http://dx.doi.org/10.4322/rbcv.2014.106

Laboratorial diagnosis of animal brucellosis

Diagnóstico laboratorial da brucelose

Juliana Pinto da Silva Mol,* Sílvia 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 - *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) (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.

* Departamento de Clínica e Cirurgia Veterinárias, Escola de Veterinária da Universidade Federal de Minas Gerais, MG, Brasil. Corresponding author: rsantos@vet.ufmg.br

** Departamento de Patologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, MG, Brasil

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 laboratorial 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 (Chosewood 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 ºC, 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 CO_2 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 CO_2 atmospheric, H_2S 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.,

Table 1: Phenotypic features of *Brucella* species and biotypes, and the tests for their identification and differentiation*

Species	Biotype	CO_2	H_2S	Biochemical tests					Growth on dyes **			Agglutination in sera†			Lysis by phage (RTD)Tb#	Motility	Acriflavin test
				Urease	Oxidase	Catalase	Citrate	Nitrate	Thionin ***		Basic fuchsin	Anti -A	Anti -M	Anti -R	Tb 10 000 x RTD		
									a	b							
B. melitensis	1	-	-	+	+	+	-	+	-	+	+	-	+	-	-	-	-
	2	-	-	+	+	+	-	+	-	+	+	+	-	-	-	-	-
	3	-	-	+	+	+	-	+	-	+	+	+	+	-	-	-	-
B. abortus	1	(+)	+	+	+	+	-	+	-	-	+	+	-	-	+	-	-
	2	(+)	+	+	+	+	-	+	-	-	-	+	-	-	+	-	-
	3	(+)	+	+	+	+	-	+	+	+	+	+	-	-	+	-	-
	4	(+)	+	+	+	+	-	+	-	-	(+)	-	+	-	+	-	-
	5	-	-	+	+	+	-	+	-	+	+	-	+	-	+	-	-
	6	-	-or+	+	+	+	-	+	-	+	+	+	-	-	+	-	-
	7	-	-or+	+	+	+	-	+	-	+	+	+	+	-	+	-	-
	8	+	-	+	+	+	-	+	-	+	+	-	+	-	+	-	-
	9	-or+	+	+	+	+	-	+	-	+	+	-	+	-	+	-	-
B. suis	1	-	++	+	+	+	-	+	+	+	(-)	+	-	-	+	-	-
	2	-	-	+	+	+	-	+	-	+	-	+	-	-	+	-	-
	3	-	-	+	+	+	-	+	+	+	+	+	-	-	+	-	-
	4	-	-	+	+	+	-	+	+	+	(-)	+	+	-	+	-	-
	5	-	-	+	+	+	-	+	+	+	-	-	+	-	+	-	-
B. neotomae		-	+	+	-	+	-	+	-	-	-	+	-	-	(+)	-	-
B. ovis		+	-	-	-	-	-	-	+	+	(-)	-	-	+	-	-	+
B. canis		-	-	+	+	+	-	+	+	+	(-)	-	-	+	-	-	+
B. ceti		-	-	+	+	+	-	+	+	+	+	+	+or-	-	-	-	-
B. pinnipedialis		-	-	+	+	+	-	+	+	+	+	+	+or-	-	-	-	-
B. microti		-	-	+	+	+	-	+	+	+	+	-	+	-	+	-	-
B. inopinata		-	+	+	+	+	-	+	+	+	+	-	-	+	+	-	-

+ 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.

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).

Indirect methods for diagnosis of brucellosis

Serological tests

Serological tests are crucial for laboratorial 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

