



Original Research Article

<https://doi.org/10.20546/ijcmas.2020.910.161>

Epidemiology and Diagnosis of Brucellosis in Large Ruminants

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A B S T R A C T

Keywords

Brucellosis, Cattle, Human, Serological and molecular methods

Article Info

Accepted: 12 September 2020
Available Online: 10 October 2020

Brucellosis is most common contagious and communicable zoonotic diseases with high rates of morbidity and life time infertility. There has been a major increase over the recent years in intra/interspecific infection rates, due to poor management problems and limited resources, especially in developing countries like India. Abortion in the last trimester is a predominant sign, which is followed by reduced milk output and pyrexia in cattle, while in humans it is characterized by undulant fever, general malaise, and arthritis. While the clinical picture of brucellosis in humans and cattle is not that clear and often misleads with the classical serological diagnosis, efforts here have been made to overcome the limitations of current serological assays through the development of PCR-based diagnosis and other diagnostic methods. Due to its complex nature, brucellosis remains a major threat to public health and livestock in developing countries. In this review, we summarized the recent literature, significant advancements, and challenges in the treatment and vaccination against brucellosis, with a special focus on developing countries.

Introduction

Brucellosis is a highly contagious and economically important bacterial disease of livestock worldwide. It is one of the five common bacterial zoonoses in the developed and developing world and is caused by organisms belonging to the genus *Brucella*: a gram-negative, non-spore-forming, intracellular bacterium. The genus *Brucella* consists of 9 species at present, of which 7, viz. *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*,

B. canis, *B. neotomae* and *B. microtiare* terrestrial (Scholz *et al.*, 2008) while *B. ceti* and *B. pinnipedialis* are marine species (Foster *et al.*, 2007). Recently, a novel species, *B. inopinata*, isolated from a wound associated with a breast implant has also been included in the genus *Brucella* (Scholz *et al.*, 2010).

The World Health Organization (WHO) laboratory biosafety manual classifies *Brucella* in Risk group III. Brucellosis is readily transmissible to humans, causing

acute febrile illness – undulant fever – which may progress to a more chronic form and can also produce serious complications affecting the musculo-skeletal, cardiovascular, and central nervous systems. Precautions should be taken to prevent human infection. Infection is often due to occupational exposure and is essentially acquired by the oral, respiratory, or conjunctival routes, but ingestion of dairy products constitutes the main risk to the general public where the disease is endemic.

It is a serious disease affecting livestock worldwide with the rates of infection varying greatly from one country to another and between regions within a country. The highest prevalence is seen in dairy cattle. In India, brucellosis was first recognized in 1942 and is now endemic throughout the country. The disease has been reported in cattle, buffaloes, sheep, goats, pigs, dogs and humans. Despite the advances made in the diagnosis and therapy, brucellosis is still wide spread and its prevalence in many developing countries is increasing. Economic losses by brucellosis in animals owing to abortions, premature births, decreased milk production and repeat breeding and may lead to temporary or permanent infertility in infected livestock. Economic losses due to brucellosis in livestock are considerable in an agrarian country like India.

Epidemiology of Brucellosis in large animals

The infection in cattle is usually caused by *B. abortus*. However, *B. melitensis* and rarely *B. suis* can also establish themselves in cattle. These infections are particularly dangerous to humans because of the high virulence of most *B. melitensis* and *B. suis* strains and due to the large numbers of bacteria that are excreted. In cattle and other *Bovidae*, Brucella is usually transmitted from animal to animal by contact following an abortion. Pasture or hay may be

contaminated and the organisms are hence most frequently acquired by ingestion. Other routes like inhalation, conjunctival inoculation, skin contamination and udder inoculation from infected milking cups are possibilities. The use of pooled colostrums for feeding newborn calves may also transmit infection. Sexual transmission usually plays little role in the epidemiology of bovine brucellosis. However, artificial insemination can transmit the disease and semen must only be collected from animals known to be free of infection. Safe and recommended embryo transfer procedures are to be followed. Transmission to people can occur through the usual routes. However, ingestion of raw or undercooked bone marrow has also been implicated as a source of human infection. In cattle, sheep, goats and swine, susceptibility to brucellosis is greatest in sexually mature animals. Young animals are often resistant, although it should be noted that latent infections can occur and such animals may present a hazard when mature. Breed may also affect susceptibility with the milking breeds seem to be the most susceptible to *B. melitensis*. Breed differences in susceptibility have not been clearly documented in cattle although genetically determined differences in susceptibility of individual animals have been demonstrated. However, management practices are far more important in determining the risk of infection. Latent or inapparent infections can occur in all farm animal species. These usually result from infection in-utero or in the early post-natal period. Such animals can retain the infection for life and may remain serologically negative until after the first abortion or parturition. Latent infection has been estimated to occur in the progeny of about 5% of infected cows. The extent of the problem in other species is not known, but latency has been documented in sheep. Acquired immunity has a substantial effect on susceptibility. In India calf-hood vaccination is practiced using *B. abortus*

strain 19. Female calves aged between 6-12 months are subjected to vaccination.

