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Some Serological Analytic Approaches and Test Execution in Determination of Bovine Brucellosis: Overview

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SUMMARY
Bovine brucellosis is a highly contagious, zoonotic, and economically significant bacterial disease. The purpose of this paper was to review available scientific data on serological tests for the diagnosis of bovine brucellosis and compare test performance based on assay sensitivity (Se) and specificity (Sp) values. Abortion, placentitis, epididymitis, and orchitis are symptoms of the disease. The clinical picture of brucellosis is not pathognomonic, and the patient's clinical history, particularly the occurrence of abortion, is crucial for diagnosis. Although the causative agent is isolated and identified for a definitive diagnosis of bovine brucellosis, serological tests are usually preferred. The host defense mechanism against Brucella is functionally divided into innate or non-specific immunity and adaptive or specific immunity. The pathogenic brucella organism has evolved a slew of mechanisms for evading and/or modulating both innate and adaptive immune responses in its host. Serological assays are based on the fact that Brucella abortus and other smooth Brucella have a unique O-polysaccharide that induces a humoral response with the production of IgM first, followed by IgG1, and then IgG2/IgA. Screening serological tests (Milk ring test, Rose Bengal Test (RBT), and Buffered plate agglutination test (BPAT)) and confirmatory serological tests (complement fixation test (CFT), serum agglutination test (SAT), 2-mercaptoethanol test (2ME), indirect-enzyme-linked immunosorbent assay (iELISA), and, more recently, competitive-ELISA (cELISA) and Brucella fluorescent pol (FPA). CFT, RBT, and iELISA tests are currently used in EU legislation for intra-community cattle trade and show comparable Se and Sp. The new FPA test demonstrated Se and Sp levels comparable to standard tests and was proposed for inclusion as a standard test in EU legislation. Similarly, the OIE considers the four tests to be a mandatory requirement for international trade. Several methods, however, produce both false-positive and false-negative results. There is no single serological test that is 100% accurate and applicable in all epidemiological situations. In general, confirmatory serological procedures involve testing each serum with multiple tests, usually a screening test with high sensitivity followed by a confirmatory test with high specificity. As a result, the development of rapid, low-cost serological tests with high sensitivity and specificity for brucellosis detection is a major issue that requires further investigation.
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1. INTRODUCTION

Brucellosis is a highly contagious, zoonotic, and economically significant bacterial disease that affects animals worldwide (Schelling *et al.,* 2003). 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 the mononuclear phagocytic system (Jarvis *et al.,* 2002). Bovine brucellosis is characterized by late-term abortion, infertility, and reduced milk production as a result of retained placenta and secondary endometritis, as well as organism excretion in uterine discharges and milk. Abortion rates in fully susceptible herds can range from 30 to 80 percent (Anonymous, 2007).

Infection is spread through dairy products such as milk and cheese, as well as contact with infected animals and aerosols (Hatami *et al.,* 2010). Because of the large volume of infected milk that a single animal can produce, as well as the extensive environmental contamination that even single abortions or infected births can cause, bovine infection is a particularly serious problem (Radostist *et al.,* 2000). Although developed countries have successfully controlled brucellosis, many developing countries, such as Ethiopia, have failed to respond adequately, and the disease remains a major public and animal health concern. Control and eradication of brucellosis are almost entirely based on serological testing of animals and culling of those who test positive for antibodies to Brucella species (OIE, 2009).

The disease has a wide range of clinical manifestations (not pathognomonic), making clinical diagnosis difficult. Because of the lack of specific symptoms, it is difficult to distinguish brucellosis from other febrile conditions that frequently occur in the same areas. As a result, the diagnosis must be confirmed either directly by Brucella isolation, mostly from blood cultures, or indirectly serologically by detecting an immune response to its antigens (Orduna *et al.,* 2000). The gold standard for brucellosis diagnosis is the isolation and identification of causative bacteria, but this requires a high-security laboratory, highly skilled personnel, a lengthy turnaround

time for results, and is considered a hazardous procedure (Nielsen, 2002). As a result, diagnoses are primarily based on the detection of antibodies in serum using serological tests such as the Rapid Plate Agglutination Test (RPAT), the Rose Bengal Test (RBT), the Standard Agglutination Test (SAT), the 2-Mercapto Ethanol Test (2ME), and the Complement Fixation Test (CFT) (McGiven *et al.,* 2003). They are relatively simple to carry out and provide a useful advantage in determining the prevalence of Brucella infection (Abubakar *et al.,* 2011).

Body fluids such as serum, uterine discharge, vaginal mucus, milk, and sperm plasma from suspect cattle may contain varying amounts of IgM, IgG1, IgG2, and IgA antibodies directed against Brucella (OIE, 2000). IgM is usually the first to appear in the blood at the end of the first week of the disease, followed by IgG. (Lucero *et al.,* 2003). As a result, serological methods are recommended for obtaining quick indirect proof of diagnosis. Each test has its own set of drawbacks, and the presence of antibodies does not always indicate the presence of active brucellosis infection (Clavijo *et al.,* 2003).

