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Review On Serological And Molecular Diagnostics Techniques Of Brucellosis

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ABSTRACT: Brucellosis is one of the most important zoonoses widely distributed in developing countries. Since there is no clear clinical manifestation of brucellosis, laboratory testing is critical for initiating treatments. Conventional diagnostic approaches have been developed and applied for many decades for the diagnosis and study of brucellosis, including several bacteriological and serological methods. Although these methods enabled us to understand the epidemiology and biology of Brucella species, they have different limitations. Serological methods are suffering from low specificity and sensitivity, while bacteriological methods take a longer processing time to generate test results. The recent advances in biotechnological or molecular techniques are increasingly expanding our understanding of Brucella species at the molecular level and significantly shortened the turn-around time required for testing and enabled early diagnosis. If widely applied in conjunction with conventional methods, molecular methods can facilitate control and intervention strategies in developing countries including Ethiopia. However, it is currently unclear when and how we can use combinations of available methods in different settings for different purposes. This review describes the principles and applications of conventional bacteriological and serological techniques with their strengths and limitations. Then discuss the current advances in molecular methods and their applications in the diagnosis of brucellosis in humans and animals. Moreover, we outline the future direction of Brucella diagnostics. To gain better outcomes, using serial combinations of different test methods should be recommended based on the investigations during surveillance, outbreak investigation, researches, and others.

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1. INTRODUCTION

Brucellosis is a zoonotic disease-causing high economic and public health impacts, particularly in developing countries. The disease is caused by *Brucella* species, which are facultative, intracellular, Gram-negative, non-spore-forming, and non-capsulated, partially acid-fast coccobacilli that lack capsules, endospores, or native plasmids. The bacterium is 0.5-0.7µ in diameter and 0.6-1.5µ in length. They are oxidase, catalase, and urease positive. Although *Brucella* species are described as non-motile, they carry all genes except the chemotactic system necessary to assemble a functional flagellum(Fretin *et al.*, 2005). They belong to the alpha-2 subdivision of the Proteobacteria, alongside Ochrobactrum, Rhizobium, Rhodobacter, Agrobacterium, Bartonella, and Rickettsia(Yanagi and Yamasato, 1993). Currently, there are ten species described within the genus *Brucella*. Each one may infect different host spps, but each *Brucella* spp. has a preference for its host spp.*B. melitensis*(sheep and goats), *B. abortus* (cattle), *B. suis*(pigs), *B. ovis* (sheep), *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)(De Jong and Tsolis, 2012; Hadush and Pal, 2013). Three of this *Brucella* spp. can be subdivided into biotypes (Bricker, 2002; Ocampo-Sosa *et al.*, 2005). 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).

In cattle, the disease is usually caused by Brucella *abortus*, less frequently by *Brucella melitensis,* and occasionally by *Brucella suis*(Dadar *et al.*, 2020). Conservative estimates are that >300 million of the 1.4 billion worldwide cattle population

is infected with the pathogen(Deka *et al.*, 2018). The disease in animals can generally cause significant loss of productivity through abortion, stillbirth, low herd fertility, and comparatively low milk production (Dadar *et al.*, 2020).

In humans, brucellosis is clinically characterized by an acute or insidious onset of fever and one or more of the following signs: night sweats, arthralgia, headache, fatigue, anorexia, myalgia, weight loss, arthritis, spondylitis, meningitis, or focal organ involvement (endocarditis, orchitis, epididymitis, hepatomegaly, splenomegaly)(Pal *et al.*, 2017). *B. abortus*, *B. melitensis,* and *B. suis* are highly pathogenic species for humans, and infection occurs mainly from exposure to contaminated animal products such as unpasteurized milk or contact to uterine discharges and infected tissues (AU-IBAR, 2014). Except *B. neotomae*, *B. microti*, and *B. ovis*all *Brucella* spp. are considered potentially pathogenic for humans(Xavier *et al.*, 2009; Hadush and Pal, 2013).

The diagnosis of brucellosis is difficult on clinical grounds alone and hence invariably based on microbiological and serological laboratory tests(Shenoy *et al.*, 2016). Microbiological (cultural) examinations are the "gold standard" remaining isolation and identification of the bacterium but time-consuming, hazardous, and not sensitive(Al Dahouk *et al.*, 2013). Despite the vigorous attempt for more than one century to come up with a definitive diagnostic technique for brucellosis, the diagnosis still relies on the combination of several tests to avoid false-negative results(Yagupsky *et al.*, 2019). However, therapid development of biotechnological techniques has resulted in animproved understanding of *Brucella* diversity at the molecular level. For instance, high-resolution molecular approaches have been developed for *Brucella* speciation, bio-typing, and illuminate new light to the *Brucella diversity*(Le Flèche *et al.*, 2006; Lopez-Goñi *et al.*, 2008).

The availability of complete genome sequences and the advancement of genomics and proteomics have enabled scientists to understand the disease and its pathogenic mechanisms(Christopher *et al.*, 2010). Thus, the application of these methods in endemic areas may enable the early detection and characterization of *Brucella*species,which is critical for disease prevention and control mechanisms. Therefore, this systematic review summarizes the current state of knowledge and skills in serological and molecular diagnostic techniques and outlines the future directions of brucellosiswith particular emphasis on molecular diagnostic methods.

2. BIOLOGY OF BRUCELLA SPECIES

2.1 Structural and antigenic characteristics

Brucella follows a Gram-negative architecture: a cytoplasm encased in a cell envelope made of an inner membrane, a periplasm, and an outer membrane (OM). The OM contains free lipids, proteins (Omp), and lipopolysaccharide (LPS). Lps is the dominant OM molecule and is critical in *Brucella*'s virulence and as an antigen. *B. ovis*and *B. canis* have a rough type LPS (R-LPS) made of a lipid A (containing two types of aminoglycosides) linked to an oligosaccharide, while other *Brucella* spp. have a smooth (S) type LPS with an O-polysaccharide linked to the oligosaccharide(Moreno, 2020)(Corbel, 1997). This is manifested in the surface of the colonies: R in *B. ovis* and *B. canis* and S in other *Brucella*spps. The S-*brucella* can dissociate to yield mixtures of S and R colonies and cells as a result of mutations affecting the O-polysaccharide. Dissociation hampers species identification and its control is essential in vaccine and antigen production (Manual, 2009; Moreno, 2020). *Brucella* O-polysaccharides create three basic epitopes: A (A= *Abortus* 5 contiguous sugars in α 1- 2 linkages); C (A=M; common to all S-brucella); and M=*Melitensis.* They are distributed in various proportions among S species and biovars so that neither A nor M is characteristic of *B. abortus* and *B. melitensis,* respectively(Baljinnyam, 2014). In addition to the S-LPS, S-*brucella* produces a free polysaccharide called native hapten (NH)(Nicoletti *et al.*, 1967; Moreno, 2020). Bacteria cross-reacting with S-*brucella* include *Stenotrophomonas maltophilia*, group N (0:30), *Salmonella*spp, *Vibrio cholerae, E.coli* 0:157, some *Escherichia hermanii* strains, and *Yersinia enterocolitica* 0:9. The soluble fraction proteins are common to all except the S-LPS cross-reacting bacteria, which make them useful for discriminating *Brucella* spp. infections from false-positive serological reactions caused by the latterMoreno (2020) and table1: Brucella species, biovars, colony morphology, preferential hosts, and pathogenicity in humans adapted from.*(Godfroid et al., 2010).*

