Review Article

MALDI-TOF Mass Spectrometry: In Search of the Most Reliable Approach to Diagnose *Brucella* in Bovines

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Abstract

The current research on the epidemiology and diagnosis of Brucella, also stimulated by the zoonotic and bioterrorism aspect, is exploiting novel methods to develop more rapid, reliable and safe diagnostic tools. This paper evaluates the various diagnostic strategies to suggest the most reliable technique to diagnose Brucella spp. infection in bovines. The pros and cons of phenotypic identification, serological diagnosis as well as genotypic identification have been discussed. MALDI-TOF-MS technology has been found to have significant advantages over other conventional and molecular identification methods and can be used as a first-line screening tool for epidemiological studies and outbreak investigations of bovine brucellosis with minimal time, labor and cost.

Key words: Bovine, Brucella, Diagnosis, MALDI-TOF-MS

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Introduction

Brucellosis is a highly contagious disease impacting the dairy sector in India as it causes reproductive impairment in the form of abortion storms, retained placentae and infertility (Priyanka *et al.*, 2019a, b). A precise diagnosis of *Brucella* spp. infection is important for the control of the disease in animals including the bovines. Besides, brucellosis being a highly contagious zoonotic disease, may also be transmitted to human beings, thus the risk of exposure of human beings to brucellosis can be indirectly assessed by finding the status in animals (Priyanka *et al.*, 2017, 2018a). A presumptive diagnosis of brucellosis can be made by the clinical history of reproductive failures in livestock, but it must be confirmed by laboratory methods. The factors determining the diagnosis of bovine brucellosis are: the absence of clinical signs other than abortion, the incubation period, the high proportion of in apparent infections, the degree of resistance, either natural or resulting from vaccination, and the presence of natural or nonspecific agglutinins (Morgan, 1982).

The diagnosis should be based upon the disease history of the herd, demonstration of the causal organism epidemiological observations, serum antibody tests and the cell-mediated immunity. Despite the vigorous attempts 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 (Poiester *et al.*, 2010). The reviews of the various diagnostic strategies have been mentioned below.

Phenotypic Identification

The conventional method of bacterial isolation is still the unequivocal method of reliable and definitive diagnosis in livestock as well as human beings. Isolation from a single animal is sufficient evidence to establish the infection status of a herd. The bacteriological method includes culturing of samples such as aborted fetal stomach contents, milk, blood, lymph nodes and vaginal discharges from suspected cases for isolation and identification of the infecting *Brucella* organisms (Quinn *et al.*, 1999). As per OIE (2009), brucellae can also be isolated from vaginal swabs, discharges from the uterus, stomach contents, spleen, and lung collected from aborted fetuses, placental bits, semen, and synovial fluids. Associated lymph nodes and spleen, udder tissues, testes and epididymis are useful samples for culture from animal carcasses (Liu, 2009).

The isolated organisms are further tested using molecular-based tests. This sequence of confirmatory procedures is referred to as the "gold standard" method of identifying *Brucella* (Keid *et al.*, 2007). However, in spite of its high specificity, the culture of *Brucella* spp. is challenging. *Brucella* spp. is a fastidious bacterium, thus it requires rich media for primary cultures. Furthermore, its isolation requires a large number of viable bacteria in clinical samples, proper storage and quick delivery to the diagnostic laboratory and it requires BSL3 facilities which are not available in most developing countries (Refai, 2003; Seleem *et al.*, 2010; Hadush and Pal, 2013). A wide range of selective media can be used for the cultivation of *Brucella* spp. The suitable media include *Brucella* agar medium (BAM) base, trypticase soy agar, modified Thayer-Martin medium, Farrell's medium, serum dextrose agar (SDA), glycerol dextrose agar and Castaneda's medium (OIE, 2004). The culture media are then incubated in an atmosphere of 5 to 10% CO2 at 37ºC for a period of 7 to 14 days till the colonies start to appear (Alton *et al.*, 1988; Bridgewater, 1989; Meyer *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; samples must be immediately sent to the laboratory, preferentially frozen at -20 ºC (+4 ºC for milk sample), and they must be identified as suspect of *Brucella* spp. infection (Poiester *et al.*, 2010). Vaginal swabs, semen and seminal fluid have low numbers of viable organisms, therefore enrichment media containing selected antibiotics can improve the sensitivity in these cases (De Miguel *et al.*, 2011). After 3-5 days of incubation on selective

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agar, growth appears as pinpoint, smooth, glistening, bluish, translucent colonies. As they age the colonies become opaque and measure about 2-3 mm in diameter. Smooth colonies in a clear growth medium such as serum-dextrose agar, are convex with entire-edge, and a smooth shiny surface and are pale yellowish-brown when viewed under transmitted light. Smooth forms are often markedly pathogenic whereas the rough variants are usually less pathogenic (Quinn *et al.,* 1999). The smears from suspected colonies stained with Modified Ziehl Neelsen's stain show small red-staining i.e. MZN positive coccobacilli. *Brucella* is Gram-negative, non-motile, catalase-positive, oxidase-positive, indole-negative, gives a rapid urease activity (except some *B. melitensis* strains) and reduce nitrates to nitrites.

For routine identification, a combination of growth characteristics, colonial and cellular morphology, staining properties, agglutinating antiserum and biochemical reactions allow an accurate identification (Quinn *et al*., 1999). Presently, the fully automated microbial identification systems like Vitek 2 Compact system are also available which perform bacterial identification by biochemical analysis using colorimetry. The Vitek 2 Compact has been used for the identification of isolates of different *Brucella* species by various researchers including Borriello *et al*. (2013); Xu *et al*. (2013); Maymona *et al*. (2014); Alsharabasi (2015), Deshmukh *et al*. (2015); Eisenberg *et al*. (2017); Jia *et al*. (2017) and Paul *et al*. (2017). It provides more precise and rapid identification with minimum handling of cultures by simultaneous application of a multitude of biochemical tests, followed by the precise prediction of *Brucella* upto species level.

Classical differentiation between the different *Brucella* species and biovars is based on phage typing, sensibility to dyes, oxidative metabolic profiles, CO_2 requirements, H_2S production and agglutination with monospecific antisera (Alton *et al*., 1988). These typing methods are time-consuming, often subjective and risk for the laboratory personnel; that is why methodological improvements are desirable. Because of the costs inducted, lack of sensitivity and difficulty in performing the culture procedures, there is an indirect method of diagnosis by way of serological tests. The current epidemiological and diagnostic *Brucella* research, also stimulated by the bioterrorism debate, is further exploiting novel molecular typing methods to develop more rapid, reliable and safe diagnostic tools.

Serological Diagnosis

Major *Brucella* species (*B. abortus*, *B. melitensis* and *B. suis*) contain O-polysaccharide on their cell surface, which is a part of the lipopolysaccharide. The O-polysaccharide is lacking in other species (*B. ovis* and *B. canis*) of *Brucella*. The major species which contain O-polysaccharide are diagnosed serologically using either whole cell antigen or smooth lipopolysaccharide. *B. abortus* antigens were the major antigens used in most of the serological tests. The serological tests rely on a reaction between the *Brucella* antigen and antibodies produced in the host in response to the infection. A number of classes and subclasses of

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antibody (isotypes) may occur in positive sera and the various serological tests vary in their ability to detect the different isotypes.

There are many serological tests for demonstrating that *Brucella* antibodies exist in serum, milk, whey, vaginal mucus, semen, and muscle juice. The commonly used tests are the Milk Ring Test (MRT), Serum Agglutination Test (SAT), Rose Bengal Plate Test (RBPT), Anti-globulin (Coombs) Test, 2-Mercaptoethanol, Rivanol, and the Enzyme-linked Immunosorbent Assay (ELISA) (Morgan, 1982). The reliability of serological tests to detect brucellosis depends on the antibodies present at the time of examination thus the infected animals may escape from detection. Serological tests may show cross-reactions with other Gram-negative organisms such as *Salmonella* group N, *Eschericia coli* O:157, *E. coli* O:116, and *Pseudomonas maltophilia*; however, the most notable cross-reaction is between smooth lipopolysaccharide (S-LPS) found in *Brucella* and *Yersinia enterocolitica* O:9 making diagnosis difficult due to the sharing of antigenic determinants in the O-polysaccharide (O-PS) molecule, which is the basis for most serological tests (Corbel *et al*., 1983; Muñoz *et al*., 2005; Nielsen *et al*., 2006). Several countries have reported such cross-reactions during serological screening (Weynants *et al*., 1996; Bercovich, 1998), and false positives in addition to false negatives have often limited accurate diagnosis and disease eradication programmes.

Historically, the Standard Tube Agglutination Test (STAT) for brucellosis described by Wright and Smith (1897), has been recognized as the principal serological test used for the diagnosis of brucellosis. The test may give a false positive reaction due to the cross-reacting antibody (IgM). Therefore, its discontinuation is recommended by the OIE (OIE, 2000). However, the test is still in use after a large number of modifications to inactivate IgM agglutination, such as the incorporation of the acidified antigen, Rivanol precipitation and 2-mercaptoethanol.

The Milk Ring Test (MRT) is a screening test for bulk milk used to detect infected animals on a herd basis or to monitor the clean herds. Herds of which the MRT is positive should be examined by serological tests to identify the infected animals (Alton *et al*., 1988). Although it is a relatively insensitive test subject to wrong interpretation caused by various milk conditions such as mastitis, colostrum and milk at the end of the lactating cycle, it is recommended by the OIE as a screening test for bovine brucellosis.

The Rose Bengal Plate Test (RBPT) is a spot agglutination test used for rapid herd screening. In RBPT, the antigen is used at a low pH of 3.65 which prevents some agglutination by IgM and encourages agglutination by IgG1 thereby reducing nonspecific interactions (Corbel, 1972). The drawbacks of RBPT include: low sensitivity, particularly in chronic cases, relatively low specificity in endemic areas and prozones make strongly positive sera appear negative in RBPT (Diaz *et al*., 2011). The present World Health Organization (WHO) guidelines recommend the confirmation of the RBPT by other assays such as serum agglutination tests (Ruiz Mesa *et al*., 2005; Diaz *et al*., 2011).

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The Complement Fixation Test (CFT) is a very specific and sensitive test. According to Radostits *et al.* (1994a; b), the CFT rarely exhibits non-specific reaction and is useful in differentiating the titres of calfhood vaccination from those due to infection. However, this method has certain disadvantages like high cost, complexity for execution, and the requirement for special equipment and trained laboratory personnel.

The Indirect Enzyme Linked Immunosorbent Assay (I-ELISA) is a highly sensitive test, but sometimes it is not capable of differentiating between antibody resulting from S19 vaccination or other false-positive reactions from those induced by pathogenic *Brucella* strains. Therefore, it should be considered more as a screening test than a confirmatory test in the testing of vaccinated herds affected by false-positive results (Nielsen, 2002; OIE, 2004). Competitive ELISA (c-ELISA) can distinguish vaccine antibody, whereas both conventional serological tests and the indirect ELISA can't (Nielsen, 2002). This test can be invariably employed in the diagnosis of brucellosis in livestock in the endemic areas where vaccination is routinely carried out.

Other serological tests include fluorescence polarization assay (FPA), agar gel immunodiffusion test (AGID), Coombs test, Dipstick assay, immunocapture agglutination for anti-Brucella (BCAP) assay, lateral flow assay and rapid slide agglutination assay test (RSAT).

Genotypic Identification

The brucellae genome is encoded on two circular chromosomes with sizes close to 2.05 Mb and 1.15 Mb for each species (Michaux-Charachon *et al.,* 1997). Only the small chromosomes of *B. suis*, *B. canis* and *B. neotomae* are 50 kb longer. The Guanine / Cytosine (G + C) contents in the DNA of various members of the genus *Brucella* are 55 to 58 per cent (Hoyer and McCullough, 1968 and Verger *et al.,* 1987). Almost identical proportions of potential coding regions (1028 and 1035, respectively) are present in both chromosomes. Housekeeping genes are evenly distributed all over the genome, which makes a highly probable coexistence (Moreno and Moriyon, 2002). A high conservation of restricted sites and genes order has been revealed by chromosomal mapping. Variability is localized to certain regions, most often on the small chromosome. The nucleotide sequence similarity between all *Brucella* species is also high and DNA-DNA homology exceeds 90 per cent (Patel, 2007).