There are currently several serological methods available; these tests are classified as screening tests (e.g., buffered antigen plate agglutination (BPAT), Rose Bengal plate test (RBPT), monitoring or epidemiological surveillance tests (e.g., milk ring test), and complementary or confirmatory tests (e.g., 2-mercapto-ethanol, complement fixation, ELISAs, and fluorescence polarization assay). The species affected, as well as local regulations, should be considered when selecting a given test (Poester *et al.,* 2010). As a result, the goals of this review paper are as follows:

- To review the serological diagnostic tests available for the diagnosis of Brucella infections in cattle and
- To compare the sensitivity and specificity values of the available serological diagnostic tests.

2. HOST IMMUNE RESPONSES IN BRUCELLA INFECTION

The host immune response is classified into two types: innate or non-specific immunity and adaptive or specific immunity (Parksin and C-ohen, 2001). Infection with Brucella typically induces both humoral and cell-mediated immune responses, but the magnitude and duration of these responses are influenced by a variety of factors, including the virulence of the infecting strain, the size of the infecting inoculum, pregnancy, and the host's sexual and immune status (skendros, 2013).

Brucella abortus' strategy is to evade the innate immune system and remain in the host long enough to be transmitted. The bacterium contains an unusual lipid A, which forms the LPS molecule and is critical for evading the host immune system during the early stages of infection (Parent *et al.,* 2007). The adaptation to living inside a macrophage is managed by its ability to block innate immunity receptors, inhibit phagolysosome fusion, inhibit apoptosis, and downregulate antigen presentation, all of which contribute to their escape from effector immune responses (Martirosyan and Gorvel, 2013).

2.1 Innate Immunity

Innate immunity is a non-specific, non-memory immune response to invading brucella pathogens. It consists of physical barriers on the body's surface, humoral components such as complement proteins, and cellular components such as macrophages, dendritic cells, granulocytes (basophils, eosinophils, and neutrophils), and natural killer cells (Dranoff, 2004).

2.1.1. Physical barriers

The body's physical barriers, which include the skin and "self-cleaning" processes like sneezing and coughing, provide the first level of protection. Physical barriers, however, are not always completely effective, and pathogens can sometimes overcome them. As a result, animals have an immune system that consists of a network of cells and molecules that can fight infection (Tigard, 2013)

2.1.2 Humoral components

Complement is a systemic plasma protein that performs a variety of functions such as opsonization by binding to antibodies or bacterial surfaces or direct killing of brucella pathogens via the formation of a membrane attack complex, resulting in bacterial lysis (Tigard, 2012).

2.1.3. Cellular components

Macrophages and dendritic cells are the first cells to respond to invading microbes, and they are responsible for inducing the adaptive immune response by presenting antigen epitopes to T helper (Th) cells. Pathogen recognition is accomplished through the expression of pattern recognition receptors (PRRs) on antigen-presenting cells, which recognize pathogen-associated molecular patterns (PAMPs) of invading microbes (Pasquevich *et al.,* 2010). Bovine natural killer cells may act directly by secreting IFNc, a cytokine that stimulates macrophage bactericidal activity (Oliveira *et al.,* 2002). When neutrophils phagocytose, their antimicrobial granules fuse with the phagosome and kill microbes intracellularly. They also release lytic enzymes and reactive oxygen species (ROS) that kill pathogens (Nauseef, 2007).

2.2. Adaptive Immunity

The second line of defense in the host arm is adaptive immunity, also known as antigen-specific immune

response or specific immunity. T lymphocytes are responsible for cytokine production and cytotoxicity, which is known as cell-mediated immunity (CMI), and B lymphocytes are responsible for antibody production, which is known as humoral immunity or antibody-mediated immunity (AMI) (Parkin and Cohen, 2001).

2.2.1. Antibody-mediated immunity (AMI)

The humoral arm of adaptive immunity, which is characterized by the production of antigen-specific antibodies, is governed by B lymphocytes. In addition to neutralizing bacteria, antibodies act as opsonins, assisting antigen-presenting cells in phagocytosis, activating complement, and promoting antibody-dependent cell-mediated cytotoxicity (ADCC) by macrophages, neutrophils, and natural killer cells (Baldwin and Goenka, 2006). The presence of anti-Brucella antibodies suggests exposure to Brucellaspp., but it does not specify which Brucellaspecies caused the antibodies to be produced. Because infected animals do not always produce all antibody isotypes in detectable quantities, the results of multiple serological tests should be used as presumptive evidence of infection (FAO, 2005).

