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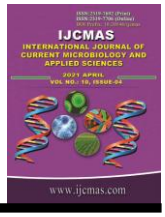

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Clinical, Serological and Molecular Diagnosis of Brucellosis in Domestic Animals

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A B S T R A C T

Brucellosis is an important zoonotic disease prevalent worldwide. It is caused by different species of *Brucella* in different animal species and humans. Early and accurate diagnosis of the disease is crucial for its control. Here we discuss the current trends in clinical, serological and molecular diagnosis of the disease in the common domestic animals.

Introduction

Brucellosis is a zoonotic disease of serious public health concern worldwide that affects all domestic animals and man. Brucellosis is endemic (Boral *et al.*, 2009) all over the world except a few countries which have eradicated the disease (Basappa and Amarnath, 2008). It is still endemic in India (Renukaradhya *et al.*, 2002).

Animals that are mostly infected by this bacterium include cattle, goats, sheep, dogs, pigs and camels among others. Humans act as accidental host to this disease while animals are its natural reservoirs (CDC, 2019). In animals, it leads to abortion, stillbirth, epididymitis, orchitis and placentitis (OIE, 2009). Early and accurate diagnosis is the key to control Brucellosis. Here we discuss various aspects of diagnosis of Brucellosis in domestic animals.

Aetiology of Brucellosis

Brucellosis is caused by bacteria of the genus *Brucella*. There are six main species of *Brucella*. They are *B. suis*, *B. abortus*, *B. melitensis*, *B. ovis*, *B. neotomae*, and *B. canis* (WHO, 2006). Some of these are pathogenic to man as well. They are *B. canis*, *B. abortus*, *B. suis*, and *B. melitensis* in the ascending order of pathogenicity (Corbel, 2006). Different species of *Brucella* infect different animals, for example *B. canis* is infectious to canines, cattle is infected by *B. abortus*, sheep are infected by *B. ovis* and *B. melitensis*, goats and camels by *B. melitensis*, swine by *B. suis*

and marine mammals are infected by *B. neotomae* (OIE, 2009). *B. pinnipediae* and *B. cetaceae* were isolated from marine hosts.

Clinical signs and symptoms of Brucellosis in animals

Bovines

Bovine Brucellosis is characterized by delayed conception, late-term abortions and retention of placenta and temporary or permanent infertility (Kollannur *et al.,* 2007) in females and orchitis and epididymitis in males, with excretion of organisms in semen, uterine discharges and in milk (Godfroid *et al.,* 2013).

Once infected, the animal may continue to shed bacteria and remains a source of infection to others for long period (Pandeya *et al.,* 2013). Sometimes the vaccinated animals may also suffer from Brucellosis due to inadequate immunity induced by the vaccine (Mohan *et al.,* 2017).

Goats

Caprine Brucellosis is endemic in India and is prevalent in all parts of the country. Brucellosis causes abortions, placentitis, epididymitis, orchitis and reproductive losses in the animals.

The causative agent of Brucellosis in goats is *Brucella melitensis.* Humans are exposed to Brucellosis by handling raw milk and meat from infected goats and animals get infected by direct contact with them or through common attendants.

Sheep

Brucellosis causes abortions, placentitis, epididymitis, orchitis and reproductive losses in animals. The causative agents of Brucellosis in sheep are *Brucella ovis* and *Brucella melitensis*. Ovine Brucellosis is endemic in India and is prevalent in all parts of the country.

Camel

Brucellosis is insidious in camels as it is mostly asymptomatic. It is difficult to estimate the prevalence of Brucellosis in camels, since they show very few clinical signs compared to cattle (Khadjeh *et al.,* 1999; Dawood, 2008; Musa *et al.,* 2008).

The clinical manifestations of Brucellosis may vary from abortion to asymptomatic in camels (Musa *et al.,* 2008). Symptoms in dams are hydrobursitis, granulomatous endometritis and adhesions in ovario-bursal region. Retention of placenta and delay in sexual maturity along with infertility have been reported (Musa *et al.,* 2001).

In males, orchitis has been reported (Abbas and Agab, 2002). Acute lameness along with hygroma and arthritis has been reported (Musa *et al.,* 2008).

Canines

Canine brucellosis is found worldwide. The disease in dogs can be subclinical or associated with acute or chronic signs, such as reproductive disease, e.g. abortion (typically late term), weak pups who die shortly after birth, infertility (in bitch or stud) or neurologic disease, e.g. back or neck pain. *Brucella canis* is the most common species found in dogs.

