a spot agglutination test where a stained *B. abortus* antigen is used. Two staining solutions are required: brilliant green (2 g/100 mL) and crystal violet (1 g/100 mL) mixed together in equal volumes to prepare a stained-cell suspension with a blue–green color. This antigen is mixed on a plate with serum and any resulting agglutination signifies a positive reaction (OIE, 2016).

Serum Agglutination Test (SAT)

The SAT has been used with success for many years in surveillance and control programs for bovine brucellosis. The first serological test for brucellosis, was described in 1897 and it was based on the sedimentation of the complexes of IgM antibodies with *B. abortus* antigens (Wright and Smith, 1897). The reaction is slow since it requires an overnight incubation at 37˚C. SAT, lacks specificity and sensitivity, although it is inexpensive and easy to perform. This test is only appropriate for cattle (OIE, 2016).

Complement Fixation Test (CFT)

The CFT detects anti-*Brucella* antibodies that are able to activate the complement. The complement system consists of a complex series of proteins, which, if triggered by an antigen-antibody complex, react in a sequential manner to cause cell lysis (Hill, 1963). This test is widely used but it is complex to perform and requires good laboratory facilities and adequately trained staff to accurately titrate and maintain the reagents.

There are numerous variations of the CFT in use, but this test is most conveniently carried out in a microtitre format.

The CFT is usually very specific but less sensitive than RBT and ELISA and the absence of anti-complementary activity must be checked for each serum. Moreover, like most serological tests, the CFT can show positive results in ruminants after *B. abortus* S19 or *B. melitensis* Rev.1 vaccination and it is not specific enough in the presence of false positive serological reactions (FPSR). Therefore, CFT results should be investigated using suitable confirmatory or complementary strategies (OIE, 2016).

Indirect Enzyme-Linked Immunosorbent Assays (i-ELISA) or Competitive Enzyme-Linked Immunosorbent Assay (c-ELISA)

Serological tests like ELISA are capable of readily identifying individual IgM and IgG antibody allowing for a better correlation with the clinical situation (Naik et al., 2017). ELISA is recommended for screening cattle as well as humans due to its sensitivity and specificity compared with RBT (Khan et al., 2017).

The i-ELISA was developed originally to allow large-scale assaying of antibodies in bovine serum and milk. Most i-ELISA use purified smooth LPS as the antigen, but a good deal of variation exists in the anti-bovine Ig conjugate used to detect mainly IgG or IgG sub-classes. Their best quality is their high sensitivity but they are also more vulnerable to non-specific reactions (McGiven et al., 2003; Saegerman et al., 2004). The diagnostic sensitivity should be equal to, or greater than that of the BBATs (RBT/BPAT), or the CFT when testing infected cattle, small ruminants or pigs. However, the specificity may be lower (Praud et al., 2012).

These cross-reactions seen in i-ELISA led to the development of c-ELISA. The c-ELISA for detection of specific antibodies has largely replaced the i-ELISA for large-scale screening and serosurveillance. In this method, sample antibody competes for binding to antigen bound to microtiter plate wells with a limited amount of labeled antibody. The

higher the sample antibody concentration, the weaker the output signal, indicating that the signal output inversely correlates with the amount of antigen in the sample. The c-ELISA offers significant advantages over the indirect assay since samples from many species may be tested without the need for species-specific enzyme-labelled conjugates for each species under test. Besides, c-ELISA reduces, but not fully eliminates, the reactions caused by antibodies produced in response to vaccination. It is highly probable that much of the specificity improvement is due to a reduction in sensitivity of the c-ELISA compared with BBAT and i-ELISA. (OIE, 2016).

Fluorescence Polarization Assay (FPA)

The FPA is a homogeneous assay in which analytes are not separated and it is therefore very rapid. It is a simple technique for measuring antigen/antibody interaction and may be performed in a laboratory setting or in the field. This method is based on a physical principle: how quickly a molecule spins in a liquid medium correlates with its mass. Molecules of small size spin faster and depolarize a polarized light beam more, while bigger molecules spin more slowly and, consequently, depolarize light less. FPA measures the degree of depolarization in milli-polarization units (mP). During the test, serum samples are incubated with a specific antigen of *B. abortus* labeled with fluorescein isothiocyanate. In the presence of antibodies against *Brucella* spp., large fluorescent complexes are formed. In negative samples, the antigen remains uncomplexed. These smaller molecules spin more quickly and therefore cause greater depolarisation of the light than do the samples positive for *Brucella* spp. (Godfroid et al., 2010; Banai et al., 2017).

