Laboratorial diagnosis of animal brucellosis
Diagnóstico laboratorial da brucelose

Juliana Pinto da Silva Mol,* Silvia de Araújo França,* Tatiane Alves da Paixão,** Renato Lima Santos*

Abstract
Brucellosis, originally known as a Malta fever or undulant fever, is a disease caused by bacteria of the genus *Brucella* that are host restricted and affect several mammalian species, including humans. It is a zoonosis widely distributed around the world, which causes great economic losses in farm animals due to abortion, the slaughter of infected animals, birth of weak animals, decrease in milk production, and infertility. In humans, brucellosis is a debilitating disease with variable clinical manifestations that can result in death in some cases. Control of brucellosis in animals requires a correct diagnosis, culling of infected animals, and permanent monitoring of brucellosis-free herds. Although a clinical presumptive diagnosis is important, it is subjective, and therefore, laboratorial tests including direct and indirect methods are extremely important for an accurate diagnosis. This review discusses current methods for laboratorial diagnosis of brucellosis using clinical samples, from animals or humans.

Keywords *Brucella*, bacteriology, PCR, diagnosis, serology.

Resumo
A brucelose, originalmente conhecida como febre de Malta ou febre ondulante, é uma doença causada por bactérias do gênero *Brucella* que possuem preferência quanto ao hospedeiro principal e que acomete diferentes espécies de mamíferos, inclusive o homem. É uma zoonose amplamente distribuída pelo mundo que provoca grandes perdas econômicas advindas de abortamentos e abate de animais infectados, do nascimento de animais fracos, diminuição na produção de leite e infertilidade. No homem provoca doença debilitante com diferentes manifestações clínicas e que pode, em alguns casos, causar a morte. O controle da doença nos animais está diretamente relacionado com um diagnóstico correto, ao sacrifício dos animais infectados e ao constante monitoramento dos rebanhos livres da doença. O diagnóstico clínico, apesar de ser importante para se estabelecer a suspeita inicial da doença, é subjetivo, e desta forma, os testes laboratoriais, que são divididos em exames diretos e exames sorológicos ou indiretos, são extremamente importantes para confirmação da infecção. Diante disso, este trabalho faz uma revisão atualizada sobre os métodos de diagnóstico laboratorial da brucelose a partir de diferentes amostras clínicas, de origem animal ou humana.

Palavras-chave: *Brucella*, bacteriologia, PCR, diagnóstico, sorologia.

Introduction
Brucellosis is a zoonotic disease caused by Gram-negative coccobacilli of the genus *Brucella* (Godfroid et al., 2005). In livestock, the disease results in significant economic losses due to reproductive impairment caused by abortion, stillbirth or weak calves and neonatal mortality, infertility. (Xavier et al., 2009a). 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 (Cutler et al., 2005).

Currently, there are ten species described in the genus *Brucella*. Each one may infect different host species, but each *Brucella* species has a preference for its host species: *B. melitensis* (sheep and goats), *B. abortus* (cattle), *B. suis* (pigs), *B. ovis* (rams), *B. canis* (dogs), *B. microti* (rodents - *Micrurus 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) (Xavier et al., 2009a, Scholz et al., 2010; de Jong e Tsolis, 2012). Three of these *Brucella* species can be subdivided in biotypes (Vizcaíno et al., 2000; Bricker, 2002a; Ocampo-Sosa et al., 2005). 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* (Whatmore, 2009). All *Brucella* species are considered potentially pathogenic for humans, with the exceptions of *B. neotomae*, *B. microti*, and *B. ovis* (Xavier et al., 2009a).

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 historic of reproductive failures in livestock, but it is a presumptive diagnosis (Poester et al., 2010) that must be confirmed by laboratorial methods (Nielsen, 2002; Poester et al., 2010).

The goal of this review is to describe laboratory methods available for diagnosing *Brucella* spp. infections.

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Direct diagnosis

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 (Bricker, 2002a; Al Dahouk et al., 2003). However, in spite of its high specificity, culture of Brucella spp. is challenging. Brucella spp. is a fastidious bacterium and require rich media for primary cultures (Alton et al., 1975). Furthermore, its isolation requires a large number of viable bacteria in clinical samples, proper storage and quick delivery to the diagnostic laboratory (Refai, 2003; Seleem et al., 2010).

