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Review

A review on diagnostic techniques for brucellosis

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Brucellosis presents with many clinical manifestation that make its diagnosis a difficult task. Ever since the report of the first serologic test for brucellosis, a definitive diagnostic technique has been actively pursued. The most widely used methods of diagnosis are based on serology, which measures the ability of the serum (antibody) to agglutinate a standardised amount of killed *Brucella abortus* (antigen) containing O-side chain. These tests are most commonly used because they are safe. However, they are prone to false-positive results due to other cross-reacting bacteria, and also, they are not useful in the detection of *Brucella canis* and *Brucella ovis* which lack the O-side chain. Other useful tests include the direct smear examination which is a presumptive method that involves making smears from vaginal swabs, placentas or aborted foetuses, stained with the stamp modification of the Ziehl-Neelsen method. Morphologically related micro-organisms such as *Chlamydia psittasi, Chlamydophila abortus* or *Coxiella burnetti* can mislead the diagnosis, therefore, confirmation on appropriate culture and selective media is recommended. Culture and isolation of the organism from blood or tissue samples has remained the only "unequivocal" method but lacks sensitivity, and its outcome depends on individual laboratory practices, and how actively the obtaining of cultures is pursued. Laboratory animal inoculation has also been a useful tool, but is also subject to interference with gastric acids. More recently, the polymerase chain reaction (PCR) has been found to be a useful and more sensitive test, but has not been validated for standard laboratory use. This paper highlights useful samples and, especially the different conventional to more sophisticated molecular techniques for the diagnosis of brucellosis.

Key words: Brucellosis, diagnosis, techniques.

INTRODUCTION

Brucellosis is an infectious disease caused by bacteria of the genus *Brucella*. It affects many mammalian species and is transmissible to humans, thus giving it an important socio-economic impact. Making a diagnosis of brucellosis may be difficult because of the unspecific symptoms and signs shared with other febrile illnesses, slow growth rate of the causative agent in blood culture, and the complexity of its sero-diagnosis (Colmenero et al., 1990; Memish et al., 2000; Al Dahouk et al., 2003). Presumptive diagnosis of brucellosis can be made by the use of several serological tests to *Brucella* antibodies, but the "gold standard" remains isolation and identification of

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Abbreviations: CFT, Complement fixation test; **OPS,** O-polysaccharide; **S-LPS,** smooth lipopolysaccharide; **MRT,** milk ring test; **SAT,** serum agglutination test; **SPAT,** standard plate agglutination test; **2-MET,** 2-mercaptoethanol test; **BPAT,** buffered antigen test; **RBPT,** Rose Bengal plate test; **IFAT,** immune-flourescent test; **HIT,** heat inactivation test; **EDTA,** ethylenediaminotetracetic acid; **RPT,** Rivanol plate test; **PCR,** polymerase chain reaction.

the bacterium. Isolation and identification of the bacterium was first reported by Bruce and co-workers, when they isolated *Brucella melitensis* from military personnel in Malta (Bruce, 1887). However, cultural examinations are time-consuming, hazardous and not sensitive. Despite the vigorous attempt for more than one century to come up with a definitive diagnostic technique for brucellosis, diagnosis still relies on the combination of several tests to avoid false negative results (Poiester et al., 2010).

AGGLUTINATION TEST

The first agglutination test for the detection of antibody to *Brucella* infection was reported by Wright and Smith over 100 years ago (Nielsen, 2002; Pabuccuoglu et al., 2011). In this test, a mixture of bacterial cell antigens and human patient serum was incubated in a glass tube. If a “mantle” pattern of cell sediment was observed, it was considered positive, while a “button” pattern was considered as negative. The test which is still in use today is based on a similar concept, except that only *Brucella abortus* cells are used as the antigen (Nielsen and Yu, 2010).