The antibody response to Brucella abortus in cattle begins with the production of IgM, then quickly progresses to the production of IgG2, and finally produces small amounts of IgG1 and IgA. (Goenka *et al.,* 2012). Thus, the presence of IgM indicates an early immune response (acute infection) to brucellosis, whereas the presence of IgG indicates chronic infection or relapse. Anti-Brucella antibodies have been shown to cross-react with antibodies raised against heterologous Brucella strains or some enteric bacteria. Many of the serological tests used in the diagnosis of brucellosis were hampered by this cross-reactivity (Young,2005).

2.2.2. Cell-mediated immunity (CMI)

T cells from the cluster distinctia (cd4) and cluster distinctia (cd8) have been shown to participate in the adaptive immune response to brucella (Carvalho *et al.,* 2010). T helper cells are CD4+ cells that include T helper type 1 (Th1) and T helper type 2 (Th2) cells that primarily act as helper cells for cell-mediated inflammatory reactions such as delayed hypersensitivity and macrophage activation and stimulate antibody production, specifically IgE, IgG1, and IgA. The Fas ligand on CTLs first interacts with its Fas receptor on target cells, activating a suicide pathway in the target cells; and second, CTLs exocytose granules containing perforin and granzymes, which form pores in the target cell membrane and eventually cell death (Janeway at el., 2001).

3. SEROLOGICAL DIAGNOSTIC TESTS IN BOVINE BRUCELLOSIS

Serological tests have a long history of use in the diagnosis of many infectious diseases (for example, HIV, syphilis, and viral hepatitis) (Steingart *et al.,* 2012). More than a century ago, serological diagnostic tests for brucellosis were developed. However, the ideal test has yet to be developed. Traditional Brucella diagnostic methods rely on the detection of antibodies specific to the surface LPS. (He, 2012).

Serological tests are crucial for laboratory diagnosis of brucellosis since most control and eradication programs of brucellosis depend on these methods. Inactivated whole bacteria or purified fractions (i.e., lipopolysaccharide or membrane proteins) are used as antigens for detecting antibodies generated by the host during the infection. Smooth Brucella antibodies (e.g., B. abortus, B. melitensis, and B. suis) cross-react with antigen preparations from B. abortus, whereas rough Brucella antibodies (e.g., B. ovis and B. canis) cross-react with antigen preparations from B. ovis (Nielsen, 2002).

Serological tests are inexpensive and reliable diagnostic tools because there is a strong correlation between Brucella isolation and positive serological and milk tests. When tests for detecting Brucella antibodies in milk and serum are considered, the serological tests, which are primarily used for brucellosis diagnosis, are the primary methods for detecting infected herds and diagnosing brucellosis in individual animals (Noriello, 2004).

The serological diagnostic method used is determined by the overall epidemiological situation in the region as well as the study's objectives: validation of the diagnosis, screening (monitoring), cross-sectional studies, or confirmation of the region's brucellosis-free status (God Froid *et al.,* 2010). The following factors should always be considered when interpreting test results: percentage of positive test results; disease prevalence and incidence; the presence of clinical signs (abortion); vaccination strategy; known risk factors; herd, area, and country status (European Commission, 2009). The sensitivity and specificity of serological tests are influenced by the external environment, such as the temperature conditions under which the test is performed, the disease endemic status, animal vaccinations, and the presence of cross-reacting antibodies from other gram-negative bacteria that share similar epitopes with Brucella species. Although several serological tests have been used for brucellosis laboratory testing, no single test is suitable for all epidemiological investigations due to sensitivity and/or specificity issues (Matope *et al.,* 2011). Depending on the antibodies being studied, various serological techniques can be used (Ryan and Ray, 2004). They are broadly classified into two types: screening tests and confirmatory tests (FAO, 2003). ELISA is the most sensitive and specific of the Brucella serologic routine tests, and it can be used to monitor antibodies in patients undergoing treatment, determine isotypes, and phases of the disease, and it can be positive even when other tests are negative (Esmaeilzadeh, 2004).

3.1. Screening Serological Tests

Screening tests are quick and low-cost methods with high sensitivity for ensuring that infected animals are not overlooked. It is recommended that all animals in an infected herd, including those that tested negative during screening, be evaluated using confirmatory tests in the following order (Adone and Pasquali, 2013). There are numerous screening tests available to diagnose brucellosis in cattle. The Rose Bengal test, Buffered Plate Agglutination Test (BPAT), and Milk Ring Test are some screening tests used in field clinics or regional laboratories (MRT). The Rose Bengal Plate Test (RBPT) is the most commonly used screening serological test for Brucella agglutinin detection. The Rose Bengal Plate Test (RBPT) is extremely sensitive, ensuring that infected animals are not overlooked. The milk ring test is also an excellent dairy cattle screening test (FAO, 2003).