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; cycloheximide 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. (Alton et al., 1975; De Miguel et al., 2011).

Another limiting factor for culturing Brucella spp. is the requirement for appropriate laboratory conditions and personnel training so the procedure can be performed safely (Nielsen and Ewalt, 2004). Brucella spp. is classified as a biosafety level 3 organism, whose manipulation should be performed in biosafety level 3 laboratories (Choosewood and Wilson, 2007; Lage et al., 2008). Importantly, brucellosis is one of the most common accidental laboratory infections, particularly in research laboratories (Esel et al., 2003; Singh, 2009; Seleem et al., 2010; Sam et al., 2012).

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 (Alton et al., 1975; Poester et al., 2006; Lage et al., 2008). 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 (Poester et al., 2010). Milk samples should be a pool from all four mammary glands. Non pasteurized dairy products can also be sampled for isolation (Lage et al., 2008; Poester et al., 2010).

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 (Poester et al., 2010).

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 (Alton, 1975; Ewalt 1989; Her et al., 2010; De Miguel et al., 2011).

Culture of blood samples is a useful diagnostic approach in the case of canine brucellosis since dogs infected with B. canis often develop bacteremia reaching 10^4 CFU (colony forming unit) per mL of blood (Carmichael and Greene, 1990). However, even in the case of canine brucellosis blood culture should not be the only diagnostic test since during the chronic phase of the infection bacteremia becomes intermittent (Alton et al., 1975).

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 species require CO₂ for growth (Alton et al., 1975). 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. False negative results should be considered in the absence of bacterial growth since the sensitivity of culture is low (Alton et al., 1975, Poster et al., 2010).

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 (Alton et al., 1975; Poester et al., 2010). In the case of blood or milk, biphasic media such as Castañeda’s medium is recommended for improving sensitivity (Alton et al., 1975; Poester et al., 2010).

Isolates must be characterized to identify species and for those species that have biotypes, as B. abortus, B. melitensis and B. suis, the biotype is determined by tests of growth in CO₂ atmospheric, H₂S production, growth in the presence of thionine and basic fuchsin and serum agglutination against anti-A, anti-M and anti-R. Such characterization is based on approximately 25 phenotypic features. Table 1 describes phenotypic features of each Brucella species and biotypes, and the test more commonly employed for their identification and differentiation.

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 (Santos et al., 1998; Xavier et al., 2009b). An advantage of this technique is that it does not require viable bacteria and allow retrospective studies (Santos et al., 1998). Although immunohistochemistry is simple, several factors may affect the result, including the fixation protocol and selection of the primary antibody (Ramos-Vara, 2005).

Molecular diagnosis

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 (Baily et al., 1992; Bricker and Halling, 1994; Bricker and Halling, 1995; Ocampo-Sosa et al., 2005; Le Flèche et al., 2006; López-Goñi et al., 2008, Xavier et al., 2010). Molecular detection of Brucella sp. 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 (Bricker et al., 2002b).

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 (Baily et al., 1992, Leal-Klevezas et al., 1995; Bricker, 2002a; Xavier et al., 2010).
Table 1: Phenotypic features of *Brucella* species and biotypes, and the tests for their identification and differentiation*

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<tr>
<th>Species</th>
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<th>Citrate</th>
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<th>Growth on dyes **</th>
<th>Agglutination in sera†</th>
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** positive; - negative; (+) usually positive; (-) usually negative; ** Species differentiation is obtained on Trypticase-soy agar or tryptose agar with the concentrations of dyes: (a)1:25 000, (b)1:50 000. ***B. abortus. 1, 2 and 4 do not grow in 1:100 000 thionine; † Monospecific antiserum: A-abortus; M-melitensis; R-rough; d-doubtful; # Tbilisi; RTD-routine test dilution

* Adapted from Alton et al. 1975; Foster et al., 2007; Jacques et al. 2007; Poester et al., 2010.
The technique is chosen based on the type of biological sample and the goal, i.e. diagnosis or molecular characterization or epidemiological survey.

The most common techniques and their applications and limitations are summarized in Table 2. 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 (Queipo-Ortuno et al., 1997; Morata et al., 1998; Navarro et al., 1999; Zerva et al., 2001; Al-Nakkas et al., 2002; Morata et al., 2003; Navarro et al., 2004; Mitka et al., 2007).