Since then, a great deal of work has been done to improve diagnostic methods and accuracy, which has lead to the production of primary-binding assays (Gall and Nielsen, 2004; Poiester et al., 2010). The primary-binding assays directly measure the interaction of antibody and antigen, while conventional serological tests, such as acidified agglutination tests or the complement fixation test (CFT), measure secondary phenomena including the aggluti-nation or activation of complement (Nielsen et al., 1996). The *Brucella* organism contains O-polysaccharide (OPS) on its cell surface, which is part of the Smooth lipopolysaccharide (S-LPS) and has been chemically defined as a homopopymer of 4, 6-dideoxy-4-formamide-alpha-D-mannose, linked via 1, 2-glycosidic linkages (Bundle et al., 1987). *Brucella ovis* and *Brucella canis* have no measurable OPS on their cell surface (Nielsen, 2002). The three main *Brucella* species, namely: *B. abortus*, *B. melitensis* and *Brucella suis* have common epitopes in their S-LPS. Thus, they can be diagnosed using whole antigen or S-LPS prepared by chemical extraction of *B. abortus* (OIE, 2009). *B. abortus* antigen has also been used in the serological diagnosis of *Brucella neotome,* found in wood rats and brucellae of marine animals (Godfroid et al., 2010).

The serological tests capable of detecting the S-LPS are the most sensitive for detecting cattle and small ruminant brucellosis, but they may yield false positive results, if the animals have been previously vaccinated or exposed to Gram-negative bacteria with LPS O-chains similar to those of brucellae (Godfroid et al., 2002; See et al., 2012; Adone and Pasquali, 2013). These bacteria include *Vibrio cholerae* O1, *Escherichia coli* O: 157, some strains of *Escherichia hermannii* and *Stenotrophomonas maltophilia*, *Salmonella* group N (O: 30), and *Yersinia enterocolitica* O: 9. However, only *Yersinia enterocolitica* O: 9 is a significant cause of false-positive serological reaction in the diagnosis of brucellosis (Gerbier et al., 1997; Otto et al., 2000).

The immune response to *B.abortus* in cattle, which has been most studied in details is the IgM isotype. The time of appearance of antibodies depends on the infecting dose of the *Brucella*, route of exposure, and the immune status of the host animal (Beh, 1973, 1974; Allan et al., 1976; Neilsen et al., 1984). It is immediately followed by production of IgGI isotype, then subsequently with small quantities of IgG2 and IgA (Diaz et al., 2011). Immune response due to other Gram-negative bacteria with LPS O-chains, similar to those of brucellae is mainly the IgM isotype. This fact makes assays that detect IgM isotype prone to false positive results leading to low assay sensitivity, and those that detect IgG1 most useful (Neilsen, 2002). In addition to cross-reacting Gram-negative organisms, vaccinal antibodies due to *B. abortus* S19 are capable of being detected by serological tests, thus resulting in false positive. This problem can be checked by vaccinating calves at three to eight months of age (calf-hood vaccination); which gives time for the antibody level to wane before maturity, and thereby preventing diagnostic problems. Despite the calfhood vaccination, some animals maintain a high antibody titre at adulthood. This problem has lead to the development of improved serological tests, like competitive enzyme immunoassay and fluorescence polarisation assay (Neilsen, 2002), and a live vaccine which contains no OPS, that is, the *B. abortus* RB51, obtained by Schurig (Poiester et al., 2010).

USEFUL SPECIMEN FOR THE DIAGNOSIS OF BRUCELLOSIS

For the diagnosis of brucellosis, the organism may be recovered from a variety of materials which usually depends on the presenting clinical signs (OIE, 2009). In animals, the placenta is the most infective and contains the greatest concentration of bacteria; this is followed by the lymph nodes and milk; and from blood in humans (Poiester et al., 2010). Furthermore, other materials rich in the organism include: Stomach contents, spleen and lungs from aborted foetuses, vaginal swabs, semen, and arthritis or hygroma fluids from adult animals. From animal carcasses, the preferred tissues for culture are the mammary gland, supramammary, medial and internal iliac, retropharyngeal, parotid and prescapular lymph nodes and spleen (OIE, 2009; Ahmed et al., 2010). All specimens must be packed separately, cooled and transported immediately to the laboratory in leak proof containers. For humans, blood for culture is the material of choice, but specimens need to be obtained early in the disease. The samples should be frozen until required for culture (OIE, 2009). There is no ideal tissue for the isolation of *Brucella* from marine mammals, unless gross

lesions are found in the tissues. However, the recommended tissues for the recovery of *Brucella* in marine mammals are the spleen, the mammary gland, the mandibular, gastric, external and internal iliac and colorectal lymph nodes, the testes and blood (Foster et al., 2002).