The National Control Programme on Brucellosis has been initiated since August, 2010 in India. It aims at mass screening followed by vaccination in areas of high endemicity.

Diagnosis

The information on the international standards of diagnostic tests is available from the Manual of diagnostic tests and vaccines for terrestrial animals, OIE, 2009. The diagnostic tests can be divided into 3 broad types based on the target of detection:

Identification of the agent

Staining methods

Brucella are Gram negative coccobacilli or short rods measuring from 0.6 to 1.5 μm long and from 0.5 to 0.7 μm wide. They are usually arranged singly, and less frequently in pairs or small groups. They do not form spores, and lack flagella, pili, or true capsules are not produced. They are not truly acid-fast, but are resistant to decolorisation by weak acids and thus stain red by Ziehl-Neelsen's method. This is the usual procedure for the examination of smears of organs or biological fluids that have been fixed with heat or ethanol. Brucella organisms stain red against a blue background.

A fluorochrome or peroxidase-labelled antibody conjugate based technique could also be used. However, these methods have a low sensitivity in milk and dairy products where Brucella are often present in small numbers, and interpretation is frequently impeded by the presence of fat globules. The results, whether positive or negative, should be confirmed by culture.

Culture

Collection of samples for culture

For the diagnosis of animal brucellosis by cultural examination, the choice of samples usually depends on the clinical signs observed. The most valuable samples include aborted fetuses (stomach contents, spleen and lung), fetal membranes, vaginal secretions (swabs), milk, semen and arthritis or hygroma fluids. From animal carcasses, the preferred tissues for culture are those of the reticuloendothelial system (i.e. head, mammary and genital lymph nodes and spleen), the late pregnant or early post-parturient uterus, and the udder.

Tissues: Samples are removed aseptically with sterile instruments. The tissue samples are prepared by removal of extraneous material (e.g. fat), cut into small pieces, and macerated using a Stomacher or tissue grinder with a small amount of sterile phosphate buffered saline (PBS), before being inoculated on to solid media.

Vaginal discharge: A vaginal swab taken after abortion or parturition is an excellent source for the recovery of Brucella and far less risky for the personnel than abortion material. The swab is then streaked on to solid media.

Milk: Samples of milk must be collected cleanly after washing and drying the whole udder and disinfecting the teats. It is essential that samples should contain milk from all quarters, and 10–20 ml of milk should be taken from each teat. The first streams are discarded and the sample is milked directly into a sterile vessel.

Dairy products: Dairy products, such as cheeses, should be cultured on the media described above. As these materials are likely to contain small numbers of organisms,

enrichment culture is advised. Samples need to be carefully homogenised before culture, after they have been ground in a tissue grinder or macerated and pounded in a ‘Stomacher’ or an electric blender with an appropriate volume of sterile PBS.

Culture of samples

Direct isolation and culture of Brucella are usually performed on solid media. This is generally the most satisfactory method as it enables the developing colonies to be isolated and recognised clearly. A wide range of commercial dehydrated basal media is available, e.g. Brucella medium base, tryptose (or trypticase)-soy agar (TSA). The addition of 2–5% bovine or equine serum is necessary for the growth of strains such as *B. abortus* biovar 2, and many laboratories systematically add serum to basal media, such as blood agar base or Columbia agar, with excellent results. Other satisfactory media, such as serum-dextrose agar (SDA) or glycerol dextrose agar, can be used. Selective media are prepared using all the basal media mentioned above. Appropriate antibiotics are added to suppress the growth of organisms other than Brucella. The most widely used selective medium is the Farrell’s medium. Another commonly used selective media is Thayer-Martin medium.

Some samples need enrichment owing to lower number of bacteria as in milk, colostrum and some tissue samples. Enrichment can be carried out in liquid medium consisting of serum-dextrose broth, tryptose broth (or trypticase)-soy broth (TSA) or Brucella broth. The enrichment medium should be incubated at 37°C in air supplemented with 5–10% (v/v) CO₂ for up to 6 weeks, with weekly subcultures on to solid selective medium.

On suitable solid media, Brucella colonies can be visible after a 2–3-day incubation period.

After 4 days incubation, Brucella colonies are round, 1–2 mm in diameter, with smooth margins. They are translucent and a pale honey colour when plates are viewed in the daylight through a transparent medium. When viewed from above, colonies appear convex and pearly white. Later, colonies become larger and slightly darker.

Identification and typing

Identification of Brucella organisms can be carried out by a combination of the following tests: organism morphology after Gram staining, colonial morphology, growth characteristics, urease, oxidase and catalase tests, and the slide agglutination test with an anti-Brucella polyclonal serum. The simultaneous use of several phages e.g. Tbilissi (Tb), Weybridge (Wb), Izatnagar (Iz) and R/C provides a phage-typing system that, in experienced hands, allows a practical identification of smooth and rough species of Brucella. However, several characteristics, for example added CO₂ requirement for growth, production of H₂S (detected by lead acetate papers), and growth in the presence of basic fuchsin and thionin at final concentrations of 20 µg/ml, are revealed by routine tests that can be performed in moderately equipped non-specialised laboratories.