This test is capable of reducing but not fully eliminating the reactions due to residual antibody produced in response to vaccination (Nielsen et al., 1996, 2000; Gall et al., 2002). Moreover, the specificity of FPA in FPSR conditions is currently unknown in cattle and small ruminants, but it

has been clearly shown that it does not resolve the FPSR problem in swine (Praud et al., 2012).

Native Hapten Test (NH)

Native hapten (NH) are free polysaccharides, produced by at least *B. melitensis* and *B. abortus* that are almost or totally identical to the O-polysaccharide. NH are suitable antigens for immunoprecipitation tests (Ducrotoy et al., 2016). In cattle, NH tests are highly specific in *B. abortus* S19 vaccination contexts and have been used successfully in combination with the RBT as a screening test (OIE, 2016). The optimal sensitivity (close to that of CFT but significantly lower than that of RBT and S-LPS based i-ELISAs) is obtained in a reverse radial immunodiffusion (RID) system in which the serum diffuses into a hypertonic gel containing the polysaccharide, but the double gel diffusion assay is also useful (Muňoz et al., 2005).

These native hapten tests are also of interest to use in sheep and goats as they are very specific for discriminating the serological responses of infected animals (positive) from those induced in *B. melitensis* Rev.1 vaccinated animals (usually negative after a given time post-vaccination). The optimal diagnostic sensitivity (around 90%) is obtained in the double gel diffusion or RID tests for sheep and goats, respectively (OIE, 2016).

Lateral Flow Assay (LFA)

The lateral flow assay (LFA) is a simplified version of ELISA consisting of a nitrocellulose detection strip, contain *Brucella* LPS as well as a *Brucella*-specific capture probe, flanked at one end by a reagent pad, consisting of a colloidal gold immune conjugate, and at the other end by an absorption pad. The flow assay is simply performed by the addition of 5 µl of serum directly onto the sample application pad, followed by the addition of some test liquid. The result is read 10 to 15 minutes later by visual

inspection for staining. The assay is based on the binding of specific antibodies present in the clinical specimen to LPS antigen and staining of the bound antibodies by a colloidal gold-labeled antibody conjugate (Smits et al., 2003).

Abdoel et al. (2008) developed a lateral flow immunochromatography device for the serodiagnosis of brucellosis in cattle, goat, sheep and swine. The sensitivity of the bovine *Brucella* LFA was calculated to be 90%, that of the caprine LFA 100%, that of the ovine LFA 77%, and that of the swine LFA 73%. No reactivity in the *Brucella* LFAs was observed for samples from animals known to be free of brucellosis indicating a high (100%) specificity.

Interferon-Gamma (IFN-γ) Release Assay

The IFN-γ release assay involves stimulation of lymphocytes in whole blood with a suitable antigen such as brucellin. The resulting IFN-γ production is detected through a capture ELISA (OIE, 2016, 2018). This is a relatively sophisticated assay that is performed by mixing heparinized blood with brucellin (phosphate buffered saline is used as a negative control) followed by incubation. IFN-γ (an important cytokine in the response against *Brucella*) is then measured using an ELISA. The method was developed in an attempt to find alternative methods to identify FPSR animals. However, when studied in *Yersinia enterocolitica* O:9 and *B. abortus* infected cattle, it fails to provide satisfactory discrimination (Ducrotoy et al., 2016).

Brucellin Skin Test (BST)

An alternative immunological assay is the brucellin skin test (BST), which can be used for screening unvaccinated herds, provided that a purified (free of S-LPS) and standardized antigen preparation is used. This

antigen is a *Brucella* protein mixture containing up to 20 different proteins (Ducrotoy et al., 2016).

The BST has a very high specificity, such that serologically negative unvaccinated cattle that are positive reactors to the brucellin test should be regarded as infected animals (Pouillot et al., 1997). This test also has a high sensitivity for the diagnosis of *B. melitensis* infection in small ruminants and, in the absence of vaccination, is considered one of the most specific diagnostic tests (OIE, 2016).