It is most often transmitted through direct dog-to-dog contact via infected body fluids and tissues (e.g. vaginal discharge, aborted fetus, placenta, semen and urine).

Other *Brucella* spp. can also infect dogs (including *Brucella abortus* and *Brucella suis*) after dogs consume placenta, aborted fetuses, or uncooked meat from infected livestock or have contact with wild (feral) swine.

Common laboratory diagnostic tests for Brucellosis

Isolation of Brucella organisms from clinical samples

Bovine foetal stomach contents, cotyledon, placenta, uterine discharge, vaginal swabs, milk, hygroma fluid, blood and visceral organs of foetus (lung, liver, spleen etc.) have been used for isolation of *Brucella* spp. by Fekete *et al.,* (1992), Nagal *et al.,* (1994), Chatterjee *et al.,* (1995), Chahota *et al.,* (2003) and O'Leary *et al.,* (2006).

Brucella is a slow growing organism and the use of selective media is recommended for primary isolation from most clinical specimens because of the high numbers of overgrowing contaminants (Marin *et al.,* 1996). An example is Farrell's medium (Oxoid), prepared by adding six antibiotics; bacitracin, vancomycin, nalidixic acid, polymixin B, nystatin and cycloheximide onto sucrose dextrose agar for the isolation of *B. abortus* from contaminated milk samples (Farrell 1974).

Most *Brucella* spp. can be isolated in unsupplemented, enriched peptone based media, or blood agar (Alton *et al.,* 1988, Quinn *et al.,* 1999). Good growth is obtained on *Brucella* medium base (Oxoid), sucrose dextrose agar (Oxoid), tryptone soy agar or glycerol dextrose agar (Oxoid) supplemented with 5% bovine or horse serum (OIE 2004, Moyer and Holocomb 1995). Moyer and Holocomb (2005) reported the use of chocolate agar containing selective supplements for the isolation of *Brucella* spp. Terzolo *et al.,* 1991 used Skirrow's agar to isolate *B. abortus*, *B. suis*, *B. melitensis*, *B. canis* and *B. ovis* from contaminated vaginal exudates and milk. Growth is inhibited on media containing bile salts, tellurite or selenite (Alton *et al.,* 1988).

Cetinkaya *et al.,* (1999) isolated *Brucella* from foetal stomach content by inoculation on *Brucella* agar base containing 10% sterile defibrinated sheep blood and *Brucella* supplement and on serum dextrose agar base and blood agar and cultures were incubated at 37^0C for 4-7 days.

Ocholi *et al.,* (2004) isolated *Brucella* from aborted foetuses, hygroma fluids, milk and vaginal swabs which were obtained from aborted cattle, sheep, goats, pigs and horses. Sanchez *et al.,* (2001) isolated *Brucella melitensis* from the sample of foot abscess lesion by 7 day culturing on Lowenstein–Jensen medium.

Identification of Brucella organisms

Brucella spp. produce colonies that are round, glistening, pin-point, 1-2 millimetres in diameter, with smooth margins (Alton *et al.,* 1988, Quinn *et al.,* 1999). Later they become larger and darker but remain clear (Alton *et al.,* 1988). On serum dextrose agar, or any other clear medium, when examined under low power microscope, *Brucella* colonies have a raised surface, translucent with entire margins and displaying a characteristic pale "honey drop-like" appearance (Corbel and Brinley-Morgan, 1984). On sheep blood agar, smooth strains of *Brucella* spp. produce small, glistening, smooth and non-haemolytic colonies which become opaque with age (Quinn *et al.,* 1999).

Brucella spp. are microscopically observed as Gram-negative cocci, coccobacilli or short rods. They are usually arranged individually and less frequently in pairs, short chains or small groups and do not usually exhibit bipolar staining (Holt *et al.,* 1994 and Garritty *et al.,* 2005).

On the basis of biochemical tests, members of the genus *Brucella* are broadly defined as

catalase positive, oxidase positive (except *B. ovis*), urease positive (except *B. ovis* and *B. neotomae*), reduce nitrates to nitrites and do not exhibit motility in semi-solid media (Alton *et al.*, 1988, Quinn *et al.*, 1999). In addition, they do not produce indole, gelatinases, haemolysins, acetylmethyl carbinol (Voges Proskauer test), formic and acetic acids from glucose (Methyl Red test) (Holt *et al.*, 1994).