DIRECT SMEAR MICROSCOPIC EXAMINATION

Marin et al. (1996) reported that a presumptive bacteriological diagnosis of *Brucella* can be made by means of the microscopic examination of smears from vaginal swabs, placentas or aborted foetuses, stained with the Stamp modification of the Ziehl-Neelsen staining method. However, morphologically-related micro-organisms, such as *Chlamydophila abortus, Chlamydia psittaci* and *Coxiella burnetti* can mislead the diagnosis because of their superficial similarity (Marin et al., 1996; Poiester et al., 2010). Accordingly, the isolation of *B. melitensis* on appropriate culture media such as Farrell's selective media is recommended for an accurate diagnosis (Farrell, 1974). Vaginal swabs and milk samples are the best samples to use in isolating *B. melitensis* from sheep and goats (Marin et al., 1996).

Cultural isolation of *Brucella* organism

This procedure may be performed by culturing body tissues or secretions like blood, milk and virginal discharge (Poiester et al., 2010). Higher sensitivity and faster culture times may be achieved in patients with previous antibiotic intervention, when the bone marrow is cultured (Mantur et al., 2006). *Brucella* species can also be cultured from pus, cerebro-spinal fluid, and pleural, joint and ascitic fluids. Growth of the bacteria in culture media is an unequivocal proof of infection (OIE, 2009a; Poiester et al., 2010). Blood cultures are useful only in animales with bacteraemia, which may not always occur. However, milk has often been found to contain *Brucella* by this test. Samples like lymph nodes, liver, spleen, udder and other organs at post-mortem can present positive culture results with negative serological tests. In this respect, the culture test has been widely used in research.

The identification of *Brucella* species in culture depends on a great deal of phenotypic traits such as: CO_2 requirement, phage typing and biochemical tests, which, among other problems, involve time, bio-safety, trained personnel and somewhat ambiguous results (Bricker, 2002). Broth or agar can be prepared from powder media for culture of *Brucella* organisms. Due to the low *Brucella* load in the blood and other body fluids, broth or a biphasic medium are preferable for their culture.

However, for other specimens, solid media with 2.5% agar facilitates the recognition of colonies and discourage bacterial dissociation (Poiester et al., 2010). Optimum pH for growth of *Brucella* varies from 6.6 to 7.4, and culture media should be adequately buffered near pH 6.8 for optimum growth. The optimum growth temperature is 36-38°C. However, most strains grow between 20 and 40°C (European Commission, 2001).

Most *Brucella* strains, particularly *B. abortus* biovar 2 and *B. ovis*, grow better in media containing 5-10% of sterile (equine or bovine) serum, free from *Brucella* antibodies. To avoid the growth of contaminants which is mostly the case with field samples, selective media should be used. The most widely used selective media are the Kuzdas and Morse (Kuzdas and Morse, 1953) and the Farrell's media (Farrell, 1974). The Kuzdas and Morse use the following antibiotics and quantities per liter of basal medium: 100 mg of cycloheximide (fungistat), 25,000 units of bacitracin (active against gram-positive bacteria) and 6,000 units of polymyxin B (active against Gram-negative bacteria). The Farrell´s medium is prepared by the addition of the following antibiotics and quantities per liter of basal medium: bacitracin (25 mg), polymyxin B sulphate (5 mg), nalidixic acid (5 mg), nystatin (100,000 units), vancomycin (20 mg), natamycin (50 mg).