3.1.1. Rose-Bengal plate test (RBT)

The Rose-Bengal Test (RBT) is the most cost-effective and widely used laboratory test for diagnosing bovine brucellosis, but its interpretation is highly subjective (Konstantinidis 2007). It is a slide-type agglutination assay using a stained B. abortus suspension and plain serum at a pH of 3.6-3.7. The low PH (3.6) of the antigen improves the test's specificity, and the temperature of the antigen and the ambient temperature at which the reaction occurs may influence the test's sensitivity and specificity (AUSVETPLAN,2005). The sensitivity is extremely high (> 99%), but the specificity is disappointingly low (68.8%) (Barrsol *et al.,* 2002). However, it is useful as a screening test in high-risk rural areas where the tube agglutination titration test is not always possible (Mantur *et al.,* 2006).

Because of its simplicity, it is an excellent screening test for small laboratories with limited resources. The serum collected from animals stored at –20 ºC was removed from the refrigerator and allowed to come to room temperature for at least 30 minutes before the test was performed. A toothpick is used to mix 30 mL of plain serum with an equal volume of RBT antigen (previously equilibrated at room temperature and shaken to resuspend any bacterial sediment) for this test. The tile is then rocked at room temperature for 4 minutes (rather than the recommended 8 minutes for human brucellosis), and any visible agglutination and/or the appearance of a typical rim is considered positive (Araj, 1999).

Because of its ease of use and apparent simplicity of reading, the Rose Bengal plate test (RBPT) is the most widely used screening test for brucellosis in both humans and animals. Personal experience, however, can influence how the RBPT results are interpreted (Cho *et al.,* 2010). The disadvantages of RBT include low sensitivity, especially in chronic cases; low specificity in endemic areas; and prozones, which cause strongly positive sera to appear negative in RBT (Diaz, 2011).

In cattle, RBPT-positive sera must be confirmed in areas with little or no infection, particularly where strain 19 vaccination has been extensive. In heavily infected herds, it may be cost-effective to remove all animals positive for this test because many of these animals, while negative to confirmatory tests, may be in the early stages of infection and may later become dangerous in spreading brucellosis (Brinley *et al.,* 1996). In the RBT, false-negative reactions occur. These tests, however, are considered adequate screening tests for brucellosis, followed by confirmatory testing. These tests will detect Brucella abortus S19 vaccination-induced antibodies (OIE, 2008).

3.1.2. Milk ring test (MRT)

The milk ring test (MRT) is a simple and effective serological test, but it can only be performed on cow's milk. In a glass or plastic tube, a drop of hematoxylin-stained antigen is mixed with a small amount of milk. If the milk contains a specific antibody, 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 remains buff (while the cream remains colorless), and the purple antigen is distributed throughout the milk. This test can be performed on individual animals or pooled milk samples by using a larger volume of milk about the pool size. The test is fairly sensitive, but it may miss a small number of infected animals in a large herd. Non-specific reactions are common with this test, particularly in areas free of brucellosis (Corbel *et al.,* 2006).

Currently, veterinary diagnostic laboratories use the Milk Ring Test in bovine milk samples to diagnose brucellosis, which indirectly identifies Brucella species in the host (Chimana *et al.,* 2010). The Brucella Milk Ring Test can be used to screen a herd and determine the level of infection. The test can be used to monitor dairy herds regularly. This test, despite being relatively inexpensive and simple to perform, does not provide accurate results. There is a significant number of false-positive results. Notably, the number of false positives is proportional to the number of cows secreting acidic milk as a result of colostrum or mastitis (OIE, 2009).

The milk ring test is prone to false positives due to mastitis-related abnormal milk, the presence of colostrum, and milk from late lactation. Despite these drawbacks, it can be used as a low-cost screening test in conjunction with other tests (Fernando *et al.,* 2010). This test is not considered sensitive, but this lack of sensitivity is offset by the fact that it can be repeated, usually monthly, due to its low cost. The OIE recommends that this test be used only with cow milk (OIE, 2009).

3.1.3. Buffered plate agglutination test

BPA tests are well-known buffered Brucella antigen tests. These are 4-minute rapid agglutination tests performed on a glass plate using an acidic-buffered antigen (pH 3.65 0.05). Because they are simple and thought to be more sensitive than the SAT, these tests have been adopted as standard screening tests in many countries (Greiner *et al.,* 2009). 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 IgM agglutination while encouraging IgG1 agglutination, reducing cross-reaction. Antibodies resulting from B. abortus s19 vaccination will be detected by these tests. These tests are recommended as a screening test for brucellosis, followed by confirmatory tests such as CFT (Migue *et al.,* 2011).

