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Diagnosis of ovine brucellosis using conventional and molecular tools

Yosef Deneke, Rajib Deb*, Ramesh D.¹

Division of Animal Biotechnology, ¹Division of Animal Biochemistry, Indian Veterinary Research Institute, Izatnagar-243122, UP, India

*drrajibdeb@gmail.com

Summary: Ovine brucellosis which is caused by *Br. melitensis* is a zoonotic infection . The control programs are hampered by the lack of simple and efficient diagnostic tests, especially for subclinically infected animals. Various conventional diagnostic tools are available like bacteriological examination, Card Test (CT) , Milk Ring Test (MRT), Allergic Skin Test (AST) , Serum Agglutination Tube Test (SATT), Complement Fixation Test (CFT), Enzyme linked Immunosorbent Assay(ELISA), Native hapten (NH) gel precipitation test and Fluorescence polarisation assay (FPA) . Molecular tools like Outer membrane protein typing, IS711 typing, Amplified fragment length polymorphism, Variable number tandem repeat typing are very much promising and challengeable. In future Genome-Wide Screening, Gene Arrays and Proteomics, Green Fluorescent Protein-encoded Brucella Strains, In Vivo Imaging System , Dominant Negative Cells and Gene Knockout Mice system and Gene-to-Gene or Function-to-Function Interactions based assay can be useful for precise detection of the disease.

Key Words : Ovine brucellosis, diagnosis, molecular

Introduction

Ovine brucellosis which is caused by *Br. melitensis* is a zoonotic infection with important effects on both public health and animals' health, where production is widespread in many areas of the world, particularly in some Mediterranean and Middle Eastern countries (Ainseba et al., 2010, Edgardo and Moriyon. 2002). *Br. melitensis* the main etiological agent of brucellosis in small

ruminants was the first species in the genus brucella described (Alton, 1990). According to Alton, (1990), Bruce first isolated it in 1887 from the spleen of soldiers dying of Mediterranean fever on the island of Malta; he called it *Micrococcus melitensis*.

The genome of *Br. melitensis*, *Br. suis* and *Br. abortus* have been sequenced completely while *Br. Ovis* is in progress (Cutler *et al* 2005). Reports of comparative genomic studies of *Br. melitensis* and *Br suis* shows only 42 unique gene in *Br suis* and 32 for *Br. melitensis* which are largely associated with genomic island suggesting a possible origin through lateral gene transfer (Rajshekara *et al* 2004, Halling *et al.*, 2005). The *B. ovis* type strain ATCC25840 (also known as 63/290 or NCTC10512) used for sequencing was isolated from a sheep in Australia in 1960. The genome of this strain consists of two circular chromosomes of 2,111,370 bp (Chromosome I; NCBI Accession Number NC_009505) and 1,164,220 bp (Chromosome II; NCBI Accession Number NC_009504), which are predicted to encode a total of 2890 proteins, 1928 on ChrI and 962 on ChrII. Comparison with the sequenced genomes of *B. suis* (GenBank Accession numbers NC_004310 and NC_004311) (Paulsan *et al.* 2002), and *B. melitensis* 16M (GenBank Accession numbers NC_003317 and NC_003318) (DeIVecchio *et al* 2002) shows a large degree of conservation, particularly in the % G+C content and size of the chromosomes. This comparison also revealed several species-specific differences, including regions missing from *B. ovis* relative to the other sequenced Brucella genomes and genes unique to *B. ovis* (Tsolis *et al.* 2009). It is postulated that due to such uniformity among brucella elucidation of pathogenicity related gene and discovery of reliable epidemiological markers through comparative genomic study can be achieved (Rajshekara *et al* 2005).

Diagnostic overview

The diagnosis of ovine brucellosis is based on three examinations, a complement fixation test on serum, physical palpation of the contents of the scrotum and cultural examination of semen or aborted material. The complement fixation, or other serological test is by far the most important; many infected rams have palpably normal scrotal contents and microbiologically negative semen. Many rams with abnormalities of intrascrotal tissues do not have brucellosis (Blood and Radostits, 1989).