Despite the high genetic similarities, a range of different molecular techniques has been established for the differentiation of *Brucella* species and to some extent the biovars. DNA-based methods such as gene probes and PCR utilize primers derived from different polymorphic regions in the genomes of *Brucella* spp. These molecular approaches include the analysis of outer-membrane genes and PCR-RFLP (Ficht *et al*., 1989; 1996; Cloeckaert *et al*., 1995, 1996, 2001; Vizcaino *et al*., 1997; 2004; Garcia-Yoldi *et al*., 2005; Al Dahouk *et al*., 2005), locus specific conventional and real-time PCR assays targeting different polymorphic regions (Fekete *et al*., 1990; Bricker and Halling, 1994; Redkar *et al*., 2001; Ferrao-Beck *et al*., 2006; Garcia-Yoldi

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et al., 2006; Ratushna *et al*., 2006), arbitrary primed and rep-PCR (Mercier *et al*., 1996; Tcherneva *et al.*, 1996; Fekete *et al*., 1992; Tcherneva *et al*., 2000), 'infrequent restriction site' (IRS)-PCR (Cloeckaert *et al*. 2003), multilocus sequence typing (Whatmore *et al*., 2007), multilocus variable number tandem-repeat analysis (Bricker *et al*., 2003; Le Flèche *et al*., 2006; Whatmore *et al*., 2006; Garcia-Yoldi *et al*., 2007; Al Dahouk *et al*., 2007), AFLP (Whatmore *et al*., 2005), and SNP-PCR (Marianelli *et al*., 2006; Scott *et al*., 2007; Fretin *et al*., 2008; Foster *et al*., 2008).

The identification of *Brucella* at genus, species and even biovar levels has improved with the application of molecular methods, especially PCR. Molecular detection of *Brucella* spp. can be done directly on the clinical samples without the previous isolation of the organism. In addition, these techniques can be used to complement the results obtained from phenotypic tests (Bricker, 2002). The technique is chosen as per the type of biological sample and the goal, i.e. diagnosis or molecular characterization or epidemiological survey. Each type of clinical samples has inherent and unique difficulties for adequate sample preparation. The most common difficulties arose from co-purification of PCR inhibitors with the DNA and from interference by excessive host DNA (Morata *et al*., 1998).

Since the routine identification and differentiation of brucellosis suspected specimens, based on culture isolation and phenotypic characterization, requires a biosafety level-3 (BSL3) protocols for the high risk of laboratory-acquired infections (Boschiroli *et al*., 2001), molecular methods have been explored in order to overcome these difficulties. A number of PCR methods developed for the detection of *Brucella* are more and more used in the diagnosis of brucellosis owing to their more sensitivity than conventional culture methods and more specificity than serological methods (Al Dahouk *et al.*, 2013). These are currently being used for the diagnosis of several infectious diseases caused by fastidious or slowly growing bacteria. Thus, the speed and sensitivity of the PCR assay coupled with the reduced risk to the laboratory workers, made this technique a very useful tool for the diagnosis of brucellosis.

However, molecular methods are relatively expensive, with variable sensitivity (Al Dahouk and Nockler, 2011), and their efficiency is highly dependent on primers specificity (Wang *et al.*, 2014). Thus, they are more appropriate for the differential diagnosis rather than for establishing prevalence. The DNA extraction protocol, type of clinical sample, and detection limits of each protocol, are other factors that can influence the efficiency of the technique (Mitka *et al*., 2007). Also, the presence of large amounts of bovine genomic DNA may have inhibitory effects on the PCR assay (Priyanka *et al.*, 2018b)

Conventional PCR

The methods based on PCR are becoming useful and to date, considerable progress has been made in the development of more sensitive, specific, easier and cheaper PCR techniques for *Brucella* detection (Yu and Nielsen, 2010). PCR had been successfully used for the identification of *Brucella* in bovine blood and milk

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(Leal-Klevezas *et al*., 1995; Romero and López-Goni, 1999; Romero *et al*., 1995b), aborted foetuses and associated maternal tissues (Fekete *et al*., 1992; Gallien *et al*., 1998; Cetinkaya *et al*., 1999, Cortez *et al*., 2001 and O'Leary *et al*., 2006), nasal secretion (Sreevatsan *et al*., 2000) and goats or sheep milk and cheese (Serpe *et al*., 1999; Tantillo *et al*., 2001,2003). For identification of *Brucella* spp. at the genus- level, the primers for sequences encoding *bcsp31* (B4/B5) (Baily *et al*., 1992), 16S rRNA (F4/R2) (Romero *et al*., 1995a), 16S-23S intergenic transcribed spacers (ITS) (Rijpens *et al*., 1996; Bricker, 2000), 16S-23S rDNA interspace region (ITS66/ITS279) (Keid *et al*., 2007), *IS711* (IS313/IS639) (Hénault *et al*., 2000), *per* (bruc1/bruc5) (Bogdanovich *et al*., 2004), *omp2* (JPF/ JPR) (Leal-Klevezas *et al*., 1995), outer membrane proteins (Imaoka *et al*., 2007), proteins of the omp25/omp31 family (Vizcaino *et al*., 2004) have been used. By increasing the number of molecular markers, both sensitivity and specificity can be increased accordingly. Molecular assays targeting the *IS711* insertion element, which is found in multiple copies within *Brucella* chromosomes, also improve analytical sensitivity (Bounaadja *et al.,* 2009). Genus-specific PCR assays are generally adequate for the molecular diagnosis of human brucellosis (Al Dahouk and Nöckler, 2011). The *bcsp31* gene, coding for a 31-kDa immunogenic outer membrane protein conserved among all *Brucella* spp. is the most common molecular target in clinical applications (Baily *et al.,* 1992). Such a genus-specific PCR can help to avoid false-negative results in individuals infected with unusual species and biovars.

Several studies have described PCR assays that make use of the specific occurrence of the multiple insertion element IS*711* which was described by Halling *et al.* (1993) and is stable in number and position in the *Brucella* chromosomes (Bricker and Halling 1994; 1995; Bricker *et al.*, 2000; Cloeckaert *et al.*, 2000; 2003; Ohishi *et al.*, 2004; Ocampo-Sosa *et al.*, 2005). There has been steady progress towards more sophisticated differential assays despite the high level of conservation among *Brucella* species and strains. Hence, in the present study, primers targeting *IS711* gene were employed for *Brucella abortus* species-specific PCR (Doust *et al*., 2007). The 16S-23S genes, the *IS*711 insertion sequence and the *bcsp*31 gene of *Brucella* spp. are validated for the detection of *Brucella* (Ouahrani-Bettache *et al*., 1996). Comparison of sensitivity of 3 pairs of primers amplifying 3 different fragments including a gene encoding the *bcsp31*, a sequence of 16S rRNA of *B. abortus*, and a gene encoding *omp2* revealed the sensitivities of the *bcsp31*, omp2 and 16S rRNA to be 98%, 88.4% and 53.1%, respectively (Baddour and Alkhalifa, 2008). Navarro *et al.* (2002) compared detection ability of three primer pairs specific for the *bcsp31*, 16SrRNA and *omp2* genes of *Brucella* in human blood samples and variation in sensitivity for detecting purified *Brucella* DNA was reported with the *bcsp31* gene to be the most sensitive for detecting *Brucella* DNA.

Various methods have been optimized for a number of *Brucella* spp. using tissues, and blood or milk samples. The first published PCR based diagnostic assay was developed by Fekete *et al*. (1990) which was specific for brucellae, applicable to all species and biovars and very sensitive. This assay targeted a 635 bp

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sequence from a gene encoding a 43 kDa outer membrane protein of *B. abortus* strain 19. Fekete *et al.* (1992) also processed 105 aborted bovine tissues comprising of fetal tissues *viz*., lung, liver, spleen and stomach contents and maternal tissues *viz*., uterine exudates and placenta and compared the PCR sensitivity and specificity with bacteriological examination which were found to be 98 per cent and 96 per cent, respectively. *Brucella* gene *16S rRNA* published by Dorsch *et al.* (1989) was explored to yield 800bp amplicon from *B. abortus* and other species of *Brucella* (Herman and De Ridder, 1992). In their opinion, the sequence was highly conserved and the test could be extended to the entire genus. A new PCR assay based on *bcsp-31* gene of *Brucella* organisms was published by Baily *et al.* (1992) which contained a single pair of oligonucleotide primers designed to amplify a 223bp product.

Another PCR assay with primer derived from the *16S rRNA* sequence of *B. abortus*, was developed by Romero *et al.* (1995a, b). In the study, they included all the representative strains of the species and biovars of *Brucella* and other non-*Brucella* species, which are related to *Brucella* species either phylogenetically or serologically. A 905bp fragment amplified from all *Brucella* species but not non-*Brucella* species except *Ochrobactrum anthropi* biotype D. They concluded that the assay was having high specificity and sensitivity and might provide a valuable tool for the diagnosis of brucellosis. The assay was evaluated with milk ELISA for the diagnosis of brucellosis in dairy cattle and a proportion of 0.91 agreement was found between the two tests. Finally, they opined that though ELISA was a better screening test than PCR, the combined sensitivity of the two assays was 100 per cent and their simultaneous application was found to be more useful than a single test for rapid screening of brucellosis in dairy cattle.

The PCR assay based on a gene encoding *omp2* of brucellae was designed by Leal- Klevezas *et al*. (1995) for the detection of *Brucella* species from body fluids of infected animals. The test was found to be sensitive to detect the brucellae in clinical samples. Da Costa *et al*. (1996) thoroughly investigated the assay in which all the *Brucella* species, biovars and non-*Brucella* organisms were tested and found that all six *Brucella* species and one strain of *Ochrobactrum* were positive for amplification. The assay was reported to be robust, sensitive and specific.

The PCR technique provides a promising option in diagnosing brucellosis with high sensitivity in detecting *Brucella* from pure cultures. Vaid *et al.* (2004) applied PCR for detection of brucellosis using primers derived from the 43 kDa outer membrane protein gene of *B. abortus*, the *16S rRNA* gene, insertion sequence *IS711*, *BCSP31* (*Brucella* Cell Surface Protein) gene. PCR for detection of *B. abortus* infection in blood, milk and lymph tissues by using different primers that amplify various regions of the *Brucella* genome, IS711 genetic element, 31 kDa outer membrane protein and 16S rRNA was used by O'Leary *et al.* (2006) and they found that there was no amplification when PCR assays was applied to the blood samples, but obtained amplicons in a proportion of the culture-positive milk (44%) and lymph tissue samples by the

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same methods. Scholz *et al.* (2008b) described an assay targeting the conserved gene *recA* that detects and differentiates *O. anthropi*, *O. intermedium* and *Brucella* by conventional PCR in a single reaction.

A differentiation between some of the classical *Brucella* species was achieved by the use of the relatively time-consuming 'infrequent restriction site' (IRS)-PCR and even characteristic IRS-PCR patterns for *B. suis* biovar 2 could be found (Cloeckaert *et al*., 2003). In another study, DNA polymorphisms in the genes for the different outer membrane proteins (OMPs; *omp2*, *omp25*, *omp31*) were examined and tested for differentiation purposes. So it was shown that strains of *B. melitensis* are characterized by the lack of the signature sequence for the restriction enzyme *Eco*RV in the *omp25* PCR product and *B. ovis* strains by a 50 bp deletion in the same gene. Furthermore, in the restriction patterns for the *omp2* PCR product, species- and also biovar-specific features were found (Cloeckaert *et al*., 1995). A high variability was also found for the restriction patterns of this PCR product in marine brucellae which does not only hint at the existence of different species but also of different biovars (Cloeckaert *et al*., 2001).

The target sequence of another PCR based diagnosis of human brucellosis is *omp31*, encoding the 31-kDa *Brucella* outer membrane protein (Casañas *et al*., 2001). In this assay, which has already been adapted to a real-time PCR (Queipo-Ortuño *et al*., 2005), *Brucella* species cannot be differentiated from all members of the closely related genus *Ochrobactrum.* A comprehensive microarray study by Rajashekara *et al*. (2004) revealed several differences in the genomes of the classical *Brucella* species most of which were insertions or deletions Marianelli *et al.* (2006) reported that the *rpoB* gene contains enough DNA polymorphisms for the identification of *Brucella* spp.