Table 2: Brucella species, biovars, colony morphology, preferential hosts, and pathogenicity in humans adapted from. *(Godfroid et al., 2010)*

Species	biovars	Colony morphology	Preferential host(s)	Pathogenicity in humans
B.melitensis	1-3	Smooth	Sheep, goat	High
B.abortus	1-6,9	Smooth	Cattle	High
	1,3	Smooth	Pig	High
B.suis	2	Smooth	Wild boar, hare	High
	4	Smooth	Reindeer, caribou	High
	5	Smooth	Rodent	No
B.neotomoe	-	Smooth	Desert rat	Moderate
B.ovis	-	Rough	Ram	No
B.canis	-	Rough	Dog	Moderate
B.pinnipedialis	-	Smooth	Seal	?
B.ceti	-	Smooth	Cetacean	?
B.microti	-	Smooth	Soil, vole, fox	?
B.inopinota	-	Smooth	Human	?

2.2. Genome of Brucella

The traditional view on *Brucella* taxonomy was challenged some time ago based on the high level of genetic relatedness indicated by DNA hybridization experiments(VERGER *et al.*, 1985). This genetic conservation has since been confirmed by a variety of approaches including multilocal enzyme electrophoresis (MLEE) (Gándara *et al.*, 2001) and 16S rRNA sequencing(Gee *et al.*, 2004). Reflecting this, a comparison of single nucleotide polymorphisms (SNPs) present in three complete *Brucella* genome sequences (representing three distinct classical species) indicates a mean diversity between genomes of around 0.22%(Halling *et al.*, 2005). It was proposed that only one species, *B. melitensis*, should be recognized within the genus Brucella (Corbel, 1997). However, reflecting practical considerations, this feature has not found widespread support with most opting to retain the none species designation.

The genetic conservation within Brucella has resulted in past difficulties in determining the true relationships between some classical Brucella species and biovars and in defining molecular markers for some groups. For instance, *B. canis* closely related to *B. suis*based on chromosomal maps(Michaux-Charachon *et al.*, 1997), *omp* profiling (Cloeckaert *et al.*, 2001), MLEE(Gándara *et al.*, 2001), AFLP (Whatmore *et al.*, 2006), and insertion sequence typing (Ouahrani *et al.*, 1993), and its status as a distinct species has been questioned. Similarly, Similarly, experiments using AFLP and MLEE have shown that *B. suis* biovar 5 is different from other *B. suis* isolates (Gándara *et al.*, 2001; Whatmore *et al.*, 2006) and therefore it is not clear if there is reason for adding *B. suis* biovar 5 in a taxonomic community with *B. suis*. Indeed, the status of *B. suis* as a single genus has been debated in terms of greater host specificity, and since, in comparison to other classical species, no species-specific markers for *B. suis* have been identified(Moreno *et al.*, 2002).

The sequencing of multiple genetic loci in bacteria, usually but not exclusively housekeeping genes, multilocal sequence typing (MLST) has rapidly gained acceptance as a tool for the characterization of microbial populations. The approach has been applied widely to microbial typing and epidemiological studies at global levels as well as generating data that is ideal for studies of population structure and phylogenetic relationships (Urwin and Maiden, 2003). In light of the conserved nature of the *Brucella* genomes, MLST is likely to be of little value for local epidemiological studies. Tools such as variable number tender repeat (VNTR) based typing (Bricker *et al.*, 2003; Le Flèche *et al.*, 2006; Whatmore *et al.*, 2006), indexing variation of more rapidly evolving markers, are likely to be far more informative in such scenarios. Ten gene sequences describing five species of *Brucella* (*B. melitensis, B. suis, B. abortus, B. ovis,* and *B. canis*) are available and approximately 25 additional Brucella strains/species are being sequenced. The genomes of the members of *Brucella* are very similar in size and gene makeup(Sriranganathan *et al.*, 2009) Each species within the genus has an average genome size of approximately 3.29Mb and consists of two circular chromosomes, Chromosome I which is approximately on average 2.11 Mb, and Chromosome II is approximately 1.18 Mb. The G + C content of all *Brucella* genomes is 57.2% for Chromosome I, and 57.3% for Chromosome II (Halling *et al.*, 2005).

The *Brucella* have no classical virulence genes encoding capsules, plasmids, pili, or exotoxins, and compared to other bacterial pathogens, relatively little is known about the factors contributing to the persistence in the host and multiplication within phagocytic cells. Moreover, many aspects of the interaction between *Brucella* and its host remain unclear (Seleem *et al.*, 2008).

3. DIAGNOSTICS OF BRUCELLOSIS

3.1 Isolation and identification

The "gold standard" of brucellosis diagnosis is the direct bacteriological testing, cultivation of Brucella, isolated from body fluids (blood, cerebrospinal fluid, urine, and others) or tissues (Yagupsky, 1999). However, Brucellais one of the slow-growing organisms and the culture result may not become available for several days or weeks. Besides, the bacteria also need special media with carboxyphilic environment(Sabour *et al.*, 2020). The modern automated blood culture systems have improved the speed of detection but are still too slow to make a rapid diagnosis(Bannatyne *et al.*, 1997). Bone marrow cultures are considered the golden norm for the diagnosis of brucellosis because the comparatively high abundance of *Brucella* in the reticuloendothelial system makes it easy to diagnose the organism. Furthermore, bacterial removal from the bone marrow is similar to microbial eradication (Pappas *et al.*, 2006). However, in some studies the findings have not been uniformly reproducible, indicating that bacteremia is as unstable as clinical manifestations, particularly in human brucellosis (Shehabi *et al.*, 1990).

The staining of bacterial isolates using the stamp method is that the classical direct method of brucellosis diagnosis(Porter *et al.*, 2011). It consists of the essential fuchsine staining followed by de-colorization with diluted ethanoic acid.Even though this method is nonspecific and some other pathogenic bacteria such as *Chlamydophilaabortus* and *Coxiella burnrti*i will be colored similarly, it is often used to obtain preliminary results(Porter *et al.*, 2011).

Identification of *Brucella* strains was done using standard classification tests, including Gram stain, modified Ziehl-Neelsen (ZN) stain, growth characteristics, oxidase activity, urease activity, H_2S production (four days), dye tolerance such as basic fuchsin (1: 50000 and 1: 100000) and thionin (1:25000, 1:50000 and 1:100000) and sero-agglutination(Urwin and Maiden, 2003). Laboratory detection of Brucella and species recognition is focused largely on culture isolation and phenotypic characterization (Yagupsky *et al.*, 2019). This phase is extensive and labor-intensive and has been associated with a heightened risk of laboratory-acquired infections. To surmount these problems, nucleic acid amplification has been explored for rapid detection and confirmation(Mantur *et al.*, 2006).

