

Bovine paratuberculosis: a review of the advantages and disadvantages of different diagnostic tests

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ABSTRACT

Paratuberculosis (PTB), or Johne's disease, is a chronic infectious granulomatous enteritis of ruminants, caused by *Mycobacterium avium* subspecies *paratuberculosis* (Map). It is characterized by diarrhea and progressive cachexia, which may cause the death of the animal. Calves are the most susceptible to infection. Infected animals excrete Map mainly by the feces. PTB is endemic worldwide, with high prevalence levels, strong economic impact and public health relevance because of its possible association with Crohn's disease. Although the current reference diagnostic test is identification of Map in the bacterial culture, there are different diagnostic tests to identify infected individuals and/or herds. The sensitivity and specificity of these tests vary according to the stage of the disease in the animals to be evaluated. The correct choice and application of each of these diagnostic tests will ensure their success and may allow to establish a control program. The aim of this work is to review and discuss the different diagnostic tests used in the detection of Map-infected animals, focusing on their advantages and disadvantages.

Key words: paratuberculosis, *Mycobacterium avium* subsp. *paratuberculosis*, diagnostic tests

RESUMEN

Paratuberculosis bovina: una revisión sobre las ventajas y desventajas de las diferentes pruebas diagnósticas. La paratuberculosis (PTBC) o enfermedad de Johne es una enteritis granulomatosa crónica de rumiantes, causada por *Mycobacterium avium* subsp. *paratuberculosis* (Map). Se caracteriza por producir diarrea y caquexia progresiva, la cual conduce a la muerte del animal. Los terneros son los animales más proclives a la infección. Los animales infectados excretan Map, principalmente por las heces. La PTBC es una enfermedad endémica a nivel mundial, con altos niveles de prevalencia, fuerte impacto económico e importancia en salud pública, debido a su posible asociación con la enfermedad de Crohn. Aunque la prueba de referencia diagnóstica es la identificación de Map en cultivo bacteriológico, existen diferentes pruebas diagnósticas para detectar animales o rodeos infectados. La sensibilidad y especificidad de estas pruebas varían según el estadio de la enfermedad de los animales que se evalúan. La correcta elección y aplicación de cada una de estas pruebas asegura el éxito del diagnóstico y permite establecer un programa de control. El objetivo de este trabajo es la recopilación y discusión de las diferentes pruebas diagnósticas utilizadas en la detección de los animales infectados por Map, concentrándose en sus ventajas y desventajas.

Palabras clave: paratuberculosis, *Mycobacterium avium* subsp. *paratuberculosis*, pruebas diagnósticas

1. INTRODUCTION

Paratuberculosis (PTB), or Johne's disease, is a chronic, progressive, infectious granulomatous enteritis caused by *Mycobacterium avium* subspecies *paratuberculosis* (Map), which affects ruminants, especially dairy cattle and a variety of domestic species (6, 48).

Clinically ill and asymptomatic animals are the primary source of infection. Transmission is primarily oral, through the colostrum and milk of infected animals, and through pasture and drinking water contaminated with feces. Several authors have also

suggested vertical transmission through placenta and semen (13, 44, 84). The high density of animals, the poor hygienic conditions and the quality of soils (acidic and wet soils) favor the spread of infection. The fact that Map is a ubiquitous microorganism highly resistant to adverse environmental factors constitutes a risk for the spread of the disease.

According to the severity of the clinical signs and the likelihood of diagnosis, bovine PTB is divided into four stages: silent, subclinical, clinical, and advanced (91). In addition, according to the fecal shedding of Map by cows in the subclinical stage,

they can be subclassified into low (<10 CFU/g), moderate (10-50 CFU/g) and high (> 50 CFU/g) fecal shedders (17) (Table 1). Apparently, there is a seasonal effect on the presence of viable Map in retail milk and clinical cases during the winter months (24).

The various PTB diagnostic tests used have certain limitations because of their different sensitivity and specificity according to the age of the animal to be evaluated and the stage of the disease. This fact makes the diagnosis of PTB a major challenge.

2. DIAGNOSTIC METHODS

The effectiveness of a diagnostic test is given by its sensitivity and specificity, as well as by its positive and negative predictive value (value dependent on the prevalence), compared to a reference test that identifies animals as truly infected or truly non-infected (45). In the case of PTB, the gold standard is the identification of the microorganism by bacterial culture. However, this test does not allow accurate identification of truly infected animals because of lack of shedding in early phases, intermittent shedding, limitations of culture protocols, etc. It is therefore necessary to consider other diagnostic tests.

Other PTB diagnosis alternatives are the observation of clinical signs, the detection of the host immune

response (cellular and/or humoral) and the identification of microscopic lesions by histopathological studies. The criteria for clinical diagnosis suggestive of PTB is the finding of 3-5 year-old bovine animals with diarrhea and wasting in spite of preserved or reduced appetite.

2.1 DIRECT DIAGNOSIS OF THE CAUSATIVE AGENT

The identification of the causative agent can be achieved by bacterioscopy, microbiological culture, or detection of genetic material.