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

Molecular technique	Applications	Limitations	Reference
PCR based on *bcsp*31 gene	Identification of *Brucella* spp.	False-positive results by amplification of sequences from genus *Ochrobactrum*	Baily et al., 1992.
PCR based on 16S ribosomal RNA gene	Identification of *Brucella* spp.		Herman and Ridder, 1992; Romero et al., 1995.
Multiplex-PCR based on *rec*A gene	Detection and differentiation of *O. intermedium, O. anthropi* e *Brucella* spp.	Does not identifies the species of *Brucella*	Scholz et al., 2008.
PCR based on ORF AO512	Reaction species-specific for *B. ovis*	Does not identifies the other species of *Brucella*	Xavier et al., 2010.
AMOS-PCR (Multiplex-PCR based on repetitive element IS711)	Identification and differentiation of *B. abortus* (biotypes 1, 2 e 4), *B. melitensis*, *B. ovis* and *B. suis* (biotype 1)	Does not differentiate vaccinal and field samples. Does not identifies all *B. abortus* and *B. suis* biotypes and the other species of *Brucella*	Bricker and Halling, 1994.
AMOS-Enhanced (Multiplex-PCR based on repetitive element IS711 and *eri* gene)	Identification and differentiation of *B. abortus* (biotypes 1, 2 e 4), *B. melitensis*, *B. ovis* e *B. suis* (biotype 1), S19 and RB51	Does not identifies all *B. abortus* and *B. suis* biotypes and the other species of *Brucella*	Bricker and Halling, 1995.
AMOS-ERY-PCR (Multiplex-PCR based on repetitive element IS711 and *ery*C-*ery*D and DEL569 genes)	Identification and differentiation of *B. abortus* (biotypes 1, 2, 3b, 4, 5, 6 e 9), *B. melitensis*, *B. ovis* and *B. suis* (biotypes 1)	Does not identifies the other species of *Brucella*	Ocampo-Sosa et al., 2005.
Bruce-Ladder (Multiplex-PCR based on repetitive element IS711 and 9 different genes)	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	Does not identifies the biotypes of *Brucella* species	Lopez-Goñi et al., 2008; Lopez-Goñi et al., 2011; Kang et al., 2011
Suis-Ladder PCR (Multiplex-PCR based on VNTR Bruce 11)	Identification of *B. suis* 1, 2, 3, 4, 5 and *B. canis*	Identifies and differentiates only the species *B. suis* (and their biotypes) and B. *canis*	Lopez-Goñi et al., 2011.
PCR HOOF-Prints (PCR based on 8 VNTR)	Distinguishes all *Brucella* species	Does not identifies the biotypes of *Brucella* species	Bricker et al., 2003; Bricker and Ewald, 2005
MLVA16 (PCR based on 16 VNTR)	Distinction all *Brucella* species and their biovariedades	It is necessary to use program analysis to interpret the results	Le Fleche et al., 2006; Al Dahouk et al., 2007; Maquart et al., 2009
RAPD-PCR (PCR using arbitrary primers)	Production of a characteristic profile for each *Brucella* sample	Presents low reproducibility, which makes limited their realization	Fekete et al., 1992; Huber et al., 2009
PCR-RFLP (PCR-RFLP based on outer membrane proteins)	Identification of polymorphisms in genes encoding outer membrane proteins	Method expensive and laborious	Cloeckaert et al., 1995; Vizcaíno et al., 1997; Cloeckaert et al., 2001; Garcia-Yoldi et al., 2005.
Real time PCR (HRM, SNP and Single-PCR analysis based on *bcsp*31 gene and 16S ribosomal RNA gene. Multiplex-PCR based on repetitive element IS711)	Rapid identification of the genus *Brucella* and species differentiation. Used for human brucellosis diagnosis	This method must be better standardized and optimized before being incorporated into diagnostic laboratories of brucellosis	Probert et al., 2004; Queipo-Ortuno et al., 2005 ; Kattar et al., 2007; Gopaul et al., 2008; Hinic, et al., 2008; Queipo-Ortuno et al., 2008; Bounaadja et al., 2009; Winchell et al.; 2010.

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 *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 biding, favoring agglutination with IgG, increasing specificity for detection of *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 *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 heard 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-*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" – PNCEBT), 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 *B. abortus*, *B. melitensis*, and *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, 2009ab). 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 immunoenzimatic 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 ELISAi has been usually used for smooth LPS *Brucella* species, 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 (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 *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% (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 *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 *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; Estein 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 *Brucella* infections. *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

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

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.

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.

Acknowledgements

Work in RLS lab is supported by CNPQ (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil) and FAPEMIG (Fundação de Amparo a Pesquisa do Estado de Minas Gerais, Brazil).

References

ABERNETHY, D.A.; MENZIES, F.D.; MCCULLOUGH, S.J.; MCDOWELL, S.W.; BURNS, K.E.; WATT, R.; GORDON, A.W.; GREINER, M.; PFEIFFER, D.U. Field trial of six serological tests for bovine brucellosis. *Veterinary Journal,* v.191, n. 3, p. 364-370, 2012.

AL DAHOUK, S.; TOMASO, H.; NÖCKLER, K.; NEUBAUER, H.; FRANGOULIS, D. Laboratory-based Diagnosis of Brucellosis – A Review of Literature. Part I, Techniques for Direct Detection and Identification of *Brucella* sp., *Clinical laboratory,* v. 49, p. 487-505, 2003.

AL DAHOUK, S.; LE FLECHE, P.; NOCKLER, K.; JACQUES, I.; GRAYON, M.; SCHOLZ, H.C.; TOMASO, H.; VERGNAUD, G.; NEUBAUER, H. Evaluation of *Brucella* MLVA typing for human brucellosis. *Journal of Microbiological Methods*, v. 69, n.1, p. 137-145, 2007.

AL-NAKKAS, A.F.; WRIGHT, S,G.; MUSTAFA, A.S.; WILSON, S. Single tube, nested PCR for the diagnosis of human brucellosis in Kuwait. *Annals of Tropical Medicine and Parasitology,* v. 96, p. 397-403, 2002.

ALTON, G.G.; JONES, L.M; PIETZ, D.E. *Laboratory Techniques in Brucellosis*. WHO Monograph Series no. 55. Geneva, Switzerland: WHO. 1975, 188p.

BAILY, G.G.; KRAHN, J.B.; DRASAR, B.S.; STOKER, N.G. Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. *Journal of Tropical Medicine Hygiene*, v. 95, p. 271-275, 1992.

BALDI, P.C.; WANKE, M.M.; LOZA, M.E.; MONACHESI, N.; FOSSATI, C.A. Diagnosis of canine brucellosis by detection of serum antibodies against an 18 kDa cytoplasmic protein of *Brucella* spp. *Veterinary Microbiology.* v.57, n. 2-3, p. 273-281, 1997.