As Farrell´s medium is rather inhibitory for some strains of *B. abortus*, *B. melitensis*, and *B. ovis*, a modified Thayer-Martin medium may be used together with Farrell's to enhance the growth of these species. This medium can be prepared with GC medium as basal medium supplemented with 1% haemoglobin and the following antibiotics per litre of medium: Colistin methanesulphonate (7.5 mg), vancomycin (3 mg), nitrofurantoin (10 mg), nystatin (100,000 units) and amphotericin B (2.5 mg) (Marin et al., 1996).

Laboratory animal inoculation

Mice have been reported to be the animal model most frequently used in brucellosis research (Mense et al., 2001; Silva et al., 2011). Nevertheless, it has been reported that guinea pigs are also susceptible and can be used (Avong, 2000; Ocholi, 2005; OIE, 2009a). Animal inoculation may be either subcutaneously or through abraded skin in guinea-pigs or, preferably, intravenously, intraperitoneally, or through the digestive tract or nasal (aerosol) routes in mice (OIE, 2009; Silva et al., 2011). The spleen of mice is cultured 7 days after inoculation, while serum samples of guinea pigs are subjected to specific tests 3 and 6 weeks after inoculation (OIE, 2009a). It is noteworthy however, that gastric acid can interfere with the infectivity of *Brucella* in laboratory animals (Silva et al., 2011).

USE OF SEROLOGY IN THE DIAGNOSIS OF BRUCELLOSIS

Body fluids such as serum, uterine discharge, vaginal mucus, milk, or semen plasma from a suspected animal

may contain different quantities of antibodies of the M, G1, G2, and A isotypes directed against *Brucella* (Beh, 1974). 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 (FAO, 2005). In addition, depending on the sensitivity and specificity, serological tests can be used to screen for, or confirm brucellosis.

Traditionally, screening tests are inexpensive, fast and highly sensitive, but most of the time, lack specificity. However, confirmatory tests are required to be both sensitive and specific, thereby eliminating some false positive reactions. Most confirmatory tests are more complicated and more expensive to perform than the screening tests (Diaz et al*.,* 2011). The commonly used serological tests include milk ring test (MRT), serum agglutination test (SAT), standard plate agglutination test (SPAT), complement fixation test (CFT), 2-mercapto-ethanol test (2-MET), buffered antigen test (BPAT), and rose Bengal plate test (RBPT). Others include the card test (CARD), Rivanol test, Coombs test, indirect immune-flourescent test (IFAT), heat inactivation test (HIT), skin test, immune-assay and molecular biology technique.

MILK RING TEST (MRT)

The MRT is basically a rapid agglutination test carried out on whole milk or cream. Haematoxylin stained *Brucella* cells are added to whole milk and incubated for reaction to take place (McCaughey, 1972; Hubber and Nicoletti, 1986). Immunoglobulins present in the milk will, in part, be attached to fat globules *via* the Fc portion of the fat molecule (Poiester et al., 2010). The immunoglobulins detected by MRT are IgM and IgA. This test may be applied to individual animals or to pooled milk samples using a larger volume of milk, relative to the pool size (MacMillan et al., 1990). 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 (Al-Mariri and Haj-Mahmoud, 2010). False negatives may also occur in milk with a low concentration of lacteal antibodies or lacking fat-clustering factors (Bercovich, 1998). In spite of these problems, the MRT has been found to be extremely effective, and is usually the method of choice in dairy herds, and may be used as an inexpensive screening test in conjunction with other tests (Corbel, 2006).