2.1.1. Bacterioscopy

The bacterioscopic method used for PTB identification is Ziehl-Neelsen (ZN) staining, which is based on the resistance of mycobacteria to decolorizing by acid alcohol after staining with fuchsin. The results are qualitative. This method has the advantage of being simple, fast and inexpensive, but has the disadvantage of having low sensitivity and specificity in feces, colostrum and milk samples. In cases of severe diarrhea, Map concentration decreases relative to the amount of feces, thus increasing the likelihood of false negative results. A similar condition occurs in animals with subclinical PTB, which have a low rate of fecal excretion. In smears or sections of tissues,

Table 1. Description of the stages of bovine PTB related to clinical signs and likelihood of diagnosis

Stages	Clinical signs	Fecal shedder (CFU/g)		Diagnosis probability
Silent infection	Not evident	Not evident		Rare
Sub-clinical	Not evident	Low	< 10	+
		Moderate	10-50	
		High	> 50	
Clinical	Normal appetite, weight loss, decreased milk production, intermittent diarrhea	+		+
Advanced	Lethargy submaxillary edema, cachexia, persistent diarrhea	+		+

especially from the ileocecal valve or the intestinal lymph nodes with gross lesions, the visualization of groups of 10 to 20 brightly colored bacilli within the resident macrophages in the lesion is highly suggestive of PTB (69).

2.1.2. Bacterial culture: liquid media and solid media

The identification of viable Map by bacterial culture is considered the reference diagnostic test (*i.e.*, the gold standard). Feces, colostrum, milk or intestinal mucosal scrapings can be used as samples (50). In order to reduce costs, the fecal culture can be made in groups of 3-5 individual samples, without losing too much sensitivity (40, 88). In dairy cattle, the samples for isolation can also be collected from the filters of milk collection systems, the milk tank and/or the four quarters of the same animal. Due to the intermittent excretion of Map, it is advisable to take serial samples over time (70). Since infected cattle can excrete 10^{8-12} CFU/g of feces and contaminate the environment, culturing pasture soil samples from manure and/or delivery areas is also recommended (95, 97).

In cases of necropsy, the culture of the lymph node close to the macroscopic lesion, scrapings from the ileocecal valve and ileocecal or jejunal lymph nodes or from the colon and rectum are the best samples for the isolation of Map (49). Cultures of intestinal tissue and/or regional lymph nodes have a sensitivity of 70 % and a specificity of 95 % (86).

Due to the differential growth speed of paratuberculosis and other bacteria, it is possible to kill the fast-growing competitors by decontaminating the samples prior to inoculation into culture media. There are different methods to do this, but the most popular is hexadecylpyridinium chloride (HPC) (21). This chemical decontamination demands a day of work and can reduce the number of Map in a range of 10^3 CFU (72). It is estimated that after the decontamination procedure, the expected contamination rate in the feces culture is 7 % and in tissue culture 0.2 % (96). A combination of antibiotics (PANTA: polymyxin B, amphotericin B, nalidixic acid, and trimethoprim sulfamethoxazol, supplemented with vancomycin and fungizone, among others) is also added to the culture medium. The contamination of fecal samples is partly related to the type of feeding of the animals at the time of sampling. For example, dairy cattle are fed grains and silos, which predispose the rapid development of fungi on the surface of the culture medium,

making it difficult to distinguish the colonies of Map.

There are several Map culture media, all supplemented with mycobactin J, sodium pyruvate, antibiotics and antifungals. A preliminary step of culture in liquid medium (Middlebrook 7H9, 7H12) supplemented with oleic acid, bovine albumin, dextrose and catalase (OADC) may be used to accelerate the detection of Map and the activation of Map prior to the culture on solid medium.

Automated systems for the detection of bacterial growth in a liquid medium have been developed. An example of this is the radiometric BACTEC 460 system (Becton Dickinson Inc.), which contains a precursor radiolabeled with C^{14} that detects changes in CO_2 concentration due to bacterial respiration. Other automated systems use fluorometric, barometric and colorimetric methods (74, 82).

Cultures on solid media, such as Herrold egg yolk medium (HEYM) with mycobactin J, Löwenstein-Jensen, or synthetic media, such as Middlebrook (7H10, 7H11) are used. The colonies formed are small, hemispherical, about 1 mm in diameter, smooth and shiny. The criteria to identify Map are the slow growth rate, the morphology of the colonies, the ZN staining and mycobactin dependence, mainly in primary culture.

Map strains have been divided into three clusters named type I or sheep strains (S), type II or cattle strains (C) and type III or intermediate strains. This classification is based on the characteristics of the culture and the restriction fragment length polymorphism analysis with hybridization to IS900 (RFLP) and the molecular characterization by pulsed field gel electrophoresis (PFGE). Other molecular techniques like PCR-restriction enzyme analysis (PCR-REA) of *gyrB* and *inhA*, PCR sequencing of *recF*, and comparative genomic hybridization analyses were proposed, although these results, the Map subdivision of strains into clusters, are still controversial (11).

As the bacterial strain cannot be known prior to the culture, it is recommended to carry out the culture in HEYM- mycobactin-sodium pyruvate, Löwenstein-Jensen mycobactin, and Middlebrook 7H11. The use of these three media allows to detect 100 % of type I/III strains and 98 % of type II strains (18).

The bacterial culture requires at least 100 CFU/g of feces (minimum detection limit) (53). Since this amount is exceeded by animals with clinical PTB, but not by subclinical low and/or moderate fecal shedders,

only 15-25 % of them can be detected by bacterial culture (91). Bacterial culture of milk from animals at this stage is difficult because excretion is 2-8 CFU/50 ml (54, 84). Map excretion in feces and milk may not be simultaneous; therefore, a significant proportion of positive animals may not be diagnosed if samples are taken from only one of these sources. For this reason, the simultaneous cultivation of both excretions is recommended (30). It is also recommended that the bacterial culture is carried out in samples collected from the same animal over several days to increase the sensitivity of the method. The sensitivity of the bacterial culture in clinical stages can be 91 % (2), a value that can be reduced to 45 % to 72 % (2, 17) in subclinical stages, whereas the specificity is very good (100 %) in all stages (5). These strategies are too expensive as to be routinely applied for field diagnostic or control of infection.

