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Serological Diagnostic Methods and Test Performance for the Diagnosis of Bovine Brucellosis: A Review

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Abstract

Bovine brucellosis is highly contagious, zoonotic and economically important bacterial disease characterized by abortion, placentitis, epididymitis and orchitis. The clinical picture of brucellosis is not pathognomonic and the clinical history of the patient, particularly occurrence of abortion, is paramount diagnostic importance. Although definitive diagnosis of bovine brucellosis is done by isolation and identification of the causative agent, serologic al tests are usually the most preferred. Therefore, the aim of this paper is to review available scientific data on serological tests for the diagnosis of bovine brucellosis and compare their test performance based on sensitivity (Se) and specificity (Sp) values of assays. The host defense mechanism against Brucella can functionally be divided into innate or non-specific and adaptive or specific immunity. The pathogenic brucellae organism has developed a battery of mechanisms to evade and/or modulate both innate and adaptive immune response in their host. Serological assays are based on the fact that Brucella abortus as well as other smooth Brucella have distinctive O-polysaccharide (OPS) that induces a humoral response with an initial production of IgM, followed by IgG1 and IgG2/IgA.

Serological methods available for the diagnosis of bovine brucellosis include the complement fixation test (CFT), serum agglutination test (SAT), Rose-Bengal test (RBT), 2-mercaptoethanol test (2ME), buffered plate agglutination test (BPAT), indirect-enzyme-linked immunosorbent assay (iELISA) and more recently the competitive-ELISA (cELISA) and fluorescent polarization assay (FPA). The RBT and CFT are the most widely used tests for the serological diagnosis of bovine brucellosis. The tests like CFT, RBT and iELISA are currently the standard tests in the EU legislation for intra-Community trade of cattle and showed comparable Se and Sp. The new test, FPA, showed Se and Sp comparable to that of standard tests and is suggested for inclusion as standard test in the EU legislation. Similarly, the OIE regards the FPA tests as a prescribed test for international trade. However, false positive and false negative results are common for several methods. There is no single serological test that is appropriate in each and all epidemiological situations and 100% accurate. Therefore, the development of serological tests that are rapid, cheap and having high sensitivity and specificity for brucellosis detection are major issues which need further researches.

Keywords: Bovine brucellosis; Brucella abortus; Diagnosis; Sensitivity; Specificity; Serological tests; Test performance

Abbreviations: 2-MET: 2-Mercaptoethanol Test; AU-IBAR: African Union-Interafrican Bureau for Animal Resources; AUSVETPLAN: Australian Veterinary Emergency Plan; BPAT: Buffered Plate Agglutination Test; CFT: Complement Fixation Tests; CTLs: Cytotoxic T lymphocytes; DSe: Diagnostic Sensitivity; DSp: Diagnostic Specificity; ELISA: Enzyme Linked Immune Sorbent Assay; cELISA: Competitive Enzyme Linked Immune Sorbent Assay; IELISA: indirect Enzyme Linked Immune Sorbent Assay; FAO: Food and Agricultural Organization; FPA: Fluorescence Polarization Assay; IgA: Immunoglobulin A; IgG: Immunoglobulin G; IgM: Immunoglobulin M; LPS: Lipopolysaccharide; MRT: Milk Ring Test; OIE: Office International des Epizooties (World Organization for Animal Health); OPS: O-Polysaccharide; RBPT: Rose Bengal Plate Test; Sat: Serum Agglutination Test; Se: Sensitivity; Sp: Specificity; Th: T helper; WHO: World Health Organization

Introduction

Brucellosis is a highly contagious, zoonotic and economically important bacterial disease of animals worldwide [1]. The disease is caused by various species of the genus Brucella, which are facultative, intracellular bacteria capable of surviving and multiplying inside the cells of mononuclear phagocytic system [2]. The disease in cattle (bovine brucellosis), usually caused by Brucella abortus and occasionally by Brucella melitensis and Brucellosis, is characterized by late term abortion, infertility and reduced milk production as a result of retained placenta and secondary endometritis and excretion of the organisms in uterine discharges and milk [3]. Infection is transmitted by consumption of dairy products like milk, cheese and contact with infected animal and aerosol [4,5]. The bovine infection presents a particularly serious problem because of the large volume of infected milk that can be produced by an individual animal and because of the extensive environmental contamination that even single abortions or infected births can produce [6].