SERUM AGGLUTINATION TEST

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 (Afify et al., 2013). The test is performed at a near neutral pH, which makes it more efficient in detecting IgM antibody. Hence, it is best used to detect acute infections. It is less effective for IgG, resulting in low assay specificity (Corbel, 1972; Nielsen et al., 1984). Due to this fact, the SAT, despite being sensitive, is generally not used as a single test, but rather it is used in combination with other tests. Other short comings of the test include false positive and false negative results (Poiester et al., 2010). For this reason, the test is only suitable for herd testing, rather than for testing individual animals. Furthermore, the presence of post-vaccinal antibody can confuse the results (Corbel and Brinley-Morgan, 1984) http://en.wikipedia.org/wiki/User:Dgrtvwnp - cite_note-308. The SAT does not detect antibodies to *B. canis and B. ovis* because these rough strains of the organism do not have OPS on their surfaces (Ndyabahinduka and Chu, 1984; Poiester et al., 2010).

Standard plate agglutination test (SPAT)

The SPAT was standardised to give similar results with the SAT titre of 1:100 (positive), but does not use series of dilutions like the SAT. Stemshorn et al. (1985) http://en.wikipedia.org/wiki/User:Dgrtvwnp - cite_note-311 reported that, due to the use of high saline (8%) and higher serum concentrations in this test, which makes it resistant to the prozone effect, this test can give positive results when SAT is negative.

Serum agglutination test with ethylenediaminotetracetic acid (SAT-EDTA)

An adaptation of the SAT, which involves the addition of EDTA, has proven to significantly increase the test specificity by reducing the chances of cross-reactions, it can, therefore, be used as an alternative to routine SAT (Nasir et al., 2005; Poiester et al., 2010; Kaltungo et al., 2013). The mechanism by which EDTA reduces non-specificity is not well understood; however, it is postulated that, it eliminates attachment of immunoglobulins to the *Brucella* cell wall via the Fc piece (Neilsen and Yu, 2010). This test works on the principle that the pH of the serum is altered to the isoelectric point of IgM to prevent its agglutination.

Buffered plate agglutination test (BPAT)

BPAT is a standardized agglutination test that is simple and inexpensive to perform. It uses antigen at pH of 3.65 which is made from *Brucella abortus* S119.3 whole cells, and stained with crystal violet and brilliant green dyes. This test is liable to false positives results due to vaccinal antibodies and prozoning effect (Nielsen, 2002). The main advantage of this test is the reduction of non-specific

test reactions. It is directed at testing for IgG (Corbel, 1972, 1973; Allan et al., 1976; Angus and Barton, 1984).

Rose bengal plate test (RBPT)

The RBPT is a spot agglutination technique which is also known as the card test or buffered *Brucella* antigen test (Stemshorn et al., 1985). It uses a suspension of *B. abortus* smooth cells stained with the Rose Bengal dye, buffered to pH 3.65. At neutral pH, this test can measure the presence of IgM, IgG1 and IgG2. However, IgM appears to be the most active. At the buffered pH of 3.65, RBPT, prevents agglutination with IgM, and apparently, measures only IgG1 (Corbel, 1972). It was considered that while the test gave few false negative results, it gave many false positives, possibly due, in significant part, to reaction with IgM in animals with previous vaccination. In situations where vaccination is not routinely conducted, the use of this test can give a good record of exposure of animals to *Brucella* organisms.

It is an internationally recommended test for the screening of brucellosis in small ruminants, but lacks standardisation of the antigen. Low pH of the antigen enhances the specificity of the test, while the temperature of the antigen and the ambient temperature at which the reaction takes place may influence the sensitivity and specificity of the test (Alton, 1981; Macmillan et al., 1990). Corbel (1972) observed that the sensitivity of the test was associated with frac-tions containing immuno-globulin IgG, especially the IgG1.

Rivanol plate test (RPT)

The test is 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 (Poiester et al., 2010; Montasser et al., 2011). Acrydine dye such as rivanol (2-ethoxy-6, 9-diaminoacridine lactate) is used to achieve the preci-pitation 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 (Nielsen, 2002; Poiester et al., 2010). The test is capable of distinguish-hing between vaccinated and infected animals, and also chronic carriers. Interpretation of the test is, however, difficult (Abdulkadir, 1989).