Some researchers have postulated the probability of positive bacterial culture due to the phenomenon known as *passage*, based on the assumption that, in certain animals, Map cannot colonize the gastrointestinal tract, and is thus shed in the feces 1 to 7 days after intake. This phenomenon has been held responsible for the presence of animals categorized as low fecal excretors in highly infected herds (84). However, it seems highly unlikely that enough bacteria pass the whole complex ruminant digestive system (reticulum-rumen-omasum-abomasum-small intestine-large intestine) without degradation.

The advantages of bacterial culture are the accurate diagnosis by isolation of Map and its quantification as colony forming unit per ml (CFU/ml), which allows classifying the animals according to their level of excretion, a useful way of establishing a program for removal of infected animals from the herd. The disadvantages are the high cost and the long incubation period that causes an epidemiologically dangerous delay in taking measures.

The use of automated systems such as BACTEC MGIT 960 shortens the time of detection (4 to 7 weeks) and can become positive from 10 CFU/ml (77). The main disadvantage is the need of expensive equipment and the high cost of the media. Furthermore, it is difficult to identify the bacteria due to the possible development of contaminant microorganisms (96). Automated systems require special equipment, specialized personnel and antibiotic combinations, all of which increases the costs.

In summary, when the bacterial culture is positive in the clinical samples, its specificity is 100 %, but the identity of bacterial growth usually needs to be confirmed by molecular methods (93). The disadvantages of solid culture media are their long incubation time, the likely environmental dehydration and the possible reduction of viable microorganism by chemical decontamination, all these being important data to interpret negative results, especially in low intensity fecal shedders (72).

2.1.2.a. Techniques for microbial concentration

Due to the quantitative variability of excretion of Map in feces and/or milk, which directly affects the sensitivity of diagnostic tests, the concentration of microorganisms in the sample is an important variable for isolation methods. Several bacterial concentration techniques, such as centrifugation, sedimentation, filtration and immunomagnetic separation (IMS) have been described.

Centrifugation and sedimentation techniques partly improve the detection of Map but increase the number of contaminants. In milk samples, centrifugation allows to obtain three fractions with different concentrations of Map. According to studies by Grant *et al.*, centrifugation at 2500 x g for 15 minutes yields the highest concentration of Map in the sediment (69.4 %), 17.6 % remaining in the whey portion and 13 % in the fat (32). However, Gao *et al.* claim that Map has different affinity for the different fractions of milk according to their processing (27). Thus, in milk subjected to heat and cold before centrifugation, the highest concentration of Map is found in the fraction of sediment, whereas in untreated milk, the bacteria are primarily found in the fat fraction, in the sediment fraction to a lesser extent and negligibly in the whey fraction.

Filtration is based on the tendency of Map to form clumps larger than other bacteria and fungi, which allows a good differential concentration.

IMS is based on the use of magnetic nanoparticles (beads) bound to antiMap antibodies (Ab) that interact with surface antigens of Map, allowing the separation and concentration of the microorganism from a heterogeneous bacterial suspension through a magnetic field. This technique has been used especially in milk samples where the bacterial concentration is relatively low (29, 32, 59). The limits of detection reported for feces and milk samples are of 1-100 CFU/g and 10-20 CFU/ml, respectively (32).

This immunoadhesion avoids losing time and prevents the harmful effects of chemical decontamination. The disadvantage of polyclonal antibodies specific to Map surface antigens (antiMap Ab) is the variability in their composition, which may lead to changes in the binding to Map between batches of sera, and to the probability of reacting with other microorganisms having antigenic similarity, thus giving unsatisfactory results. In contrast, the use of antiMap monoclonal antibodies (antiMap mAb) improves the results, since they are chemically homogeneous and have the same specificity. By means of the joint use of an antiMap Ab and an antiMap MAb, high adhesion efficiency may be achieved (29). A similar magnetic separation technique was developed by Stratmann *et al.* (83), who characterized a peptide aMptD, which, bound to magnetic bead, binds to type I and II Map. Recently, Foddai *et al.* (26), used beads coated with the specific peptides biotinylated aMp3 and biotinylated aMptD in spiked milk. With this technique they showed a sensitivity to reach 85 % to 100 % capture of *M. avium* subsp. *paratuberculosis* and minimal (< 1 %) nonspecific recovery of other *Mycobacterium* spp. Naturally contaminated bovine bulk tank milk and feces were tested by peptide-mediated magnetic separation and viable Map count ranging from 1-110 PFU/50 ml milk and 6-41,11 PFU/g feces were detected (26).

2.1.3. Other methods

Fluorescent microscopy: Fluorescent microscopy is based on the use of a fluorogenic compound, which, transformed into carboxyfluorescein by enzymatic action of viable cells, allows to identify bacterial viability (20). This method, used in pasteurized milk spiked with 10^2 CFU/ml, has been shown to have a sensitivity of 73 % (20). However, this methodology is not yet used routinely by most laboratories.