3.2. Confirmatory Serological Tests

The confirmatory serological test has high sensitivity but low specificity, which eliminates some false positive reactions. As there are false-positive test results, all screening test results that show a positive test result must be confirmed by a confirmatory serological test. Most confirmatory tests are more difficult and costly to perform (Fernando *et al.,* 2010). Numerous serological tests can be used as confirmatory tests for bovine brucellosis. The most common is the Complement Fixation Test (CFT), Enzyme-Linked Immune Sorbent Assay (ELISA), Serum Agglutination Test (SAT), 2-mercaptoethanol test (2MT), Fluorescence Polarization Assay (FPA), and Brucellin allergic skin test (BAST). ELISA and CFT are the most commonly used confirmatory serological tests (FAO, 2003).

3.2.1. Complement fixation test

A common confirmatory test for brucellosis is the complement fixation test. B. abortus antigen, usually whole cells, is incubated with dilutions of heat-inactivated (to destroy indigenous complement) serum and a titrated complement source, usually guinea pig serum, in a basic test. It's the gold standard test for brucellosis in cattle serological diagnosis. The Complement Fixation Test (CFT) allows anti-Brucella antibodies that can activate complement to be detected. The IgG and IgM immunoglobulins (Ig) in cattle are capable of activating bovine complement. The CFT test is extremely specific, but it necessitates highly trained personnel as well as specialized laboratory equipment. It detects a higher number of IgG1 antibodies than IgM antibodies (Nielson, 2001). The test is based on the principle that activation of the complement system by antigen-antibody complexes in the presence of red blood cells causes hemolysis of red blood cells, which can be visually assessed. Before adding the whole-cell brucella CFT antigen and incubating, the complement in the test serum is heat-inactivated to allow the complement cascade to occur if anti-Brucella antibodies are present, a process known as complement fixation (Au IBAR, 2013).

Although the Complete fixation test is widely used and accepted as a confirmatory test, it is difficult to perform because it requires good laboratory facilities and adequately trained personnel to accurately titrate and maintain the reagents (Xavler *et al.,* 2009). The sensitivity range for complement fixation is 77.1 to 100 percent, and the specificity range is 65 to 100 percent (Perrett *et al.,* 2010). It is highly efficient and thus widely accepted around the world (Nielsen, 2002).

The complement fixation test is technically difficult because a large number of reagents must be titrated daily and a large number of controls of all reagents are required. It is also an expensive test, due to the large number of reagents required and the labor involved. However, because only the IgG1 isotype of antibody fixes complement well, the test specificity is high. Unfortunately, the test does not distinguish between antibodies derived from B. abortus S19. Other issues include the test's inability to be used with haemolyzed serum samples, the subjectivity of the interpretation of results, the occasional direct activation of complement by serum (anticomplementary activity), and the test's inability to be used with haemolyzed serum samples. Despite its shortcomings, the complement fixation test has been and continues to be an important confirmatory test in brucellosis control/eradication programs (Fernando *et al.,* 2010).

3.2.2 2-Mercaptoethanol (2ME) test

The 2-mercaptoethanol (2ME) test is a confirmatory serological test that allows for the selective quantification of IgG anti-Brucella antibodies due to IgM inactivation in the test sample. Because IgG production is usually associated with chronic infection, a positive result with this test is a strong indicator of brucellosis. However, there are some drawbacks to this test, including the toxicity of mercaptoethanol, which necessitates its manipulation in a fume hood, and the possibility of IgG degradation caused by 2-mercaptoethanol, which may result in false-negative results. Because IgM's disulfide bridge is reduced to monometric molecules and thus unable to agglutinate, the test primarily measures IgG. However, immunoglobulin G (IgG) levels can fall during the process, leading to false-negative results. However, decreasing IgM generally increases specificity (Poester*et al.,* 2010).

The 2-mercaptoethanol (2-ME) test can be used to forecast the progression of the disease (Aliskan, 2008). The sensitivity of the 2-mercaptoethanol test ranges between 88.4 and 99.6 percent, and its specificity ranges between 91.5 and 99.8 percent (Nielsen, 2004). Because the test does not eliminate vaccinal antibodies, it is not

recommended for international trade. The 2-MET, on the other hand, is widely used in national control and eradication programs (Nielsen, 2002).

3.2.3 Enzyme-linked immunosorbent assay (ELISA)

The Enzyme-linked immunosorbent assay is a rapid serological diagnostic test with an 80% sensitivity and specificity for detecting IgM, IgG, and IgA antibodies related to brucella in the blood (Kostoula *et al.,* 2002). It works on the principle that, as the name implies, it uses an enzyme system to demonstrate the specific combination of an antigen and its antibody. The enzyme system is made up of an enzyme that has been labeled or linked to a specific antibody or antigen, as well as a substrate that is added after the antigen-antibody reaction. This substrate is acted on (usually hydrolyzed) by the enzyme attached to the antigen-antibody complexes, resulting in a color change. The intensity of the color indicates the amount of bound antigen or antibody (Beker and Kedir, 2008). This test can detect an incomplete antibody, which is commonly seen in chronic brucellosis patients, so it is recommended for such patients (Ertek *et al.,* 2006).