Complement fixation test (CFT)

The CFT detects mainly the IgG1isotype antibody, as the IgM isotypes are partially destroyed during the inactivation process. Since antibodies of the IgG1 type usually appear after antibodies of the IgM type, control and surveillance of this disease is best done with SAT and CFT (WHO/MZCP, 1998). The test shows good correlations with the recovery of *Brucella* organisms from artificial recovery or naturally-infected animals (Madsen, 1994). Although the test is fast and accurate, it does not allow for discrimination between antibodies due to infection from vaccinal antibodies (Nielsen, 2002; Poiester et al., 2010). Other problems include large number of reagents and controls needed to carry out the test. Furthermore, each time the assay is set up, a large number of titrations are needed, and interpretation of the results is subjective due to differences in techniques (Madsen, 1994). Occasionally, there is direct activation of complement by serum (anti-complementary activity) and the inability of the test to be amenable for use with haemolysed serum samples. The laborious nature of this test and the requirement of highly- trained personnel and suitable laboratory facilities make the CFT less suitable for use in developing countries (FAO, 2005). The CFT may also test false negative, when antibodies of the IgG2 type hinder complement fixation (Nielsen et al., 1988; MacMillan et al., 1990). Despite these inherent problems, the CFT is a widely used test, and has been regarded as the most specific and accepted serological test for diagnosis of brucellosis. Thus, it is a recommended test for international trade (OIE, 2009).

The 2-mercaptoethanol test

The 2-MET is an adaption of the SAT titre. There are two forms of this test, which uses either 2-mercaptoethanol (Rose and Roepke, 1964) or dithiothreitol (Klein and Behan, 1981). Dithiothreitol is preferable because of the toxicity of 2-mercaptoethanol. 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 (Poiester et al., 2010). 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 programmes (Nielsen, 2002).

Anti-globulin (Coombs) test

The Coombs test is used to confirm SAT result from animals that give negative, suspicious or non-conclusive responses (Farina, 1985). It is a useful test in the epide-miological survey of brucellosis because of the advan-tage of detecting incomplete antibodies of the IgG types that combine with cellular antigens, but do not give rise to

an agglutination reaction (MacMillan et al., 1990; WHO/MZCP, 1998). The test has been adapted to micro-titer plate set-up to save time (Otero et al., 1982). The main limitation of the test is that, it is not recommended for testing vaccinated animal (Farina, 1985; MacMillan et al., 1990).

Heat inactivation test

In an experimental set-up, the HIT is very sensitive at early stages of infection. The test is based on the observation that two types of *Brucella* agglutinins IgM and IgG are found and can be differentiated on the basis of stability at 65°C for 15 min, and cooled to 18°C. The agglutinins that were not inactivated at this temperature are regarded as positive for *Brucella*. The test is particularly important within the first sixty days of infection (Manthei, 1964).

Whey agglutination test

This test has been an important test in detecting sero-logically negative or suspect animals with udder infection. These animals usually are chronically or recently infected animals in herds, from which it has been difficult to eradicate brucellosis (Stiles et al., 1958; Cameron, 1959; Biberstein et al., 1961).

The skin test

The skin test uses a protein antigen derived from *Brucella* (brucellergene or brucellin) (Godfroid et al., 2010). Brucellosis is capable of eliciting both cellular and anti-body mediated responses in the host; the brucellin skin test should therefore be considered in cases of false positive serological reactions. The test has a very high specificity such that latently-infected animals devoid of measurable antibody, and unvaccinated animals that are positive reactors to this test, should be regarded as infected (Pouillot et al., 1997; Saergerman et al., 1999; Neilsen and Yu, 2010). Therefore, results of this test may aid in the interpretation of serological reactions thought to be false positive serologically due to infection with cross-reacting bacteria, especially in brucellosis-free areas (Pouillot et al., 1997; Saegerman et al., 1999; EC 2001; De Massis et al., 2005).