The disease presents a great variety of clinical manifestations (not pathognomonic), making it difficult to diagnose clinically. This absence of specific symptoms also makes it difficult to

distinguish brucellosis from several febrile conditions that often occur in the same areas. Therefore, the diagnosis must be confirmed directly by isolation of Brucella from blood culture, or indirectly serologically by the detection of host immune response against its antigens [7]. Isolation and identify cation of causative bacterium is the gold-standard for diagnosis of brucellosis, but it requires high security laboratory, highly skilled personnel, an extended turn-around time for results and it is considered a hazardous procedure [8]. Therefore, diagnoses are mainly based on the detection of antibodies in serum by serological tests like Buffered Plate Agglutination Test (BPAT), Rose Bengal Test (RBT), Standard Agglutination Test (SAT), 2 Mercapto Ethanol Test (2 ME) and Complemen t Fixation Test (CFT) [9]. They are relatively easy to perform and provide a practical advantage in detecting the prevalence of Brucella infection [10].

Body fluids such as serum, uterine discharge, vaginal mucus and milk or semen plasma from suspected cattle may contain different quantities of antibodies of the IgM, IgG1, IgG2 and IgA types directed against Brucella antigen [11]. Antibodies usually begin to appear in the blood at the end of the first week of the disease, IgM appearing first followed by IgG [12]. Thus, serological methods are recommended as a mean to obtain fast indirect proof of the diagnosis. Each test has its own disadvantages, and the presence of antibodies does not always refer to the presence of an active infection of brucellosis [13,14].

Several serological methods are currently available; these tests can be classified as screening tests (e.g. buffered antigen plate agglutination (BPAT), Rose Bengal plate test (RBPT) and milk ring test (MRT)) and complementary or confirmatory tests (e.g. 2-mercapto-ethanol (2-MT), complement fixation (CFT), ELISAs and fluorescence polarization assay (FPA)). Selection of a given test should consider the species affected as well as local regulations [8,15]. Although developed countries have successfully controlled brucellosis, many developing countries such as Ethiopia, have not been able to react adequately and the disease continues to be a major public and animal health problem. Control and eradication of brucellosis is usually based on vaccination and almost exclusively based on the serological testing of animals and the subsequent culling of those that are seropositive for antibodies to Brucella species [16,17].

Hence, the objectives of this review paper are:

a. To review serological diagnostic tests available for the diagnosis of bovine brucellosis

b. To compile available information on the performance of serological diagnostic tests based on the sensitivity and specificity values obtained from various sources.

Host Immune Responses in Brucella Infection

Functionally, the host immune response is divided into innate or non-specific and adaptive or specific immunity [18]. Infection with Brucella usually results in the induction of both humoral and cell-mediated immune responses, but the magnitude and duration of these responses is affected by various factors including the virulence of the infecting strain, the size of infecting inoculum, pregnancy and sexual and immune status of the host [19]. The strategy of Brucella abortus to evade the innate immune system and persist in the host is long enough to be transmitted. The bacterium contains an unusual lipid A constituting the lipopolysaccharide (LPS) molecule, which is important for evading the host immune system during the early stages of infection [20]. The adaptation to live inside machrophage [21] is managed by its ability to block receptors for innate immunity [22] inhibit phagolysosome fusion, in habit apoptosis and down regulate antigen presentation [23] which collectively leads to their escape from effector immune responses [24].

Innate Immunity

Innate immunity is the rapid, non-specific, and non-memory immune response against invading brucellae pathogens. It consists of physical barriers at the surface of the body, humoral components such as complement proteins and cellular components that include macrophages, dendritic cells, granulocytes (basophils, eosinophils and neutrophils) and natural killer cells [25].

Physical Barriers: The body's physical barriers provide the first level of protection and include the skin, mucus membrane and the body's 'self-cleaning' processes such as sneezing or coughing. However, physical barriers cannot be completely effective and sometimes pathogens may overcome them. For this reason, animals possess an immune system comprising a network of cells and molecules that can fight infection [26].

Humoral components: Complement is a systemic plasma protein with a variety of functions that include opsonization by binding to antibodies or bacterial surfaces or direct killing of brucella pathogens by the formation of a membrane attack complex and causing bacterial lysis [27,28].

Cellular components: Macrophages and dendritic cells are the first cells that react to invading microbes and are responsible for induction of the adaptive immune response by the presentation of antigen epitopes to T helper (Th) cells. Pathogen recognition is achieved by pattern recognition receptors expressed by the antigen presenting cells that recognize pathogen associated molecular patterns of the invading microbes [29,30]. Bovine natural killer cells may act directly through the secretion of interferon [31] which is a cytokine that stimulates bactericidal activity of macrophages [32,33]. Neutrophils encounter and kill microbes intracellularly upon phagocytosis, when their antimicrobial granules fuse with the phagosome. Furthermore, they release lytic enzymes and reactive oxygen species that destroy pathogens [34].

Adaptive Immunity

The second barricade in the host arm is the adaptive immunity, which is otherwise known as antigen-specific immune response or specific immunity. It consists of T lymphocytes, which are responsible for cytokine production and cytotoxicity, known as cell-mediated immunity and B lymphocytes that are responsible

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for antibody production, known as humoral immunity or antibody-mediated immunity.