A diagnostic assay should have the properties of being sensitive, specific, fast and easy to perform. Though, a number of assays are available for the diagnosis of brucellosis, but none is 100% sensitive and specific. To overcome this weakness various authors have evaluated the efficacy of serological, cultural and molecular assays to diagnose brucellosis. Ferris *et al.* (1995) compared results of 6 serological tests viz Particle Concentration Fluorescence Immunoassay, ACF Assay, Card Test, Buffered Acidified Plate Antigen Assay, STAT and Rivanol Test and isolation for diagnosis of brucellosis in pigs. Leal-Klevezas *et al.* (2000) compared PCR, serological and bacteriological techniques to diagnose goat brucellosis. Amin *et al.* (2001) compared PCR and cultural isolation for detection of *Brucella melitensis* DNA in bovine and ovine semen. Leyla *et al.* (2003) evaluated detection of *Brucella* DNA directly from the stomach contents of aborted sheep fetuses with culture isolation.

While comparing PCR, RBPT and STAT for diagnosis of brucellosis in human beings Varasada (2003) found the highest number of positive results by PCR followed by RBPT and STAT. Lavaroni *et al.* (2004) comparatively evaluated isolation, blood PCR, i-ELISA and CFT for diagnosis of bovine brucellosis. Rahman (2005) evaluated serological and cultural methods for diagnosis of *B. abortus* biotype 1 infection in experimentally infected Sprague-Dawley rats. Gupta *et al.* (2006) evaluated sensitivity and specificity of

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the tissue PCR in comparison to STAT and dot-ELISA. O'Leary *et al.* (2006) assessed the viability of using conventional and real-time PCR assays as potential diagnostic tools for the detection of *Brucella abortus* in naturally infected cows.

A study comparing the culture and PCR methods for detection of *Brucella* was conducted by Kanani (2007) using semen of serologically positive 101 bulls; PCR assay detected a highest number of positive bulls (19) than cultural isolation (8). Also, the highest numbers of bulls were found positive by B4 / B5 primer pair based PCR assay (18.81 per cent) as compared to JPF / JPR (1.98 per cent) and F4 / R2 (4.95 per cent) published primer pairs, respectively. The same finding was reported by Ghodasara (2008) that the B4/B5 primer pair was more suitable than the other two pairs of primers. Out of the 10 cultured isolates from 248 samples of vaginal swabs and aborted materials from cows, buffaloes, goats and bitches, the desired product of 223 bp using B4/B5 primer pair was amplified in all the 10 isolates, 8 by JPF/JPR primer pair and 8 by F4/R2 primer pair. But, when PCR and ELISA were compared by Chothe *et al*. (2013) for the detection of *Brucella* in 200 serum samples, only three samples yielded positive results in PCR against the 75 ELISA positive samples.

The PCR assay for detection of *Brucella* DNA using *BCSP 31* target gene and IS711 locus was conducted by Garshasbi *et al*. (2014) which showed that an amplicon of 223 bp was obtained in 73.8 % (133/180) of the tested sera using primers (B4/B5) and an amplicon of 498 bp was obtained in 63.8% (115/180) of the samples using *Brucella abortus*-specific primers derived from a locus adjacent to the 3'-end of IS711. But, in another study conducted by Patel *et al*. (2015), all the samples (7 out of 33) from aborted buffaloes which yielded *Brucella* in genus specific PCR were confirmed as *Brucella abortus* in species specific PCR based on IS711 as well. Similarly, when Karthik *et al*. (2014) used the same primers i.e. *bcsp31* and *IS711* for the detection and identification of *B. abortus* in blood samples (n=370) of cattle from three states *viz.* Uttar Pradesh, Uttarakhand and Tamil Nadu, a total of 56 samples (15.03 %) were detected as positive by both the PCRs.

Multiplex PCR

Several multiplex PCRs have been developed using different primer combinations which identify *Brucella* at the species level and partly at the biovar level as well. The AMOS-PCR (*abortus-melitensis-ovis-suis*) was the first species-specific multiplex PCR which identified and differentiated *Brucella* biovars including *B. abortus* biovars 1, 2 and 4, *B. melitensis*, *B. ovis* and *B. suis* biovar 1. It is based on polymorphism due to species-specific localization of the insertion sequence *IS711* in the *Brucella* chromosome (Bricker and Halling, 1994). This PCR technique has a disadvantage of not being able to identify some *Brucella* species like *B. canis* and *B. neotomae*. Furthermore, some biovars within a given species gave negative results. The assay was improved to identify the vaccine strains S19 and RB51 by including further strain specific

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oligonucleotides into the reaction mixture (Bricker and Halling, 1995). This assay has been employed by several other laboratories with great success (Adone *et al*., 2001). A multiplex PCR for the detection of *Brucella* spp. and *Leptospira* spp. in aborted bovine fetuses was described by Richtzenhain *et al*. (2002).
In a study based on AMOS PCR, Baek *et al*. (2003) collected serum and blood sample from three dogs reared on serologically *Brucella* positive dairy farm in Korea with a high incidence for *Brucella abortus* which were serologically positive for RBPT and PAT. The amplified product with 498 bp DNA band of *B. abortus* in AMOS PCR as well as the specific primers of *B. canis* confirmed the identity of the organism. There was 100 per cent homology of the canine isolate and the bovine pathogen isolated from the farm, thus the only possible source of infection was infected cattle on the same farm. The results suggested that dogs should be routinely included in brucellosis surveillance and eradication programmes.
The AMOS PCR assay was further modified as BaSS-PCR (*Brucella abortus* Strain Specific PCR assay) which helped to identify and differentiate field strains of biotypes 1, 2 and 4 of *B. abortus,* and vaccine strains and other *Brucella* species from cattle (Bricker *et al*., 2003). Alain *et al*. (2005) developed a new PCR assay to identify B. abortus biovar 5, 6 and 9 and the subgroup 3b of biovar 3. This was the modified form of the assay developed earlier by Bricker and Halling (1994) and was named as AMOS-ERY PCR. For identification of *B. abortus* biovars 3, 5, 6 and 9 a new primer was added (Ocampo-Sosa *et al*., 2005). For recognition and discrimination of *Brucella* species and vaccine strains in a single step, a multiplex PCR assay, Bruce-ladder was developed (García-Yoldi *et al*., 2006). The multiplex was further enhanced to identify the marine strains, *B. microti* and *B. inopinata*. However, it does not differentiate at the biovar level, or below (López-Goñi *et al*., 2008; Hubber *et al*., 2009; Mayer-Scholl *et al*., 2010). Lopez-Goni *et al*. (2008) developed a multiplex PCR assay (Bruce-ladder) which could differentiate in a single step all of the classical *Brucella* species, including those found in marine animals and the S19, RB51 and Rev.1 vaccine strains. A comparable multiplex approach was described having the ability to discriminate the six classical species based on species-specific differences (Hinic *et al*., 2008), however, the approach was not as inclusive as "Bruce-ladder".
The primer pair identifying *B. microti* (Scholz *et al*., 2008a) was included in the multiplex PCR described by Garcia-Yoldi *et al*. (2006), and the assay was set up on the DNA of *Brucella* reference strains and field isolates. The assay allowed the identification of all currently known *Brucella* species, also distinguishing between the marine species *B. ceti* and *B. pinnipedialis* besides identifying the recently described species *B. microti* and *B. inopinata*. An advancement of the Garcia Yoldi protocol for the differentiation of all currently described *Brucella* species was published by Mayer-Scholl *et al*. (2010). A multiplex PCR assay (Suis ladder) was also developed recently to differentiate among biovars of *B. suis* (López-Goñi *et al*., 2011). A PCR multiplex for *Brucella* and *Leptospira* was used by Scarcelli *et al.* (2004) to analyze the samples of abomasal contents, organs and/or fetal annexes of 67 aborted bovine fetuses, besides the

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bacteriological methods. PCR-multiplex showed 50.7% (34/67) of the samples positive for *Brucella*, whereas, the *Brucella* could be isolated from 38.8% (26/67) of the samples, thus showing an 88% agreement rate between the two methods used. As per the results, PCR was found to be more sensitive than culture in bovine brucellosis cases. Another multiplex PCR (mPCR) for the detection of *Brucella* spp. and *Leptospira* spp. in aborted bovine fetuses was described by Richtzenhain *et al*. (2002) who applied the mPCR to 63 clinical samples from bovine aborted fetuses. The 31 kDa outer membrane protein gene for *Brucella* spp. (primer B4/B5) was employed in the mPCR which amplified the expected size of amplicons (223 bp).
The mPCR assays for the detection of *Brucella* spp. (PCR/Bruce) and *Salmonella abortus ovis* (PCR/SAO) was performed by Sharifzadeh *et al*. (2008) for analyzing 54 clinical samples from aborted fetal stomach contents collected from the ovine abortion. The isolates amplified amplicons of 243 bp for *Brucella* spp. and 172 bp for *Salmonella abortus ovis*. Out of the 54 samples, 14 samples were totally negative for both, 10 resulted positive for *Brucella* spp., 24 came positive for *Salmonella abortus ovis* and 6 resulted positive for both the bacteria. Lopez-Goni *et al*. (2008) also used Bruce-Ladder PCR for the confirmation of *Brucella* species from 625 *Brucella* strains from different animal and geographical origins, and they obtained the expected profile of *Brucella abortus* in field isolates compared to known strains. The multiplex PCR Bruce-ladder based typing of 153 *Brucella* strains isolated from different regions of Mexico was reported by Morales-Estrada *et al*. (2012). The three vaccine strains (*B. abortus* S19 and RB51, and *B. melitensis* Rev1) were also included in this study. The results of microbiological typing and multiplex Bruce ladder amplification were identical for all the *Brucella* isolates tested. Because of its speed, Bruce-Ladder PCR served as a useful method in the typing of *Brucella* species isolated from animal and humans. The same assay was used by Orzil *et al*. (2016) for the identification and differentiation of *Brucella* species *viz*., *Brucella abortus*, *B. suis*, *B. ovis*, *B. melitensis* in the field as well as slaughter house samples in Brazil.

Real Time PCR

So far, acceptance of molecular diagnostics has been slow. This is reasonable considering the consequences at stake. However, threats of biological warfare and agro-terrorism may accelerate the process. Already, assays specific to this circumstance are being considered (McDonald *et al*., 2001) and the real-time decision is being explored (Redkar *et al.*, 2001). The real-time PCR constitutes a further technological improvement for the molecular identification and differentiation of *Brucella* species. Many studies have shown that the conventional method for detecting *Brucella* spp. are technically time-consuming and labour intensive than real-time PCR assay (Bogdanovich *et al*., 2004; Yang *et al*., 2007). A measurable fluorescence signal is obtained during the real-time PCR process by different approaches, relying on the cleavage of fluorogenic probes, e.g. by double-stranded DNA intercalating dye (SYBR Green I), by enzymatically released fluorophores (5'exonuclease assay) or by fluorescence resonance energy transfer (hybridization probes).

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Using SYBR Green I assay, designing of probes is not required because of the non-specific intercalation of the dye in double-stranded DNA followed by fluorescence emission. However melting curve analysis is mandatory and sometimes, the amplicon sequencing can be necessary. Real-time PCR methods based on SYBR green have been shown to be less expensive than TaqMan based methods and more sensitive than conventional PCR techniques. Due to their ability to amplify larger fragments than TaqMan based real-time PCRs, these methods enable phylogenetic studies by sequencing PCR products. Finally, they can be easily adapted to conventional PCR protocols, and therefore can be used in a larger number of laboratories (Sacristán *et al*., 2015).