However, more recently, both *B. abortus*-vaccinated animals and animals infected with cross-reacting microor-ganisms gave positive reactions to skin tests for a period of time. Bercovich (1999) reported that, the skin test should be the test of choice in developing countries, as cattle in these countries are usually not tagged, so that serological test results could be related to the individual animal. The test can be relied upon for clinical survey-llance and epidemiological surveys (FAO/WHO, 1986; EC 2001). It is of great importance in areas with low pre-valence, and in areas known to be brucellosis-free (Bercovich, 1998). The test involves injecting brucellin into the flank, or intrapalpebrally, after which the skin thickness is measured (Weildman, 1991; Cheville et al., 1994). Not all infected animals react, therefore, this test alone cannot be recommended as the sole diagnostic test, or for the purposes of international trade (OIE, 2009). Similarly, it was reported by Cutler et al. (2005) that the specificity of the test is reduced following vaccination, and the necessity for two farm visits, delay between repeat tests, and subjective nature of result interpretation, make this type of test impractical for effective diagnosis.

Primary binding assays

Conventional methods of brucellosis diagnosis have limitations, which led to the development of primary binding assay techniques. These tests are capable of rapidly and accurately detecting humoral antibodies to *Brucella* (Poiester et al., 2010). Vaccination induces antibody thought to be of lower affinity due to a short exposure time to the antigen because it is eliminated by the immune system. Alternatively, antibody produced in response to natural infection is of higher affinity because the antigen is not removed quickly by the immune system and, therefore, persists for a much longer period (Nielsen et al., 1989).

Thus, the competitive enzyme-linked immu-nosorbent assay (cELISA) and the fluorescent pola-risation assay (FPA) were developed to overcome this problem. These tests are capable of distinguishing vacci-nated animals or animals infected with cross-reacting organisms such as *Salmonella urbana* O: 30, *Escherichia coli* O: 116 and O: 157, and *Yersinia enterocolitica* serotype 9 from naturally-infected animals. This capability of the test makes it possible for giving reduced the number of false-positive reactions, and subsequent trace backs, or slaughter of animals in an otherwise negative or healthy population (Gall and Nielsen, 2004).

Lateral flow assay (LFA)

The LFA is a simplified ELISA for the qualitative detection of antigen specific antibodies in serum, milk or whole blood samples (Christopher et al., 2010). The assay is based on the binding of specific antibodies to antigen immobilised on a test strip (cellulose membrane matrix). It allows the detection of specific IgM as well as specific IgG antibodies and that a high sensitivity is assured for all stages of the disease (Nielsen and Yu, 2010).

Application of the assay does not require specific expertise, equipment or electricity, and test kits may be kept in stock without the need for refrigeration, thus, making the assay a very useful one for poor resource countries including most African countries and migratory

herds/flocks (Abdoel et al., 2008, Baddour, 2012). However, its interpretation is subjective, depending on the formation of a visible coloured line of reaction, and the assay itself tends to be expensive because of the multiple ingredients/components involved (Nielsen and Yu, 2010).

Fluorescence polarization assay (FPA)

The FPA is based on the fact that, when polarised light excites fluorescent molecules, they will emit polarised light. In solution, the polarisation of the emitted light is inversely proportional to the molecule's rotational speed, which is influenced by the solution's viscosity, absolute temperature, molecular volume and the gas constant (Poiester et al., 2010).

In brucellosis serology, small molecular weight subunit of OPS is labelled with fluorescein 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 labelled antigen will be reduced at a rate which is proportional to the amount of antibody present (Muma et al., 2007). The FPA is very accurate, and the sensi-tivity/specificity can be manipu-lated by altering the cut-off value between positive and negative reactions to provide a highly sensitive screening test as well as a highly specific confirmatory test. The FPA can distinguish vaccinal antibody in most vaccinated animals, and it can as well eliminate reactivity by some cross-reacting antibodies (Nielsen and Yu, 2010).