Antibody-mediated immunity: The B lymphocytes govern the humoral arm of adaptive immunity, characterized by production of antigen-specific antibodies. In addition to their neutralizing effect, antibodies act as opsonin's that facilitate the phagocytosis of bacteria by antigen presenting cells, activate complement and promote antibody-dependent cell-mediated cytotoxicity by macrophages, neutrophils and natural killer cells [35]. The presence of anti-Brucella antibodies suggests exposure to Brucella spp., but it does not indicate which Brucella species induced production of those antibodies [36]. Infected animals may not always produce all antibody isotypes in detectable quantities; therefore, results from several serological tests should be used as a presumptive evidence of infection [37].

The antibody response to Brucella abortus in cattle consists of the early production of IgM and it almost immediately progresses to the production of IgG2 and later produces small amounts of IgG1 and IgA [38,39]. Thus, the appearance of IgM indicates an early immune response (acute infection) against brucellosis and IgG correspondingly indicates chronic infection or relapse [40,41].

Cell-mediated immunity: CD4+ and CD8+ T cells have been shown to play a role in adaptive immune response to brucella [42]. Classically, two types of effector CD4+ T helper cell responses can be induced by a professional antigen presenting cells, designated Th1 and Th2, each designed to eliminate different types of pathogens. The Th1 response is characterized by the production of Interferon-gamma, which activates the bactericidal activities of macrophages and induces B cells to make opsonizing (coating) and complement-fixing antibodies [43]. The Th2 response is characterized by the release of Interleukin 5, which induces eosinophils in the clearance of pathogens [44]. Cytotoxic lymphocytes (CTLs) kill target cells principally via two major pathways:

a. The Fas ligand on CTLs interacts with its Fas receptor on target cells and then activates a suicide pathway in the target cells;

b. The CTLs exocytose granules containing perforin and granzymes that form pores in target cell membrane and eventual cell death [45].

Serological Diagnostic Tests in Bovine Brucellosis

Serological tests have a long history and have been used successfully for the diagnosis of many infectious diseases (e.g., HIV, syphilis and viral hepatitis) [46]. Serological diagnostic tests for brucellosis have been invented more than a century ago; however, the perfect test has still not been developed. Serological diagnostic methods are based on the detection of antibodies, specific to the surface LPS [47].

Serological tests are crucial for laboratorial diagnosis of brucellosis since most of control and eradication programs of brucellosis depend on these methods. Inactivated whole bacteria or purified fractions (i.e. LPS or membrane proteins) are used as antigens for detecting antibodies generated by the host during the infection. Antibodies against smooth Brucella species (e.g. B. abortus, B. melitensis and B. suis) cross react with antigen preparations from B. abortus, whereas antibodies against rough Brucella species (e.g. B. ovis and B. canis) cross-react with antigen preparations from B. ovis [48].

Serological tests are economical and reliable tools of diagnosis as there is a good correlation between isolation of Brucella and positive tests performed with sera and milk. When tests for detecting Brucella antibodies in milk and serum are considered, the principal methods for detecting infected herds and for diagnosing brucellosis in individual animals are the serological tests which are mainly used for diagnosis of brucellosis [49]. The choice of the serological diagnostic method depends on the overall epidemiologic al situation in the region and the objectives of the study: validation of the diagnosis, screening (monitoring), cross-sectional studies or confirmation of brucellosis-free status of the region [50]. Test result interpretation should always take the following elements into account: Percentage of positive tests results, disease prevalence and incidence; presence of clinical signs (abortion); vaccination strategy; known risk factors; status of the herd, the area and the country [51]. The sensitivity and specificity of serological tests have been found to be influenced by the external environment, such as temperature conditions under which the test is performed, the disease endemic status, animals' vaccination and the presence of cross reacting antibodies from other Gram-negative bacteria which share similar epitopes with Brucella species [52,53].

Although, several serological tests have been used for the laboratory testing of brucellosis, no single test is convenient in all epidemiological investigations due to problems of sensitivity and/or specificity [54]. There are several serological techniques that can be used depending on the antibodies being studied [55]. They can be broadly divided into two groups and these are screening tests and confirmatory tests [56]. Among them ELISA is the most sensitive and specific of the Brucella serologic routine tests and is useful to monitor antibodies in patients undergoing treatment, isotype determining and phase of disease and it may be positive when other tests are negative [57].

Screening Serological Tests

Screening tests are rapid and inexpensive methods with high sensitivity to ensure that infected animals are not missed. It is recommendable that all animals in an infected herd, including those that test negative during screening, should be successively evaluated using confirmatory tests [58]. There are many screening tests which are used to diagnose brucellosis in bovines. Some screening tests used in the field clinics or in regional laboratories are the Rose Bengal test (RBT), Buffered Plate Agglutination Test (BPAT) and Milk Ring test (MRT). The Rose Bengal Plate Test (RBPT) is the most common screening serological test for

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detection of Brucella agglutinins. The RBPT has a very high sensitivity to ensure that infected animals are not missed. The MRT is also an excellent screening test for dairy cattle [56].