The real-time PCRs were described by Redkar *et al*. (2001); Newby *et al*. (2003); Probert *et al*. (2004) and Bogdanovich *et al*. (2004) as rapid, sensitive and specific diagnostic tools with a low risk of cross-contamination and the potential of automation. Using real-time PCR, the nucleic acids can be quantified and data can be automated for the individual samples. There is no requirement of post-amplification handling of PCR products and it can be performed in a very short time without electrophoretic analysis, thereby reducing the risk of laboratory contamination as well as false-positive results. Recently, the real-time PCR assays have been described for testing the *Brucella* cells (Redkar *et al*., 2001), urine (Queipo-Ortuno *et al*., 2005), blood, and paraffin-embedded tissues (Kattar *et al*., 2007). The real-time PCR assays targeting 16S-23S internal transcribed spacer region (ITS) and the genes coding o*mp25* and *omp31* (Kattar *et al*., 2007), *bcsp 31* (Colmenero *et al*., 2005; Debeaumont *et al*., 2005; Queipo-Ortuño *et al*., 2008), and *IS711* (Cerekci *et al*., 2011; Zhang *et al*., 2013) have been developed for the rapid detection and differentiation of *Brucella* species in clinical samples.

Three separate real-time PCRs were developed to specifically identify seven biovars of *B. abortus*, three biovars of *B. melitensis* and biovar one of *B. suis* using fluorescence resonance energy transfer; with the upstream primers derived from the insertion element, *IS711* whereas, the reverse primer and FRET probes were selected from unique species or biovar-specific chromosomal loci. The sensitivity of *B. abortus*-specific assay was as low as 0.25 pg DNA corresponding to 16-25 genome copies and similar detection levels were also observed for *B. melitensis* and *B. suis*-specific assays (Redkar *et al*., 2001). In spite of the *IS711* based real-time PCR assay being specific and highly sensitive, its different copy number according to each species can affect the quantification of bacterial load (Chen *et al*., 2007). Another study for evaluation of three assays (SYBR Green I, 5-exonuclease and hybridization probes) to detect *Brucella abortus* was done by Newby *et al.* (2003) and the greatest specificity was achieved with the hybridization probe assay for use in a real-time PCR assay to detect *Brucella abortus.* Whereas, in another study, the majority (>90%) of the tested *B. abortus* and *B. melitensis* strains could be correctly identified in a real-time multiplex assay, employing only one reverse primer (binding IS*711* of *B. abortus* and *B. melitensis*)

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and two specific forward primers (targeting the neighbouring *alkB* and BMEI1162 gene, respectively) (Probert *et al*., 2004).

Meanwhile, other real-time PCR assays were also developed, like the LightCycler-based real-time PCR (LCPCR) assay developed by Queipo-Ortuno *et al.* (2005) for the detection of *Brucella* based on a 223-bp gene sequence encoding an immunogenetic membrane protein (BCSP31) specific for the *Brucella* genus; melting curve and DNA sequencing analysis was performed to verify the specificity of the PCR products. This LC-PCR assay was found to be 91.9% sensitive and 95.4% specific for active brucellosis. The viability of using conventional and real-time PCR assays as potential diagnostic tools for the detection of *B. abortus* in naturally infected cows was assessed by O'Leary *et al*. (2006). In this study, PCR assays that amplified various regions of the *Brucella* genome, IS711 genetic element, 31 kDa *omp* and 16S rRNA, were optimized using nine known *Brucella* strains. To evaluate the various previously published real-time PCR assays targeting *bcsp31*, *per*, IS711, alkB/IS711 and BMEI1162/ IS711, an in-house assay was developed by Al Dahouk *et al*. (2007) using 248 *Brucella* strains representing the biotypes of all species and a large panel of clinically relevant, phylogenetically related and serologically cross-reacting bacteria. It was concluded that assays targeting the *bcsp31* gene can be recommended to screen for *Brucella*. A comprehensive approach was made by Hinic *et al*. (2008) who used unique genetic loci of the six classical species to develop seven individual reactions for detection of the *Brucella* genus and the differentiation between the six species to be used in conventional as well as real-time PCR assay based on the *Brucella*-specific insertion sequence IS*711*.

Also, the further studies done by Bounaadja *et al*. (2009) by using TaqMan probes for the detection of *Brucella* at genus level, revealed that the real-time PCR assay targeting IS711 presented an identical or a greater sensitivity than those targeting the *bcsp31* and *per* genes. The TaqMan probe-based real-time PCR with the target sequence of IS711 was carried out with the probe of *Brucella* genus Hinic Probe (IS711) by Hinic *et al*. (2009). The real-time PCR assays using such TaqMan probes have been used to study the prevalence of *Brucella* by various investigators, like Doosti and Ghasemi (2011) who performed TaqMan analysis on 425 bovine blood samples in southwest Iran and found 9, 69 and 5 of these samples to be positive for *B. melitensis*, *B. abortus*, and both bacteria respectively. A similar real-time assay was carried out by Dehkordi *et al*. (2012) in a total of 3710 DNA of abomasal contents of bovine, ovine, caprine and camel aborted fetuses. In the bovine fetuses, 281/892 (31.5%) gave positive results for *Brucella* species by conventional PCR and the TaqMan analysis confirmed that 45/281 and 231/281 were positive for *B. meltensis* and *B. abortus*, respectively.

Besides the studies being conducted in the animals, the human studies also revealed the real-time PCR assay to be an efficient monitoring tool, such as the one conducted by Sohrabi *et al*. (2014) who reported efficient diagnosis and treatment follow-up of human brucellosis by a novel quantitative TaqMan real-time

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PCR assay based on *bcsp31* gene; by monitoring the DNA load of the 37 brucellosis patients for four weeks which decreased significantly by the end of the treatment period.

Other Molecular Approaches

Genomic fingerprinting is another approach to differentiate between *Brucella* species, biovars and strains. REP- and ERIC-PCR (rep-PCR) were used to examine whether these assays could be used for a differential typing of brucellae on the basis of the resulting banding patterns (genomic fingerprints) (Mercier *et al*., 1996; Tcherneva *et al*., 1996). The two studies yielded inconsistent results and REP-PCR assays proved to be difficult to reproduce and not the method of choice for reliable differentiation of species and strains. However, both groups showed that by the one or the other technique a range of different banding patterns could be obtained and that even *B. canis* strains could be differentiated from the other species by their banding patterns after REP-PCR (Tcherneva *et al*., 1996). Complex banding patterns were also found after PCR analyses of *Brucella* when short (10mer) and randomly designed primers were used for amplification (RAPD PCR) (Fekete *et al*., 1992; Tcherneva *et al*., 2000).

A fingerprinting method with a DIG-labelled IS*711* probe was successfully used to differentiate between terrestrial and marine *Brucella* strains (Ouahrani-Bettache *et al*., 1996; Bricker *et al*., 2000). A similarly high variability in genomic fingerprints was detected in the typing of *Brucella* strains after a 'multi-locus variable number tandem repeats' analysis. However, the results showed neither species- nor biovar specificity (Bricker *et al*., 2003). The high genomic similarities between *Brucella* species is a major reason for the difficulty to differentiate between them on the basis of molecular techniques. Hence, the newer approaches including the proteomics-based (for e.g. MALDI-TOF-MS), and genomics-based techniques like fingerprinting and other approaches as the key strategy towards the identification of regions of variability in the *Brucella* genomes, are being evaluated to cover the sensitivity and specificity related challenges in the diagnosis of brucellosis.

Matrix-Assisted Laser Desorption Ionization- Time of Flight- Mass Spectrometry (MALDI-TOF-MS)

Still undisputable detection of *Brucella* is the classic isolation followed by genus and species identification with either phenotypic or nucleic acid recognition (OIE, 2012). But the molecular diagnostic methods, mainly 16S ribosomal RNA sequencing or real-time PCR detection of selected genes remain complicated and costly, and are not suited for use on the vast majority of routine samples. Further, the high genetic and phenotypic homology of *Brucella* renders its genospeciation an easier-said-than-done task. PCR based on specific genome sequences still suffer some inter-lab standardization problems (Yu and Nielsen, 2010). On the other hand, phenotypic methods reveal quantitative rather than qualitative differences among brucellae.

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Analysis of cellular proteome is a method which occupies an intermediary position with respect to the phenotypic–genotypic dichotomy, since the proteins analyzed reflect gene products and metabolic functions (Singhal *et al.*, 2015). The automation in proteomics technology, in recent years, has increased its throughput and potential use for microbial identification. The MALDI-TOF MS technique, combined with reference peptide databases and advanced software, has revolutionized microbial characterization (Seng *et al*., 2009). It is consistent with 16S rRNA gene sequencing and is expected to substitute for classic biochemical tests (Van Belkum *et al*., 2012). Its quickness and reliability make it fit for counter-bioterrorism, epidemiological tracing of field strains and detection of food contamination (Sandrin *et al*., 2013). MALDI-TOF MS is approximately two-thirds less expensive than conventional bacteriological methods (Böhme *et al*., 2012).

MALDI-TOF-MS has existed for a long time but it was in 1996 when MALDI-TOF spectral fingerprints could be obtained from whole bacterial cells for the first time (Holland *et al*., 1996). The same year, Krishnamurthy *et al*. (1996) obtained spectral fingerprints of pathogenic species such as *Bacillus anthracis, B. melitensis, Yersinia pestis,* and *Francisella tularensis* using MALDI-TOF (Carbonnelle *et al*., 2011). Ever since, the number of publications concerning the bacterial as well as mold and yeast identification have increased exponentially. However, use of MALDI-TOF in clinical microbiology as a routine first-line identification method started just during the past five years (Kostrzewa *et al*., 2013). Databases that include the main pathogenic microorganisms have been developed, thus allowing the use of this method in routine bacterial identification from plate culture. Recently, to identify *Brucella* species a reference library was constructed using 12 *Brucella* strains. With this '*Brucella* library' discrimination was not possible to the species level (Ferreira *et al*., 2010).

MALDI-TOF-MS technology has fundamentally altered well established diagnostic testing methods because of its significant advantages over other conventional and molecular identification methods (Murray, 2012). It is rapid, and reliable, it takes only few minutes for correct identification (Fenselau, 2012). In addition, the MALDI technique is simple, does not require highly skilled personnel and is cost-effective (Seng *et al*., 2009). MALDI-TOF-MS works well for many bacterial species hence has the potential to replace conventional phenotypic identification for most bacterial strains isolated in clinical microbiology laboratories (Biswas and Rolain, 2013). The intrinsic property of MALDI-TOF-MS is to detect the mass-to-charge ratio (m/z) of bacterial ribosomal proteins, providing a unique mass spectrum of the microorganism within minutes (Carbonnelle *et al*., 2011). Importantly, MALDI approach does not rely on actual identification of the biomarker ion peaks in an MS spectrum but on the characteristic mass profile generated by a set of ion peaks that constitute a bacterial "fingerprint" (Dieckmann and Malorny, 2011). In this method, the biopolymer molecules are converted into isolated ionized molecules in the gas phase which are then separated according to their molecular weight after migration in an electric field. Each molecule

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detected is characterized by the molecular mass, the charge, and the relative intensity of the signal (Carbonnelle *et al*., 2011). A mass spectrum unique to the organism is produced, get compared to a library of spectra obtained from known reference organisms, and the organism's likely identification is provided based on the closest match (Dekker and Branda, 2011). The SARAMIS (Spectral Archive and Microbial Identification System) database version 4.09 (originally developed by AnagnosTec, contains ReferenceSpectra for 1161 bacteria, and 263 mycota and yeast, and SuperSpectra for 552 bacteria, and 139 mycota and yeast (Martiny *et al*., 2012).

MALDI-TOF MS may be used to analyze samples of many types, including solutions of organic molecules, nucleic acids, proteins, and whole microorganisms, with the last two being the most useful in present clinical Microbiology applications (Dekker and Branda, 2011). Fatty acids were evaluated early on as biomarkers for bacterial identification, but rejected as they are too dependent on growth and storage condition (Fenselau, 2012). However, the most reliable MS biomarkers for bacterial identification are considered to be the major proteins, mainly ribosomal proteins which are abundant, basic, and of medium hydrophobicity, all biochemical traits that favor efficient ionization (De Carolis *et al*., 2014). A sufficient number of stable mass signals of these proteins(between 2000 Da and 20000 Da) can be detected, yielding profile spectra consisting of a series of peaks that are conserved at genus, species and subspecies as well (Barbuddhe *et al*., 2008).