Bio-typing of *Brucella* species, isolated from biological samples, provides significant epidemiological data that allow tracing the focus of infection and the ways of its spread(Raghava *et al.*, 2017). Classical bio-typing of *Brucella* species is made based on phenotypic differences of surface lipopolysaccharide (LPS) antigens, sensitivity to staining, CO_2 dependence, H_2S production, and other metabolic properties, phage lysis, as well as the ability to grow in the presence of alkaline fuchsine or thionine (Pappas, 2005). Until, the methods of agglutination with antibodies against rough or smooth LPS, such as agglutination with antibodies against the A and M epitopes of the O-polysaccharide chain, were widely used for bio-typing. However, there is a cross-reaction with the epitopes of the surface LPSs from bacteria of some other genera, such as the type of *Yersinia*. Besides, *Brucella* is a highly homomorphic genus and classical typing methods do not allow to differentiate isolates of the same species and biovars(VERGER *et al.*, 1985; Cardoso *et al.*, 2006).Moreover, the classical methods of bio-typing require systematic methods of study and highly trained professionals to perform them and hence are kept almost exclusively in reference laboratories.

3.2 Serological methods

Despite the availability of several methods of serological diagnosis of brucellosis, none of these tests are 100% reliable, and hence serological test results should always be interpreted in conjunction with patient history, clinical manifestations, and other laboratory findings(Sabour *et al.*, 2020). The serological tests like Complement Fixation Test (CFT), Serum Agglutination Test (SAT), Enzyme-Linked Immuno-Sorbent Assay (ELISA) detect antibodies against the S-LPS antigentable 3(Ducrotoy *et al.*, 2016). Most of these methods in animals have been initially developed for the testing of cattle and then were used to test domestic goats and sheep (except for the analysis of milk), and later were adapted for the monitoring of certain species of wild animals(Godfroid *et al.*, 2010).The most widely used serological tests are focused on the identification of antibodies against smooth surface LPS, as they are immunodominant antigens of Brucella. For the precise diagnosis of *B.ovis* and *B.canis* infection, antibodies against rough LPSs of Brucella are describedby Godfroid *et al.* (2010) and table *4 :*

Sensitivity and specificity of indirect tests for the diagnosis of cattle brucellosis (Diab et al.*; Mahmoud and Ahmed; Godfroid* et al.*, 2010; Madboly* et al.*, 2014).*

3.2.1 Rose Bengal plate test (RBPT)

It is often used as a rapid screening test. The sensitivity is very high (>99%), but the specificity is disappointingly as low as 68.8%(Pfukenyi *et al.*, 2020). However, this is of value as a screening test in high-risk rural areas where it is not always possible to perform the tube agglutination titration test. It uses a suspension of *B. abortus* smooth cells stained with the Rose Bengal dye. These tests have been introduced in many countries as the standard screening test because it is very simple and thought to be more sensitive than the SAT(Greiner *et al.*, 2009). The OIE considers these tests prescribed tests for trade (Manual, 2009).

3.2.2. Milk ring test (MRT)

The test consists of blending colored Brucella 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 purple ring on the surface. In the absence of antigen-antibody complexes, the cream remains colorless. This test isn't considered sensitive, but this lack of sensitivity is compensated by the very fact that the test is often repeated, usually monthly, thanks to its very low cost. This test is prescribed by the OIE for use only with cow milk(Manual, 2009).

3.2.3 Anti-globulin (Coombs) test

The Coombs test was used to confirm SAT results from animals that give negative, suspicious, or non-conclusive responses(Farina, 1985). It is a useful test in the epidemiological survey of brucellosis because of the advantage of detecting incomplete antibodies of the IgG type that combine with cellular antigens but do not give rise to an agglutination reaction (MacMillan *et al.*, 1990). The test has been adapted to a microtiter plate set-up to save time (Otero *et al.*, 1982). The main limitation of the test is that it is not recommended for testing vaccinated animals (Farina, 1985; MacMillan *et al.*, 1990).

3.2.4. Complement Fixation Test (CFT)

The Complement Fixation Test (CFT) allows the detection of anti-Brucella antibodies that can activate complement. Cattle immunoglobulins (Ig) that can activate bovine complement are IgG and IgM. The CFT is very specific but it's laborious and requires highly trained personnel also as suitable laboratory facilities that make it less suitable to be used in developing countries. According to some literature, this test is not highly sensitive but shows excellent specificity(Emmerzaal *et al.*, 2002). Because the test is difficult to standardize, it is progressively being replaced by ELISAs (Manual, 2009). This test is a "prescribed test for trade" by the OIE Manual (2009) and table 5.

3.2.5. Serum Agglutination Test (SAT)

Developed by Wright and colleagues remains the foremost popular and yet used worldwide diagnostic tool for the diagnosis of brucellosis because it's easy to perform, doesn't need expensive equipment and training. SAT measures the total number of agglutinating antibodies IgM and IgG(Young, 1991). The amount of specific IgG was determined by treatment of the serum with 0.05M 2-mercaptoethanol (2ME), which inactivates the agglutin ability of IgM. SAT titers above 1:160 are considered diagnostic in conjunction with a compatible clinical presentation. However, in areas of endemic, employing a titer of 1:320 as cut-off may make the test more specific. The differentiation within the sort of antibody is additionally important, as IgG antibodies are considered a far better indicator of active infection than IgM, and therefore the rapid fall within the level of IgG antibodies is said to be prognostic of successful therapy(Buchanan and Faber, 1980).

3.2.6. Indirect Enzyme-linked Immune Sorbent Assay (I-ELISA)

Typically, it uses cytoplasmic proteins as antigens. ELISA measures IgM, IgG, and IgA, which allows for a far better interpretation of the clinical situation. A comparison with the SAT ELISA yields higher sensitivity and specificity. ELISA is also reported to be the most sensitive test for the diagnosis of central nervous system brucellosis. Among the newer serologic tests, the ELISA appears to be the foremost sensitive; however, more experience is required before it replaces the SAT because the test of choice for brucellosisby Bulashev *et al.* (2020) and table 6. O-polysaccharides of *Brucella* are similar to those of *Yersinia enterocolitica* and other bacteria. It leads to false-positive results and thus reduces the specificity of the test(Pardon *et al.*, 1990; Nielsen *et al.*, 2004; Lopez-Goñi *et al.*, 2008).Partly this problem is solved in the competitive ELISA (cELISA), where specific epitopes of Brucella O-polysaccharides are used as antigens, but the sensitivity of cELISA is

significantly lower than the IELISA(Nielsen *et al.*, 2004).

3.2.7. Lateral Flow Assay (LFA)

The LFA is a simplified ELISA for the qualitative detection of antigen-specific antibodies in serum, milk, or whole blood samples(Christopher *et al.*, 2010). The assay is based on the binding of specific antibodies to the antigen immobilized on a test strip (cellulose membrane matrix). It allows the detection of specific IgM, as well as specific IgG antibodies, and that high sensitivity is assured for all stages of the disease(Yu and Nielsen, 2010). Application of the assay does not require specific expertise, equipment, or electricity, and test kits may be kept in stock without the need for refrigeration, thus, making the assay a very useful one for poor resource countries including most African countries and migratory herds/flocks(Abdoel *et al.*, 2008; Baddour, 2012). However, its interpretation is subjective, depending on the formation of a visible colored line of reaction, and the assay itself tends to be expensive because of the multiple ingredients/components involved(Yu and Nielsen, 2010).