Wide varieties of methods have been used for the diagnosis of brucellosis in different animal species in various countries and these are, direct smear and serological tests (Buxton and Fraser, 1977). Diagnostic methods for detecting brucellosis which are available at present are discussed in detail as follows by (Seifert, 1996).

The Direct Demonstration of the Causal Organism

Bacteriological examination

This is the absolute proof of brucella infection and it is epidemiologic studies to determine the species and biotype of the isolated strain. Therefore, bacteriologic examination on aborted fetuses or placentas should be undertaken whenever possible. Vaginal discharges, if taken shortly after parturition and milk samples are suitable for cultures. Selective media make it possible to isolate brucella from contaminated specimens (Kolar, 1982&1984). Bacteriological examination of aborted material is time consuming, laborious, costly and cannot routinely be used as a diagnostic procedure in developed or developing countries. Nevertheless, *Br. melitensis* can be isolated from the lungs, liver, kidney, stomach contents, abomasums and brain of aborted animals (Ribeiro, *et al.*, 1990). Other organs from which specimens may be collected are lymph nodes, udder tissue, male sex glands, as well as milk and seminal plasma (Seifert, 1996). Generally a primary bacteriological diagnosis can be made on smears from vaginal swabs, milk, placentas or aborted fetuses stained with stamp's method. Confirmation on appropriate culture and selective media is recommended. Spleen and lymph nodes are most reliable from necropsy material. PCR is potentially a useful method on samples containing a low number of brucella (Kolar, 1984).

By Indirect Demonstration of the Pathogen

The diagnosis of ovine brucellosis is based largely up on serologic tests. Since the clinical manifestation of brucellosis is not pathognomonic, laboratory tests are necessary for confirmation. Research efforts have produced a large variety of methods from simple screening testes to some which are complicated and sensitive. In general, the same diagnostic tests can be applied as in cattle, but their efficacy in small ruminants is considerably lower. They often fail to detect infected sheep and particularly goats. Because of the limited reliability of serologic tests to detect each individual infected sheep and goats, the results should be interpreted on a flock basis; i.e. the whole flock should be regarded as infected even if only some single animals prove to be infected. Despite these limitations, serologic and allergic tests are very useful in diagnosing brucellosis in sheep and goats. The selection of diagnostic methods from the large battery of available tests depends up on the purposes of the investigation or survey, identification of individual infected animals, size of flocks, farming and climatic conditions, laboratory facilities, costs of testing and expected prevalence (Kolar, J., 1984).

Card Test (CT)

This method is the most suitable for detecting infected flocks and for survey. It is simple and rapid and does not require laboratory facilities (Bercovich, 1998).

Milk Ring Test (MRT)

This procedure is valuable in screening dairy cows and has limitations in the diagnosis of caprine and ovine brucellosis. The smaller fat globulin of goat and sheep cream absorbs agglutinated stained brucella in positive milk samples less efficiently and do not rise to form the typical

colored ring. An additional problem with the MRT is the low content of antibodies in goat and sheep milk. The sensitivity of the MRT can be increased by performing it in hypertonic medium of 5% NaCl (Alivisatos and Edipides, 1953). A serious disadvantage of the test is that its use is limited to milking animals.

Allergic Skin Test (AST)

This method is characteristic of brucellosis in man and some animals and appears through the delayed type of hypersensitivity to brucella allergens in generalized infection and the sensitivity may persist for several years. Preference should be given to purified, non-antigenic allergens with a precisely determined protein content and sufficiently high allergic potency which is titrated on sensitized animals (Kolar and Kral, 1956). The test is highly specific and no reactions are observed in brucella-free flocks by confirming the absence of exposure to brucella as well as the extent of infection in other flocks. It is also useful in epidemiologic surveys and can be used in all farm animal species. Large flocks of sheep on pastures may be tested where blood sampling and identification of animals is laborious. No laboratory facilities are necessary and results are easy to interpret by trained veterinary workers.