The Enzyme-linked immunosorbent assay (ELISA) method offers a great opportunity for identifying all four antibody classes (IgM, IgG1, IgG2, and IgA) (Crowther, 2010). Although the ELISA technique is considered 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, variations in preparation quality, and the use of multiple endpoints make interpreting ELISA results difficult (Clavijo*et al.,* 2003). For the diagnosis of brucellosis in humans and domestic animal species, two types of immunoenzymatic tests are used: indirect ELISA (ELISAi) and competitive ELISA (ELISAc) (Di Febo *et al.,* 2012).

The indirect enzyme-linked immunosorbent assay is a highly sensitive and specific test that can be scaled up to process a large number of samples quickly. They are time and effort efficient, with sensitivity and specificity ranging from 98 to 99 percent for both serum and milk ELISA (OIE, 2004). The indirect ELISA (iELISA) method relies on the specific binding of antibodies in the test sample to immobilized antigen. Chemically or enzymatically derived fluorescent, luminescent, or colorimetric reactions indicative of the presence of antibodies in the sample are used to visualize the binding event. There are numerous iELISA tests on the market (Poester *et al.,* 2010). Because they are unable to distinguish B. abortus S19 vaccinal antibodies from cross-reacting antibodies in areas where vaccination is not practiced, the specificity of the indirect ELISA can be slightly lower than the assay specificity (Fernando *et al.,* 2010).

The Competitive Enzyme-Linked Immunosorbent Assay (cELISA) is based on the displacement of serum antibodies by a fixed concentration of a mouse monoclonal antibody (MAb) against the common (C/Y) epitope, which is the dominant epitope in the O polysaccharides of both B.abortus and B. melitensis and is the most relevant in serological diagnosis. Because the cELISA does not require the use of a specific conjugate anti-animal species immunoglobulin, it is easily adaptable to detect Brucella infections in different animal species. This cELISA employs SLPS immobilized passively on the wall of 96-well polystyrene plates. A monoclonal antibody specific for a common epitope of OPS and test serum, both appropriately diluted, are used to compete. The monoclonal antibody can be labeled directly with an enzyme, or it can be combined with a secondary anti-mouse antibody labeled with an enzyme (Thompson *et al.,* 2009). It can tell the difference between vaccinated animals and animals infected with cross-reacting organisms, reducing the number of false-positive reactions (OIE, 2009).

3.2.4. Serum agglutination test

One of the standard serological screening tests for brucellosis is the serum agglutination test (Memish and Balkhy, 2004). It is a serological test developed by Wright and colleagues that is still the most popular and widely used worldwide diagnostic tool for brucellosis diagnosis because it is simple to perform and does not necessitate expensive equipment or training. The total amount of agglutinating antibodies, IgM and IgG, is measured by SAT. This test is based on the reactivity of antibodies to Brucella smooth lipopolysaccharide. The prozone effect causes an excess of antibodies that causes false-negative reactions, which can be overcome by serial dilution of serum samples from 1:2 to 1:64, increasing test specificity (Afify *et al.,* 2013).

The amount of specific IgG is determined by treating the serum with 0.05M 2-mercaptoethanol (2ME), which inhibits IgM agglutination. When combined with a compatible clinical presentation, SAT titers greater than 1:160 are considered diagnostic. In endemic disease areas, however, using a titer of 1:320 as a cut-off may make the test more specific. The distinction in antibody type is also important, as IgG antibodies are thought to be a better indicator of active infection than IgM antibodies, and a rapid drop in the level of IgG antibodies is said to be prognostic of successful therapy. As a result, the serum agglutination test has a low sensitivity (41%), but a high specificity (66.7%) in bovines (Akhtar *et al.,* 2010).

3.2.5. Fluorescence polarization assay

The fluorescence polarization assay (FPA) is a simple technique for measuring antigen/antibody interaction that can be done in the lab or the field. It is a homogeneous, species-independent assay in which analytes are not separated, making it a very fast test for Brucella infection diagnosis (Corbel and MacMillan, 2000). The FPA was originally designed to test serum. The technology, however, has been expanded to test whole blood and milk

samples from individual animals (Supriya *et al.,* 2010). It is based on the physical principle of the mass-dependent change in the rotation speeds of molecules in a liquid medium. The smaller the molecule, the faster it rotates, causing the depolarization of a polarized light beam. FPA involves incubating a serum sample with a specific Brucella antigen conjugated with a fluorescent label. When anti-Brucella antibodies are present in the serum, a large fluorescently labeled antigen-antibody complex forms, which can be easily distinguished from the unbound antigen negative control (McGiven *et al.,* 2003). Thus, if anti-Brucella LPS antibodies bind to a fluorescent molecule (fluorophore) conjugated to the Brucella O-chain, the rotation of the fluorophore will be slowed (Montagnaro *et al.,* 2007). If the serum contains antigen antibodies, the rate of rotation slows due to an increase in the molecular weight of the antigen-antibody complex. This decrease enables the distinction between negative and positive outcomes (OIE Terrestrial Manual, 2009).