Recently, the MALDI-TOF-MS has been used as a tool for classification and subtyping of bacteria. While MALDI-TOF spectrometry-based identification of bacteria at genus and species levels has been shown clearly to be rapid and effective, the utility of this approach at the strain level has not been completely explored and lack approved guidelines for data interpretation. Identification to the more specific ''strain'' requires higher resolution approaches and tends to be more challenging, because strains within a single species are quite often extremely similar, genotypically and phenotypically (Sandrin *et al*., 2013). There has been considerable interest in using MALDI-TOF MS to identify fastidious organisms and potential agents of bioterrorism. Ferreira *et al*. (2010) reported the reliability of MALDI-TOF in the identification of *Brucella species* at genus level by studying 131 clinical isolates on the MALDI Biotyper 2.0 profiles created for type strains belonging to *B. melitensis* biotypes 1, 2 and 3; *B. abortus* biotypes 1, 2, 5 and 9; *B. suis*, *B. canis*, B. *ceti* and *B. pinnipedialis*. As stated by him, an important problem for the routine use of MALDI-TOF-MS for identification of *Brucella* species is that no reference library for *Brucella* has been incorporated to the main databases, because of the issues derived from their potential bio-terrorist use.

Later, the accurate identification of *Brucella* upto species level was achieved by Lista *et al*. (2011) using MALDI-TOF-MS by constructing a *Brucella* reference library based on multilocus variable-number tandem repeat analysis (MLVA) data. The comparison of MS-spectra from *Brucella* species against a custom-made MALDI-TOF-MS reference library could be utilized to use MALDI-TOF-MS as a rapid

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identification method for *Brucella* species. In this manner, identification of 99.3% of the 152 isolates at the species level, and *B. suis* biovar 1 and 2 at the biovar level was obtained. This result demonstrated that even minimal genomic differences between these serovars of *Brucella* translate to specific proteomic differences. The results from other studies also indicated that MALDI-TOF-MS assay is a reliable approach to identify *Brucella* genus and species and an increasing number of different *Brucella* strains in the database could provide a higher discriminatory power. A similar inference was given by De Maio *et al*. (2015) who assessed a new protein extraction protocol and constructed a home-made reference database to improve the efficiency of the method. The reliability of this database was evaluated by testing blind-coded *Brucella* field isolates and reference strains. The identification results at the genus level were always correct whereas, at the species level, a total of 94% bacterial samples were correctly identified. On the other hand, incorrect biovar assignments resulted in 23 out of 39 *B. abortus* strains and in 4 out of 53 *B. melitensis* strains.

In further studies, the biovar delineation of *Brucella* was further assessed. Sayour and Sayour (2015) evaluated the MALDI-TOF-MS for biotyping of 124 *Brucella* isolates from raw milk and tissues of cattle, buffaloes, sheep and goats in 9 governorates and unknown areas in Egypt for way faster and reliable genospeciation based on protein profiles. It was concluded that despite the high intrageneric similarity of *Brucella*, the MALDI Biotyper had enough resolution for binomial identification with good matching scores but MALDI identification at the biovar level was accurate in only *B. melitensis* bv. 3. Due to the limitation of the library created, it was hard to judge for the other *Brucella* species tested.

Lastly, for the routine application of MALDI-TOF-MS to unidentified bacteria as well as the safety of laboratory workers, a simple and safe protocol was needed which could allow storage, and eventual shipping, of inactivated samples. Mesureur *et al*. (2016) described such protocol for preparing *Brucella* samples prior to their analysis by matrix-assisted laser desorption ionization–time of flight mass spectrometry, which was also effective for several other bacterial pathogens.

Conclusion

MALDI-TOF-MS is a rapid as well as a reliable technique for the identification of bovine brucellosis and has the potential to become a first-line screening tool for epidemiological studies and outbreak investigations with minimal time, labor and cost, making it an attractive alternative to the relatively high investment required for other conventional and molecular settings.

References

1. Adone R, Ciuchini F, La Rosa G, Marianelli C and Muscillo M. (2001). Use of polymerase chain reaction to identify *Brucella abortus* strain RB51 among *Brucella* field isolates from cattle in Italy. *Journal of Veterinary Medicine. B, Infectious Diseases and Veterinary Public Health,* 48(2): 107-113.
2. Al Dahouk S and Nockler K. (2011). Implications of laboratory diagnosis on brucellosis therapy. *Expert Review Anti-Infective Therapy,* 9: 833-845.

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DOI 10.5455/ijlr.20190413030405

3. Al Dahouk S, Le Flèche P, Nöckler K, Jacques I, Grayon M, Scholz HC, Tomaso H, Vergnaud G and Neubauer H. (2007). Evaluation of *Brucella* MLVA typing for human brucellosis. *Journal of Microbiological Methods,* 69: 137-145.
4. Al Dahouk S, Sprague DL and Neubauer H. (2013). New developments in the diagnostic procedures for zoonotic brucellosis in humans. *Revue scientifique et technique (International Office of Epizootics),* 32:177-188.
5. Alain A, Ocampo-Sosa JA, Juan, M and Garcialobo. (2005). Development of a new PCR assay to identify *Brucella abortus* biovars 5, 6 and 9 and the new sub group 3b of biovar 3. *Veterinary Microbiology,* 110: 41- 51.
6. Alsharabasi OA. (2015). Application of MALDI-TOF mass spectrometry as a tool for biotyping of *B. melitensis.* M.Sc. Thesis submitted to the Qatar University, Doha, Qatar.
7. Alton GG, Jones LM, Angus RD, Verger JM. (1988). Techniques for the brucellosis laboratory. Institut National de la Recherche Agronomique, Paris, France.
8. Amin AS, Hamd, ME and Ibrahim AK. (2001). Detection of *Brucella melitensis* in semen using the polymerase chain reaction assay. *Veterinary Microbiology,* 83: 37-44.
9. Baddour MM. and Alkhalifa DH. (2008). Evaluation of three polymerase chain reaction techniques for detection of *Brucella* DNA in peripheral human blood. *Canadian Journal of Microbiology,* 54(5): 352-357.
10. Baek BK, Lim CW, Rahman MS, Kim CH, Oluoch A and Kakoma I. (2003). *Brucella abortus* infection in indigenous Korean dogs. *Canadian Journal of Veterinary Research,* 67(4): 312-314.
11. Baily GG, Krahn JB, Drasar BS and Stoker NG. (1992). Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. *Journal of Tropical Medicine and Hygiene,* 95: 271-275.
12. Barbuddhe SB, Maier T, Schwarz G, Kostrzewa M, Hof H, Domann E, Chakraborty T and Hainm T. (2008). Rapid identification and typing of *Listeria* species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Applied and Environmental Microbiology,* 74: 5402-5407.
13. Bercovich Z. (1998). Maintenance of *Brucella abortus*-free herds: a review with emphasis on the epidemiology and the problems in diagnosing brucellosis in areas of low prevalence. *Veterinary Quarterly,* 20: 81-88.
14. Biswas S and Rolain, JM. (2013). Use of MALDI-TOF mass spectrometry for identification of bacteria that are difficult to culture. *Journal of Microbiological Methods,* 92: 14-24.
15. Bogdanovich T, Skurnik M, Lübeck PS, Ahrens P and Hoorfar J. (2004). Validated 5' nuclease PCR assay for rapid identification of the genus *Brucella. Journal of Clinical Microbiology,* 42(5): 2261-2263.
16. Böhme K, Fernández-No IC, Barros-Velázquez J, Gallardo JM, Cañas B and Calo-Mata P. (2012). SpectraBank: an open access tool for rapid microbial identification by MALDI-TOF MS fingerprinting. *Electrophoresis,* 33(14): 2138-2142
17. Borriello G, Peletto S, Lucibelli MG, Acutis PL, Ercolini D, and Galieroa G. (2013). Link between geographical origin and occurrence of *Brucella abortus* biovars in cow and water buffalo herds. *Applied and environmental microbiology,* 79(3): 1039-1043.
18. Boschiroli L, Foulongne, V and O'Callaghan D. (2001). Brucellosis: A worldwide zoonosis. *Current Opinion in Microbiology,* 4: 58-64.
19. Bounaadja L, Albert D, Chénais B, Hénault S, Zygmunt MS, Poliak S and Garin-Bastuji B. (2009). Real-time PCR for identification of *Brucella* spp.: a comparative study of IS*711, bcsp31*and *per* target genes. *Veterinary Microbiology,* 137: 156-164.
20. Bricker BJ and Halling SM. 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.

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DOI 10.5455/ijlr.20190413030405

21. Bricker BJ and Halling SM. (1995). Enhancement of the Brucella AMOS PCR assay for differentiation of *Brucella abortus* vaccine strains S19 and RB51. *Journal of Clinical Microbiology,* 33: 1640-1642.
22. Bricker BJ, Ewalt DR and Halling SM. (2003). *Brucella* 'HOOF-Prints': strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). *BMC Microbiology,* 3:15.
23. Bricker BJ, Ewalt DR and Halling SM. (2003). *Brucella* 'HOOF-Prints': strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). *BMC Microbiology,* 3:15.
24. Bricker BJ, Ewalt DR, MacMillan AP, Foster G and Brew S. 2000. Molecular characterization of Brucella strains isolated from marine mammals. *Journal of Clinical Microbiology,* 38: 1258-1262.
25. Bricker BJ. (2000). Characterization of the three ribosomal RNA operons rrnA, rrnB, and rrnC, from *Brucella melitensis*. *Gene,* 255: 117-126.
26. Bricker BJ. (2002). PCR as a diagnostic tool for brucellosis. *Veterinary Microbiology,* 90: 435-446.
27. Bridgewater DR. (1989). Epidemiological report, Western regional epidemiologist U.S. *Department of Agriculture's* (USDA) *Animal and Plant Health Inspection Service* (APHIS).
28. Carbonnelle E, Mesquita C, Bille E, Day N, Dauphin B, Beretti JL, Ferroni A, Gutmann L and Nassif X. (2011). MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. *Clinical Biochemistry,* 44: 104-109.
29. Casañas MC, Queipo-Ortuño M I, Rodriguez-Torres A, Orduña A, Colmenero JD and Morata P. (2001). Specificity of a polymerase chain reaction assay of a target sequence ond the 31-kilodalton *Brucella* antigen DNA used to diagnose human brucellosis. *European Journal of Clinical Microbiology and Infectious Diseases,* 20: 127-131.
30. Cerekci A, Kılıç S, Bayraktar M, Uyanık MH, Yaşar E, Esen B. (2011). Comparison of conventional methods and real-time multiplex polymerase chain reaction for identification and typing of *Brucella* isolates of human origin. *Mikrobiyoloji Bülteni,* 45(3): 392-400.
31. Cetinkaya B, Ongor H, Muz A, Ertas H B, Kalender H and Erdogan HM. (1999). Detection of *Brucella* species DNA in the stomach content of aborted sheep fetuses by PCR. *Veterinary Record,* 144: 239-240.
32. Chen J, Kadlubar FF and Chen JZ. (2007). DNA supercoiling suppresses real-time PCR: a new approach to the quantification of mitochondrial DNA damage and repair. *Nucleic Acids Research,* 35:1377-88.
33. Chothe S, Saxena HM and Chachra D. (2013). Comparative Evaluation of PCR and ELISA for diagnosing Bovine Brucellosis. *Intas Polivet,* 14(1): 80-84.
34. Cloeckaert A, Grayon M and Grepinet O. (2000). An IS711 element downstream of the bp26 gene is a specific marker of *Brucella* spp. isolated from marine mammals. *Clinical and Diagnostic Laboratory Immunology,* 7: 835-839.
35. Cloeckaert A, Grayon M, Grepinet O. and Boumedine KS. (2003). Classification of *Brucella* strains isolated from marine mammals by infrequent restriction site-PCR and development of specific PCR identification test. *Microbes and Infection,* 5: 593-602.
36. Cloeckaert A, Verger JM, Grayon M and Grepinet O. (1995). Restriction site polymorphism of the genes encoding the major 25 kDa and 36 kDa outer-membrane proteins of *Brucella*. *Microbiology,* 141: 2111-2121.
37. Cloeckaert A, Verger JM, Grayon M and Vizcaino N. (1996). Molecular and immunological characterization of the major outer membrane proteins of *Brucella*. *FEMS Microbiology Letters,* 145: 1-8.
38. Cloeckaert A, Verger JM, Grayon M, Paquer, JY, Garin-Bastuji B, Foster G and Godfroid J. (2001). Classification of *Brucella* spp. isolated from marine mammals by DNA polymorphism at the *omp2* locus. *Microbes and Infection,* 3: 729-738.
39. Colmenero JD, Queipo-Ortuño MI, Reguera JM, Baeza G, Salazar JA, Morata P. (2005). Real time polymerase chain reaction: a new powerful tool for the diagnosis of neurobrucellosis. *Journal of Neurology, Neurosurgery and Psychiatry,* 76(7): 10251027.