Rose bengal plate test (RPBT): The Rose-Bengal Test (RBT) is the most economical and most widely used laboratory test in diagnosis of the bovine brucellosis but the interpretation of the result is largely subjective [59,60]. It is a rapid, slide-type agglutination assay performed with a stained B. abortus suspension at pH of 3.6-3.7 and plain serum. Although the low PH (3.6) of the antigen enhances the specificity of the test and temperature of the antigen and the ambient temperature at which the reaction takes place may influence the sensitivity and specificity of the test [61]. The sensitivity is very high (>99%) but the specificity is disappointingly as low as 68.8% [62]. However, this is of value as a screening test in high risk rural areas where it is not always possible to perform the tube agglutination titration test [63].

The simplicity of RBT made it an ideal screening test for small laboratories with limited resources. The principle of the test is in that the sera collected from animals that were stored at –20 °C were removed from the refrigerator and left at room temperature for at least 30 minutes before the test was performed. For this test 30 l of plain serum is dispensed on a white glossy ceramic tile and mixed with an equal volume of RBT antigen (previously equilibrated at room temperature and shaken to resuspend any bacterial sediment) using a toothpick. The tile is then rocked at room temperature for 4 minutes and any visible agglutination and/or the appearance of a typical rim is taken as a positive result [64].

The Rose Bengal plate test (RBPT) is the most widely used screening test for brucellosis in both humans and animals for its easy application and apparent simplicity of reading. However, interpretations of the RBPT results can be affected by personal experience [65]. The drawbacks of RBT include low sensitivity particularly in chronic cases, relatively low specificity in endemic areas and prozone s make strongly positive sera appear negative in RBT [66]. In cattle, in areas where there is little or no infection and particularly where there has been much strain 19 vaccination, the RBPT positive sera have to be subjected to confirmatory tests. In heavily infected herds, it may prove economical to remove all animals positive to this test, since many such animals, although negative to confirmatory tests, may be in the early stages of infection and likely to become dangerous in spreading brucellosis later [67]. False negative reaction s occurs in the RBT, however, these tests are considered as suitable screening tests for brucellosis, followed by confirmatory testing. Antibody resulting from Brucella abortus S19 vaccination will react in these tests [68].

Milk ring test (MRT): The milk ring test (MRT) is a simple and effective serological method but can only be used with cow's milk. A drop of haematoxylin-stained antigen is mixed with a small volume of milk in a glass or plastic tube. If specific antibody is present in the milk it will bind to the antigen and rise with the cream to form a blue ring at the top of the column of milk. If no antibody is present, the fat layer will remain a buff color and the purple antigen will be distributed throughout the milk. This test may be applied to individual animals or to pooled milk samples using a larger volume of milk relative to the pool size. The test is reasonably sensitive but may fail to detect a small number of infected animals within a large herd. Non-specific reactions are common with this test, especially in brucellosis free areas [69,70].

Currently, veterinary diagnostic laboratories utilize MRT for diagnosis of brucellosis in bovine milk samples, which indirectly identifies Brucella species in the host [71]. It can be used for screening the herd and to indicate level of infection in a herd. The test can also be applied to monitor the dairy herds at regular intervals. Although relatively cheap and easy to perform, this test does not give accurate results. There are a high percentage of false positive results. Importantly, the number of false positive results is proportional to the number of cows secreting acidic milk due to colostrum's or mastitis [72].

The milk ring test is prone to false reactions caused by abnormal milk due to mastitis, presence of colostrum and milk from the late lactation. Still, in spite of this problems, it may be used as an inexpensive screening test in conjunction with other tests [73,74]. This test is not considered sensitive but this lack of sensitivity is compensated by the fact that the test can be repeated, usually monthly, due to its very low cost. This test is prescribed by the OIE for use only with cow milk.

Buffered plate agglutination test (BPAT): Buffered plate agglutination (BPA) tests are the well-known buffered Brucella antigen tests. These tests are rapid agglutination tests lasting 4 minutes and it is done on a glass plate with the help of an acidic-buffered antigen (pH 3.65 ± 0.05). These tests have been introduced in many countries as the standard screening test because it is very simple and thought to be more sensitive than the SAT [75]. In BPAT, the cells are stained with Crystal Violet and suspended in a buffer which when mixed with the appropriate volume of serum results in a final PH of 3.65. This PH discourages agglutination by IgM but encourages agglutination by IgG1, reducing cross reaction. Antibody resulting from B. abortus s19 vaccination will react in these tests. These testes are considered as a suitable screening test for brucellosis followed by confirmatory tests like CFT [74,76].