Animals vaccinated with *B. melitensis* Rev.1, *B. abortus* S19 or RB51 can give positive results in this test for years (De Massis et al., 2005). Although the BST is probably the most specific indirect assay for diagnosing brucellosis (in unvaccinated animals), the final diagnosis should not be made solely based on positive intradermal reactions given by a few animals in the herd and should be supported by a complementary diagnostic test.

For sheep and goats, 0.1 mL of brucellin (2000 Units/mL) is injected intra-dermally into the lower eyelid, after 48 hours any visible or palpable reaction of hypersensitivity, such as an oedematous reaction leading to an elevation of the skin or thickening of the eyelid (≥ 2 mm), should be interpreted as a positive reaction (OIE, 2016).

SENSITIVITY AND SPECIFICITY OF INDIRECT TESTS FOR BRUCELLOSIS DIAGNOSIS

Diagnostic sensitivity (DSe) describes the ability of a test to detect the disease. Diagnostic specificity (DSp) indicates the accuracy of the test to detect non-diseased animals.

B. melitensis and *B. abortus* S-LPS and core-O-polysaccharide are present in most currently used immunological tests. This antigen is highly effective for detecting the presence of specific antibodies, such as S-LPS, which is immunodominant in the antibody response, and O-polysaccharide antibodies, as the respective epitopic density of the antigene is high. On the

other hand, *Brucella* O-polysaccharide cross-reacts with other gram-negative bacteria, mainly *Yersinia enterocolitica* serotype O:9, which generates the strongest cross-reactivity, and other bacteria such as *Escherichia coli* 0157 and the *Salmonella* group N (O:30). None of the above-mentioned tests are *Brucella* species specific. Nevertheless, some of those methods, specifically buffered agglutination tests, set a high standard with regards to the DSe/DSp if no vaccination is practiced or when the FPSR is not significant. These tests are very inexpensive. When interference of vaccination is expected, a cautious use of the smooth vaccines combined with NH immunoprecipitation or c-ELISA testing remain as the best strategy available (Ducrotoy et al., 2016).

The sensitivity and specificity of diagnosis tests of brucellosis are presented in Table 8.2. The i-ELISA and FPA proved to be the most sensitive, while the highest specificity was determined for the brucellin skin test, CFT and also, i-ELISA. The less specific was the MRT.

Table 8.2. Sensitivity and specificity of indirect tests for the diagnosis of cattle brucellosis. Adapted from Godfroid et al. (2010)

Tests	**Sensitivity (%)**	**Specificity (%)**
Serological tests		
SAT (SAW)/MAT	81.5	98.9
CFT	90-91.8	99.7-99.9
BBAT	87	97.8
i-ELISA	97.2	97.1-99.8
c-ELISA	95.2	99.7
FPA	96.6	99.1
Milk tests		
MRT	88.5	77.4
FPA	76.9	100
i-ELISA	98.6	99.0
Cellular tests		
Brucellin Skin test	78-93	99.8

Abbreviations: slow agglutination test (SAT); slow agglutination of wright (SAW); micro agglutination test (MAT); complement fixation test (CFT); buffered *Brucella* antigen test (BBAT); indirect ELISA (I-ELISA); competitive ELISA (C-ELISA); fluorescence polarization assay (FPA); milk ring test (MRT).

A 100% DSp is displayed by i-ELISA, with S-LPS or OPS-core antigenes, in *Brucella*-free animals not exposed to *Y. enterocolitica* O:9, though they offer poor DSp in cattle exposed experimentally to *Y. enterocolitica* O:9 or from herds with FPS. To circumvent the FPSR problem, the approach may be the use of cellular immunity tests, in which antigen is a cellular fraction consisting of non-denatured water-soluble proteins resulting in superb DSp in the skin test, in the absence of vaccination. Although its DSp is excellent, the protein skin test is only useful at herd level and requires two consecutive visits to herds (Ducrotoy et al., 2016, 2018).

Although no current serological test provides enough DSe for the 100% DSp required, some test combinations can be helpful. According to Ducrotoy et al. (2018), the best test when DSe/DSp balances are considered is the reverse radial immunodiffusion-native hapten (RID-NH)

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