Table 2: Applications and limitations of the most commonly used molecular techniques for the diagnosis of brucellosis

<table>
<thead>
<tr>
<th>Molecular technique</th>
<th>Applications</th>
<th>Limitations</th>
<th>Reference</th>
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<tbody>
<tr>
<td>PCR based on 16S ribosomal RNA gene</td>
<td>Identification of Brucella spp.</td>
<td></td>
<td>Herman and Ridder, 1992; Romero et al., 1995.</td>
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<tr>
<td>Multiplex-PCR based on recA gene</td>
<td>Detection and differentiation of O. inermis, O. antropi e Brucella spp.</td>
<td>Does not identifies the species of Brucella</td>
<td>Scholz et al., 2008.</td>
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<tr>
<td>PCR based on ORF AO512</td>
<td>Reaction species-specific for B. ovis</td>
<td>Does not identifies the other species of Brucella</td>
<td>Xavier et al., 2010.</td>
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<td>AMOS-PCR (Multiplex-PCR based on repetitive element IS711)</td>
<td>Identification and differentiation of B. abortus (biotypes 1, 2 e 4), B. melitensis, B. ovis and B. suis (biotype 1)</td>
<td>Does not differentiate vaccinal and field samples. Does not identifies all B. abortus and B. suis biotypes and the other species of Brucella</td>
<td>Bricker and Halling, 1994.</td>
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<tr>
<td>AMOS-Enhanced (Multiplex-PCR based on repetitive element IS711 and eri gene)</td>
<td>Identification and differentiation of B. abortus (biotypes 1, 2 e 4), B. melitensis, B. ovis e B. suis (biotype 1), S19 and RB51</td>
<td>Does not identifies all B. abortus and B. suis biotypes and the other species of Brucella</td>
<td>Bricker and Halling, 1995.</td>
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<tr>
<td>AMOS-ERY-PCR (Multiplex-PCR based on repetitive element IS711 and eryc-eryD and DEL569 genes)</td>
<td>Identification and differentiation of B. abortus (biotypes 1, 2, 3b, 4, 5, 6 e 9), B. melitensis, B. ovis and B. suis (biotypes 1)</td>
<td>Does not identifies the other species of Brucella</td>
<td>Ocampo-Sosa et al., 2005.</td>
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<td>Bruce-Ladder (Multiplex-PCR based on repetitive element IS711 and 9 different genes)</td>
<td>Identification and differentiation of B. melitensis B. abortus, B. suis, B. ovis, B. canis, B. neotomae, B. microti, B. pinnipedialis e B. ceti, S19, RB51and Rev1</td>
<td>Does not identifies the biotypes of Brucella species</td>
<td>Lopez-Goñi et al., 2008; Lopez-Goñi et al., 2011; Kang et al., 2011</td>
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<td>Suis-Ladder PCR (Multiplex-PCR based on VNTR Bruce 11)</td>
<td>Identification of B. suis 1, 2, 3, 4, 5 and B. canis</td>
<td>Identifies and differentiates only the species B. suis (and their biotypes) and B. canis</td>
<td>Lopez-Goñi et al., 2011.</td>
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<tr>
<td>PCR HOOF-Prints (PCR based on 8 VNTR)</td>
<td>Distinguishes all Brucella species</td>
<td>Does not identifies the biotypes of Brucella species</td>
<td>Bricker et al., 2003; Bricker and Ewald, 2005.</td>
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<tr>
<td>MLVA16 (PCR based on 16 VNTR)</td>
<td>Distinction all Brucella species and their biovariedades</td>
<td>It is necessary to use program analysis to interpret the results</td>
<td>Le Fleche et al., 2006; Al Dahouk et al., 2007; Maquart et al., 2009.</td>
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<tr>
<td>RAPD-PCR (PCR using arbitrary primers)</td>
<td>Production of a characteristic profile for each Brucella sample</td>
<td>Presents low reproducibility, which makes limited their realization</td>
<td>Fekete et al., 1992; Huber et al., 2009.</td>
</tr>
<tr>
<td>PCR-RFLP (PCR-RFLP based on outer membrane proteins)</td>
<td>Identification of polymorphisms in genes encoding outer membrane proteins</td>
<td>Method expensive and laborious</td>
<td>Cloeckaert et al., 1995; Vizcaino et al., 1997; Cloeckaert et al., 2001; Garcia-Yoldi et al., 2005.</td>
</tr>
<tr>
<td>Real time PCR (HRM, SNP and Single-PCR analysis based on bcap31 gene and 16S ribosomal RNA gene. Multiplex-PCR based on repetitive element IS711)</td>
<td>Rapid identification of the genus Brucella and species differentiation. Used for human brucellosis diagnosis</td>
<td>This method must be better standarized and optimized before being incorporated into diagnostic laboratories of brucellosis</td>
<td>Probert et al., 2004; Queipo-Ortuno et al., 2005; Kattar et al., 2007; Gopaul et al., 2008; Jinic et al., 2008; Queipo-Ortuno et al., 2008; Bounaadia et al., 2009; Winchell et al., 2010.</td>
</tr>
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</table>