Competitive immunoassays

The competitive enzyme immunoassays were developed in order to eliminate some, but not all of the problems arising from residual vaccinal antibody, and from cross-reacting antibodies, the assays are carried out by selecting a monoclonal antibody with slightly higher affinity for the antigen than most of the vaccinal/cross-reacting antibody, but with lower affinity than antibody arising from infection (Munoz et al., 2005; OIE, 2009; Poiester et al. 2010). The specificity of the competitive enzyme immunoassay is very high and is able to detect all antibody isotypes (IgM, IgG1, and IgG2 and IgA) (Nielsen, 2002). However, it is slightly less sensitive than the indirect enzyme immunoassay. This assay is an outstanding confirmatory assay for the diagnosis of brucellosis in most mammalian species.

Molecular biology techniques

The polymerase chain reaction (PCR) is a recent and promising technique that allows for rapid and accurate diagnosis of brucellosis without the limitations of conventional methodology (Baddour, 2012). Several genus-specific PCR systems using primer pairs that target 16SRNA sequences and genes of different outer membrane proteins have been developed (Zerva et al, 2001; Queipo-Ortupo et al., 2005). The first brucellosis PCR-based test was introduced in 1990 (Fekete et al., 1990). The first species-specific multiplex PCR was called *Abortus-Melitensis-Ovis-Suis* (AMOS-PCR) assay, which is used to identify and differentiate *B. abortus* biovars 1, 2 and 4, *B. melitensis*, *B. ovis* and *B. suis* biovar 1. The PCR is based on the polymorphism arising from species-specific localisation of the insertion sequence IS711 in the *Brucella* chromosome (Bricker and Halling, 1994). This PCR technique has a disad-vantage of not being able to identify some species like *B. canis* and *B. neotomae*.

Furthermore, some biovars within a given species gave negative results (Nielsen and Yu, 2010; Scholz and Vergnaud, 2013). A Bruce-ladder multiplex PCR assay was also developed for identi-fication and differentiation of *Brucella* species and vaccine strains in a single step (Weiner et al., 2011). The PCR was further enhanced to identify the marine strains like *B. microti* and *B. inopinata*. However, it does not differentiate at the biovar level, or below (Lopez-Goni et al., 2008; Hubber et al., 2009; Mayer-Scholl et al., 2010). More recently, a multiplex PCR assay (suis ladder) was developed to differentiate among *B. suis* biovars 1 to 5 (López-Goñi et al., 2011).

Other tests for brucellosis

Urinalysis may likely demonstrate a sterile pyuria similar to tuberculosis while arthrocentesis can be performed for septic arthritis. The joint aspirate can demonstrate an exudative fluid with low cell count and predominance of mononuclear cells. Radiographic evaluations in infected animals may reveal evidence of acute or chronic *Brucella* leptomeningitis, subarachnoid haemorrhage or cerebral abscess following cranial radiography.

Similarly, echocar-diography can also be used to eva-luate possible endo-carditis. Mycotic aneurysms of the aorta or carotids may be observed on duplex arterio-graphy. Furthermore, bone marrow biopsy and liver biopsy may also be performed to obtain specimen for diagnosis, especially during the acute phase of the disease (Maloney and Fraser, 2006).

CONCLUSION

Definitive diagnosis of brucellosis remains a difficult task. The only finite diagnosis, which is the 'gold standard', is the recovery of the causative agent from the host. However, it is associated with some problems: low sensitivity, cost and danger due to laboratory infection of personnel. Indirect testing of anti-*Brucella* spp. antibodies in serum, milk and other clinical specimen are routinely

used in brucellosis control and eradication programmes. These tests have, however, been shown to be inconclusive, leading to culling of *Brucella*-free animals and subsequent economic loss. This makes a complete eradication programme a difficult task. Thus, the specificity of the test employed is of paramount importance. Most likely, the solution to the problems with accurate diagnosis will involve several tests for different functions of the immune response. Molecular biology with random or selected primers as a diagnostic tool is advancing with promising results, and may soon be at the point of replacing actual bacterial isolation. It is rapid, safe and cost-effective, the only real problems, being some uncertainties regarding specificity. Standardisation and further evaluation are therefore, needed, especially in chronic cases.

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