The sensitivity and specificity of the fluorescence polarization assay range from 87.5 to 100%. (Godfroid *et al.,* 2010). It is very accurate, and the sensitivity and specificity can be adjusted by changing the cutoff value between positive and negative reactions, resulting in a very sensitive screening test as well as a highly specific confirmatory test. The test is technically simple and inexpensive because it requires only two reagents, antigen and diluent buffer. It does necessitate the use of a fluorescence polarization analyzer, of which several are available at various prices. Diagnostic kits are also commercially available from a variety of sources. The FPA can distinguish vaccinal antibodies in the majority of vaccinated animals and can also eliminate some cross-reactions (Nielsen, 2002).

The small molecular weight subunit of OPS is labeled with fluorescein isothiocyanate and used as the antigen in brucellosis serology. If an antibody to the OPS is detected in serum, blood, or milk, the rate of rotation of the labeled antigen will be slowed. The rate of reduction varies with the amount of antibody present (Montagnaro, 2008).

3.2.6. Brucellin allergic skin test

The 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, causes a local inflammatory response in a sensitized animal. This delayed-type hypersensitivity reaction is measured by an increase in skin thickness at the site of inoculation. Ten square centimeters of healthy, clean skin on the side of the neck were shaved with scissors or electric clippers. 100 l of brucellin was injected intradermally with a tuberculin syringe and a 4 mm needle, and the reaction was measured three days later. The primary technique for assessing the reaction was palpation of the injection site. The diameter of the swelling was used to quantify the visible and/or palpable reaction. The difference in skin thickness at the injection site was compared to a fold of healthy skin adjacent to the site using an Aesculap spring meter. A positive reaction would be skin thickening of 1.5–2 mm (Saegerman *et al.,* 1999).

The brucellin skin test has such a high specificity that serologically negative unvaccinated animals that respond positively to the brucellin test should be considered infected (Neilsen and Yu, 2010a). As a result, this test could be used as a confirmatory test on cattle that have not been immunized against brucellosis. For these reasons, this test should not be used as the sole diagnostic test or for international trade. The OIE recommends this test as an alternative (OIE, 2009).

3.2.7. Rivanol plate test

The test is designed to eliminate non-specific reactions based on the precipitation of high molecular weight serum glycoprotein from serum solutions, which in this case is primarily IgM, leaving mostly IgG in the serum (Monasser *et al.,* 2011). To precipitate the precipitate, an acridine dye such as rivanol (2-ethoxy-6, 9-diaminoacridine lactate) is used, and the precipitate is removed by centrifugation. A rapid plate agglutination test with undiluted serum or a tube test with serum dilutions of 1:25, 1:50, 1:100, and 1:200 are used to test the supernatant. Because of their time-consuming protocols, precipitation tests are typically used as confirmatory tests (Poiester *et al.,* 2010).

3.2.8. Antiglobulin or Coomb's test

The Coomb's test is the most appropriate and sensitive test for confirming relapsing patients with chronic disease (Supriya *et al.,* 2010). The Coombs test is useful for detecting antibodies, such as IgG blocking in chronic disease patients (Galinska and Zagorski, 2013). False-negative SAT results are caused by a block on an antibody or the Prozone phenomenon, and thus the Coombs test is an ideal method to overcome this problem. The SAT is the most reliable in brucellosis diagnosis; however, in some patients with obvious clinical symptoms and negative SAT results, the Coombs and enzyme-linked immunosorbent assay (ELISA) methods are preferable (Serra and Vinas, 2004).

3.2.9. Native hapten and poly B tests

Native hapten and poly B tests are confirmatory tests that have been used successfully in an eradication program in conjunction with the RBT as a screening test (Carrasco, 1995). Conjunctival vaccination (both in children and adults) shortens the time required to obtain a negative response in native hapten tests. A notable feature of the radial immunodiffusion test is that a positive result correlates with Brucellashedding in experimentally infected

cattle and naturally infected cattle undergoing antibiotic treatment (Joint FAO/WHO Expert Committee on Brucellosis, 1986). Precipitin tests with native hapten or Brucella cytosol proteins have also been shown to eliminate FPSR reactions caused by Yersinia enterocolitica O:9 and FPSR of unknown origin in the majority of cases (Munoz *et al.,* 2005).