Confirmatory Serological Tests

The confirmatory serological test is a test which provides good sensitivity but higher test specificity, thereby eliminating some false positive reactions. All screening test results which show positive test result must be confirmed by confirmatory serological test as there are false positive test results. Most confirmatory tests are more complicated and more expensive to perform [74]. There are many serological tests that can be used as confirmatory serological tests for bovine brucellosis. The common confirmatory tests are; Complement Fixation Test (CFT), Enzyme Linked

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Immunosorbent Assay (ELISA), Serum Agglutination Test (SAT), 2mercapto ethanol test (2MT), Fluorescence polarization assay (FPA) and Brucellin allergic skin test (BAST). Among them ELISA and CFT are the most commonly used confirmatory serological tests.

Complement fixation test (CFT): Complement fixation test (CFT) is a widely used confirmatory test for brucellosis. The basic test consists of B. abortus antigen, usually whole cells, incubated with dilutions of heat inactivated (to destroy indigenous complement) serum and a titrated source of complement, usually guinea pig serum [77]. It is the gold standard test for serological diagnosis of brucellosis in cattle. The CFT allows the detection of anti-Brucella antibodies that are able to activate complement. Cattle immunoglobulins that can activate bovine complement are the IgG and the IgM. The CFT test is highly specific but it requires highly trained personnel as well as suitable laboratory facilities. It measures more antibodies of the IgG1 type than antibodies of the IgM type [78]. The test is based on the principle that activation of the complement system by antigen-antibody complexes in the presence of red blood cells will lead to haemolysis of red blood cells which can be appraised visually. Complement in the test serum is heat inactivated before the addition of the whole cell brucella CFT antigen and incubation to allow the complement cascade to occur if anti-Brucella antibodies are present so-called complement fixation [79,80].

The CFT is widely used and accepted as a confirmatory test although it is complex to perform, requiring good laboratory facilities and adequately trained staff to accurately titrate and maintain the reagents. Sensitivity of complement fixation ranges from 77.1 to 100% and its specificity from 65 to 100% [81,82]. It is highly efficient and therefore accepted worldwide. The complement fixation test is technically challenging because a large number of reagents must be titrated daily and a large number of controls of all the reagents is required. It is also an expensive test again because of the large number of reagents needed and because it is labor intensive. However, since only IgG1 isotype of antibody fixes complement well, the test specificity is high. Unfortunately, the test does not allow for discrimination of B. abortus S19 derived antibody. Other problems include the subjectivity of the interpretation of results, occasional direct activation of complement by serum (anticomplementary activity) and the inability of the test for use with hemolyzed serum samples. In spite of the shortcomings, the complement fixation test has been and is a valuable asset as a confirmatory test in control/eradicati on programs of brucellosis [74].

2-Mercaptoethanol (2ME) test: The 2-mercaptoethanol (2ME) test is a confirmatory serological test that allows selective quantification of IgG anti-Brucella due to inactivation of IgM in the test sample. Production of IgG is usually associated with chronic infection and therefore, a positive result with this test is a strong indicator of brucellosis. However, this test has some drawbacks including the toxicity of mercaptoethanol, which requires a fume hood for its manipulation, and the possibility of IgG degradation caused by the 2-mercaptoethanol, which may result in false negative results. The test measures mainly IgG, because the disulphide bridge of IgM is being reduced to monometric molecules, and, therefore, unable to agglutinate. However, IgG can also be reduced in the process, giving false negative results. Though in general, reduction of IgM increases specificity.

The 2- mercaptoethanol (2-ME) test can be used to predict the course of the disease [83]. Sensitivity of the 2-mercaptoethanol test varies from 88.4 and 99.6%, and its specificity from 91.5 and 99.8% [84]. The test does not eliminate vaccinal antibodies, therefore is not recommended for international trade. The 2-MET is, however, used extensively for national control and or eradication programmed.

Enzyme-linked immunosorbent assay (ELISA): The ELISA is a fast-serological diagnostic test and has a high sensitivity and specificity of about 80% for the diagnosis of IgM, IgG and IgA antibodies related to brucella in blood [85-87]. It is based on the principle that as its name suggests; it uses an enzyme system to show the specific combination of an antigen with its antibody. The enzyme system consists of an enzyme which is labeled or linked to a specific antibody or antigen and a substrate which is added after the antigen antibody reaction. This substrate is acted on (usually hydrolyzed) by the enzyme attached to the antigen antibody complexes, to give a colour change. The intensity of the colour gives an indication of the amount of bound antigen or antibody. The more intense the colour is, the higher the concentration of antibody in the serum [88]. This test can diagnose an incomplete antibody and this antibody is generally observable in chronic patients with brucellosis, there for recommending this test for such patients [89].