Nucleic acid recognition methods

The PCR, including the real-time format, provides an additional means of detection and identification of Brucella spp. Despite the high degree of DNA homology within the genus Brucella, several molecular methods, including PCR, PCR restriction fragment length polymorphism (RFLP) and Southern blot, have been developed that allow differentiation between Brucella species and some of their biovars to a certain extent. Pulse-field gel electrophoresis has been developed that allows the differentiation of several Brucella species. An assay named

AMOS-PCR which can distinguish the abortus, melitensis, ovis and canis spp. has been devised. A new multiplex PCR assay (Bruce-ladder) has been proposed for rapid and simple one-step identification of *Brucella*. The major advantage of this assay over previously described PCRs is that it can identify and differentiate in a single step most *Brucella* species as well as the vaccine strains *B. abortus* S19, *B. abortus* RB51 and *B. melitensis* Rev.1. In contrast to other PCRs, Bruce-ladder is also able to detect DNA from *B. neotomae*, *B. pinnipedialis* and *B. ceti*.

Serology

No single serological test is appropriate in all epidemiological situations; all have limitations especially when it comes to screening individual animals. It should be stressed that the serum agglutination test (SAT) is generally regarded as being unsatisfactory for the purposes of international trade. The complement fixation test (CFT) is diagnostically more specific than the SAT. The diagnostic performance characteristics of the ELISAs are comparable with or better than that of the CFT, and as they are technically simpler to perform and more robust, their use may be preferred. For the control of brucellosis at the national or local level, the buffered *Brucella* antigen tests, i.e. the Rose Bengal test (RBT) as well as the ELISA are suitable screening tests. Positive reactions should be retested using a suitable confirmatory and/or complementary strategy.

Rose Bengal plate test (RBT): The RBT is one of a group of tests known as the buffered *Brucella* antigen tests which rely on the principle that the ability of IgM antibodies to bind to antigen is markedly reduced at a low pH. The RBT and other tests such as the buffered plate agglutination tests and the card test play a major role in the serological

diagnosis of brucellosis worldwide. The RBT is a simple spot agglutination test where drops of stained antigen and serum are mixed on a plate and any resulting agglutination signifies a positive reaction. The test is an excellent screening test but may be oversensitive for diagnosis in individual animals, particularly vaccinated ones.

ELISA tests: These tests offer excellent sensitivity and specificity whilst being robust, fairly simple to perform with a minimum of equipment and readily available from a number of commercial sources in kit form. They are more suitable than the CFT for use in smaller laboratories and ELISA technology is now used for diagnosis of a wide range of animal and human diseases. Although in principle ELISAs can be used for the tests of serum from all species of animal and man, results may vary between laboratories depending on the exact methodology used. Not all standardization issues have yet been fully addressed. For screening, the test is generally carried out at a single dilution. It should be noted, however, that although the ELISAs are more sensitive than the RBT, sometimes they do not detect infected animals which are RBT positive. It is also important to note that ELISAs are only marginally more specific than RBT or CFT.

Serum agglutination test (SAT): The SAT has been used extensively for brucellosis diagnosis and, although simple and cheap to perform, its lack of sensitivity and specificity mean that it should only be used in the absence of alternative techniques.

Complement fixation test (CFT): The sensitivity and specificity of the CFT is good, but it is a complex method to perform requiring good laboratory facilities and trained staff. If these are available and the test is carried out regularly with good attention to quality assurance, then it can be very

satisfactory for diagnosis of brucellosis in humans and animals.

Other tests

Many other serological tests have been employed. Some, such as the Rivanol or 2-ME test, are variations of the SAT and, although more specific, share many of its disadvantages. At present, the use of such procedures in the place of the standard test is not advised.

Milk testing: In dairy herds, milk is an ideal medium to test as it is readily and cheaply obtained, tests can be repeated regularly and give a good reflection of serum antibody. Milk from churns or the bulk tank can be screened to detect the presence of infected animals within the herd which can then be identified by blood testing. This method of screening is extremely effective and is usually the method of choice in dairy herds.

Milk ring test: The milk ring test (MRT) is a simple and effective 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. 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.

The milk ELISA is far more specific than the MRT.

Milk ELISA: The ELISA may be used to test bulk milk and is extremely sensitive and specific, enabling the detection of single infected animals in large herds in most circumstances.

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How to cite this article:

Solanki, S. and Durga Devi. 2020. Epidemiology and Diagnosis of Brucellosis in Large Ruminants. *Int.J.Curr.Microbiol.App.Sci*. 9(10): 1343-1348.
doi: <https://doi.org/10.20546/ijcmas.2020.910.161>