Bioluminescence: Sasahara *et al.* (73) proposed the use of plasmid-phages labeled with luciferase for rapid detection of viable Map. The oxidation of protein luciferin is catalyzed by the enzyme luciferase, resulting in oxyluciferin. This reaction requires ATP, which is provided only by viable bacteria. Among the three mycobacteriophages evaluated, pHAE85 was shown to be the most efficient, being able to detect 10^2 CFU/ml in skim milk and 10^3 CFU/ml in whole milk within 24-48 h. Although it is a rapid and sensitive method for food samples, its use requires facilities which are usually not available in diagnostic laboratories.

2.2 MOLECULAR TECHNIQUES

2.2.1. Detection of genetic material

The characterization of the IS900 insertion sequence (15) which has 1,451 base pairs and is present with 15 to 20 copies in the Map genome, has enabled the specific identification of minimum amounts of bacterial DNA by the polymerase chain reaction (PCR) technique (15, 47). By means of the technique of restriction enzyme digestion of IS900 (IS900-RFLP), three Map patterns, known as bovine type, sheep type, and intermediate type, according to their apparent species preference, have been identified and isolated from domestic ruminants as well as from American bisons and Indian goats (16). Although some researchers have described elements similar to IS900 (IS900-like sequences) in other bacteria, they can be differentiated through the characterization of the amplified segment by sequencing or genotyping by methylation-restriction (59). Other 58 insertion elements, including *f57*, ISMav2, ISMAPO2, ISMAPO4 and IS1311, have been identified in the Map genome, in a variable number of copies (47, 67, 69).

Patterns that allow to identify genetic differences in mycobacteria by analysis of different loci with interspersed repetitive units (MIRU) and DNA sequences in a tandem repeat (VNTR) showing variations in the number of repeats between different isolates, have also been described (69, 87).

A novel methodology was developed by Gazouli *et al.* (28) for specific detection of DNA using fluorescent semiconductor quantum dots and magnetic beads for fast and specific detection of *Mycobacterium* spp., dispensing with the need for DNA amplification.

2.2.1.a. Samples for PCR

PCR is a powerful method for specific detection of DNA sequences, for which samples can be taken from colostrum, milk, feces, and tissues from the ileocecal valve, ileum, or jejunum, or jejunal or ileocecal lymph nodes (10). Consideration should be given to the inhibitory effects of certain components of the samples on Taq polymerase, which could cause false negative results (86). As bacteria are present at the initial stages of the infection, performing PCR in blood is an interesting option, despite its low sensitivity.

2.2.1.b. Variants of conventional PCR

Among the variants of the conventional PCR technique, we can mention: i) nested PCR, which involves two rounds of amplification of the same sequence with different primer pairs each and thus allows to increase the sensitivity of the reaction; ii) multiplex PCR, which uses several pairs of primers in the same reaction to amplify multiple target sequences of the bacterial genome simultaneously. This variant of PCR was used by Moravkova *et al.* (58) to differentiate mixed Map mycobacterial infections (*M. avium* subsp. *hominissuis*, *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum*) by simultaneously amplifying sequences IS900, IS901 and IS1245 and the *dnaJ* gene (58); iii) real-time or quantitative PCR (RT-PCR), which uses a fluorochrome-labeled probe complementary to an intermediate fragment of the target sequence that is amplified. The quantification of fluorescence emitted during each PCR cycle is proportional to the amount of DNA. The application of RT-PCR using the insertion sequences ISMav2 showed a 76 % sensitivity in feces samples from large fecal shedders, and very low sensitivity (4 %) for low and moderate fecal shedders (90). Similar results were obtained in another study by Alinovi *et al.* (2).

Other variants are the PCR amplification system called loop-mediated isothermal amplification (LAMP), which does not require the use of a thermocycler (25), and the triple real-time PCR (TRT-PCR), designed by Irenge *et al.* (38). LAMP was used by Enosawa *et al.* to identify IS900 in cultures of *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. smegmatis*, *M. bovis* and *M. kansasii* (25). TRT-PCR of IS900, *f57* and ISMAP02 was used in fecal samples and showed a detection limit of 2.5×10^2 CFU/g of feces, thus showing a higher sensitivity than bacterial culture and ZN.

The advantage of PCR is the timely detection of Map, without the need of viable bacteria in the sample. Multiplex PCR provides information from several loci in a single reaction. The advantage of RT-PCR is that it allows the immediate observation of the target amplification, quantification and has greater sensitivity than bacterial culture (7). The use of IS900 in this type of PCR is sensitive to detect very low numbers of Map, but inadequate for accurate quantification of CFU in the sample, since it is present in many copies within the bacterial genome. Therefore, the *f57* sequence is used for real-time PCR. LAMP, on the other hand, has high sensitivity and specificity,

is not laborious, and does not require special equipment, all of which make it an inexpensive diagnostic tool. According to the results obtained by Irenge (38) with TRT-PCR, it can be stated that it is a rapid and specific technique to evaluate fecal samples from animals in the subclinical stage, although it must be validated with a higher number of samples from different herds.

The disadvantage of PCRs is their high cost. The possibility of false positive results (by contamination during the development of the technique) and/or false negatives (by possible inhibitory components on the Taq polymerase), required control by use of appropriate internal negative and positive controls within each batch of samples. All different types of PCR previously described show risks of contamination (2, 8, 25, 38, 57, 58).