BOUNAADJA, L.; ALBERT, D.; CHÉNAIS, B.; HÉNAULT, S.; ZYGMUNT, M.S.; POLIAK, S.; GARIN-BASTUJI, B. Real-time PCR for identification of *Brucella* spp.: a comparative study of IS711, *bcsp*31 and per target genes. *Veterinary Microbiology*, v. 137, n. 1-2, p. 156-164, 2009.

BRICKER, B.J. Diagnostic strategies used for the identification of *Brucella*. *Veterinary Microbiology*, v. 90, p. 433-434, 2002a.

BRICKER, B.J. PCR as a diagnostic tool for brucellosis. *Veterinary Microbiology*, v. 90, p. 435-446, 2002b.

BRICKER, B.J.; HALLING, S.M. Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* bv. 1 by PCR. *Journal of Clinical Microbiology*, v. 32, n.11, p. 2660-2666, 1994.

BRICKER, B.J.; HALLING, S.M. Enhancement of the *Brucella* AMOS PCR Assay for differentiation of *Brucella abortus* Vaccine Strains S19 and RB51. *Journal of Clinical Microbiology*, v. 33, n. 6, p.1640-1642, 1995.

BRICKER, B.J.; EWALT, D.R.; HALLING, S.M. *Brucella* 'HOOF-Prints': strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). *BMC Microbiology*, v. 3, p. 15, 2003.

BRICKER, B.J.; EWALT, D.R. Evaluation of the HOOF-print assay for typing *Brucella abortus* strains isolated from cattle in the United States: results with four performance criteria. *BMC Microbiology,* v. 5, p. 37, 2005.

CARMICHAEL, L.E.; GREENE, C.E. *Canine Brucellosis* In: GRENNE, C.E. Infectious Diseases of the Dog and Cat. Philadelphia: WB. Saunders Company, 1990. p. 573-584.

CHIN, J.C.; PANG, B.; CARRIGAN, M. Comparison of seroreactivity of rams with brucellosis in a complement fixation test, whole cell ELISA and by immunoblotting. *Veterinary Microbiology*, v. 26, p. 291-299, 1991.

CHOSEWOOD, L.C.; WILSON, D.E. *Biosafety in microbiological and biomedical laboratorios*. Washington: US Government Printing Office, 2007. 409 p.

CLOECKAERT, A.; VERGER, J.M.; GRAYON, M.; GREPINET, O. Restriction site polymorphism of the genes encoding the major 25 kDa and 36 kDa outer-membrane proteins of *Brucella. Microbiology*. v. 141, p. 2111-2121, 1995.

CLOECKAERT, A.; VERGER, J.M.; GRAYON, M.; PAQUET, J.Y.; GARIN BASTUJI, B.; FOSTER, G.; GODFROID, J. Classification of *Brucella* spp. isolated from marine mammals by DNA polymorphism at the omp2 locus. *Microbes and infection,* v. 3, p. 729-738, 2001.

COSTA, E.A.; SANT'ANA, F.M.; CARVALHO, C.J.S.; MOUSTACAS, V.S.; SILVA, S.M.M.S.; PAIXÃO, T.A.; SANTOS, R.L. Diagnosis of *Brucella ovis* infection by serology and PCR in urine samples from naturally infected rams in the State of Piauí. *Arquivo. Brasileiro de Medicina Veterinária e. Zootecnia*., v. 64, n. 3, p. 751-754, 2012.

CUTLER, S. J.; WHATMORE, A. M.; COMMANDER, N. J. Brucellosis-new aspects of an old disease. *Journal of Applied Microbiology,* v. 98, n. 6, p. 1270-1281, 2005.

DE JONG, M.F.; TSOLIS, R.M. Brucellosis and type IV secretion. *Future Microbiology*. v. 7, p. 47-58, 2012.

DE MIGUEL, M.J.; MARÍN, C.M.; MUÑOZ, P.M.; DIESTE, L.; GRILLÓ, M.J.; BLASCO, J.M. Development of a selective culture medium for primary isolation of the main *Brucella* species. *Journal of Clinical Microbiology.* v.49, p.1458-1463, 2011. DI FEBO, T; LUCIANI, M.; PORTANTI, O.; BONFINI, B.; LELLI, R.; TITTARELLI, M. Development and evaluation of diagnostic tests for the serological diagnosis of brucellosis in swine. *Veterinaria Italiana*, v. 48, n. 2, p. 145-156, 2012.

DIAZ-APARICIO, E.; MARIN, C.; ALONSO-URMENETA, B.; ARAGON, V.; PEREZ-ORTIZ, S.; PARDO, M.; BLASCO, J.M.; DIAZ, R. AND MORIYON, I. Evaluation of serological tests for diagnosis of *Brucella melitensis* infection of goats. *Journal of Clinical Microbiology,* v. 32, n. 5, p.1159-1165, 1994.

EBANI, V.V.; CERRI, D.; FRATINI, F.; BEY, R.F.; ANDREANI, E. Serological diagnosis of brucellosis caused by *Brucella canis*. *New Microbiology*, v. 26, n.1, p. 65-73,2003.

EOH, H.; JEON, B.Y.; KIM, Z. KIM, S.C.; CHO, S.N. Expression and validation of D-erythrulose 1-phosphate dehydrogenase from *Brucella abortus*: a diagnostic reagent for bovine brucellosis. *Journal of Veterinary Diagnostic Investigation*, v. 22, p. 524-530, 2010.