Immunodiagnosis of Brucellosis

There are many serological tests for demonstrating *Brucella* antibodies 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 classical serology tests conducted for the *Brucella* spp in livestock include Complement Fixation Test (CFT), Rose Bengal Test (RBT) and Serum Agglutination Test (SAT). The CFT is required by the World Health Organization for compulsory international trade inspection. All three tests are specific, i.e. they give correct identification of true positive animals (Godfroid *et al.*, 2002). RBPT, ELISA and polymerase chain reaction (PCR) are the most widely used tests for the laboratory diagnosis of Brucellosis in cattle, camel and humans (Alton, 1990). The serological tests used for *B. abortus* infection in cattle are also adequate for serodiagnosis of *B. melitensis* infection in camels and humans.

Serological tests

Rose Bengal Plate Test (RBPT)

Rose Bengal Plate Test (RBPT) or Rose Bengal Test (RBT) is internationally acknowledged as the test of choice for the screening of Brucellosis in camels and humans (Garin-Bastuji and Blasco, 2004). However, standardization conditions suitable for diagnosing cattle infection (MacMillan, 1990; European Commission, 2002; Garin-Bastuji and Blasco, 2004) are not adequate in camels and humans (Blasco *et al.*, 1994a, b) and account for the low sensitivity of RBT antigens in them (Blasco *et al.*, 1994a; Falade, 1978, 1983) along with the fact that a high proportion of animals in infected areas give results negative in RBT, but positive in CFT, question the efficacy of the RBT as an individual test (Blasco *et al.*, 1994a).

Enzyme linked immunosorbent assay (ELISA)

Good diagnostic results have been obtained in camels and humans with ELISA or, at a lesser degree, ELISA using various antigens, but generally those with a high content of smooth lipopolysaccharide (LPS) are the most reliable. These ELISAs provide similar or better sensitivity than both RBT and CFT, but ELISAs are unable to differentiate infected animals from animals recently vaccinated with the Rev-1 vaccine (Jimenez´ de Bagu¨es´ *et al.*, 1992; Blasco *et al.*, 1994b; D´ıaz-Aparicio *et al.*, 1994; Delgado *et al.*, 1995; Ficapal *et al.*, 1995; Marin *et al.*, 1999; Ferreira *et al.*, 2003) or animals infected with cross-reacting bacteria. However, the association of the conjunctival vaccination procedure and the presence of a moderate interval after vaccination minimize or abrogate the specificity problems. A similar indirect technique has been also proposed for diagnosing Brucellosis in individual or pooled milk samples (Alonso-Urmeneta *et al.*, 1998), but the test lacks sensitivity when compared with serological tests.

A highly immunogenic periplasmic protein from *B. abortus* (Rossetti *et al.*, 1996) or *B.*

melitensis (Cloeckaert *et al.,* 1996a) has been applied to Brucellosis diagnosis in different host species. Indirect and competitive ELISA with this antigen could be sensitive and specific tests for diagnosing *B. melitensis* infection in camels and humans and have been reported to be useful in differentiating Rev-1 vaccinated from infected animals (Debbarh *et al.,* 1995, 1996b; Cloeckaert *et al.,* 1996b). All these ELISAs have potential advantages in sensitivity and specificity with respect to both RBT and CFT, but their diagnostic efficacy at large scale is unknown and a great deal of standardization work is required (Garin-Bastuji and Blasco, 2004).

Comparison of different laboratory diagnostic methods

Different diagnostic tests have been validated for diagnosing Brucellosis in camels and humans, but only the Rose Bengal test (RBT) and the complement fixation test (CFT) are approved for diagnosis of camel and human Brucellosis in the European Union (EU) legislation on intra-community trade (Council Directive 91/68/EEC). However, there is evidence that both tests are less sensitive and specific for the diagnosis of Brucellosis in camels and humans than in cattle (Blasco *et al.,* 1994; Garin-Bastuji *et al.,* 1998).

According to Biancifiori *et al.,* (2000), the ELISA has a diagnostic sensitivity (99.4%) and specificity (98.9%) in camels and humans comparable to that of many standard indirect ELISA methods. In addition, the test proved able to distinguish between vaccinated and infected animals with an accuracy of up to 90% and result reproducibility of 93%. They concluded that ELISA could be useful for differentiation of Rev.1 vaccinated and naturally infected camels and humans.