2.2.2. Combination of microbial concentration and PCR

The use of IMS prior to PCR in milk samples has a sensitivity of 100 % and a specificity of 95 %, while the sensitivity of PCR alone is of 23 % (29, 32, 59). The advantage of this technique is increased specificity in the concentration of the microorganism, allowing high repeatability of the assays, and the elimination of potential Taq polymerase inhibitors in the samples (32, 54).

2.2.3. *In situ* hybridization (ISH)

ISH is a molecular technique that uses a labeled probe to specifically detect a nucleic acid sequence (DNA or RNA) on a histologically processed tissue section, allowing their tissue localization. ISH in PTB diagnosis uses a specific DNA probe of variable size. The use of a small probe easily penetrates tissues and reaches the target sequence, but may induce no specific reactions or weak staining that may impair the reading of the assay. In contrast, a larger probe may have difficulty in penetrating the tissue and finding the target sequence. Among the markers used are radioactive and fluorescent compounds, which allow to detect the sequence of interest but with loss of detail of the tissue structure, and enzymatic markers, which allow better observation. ISH is a technique that has been used primarily to detect spheroplasts in animal samples, samples from patients with Crohn's disease (76) and unicellular parasites where Map can grow (61).

The advantage of the technique is the identification of Map in tissue. The disadvantages are those

concerning the diversity of methodologies (how to preserve the sample, the probe length, etc.), the accreditation of laboratories to work with radioactive markers and the need for trained personnel.

2.3 ANATOMOHISTOPATHOLOGICAL DIAGNOSIS

2.3.1. Anatomopathological diagnosis

The macroscopic lesion characteristic of bovine PTB is a thickening of the intestinal wall, as well as the corrugation of the mucosa affecting different intestinal locations (31). However, the clinical signs may not present a linear relationship with the findings at necropsy. The intestinal wall presents a “cerebroid” aspect due to the presence of numerous 5-8 mm folds, which do not disappear when pulling. These folds are due to thickening of the wall by infiltration of macrophages, and epithelioid and giant cells containing acid-fast bacilli (AFB) in variable numbers depending on the specific immunopathological form. There is also lymphadenomegaly and edema of the mesenteric lymph nodes, together with lymphangiectasia. The intestinal lymph flow is restricted by the presence of macrophages that obstruct the subcapsular sinus, the trabecules and the afferent lymphatic vessels. Although it is rare to find lesions outside the intestinal tract, liver injury, atherosclerosis of the aorta, myoatrophy, emaciation, atrophy of body fat, alopecia, renal infarction, edema, serous exudates in body cavities and anemia can occur in advanced PTB (86).

PTB lesions are classified as tuberculoid (focal, multifocal and lymphocytic or paucibacillary), lepromatous (diffuse multibacillary) and intermediate type, according to their size, and the type and number of cells involved (31). Focal lesions are supposed to be the first to appear and are associated with a strong cellular immune response. Tuberculoid and multifocal lesions progress until confluence, compressing and obliterating the intestinal crypts. The face of villi is fused, causing a decrease in the absorptive surface, which leads to weight loss, resulting in hypo-proteinemia and edema. The lepromatous type appears in some animals, related to the changing profile of the immune response.

2.3.2. Histopathological diagnosis

Tissue samples (2-4 grams) can be obtained from distal portions of the ileum, ileocecal valve, mesenteric

lymph nodes and biopsy or scraping of the rectal mucosa, although the latter is not frequently used because it does not present detectable lesions in most animals (9). Microscopically, the characteristic lesion is a chronic diffuse catarrhal enteritis characterized by hyperplasia of macrophages, lymphocytes, plasma cells, epithelioid and multinucleated giant Langhans cells in the lamina propria, intestinal submucosa and paracortical region of regional lymph nodes, atrophy and fusion of intestinal villi with thickening of the mucosa. In some cases, granulomatous lymphangitis can also be observed. In the lymph nodes, the sub-capsular and peritrabecular cortical sinuses contain numerous macrophages. Optical microscope observation after ZN shows 1.5 x 0.5 μ acid-fast bacilli, in clumps or within macrophages.

The advantage of the anatomohistopathological diagnosis is that it allows to identify animals with focal lesions associated with subclinical stages, whose fecal and/or milk excretion is insufficient for bacterial culture or PCR. However, its disadvantages are that it requires trained personnel for sample study and that it has a high cost (49, 94), especially if it is considered that Whitlock *et al.* (91) recommend that in order to establish the true stage of the disease, samples should be taken from at least 100 sites of the gastrointestinal tract of each animal.

2.3.3. Immunohistochemistry (IHC)

This technique uses a MAP-specific antibody (antiMap Ab) marked with enzymes, which allows to visualize the reaction on the enzymatic substrate (19). The advantage of this method is that it enables to identify spheroplasts and Map in tissue (14). It has good sensitivity in animals with subclinical PTB, but can cross-react with *Mycobacterium smegmatis*, *Mycobacterium bovis*, *Mycobacterium tuberculosis* and *Mycobacterium leprae*. The efficiency of the method depends on the antiMap Ab used (60) and the sensitivity is low as compared with bacterial culture (51).

2.4 INDIRECT DIAGNOSIS: HOST IMMUNE RESPONSE

Indirect diagnosis can be made by assessing the animal's immune response, which depends on the stage of disease. Subclinical stages are typically characterized by high cellular immune response, clinical stages by a humoral immune response (80, 81) and advanced stages by anergy, where diagnostic

tests of cellular immunity become negative and serological tests are less reliable (49). The ELISA is, at present, the most sensitive and specific test for serum antibodies to Map, and several absorbed ELISA kits are commercially available (50).