40. Corbel MJ, Stuart FA, Brewer RA. (1983). Observations on serological cross-reactions between smooth *Brucella* species and organisms of other genera. 3rd International Symposium on Brucellosis; In Develop Biology Standard. 56: 341-348.
41. Corbel MJ. (1972). Characterization of antibodies active in Rose Bengal Plate Test. *Veterinary Record,* 90: 484-485.
42. Cortez A, Scarcelli E, Soares RM, Heinemann MB, Sakamoto SM, Genovez ME, Ferreira F and Richtzenhaim LJ. (2001). Detection of *Brucella* DNA from aborted bovine foetus by polymerase chain reaction. *Australian Veterinary Journal,* 79: 500-501.
43. Da Costa M, Guillou JP, Garin-Bastuji B, Thieband M and Dubray G. (1996). Specificity of six gene sequences for the detection of the genus *Brucella* by DNA amplication. *Journal of Applied Bacteriology,* 81: 267-275.
44. De Carolis E, Vella A, Vaccaro L, Torelli R, Spanu T, Fiori B, Posteraro, B and Sanguinetti M. (2014). Application of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *The Journal of Infection in Developing Countries,* 8: 1081-1088.
45. De Maio F, Sali M, Tarantino M, Garofolo G, Pasquali P, Petrucci P, Sanguinetti M, Di Giannatale E, Tittarelli M and Adone R. (2015). Identification of *Brucella* spp. Isolates by MALDI-TOF mass spectrometry. *Journal of Bacteriology and Parasitology,* 6:4.
46. De Miguel J, Marín M, Muñoz M, Dieste L, Grilló J and Blasco M. (2011). Development of a selective culture medium for primary isolation of the main *Brucella* spp. *Journal of Clinical Microbiology,* 49: 1458-1463.
47. Debeaumont C, Falconnet PA, Maurin M. (2005). Real-time PCR for detection of *Brucella* spp. DNA in human serum samples. *European Journal of Clinical Microbiology and Infectious Diseases,* 24(12): 842-845.
48. Dehkordi FS, Saberian S and Momtaz H. (2012). Detection and identification of *Brucella abortus* and *Brucella melitensis* in aborted bovine, ovine, caprine, buffaloes and camelid fetuses by application of conventional and real time polymerase chain reaction. *Thai Journal of Veterinary Medicine,* 42(1):13-20.
49. Dekker JP and Branda JA. (2011). MALDI-TOF mass spectrometry in the clinical microbiology laboratory. *Clinical Microbiology Newsletter,* 33: 87-93.
50. Deshmukh A, Hagen F, Alsharabasi OA, Mariamma A, Godwin W, Doiphode S, Maslamani MA and Meis JF. (2015). In vitro antimicrobial susceptibility testing of human *Brucella melitensis* isolates from Qatar between 2014-2015. *BMC Microbiology,* 15: 121.
51. Diaz R, Casanova A, Ariza J and Moriyon I. (2011). The rose Bengal test in human brucellosis: A neglected test for the diagnosis of a neglected disease. *PLoS Neglected Tropical Diseases,* 5: 1-7.
52. Dieckmann R and Malorny B. (2011). Rapid screening of epidemiologically important *Salmonella enterica subsp. enterica* serovars by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Applied and Environmental Microbiology,* 77: 4136-4146.
53. Doosti A and Ghasemi DP. (2011). Application of real-time PCR for identification and differentiation of *Brucella abortus* and *Brucella melitensis* in cattle. *Bulgarian Journal of Veterinary Medicine,* 14(2): 109-115.
54. Dorsch M, Moreno E and Stackbrandt E. (1989). Nucleotide sequence of the 16S rRNA from *Brucella abortus*. *Nucleic Acids Research,* 17: 1765.
55. Doust SRH, Ahamdi Z, Ahamdi A, Hajia M, Izadi M and Mobarez AM. (2007). Detection of *Brucella abortus* by alkB and *IS711* based primers. *Journal of Research in Medical Sciences,* 12: 62-67.
56. Eisenberg T, Ribe K, Schauerte N, Geiger C, Blom J and Scholz HC. (2017). Isolation of a novel 'atypical' *Brucella* strain from a bluespotted ribbontail ray (*Taeniura lymma*). *Antonie Van Leeuwenhoek,* 110 (2): 221-234.
57. Fekete A, Bantle JA and Halling SM. (1992). Detection of *Brucella* by polymerase chain reaction in bovine fetal and maternal tissues. *Journal of Veterinary Diagnostics and Investigations,* 4: 79-83.

CC BY NC SA
DOI 10.5455/ijlr.20190413030405

58. Fekete A, Bantle JA, Halling SM and Sanborn MR. (1990). Preliminary development of a diagnostic test for *Brucella* using polymerase chain reaction. *Journal of Applied Bacteriology,* 69: 216-227.
59. Fekete A, Bantle JA, Halling SM and Stich RW. (1992). Amplification fragment length polymorphism in *Brucella* strains by use of polymerase chain reaction with arbitrary primers. *Journal of Bacteriology,* 174: 7778-7783
60. Fenselau C. (2012). Rapid Characterization of Microorganisms by Mass Spectrometry-What Can Be Learned and How? *Journal of the American Society for Mass Spectrometry,* 24, 1161-1166.
61. Ferrao-Beck L, Cardoso R, Munoz PM, De Miguel MJ, Albert D, Ferreira AC, Marin CM, Thiebaud M, Jacques I, Grayon M, Zygmunt MS, Garin-Bastuji B, Blasco JM and Sa MI. (2006). Development of a multiplex PCR assay for polymorphism analysis of *Brucella suis* biovars causing brucellosis in swine. *Veterinary Microbiology,* 115: 269-277.
62. Ferreira L, Vega S, Sanchez-Juanes F, Gonzalez-Cabrero S and Menegotto F. (2010). Identification of *Brucella* by MALDI-TOF mass spectrometry. Fast and reliable identification from agar plates and blood cultures. *PLoS One,* 5: 14235.
63. Ferris RA, Schoenbaum MA and Crawford RP. (1995). Comparison of serologic tests and bacteriologic culture for detection of brucellosis in swine from naturally infected herds. *Journal of the American Veterinary Medical Association,* 207: 1332-1333.
64. Fitch TA, Bearden SW, Sowa BA and Admas LG. (1989). DNA sequence and expression of the 36-kDa outer membrane protein gene of *Brucella abortus*. *Infection and Immunity,* 57: 3281-3291.
65. Foster JT, Okinaka RT, Svensson R, Shaw K, De BK, Robison RA, Probert WS, Kenefic LJ, Brown WD and Keim P. (2008). Real-time PCR assays of single-nucleotide polymorphisms defining the major *Brucella* clades. *Journal of Clinical Microbiology,* 46: 296-301.
66. Fretin D, Whatmore AM, Al Dahouk S, Neubauer H, Garin-Bastuji B, Albert D, Van Hessche M, Ménart M, Godfroid J, Walravens K and Wattiau, P. (2008). *Brucella suis* identification and biovar typing by real-time PCR. *Veterinary Microbiology,* 131: 376-385.
67. Gallien P, Dorn C, Alban G, Staak C and Protz D. (1998). Detection of *Brucella* species in organs of naturally infected cattle by polymerase chain reaction. *Veterinary Record,* 142(19): 512-514.
68. García-Yoldi D, Flèche PL, De Miguel M, Muñoz PM, Blasco JM, Cvetnik Z, Marín CM, Vergnaud G and López-Goñi I. (2007). Comparison of multiple-locus variable-number tandem-repeat analysis with other PCR-based methods for typing *Brucella suis* isolates. *Journal of Clinical Microbiology,* 454070-4072.
69. Garcia-Yoldi D, Marin CM and Lopez-Goni I. (2005). Restriction site polymorphisms in the genes encoding new members of group 3 outer membrane protein family of *Brucella* spp. *FEMS Microbiology Letters,* 245: 79-84.
70. Garcia-Yoldi D, Marin CM, de Miguel MJ, Munoz PM, Vizmanos JL and Lopez-Goni 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.
71. Garshasbi M, Ramazani A, Sorouri R, Javani S and Moradi S. (2014). Molecular detection of Brucella species in patients suspicious of brucellosis from Zanjan. *Brazilian Journal of Microbiology,* 45(2): 533-538.
72. Ghodasara S. (2008). Isolation, identification, molecular characterization of *Brucella* from reproductive disorders of animals and serodetection of *Brucella* antibody. A thesis submitted to Anand Agricultural University, Gujarat, India.
73. Gupta VK, Kumari, R, Verma DK, Singh K, Singh SV and Vihan VS. (2006). Detection of *Brucella melitensis* from goat tissues employing PCR. *Indian Journal of Animal Sciences,* 76: 793-795.
74. Hadush A and Pal M. (2013). Brucellosis: An infectious re-emerging bacterial zoonosis of global importance. *International Journal of Livestock Research,* 3: 28-34.
75. Halling SM, Tatum FM and Bricker BJ. (1993). Sequence and characterization of an insertion sequence, IS*711*, from *Brucella ovis*. *Gene,* 133: 123-127.

CC BY NC SA
DOI 10.5455/ijlr.20190413030405

76. Hénault S, Calvez D, Thiébaud M, Bouličre M, Garin-Bastuji B. (2000). Validation of a nested-PCR based on the *IS6501/711* sequence for the detection of *Brucella* in animal samples. Proceedings of the Brucellosis 2000 International Research Conference 9I including the 53rd Brucellosis Research Conference. Nimes, France.
77. Herman L and Herman DR. (1992). Identification of *Brucella* spp. by using the polymerase chain reaction. *Applied and Environmental Microbiology,* 58: 2099-2101.
78. Hinic V, Brodard I, Thomann A, Cvetnic Z and Frey J. (2008). Novel identification and differentiation of *B.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.
79. Hinic V, Brodard I, Thomann A, Holub M, Miserez R and Abril C. (2009). IS711-based real-time PCR assay as a tool for detection of *Brucella* spp. in wild boars and comparison with bacterial isolation and serology. *BMC Veterinary Research,* 5: 22.
80. Holland RD, Wilkes JG, Rafii F, Sutherland JB, Person CC, Voorhees KJ and Lay JO. (1996). Rapid identification of intact whole bacteria based on spectral patterns using MALDI-TOF-MS. *Rapid Communications in Mass Spectrometry,* 10:1227-1232.
81. Hoyer BH and McCullough NB. (1968). Polynucleotide homologies of *Brucella* deoxyribonucleic acids. *Journal of Bacteriology,* 95: 444-448.
82. Hubber B, Scholz HC and Lucero N. (2009). Development of a PCR assay for typing and subtyping of *Brucella* species. *International Journal of Biological Sciences,* 63:563-73.
83. 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(2-3): 137-139.
84. Jia B, Zhang F, Lu Y, Zhang W, Li J, Zhang Y and Ding J. (2017). The clinical features of 590 patients with brucellosis in Xinjiang, China with the emphasis on the treatment of complications. *PLOS Neglected Tropical Diseases,* 11(5): E0005577.
85. Kanani AN. (2007). Serological, cultural and molecular detection of *Brucella* infection in breeding bulls. Ph.D. Thesis, Anand Agricultural University, Gujarat, India.
86. Karthik K, Rathore R, Thomas P, Arun TR, Viswas KN, Agarwal RK, Manjunathachar HV and Dhama K. (2014). Loop-mediated isothermal amplification (LAMP) test for specific and rapid detection of *Brucella abortus* in cattle. *Veterinary Quarterly,* 34(4): 174-179.
87. Kattar MM, Zalloua PA, Araj GF, Samaha-Kfoury J, Shbaklo H, Kanj SS, Khalife S and Deeb M. (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 Diseases,* 59(1): 23-32.
88. Keid LB, Soares RM, Vasconcellos SA, Chiecao DP, Megid JR, Salgado VR and Richtzenhain LJ. (2007). A polymerase chain reaction for the detection of *Brucella* canis in semen of naturally infected dogs. *Theriogenology,* 67: 1203-1210.
89. Kostrzewa M, Sparbier K, Maier T and Schubert S. (2013). MALDI-TOF MS: an upcoming tool for rapid detection of antibiotic resistance in microorganisms. *Proteomics Clinical Application,* 7: 767-778.
90. Krishnamurthy T, Ross PL and Rajamani U. (1996). Detection of Pathogenic and Non-Pathogenic Bacteria by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *Rapid Communications in Mass Spectrometry,* 10(8):883-888.
91. Lavaroni O, Aguirre N, Vanzini V, Lugaresi C and Torioni de Echaide S. (2004). Assessment of polymerase chain reaction (PCR) to diagnose brucellosis in a *Brucella* infected herd. *Revista Argentina de Microbiología,* 36: 101-106.
92. Le Flèche P, Jacques I, Grayon M, Al Dahouk S, Bouchon P, Denoeud F, Nöckler K, Neubauer H, Guilloteau LA and Vergnaud G. (2006). Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. *BMC Microbiology,* 6: 9.