ESEL, D.; DOGANAY, M.; ALP, E.; SUMERKAN, B. Prospective evaluation of blood cultures in a Turkish university hospital: epidemiology, microbiology and patient outcome. *Clinical Microbiology and Infection*, v. 9, p. 1038-1044, 2003.

ESTEIN, S.M.; BALDI, P.C.; BOWDEN, R.A. Comparison of serological tests based on outer membrane or internal antigens for detecting antibodies to *Brucella ovis* in infected flocks. *Journal of Veterinary Diagnostic Investigation*, v. 14, p. 407-411, 2002.

EWALT, D.R., Comparison of three culture techniques for the isolation of *Brucella abortusm* from bovine supramammary lymph nodes. *Journal of Veterinary Diagnostic Investigation,* v. 1, p. 227-230, 1989.

FEKETE, A., BANTLE, J.A., HALLING, S.M., STICH, R.W. Amplification fragment length polymorphism in *Brucella* strains by use of polymerase chain reaction with arbitrary primers. *Journal of Bacteriology*, v. 174, p. 7778-7783, 1992.

FICAPAL, A.; JORDANA, J.; BLASCO, J. M.; MORIYÓN, I. Diagnosis and epidemiology of *Brucella ovis* infection in rams. *Small Ruminant Research*, v. 29, p. 13-19, 1998.

FOSTER, G.; OSTERMAN, B.S.; GODFROID, J.; JACQUES, I.; CLOECKAERT, A. *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred host. *International Journal of Systematic and Evolutionary Microbiology*, v. 57, p. 2688-2693, 2007.

GALL, D.; NIELSEN, K.; FORBES, L.; DAVIS, D.; ELZER, P.; OLSEN, S.; BALSEVICIUS, S.; KELLY, L.; SMITH, P.; TAN, S.; JOLY, D. Validation of the fluorescence polarization assay and comparison to other serological assays for the detection of serum antibodies to *Brucella abortus* in bison. *Journal of Wildlife Disease*, v. 36, n. 3, p. 469-476, 2000.

GALL, D.; NIELSEN, K.; FORBES, L.; COOK, W.; LECLAIR, D.; BALSEVICIUS, S.; KELLY, L.; SMITH, P.; MALLORY, M. Evaluation of the fluorescence polarization assay and comparison to other serological assays for detection of brucellosis in cervids. *Journal of Wildlife Disease*, v. 37, n. 1, p. 110-118, 2001.

GALL, D.; NIELSEN, K.; VIGLIOCCO, A.; SMITH, P.; PEREZ, B.; ROJAS, X.; ROBLES, C. Evaluation of an indirect-linked immunoassay for presumptive serodiagnosis of *Brucella ovis* in sheep. *Small Ruminant Research*, v. 48, p. 173-179, 2003.

GALL, D.; NIELSEN, K. Serological diagnosis of bovine brucellosis: a review of test performance and cost comparison. *Revue Scientifique et Technique*. v. 23, n. 3, p. 989-1002, 2004.

GARCÍA-YOLDI, D.; MARÍN, C.M.; LÓPEZ-GOÑI, I. Restriction site polymorphisms in the genes encoding new members of group 3 outer membrane protein family of *Brucella* spp. *FEMS Microbiology Letters*, v. 245, n. 1, p. 79-84, 2005.

GODFROID, J.; CLOECKAERT, A.; LIAUTARD, J.P.; KOHLER, S.; FRETIN, D.; WALRAVENS, K.; GARIN-BASTUJI, B.; LETESSON, J.J. From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. *Veterinary Research.* v. 36, p. 313-326, 2005.

GODFROID, J.; NIELSEN, K.; SAEGERMAN, C. Diagnosis of brucellosis in livestock and wildlife. *Croatian Medical Journal,* v.5 1, n. 4, p. 296-305, 2010.

GOPAUL, K.K.; KOYLASS, M.S.; SMITH, C.J.; WHATMORE, A.M. Rapid identification of *Brucella* isolates to the species level by real time PCR based single nucleotide polymorphism (SNP) analysis. *BMC Microbiology*, v. 8, p. 86, 2008.

HER, M.; CHO, D.H.; KANG, S.I.; CHO, Y.S.; HWANG, I.Y.; BAE, Y.C.; YOON, H.; HEO, Y.R.; JUNG, S.C.; YOO, H. The development of a selective medium for the *Brucella abortus* strains and its comparison with the currently recommended and used medium. *Diagnostic Microbiology and Infectious Disease.* v. 67, p. 15-21, 2010.

HERMAN, L.; DE RIDDER, H. Identification of *Brucella* spp. by using the polymerase chain reaction. *Applied and Environmental Microbiology*, v. 58, n. 6, p. 2099-2101, 1992.

HILBINK, F.; WRIGHT, M.; ROSS, G. Use of the double immune gel diffusion test and the enzyme-linked immunosorbent assay to distinguish false from true reactors in the complement fixation test for *Brucella ovis*. *New Zealand Veterinary Journal*, v. 41, p. 111-115, 1993.

HINIC, V.; BRODARD, I.; THOMANN, A. Novel identification and differentiation of *Brucella melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* suitable for both conventional and real-time PCR systems. *Journal of Microbiological Methods*, v. 75, p. 375-378, 2008.