2.4.1. Cellular immune response

The first immune response after Map entrance is mediated by cells, specifically T lymphocytes. The diagnostic tests that evaluate this response are the intradermal reaction (*in vivo*) and the detection of gamma interferon production (*in vitro*). The discriminatory power of both tests is low due to their cross-reaction with other environmental mycobacteria.

2.4.1.a. *In vivo*: intradermal reaction (IDR)

After the animal first comes into contact with Map, it develops a type IV delayed hypersensitivity which can be detected by IDR. The test is performed by intradermal inoculation of 0.1 mL (0.5 mg/ml, 25,000 UI) of PPD-A (purified protein derivative of *M. avium*) or PPD-J (Johnine) (both with comparable sensitivity and specificity) in the middle third of the neck or anocaudal fold. The skin thickness is measured with a caliper before and 72 hours after inoculation. An increase in skin thickness greater than 2 mm (70) or 3 mm (49) is considered positive. Development of cutaneous hypersensitivity to johnin PPD occurred in the majority of orally inoculated calves by the second month after administration (46).

A comparative IDR variant can be performed by simultaneous inoculation of PPD-B (purified protein derivative of *M. bovis*) and PPD-A in two separate areas in the neck. The animals with PTB react positively to both PPDs, but with greater intensity to PPD-A, because of antigenic similarity between Map and *M. bovis*. IDR allows to identify cattle carrying Map, without interfering with the controls of health prophylaxis and eradication of tuberculosis. IDR has an estimated sensitivity of 54 %, a specificity of 79 % (5, 42), low positive predictive power (22 %), and good negative discriminatory power (95 %) (12)

The advantages are that it is easy to perform in the field, and that there is a chance of early detection of infected animals, since cellular immunity is developed before bacilli excretion and development of the humoral immune response, allowing the detection of infected animals in subclinical stages much earlier than the serological tests or bacterial culture. The disadvantages are its low sensitivity and

its low specificity (due to probable cross-reactions). Since the positive reactions indicate sensitization of the animals to Map or to the *M. avium* complex, this technique should be used only as a preliminary test, before the initiation of control programs (49, 50).

2.4.1.b. *In vitro*: detection of interferon gamma (IFN- γ)

This test evaluates the specific production of cytokine IFN- γ by T lymphocytes after stimulation with PPD. Quantitative detection of IFN- γ can be used in animals aged 1 to 2 years old (3, 5, 35-37, 39, 78). In animals in the subclinical stage, the sensitivity of this test is higher than that of the serological tests, but low in absolute terms (41 %) (33, 79). It can even decrease to about 20 % in herds with mixed infections (tuberculosis and PTB), these differences could be due to the host species or the strains present in each herd (3, 4). Walravens *et al.* (89) compared the IFN- γ response in cattle inoculated with *M. bovis*, Map and *Mycobacterium phlei*, and obtained a response of low intensity and slow onset (4th to 5th week post-inoculation) in those inoculated with Map. These authors concluded that this test does not allow an accurate diagnosis in the first six months post-infection.

The advantage of the IFN- γ test is the significant secretion of IFN- γ during the early stages of PTB and may thus be an attractive tool to detect animals in the subclinical stage. However, it has several disadvantages: i) the possible cross-reactions, ii) the need to process the sample quickly since cells must be alive (79), iii) its high cost and iv) its low sensitivity. For all these reasons, this test is not widely used, although it can be used in control programs in order to reduce transmission to adult animals and to identify infected animals before they develop the disease (39, 68, 79).

2.4.2. HUMORAL IMMUNE RESPONSE

Cattle in the later stages of the disease, and especially with lepromatous lesions show high concentration of antibodies specific to Map, which can be detected by complement fixation (CF), agar gel immunodiffusion (AGID), and enzyme-linked immunosorbent assay (ELISA). The last two techniques are fast, inexpensive, easy to implement and do not require much equipment. In addition, ELISA may be automated. In contrast, CF is difficult to perform and is carried out only by reference

laboratories. In cattle, ELISA is more sensitive than CF and AGID. In general, the tests to assess the humoral immune response have the disadvantage of being variable in individual responses due to the stage of disease and anergy (48, 94). The sensitivity is high in animals in the advanced clinical stage and large fecal shedders, but irrelevant to identify animals in the subclinical stage (66).

2.4.2.a. Complement fixation

This test has been widely used in the past, being adequate to identify animals with clinical signs suggestive of PTB, but not specific enough to be used in control programs. However, it is often applied in international export of cattle (50). The technical protocols are variable, but it is generally dilution of sera samples plus specific antigen (49, 50).

2.4.2.b. Agar Gel Immunodiffusion

AGID is based on the precipitation of immune complexes formed by the antibodies of infected animals with a soluble antigen from a protoplasmic extract of Map in a gel matrix of agar. It is a simple, fast and relatively inexpensive method, but has low sensitivity in the early stages of PTB and therefore it is considered a good diagnostic method in animals in advanced clinical stages. It can be used as a rapid confirmatory test of suspected cases. The sensitivity is good in advanced clinical PTB (90 % - 95 %), but low in subclinical stages (30 % - 18.9 %) (5).