An additional advantage of the AST is that results can be seen and understood by owners of animals resulting in better cooperation. Owners who oppose blood collections from their animals willingly accept injection of an allergen. In animals vaccinated with Rev 1 vaccine, the skin test sensitivity usually disappears in 2-4 months. Therefore, use of the AST in vaccinated flocks for survey purposes is superior to serologic tests (Kolar, J., 1984).

Serological Tests

Serum Agglutination Tube Test (SATT)

This is the oldest and most widely used procedure in brucellosis diagnosis. Its diagnostic value in small ruminants is less than in cattle. It is usually positive in the early stages of infection but often fails to detect chronic brucellosis. The sensitivity of the SATT in sheep can be increased if it is performed in a hypertonic medium of 5% NaCl (Alivisatos and Edipides, 1953; Kolar and Kral, 1956).

Complement Fixation Test (CFT)

This serologic test has a relatively high sensitivity and specificity and is superior to the SATT. The CFT indicates active brucella infection better than any other serologic test. It detects mostly IgG antibodies which are present in both acute and chronic stages of brucellosis. The CFT is also superior for the differentiation of post vaccination antibodies from those of infection and in animals vaccinated with Rev 1 vaccine the CFT become negative in 2-4 months. The disadvantage of the CFT is that it requires laboratory facilities and trained personnel which are absent or deficient in many countries sheep brucellosis is prevalent (Kolar, J., 1984). By means of serological techniques, only the question about whether the animal has produced an immunological response to the agent can be answered. It is not possible to determine whether

this reaction is a sterile or unsterile immunity; nor is the varied serological techniques able to detect all stages of infection (Seifert, 1996).

As abortion is the only symptom that indicates *Br. melitensis* infection, serological tests are used to confirm brucellosis in suspect animals. The use of the RBPT, which is easy to perform and is considered a valuable screening test (Farina, 1985) is less effective than the CFT at detecting brucellosis in individual sheep and goats (FAO/WHO, 1986). The CFT is considered to be the most effective test for diagnosing brucellosis in small ruminants (FAO/WHO, 1989,1997) but has no particular advantage over the SAT performed in a hypertonic environment of 5-20% NaCl (Levchenko and Drozhzhin, 1958).

Enzyme linked Immune sorbent Assay(ELISA)

Since neither a single serological test nor a combined use of several serological tests detects all infected animals in a flock, detection of brucellosis remains a major problem in areas of low prevalence of brucellosis. Most studies agree that the ELISA is as specific as the CFT but it is more sensitive. Yet, for a reliable diagnosis of infected animals studies suggest using the ELISA in combination with other tests (Bercovich *et al.*, 1998). Other studies consider the ELISA suitable for screening flocks of sheep and goats for brucellosis (Biancifiori *et al.*, 1996). Nevertheless, small ruminants should be tested with the ELISA and CFT tests to prevent the spread of brucellosis after an outbreak of the disease in an area with low prevalence of brucellosis or in an area free from brucellosis (Bercovich *et al.*, 1998).

Native hapten (NH) gel precipitation tests

In the case of recent vaccination with S19, only the gel diffusion (GD) or radial immunodiffusion (RID) tests with Native Hapten antigen (Díaz *et al.*, 1979) and the cELISA (Nielsen *et al.*, 1996) appear to be useful for diagnosis, allowing differentiation of infection by *B. abortus* from S19 vaccination. However, only the GD test with NH (Díaz *et al.*, 1979) was able to discriminate, at a given time after vaccination, the immune responses of *B. melitensis* infected from vaccinated sheep (Marin *et al.*, 1999).

Fluorescence polarisation assay (FPA)

This test, already regarded as an official test for the diagnosis of bovine brucellosis by the OIE (Collective, 2004 and McGiven *et al.*, 2003), is currently under evaluation in sheep and goats.