HUBER, B; SCHOLZ, H.C.; LUCERO, N. BUSSE, H.J. Development of a PCR assay for typing and subtyping of *Brucella* species. *International Journal Medical Microbiology,* v. 299, p. 563-573, 2009.

JACQUES, I.; GRAYON, M.; VERGER, J.-M. Oxidative metabolic profiles of *Brucella* strains isolated from marine mammals: contribution to their species classification. *FEMS Microbiology Letters*, v. 270, p. 245-249, 2007.

KANG, S.I.; HER, M.; KIM, J.W.; KIM, J.Y.; KO, K.Y.; HA, Y.M.; JUNG, S.C. Advanced multiplex PCR assay for differentiation of *Brucella* species. *Applied Environmental Microbiology,* v. 77, n. 18, p. 6726-6728, 2011.

KATTAR, M.M.; ZALLOUA, P.A.; ARAJ, G.F.; SAMAHA-KFOURY, J.; SHBAKLO, H.; KANJ, S. S.; KHALIFE, S.; DEEB, M. Development and evaluation of real-time polymerase chain reaction assays on whole blood and paraffin-embedded tissues for rapid diagnosis of human brucellosis. *Diagnostic Microbiology and Infectious Disease*, v. 59, p. 23-32, 2007.

KO, K.Y.; KIM, J.W.; HER, M.; KANG, S.I.; JUNG, S.C.; CHO, D.H.; KIM, J.Y. Immunogenic proteins of *Brucella abortus* to minimize cross reactions in brucellosis diagnosis. *Veterinary Microbiology*, v. 156, n. 3-4, p. 374-380, 2012.

KOVÁČOVÁ, D.; ZUBRICKÝ, P.; BABINČÁKOVÁ, M. TRÁVNIČEK, M. Importance of serological diagnostics in ovine epididymitis caused by *Brucella ovis*. *Bulletin of the Veterinary Institute in Pulawy,* v. 51, p. 219-224, 2007.

LAGE, A. P.; POESTER, F. P.; PAIXÃO, T. A.; SILVA, T. M. A.; XAVIER, M. N.,; MINHARRO, S.; MIRANDA, K. L.; ALVES, C. M.; MOL, J. P. S.; SANTOS, R. L. Brucelose bovina: uma atualização. *Revista Brasileira de Reprodução Animal*, v. 32, n. 3, p. 202-212, 2008.

LE FLÈCHE, P.; JACQUES, I.; GRAYON, M.; AL DAHOUK, S.; BOUCHON, P.; DENOEUD, F.; NOCKLER, K.; NEUBAUER, H.; GUILLOTEAU, L.A.; VERGNAUD, G. Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. *BMC Microbiology*, v. 6, p. 9, 2006.

LEAL-KLEVEZAS, D.S.; MARTINEZ-VAZQUEZ, I.O.; LOPEZ-MERINO, A.; MARTINEZ- SORIANO, J. P. Single-step PCR for detection of *Brucella* spp. from blood and milk of infected animals. *Journal of Clinical Microbiology*, v. 33, p. 3087- 3090, 1995.

LETESSON, J.J.; TIBOR, A.; EYNDE, G.; WANSARD, V.; WEYNANTS, V.; DENOEL, P.; SAMAN, E. Humoral immune responses of *Brucella*-infected cattle, sheep, and goat to eight purified recombinant *Brucella* proteins in an indirect enzyme-linked immunosorbent assay. *Clinical and diagnostic laboratory immunology*, v. 4, n. 5, p. 556-564, 1997.

LIM, J.J.; KIM, D.H.; LEE, J.J.; KIM, D.G.; MIN, W.; LEE, H. J.; RHEE, M. H.; CHANG, H. H.; KIM, S. Evaluation of recombinant 28 kDa outer membrane protein of *Brucella abortus* for the clinical diagnosis of bovine brucellosis in Korea. *Journal of Veterinary Medical Science*, v.74, n. 6, p. 687-691, 2012.

LÓPEZ-GOÑI, I.; GARCÍA-YOLDI, D.; MARÍN, C.M.; DE MIGUEL, M.J.; MUÑOZ, P. M.; BLASCO, J.M.; JACQUES, I.; GRAYON, M.; CLOECKAERT, A.; FERREIRA, A.C.; CARDOSO, R.; CORRÊA DE SÁ, M.I.; WALRAVENS, K.; ALBERT, D.; GARIN-BASTUJI, B. Evaluation of a multiplex PCR assay (Bruce ladder) for molecular typing of all *Brucella species*, including the vaccine strains. *Journal of Clinical Microbiology*, v. 46, p. 3484-3487, 2008.

LÓPEZ-GOÑI, I.; GARCÍA-YOLDI, D.; MARÍN, C.M.; DE MIGUEL, M.J.; BARQUERO-CALVO, E.; GUZMÁN-VERRI, C.; ALBERT, D.; GARIN-BASTUJI, B. New Bruce-ladder multiplex PCR assay for the biovar typing of *Brucella suis* and the discrimination of *Brucella suis* and *Brucella canis*. *Veterinary Microbiology,* v. 154, n. 1-2, p. 152-155, 2011.