4. TEST PERFORMANCE AND COST COMPARISON OF SEROLOGICAL TESTS

The available serological tests for bovine brucellosis perform differently under different conditions. The diagnostic sensitivity and specificity of an assay are indicators of its diagnostic performance, as defined in the OIE Terrestrial manual as follows: The proportion of samples from known infected reference animals that test positive in an assay is referred to as diagnostic sensitivity. The proportion of samples from known uninfected reference animals that test negative in an assay is referred to as diagnostic specificity (OIE, 2013).

The host's stage of infection or immunity, as well as the combination of cases tested, may influence sensitivity in the individual animal. Depending on the purpose of the testing, maternal antibodies, antibody persistence after recovery, or vaccination may affect diagnostic specificity. The sensitivity can be calculated by dividing the true positives by the sum of true positives and false negatives. There are several methods for determining an assay's diagnostic performance. Diagnostic sensitivity and specificity are typically determined by testing samples from animals with known status (Greiner and Gardner, 2000). Another method for estimating diagnostic sensitivity and specificity in the absence of true disease status necessitates the use of complex statistical concepts and formulas. It is based on the use of two tests, where one test's diagnostic sensitivity is imperfect but known, or where the sensitivity and specificity of both tests are unknown (Enoe *et al.,* 2000).

The complement fixation test (CFT) is more diagnostically specific than the SAT and has a standardized unit system. Some enzyme-linked immunosorbent assays and the fluorescence polarization assay (FPA) have diagnostic performance characteristics comparable to or better than the CFT, and their use may be preferred because they are technically simpler to perform and more robust (Wright *et al.,* 1997). When compared to the SAT and ELISA, the SAT has higher sensitivity and specificity. ELISA is also said to be the most sensitive test for brucellosis of the central nervous system. The FPA has nearly identical diagnostic sensitivity and specificity for bovine brucellosis to the cELISA. The ELISA appears to be the most sensitive of the newer serologic tests. However, more experience is required before it can replace the SAT as the preferred test for brucellosis (Almunneef and Memish, 2003).

Traditionally, screening tests are low-cost, quick, and highly sensitive, but not always highly specific. Confirmatory tests must be sensitive as well as specific. Due to differences in sensitivity and specificity of serological tests, it is clear that no single test is capable of identifying all positive cases of Brucella-infected animals (Salama *et al.,* 2011).

The following table summarizes the sensitivity and specificity ranges for commonly used serological tests. These figures were compiled from a variety of sources in the literature (Nielsen, 2002). By adding the sensitivity and specificity values, the Performance Index provides an overall estimate of the test's accuracy. The Min and Max values in Table 1 represent the lowest and highest indexes, respectively.

The sensitivity, specificity, and performance index of commonly used bovine brucellosis serological tests are shown in Table 1.

Serological Test	% Sensitivity	% Specificity	Performance Index (Min-Max)
SAT	29.1 – 100	99.2 – 100	128.3 - 200
RBT	21.0 - 98.3	68.8 – 100	89.8 - 198.3
BPAT	75.4 - 99.9	90.6 – 100	166.0 - 199.9
RIV	50.5 – 100	21.9 – 100	72.4 - 200
2ME	56.2 – 100	99.8 – 100	156.0 - 200
CFT	23.0 - 97.0	30.6 – 100	53.6 - 197.0
iELISA	92.0 – 100	90.6 – 100	182.6 - 199.8
cELISA	97.5 – 100	99.7 - 99.8	197.3 - 199.8
FPA	99.0 - 99.3	96.9 – 100	195.9 - 199.3

Source: (Nielsen, 2002): Based on a meta-analysis of sensitivity (Se) and specificity (Sp) values from several sources published in peer-reviewed journals, especially journals of animal and veterinary science,

5. CONCLUSIONS AND RECOMMENDATIONS

Due to the complicated nature of the infection and its innate and acquired immune response, the use and understanding of various serological tests for the diagnosis of bovine brucellosis under various circumstances are required. The most commonly used tests for the serological diagnosis of bovine brucellosis are the Rose Bengal (RB) and complement fixation (CF) tests. There is no single serological test that is applicable in all epidemiological situations; they all have limitations, particularly when screening individual animals. Despite

these limitations, serologic diagnostic tests for bovine brucellosis in cattle are extremely useful. In all cases, a blood sample should be collected from the patient and laboratory testing should be requested, as brucellosis cannot be diagnosed without laboratory confirmation. For this review, the serological methods described represent standardized and validated methods with appropriate performance characteristics to be designated as prescribed or alternative tests for international trade. This does not preclude the use of modified or similar test methods, as well as the use of various biological reagents. The solution to the problem of accurate serological diagnosis of bovine brucellosis will almost certainly involve several tests for different functions of the host immune response.

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