2.4.2.c. Enzyme-linked immunosorbent assay

ELISA is the diagnostic test most commonly used for serological diagnosis of PTB. Various antigens, including the soluble antigen from Map protoplasmic extract marketed by Allied Monitor (Fayette, MO, USA) and glycolipid extracts from the walls of mycobacteria such as lipoarabinomannan may be used (65). It can be applied in blood serum and milk, there being a moderate correlation between them because the concentration of antibodies in milk depends not only on the levels of serum antibodies, but also on genetic milk production level, days in lactation, and number of calvings (71). Based on these parameters, ELISA carried out in milk can detect about 12 % fewer positive animals than that carried out in serum (34).

Therefore, it is recommended that at least two ELISA determinations are carried out at different

times of lactation to establish not only the level of antibodies but also the stability of the result, so as to have good sensitivity without loss of specificity. Thus, the combination of ELISA and bacterial culture, in order to interpret the results in parallel (bacterial culture level and stability of ELISA) provides high sensitivity in low-prevalence herds (1, 63, 65, 66).

When ELISA yields positive results in apparently healthy or low-prevalence herds, a bacterial culture should be carried out to confirm the stage of infection. If the results are negative, the positive ELISA should be re-examined in 6-12 months, since it may be a false positive or it may be that at that time the animal was not shedding Map in the feces in detectable amounts (86). It has been suggested that the results are categorized as negative, positive or suspicious according to their optical density (1, 41). The pre-absorption of sera with *M. phlei* allows to eliminate cross-reactions against other microorganisms and increase sensitivity and specificity (55, 56).

The ELISA test is applied mainly in animals older than two years or as from the second or third delivery, a period where the fecal and/or milk excretion of Map is quantitatively important, with diagnostic certainty when the animal shows signs of diarrhea and submandibular edema (43, 63, 64). The sensitivity of ELISA in serum of animals is of 7 % in the silent stage, 15 % in the subclinical stage, and between 85 % to 98 % in the clinical stage (5, 23, 52, 62, 65, 85).

Speer *et al.* (78) developed a variant of ELISA called SELISA, by sensitizing plates with Map antigens treated with formaldehyde and sonication, with which they obtained 96 % sensitivity and 100 % specificity in calves experimentally infected or especially in low shedders. Other researchers performed another variant of ELISA, known as EVELISA (23), using a Map antigen extract obtained by extraction with ethanol and highlighted its lower risk in the production of antigen and its higher stability, since plates may be conserved for 7 weeks without changing its sensitivity or specificity. By testing serum samples from fecal culture-positive cattle, these authors categorized as low (<10 colonies), middle (10 to 50 colonies) and high (>50 colonies) shedders, the sensitivity of the EVELISA was 96.6 % for low shedders and 100 % for middle and high shedders. However, four years later, the same researches (75) observed some cases of serological false-positive reactions. Antibodies in the serum samples reacted strongly with antigens of various environmental mycobacteria, suggesting the presence of cross-

reactive antibodies in the samples. This possibility in the EVELISA was inhibited markedly by *M. phlei* antigen absorption (EVA-ELISA). The sensitivity and specificity of the EVA-ELISA were estimated to be 97 % and 100 %, respectively. The sensitivity of commercial ELISA kits is between 9 % and 32 % for low fecal shedders and between 47 % and 63 % for moderate fecal shedders (92).

The traditional ELISA test has several advantages, such as easy automation, repeatability, objective interpretation of the results, possibility to evaluate multiple samples together and possibility to modify the cutoff according to the sensitivity or specificity required. It has very good sensitivity and specificity in clinical stages and is relatively inexpensive. It is a good method to assess the prevalence of PTB in the herd, although several researchers have found that the prevalence of bovine tuberculosis decreases the sensitivity and specificity of the test for PTB (49). The disadvantage is that the antigenic variability in different ELISA tests of serum and the different ages of the animals tested can lead to errors in sensitivity and specificity.

2.4.2.d. Flow cytometry

This technique allows to detect animals with subclinical infection and differentiate between Map, *Mycobacterium scrofulaceum* and *M. avium* subsp. *avium*. Using this technique, Eda *et al.* detected antiMap IgG in calves at 240 days post-experimental inoculation, without cross-reactions, and with a sensitivity of 95 % and a specificity of 97 % (22). This technique is rapid (less than 4 hours) and objective, but expensive and complex to execute given the kind of sophisticated equipment required.

3. DIAGNOSIS FOR SUBCLINICAL PARATUBERCULOSIS

Bacterioscopy has low sensitivity and specificity and is at risk of obtaining false positive results due to the presence of other environmental mycobacteria. Since Map excretion levels in colostrum, milk and feces at the subclinical stage are below the minimum detection limit, the sensitivity of the bacterial culture is low, and negative results should be interpreted with caution. The use of the combined IMS-PCR technique in these samples improves both sensitivity and specificity. Because Map colonization of the intestinal mucosa occurs within the first hours of exposure to

the bacterium and is the first sign of infection, both the anatomohistopathological diagnosis and the bacterial culture of intestinal tissue (ileocecal valve and regional lymph nodes) and/or of lymph nodes close to macroscopic lesions are early and accurate diagnostic methods, but impractical and expensive. Immunohistochemistry (IHC) is a good diagnostic alternative having good sensitivity but implying possible cross-reactions. The IHC technique has the disadvantages already discussed.