The serological tests used in earlier studies were the Rose Bengal Test (RBT), Standard Tube Agglutination Test, ELISA or Plate Agglutination Test. None of the aforementioned tests are perfect. So, the prevalence reported using these tests are not true prevalence due to misclassification of some of the tested animals. Moreover, the performance of these tests has not been validated in naturally infected camels and humans. Tests are normally validated by comparing with the gold standard or perfect test. However, the gold standard for the diagnosis of Brucellosis is isolation and identification of the organism (Alton *et al.,* 1988; OIE, 2008).

The Tube Agglutination test (TAT) among all tests used in a study gave the highest rate of positive animals compared with other traditional serological tests. This was explained by the fact that the test has a high sensitivity in respect of IgM rather than IgG as reported by Alton (1977). On the other hand, MacMillan (1990) reported that TAT failed to show significant titres in recent and chronic *Brucella* infections. While Corbel (1972) reported that TAT gives false positive reaction as a result of cross reaction between the antigens of *Brucella* and other organisms or due to the presence of non specific agglutinins in bovine sera. The presence of some samples collected from non reactors (*Brucella* - free cows) reacted positively with RBPT, BAPT and TAT. This may be attributed to the presence of some bacteria as *Escherichia coli*, *Salmonella* Dublin, *Yersinia enterocolitica* 0:9 and *Pasteurella tularensis* in the body fluids and secretions which react positively with the tests used in diagnosis of Brucellosis causing faults or error in the interpretation of the results.

The Fluorescence Polarization Assay (FPA) was reported to be the test with the smallest sensitivity and I-ELISA that with the smallest specificity. I-ELISA was found to be the most sensitive test, whilst cELISA the most

specific. The cELISA had the best and FPA the second best positive predictive value. ELISA had the best negative predictive value, which is expected due to the use of monoclonal antibodies. This test may be ultimately used for confirming negative samples, as it is not influenced by non-specific reactions (Nielsen *et al.,* 1989). I-ELISA would be valuable for use at the early stage of a control program, whilst ELISA at its final stage. Although the FPA did not appear to be as accurate when compared to the other two methods when testing vaccinated animals, it was the easier, cheaper (use of reagents is minimal) and quicker to perform; further investigation should be carried to establish its value in large scale surveys, where a great number of samples need to be tested.

Furthermore, Nielsen *et al.,* (2001) have reported its use in testing stored whole blood samples with an almost 100% sensitivity and specificity; this can be an added advantage. The test may also be used in samples from all animal species, as well as in poor quality samples (Nielsen *et al.,* 2001).

Reddy *et al.,* (2014) applied multiple tests like RBPT, I-ELISA and Dot-ELISA to determine the apparent prevalence of camel and human Brucellosis in the Indian state of Karnataka and compare the relative sensitivities and specificities among the different tests applied. Test-wise, the seroprevalence in camels and humans was 5.15% by RBPT, 6.34% by STAT, 1.98% by 2-MET, 9.52% by I-ELISA and 7.14% by Dot-ELISA, respectively. The prevalence of Brucellosis was found to be highest among camels and humans of northeast Karnataka followed by northwest Karnataka, central Karnataka and south Karnataka. I-ELISA detected maximum number of positive samples. Taking I-ELISA as the reference, the tests revealed the relative sensitivity values in the following order: Dot-ELISA>STAT>RBPT.

Damp *et al.,* (1973) utilized Macro-tube agglutination (TA) methods in the study of the agglutinins present in the sera of humans and animals infected with *B. canis*. A Microtiter plate Agglutination Test was formulated which provides results comparable to the TA method, using a minimal amount of equipment, time, reagents, and serum.

Sareyyupoglu *et al.,* (2010) investigated *Brucella* antibodies in bovine sera by RBPT, serum agglutination test (SAT), Microtitre Plate Agglutination Test (MAT) and 2-Mercaptoethanol MAT (2-ME-MAT) and determined MAT as a fast, reliable and economical method for routine laboratories and serological diagnosis.

In another study, 141 bovine sera were screened for Brucellosis using RBPT and STAT. The relative sensitivity and specificity of STAT and RBPT was found to be 88.61% and 98.59% for STAT and 56.96% and 96.77% for RBPT, respectively, classification being based on ELISA (Chakraborty *et al.,* 2000).