LUCERO, N.; ESCOBAR, G.I.; AYALA, S.M.; SILVA, P.P.; NIELSEN, K. Fluorescence polarization assay for diagnosis of human brucellosis. *Journal of Medical Microbiology*, v.52, n.10, p. 883-887, 2003.

MANUAL TÉCNICO DO PROGRAMA NACIONAL DE CONTROLE E ERRADICAÇÃO DA BRUCELOSE E DA TUBERCULOSE – PNCEBT. Brasília, Ministério da Agricultura, Pecuária e Abastecimento/DAS, 2006. 184 p.

MAQUART, M.; LE FLECHE, P.; FOSTER, G.; TRYLAND, M.; RAMISSE, F.; DJONNE, B.; AL DAHOUK, S.; JACQUES, I.; NEUBAUER, H.; WALRAVENS, K.; GODFROID, J.; CLOECKAERT, A.; VERGNAUD, G. MLVA-16 typing of 295 marine mammal *Brucella* isolates from different animal and geographic origins identifiifies 7 major groups within *Brucella ceti* and *Brucella pinnpedialis. BMC Microbiology*, v. 9, p. 145, 2009.

MARIN, C.M.; BAGUES, M.P.J.; BLASCO, J.M. Comparison of three serological tests for *Brucella ovis* infection of rams using different antigenic extracts. *Veterinary Record*, v.125, p. 504-508, 1989.

MERT, A.; OZARAS, R.; TABAK, F.; BILIR, M.; YILMAZ, M.; KURT, C.; ONGOREN, S.; TANRIVERDI, M.; OZTURK, R. The sensitivity and specificity of *Brucella* agglutination tests. *Diagnostic Microbiology and Infectious Disease*, v. 46, n. 4, p. 241-243, 2003.

MITKA, S.; ANETAKI, S. C.; SOULIOU, E.; DIZA, E.; KANSOUZIDOU, A. Evaluation of different PCR assays for the early detection of acute and relapsing human brucellosis in comparison with conventional methods. *Journal Clinical of Microbiology* v. 45, p. 1211-1218, 2007.

MORATA, P.; QUEIPO-ORTUNO, M.I.; COLMENERO, J.D. Strategy for optimizing DNA amplification in a peripheral blood PCR assay used for diagnosis of human brucellosis. *Journal Clinical of Microbiology*; v. 36, p. 2443-2446, 1998.

MORATA, P.; QUEIPO-ORTUNO, M.I.; REGUERA, J.M.; GARCIA-ORDONEZ, M.A.; CARDENAS, A.; COLMENERO, J.D. Development and evaluation of a PCR-enzyme linked immunosorbent assay for diagnosis of human brucellosis. *Journal Clinical of Microbiology,* v. 41, p.144-148, 2003.

MYERS, D.M.; JONES, L.M.; VARELA-DIAZ, V.M. Studies of antigens for complement fixation and gel diffusion tests in the diagnosis of infections caused by *Brucella ovis* and other *Brucella*. *Applied Microbiology*, v. 23, p. 894-902, 1972.

NAVARRO, E.; FERNANDEZ, J.A.; ESCRIBANO, J.; SOLERA, J. PCR assay for diagnosis of human brucellosis. *Journal Clinical of Microbiology*, v. 37, p. 1654–1655, 1999.

NAVARRO, E.; CASAO, M.A.; SOLERA, J. Diagnosis of human brucellosis using PCR. *Expert Review of Molecular Diagnostics*, v. 4, n. 1, p.115-123, 2004.

NIELSEN, K.; GALL, D.; SMITH, P.; BALSEVICIUS, S.; GARRIDO, F.; FERRER, M.D.; BIANCIFIORI, F.; DAJER, A.; LUNA, E.; SAMARTINO, L.; BERMUDEZ, R.; MORENO, F.; RENTERIA, T.; CORRAL. A. Comparison of serological tests for the detection of ovine and caprine antibody to *Brucella melitensis*. *Revue Scientifique et Technique*, v. 23, n. 3, p. 979-87, 2004.

NIELSEN, K.; LIN, M.; GALL, D.; JOLLEY, M. Fluorescence polarization immunoassay: detection of antibody to *Brucella abortus*. *Methods*, v. 22, p. 71-76, 2000.

NIELSEN, K. Diagnosis of brucellosis by serology. *Veterinary Microbiology*, v. 90, p. 447-459, 2002.

NIELSEN, K.H.; EWALT, D.R. *Bovine Brucellosis*. In: Office International des Epizooties. Manual of Standards for Diagnostic Tests and Vaccines. 5. ed. Paris: Office international des Epizooties, 2004, p. 328-345.

OCAMPO-SOSA, A.A.; BALBÍN, J.A.; GARCIA-LOBOI, J.M. Development of a new PCR assay to identify *Brucella abortus* biovars 5, 6 and 9 the new subgroup 3b of biovar 3. *Veterinary Microbiology*, v. 110, p. 41-51. 2005.

OIE Terrestrial Manual 2009. Bovine brucellosis. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.03_BOVINE_BRUCELL.pdf. Acesso em 11 de fev. 2012. 2009a.

OIE Terrestrial Manual 2009 – Ovine epididymitis (*Brucella ovis*). http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.07.09_OVINE_EPID.pdf. Acesso em 11 de fev. 2012. 2009b.