The tests that evaluate the cellular immune response allow to detect subclinical infected animals much earlier than serological tests or the bacterial culture. The intradermal reaction, although being easy to perform in the field, has low sensitivity and specificity, with possible cross-reactions between Map and other members of the *M. avium* complex infections. The IFN- γ technique is not recommended in the first six months post-infection, as it can yield false negative and positive results, especially in calves, heifers and even females until the first lactation (89).

The various diagnostic tests that evaluate the humoral immune response in the subclinical stage of PTB have low sensitivity and specificity due to the late appearance of antibodies. Although Eda *et al.* (28) state that they have detected antiMap IgG early in experimental conditions, they also state that the antibodies detected may be of colostral origin.

4. DIAGNOSIS OF CLINICAL PARATUBERCULOSIS

At the clinical stage, the microbiological culture is a good, sensitive and specific diagnostic method since the animals are usually large fecal shedders of Map. Diagnostic bacterioscopy has the inconvenience already described in the previous stage. The combined technique of IMS-PCR is still a very good diagnostic option. Buerget *et al.* have demonstrated that at this stage there is a positive correlation between the results of nested PCR in milk and serum and those obtained by ELISA in serum (10). The anatomohistopathological diagnosis or tissue culture is a good tool because the lesions are pathognomonic of PTB. The disadvantages of IHC and ISH have been described above.

The diagnostic tests of the cellular immune response evaluated have no value in this stage. There are a number of commercial ELISA tests that are widely used in laboratories throughout the world; also, home-made PPD-A ELISA tests are routinely used

in laboratories due to their very good sensitivity and specificity, in addition to diagnostic certainty in animals with diarrhea and submandibular edema. The CF test having very good sensitivity and specificity at this stage, is used in some diagnostic laboratories, but it is laborious and expensive. AGID is a diagnostic alternative, which is technically easy and has low cost and good sensitivity. Table 2 shows the sensitivity and specificity of the different PTB diagnostic techniques, expressed as percentage for each stage, according to the different authors.

5. CONCLUSION

Map detection by means of bacterial culture in solid medium is still the reference diagnostic method because it also allows to categorize the animals as low, moderate or large fecal shedders. However, it is slow and insensitive, especially at the early stages. These limitations prevent a rapid identification of Map, thus delaying the decision to remove the infected animals, and allowing the pathogen to circulate in the herds.

Another diagnostic alternative is to evaluate the cellular and/or humoral immune responses, whose sensitivity and specificity depend on the stage of the disease. The humoral immune response against Map

in subclinical animals may vary over time, even day to day, probably due to fluctuations in the production of antibodies. The sensitivity of these tests increases with the magnitude of fecal shedding of Map and the degree of spread of lesions (clinical and advanced stage), while in the silent and subclinical periods, the cellular immune response is the one having diagnostic value. Map detection by PCR is rapid and specific and does not require bacterial viability. In addition, the concentration mediated by immunomagnetic separation and the use of antiMap specific antibodies increase the sensitivity and specificity of the test.

Definitive diagnosis is made post-mortem by the signs found in the gastrointestinal tract, for which older animals are preferably selected.

Due to the immunological complexity and the prolonged subclinical period of the disease, it is difficult to determine only one reference diagnostic test, especially if a diagnostic test with high sensitivity and specificity is expected. The limitations of each diagnostic test determine the use of two or three of them, repeated in time in the same animal to establish the stage of the disease both in the animal and the herd. For this reason, and to prevent PTB transmission, detection of infected animals in the silent or subclinical periods is the key to the initiation of control programs of the disease and to establish biosecurity standards.

Table 2. Sensitivity (*Se*) and specificity (*Ep*) of different PTB diagnostic tests, expressed as rate for each stage, according to different authors

Stage	Diagnostic test											
	ZN staining ⁽⁵³⁾	IDR ^(6,42)	IFN- γ ⁽⁶⁵⁾	Culture		ELISA		SELISA ⁽⁷⁸⁾	EVA-ELISA ⁽²³⁾	CF ⁽²²⁾	PCR ⁽³²⁾	IMS-PCR ^(32,59)
				Feces ^(2,3)	Tissues ⁽⁶⁶⁾	Serum ⁽⁶⁵⁾	Milk ⁽⁶⁵⁾					
Silent infection	NO	YES	YES	NO	YES <i>Se</i> : 70 <i>Ep</i> : 95	YES <i>Se</i> : 7	ND	YES <i>Se</i> : 95.6 <i>Ep</i> : 100	YES <i>Se</i> : 97.4 <i>Ep</i> : 100	YES <i>Se</i> : 95.2 <i>Ep</i> : 96.7	ND	ND
Sub-clinical detectable	Not	YES <i>Se</i> : 54 <i>Ep</i> : 79	YES <i>Se</i> : 41 <i>Ep</i> : 98	YES <i>Se</i> : 45-72		YES <i>Se</i> : 15 <i>Ep</i> : 98,9	<i>Se</i> : 29-61					
Clinical	YES	ND	ND	YES <i>Se</i> : 91 <i>Ep</i> : 100		YES <i>Se</i> : 85/98 <i>Ep</i> : 98,9	YES <i>Se</i> : 83-100				<i>Se</i> : 23 <i>Ep</i> : 95	YES <i>Se</i> : 100 <i>Ep</i> : 95
Advanced	YES	ND	ND			Energy ^(80,81)						

The author's references are indicated in brackets; ND: no data; IDR: intradermal reaction; CF: complement fixation; AGID: agar gel immunodiffusion; IFN- γ : interferon gamma; IMS: immunomagnetic separation.

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