3.2.8. Fluorescence polarization assay (FPA)

It is based on the physical principle of the mass-dependent change of the molecule's rotation speed in a liquid medium. The bigger the particle, the more it rotates and the depolarization of a polarized beam of light happens. In FPA, the serum sample is incubated with a particular Brucella antigen, conjugated with a fluorescent marker. In case there are anti-Brucella antibodies in the serum, a large fluorescently labeled antigen-antibody complex is produced, which can easily be separated from the unbound antigen negative regulation. FPA method has high precision but less sensitivity than ELISA (McGiven *et al.*, 2003).In Europe and the USA, the FPA method is used in systems to detect and manage the spread of brucellosis, but it requires special equipment and it is not ideal for quick and simple monitoring.

The Brucella specific seropositive response is the proof of the infection, but it doesn't include much detail about the nature of Brucella species, the time of infection, the severity of the disease, or even that the animal has the disease at the time of sample collection because the antibody titers can be very large for a long period after the acute phase of the disease in the acute process of brucellosis, the IgM antibodies are mainly produced, and then, after a brief time, IgG antibodies are produced(Padilla Poester *et al.*, 2010).The number of IgG antibodies could also be reduced after treatment, however, a high level of IgG antibodies circulating within the blood can continue the absence of the acute phase of the disease. In the chronic form of brucellosis, IgG antibodies dominate in blood samples, while IgM antibodies are not detected or found only in small amounts. Most iELISA methods predominantly detect IgG and its subclasses, and Wright's reaction mainly detects IgM (Godfroid *et al.*, 2010).

Thus, using the mixture of those methods, i t's possible to get the kinetics of the immune reaction and to differentiate between acute and chronic phases of the disease. Another group of tests is allergic or skin-allergic tests for brucellosis (Padilla Poester *et al.*, 2010). It identifies specific cellular immune responses to the under-the-skin administration of *Brucella* antigen. This test confirms the actual cases of brucellosis and allows distinguishing them from the false-positive results of other tests (Padilla Poester *et al.*, 2010).

Table 7: Sensitivity and specificity of indirect tests for the diagnosis of cattle brucellosis (Diab et al.*; Mahmoud and Ahmed; Godfroid* et al.*, 2010; Madboly* et al.*, 2014)*

Serological tests	Sensitivity (%)	Specificity (%)
SAT(SAW)/MAT	81.5	98
CFT	90-91.8	99.7-99.9
BAT	87	97.8
iELISA	97.2	97.1-99.8
cELISA	95.2	99.7
FPA	96.2	99.1
MRT	88.5	77.4
FPA	76.9	100
Skin test	78-93	99.8

3.3. Molecular methods

3.3.1. An overview of Molecular approaches

To avoid the difficulties of bacteriological testing, molecular techniques, often based on polymerase chain reaction (PCR) amplification are successfully used for Brucella identification and typing (Yu and Nielsen, 2010). Initially, PCR-based identification has been developed for the determination of bacterial isolates (Ouahrani‐Bettache *et al.*, 1996),but now these methods also are also used for the detection of *Brucella* species in clinical samples of humans and animals (Baddour and Alkhalifa, 2008). The foremost simple and reliable method of Brucella identification is PCR with a single pair of primers, specific to bacterial DNA sequences, such as the 16S - 23S rRNA operon, IS711, or BCSP31 genes(Baddour and Alkhalifa, 2008; Godfroid *et al.*, 2010).Using a combination of several primer pairs for amplification of BCSP31, OMP2B, OMP2A, OMP31 genes, encoding external membrane proteins, it is possible to identify four Brucella species: *B.melitensis, B.suis, B. abortus,* and *B.canis*(Imaoka *et al.*, 2007). Another method, based on the combination of seven PCR reactions, allows discrimination between six Brucella species (Hinić *et al.*, 2008). There are PCR methods for the identification of some *Brucella abortus* biovars stated by Leal-Klevezas *et al.* (2000) and distinguishing between S19 and RB51 strains of *Brucella abortus*, used for vaccination against pathogenic strains(Sangari and Agüero, 1994).A more effective method of diagnosis and identification of Brucella is multiplex PCR. The identification of *Brucella* was precisely performed with various PCR assays like AMOS, Multiplex, and real-time PCR (Sabour *et al.*, 2020).

3.3.2 AMOS PCR Assay for Brucella

*The first multiplex PCR-based test for Brucella detection was developed in 1994(Bricker and Halling, 1994). It allowed the identification of four Brucella species (*B. abortus, B. melitensis, B. ovis, *and* B. suis*) and was named AMOS PCR for the first letter of species name listed in table 8. AMOS PCR identifies only a couple of biovars of every of the four species and can't distinguish individual biovars of an equivalent species. Later on, this method has been improved to detect more biovars and identify Brucella S19 and RB51 vaccine strains (Bricker* et al.*, 2000; Ocampo-Sosa* et al.*, 2005). However, the disadvantage of this PCR was that not all species could be identified (i.e., B.* canis *and* B. neotomae*) and that some biovars within a given species gave negative resultsin table* Table 9**:** The molecular markers employed in *Brucella* AMOS PCR assay Adopted from(Gupta *et al.*, 2014).

Table 10**:** The molecular markers employed in *Brucella* AMOS PCR assay Adopted from (Gupta *et al.*, 2014).

S/n	Species-specific primer	Primer sequence (5' ▶ 3')	Size of amplicon (bp)
1	*B. abortus*	F: GACGAACGGAATTTTTCCAATCCC R: TGCCGATCACTTAAGGGCCTTCAT	498
2	*B. melitensis*	F: AAATCGCGTCCTTGCTGGTCTGA R: TGCCGATCACTTAAGGGCCTTCAT	731
3	*B. ovis*	F: CGGGTTCTGGCACCATCGTCG R: TGCCGATCACTTAAGGGCCTTCAT	976
4.	*B. suis*	F: GCGCGGTTTTCTGAAGGTTCAGG R: TGCCGATCACTTAAGGGCCTTCAT	285
Additional oligonucleotides for vaccine strains differentiation			
5	RB51/2308	F: CCCCGGAAGATATGCTTCGATCC R: TGCCGATCACTTAAGGGCCTTCAT	364(2308strains) 498 (RB51)
6	eri primers	F: GCGCCGCGAAGAACTTATCAA R: CGCCATGTTAGCGGCGGTGA	178 eri

A= adenine C= Cysteine G= Guanine T=Thymine F= Forward sequence R= Reverse sequence bp= base pair

3.3.3. Multiplex PCR for one-step identification of Brucella spp. (Bruce–Ladder)

For the rapid and one-step identification of Brucella, a novel multiplex PCR assay (Bruce–ladder) has been developed(García-Yoldi *et al.*, 2006). It provides identification of all known *Brucella* species, including pathogens of marine mammals, at the species or maybe biovars level by using certain combinations of primer pairs. This multiplex PCR assay has the cutting-edge advantage compared to the previously described PCR assays, for the identification and differentiation of most *Brucella* spp. including vaccine strains in a single tube *table 11: The molecular markers employed in the multiplex PCR assay (Bruce–ladder)Adopted from Gupta* et al. *(2014).*

Table 12: The molecular markers employed in the multiplex PCR assay (Bruce–ladder) Adopted from Gupta et al. *(2014)*