OIE Terrestrial Manual 2009. Bovine brucellosis. Caprine and ovine brucellosis (excluding *Brucella ovis*). http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.07.02_CAPRINE_OVINE_BRUC.pdf. Acesso em 11 de fev. 2012. 2009c.

PABUCCUOGLU, O.; ECEMIS, T.; EL, S. COSKUN, A.; AKCALI, S.; SANLIDAG, T. Evaluation of serological tests for diagnosis of brucellosis. *Japanese Journal of Infectious Diseases*, v. 64, p. 272-276, 2011.

PERRETT, L.L.; MCGIVEN, J.A.; BREW, S.D.; STACK, J.A. Evaluation of competitive ELISA for detection of antibodies to *Brucella* infection in domestic animals. *Croatian Medical Journal*, v.51, n. 4, p. 314-319, 2010.

POESTER, F.P.; GONÇALVES, V.S.; PAIXÃO, T.A.; SANTOS, R.L.; OLSEN, S.C.; SCHURING, G.G.; LAGE, A.P. Efficacy of strain RB51 vaccine in heifers against experimental brucellosis. *Vaccine,* v. 24, p. 5327-5334, 2006.

POESTER, F.P.; NIELSEN, K.; SAMARTINO, L.E. Diagnosis of brucellosis. *Open Veterinary Science Journal*, v. 4, p. 46-60, 2010.

PRAUD, A.; GIMENEZ, O.; ZANELLA, G.; DUFOUR, B.; POZZI, N.; ANTRAS, V.; MEYER, L.; GARIN-BASTUJI, B. Estimation of sensitivity and specificity of five serological tests for the diagnosis of porcine brucellosis. *Preventive Veterinary Medicine*, v. 104, p. 94-100, 2012.

PROBERT, W.S.; SCHRADER, K.N.; KHUONG, N.Y.; BYSTROM, S.L.; GRAVES, M.H. Real-time multiplex PCR assay for detection of *Brucella* spp., *B. abortus,* and *B. melitensis*. *Journal of Clinical Microbiology*, v. 42, n. 3, p. 1290-1293, 2004.

QUEIPO-ORTUNO, M.I.; MORATA, P.; OCON, P.; MANCHADO, P.; COLMENERO, J.D. (1997) Rapid diagnosis of human brucellosis by peripheral blood PCR assay. *Journal Clinical of Microbiology* v. 35, p. 2927-2930, 1997.

QUEIPO-ORTUÑO, M.I.; COLMENERO, J.D.; REGUERA, J.M.; GARCÍA-ORDOÑEZ, M.A.; PACHÓN, M.E.; GONZALEZ, M.; MORATA, P. Rapid diagnosis of human brucellosis by SYBR Green I-based real-time PCR assay and melting curve analysis in serum samples. *Clinical Microbiology and Infection*, v. 11, p. 713-718, 2005.

QUEIPO-ORTUÑO, M.I.; COLMENERO, J.D.; BRAVO, M.; GARCÍA-ORDOÑEZ, M.A.; MORATA, P. Usefulness of a quantitative real-time PCR assay using serum samples to discriminate between inactive, serologically positive and active human brucellosis. *Clinical Microbiology and Infection,* v. 14, p. 1128-1134, 2008.

RAMOS-VARA, J.A. Technical aspects of immunohistochemistry. *Veterinary Pathology*, v. 42, n. 4, p. 405-426, 2005.

REFAI, M. Application of biotechnology in the diagnosis and control of brucellosis in the Near East Region. *World Journal of Microbiology & Biotechnology***,** v. 19, p. 443-449, 2003.

RIS, D.R.; HAMEL, K.L.; LONG, D.L. Comparison of an enzyme-linked immunospecific assay (ELISA) with the cold complement fixation test for the serodiagnosis of *Brucella ovis* infection. *New Zealand Veterinary journal*, v. 32, p. 18-20, 1984.

ROMERO, C.; GAMAZO, C.; PARDO, M.; LOPEZ-GONI, I. Specific detection of *Brucella* DNA by PCR. *Journal of Clinical Microbiology*, v. 33, p. 615-617, 1995.

SAM, I.C.; KARUNAKARAN, R.; KAMARULZAMAN, A.; PONNAMPALAVANAR, S.; SYED OMAR, S.F.; NG, K.P.; MOHD YUSOF, M.Y.; HOOI, P.S.; JAFAR, F.L.; ABUBAKAR, S. A large exposure *to Brucella melitensis* in a diagnostic laboratory. *The Journal of Hospital Infection*. v. 80, p. 321-325, 2012.

SANTOS, R.L.; PEIXOTO, M.T.D.; SERAKIDES, R.; COSTA, G.M.; MARTINS, N. E. Detección de *Brucella abortus* (muestra B19) por el complejo inmunoenzimático avidina-biotina-peroxidasa en el testículo y en el epidídimo de bovinos inoculados experimentalmente. *Archives Reproduction Animal*, p. 34-41, 1998.

SARAVI, M.A.; WRIGHT, P.F.; GREGORET, R.J.; GALL, D.E. Comparative performance of the enzyme-linked immunosorbent assay (ELISA) and conventional assays in the diagnosis of bovine brucellosis in Argentina. *Veterinary Immunology and Immunopathology,* v. 47, n. 1-2, p. 93-99, 1995.