Diaz *et al.,* (1967) compared *Brucella* antigens capable of sensitizing normal and tanned sheep RBC's for IHA with antigens involved in agglutination, gel diffusion and immuno-electrophoresis. Sawada *et al.,* (1982) described a new modification in Indirect Haemagglutination (IHA) test that uses glutaraldehyde - fixed sheep RBCs for detection of antibodies.

Saxena and Kaur (2013) reported that only 5.55% of suspected samples were found positive by STAT while 50% samples were positive by RBPT and Dot ELISA could detect antibodies in all (100%) samples. A study of the plate agglutination test with Rose Bengal antigen for the diagnosis of Brucellosis was carried out by Cernyseva *et al.,* (1997). They opined that RBPT holds a greater

promise for animal screening. They found that RBPT had higher specificity and sensitivity.

On evaluation of canine Brucellosis by MAT, Kimura *et al.,* (2008) stated that MAT was more sensitive, simpler to perform and easier than Tube Agglutination Test. It allows handling of large number of samples at once. Damp *et al.,* (1973) described Plate agglutination test as a valid screening method for *Brucella canis* agglutinins. They concluded that the micro method provided an economical means of screening sera for presence of antibodies.

In a study by Saz *et al.,* (1987), the performance of an ELISA for detection of total antibodies to *Brucella* spp. was compared with that of the Rose Bengal Plate Test, standard tube agglutination test and Coombs' test in the diagnosis of Brucellosis. Sera tested were from 208 patients from whom *Brucella melitensis* had been isolated, 177 patients with significant results in at least two conventional tests, and 107 patients with fever from whom no *Brucella* spp. had been isolated and in whom all conventional tests were negative. ELISA was the most sensitive test (97%), showing greater specificity (96%) and good predictive positive and negative values (98% and 94%, respectively). ELISA was the only positive test in 6% of patients in whom Brucellosis had been confirmed by culture.

Chachra *et al.,* (2009) carried out a study to compare the efficacy of RBPT, STAT and Dot ELISA in immunological detection of antibodies to *Brucella abortus* in sera. The study revealed that Dot ELISA was the most sensitive of the three tests used. The authors suggested that in order to get confirmatory diagnosis of *Brucella* infection, a combination of RBPT and Dot ELISA should be used, especially for the samples which are found negative by RBPT or STAT used alone or in combination.

ELISA has been claimed to be more sensitive followed by RBPT and STAT when applied to cattle sera, whereas RBPT was found to be more sensitive followed by STAT and ELISA when applied to buffalo sera. Comparison of dot-ELISA and ELISA for diagnosis of bovine Brucellosis proved dot-ELISA to be more sensitive (Ganesan and Anuradha 2006).

Versilova *et al.,* (1974) diagnosed human and animal Brucellosis by Indirect Haemagglutination (IHA) test. They showed that the use of sheep erythrocytes sensitized with a specific lipopolysaccharide antigen in the IHA test provided a specific method, which is more sensitive than the agglutination test, for the diagnosis of Brucellosis in man and farm animals. They opined that the IHA test was more specific and sensitive than the agglutination test and justified its use in diagnosis of human Brucellosis, the study of immunological status of the population and examination of animals for Brucellosis.

Chand and Sharma (2004) advocated the use of ELISA in comparison to RBPT and STAT for assessing the situations of Brucellosis in cattle, to have better results because chances of non detection of an infected animal in ELISA is minimum. ELISA can be used to eliminate false positive results amongst RBPT positive sera (Erdenebaatar *et al.,* 2004).

Singh *et al.,* (2010) used PCR in diagnosis of sheep Brucellosis employing serum as the sample and the results were compared with those of RBPT. Out of 36 samples tested, 19 were positive by RBPT whereas PCR revealed 13 samples to be positive. Malik *et al.,* (2013) evaluated i-ELISA, RBPT, MAT and PCR for diagnosis of Brucellosis in buffaloes and concluded that indirect ELISA detected more samples as positive among these tests. They suggested that i-ELISA can be used for routine sero-diagnosis of *Brucella* infection in buffaloes.