Molecular targets	Primers sequences (5' 3')	Size of amplicon (bp)
Glycosyltransferase, gene wboA	F: ATC CTA TTG CCC CGA TAA GG R: GCT TCG CAT TTT CAC TGT AGC	1682
Immunodominant antigen, gene bp26	F: GCG CAT TCT TCG GTT ATG AA R: CGC AGG CGA AAA CAG CTA TAA	450
Outer membrane protein, gene omp31	F: TTT ACA CAG GCA ATC CAG CA R: GCG TCC AGT TGT TGT TGA TG	1071
Outer membrane protein OMP-2	F: GCG CTC AGG CTG CCG ACG CAA R: ACC AGC CAT TGC GGT CGG TA	193
Polysaccharide deacetylase	F: ACG CAG ACG ACC TTC GGT AT R: TTT ATC CAT CGC CCT GTC AC	794
Erythritol catabolism, gene eryC (derythrulose-1-phosphate dehydrogenase)	F: GCC GCT ATT ATG TGG ACT GG R: AAT GAC TTC ACG GTC GTT CG	587
ABC transporter binding protein	F: GGA ACA CTA CGC CAC CTT GT R: GAT GGA GCA AAC GCT GAA G	272
Ribosomal protein S12, gene rpsl	F: CAG GCA AAC CCT CAG AAG C R: GAT GTG GTA ACG CAC ACC AA	218
Transcriptional regulator, CRP family	F: CGC AGA CAG TGA CCA TCA AA R: GTA TTC AGC CCC CGT TAC CT	152

A= adenine C= Cysteine G= Guanine T=Thymine F= Forward sequence R= Reverse sequence bp= base pair

3.3.4. Real-time PCR

Real-time PCR is quicker and more fragile than conventional PCR. It doesn't require post-amplification handling of PCR products, thereby reducing the danger of laboratory contamination and false-positive results. Real-time PCR assays have been recently described to test *Brucella* cells, urine (Queipo-Ortuño *et al.*, 2005), blood, and paraffin-embedded tissues(Kattar *et al.*, 2007).

To precisely recognize seven biovars of B, three distinct real-time PCRs were created. Three biovars of B. *abortus, B. melitensis,* and one B.suis biovar. Using the conversion of fluorescence resonance energy. The upstream primers used are extracted from the insertion factor, IS711, in these real-time PCRs, while the reverse primer and FRET probes are chosen from unique species or biovar-specific chromosomal loci. The sensitivity of B. abortus -specific assay was as low as 0.25 pg DNA like 16-25 genome copies and similar detection levels were also observed for *B. melitensis* and *B. suis* specific assaysRedkar *et al.* (2001) and *table 13: The molecular targets employed in routine Brucella PCR assay, Adopted from(Gupta* et al.*, 2014).*

Table 14: The molecular targets employed in routine Brucella PCR assay, Adopted from(Gupta *et al.*, 2014).

Molecular targets	Primers sequences (5' 3')	Size of amplicon (bp)
Outer membrane protein OMP-2 of Brucella spp.	F: GCG CTC AGG CTG CCG ACG CAA R: ACC AGC CAT TGC GGT CGG TA	1**93**
Single-step PCR for *B.abortus* protein BCSP31	F: TGG CTC GGT TGC CAA TAT CAA R: CGC GCT TGC CTT TCA GGT CTG	223
16S rRNA detection of *B.abortus*	F: TCG AGC GCC CGC AAG GGG R: ACC ATA GTG TCT CCA CTA A	905
***Brucella abortus* vaccine RB51 detection**		
For *wboA* gene	F: TTA AGC GCT GAT GCC ATT TCC TTC AC R: GCC AAC CAA CCC AAA TGC TCA CAA	-1300bp (RB51) approx 400 bp
For *wboA* gene with part of IS711	F: TTT AGT TTG CCG TAA TAT AGG TCT AGA ACC TGT C R: GCC AAC CAA CCC AAA TGC TCA CAA	900

A= adenine C= Cysteine G= Guanine T=Thymine F= Forward sequence R= Reverse sequence bp= base pair

3.3.5. High-resolution melt

The development of a molecular technique that utilizes real-time PCR followed by high-resolution melt (HRM) curve analysis to reliably type members of this genus has been described by Winchell *et al.* (2010).The assay targeted discriminating loci within the genomes of Brucella spp. and through the dissociation curve analysis, allowed the accurate identification of Brucella isolates at the species level and of bizarre Brucella isolates like BO1 and BO2.This assay also proved successful for discriminating *B.suis* from *B. canis*, but was unable to accurately differentiate a *B.suis* biovar 4 from *B. canis*. However, this particular *B. suis biovar* has previously been reported to exhibit a genotypic pattern just like*B. canis* and it is still debated as to whether this is the truly unique biovar of *B. suis*(Whatmore *et al.*, 2007; Huynh *et al.*, 2008).

Other methods of PCR-based identification of Brucella include a multi-locus analysis of genome regions with a variable number of tandem repeats (MLVA) (Bricker *et al.*, 2003) and multi-locus sequencing of genome regions of bacterial isolates (MLSA)(Le Flèche *et al.*, 2006).These approaches are based on the quantification of the number of tandem repeats in a single locus of the bacterial genome and are used not only at the level of genus and organisms, but also at the level of biovars for Brucella genotyping.

3.3.6. Restriction Fragment Length Polymorphism Based Approaches

PCR-restriction fragment length polymorphism (PCR-RFLP) may be a common approach for typing Brucella spp., providing an honest tool for taxonomic, epidemiological, evolutionary, and diagnostic studies. The method has especially been utilized in studies of various outer membrane protein (omp) genes(Dahouk *et al.*, 2005).

3.3.7. Single Nucleotide Polymorphisms Typing

Single nucleotide polymorphisms (SNPs) represent powerful markers that allow accurately describing the phylogenetic structure of a population, especially in a genetically conserved group as Brucella. The method is focused on a sequence of discrimination assays interrogating SNPs that are seen to be unique to a particular Brucella spp.(Scott *et al.*, 2007)identified the use of SNPs to establish a multiplex SNP detection assay based on primer extension technology that can easily and unambiguously classify an isolate as a member of one of the six classical Brucella spp. Or as a member of the marine mammal group that has recently been described. An alternative method was identified based on minor groove binding protein (MGB) samples added to a real-time PCR platform(Foster *et al.*, 2008).Both members of the classical species are differentiated by the assay, but the *B. suis*distinction and *B. canis* was difficult as no *B. suis* specific SNP has been identified. However, as a specific *B. canis* SNP has been identified(Whatmore *et al.*, 2007), it is possible discrimination with *B. suis*, *B. canis* specific SNP and the *B. canis* specific SNP(Whatmore, 2009).

3.3.8. Tandem repeat-based typing

In the last years, the availability of microbial genome sequences has facilitated the development of multilocal sequence-based typing approaches such as multiple-locus variable number of tandem repeats (VNTR) analysis (MLVA). The VNTR, allelic hypervariability related to variation in the number of tandemly repeated sequences observed at several genomic loci in the *Brucella* genomes, were used for the discrimination of bacterial species that display very little genomic diversity(Whatmore, 2009).