The ELISA method poses a great opportunity of identification of all of the four antibody classes (IgM, IgG1, IgG2 and IgA) [90]. Although the ELISA technique is considered as one of the most sensitive serological tests and is a useful method for monitoring antibodies in patients undergoing treatment, the lack of a standard antigen, the variations in the quality of preparations and the use of various endpoints make difficult the interpretation of ELISA results. There are basically two different kinds of immunoenzymatically essays that are used for diagnosis of brucellosis in humans and domestic animal species: the indirect ELISA (iELISA) and the competitive ELISA (cELISA) [91,92].

The indirect Enzyme-linked immunosorbent assay (iELISA) is highly sensitive and specific tests and can be adapted to process a large number of samples in a short time [93]. They are economical in terms of time and effort, with sensitivity and specificity ranging between 98 to 99% for both serum and milk ELISA [94]. Indirect ELISA (iELISA) method is based on the specific binding of antibodies present in the test sample with immobilized antigen. The binding event is visualized using chemically or enzymatically derived fluorescent, luminescent or colorimetric reaction indicative of the presence of antibody in the sample. Many iELISA tests are available on the market. The indirect ELISA generally

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have very high sensitivity but because they are largely unable to distinguish B. abortus S19 vaccinal antibody and cross reacting antibody, the specificity can be slightly lower than the assay specificity in areas where vaccination is not practiced [74].

The Competitive Enzyme-linked immunosorbent assay (cELISA) is based on the displacement of serum antibodies by a fixed concentration of a mouse monoclonal antibody against the common epitope, which is the dominant epitope in the OPS of both B. abortus and B. melitensis and is the most relevant in serological diagnosis. Since the cELISA does not involve the use of a specific conjugate anti-animal species immunoglobulin, this assay can be easily adapted to detect Brucella infections in different animal species [95]. This cELISA uses SLPS passively immobilized on the wall of 96 well polystyrene plates. Competition between a monoclonal antibody specific for a common epitope of OPS and test serum, both appropriately diluted are added. The monoclonal antibody may be labeled directly with enzyme or a secondary anti-mouse antibody labelled with enzyme may be added [96]. It is capable of distinguishing vaccinated animals or animals infected with cross-reacting organisms from naturally infected animals, thereby reducing the number of false-positive reactions [97].

Serum agglutination test (SAT): Serum agglutination test (SAT) is one of standard serological tests, used for the diagnosis of brucellosis. It is a serological test developed by Wright and colleagues remains the most popular and yet used worldwide diagnostic tool for the diagnosis of brucellosis because it is easy to perform, does not need expensive equipment's and training. SAT measures the total quantity of agglutinating antibodies IgM and IgG [98]. This test is based on the reactivity of antibodies against the smooth lipopolysaccharide of Brucella. Excess of antibodies resulting in false negative reaction due to prozone effect can be overcome by applying a serial dilution of 1:2 through 1:64 of the serum samples thus increasing the test specificity [99]. The quantity of specific IgG is determined by treatment of the serum with 0.05M 2-mercaptoethanol (2ME), which inactivates the agglutin ability of IgM. SAT titers above 1:160 are considered diagnostic in conjunction with a compatible clinical presentation. However, in areas of endemic disease, using a titer of 1:320 as cut off may make the test more specific. The differentiation in the type of antibody is also important, as IgG antibodies are considered a better indicator of active infection than IgM and the rapid fall in the level of IgG antibodies is said to be prognostic of successful therapy [100]. The serum agglutination test has low sensitivity (41 %), while its specificity was 66.7% in bovines [101].

Drawbacks of the serum agglutination test include the inability to diagnose B. canis infections; the appearance of cross-reactions of class M immunoglobulins with Francisella tularensis, Escherichia coli O116 and O157, Salmonella urbana, Yersinia enterocolitica O: 9. Some of these shortcomings can be overcome by modifications such as the addition of EDTA, 2-mercaptoethanol, or antihuman globulin [98].

Fluorescence polarization assay (FPA): The fluorescence polarization assay (FPA) is a simple technique for measuring antigen/antibody interaction and may be performed in a laboratory setting or in the field. It is a homogeneous species-independent assay in which analytes are not separated and it is therefore very rapid test for the diagnosis of Brucella infection [102]. The FPA was initially developed for testing serum. However, the technology has been extended to testing whole blood and milk samples from individual animals [103,104]. It is based on the physical principle of the mass-dependent change of the molecule's rotation speed in a liquid medium. The smaller the molecule, the faster it rotates and the depolarization of a polarized beam of light occurs. In FPA, the serum sample is incubated with a specific Brucella antigen and conjugated with a fluorescent label. In case there are anti-Brucella antibodies in the serum, large fluorescently labelled antigen-antibody complex is formed, which can easily be distinguished from the unbound antigen negative control. Thus, the rotation of a fluorescent molecule (fluorophore) conjugated to Brucella O-chain will slow if bound by anti-Brucella LPS antibodies [105]. If serum contains antibodies to antigen there is a decrease in the rate of rotation due to an increase in the molecular weight of antigen -antibody complex. It is this decrease, which enables the distinction between negative and positive results.