Chachra *et al.,* (2009) compared the efficacy of RBPT, STAT and Dot ELISA in immunological detection of antibodies to *Brucella abortus* in sera. The study revealed that Dot ELISA was the most sensitive of the three tests used. The authors suggested that in order to get a fool proof diagnosis of *Brucella* infection, a combination of RBPT and Dot ELISA should be used, especially for the samples which are found negative by RBPT or STAT used alone or in combination.

A study was carried out on 180 serum samples by Ghodasara *et al.,* (2010) with an aim of comparing the RBPT, STAT and i-ELISA for detection of *Brucella* antibodies in cows and buffaloes. The seropositivity was found highest by i-ELISA (25%), followed by STAT (14.45%) and RBPT (10.56%).

Cernyseva *et al.,* (1977) carried out a study of the plate agglutination test with Rose Bengal antigen for the diagnosis of brucellosis. They opined that the RBPT holds a great promise for animal screening. The purpose of the study was to compare the RBPT with other serological tests. They found that the RBPT had high specificity and sensitivity.

RBPT, CFT and i-ELISA were compared in a study on a herd of unvaccinated cattle. It was found that the ELISA titers (≤ 20) accurately predicted all the negative sera in herds that were also negative by the CFT, the number of seropositive animals was higher by ELISA in herds that had positive animals. It was also suggested that the serum samples which give higher degrees of agglutination with the RBPT need not be re-tested with CFT.

Diagnostic sensitivity of i-ELISA and CFT was found to be 100% and 83%, respectively when 4803 cattle sera were tested against them, whereas their specificities were 99.8% and 100% respectively (Paweska *et al.,* 2002). In another study, 141 bovine sera were screened for brucellosis using RBPT and STAT. The relative sensitivity and specificity of STAT and RBPT was found to be 88.61% and 98.59%, respectively for STAT and 56.96% and 96.77% for RBPT, respectively, classification being based on ELISA. (Chakraborty *et al.,* 2000).

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Vivekananda *et al.,* (2012) carried out a study on a sheep flock with a history of reproductive disorders. Among RBPT, STAT and iELISA, iELISA detected highest positive samples (14.8%) followed by RBPT (5.88%) and STAT (4.17%). They found that vaginal samples were most suitable for isolation and PCR, followed by serum and blood.

Keid *et al.,* (2010) carried out a comparison of a PCR assay in whole blood and serum specimens for canine brucellosis diagnosis on 72 serum samples. They reported that the sensitivities of blood PCR and serum PCR were, 97.14% and 25.71% respectively. The specificities of both were found to be 100%. The authors concluded that the serum PCR showed little value for the direct diagnosis of canine brucellosis.

Mariri *et al.*, (2011) carried out a study on serum samples from a total of 2580 unvaccinated Syrian female sheep. Presence of antibodies against *B. melitensis* was tested using four serological methods: RBPT, SAT, CFT and iELISA. Positivity by RBPT, SAT and iELISA was 66%, 64% and 60%, respectively. The CFT revealed the smallest number of positive samples.

Bovines

Mohan and Saxena (2018) carried out a study for serological diagnosis of Brucellosis in cattle from a local gaushala (cow shelter) employing common agglutination tests RBPT, STAT and MAT as well as ELISA. Out of 56 samples from cattle from the Gaushala, 37.5% samples by RBPT, 64.28% each by STAT and MAT and 49.23% samples by ELISA, respectively were found to be positive for anti-*Brucella* antibodies. Among the 29 suspected serum samples from the field cattle, samples found to be positive for anti-*Brucella* antibodies were 100% by RBPT, 79.31% by MAT and 89.65% by ELISA, respectively.

Malik *et al.*, (2013) tested 178 blood samples from buffaloes against indirect enzyme linked immunosorbent assay (I-ELISA), rose bengal plate test (RBPT), microagglutination test (MAT), modified microagglutination test (mMAT), and PCR to select the most suitable test for efficient and effective diagnosis of bovine brucellosis. I-ELISA was pair compared with all the other tests. Out of 178 samples, 102 were found positive by I-ELISA, 81 by RBPT, 85 by MAT, 79 by mMAT, and 68 by PCR. Substantial agreement was observed between I-ELISA and RBPT (κ = 0.72), I-ELISA and MAT (κ = 0.65), and I-ELISA and mMAT (κ = 0.67), respectively. The least degree of agreement was observed between I-ELISA and PCR (κ = 0.15). I-ELISA detected more samples as positive among these tests. They concluded that I-ELISA can be used for routine sero-diagnosis of *Brucella* infection in buffaloes. Furthermore, PCR can be used in combination with I-ELISA to complement the serological diagnosis, especially in the initial phase when the immune response of the animal is not detectable.