The first application of VNTR based typing to *Brucella* was the HOOF-Prints scheme (Hyper Variable Octomeric Oligonucleotide Finger-Prints) published (Bricker *et al.*, 2003; Manual, 2009). The approach was based on a comparison of the newly completed genome sequences of *B. suis* and *B. melitensis* along with a draft *B. abortus* sequence which identified an eight-base pair tandem repeat sequence at nine distinct genomic loci(Whatmore, 2009). Although up-to-date PCR-based methods of Brucella identification and genotyping have several advantages in comparison with classical bacteriological methods, they even have some significant problems. The sensitivity and accuracy of PCR-based methods strongly depend upon the methods of DNA isolation and therefore the quality of the isolated DNA (especially for multiplex PCR). There remains the problem of false-negative results because the PCR is inhibited in the presence of some admixtures, such as EDTA, RNAASES, DNAASE, heparin, phenols, urea, and many others, in clinical samples or DNA isolation and purification procedures. False-positive results may also occur as a result of sample contamination. It is further necessary to develop positive and negative controls and standardize the conditions for PCR reactions with clinical samples.

4. CONCLUSION

Brucellosis is one of the most important zoonotic trans-boundary animal diseases in developing countries including Ethiopia. Several biological, serological, and molecular diagnostic methods have been developed and implemented. Each of the approaches has its own benefits and limitations. One of the major limiting factors for the implementation of comprehensive brucellosis control and prevention strategies is the lack of affordable, and effective diagnostic techniques. However, the advances in molecular techniques and their applications in brucellosis research are increasingly expanding knowledges of *Brucella* species genetic diversity and new light on the molecular epidemiology of the disease and high sensitivity and specificity compared to serological tests. Generally, the development of modern diagnostic techniques of brucellosis is associated with an easytouse point of health care for diagnosis of the disease accurately for further treatment, prevention, and control mechanisms. Finally, researchers, research centers, and other stakeholders may concern to do increasingly on the diagnostic techniques of brucellosis to come up with economic and social problems.

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5. REFERENCES

[1]. Abdoel, T., Dias, I.T., Cardoso, R., and Smits, H.L., 2008. Simple and rapid field tests for brucellosis in livestock. *Vet. Microbiol.,* 130, 312-319.

[2]. Al Dahouk, S., Sprague, L., and Neubauer, H., 2013. New developments in the diagnostic procedures for zoonotic brucellosis in humans. *Rev Sci Tech.* 32, 177-188.

[3]. Baddour, M., 2012. Diagnosis of brucellosis in humans: a review. *J Vet Adv.* 2, 149-156.

[4]. Baddour, M.M., and Alkhalifa, D.H., 2008. Evaluation of three polymerase chain reaction techniques for detection of Brucella DNA in peripheral human blood. *Canadian journal of microbiology.* 54, 352-357.

[5]. Baljinnyam, Z. 2014. Molecular epidemiology of animal and human brucellosis in Mongolia. 2014, (University_of_Basel,

[6]. Bannatyne, R.M., Jackson, M.C., and Memish, Z., 1997. Rapid diagnosis of Brucella bacteremia by using the BACTEC 9240 system. *Journal of clinical microbiology.* 35, 2673.

[7]. Bricker, B.J., 2002. Diagnostic strategies used for the identification of Brucella. *Veterinary microbiology (Amsterdam).* 90, 433-434.

[8]. Bricker, B.J., Ewalt, D.R., and Halling, S.M., 2003. Brucella'HOOF-Prints': strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). *BMC microbiology.* 3, 15.

[9]. Bricker, B.J., Ewalt, D.R., MacMillan, A.P., Foster, G., and Brew, S., 2000. Molecular characterization of Brucellastrains isolated from marine mammals. *Journal of Clinical Microbiology.* 38, 1258-1262.

[10]. Bricker, B.J., and Halling, S.M., 1994. Differentiation of Brucella abortus bv. 1, 2, and 4, Brucella melitensis, Brucella ovis, and Brucella suis bv. 1 by PCR. *Journal of clinical microbiology.* 32, 2660-2666.

[11]. Buchanan, T.M., and Faber, L., 1980. 2-mercaptoethanol Brucella agglutination test: usefulness for predicting recovery from brucellosis. *Journal of Clinical Microbiology.* 11, 691-693.

[12]. Bulashev, A., Akibekov, O., Syzdykova, A., Suranshiyev, Z., and Ingirbay, B., 2020. Use of recombinant Brucella outer membrane proteins 19, 25, and 31 for serodiagnosis of bovine brucellosis. *Veterinary World.* 13, 1439.

[13]. Cardoso, P.G., Macedo, G.C., Azevedo, V., and Oliveira, S.C., 2006. Brucella spp noncanonical LPS: structure, biosynthesis, and interaction with host immune system. *Microbial cell factories.* 5, 13.

[14]. Christopher, S., Umapathy, B., and Ravikumar, K., 2010. Brucellosis: review on the recent trends in pathogenicity and laboratory diagnosis. *Journal of laboratory physicians.* 2, 55.

[15]. Cloeckaert, A., Verger, J.-M., Grayon, M., Paquet, J.-Y., Garin-Bastuji, B., Foster, G. *et al.*, 2001. Classification of Brucella spp. isolated from marine mammals by DNA

polymorphism at the omp2 locus. *Microbes and Infection.* 3, 729-738.

[16]. Corbel, M.J., 1997. Brucellosis: an overview. *Emerging infectious diseases.* 3, 213.

[17]. Dadar, M., Shahali, Y., Fakhri, Y., and Godfroid, J., 2020. The global epidemiology of Brucella infections in terrestrial wildlife: A meta‐analysis. *Transboundary and Emerging Diseases.*

[18]. Dahouk, S.A., Tomaso, H., Prenger-Berninghoff, E., Splettstoesser, W.D., Scholz, H.C., and Neubauer, H., 2005. Identification of Brucella species and biotypes using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). *Critical reviews in microbiology.* 31, 191-196.

[19]. De Jong, M.F., and Tsolis, R.M., 2012. Brucellosis and type IV secretion. *Future microbiology.* 7, 47-58.

[20]. Deka, R.P., Magnusson, U., Grace, D., and Lindahl, J., 2018. Bovine brucellosis: prevalence, risk factors, economic cost and control options with particular reference to India-a review. *Infection Ecology & Epidemiology.* 8, 1556548.

[21]. Diab, M., Samy, A., Shell, W., and Eljakee, J., Evaluation of Diagnostic Potential of Recombinant D-erythrulose 1-phosphate Dehydrogenase Using Indirect Enzyme-linked Immunosorbent Assay for Diagnosis of Bovine Brucellosis.

[22]. Ducrotoy, M.J., Conde-Álvarez, R., Blasco, J.M., and Moriyón, I., 2016. A review of the basis of the immunological diagnosis of ruminant brucellosis. *Veterinary immunology and immunopathology.* 171, 81-102.

[23]. Emmerzaal, A., De Wit, J., Dijkstra, T., Bakker, D., and Van Zijderveld, F., 2002. The Dutch Brucella abortus monitoring programme for cattle: The impact of false‐positive serological reactions and comparison of serological tests. *Veterinary quarterly.* 24, 40-46.

[24]. Farina, R., 1985. Current serological methods in Brucella melitensis diagnosis. *Current topics in veterinary medicine and animal science.*

[25]. Foster, J.T., Okinaka, R.T., Svensson, R., Shaw, K., De, B.K., Robison, R.A. *et al.*, 2008. Real-time PCR assays of single-nucleotide polymorphisms defining the major Brucella clades. *Journal of Clinical Microbiology.* 46, 296-301.