Sensitivity of the fluorescence polarization assay varies from 87.5 and 100%, and specificity from 84 to 100% [106-108]. It is very accurate and the sensitivity: specificity can be manipulated by altering the cutoff value between positive and negative reactions to provide a very sensitive screening test as well as a highly specific confirmatory test. Because only 2 reagents, antigen and diluents buffer are required, the test is technically simple and relatively inexpensive. It does require fluorescence polarization analyzer of which several are available at various costs. Diagnostic kits are also commercially available from several sources. The FPA is capable of distinguishing vaccinal antibody in most vaccinated animals and it can eliminate some cross reactions as well. In the case of brucellosis serology, small molecular weight subunit of OPS is labeled with fluoroescein isothiocyanate and used as the antigen. When testing serum, blood, or milk, if antibody to the OPS is present, the rate of rotation of the labeled antigen will be reduced. The rate of reduction is proportional to the amount of antibody present [109].

Brucellin allergic skin test: The Brucellin allergic skin test is an allergic test that detects the specific cellular immune response induced by Brucella infection. The injection of brucellergene, a protein extract of a rough strain of Brucella species, is followed by a local inflammatory response in a sensitized animal. This delayed type hypersensitivity reaction is measured by the increase in skin thickness at the site of inoculation. Ten square centimeters of healthy clean skin on the side of the neck can be shaved with scissors or electric clippers. A tuberculin syringe with a 4 mm needle is used to inject 100 µl of brucellin intradermally, and the reaction read three days after the injection. Palpation of the injection site was the primary technique for evaluating the reaction. The visible and/or palpable reaction was quantified by measuring the diameter of the swelling. A spring meter (Aesculap) was used to compare the difference in skin thickness at the

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injection site with a fold of healthy skin adjacent to the site. Skin thickening of 1.5-2mm would be considered as a positive reaction [110].

The brucellin allergic skin test has a very high specificity, such that serologically negative unvaccinated animals that are positive reactors to the brucellin test should be regarded as infected animals [111]. Therefore, this test could be used as a confirmatory test on cattle non-vaccinated against brucellosis. Not all infected animals react, therefore, this test alone cannot be recommended as the sole diagnostic test, or for the purposes of international trade. This test is prescribed as an alternative test by the OIE.

Rivanol plate test: The Rivanol plate test is a test aimed at eliminating some non-specific reactions which is based on precipitation of high molecular weight serum glycoprotein from serum solutions; which in this case, is mainly IgM, leaving mostly IgG in the serum. Acrydine dye such as rivanol (2-ethoxy-6, 9-diamino acridine lactate) is used to achieve the precipitation process, after which the precipitate is removed by centrifugation. The supernatant is tested, using rapid plate agglutination test with undiluted serum, or a tube test, using serum dilutions of 1:25, 1:50, 1:100, and 1:200. The precipitation tests are usually used as confirmatory tests, because of their laborious protocols.

Antiglobulin or Coombs test: The Coombs test is the most suitable and sensitive test for confirmation of relapsing patients with persistent disease [104]. The test is useful for antibodies detection, like the blocking of IgG in patients who are suffering from the chronic form of disease [112,113]. Existence of a block on an antibody or Prozone phenomenon cause false-negative SAT results, and therefore the usage of the Coombs test is an ideal method to overcome this problem [114,115]. In brucellosis diagnosis, the SAT test is the most trustworthy test, yet in some patients who have obvious clinical symptoms and negative SAT results, it is better to use the Coombs and enzyme-linked immunosorbent assay (ELISA) methods [116].

Native hapten and poly B tests: Native hapten and poly B tests are confirmatory tests that have been used successfully in an eradication programme in combination with the RBT as a screening test [117]. The conjunctival vaccination (both in young and adults) reduces the time to obtain a negative response in native hapten tests. A remarkable characteristic of the radial immunodiffusion test is that a positive result correlates with Brucella shedding in experimentally infected cattle and in naturally infected cattle undergoing antibiotic treatment. Precipitin tests using native happen or Brucella cytosol proteins have also been shown to eliminate, in most cases, false positive serological reactions caused by Yersinia enterocolitica O:9 and false positive serological reactions of unknown origin [118].