SCHOLZ, H.C.; PFEFFER, M.; WITTE, A.; NEUBAUER, H.; AL DAHOUK, S.; WERNERY, U.; TOMASO, H. Specific detection and differentiation of *Ochrobactrum anthropi, Ochrobactrum intermedium* and *Brucella* spp. by a multi-primer PCR that targets the recA gene. *Journal of Medical Microbiology*, v. 57, p. 64-71, 2008.

SCHOLZ, H.C.; NÖCKLER, K.; GÖLLNER, C.; BAHN, P.; VERGNAUD, G.; TOMASO, H.; AL DAHOUK, S.; KÄMPFER, P.; CLOECKAERT, A.; MAQUART, M.; ZYGMUNT, M. S.; WHATMORE, A. M.; PFEFFER, M.; HUBER, B.; BUSSE, H. J; DE, B. K. *Brucella inopinata* sp. nov., isolated from a breast implant infection. *International journal of systematic and evolutionary microbiology*, v. 60, p. 4, p. 801-808, 2010.

SEARSON, J.E. Sensitivity and specificity of two microtitre complement fixation test for the diagnosis of *Brucella ovis* infection in rams. *Australian Veterinary Journal*, v. 58, p. 5-7, 1982.

SELEEM, M.N.; BOYLE, S.M.; SRIRANGANTHAN, N. Brucellosis: A re-emerging zoonosis. *Veterinary Microbiology*, v.110, p. 392-398, 2010.

SINGH, K. Laboratory-acquired infections. *Clinical Infection Disease*. v. 49, p.142-147, 2009.VIGLIOCCO, A.M., PAULO, P.S.S., MESTRE, J. Development and validation of the indirect enzyme immunoassay for the detection of ovine antibody to *Brucella ovis*. *Veterinary Microbiology*, v. 54, p. 357-368, 1997.

VIZCAÍNO, N.; VERGER, J. M.; GRAYON, M.; ZYGMUNT, M.S.; CLOECKAERT, A. DNA polymorphism at the omp-31 locus of *Brucella* spp.: evidence for a large deletion in *Brucella abortus*, and other species-specific markers. *Microbiology.* v.143, p. 2913-2921, 1997.

VIZCAÍNO, N.; CLOECKAERT, A.; VERGER, J.; GRAYON, M.; FERNÁNDEZ-LAGO, L. DNA polymorphism in the genus *Brucella*. *Microbes and Infection*, v. 2, n. 9, p.1089-1100, 2000.

WEST, D.M.; BRUCE, A.N. The *Brucella ovis* complement fixation test. *New Zealand Veterinary Journal*, v. 31, p.124-126, 1983.

WHATMORE, A.M. Current understanding of the genetic diversity of *Brucella,* an expanding genusof zoonotic pathogens. *Infection, Genetics and Evolution*, v. 9, n. 9, p. 1168-1184, 2009.

WINCHELL, J.M.; WOLFF, B.J.; TILLER, R.; BOWEN, M.D.; HOFFMASTER, A.R. Rapid identification and discrimination of *Brucella* isolates by use of real-time PCR and high-resolution melt analysis. *Journal of Clinical Microbiology*, v. 48, n. 3, p. 697-702, 2010.

XAVIER, M.N., COSTA; E.A., PAIXÃO, T.A.; SANTOS, R.L. Genus *Brucella* and clinical manifestations. *Ciência Rural*, v. 39, n. 7, p. 2252-2260, 2009a.

XAVIER, M.N.; PAIXÃO, T.A.; POESTER, F.P.; LAGE, A.P.; SANTOS, R.L. Pathology, immunohistochemistry and bacteriology of tissues and milk of cows and fetuses experimentally infected with *Brucella abortus*. *Journal of Comparative Pathology*, v.140, p.149-157, 2009b.

XAVIER, M.N.; SILVA, T.M.A.; COSTA, E.A.; PAIXÃO, T.A.; MOUSTACAS, V. S.; CARVALHO, C.A. JR.; SANT'ANNA, F.M.; ROBLES, C.A.; GOUVEIA, A.M.; LAGE, A.P.; TSOLIS, R.M.; SANTOS, R.L. Development and evaluation of a species-specific PCR assay for the detection of *Brucella ovis* infection in rams. *Veterinary Microbiology*, v.145, p.158-164, 2010.

XAVIER, M.N.; SANT'ANNA, F.M.; SILVA, T.M.A.; COSTA, E.A.; MOUSTACAS, V. S.; MERLO, F.A.;. CARVALHO JÚNIOR, C.A.; DASSO, M.G.; MATHIAS, L.A.; GOUVEIA, A.M.G.; LAGE, A.P., SANTOS, R.L. A comparison of two agar gel immunodiffusion methods and a complement fixation test for serologic diagnosis of *Brucella ovis* infection in experimentally infected rams. *Arquivo brasileiro de medicina veterinária e zootecnia*, v. 63, n. 4, p.1016-1021, 2011.

ZERVA, L.; BOURANTAS, K.; MITKA, S.; KANSOUZIDOU, A.; LEGAKIS, N.J. Serum is the preferred clinical specimen for diagnosis of human brucellosis. *Journal Clinical of Microbiology,* v. 39, p. 1661-1664, 2001.