Camel

The species of *Brucella* that cause infection in camels are *B. melitensis* and *B. abortus*. The Biotype 3 of *Brucella melitensis* is the most prevalent. Diagnostic tests for Brucellosis have not been properly validated for camels causing aberrations in accurate diagnosis of this disease (Musa *et al.*, 2001). The diagnosis of Brucellosis by growing and phenotyping the organisms is not only time consuming, but also poses a high risk for the researcher towards infection. Isolation of the agent has been reported from the lymph (Abu *et al.*, 1989), aborted fetuses, vaginal swabs (Gameel *et al.*, 1993), milk (Radwan *et al.*, 1992; Radwan *et al.*, 1995), semen, and testes (Agab *et al.*, 1996). Gwida *et al.*, (2011) compared multiple serological tests in a study on 895 sera from clinically sound camels, involving RBT, SAT, CFT, cELISA and the FPA test. FPA (79.3%), followed by the CFT in descending order (71.4%), RBT (70.7%), SAT (70.6%) and the cELISA (68.8%) gave the best results for detecting the presence of anti-*Brucella* antibodies. In all serological studies, only 66.5% of samples were positive. In addition, the analysis tested the utility of a PCR in real time, which was observable in 84.8 per cent of samples positive for the gene that codes for bcsp31 kDa *Brucella* cell surface protein.

Sheep and goats

The tests commonly used for diagnosis of caprine and ovine Brucellosis in India are the milk ring test, Rose Bengal Plate Test

(RBPT), Standard Tube Agglutination Test (STAT), Microtiter Plate Agglutination Test (MAT) and ELISA. The RBPT is a rapid screening test for the diagnosis of Brucellosis. The sensitivity of RBPT is very high (> 99%) but the specificity can be low and it could sometimes give a false positive result. Its positive predictive value is low and a positive test result requires confirmation by a more specific test. Isolation and culture of *Brucella* organisms is the gold standard test for the diagnosis of Brucellosis (Saxena *et al.,* 2018).

Saxena *et al.,* (2017) investigated the prevalence of Brucellosis in sheep in Ludhiana district of Punjab state of India. Serum samples of sheep (n=171) from slaughter houses and an organized farm were analyzed with common serodiagnostic tests RBPT, STAT, MAT and ELISA. Out of 171 sera, 31 were positive by one or more of the four tests; 12 were positive and 140 were negative by all the four tests. ELISA gave maximum true positives and RBPT gave minimum of false negative results. RBPT showed the highest sensitivity (78.26%) followed by ELISA (75%), STAT (68%) and MAT (50%), respectively. STAT had the highest specificity (98.63%) followed by ELISA (96.79%), RBPT and MAT (95.89% each), respectively. STAT had the highest positive predictive value of 89.47% followed by ELISA (79.26%), RBPT (75%) and MAT (66.67%), respectively. RBPT had the highest negative predictive value of 96.55% followed by ELISA (96.18%), STAT (94.74%) and MAT (92.11%), respectively. STAT had the highest positive likelihood ratio of 49.64 while MAT gave the highest negative likelihood ratio of 0.52.

Saxena *et al.,* (2017) investigated the frequency of Brucellosis in goats in Ludhiana district of Punjab state of India. A total of 191 serum samples of goats from slaughter houses were analyzed with RBPT, STAT, MAT and ELISA. Out of these, 31 goats were positive for Brucellosis by one or more of these tests. Among positives, 14 were detected by RBPT, 17 by STAT, 21 by MAT, and 21 by ELISA; 10 samples were positive and 160 negative by all methods. ELISA and MAT detected highest number of samples followed by STAT and RBPT.

Polymerase Chain Reaction (PCR)

PCR is a very sensitive technique and is a more reliable alternative of culture and isolation. (Amin *et al.,* 2001). PCR does not cross react with closely reactive bacteria except *Orchobacterium anthropi*, the closest known relative of *Brucella* (Dorsch *et al.,* 1989). Baily *et al.,* (1992) detected *Brucella melitensis* and *Brucella abortus* by DNA amplification of the gene encoding 31kDa *B. abortus* antigen. *B. melitensis* and *B. abortus* showed no difference in the sensitivity of the reaction or in the size of amplification product (223 bp). Romero *et al.,* (1995) found 100% specificity for PCR and indirect ELISA in milk samples of bovines and developed a PCR assay using the genus specific primer pair derived from the 16S rRNA sequence of *B. abortus*. DNA from all the representative strains of *Brucella* species and its biovar from 23 different isolates analyzed and yielded exclusively the 905 bp sequence.