Molecular tools

Outer membrane protein typing

A typing approach successfully used for differentiation of microbial groups has been PCR of a target gene with a high degree of diversity and its subsequent digestion with restriction

endonucleases to produce restriction fragment length polymorphism (RFLP) profiles for differentiation based on polymorphic alleles. Several *Brucella* omp genes were considered as potential targets for typing schemes, however, the Omp2 Locus [Omp2a and Omp2b (Cloeckeaert et al. 2002), 36-kDa porin proteins (Cloeckeaert et al. 2002)] show greatest polymorphism among the brucellae, these could be utilized to differentiate brucellae.

IS711 typing

Chromosomal indexing of DNA polymorphisms can be achieved through use of the variable number and location of copies of a mobile insertion sequence believed to be specific for *Brucella*. Genomic DNA is digested with restriction endonucleases such as EcoR1, and resulting fragments probed with DIG- labelled IS711. IS711 provides a specific target able to generate data on DNA polymorphisms among the brucellae (Cloeckeaertetal.2002).

Amplified fragment length polymorphism.

Amplified fragment length polymorphism (AFLP) has recently been heralded as a useful technique to differentiate numerous bacterial species. This technique provides a valuable method to index variation over the whole genome. The AFLP technique is based on the amplification of subsets of genomic restriction fragments using PCR. DNA is cut with restriction enzymes and double stranded adaptors ligated to the ends of DNA fragments to generate template DNA for PCR amplification. The sequence of the adaptors and adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments. Selective nucleotides can be included at the 3'-ends of the PCR primers, which therefore prime DNA synthesis from only a subset of the restriction sites. Labelling of one of the primers with a fluorescent dye permits visualization of a banding pattern following electrophoresis on an acrylamide gel. Although AFLP data may be of limited epidemiological significance, it provides useful information on the taxonomy of *Brucella* and the relationship between the various biovars (Whatmore et al., 2005).

Variable number tandem repeat typing

Use of the forensic based method of variable number tandem repeat typing has proved itself as a valuable approach for profiling highly homogeneous populations of microbes (Keim et al. 1999, Yoldi et a., 2007). This method has also been shown to provide high level of discriminatory power among the brucellae (Lucero et al., 2010).

Future Trends

The 21st century is the period of postgenomics, *B. melitensis* and *B. abortus* genome projects should provide useful information for performing reverse-genetic approaches to elucidate virulence factors.

Genome-Wide Screening, Gene Arrays and Proteomics

Gene arrays and proteomics are novel computer-aided screening systems to detect changes at the transcriptional and/or translational level in the host and/or pathogen . The application of gene KO's within the pathogen and host will provide an approach to investigate the significance of the changes observed in gene arrays and proteomics. Taken together, even with the shortcomings of genome-wide screening systems, the patterns of gene expression will facilitate the elucidation of the interplay between host and pathogen (Li et al., 2010, Celis et al., 2000).

Introduction of Green Fluorescent Protein-encoded Brucella Strains

Cellular microbiology is an emerging discipline that is being used to investigate the effect of microbes on cell biology (Jinkyung and spliter, 2003). For instance, many intracellular bacteria can survive inside specialized compartments or in the cytoplasm, suggesting that these bacteria have unique invasion mechanisms. In fact, the dissection of bacterial pathogenesis provides information for understanding the dynamics of the host cell cytoskeleton, intracellular membrane, and signal transduction events. In brucella research, the movement of brucella inside of phagocytic and nonphagocytic cells is being investigated with green fluorescent protein (GFP)-expressing brucella strains. This fluorescence-based technique is a useful tool to detect the cellular location of brucella in combination with diverse monoclonal antibodies to define the molecular organelles. In addition, GFP is quite stable and can be used for immunostaining of brucella on the tissue sections with GFP-specific monoclonal antibodies (Kittelberger et al., 1995).