HOOF - hypervariable octameric oligonucleotide fingerprint, HRM - high resolution melting, MLVA - multilocus VNTR analysis, PCR - polymerase chain reaction, RAPD - random amplified polymorphic dna, RFLP - restriction fragment length polymorphism, SNP - single nucleotide polymorphism VNTR - variable number tandem repeat.
react with antigen preparations from \textit{B. ovis} (Alton et al., 1975; Nielsen, 2002).

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 (Poester et al., 2010; Nielsen, 2002).

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 (OIE, 2009a, Nielsen, 2002; Poester et al., 2010). Methods employing acidified antigens (i.e. card test, rose bengal test, and BPAT) are traditional methods that has been extensively used for diagnosis of brucellosis in cattle, pigs, and goats (Nielsen, 2002; Abernethy et al., 2012; Praud et al., 2012). Antigen acidification prevents IgM binding, favoring agglutination with IgG, increasing specificity for detection of 	extit{Brucella} spp. infection (Poester et al., 2010). These methods are used as screening tests and may result in false positive results, particularly in cattle previously vaccinated with \textit{B. abortus} strain 19 (OIE, 2009a). Sensitivity of BPAT varies from 81.2 to 100%, and its specificity range from 86.3 to 100% (Diaz-Aparicio et al., 1994, Saravi et al., 1995; Mert et al., 2003; Praud et al., 2012). Considering that these are screening tests, positive samples should be subjected to a confirmatory method.

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 (Alton et al., 1975, OIE, 2009a). Importantly, the number of false positive results is proportional to the number of cows secreting acidic milk due to colostrums or mastitis (OIE, 2009a). 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.

The 2-mercaptoethanol is a confirmatory test that allows selective quantification of IgG anti-\textit{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 (Alton et al., 1975). 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 (Poester et al., 2010). Sensitivity of the 2-mercaptoethanol test varies from 88.4 and 99.6%, and its specificity from 91.5 and 99.8% (Saravi et al., 1995; Baldi et al., 1997; Gall and Nielsen, 2004). Although vaccination with strain 19 may interfere with 2- mercaptoethanol results, this is the confirmatory test recommended by the Brazilian national eradication program (“Programa Nacional de Controle e Erradicação Brucelose e Tuberculose Bovina” – PNCETB), after use of a screening test (Manual, 2006).

The complement fixation test is highly efficient and therefore accepted worldwide (West and Bruce, 1983; Nielsen, 2002). Due to its high accuracy, complement fixation is used as confirmatory test for \textit{B. abortus}, \textit{B. melitensis}, and \textit{B. ovis} infections (West and Bruce, 1983; Ris et al, 1984, Chin et al., 1991), and it is the reference test recommended by the OIE for international transit of animals (OIE, 2008ab). 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 (Ris et al., 1984; Kováčová et al., 2007; OIE, 2009a). Sensitivity of complement fixation ranges from 77.1 to 100% and its specificity from 65 to 100% (Searson, 1982; Diaz-Aparicio et al., 1994; Gall et al., 2001; Perrett et al., 2010).