[26]. Fretin, D., Fauconnier, A., Köhler, S., Halling, S., Léonard, S., Nijskens, C. *et al.*, 2005. The sheathed flagellum of Brucella melitensis is involved in persistence in a murine model of infection. *Cellular microbiology.* 7, 687-698.

[27]. Gándara, B.n., Merino, A.L., Rogel, M.A., and Martínez-Romero, E., 2001. Limited Genetic Diversity ofBrucella spp. *Journal of Clinical Microbiology.* 39, 235-240.

[28]. García-Yoldi, D., Marín, C.M., de Miguel, M.J., Muñoz, P.M., Vizmanos, J.L., and López-Goñi, I., 2006. Multiplex PCR assay for the identification and differentiation of all Brucella species and the vaccine strains Brucella abortus S19 and RB51 and Brucella melitensis Rev1. *Clinical chemistry.* 52, 779-781.

[29]. Gee, J.E., De, B.K., Levett, P.N., Whitney, A.M., Novak, R.T., and Popovic, T., 2004. Use of 16S rRNA gene sequencing for rapid confirmatory identification of Brucella isolates. *Journal of clinical microbiology.* 42, 3649-3654.

[30]. Godfroid, J., Nielsen, K., and Saegerman, C., 2010. Diagnosis of brucellosis in livestock and wildlife. *Croatian medical journal.* 51, 296-305.

[31]. Greiner, M., Verloo, D., and de Massis, F., 2009. Meta-analytical equivalence studies on diagnostic tests for bovine brucellosis allowing assessment of a test against a group of comparative tests. *Preventive veterinary medicine.* 92, 373-381.

[32]. Gupta, V.K., Nayakwadi, S., Kumar, A., Gururaj, K., Kumar, A., and Pawaiya, R.S., 2014. Markers for the molecular diagnosis of brucellosis in animals. *Adv. Anim. Vet. Sci.* 2, 31-39.

[33]. Hadush, A., and Pal, M., 2013. Brucellosis-An infectious re-emerging bacterial zoonosis of global importance. *Int J Livest Res.* 3, 28-34.

[34]. Halling, S.M., Peterson-Burch, B.D., Bricker, B.J., Zuerner, R.L., Qing, Z., Li, L.-L. *et al.*, 2005. Completion of the genome sequence of Brucella abortus and comparison to the highly similar genomes of Brucella melitensis and Brucella suis. *Journal of Bacteriology.* 187, 2715-2726.

[35]. Hinić, V., Brodard, I., Thomann, A., Cvetnić, Ž., Makaya, P., Frey, J. *et al.*, 2008. 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.* 75, 375-378.

[36]. Huynh, L.Y., Van Ert, M.N., Hadfield, T., Probert, W.S., Bellaire, B.H., Dobson, M. *et al.* 2008. Multiple locus variable number tandem repeat (VNTR) analysis (MLVA) of Brucella spp. identifies species-specific markers and insights into phylogenetic relationships. National Institute of Allergy and Infectious Diseases, NIH, 2008, (Springer, 47-54.

[37]. Imaoka, K., Kimura, M., Suzuki, M., Kamiyama, T., and Yamada, A., 2007. Simultaneous detection of the genus Brucella by combinatorial PCR. *Japanese journal of infectious diseases.* 60, 137.

[38]. Kattar, M.M., Zalloua, P.A., Araj, G.F., Samaha-Kfoury, J., Shbaklo, H., Kanj, S.S. *et al.*, 2007. 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.* 59, 23-32.

[39]. Le Flèche, P., Jacques, I., Grayon, M., Al Dahouk, S., Bouchon, P., Denoeud, F. *et al.*, 2006. Evaluation and selection of tandem repeat loci for a Brucella MLVA typing assay. *BMC microbiology.* 6, 9.

[40]. Leal-Klevezas, D.S., Martínez-Vázquez, I.O., García-Cantú, J., López-Merino, A., and Martínez-Soriano, J.P., 2000. Use of polymerase chain reaction to detect Brucella abortus biovar 1 in infected goats. *Veterinary Microbiology.* 75, 91-97.

[41]. Lopez-Goñi, I., Garcia-Yoldi, D., Marin, C., De Miguel, M., Munoz, P., Blasco, J. *et al.*, 2008. Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all Brucella species, including the vaccine strains. *Journal of clinical microbiology.* 46, 3484-3487.

[42]. MacMillan, A., Greiser-Wilke, I., Moennig, V., and Mathias, L., 1990. A competition enzyme immunoassay for brucellosis diagnosis. *DTW. Deutsche tierarztliche Wochenschrift.* 97, 83-85.

[43]. Madboly, A., Ghazi, Y., Sokkar, S.M., Desouky, H., and Ahmed, Y., 2014. Serological, bacteriological, molecular and immunohistochemical techniques for diagnosis of brucellosis in buffaloes. *Middle East J Appl Sci.* 4, 762-770.

[44]. Mahmoud, H., and Ahmed, H., Evaluation of indirect multispecies ELISA for diagnosis of brucellosis in different farm animals.

[45]. Mantur, B.G., Biradar, M.S., Bidri, R.C., Mulimani, M.S., Veerappa, K., Kariholu, P. *et al.*, 2006. Protean clinical manifestations and diagnostic challenges of human brucellosis in adults: 16 years' experience in an endemic area. *Journal of medical microbiology.* 55, 897-903.

[46]. Manual, O.T. 2009. Bovine brucellosis. Retrieved February 02, 2012 fromhttp. 2009,

[47]. McGiven, J., Tucker, J., Perrett, L., Stack, J., Brew, S., and MacMillan, A., 2003. Validation of FPA and cELISA for the detection of antibodies to Brucella abortus in cattle sera and comparison to SAT, CFT, and iELISA. *Journal of immunological methods.* 278, 171-178.

[48]. Michaux-Charachon, S., Bourg, G., Jumas-Bilak, E., Guigue-Talet, P., Allardet-Servent, A., O'Callaghan, D. *et al.*, 1997. Genome structure and phylogeny in the genus Brucella. *Journal of bacteriology.* 179, 3244-3249.

[49]. Moreno, E., 2020. The one hundred year journey of the genus Brucella (Mayer and Shaw 1920). *FEMS Microbiology Reviews.*

[50]. Moreno, E., Cloeckaert, A., and Moriyón, I., 2002. Brucella evolution and taxonomy. *Veterinary microbiology.* 90, 209-227.

[51]. Nicoletti, P.L., Quinn, B., and Minor, P., 1967. Canine to human transmission of brucellosis. *NY State J Med.*

[52]. Nielsen, K., Smith, P., Widdison, J., Gall, D., Kelly, L., Kelly, W. *et al.*, 2004. Serological relationship between cattle exposed to Brucella abortus, Yersinia enterocolitica O: 9 and Escherichia coli O157: H7. *Veterinary microbiology.* 100, 25-30.

[53]. Ocampo-Sosa, A.A., Agüero-Balbín, J., and García-Lobo, J.M., 2005. Development of a new PCR assay to identify Brucella abortus biovars 5, 6 and 9 and the new subgroup 3b of biovar 3. *Veterinary microbiology.* 110, 41-51.