Novel Methods to Identify Brucella Virulence Factors

The most recent significant breakthrough in *Brucella* pathogenesis research is the application of STM, where mutants were tested in phagocytic and nonphagocytic cells as well as acute and chronic stages of infection in mice. Many *Brucella* attenuated mutants have been identified and are being characterized individually. In addition, differential fluorescence induction has identified several promoters induced specifically within macrophage cell lines. However, individual mutants must be generated to establish a role for these genes in *Brucella* virulence. In addition, the recent completion of the *Brucella* genome projects should provide useful information about *Brucella* physiology and pathogenesis , codifying the renaissance of reverse genetics(Jinkyung and spliter, 2003).

In Vivo Imaging System

The virulence of *Brucella* has been assessed in vivo by enumerating the residual CFU in splenic or hepatic tissues in a time-dependent manner. However, the current development of photonic detection systems promises to monitor the kinetics of bacteria in vivo without killing the infected

mice. *luxABCDE Brucella* can be used to infect mice and the infected mice can be analyzed using an intensified charge-coupled device camera and analytical software. This simple equipment will alleviate the ethical problems caused by killing mice, accelerate the detection of attenuation in *Brucella* strains, and detect the location of *Brucella* in vivo in a time-dependent manner (Rajashekara et al., 2005, Paschos et al., 2006).

Dominant Negative Cells and Gene Knockout Mice

The loss of function is a dominant dogma in genetics to analyze the function of a gene. The two major approaches to generate loss-of-function phenotypes are the use of dominant negative cell lines and the use of gene KO mice. Diverse KO mice have been used in *Brucella* research for elucidating the function of respective immune components. However, dominant negative cell lines have not been used in brucellosis research, unlike other intracellular bacterial pathogen research (Jinkyung and spliter, 2003). The generation of dominant negative cells requires a relatively short time compared to the production of gene KO mice. Besides, dominant negative cell lines will be useful for in vitro experiments in cases where the gene KO is lethal to the mice.

Gene-to-Gene or Function-to-Function Interactions

During the last several decades in brucellosis research, three different kinds of combinatorial experiments were widely performed. First, to understand the general immune responses in the host, wild-type mice were infected with wild-type *Brucella* strains. Second, to find a virulence factor or to detect the attenuation in *Brucella*, mutant *Brucella* strains were used to infect wild-type mice. Third, to understand the role of specific immune components in brucellosis, gene KO mice were infected with wild-type *Brucella* strains. However, gene KO mouse infection with mutant *Brucella* strains had not been performed in brucellosis research until the introduction of IRF-1^{-/-} mice. The virulence of *B. abortus* in IRF-1^{-/-} mice can be assessed by either the death or survival of infected mice (Eskra et al., 2005). The rapidity of death of IRF-1^{-/-} mice and the hepatic damage correlated with virulence when mice were infected with 5×10^5 CFU, suggesting that the hepatic damage contributes to the death of IRF-1^{-/-} mice. This is one example of a combinatorial experiment. Similarly, STM experiments can be performed directly with specific gene KO mice instead of wild-type mice. Each KO mouse provides a different selective pressure to the mutants, and different sets of mutants are selected compared to wild-type mice. Thus, a unique opportunity exists to identify particular bacterial genes that cope with specific immune components. Alternatively, in vitro STM can be performed using specific KO cell lines such as dominant negative cell lines or cells obtained from individual gene KO mice. By using a specific dominant negative cell line infected with *Brucella*, host responses could be analyzed with DNA chips or proteomics. Also, gene KO mice could be infected with *Brucella* and then the responses of a purified cell population could be detected by using DNA chips or proteomics. Not only wild-type *Brucella* but also diverse genetically manipulated *Brucella* mutants can be used for these kinds of combinatorial experiments (Jinkyung and spliter, 2003).

Conclusion and future perspectives

Conventional techniques to detect ovine brucellosis infection are either not precisely specific or lack an optimum degree of sensitivity which cannot be overlooked when screening of a herd is concerned. With the critical need for improved diagnostic tests to detect the infection, effort need to be concentrated on the development of simple, rapid, noninvasive tests that can perform without expensive laboratory equipment.

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