O'Leary *et al.,* (2006) detected *B. abortus* from blood, milk and lymph tissue of serologically positive cows by conventional and real time PCR assays. They amplified various regions of the *Brucella* genome, IS711 genetic element, gene for 31kDa outer membrane protein and 16S rRNA. Mukherjee *et al.,* (2007) used three independent *Brucella* specific nucleotide sequences encoding bcsp, omp 2 and 16S rRNA gene in PCR assays which resulted in the amplification of 223, 193 and 905 bp amplicons, respectively. The

bovine blood samples were insensitive to 16S rRNA PCR. Al-Mariri and Haj-Mahmoud (2010) compared Milk Ring Test and three different polymerase chain reaction techniques to identify infection in bovine milk.

They concluded that PCR on the animal's filtered milk is the best procedure to make the diagnosis of *B. abortus* infections. Ghodasara *et al.,* (2010) isolated *Brucella* from samples of vaginal swabs, aborted materials and placenta and confirmed by PCR using genus specific primer pairs B4/B5, F4/R2 and JPF/JPR. The results on comparison of sensitivity of three pairs of primers amplifying three different fragments including a gene encoding BCSP 31 (B4/B5), a sequence 16S rRNA of *Brucella abortus* (F4/R2) and a gene encoding omp2 (JPF/JPR) showed that the sensitivity of the B4/B5 primer pair was more (98%), followed by JPF/JPR primer pair and F4/R2 primer pair (88.4% and 53.1%) respectively (Baddour and Alkhalifa 2008 ; Wang *et al.,* 2014).

Chothe *et al.,* (2013) carried out a study on 200 samples from cattle suspected of Brucellosis to ascertain the effectiveness and suitability of PCR and commercially available ELISA kits. In PCR, only 3 out the 200 sample showed a band of size 905bp, typical of *Brucella* species. Other samples failed to show positive reaction by PCR. In comparison, ELISA could detect 75 out of the 200 samples as positive. They concluded that ELISA is a better confirmatory test than PCR for screening animals for Brucellosis.

Reverse Transcriptase PCR (RT-PCR)

The classical methods for the determination of bacterial viability rely on the ability of cells to actively grow and form visible colonies on solid media. But under some circumstances, the number of viable organisms may be under represented by such methods as sublethally damaged organisms, fastidious, uncultivable bacteria and viable cells that have lost the ability to form colonies under the test conditions will not be detected (Keer and Birch 2003). The presence of intact DNA sequences was initially used as an indicator of cell viability with the assumption that the DNA would be degraded in dead cell more rapidly than other cellular components (Jamil *et al.,* 1993). However, the precise correlation of cell viability with detection of DNA was shown to be poor, with DNA persisting in actively killed cells for significant periods of time (Masters *et al.,* 1994). DNA was also demonstrated to persist in a PCR- detectable form in culture-negative environmental and clinical samples (Deere *et al.,* 1996 and Hellyer *et al.,* 1999). The RNA has been found to be positively correlated with viability and the most commonly used amplification techniques for detecting RNA are RT-PCR and nucleic acid sequence based amplification (Simpkins *et al.,* 2000).

RT-PCR has historically been the amplification method of choice when analysing RNA, mainly as PCR has become a key technique underpinning many DNA based measurements (Keer and Birch 2003). Al-Ajlan *et al.,* (2011) compared the DNA and RNA detection PCR methods for identifying *Brucella* species in human blood samples. Saxena and Raj (2018) employed RT-PCR for monitoring and assessment of efficacy of immunotherapy against Brucellosis in cattle.

Several laboratory diagnostic tests for Brucellosis in domestic animals are employed worldwide. These include RBPT, ELISA, CFT, FPA, PCR, RT-PCR etc. However, they vary in accuracy, ease of operation, level of skill and competence required and cost. Different labs employ different tests depending on the availability of skilled manpower, laboratory facilities, resources and funds.

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