CC BY NC SA
DOI 10.5455/ijlr.20190413030405

93. Leal-Klevezas DS, Lopez-Merino A and Martinez-Soriano JP. (1995). Molecular detection of *Brucella* spp. rapid identification of *B. abortus* biovar I using PCR. *Archives of Medical Research,* 26(3): 263-267.
94. Leal-Klevezas DS, Martinez VIO, Garcia CJ, Lopez MA and Martinez SJP. (2000). Use of polymerase chain reaction to detect *Brucella abortus* biovar 1 in infected goats. *Veterinary Microbiology,* 75: 91-97.
95. Leyla G, Kadri G and Umran O. (2003). Comparison of polymerase chain reaction and bacteriological culture for the diagnosis of sheep brucellosis using aborted fetus samples. *Veterinary Microbiology,* 93: 53-61.
96. Lista F, Reubsaet FA, De Santis R, Parchen RR, de Jong AL, Kieboom J, derLaaken AV, Voskamp-Visser I, Jansen SF and Paauw A. (2011). Reliable identification at the species level of *Brucella* isolates with MALDI-TOF-MS. *BMC Microbiology,* 11: 267.
97. Liu D. (2009). Molecular detection of foodborne pathogens. CRC Press, Taylor and Francis Group, USA.
98. López-Goñi I, García-Yoldi D, Marín CM, de Miguel MJ, Barquero-Calvo E, Guzmán-Verri C, Albert D and Garin-Bastuji B. (2011). New Bruce-ladder multiplex PCR assay for the biovar typing of *Brucella suis* and the discrimination of *Brucella suis* and *Brucella canis*. *Veterinary Microbiology,* 154(1-2): 152-155.
99. Lopez-Goni I, Garcia-Yoldi D, Marin CM, de Miguel MJ, Munoz PM, Blasco JM, Jaques I, Grayon M, Cloeckaert A, Ferreira AC, Cardoso R, Correa de Sa MI, Walravens K, Albert D and Garin-Bastuji B. (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.
100. Marianelli C, Ciuchini F, Tarantino M, Pasquali P and Adone R. 2006. Molecular characterization of the rpoB gene in *Brucella* species: new potential molecular markers for genotyping. *Microbes and Infection,* 8: 860-865.
101. Martiny D, Busson L, Wybo I, El Haj RA, Dediste A and Vandenberg O. (2012). Comparison of the Microflex LT and Vitek MS systems for routine identification of bacteria by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Journal of Clinical Microbiology,* 50: 1313-1325.
102. Mayer-Scholl A, Draeger A, Gollner C, Scholz HC, Nockler K. (2010). Advancement of a multiplex PCR for the differentiation of all currently described *Brucella* species. *Journal of Microbiological Methods,* 80(1): 112-114.
103. Maymona AM, Mohamed TS, Abdulwahab YA and Musa TM. (2014). Phenotypic characterization of *Brucella melitensis* isolated from livestock in Abu Dhabi Emirate. *African Journal of Microbiology Research,* 8(39): 3523-3528.
104. *McDonald* R, Cao T and Borschel R. (2001). *Multiplexing* for the detection of *multiple biowarfare* agents shows promise in the field. *Military Medicine,* 166: 237-239.
105. Mercier E, Jumas-Bilak E, Allardet-Servent A, O'Callaghan D and Ramuz M. (1996).. Polymorphism in *Brucella* strains detected by studying distribution of two short repetitive DNA elements. *Journal of Clinical Microbiology,* 34: 1299-1302.
106. Mesureur J, Ranaldi S, Monnin V, Girard V, Arend S, Welker M, O'Callaghan D, Lavigne JP and Keriel A. (2016). A simple and safe protocol for preparing *Brucella* samples for matrix-assisted laser desorption ionization–time of flight mass spectrometry analysis. *Journal of Clinical Microbiology,* 54: 449-452.
107. Meyer KF, Eddie B, Veazie L, Stevens IM, Stewart B and Gelger JC. (2008). The heterogenous infection chains as occupational diseases. *International Archives of Occupational and Environmental Health,* 5: 1934.
108. Michaux-Charachon S, Bourg G and Jumas-Bilak. (1997). Genome structure and phylogeny in the genus *Brucella*. *Journal of Bacteriology,* 179: 3244-9.

CC BY NC SA

DOI 10.5455/ijlr.20190413030405

109. Mitka S, Anetakis C and Souliou E. (2007). Evaluation of different PCR assays for early detection of acute and relapsing brucellosis in humans in comparison with conventional methods. *Journal of Clinical Microbiology,* 45: 1211-1218.
110. Morales-Estrada AI, Castillo-Salto J, López-Merino A, Morales-García1 MR, Valle-Valdez JG and Contreras-Rodríguez A. (2012). Characterization of *Brucella* species in Mexico by Bruce-Ladder polymerase chain reaction (PCR). *African Journal of Microbiology Research,* 6(11): 2793-2796.
111. Morata P, Queipo-Ortuno MI, Dios D and Colmenero J. (1998). Strategy for optimizing DNA amplification in a peripheral blood PCR assay used for diagnosis of human brucellosis. *Journal of Clinical Microbiology,* 36: 2443-2446.
112. Moreno E and Moriyon I. (2002). *Brucella melitensis*: a nasty bug with hidden credentials for virulence. *Proceedings of the National Academy of Sciences,* 99: 1-3.
113. Morgan WJB. 1982. *Brucella abortus*, Handbuch der bakteriellen Infektionen bei. Tieren. Jena 53-213.
114. Muñoz PM, Marín CM, Monreal D, Garin-Bastuji B, Díaz R, Mainarjaime RC, Moriyón I and Blasco JM. (2005). Efficacy of several serological tests and antigens for diagnosis of bovine brucellosis in the presence of false-positive serological results due to Yersinia enterocolitica O:9. *Clinical and Diagnostic Laboratory Immunology,* 12(1): 141-151.
115. Murray PR. (2012). What Is new in clinical microbiology microbial identification by MALDI-TOF mass spectrometry. *Journal of Molecular Diagnostics,* 14: 419-423.
116. Navarro E, Escribano J, Fernández J and Solera J. (2002). Comparison of three different PCR methods for detection of *Brucella* spp. in human blood samples. *FEMS Immunology and Medical Microbiology,* 34(2): 147-151.
117. Newby DT, Hadfield TL and Roberto FF. (2003). Real-time PCR detection of *Brucella abortus*: a comparative study of SYBR green I, 59-exonuclease, and hybridization probe assays. *Applied and Environmental Microbiology,* 69: 4753-4759.
118. Nielsen K, Smith P, Yu W, Nicoletti P, Jungersen G, Stack J and Godfroid J. (2006). Serological discrimination by indirect enzyme immunoassay between the antibody response to *Brucella* sp. and *Yersinia enterocolitica* O:9 in cattle and pigs. *Veterinary Immunology and Immunopathology,* 109:69-78.
119. Nielsen K. (2002). Diagnosis of brucellosis by serology. *Veterinary Microbiology,* 90 (1-4): 447-459.
120. Ocampo-Sosa AA, Agüero-Balbin J and Garcia-Lobo JM. (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.
121. Ohishi K, Takishita K, Kawato M, Zenitani R, Bando T, Fujise, Y, Goto Y, Yamamoto S and Maruyama T. (2004). Molecular evidence of new variant *Brucella* in North Pacific common minke whales. *Microbes and Infection,* 6: 1199-1204.
122. OIE. (2000). Bovine brucellosis. *In:* OIE Manual of Standards for Diagnostic Tests and Vaccines. 4th Edn. Office International des Épizooties, Paris, France. pp: 328-345.
123. OIE. (2004). Bovine brucellosis. *In:* OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 5th Edn. Office International des Épizooties, Paris, France. pp: 409-438.
124. OIE. (2009). Bovine brucellosis. In: OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals: Mammals, Birds and Bees. 7th Edn. Office International des Épizooties, Paris, France. pp: 1-35.
125. O'Leary S, Sheahan M and Sweeney T. (2006). *Brucella abortus* detection by PCR assay in blood, milk and lymph tissue of serologically positive cows. *Research in Veterinary Science,* 81(2): 170-176.
126. Ouahrani-Bettache S, Soubrier MP and Liautard JP. (1996). IS6501-anchored PCR for the detection and identification of Brucella species and strains. *Journal of Applied Bacteriology,* 81: 154-160.
127. Patel BC, Chauhan, HC, Chandel BS, Dadawala AI and Jain BK. (2015). Seroprevalence and Molecular Characterization of Brucella spp. in Buffalo from North Gujarat, India. *International Journal of Current Microbiology and Applied Sciences,* 4(4): 174-180.