The Diagnostic Performance of Serological Tests

The serological tests available in bovine brucellosis show different performances under different conditions. The diagnostic performance of an assay is indicated by the diagnostic sensitivity (DSe) and specificity (DSp), which is defined in the OIE Terrestrial manual as follows: DSe is the proportion of samples from known infected reference animals that test positive in an assay. DSp is the proportion of samples from known uninfected reference animals that test negative in an assay [119]. The sensitivity (Se) in the individual animal may be influenced by the stage of infection or immunity of the host, and the mix of cases tested. Specificity (Sp) may be affected by presence of maternal antibodies, persistence of antibodies after recovery or vaccination depending on purpose of testing. Several methods can be employed to determine the diagnostic performance of an assay. Typically, the DSe and DSp are determined by testing samples from animals with known status. The sensitivity can be estimated as the true positives, divided by the sum of the true positives and false negatives [120]. Another method to estimate diagnostic sensitivity and specificity in the absence of knowledge about the true disease status necessitates the use of complicated statistical concepts and formula. It relies on the use of two tests, where the diagnostic sensitivity of one test is imperfect but known, or the sensitivity and specificity of both tests are unknown [121].

Table 1: Sensitivity, Specificity and Performance Index of the commonly used serological tests for bovine brucellosis.

Serological Test	% Sensitivity		% Specificity		Performance Index	
					Min	Max
SAT	29.1	-100	99.2	– 100	128.3	200
RBT	21	-98.3	68.8	– 100	89.8	198.3
BPAT	75.4	-99.9	90.6	– 100	166	199.9
RIV	50.5	-100	21.9	– 100	72.4	200
2ME	56.2	-100	99.8	– 100	156	200
CFT	23	-97	30.6	– 100	53.6	197.0
iELISA	92	-100	90.6	– 100	182.6	-199.8
Celisa	97.5	-100	99.7	-99.8	197.3	-199.8
FPA	99	-99.3	96.9	– 100	195.9	-199.3

Source: Nielsen (2002) based on Sensitivity (Se) and Specificity (Sp) values obtained from several sources.

The complement fixation test (CFT) is diagnostically more specific than the SAT, and also has a standardized system of unitage. The diagnostic performance characteristics of some enzyme linked immunosorbent assays and the fluorescence polarisation assay (FPA) are comparable with or better than that of the CFT, and as they are technically simpler to perform and

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more robust, their use may be preferred [122-124]. A comparison with the SAT; ELISA yields higher sensitivity and specificity. ELISA is also reported to be the most sensitive test for the diagnosis of central nervous system brucellosis. The DSe and DSp of the FPA for bovine brucellosis are almost identical to those of the cELISA. The DSp for cattle recently vaccinated with S19 is over 99% [125]. Among the newer serologic tests, the ELISA appears to be the most sensitive; however, more experience is needed before it replaces the SAT as the test of choice for brucellosis [126].

Traditionally, screening tests are inexpensive, fast and highly sensitive, but not necessarily highly specific. Confirmatory tests are required to be both sensitive and specific [127]. It is clear that no single test is capable to identify all positive cases of brucella infected animals due to variation in sensitivity and specificity of serological tests [128-135]. Published sensitivity and specificity ranges for the commonly used serological tests are tabulated below. These are values obtained from several sources in the literature. The Performance Index provides an overall estimate of the accuracy of the test by adding the sensitivity and specificity values. In Table 1, the min and max values represent the lowest and highest indexes [74].

Conclusion and Recommendations

The use and understanding of different serological tests for the diagnosis bovine brucellosis under different circumstances is necessary due to the complicated nature of the infection and its innate and acquired immune response. There is no single serological test that is appropriate in all epidemiological situations; all have limitations especially when it comes to screening individual animals. Despite these limitations, serologic diagnostic tests are very useful in diagnosing bovine brucellosis in cattle. In all cases a blood sample should be collected from the patient and laboratory testing should be requested as the definite diagnosis of brucellosis is impossible without laboratory confirmation. For the purposes of this review, the serological methods described represent standardized and validated methods with suitable performance characteristics to be designated as either prescribed or alternative tests for international trade. This does not preclude the use of modified or similar test methods or the use of different biological reagents. Most likely the solution to the problems with accurate serological diagnosis of bovine brucellosis will involve several tests for different functions of the host immune response. Generally, serological diagnostic procedures in bovine brucellosis consist of testing each serum by multiple tests, usually a screening test of high sensitivity, followed by a confirmatory test of high specificity.

Based on the above conclusion, the following recommendations are forwarded:

a. Researches should have to be done for continued improvement of serological diagnostic methods through identifying existing shortcomings.

b. Serological diagnostic tests should be validated in accordance with OIE standards.

c. Consideration should be given to all factors that impact on the relevance of the serological tests.

d. Finally, molecular biology techniques should be used as a diagnostic tool for brucellosis which are advancing with promising results and may soon be at the point of replacing actual bacterial isolation.

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