[54]. Otero, J.R., Fuertes, A., Palenque, E., and Noriega, A.R., 1982. Microtiter-adapted method that facilitates the Coombs test for brucellosis. *Journal of clinical microbiology.* 16, 737-738.

[55]. Ouahrani‐Bettache, S., Soubrier, M.P., and Liautard, J.P., 1996. IS6501‐anchored PCR for the detection and identification of Brucella species and strains. *Journal of applied bacteriology*. 81, 154-160.

[56]. Ouahrani, S., Michaux, S., Widada, J.S., Bourg, G., Tournebize, R., Ramuz, M. *et al.*, 1993. Identification and sequence analysis of IS6501, an insertion sequence in Brucella spp.: relationship between genomic structure and the number of IS6501 copies. *Microbiology*. 139, 3265-3273.

[57]. Padilla Poester, F., Nielsen, K., Ernesto Samartino, L., and Ling Yu, W., 2010. Diagnosis of brucellosis. *The Open Veterinary Science Journal*. 4,

[58]. Pal, M., Gizaw, F., Fekadu, G., Alemayehu, G., and Kandi, V., 2017. Public health and economic importance of bovine Brucellosis: an overview. *Am J Epidemiol Infect Dis*. 5, 27-34.

[59]. Pappas, G., 2005. akritidis n, Bosilkovski M, tsianos e. *Brucellosis. n engl J Med*. 352, 2325-2336.

[60]. Pappas, G., Panagopoulou, P., Christou, L., and Akritidis, N., 2006. Biological weapons. *Cellular and molecular life sciences CMLS*. 63, 2229-2236.

[61]. Pardon, P., Sanchis, R., Molenat, G., Marly, J., and Renard, D., 1990. Serological and allergic reactions of ewes after simultaneous vaccinations with two living attenuated strains of Brucella and Salmonella.

[62]. Pfukenyi, D.M., Meletis, E., Modise, B., Ndengu, M., Kadzviti, F.W., Dipuo, K. *et al.*, 2020. Evaluation of the sensitivity and specificity of the lateral flow assay, Rose Bengal test and the complement fixation test for the diagnosis of brucellosis in cattle using Bayesian latent class analysis. *Preventive Veterinary Medicine*. 181, 105075.

[63]. Porter, S.R., Czaplicki, G., Mainil, J., Guattéo, R., and Saegerman, C., 2011. Q Fever: current state of knowledge and perspectives of research of a neglected zoonosis. *International journal of microbiology*. 2011,

[64]. Queipo-Ortuño, M., Colmenero, J., Reguera, J., Garcia-Ordonez, M., Pachon, M., Gonzalez, M. *et al.*, 2005. 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*. 11, 713-718.

[65]. Raghava, S., Honnayakanahalli, M., Gowda, M., Shome, R., Kulkarni, M., and Umesha, S., 2017. Epidemiological and molecular characterization of Brucella species in cattle.

[66]. Redkar, R., Rose, S., Bricker, B., and DelVecchio, V., 2001. Real-time detection of Brucella abortus, Brucella melitensis and Brucella suis. *Molecular and cellular probes*. 15, 43-52.

[67]. Sabour, S., Arzanlou, M., Jeddi, F., Azimi, T., Hosseini-Asl, S., Naghizadeh-Baghi, A. *et al.*, 2020. Evaluating the efficiency of TaqMan real-time PCR and serological methods in the detection of Brucella spp. in clinical specimens collected from suspected patients in Ardabil, Iran. *Journal of Microbiological Methods*. 175, 105982.

[68]. Sangari, F.J., and Agüero, J., 1994. Identification of Brucella abortus B19 vaccine strain by the detection of DNA polymorphism at the ery locus. *Vaccine*. 12, 435-438.

[69]. Scott, J.C., Koylass, M.S., Stubberfield, M.R., and Whatmore, A.M., 2007. Multiplex assay based on single-nucleotide polymorphisms for rapid identification of Brucella isolates at the species level. *Applied and environmental microbiology*. 73, 7331-7337.

[70]. Seleem, M.N., Boyle, S.M., and Sriranganathan, N., 2008. Brucella: a pathogen without classic virulence genes. *Veterinary microbiology*. 129, 1-14.

[71]. Shehabi, A., Shakir, K., El-Khateeb, M., Qubain, H., Fararjeh, N., and Shamat, A.R.A., 1990. Diagnosis and treatment of 106 cases of human brucellosis. *Journal of infection*. 20, 5-10.

[72]. Shenoy, B., Jaiswal, A., and Vinod, A., 2016. Lab diagnosis of brucellosis. *Pediatric Infectious Disease*. 8, 40-44.

[73]. Sriranganathan, N., Seleem, M.N., Olsen, S.C., Samartino, L.E., Whatmore, A.M., Bricker, B. *et al.* 2009. Brucella. Genome mapping and genomics in animal-associated microbes, 2009, (Springer, 1-64.

[74]. Urwin, R., and Maiden, M.C., 2003. Multi-locus sequence typing: a tool for global epidemiology. *Trends in microbiology*. 11, 479-487.

[75]. Whatmore, A.M., 2009. Current understanding of the genetic diversity of Brucella, an expanding genus of zoonotic pathogens. *Infection, Genetics and Evolution*. 9, 1168-1184.

[76]. Whatmore, A.M., Perrett, L.L., and MacMillan, A.P., 2007. Characterisation of the genetic diversity of Brucella by multilocus sequencing. *BMC microbiology*. 7, 34.

[77]. Whatmore, A.M., Shankster, S.J., Perrett, L.L., Murphy, T.J., Brew, S.D., Thirlwall, R.E. *et al.*, 2006. Identification and characterization of variable-number tandem-repeat markers for typing of Brucella spp. *Journal of clinical microbiology*. 44, 1982-1993.

[78]. Winchell, J.M., Wolff, B.J., Tiller, R., Bowen, M.D., and Hoffmaster, A.R., 2010. Rapid identification and discrimination of Brucella isolates by use of real-time PCR and high-resolution melt analysis. *Journal of clinical microbiology*. 48, 697-702.

[79]. Xavier, M.N., Costa, É.A., Paixão, T.A., and Santos, R.L., 2009. The genus Brucella and clinical manifestations of brucellosis. *Ciência Rural*. 39, 2252-2260.

[80]. Yagupsky, P., 1999. Detection of Brucellae in blood cultures. *Journal of clinical microbiology*. 37, 3437-3442.

[81]. Yagupsky, P., Morata, P., and Colmenero, J.D., 2019. Laboratory diagnosis of human brucellosis. *Clinical microbiology reviews*. 33,

[82]. Yanagi, M., and Yamasato, K., 1993. Phylogenetic analysis of the family Rhizobiaceae and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. *FEMS Microbiology Letters*. 107, 115-120.

[83]. Young, E.J., 1991. Serologic diagnosis of human brucellosis: analysis of 214 cases by agglutination tests and review of the literature. *Reviews of infectious diseases*. 13, 359-372.

[84]. Yu, W.L., and Nielsen, K., 2010. Review of detection of Brucella spp. by polymerase chain reaction. *Croatian medical journal*. 51, 306-313.

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