 DOI 10.5455/ijlr.20190413030405

128. Patel TJ. (2007). Serological, Cultural and Molecular detection of *Brucella* infection in Bovines including quantification in milk by Real-time PCR. M.V.Sc. Thesis submitted to the Anand Agricultural University, Anand, Gujarat, India.
129. Paul E, Abdelkareem M and Malik S. (2017). Overview of Human Brucellosis in Aseer Region, Saudi Arabia. *Australasian Medical Journal,* 10(3): 202-210.
130. Poiester FP, Nielsen K, Samartino LE and Ling YW. (2010). Diagnosis of Brucellosis. *Open Veterinary Science Journal,* 4: 46-60.
131. Priyanka, Shringi BN, Patel KB, Chauhan HC, Chandel BS and Kashyap SK (2018b). Molecular Epidemiology of Brucellosis among Cattle in Western Rajasthan. *Indian Journal of Animal Research,* 6(8): 1065-1069.
132. Priyanka, Shringi BN and Kashyap SK. (2019a). Bovine brucellosis: A review on background information and perspective. *Journal of Entomology and Zoology Studies,* 7(2): 607-613.
133. Priyanka, Shringi BN, Choudhary OP and Kashyap SK. (2019b). Expression profiling of cytokine-related genes in *Brucella abortus* infected cattle. *Biological Rhythm Research,* DOI: https://doi.org/10.1080/09291016.2019.1600263
134. Priyanka, Shringi BN, Patel K, Chauhan HC, Chandel BC and Kashyap SK. (2017). Seroprevalence of Brucellosis in Cattle in Western Rajasthan. *Ruminant Science,* 6(2): 305-307.
135. Priyanka, Shringi BN, Patel K, Kumari M, Chauhan HC, Chandel BC and Kashyap SK. (2018a). Brucellosis in buffaloes in Western Rajasthan: A seroprevalence study. *Indian Veterinary Journal,* 95(8):77-79.
136. Probert WS, Schrader KN, Khuong NY, Bystrom, SL and Graves MH. (2004). Real-time multiplex PCR assay for detection of *Brucella* spp., *B. abortus*, and *B. melitensis*. *Journal of Clinical Microbiology,* 42: 1290-1293.
137. Queipo-Ortuño M I, Colmenero JD, Bravo MJ, García-Ordoñez MA and Morata P. (2008). 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,* 14(12): 1128-1134.
138. Queipo-Ortuño M, Colmenero JD, Baeza G and Morata P. (2005). Comparison between Light Cycler real-time polymerase chain reaction (PCR) assay with serum and PCR-enzyme-linked immunosorbent assay with whole blood samples for the diagnosis of human brucellosis. *Clinical Infectious Diseases,* 40: 260-264.
139. Quinn PJ, Carter ME, Markey B and Carter GR. (1999). Clinical Veterinary Microbiology, Edinburgh: Mosby International Limited. pp: 261-267.
140. Radostits OM, Blood DC and Gay CC. (1994a). Brucellosis caused by *Brucella abortus* (Bang´s disease) In: Veterinary Medicine a textbook of the diseases of cattle, sheep, pigs, goats and horses. 8th Edn, Bath Press, Avon. pp: 787-803.
141. Radostits OM, Blood DC and Gay CC. (1994b). Brucellosis caused by *Brucella melitensis* In: Veterinary Medicine a textbook of the diseases of cattle, sheep, pigs, goats and horses. 8th Edn, Bath Press, Avon. pp: 810-813.
142. Rahman MS. (2005). Serological and bacteriological diagnosis of *B. abortus* biotype 1 infection in Sprague-Dawley rats. *Indian Journal of Animal Sciences,* 75: 610-616.
143. Rajashekara G, Glasner JD, Glover DA and Splitter GA. (2004). Comparative whole-genome hybridization reveals genomic islands in *Brucella* species. *Journal of Bacteriology,* 186: 5040-5051.
144. Ratushna VG, Sturgill DM, Ramamoorthy S, Reichow SA, He Y, Lathigra R, Sriranganathan N, Halling SM, Boyle SM and Gibas CJ. (2006). Molecular targets for rapid identification of *Brucella* spp. *BMC Microbiology,* 6:13.
145. 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.
146. Refai M. (2003). Application of biotechnology in the diagnosis and control of brucellosis in the near east region. *World Journal of Microbiology and Biotechnology,* 19: 443-449.

cc BY NC SA
DOI 10.5455/ijlr.20190413030405

147. Richtzenhain LJ, Cortez A, Heinemann MB, Soares RM, Sakamoto SM, Vasconcellos SA, Higa ZM , Scarcelli E and Genovez ME. (2002). A multiplex PCR for the detection of *Brucella* spp. and *Leptospira* spp. DNA from aborted bovine fetuses. *Veterinary Microbiology,* 87: 139-147.
148. Rijpens NP, Jannes G, Van Asbroeck M, Rossau R, Herman LM. (1996). Direct detection of *Brucella* spp. in raw milk by PCR and reverse hybridization with 16S- 23S rRNA spacer probes. *Applied Environmental Microbiology,* 62(5): 1683-1688.
149. Romero C and López-Goñi I. (1999). Improved method for purification of bacterial DNA from bovine milk for detection of *Brucella* spp. by PCR. *Applied Environmental Microbiology,* 65(8): 3735-3737.
150. Romero C, Gamazo C, Pardo M and Lopez-Goni I. (1995a). Specific detection of *Brucella* DNA by PCR. *Journal of Clinical Microbiology,* 33: 615-617.
151. Romero C, Pardo M, Grillo MJ, Diaz R, Blasco JM, López -Goñi I. (1995b). Evaluation of PCR and indirect enzyme-linked immunosorbent assay on milk samples for diagnosis of brucellosis in dairy cattle. *Journal of Clinical Microbiology,* 33(12): 3198-3200.
152. Ruiz-Mesa D, Sanchez-Gonzalez J, Reguera M, Martin L and Lopez-Palmero S. (2005). Rose Bengal test: Diagnostic yield and use for the rapid diagnosis of human brucellosis in emergency departments in endemic areas. *Clinical Microbiology and Infection,* 11: 221-225.
153. Sacristán C, Carballo M, Muñoz MJ, Bellière EN, Neves E, Nogal V, Esperón FJ. (2015). Diagnosis of Cetacean morbillivirus: a sensitive one step real time RT fast-PCR method based on SYBR Green. *Journal of Virological Methods,* 226: 25–30.
154. Sandrin TR, Goldstein JE and Schumaker S. (2013). MALDI-TOF MS profiling of bacteria at the strain level: a review. *Mass Spectrometry Reviews,* 32(3): 188-217.
155. Sayour AE and Sayour HE. (2015). Binomial identification of *Brucella* isolates by MALDI-TOF mass spectrometry. 2nd Scientific Conference of Food Safety and Technology, Animal Health Research Institute. pp: 28-49.
156. Scarcelli E, Piatti RM, Cardoso MV, Miyashiro S, Campos FR, Teixeira S, Castro V and Genovez ME. (2004). Detection of bacterial agents by isolation and identification and multiplex PCR in aborted bovine fetuses. *Revista Brasileira de Reproducao Animal,* 28: 23-27.
157. Scholz C, Hubalek Z, Sedlácek I, Vergnaud G, Tomaso H and Al-Dahouk S. (2008a). *B. microti* spp. nov., isolated from the common vole *Microtus arvalis*. *International Journal of Systematic and Evolutionary Microbiology,* 58: 375-382.
158. Scholz HC, Pfeffer M, Witte A, Neubauer H, Al Dahouk S, Wernery U and Tomaso H. (2008b). 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,* 57: 64-71.
159. Scott JC, Koylass MS, Stubberfield MR and Whatmore AM. (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.
160. Seleem MN, Boyle SM and Sriranganathan N. (2010). Brucellosis: A re-emerging zoonosis. *Veterinary Microbiology,* 140: 392-398.
161. Seng, P, Drancourt M, Gouriet F, Scola BL, Fournier PE, Rolain JM and Raoult D. (2009). Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clinical Infectious Diseases,* 49: 543-551.
162. Serpe L, Gallo P, Fidanza N, Scaramuzzo A and Fenizia D. (1999). Single-step method for rapid detection of *Brucella* spp. in soft cheese by gene-specific polymerase chain reaction. *Journal of Dairy Research,* 66(2): 313-317.
163. Sharifzadeh A, Doosti A, and Khaksar K. (2008). Simultaneous detection of *Brucella* sp. and *Salmonella abortus ovis* by multiplex PCR. *Research Journal of Biological Sciences,* 3: 109-11.
164. Singhal N, Kumar M, Kanaujia PK and Virdi JS. (2015). MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. *Frontiers in Microbiology,* 6:791.

cc BY NC SA

 DOI 10.5455/ijlr.20190413030405

165. Sohrabi M, Mobarez AM, Khoramabadi N, Doust RH and Behmanesh M. (2014). Efficient diagnosis and treatment follow-up of human brucellosis by a novel quantitative TaqMan real-time PCR assay: a human clinical survey. *Journal of Clinical Microbiology,* 52(12): 4239-4243.
166. Sreevatsan, S, Jack B, Bookout, Ringpis F, Veera, Perumaalla S, Ficht TA, Adams GA, Hagius SD, Elzer PH, Betsy, Bricker J, Girish K, Kumar M, Rajasekhar, Srikrishna I and Barathur RR. (2000). A Multiplex Approach to Molecular Detection of *Brucella abortus* and/or *Mycobacterium bovis* Infection in Cattle. *Journal of Clinical Microbiology,* 38: 2602-2610.
167. Tantillo G, Di Pinto A, Vergara A and Buonavoglia C. (2001). Polymerase chain reaction for the direct detection of *Brucella* spp. in milk and cheese. *Journal of Food Protection,* 64(2): 164-167.
168. Tantillo GM, Di Pinto A and Buonavoglia C. (2003). Detection of *Brucella* spp. in soft cheese by semi-nested polymerase chain reaction. *Journal of Dairy Research,* 70(2): 245-247.
169. Tcherneva E, Rijpens N, Jersek B and Herman LM. (2000). Differentiation of *Brucella* species by random amplified polymorphic DNA analysis. *Journal of Applied Microbiology,* 88:69-80.
170. Tcherneva E, Rijpens N, Naydensky C and Herman LM. (1996). Repetitive element sequence based polymerase chain reaction for typing of *Brucella* strains. *Veterinary Microbiology,* 51: 169-178.
171. Vaid RK, Thakur SD and Barua S. (2004). *Brucella* diagnosis by PCR. *Journal of Immunology and Immunopathology*, 6: 1-8.
172. Varasada RN. (2003). Seroprevalence of brucellosis in cattle, buffalo and human being in central Gujarat. M.V.Sc. Thesis submitted to Gujarat Agricultural University, Sardar Krushinagar, Gujarat, India.
173. Verger JM, Grimont F, Grimont PDA and Grayon M. (1987). Taxonomy of the genus Brucella. *Annales de l'Institut Pasteur Microbiology,* 138: 235-238.
174. Vizcaino N, Caro-Hernández P, Cloeckaert A and Fernández-Lago L. (2004). DNA polymorphism in the omp25/omp31 family of *Brucella* spp.: identification of a 1.7-kb inversion in *Brucella cetaceae* and of a 15.1-kb genomic island, absent from *Brucella ovis*, related to the synthesis of smooth lipopolysaccharide. *Microbes and Infection,* 6(9): 821-834.
175. Wang Y, Wang Z, Zhang Y, Bai L, Zhao Y, Liu C and Yu H. (2014). Polymerase chain reaction-based assays for the diagnosis of human brucellosis. *Annals of Clinical Microbiology and Antimicrobials,* 13, 31.
176. Weynants V, Tibor A, Denoel PA, Saegerma C, Godfroid J, Thiange P and Letesson JJ. (1996). Infection of cattle with *Yersinia enterocolitica* O: 9 a cause of the false positive serological reactions in bovine brucellosis diagnostic tests. *Veterinary Microbiology,* 48: 101-112.
177. Whatmore AM, Murphy TJ, Shankster S, Young E, Cutler SJ and Macmillan, A.P. (2005). Use of amplified fragment length polymorphism to identify and type *Brucella* isolates of medical and veterinary interest. *Journal of Clinical Microbiology,* 43: 761-769.
178. Whatmore AM, Perrett LL and MacMillan AP. (2007). Characterisation of the genetic diversity of *Brucella* by multilocus sequencing. *BMC Microbiology,* 7:34.
179. Whatmore AM, Shankster S, Perrett LL, Murphy TJ, Brew SD, Thirlwall RE, Cutler SJ and MacMillan AP. (2006). Identification and characterization of variable number of tandem repeat markers for typing of *Brucella spp*. *Journal of Clinical Microbiology,* 44: 1982-1993.
180. Wright AE and Smith F. (1897). On the application of the sérum test to the differential diagnoses of Typhoid and Malta fever. *Lancet,* 1: 656-659.
181. Xu XL, Chen X, Yang PH, Liu JY and Hao XK. (2013). In vitro drug resistance of clinical isolated *Brucella* against antimicrobial agents. *Asian Pacific Journal of Tropical Medicine,* 6(11): 921-924.
182. Yang X, Hinnebusch BJ, Trunkle T, Bosio CM, Suo Z, Tighe M, Harmsen A, Becker T, Crist K, Walters N, Avci R and Pascual DW. (2007). Oral vaccination with *salmonella* simultaneously expressing *Yersinia pestis* F1 and V antigens protects against bubonic and pneumonic plague. *Journal of Immunology,* 178(2):1059-1067.
183. Yu WL and Nielsen K. (2010). Review of Detection of *Brucella* sp. by Polymerase Chain Reaction. *Croatian Medical Journal,* 51: 306-313.

CC BY NC SA

 DOI 10.5455/ijlr.20190413030405

184. Zhang B, Wear DJ, Stojadinovic A and Izadjoo M. (2013). Sequential real-time PCR assays applied to identification of genomic signatures in formalin-fixed paraffin embedded tissues: a case report about brucella-induced osteomyelitis. *Military Medicine,* 178(1): 88-94.

DOI 10.5455/ijlr.20190413030405