There are basically two different kinds of immunoenzymatic essays that are used for diagnosis of brucellosis in humans and domestic animal species: the indirect ELISA (ELISAI) and the competitive ELISA (ELISAc) (Baldi et al., 1997; Nielsen, 2002; Di Febo et al., 2012). The ELISAI 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 (Nielsen, 2002; Gall et al., 2003; Di Febo et al., 2012). O ELISAc has been usually used for smooth LPS \textit{Brucella} species, and it is sensitive and specific for \textit{B. abortus} or \textit{B. melitensis}, but it is not capable of differentiating antibodies induced by the vaccine strains S19 or Rev1 (Letesson et al., 1997; Eoh et al., 2010, Ko et al., 2012; Lim et al., 2012). Sensitivity of ELISAI varies from 96 to 100%, and its specificity from 93.8% and 100% (Marin et al., 1989; Diaz-Aparicio et al., 1994; Vigliocco et al., 1997; Gall et al., 2001; Gall and Nielsen, 2004). The ELISAc with smooth \textit{Brucella} LPS as antigen is used for detection of anti-\textit{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% (Gall et al., 2001; Gall and Nielsen, 2004; Nielsen et al., 2004; Godfroid et al., 2010; Perrett et al., 2010).

The fluorescence polarization assay has been used for the diagnosis of \textit{Brucella} spp. infection in man (Lucero et al., 2003) and several animal species, using serum, milk or whole blood in EDTA (Gall et al., 2000; Nielsen et al., 2000; Gall et al., 2001). This test can be performed under field conditions (Nielsen, 2002). Sensitivity of the fluorescence polarization assay varies from 87.5 and 100%, and specificity from 84 to 100% (Gall et al., 2000; Nielsen et al., 2000; Gall et al., 2001; Lucero et al., 2003; Godfroid et al., 2010), which is similar to the levels obtained with ELISAc (Gall et al., 2001).

The agar gel immunodiffusion test is based on precipitation of the antigen-antibody complex. This method is often used for the diagnosis of \textit{B. ovis} infection. This test has a low cost, it is easily performed and it has sensitivity levels that are comparable to complement fixation (Myers et al., 1972; Ficapal et al., 1998; Xavier et al., 2011). However, it has some disadvantages such as a marked decrease in sensitivity in chronic infections (Xavier et al., 2011), and high variability of the quality of commercially available antigens. Therefore, it is highly advisable to perform complementary diagnostic techniques such as PCR (Costa et al., 2012). Sensitivity of the agar gel immunodiffusion test varies from 50 to 92.7% and the specificity from 94.3 and 100% (Marin et al., 1989; Hilbink et al., 1993; Ficapal et al., 1998; Esteen et al., 2002; Gall and Nielsen, 2004; Xavier et al., 2011).

Table 3 summarizes screening and confirmatory serological methods for diagnosis of the most important \textit{Brucella} infections. \textit{Brucella} species that do not appear in Table 3 do not have standardized serological test.
Table 3: Screening and confirmatory tests commonly used in the serological diagnosis of Brucella spp. infection

<table>
<thead>
<tr>
<th>Species</th>
<th>Screening tests</th>
<th>Confirmatory test</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>B. abortus</td>
<td>BPAT; MRT</td>
<td>2ME, CF, ELISAc</td>
<td>Alton et al., 1975; Manual, 2006; OIE, 2009a.</td>
</tr>
<tr>
<td>B. melitensis</td>
<td>BPAT</td>
<td>BPAT, CF</td>
<td>Alton et al., 1975; OIE, 2009c.</td>
</tr>
<tr>
<td>B. suis</td>
<td>BPAT</td>
<td>2ME, CF, AGIT, ELISAi</td>
<td>Di Febo et al., 2012; Praud et al., 2012.</td>
</tr>
<tr>
<td>B. canis</td>
<td>-</td>
<td>2ME, AGIT, ELISAi</td>
<td>Baldi et al., 1997; Ebani et al., 2003.</td>
</tr>
<tr>
<td>B. ovis</td>
<td>-</td>
<td>CF, ELISAi</td>
<td>Gall et al., 2003 ; OIE, 2009b.</td>
</tr>
</tbody>
</table>

2ME - 2-mercaptethanol, 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.

Concluding remarks

Serological tests are widely used for the diagnosis of brucellosis, mostly for screening of herds. Although these methods have been extensively improved in regard to sensitivity and specificity, they have some limitations as an indirect method, and in most cases lack of possibility to differentiate vaccinated from infected animals. The gold standard for diagnosis of brucellosis is the isolation and phenotypic characterization of the organism, but this is a laborious and slow technique that requires well trained personnel and biosafety level 3 laboratories. Therefore, molecular methods have been increasingly used for a definitive diagnosis. Currently, molecular methods are used mostly for experimental or epidemiological studies, but it will probably be used as routine techniques in diagnostic laboratories in the near future.

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